Centromere Protein H Is Up-regulated in Primary Human Colorectal Cancer and Its Overexpression Induces Aneuploidy

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Abstract

Chromosomal instability (CIN) has been recognized as a hallmark of human cancer and is caused by continuous chromosome missegregation during mitosis. Proper chromosome segregation requires a physical connection between spindle microtubules and centromeric DNA and this attachment occurs at proteinaceous structures called kinetochore. Several centromere proteins such as CENP-A and CENP-H are the fundamental components of the human active kinetochore, and inappropriate expression of the centromere proteins could be a major cause of CIN. We have previously shown that CENP-A was overexpressed in primary human colorectal cancer. In this study, we show that CENP-H was also up-regulated in all of 15 primary human colorectal cancer tissues as well as in CIN tumor cell lines. Surprisingly, transient transfection of CENP-H expression plasmid into the diploid cell line HCT116 remarkably induced aneuploidy. Moreover, CENP-H stable transfectant of mouse embryonic fibroblast/3T3 cell lines showed aberrant interphase micro-nuclei, characteristic of chromosome missegregation. In these CENP-H overexpressed cells, CENP-H completely disappeared from the centromere of mitotic chromosomes, which might be the cause of the chromosome segregation defect. These results suggest that the aberrant expression and localization of a kinetochore protein CENP-H plays an important role in the aneuploidy frequently observed in colorectal cancers. (Cancer Res 2005; 65(11): 4683-9)

Introduction

A very large fraction of human cancers have an abnormal or heterogeneous number of chromosomes, a feature referred to as aneuploidy. This aneuploidy has recently been explained by chromosomal instability (CIN), an accelerated rate of gain or loss of whole or large portions of chromosomes. Evidence is accumulating that CIN plays an important role in the development and progression of cancer because aneuploidy is found in the earliest stages of tumorigenesis (e.g., carcinomas in situ or precancerous lesions of many tumor types; refs. 1–3). In addition, studies in vitro using human and rodent cells have shown that aneuploidy is required for neoplastic transformation (4, 5).

Recent compelling data has strongly suggested that chromosome missegregation during mitosis is the main cause of aneuploidy and contributes to oncogenesis. A centromere-associated kinase STK15/BTAK/aurora2 is amplified in human cancers and exogenous expression of the kinase in rodent and human cells induced unequal partitioning of chromosomes during mitosis and tumorigenic transformation of cells (6, 7). Human securin is also overexpressed in some tumors and exhibits transforming activity in NIH3T3 cells (8). A small fraction of human colorectal cancers revealed mutations in either hBub1 or hBubR1 checkpoint genes (9). Inactivation of hCDC4 in karyotypically stable colorectal cancer cells caused CIN and mutations of the gene were found in early-stage tumors (10). Recently, the gene product of adenomatous polyposis coli (APC), the most frequently mutated gene in colorectal tumors, has been observed at the plus ends of kinetochore microtubules. Mutations in APC, similar to the mutations found in tumor cells, interfere with microtubule plus-end attachments and result in mitotic abnormalities (11–13). These observations suggest that the components necessary for microtubules to attach to centromeres play an important role in CIN.

In yeast, over 30 kinetochore proteins have recently been identified; many of which are conserved among eukaryotes (14). CENP-A was one of the first kinetochore components identified in humans (15). It is a unique histone H3-like protein that has been found only at active centromeres and believed to be a central element of the kinetochore proteins (16, 17). CENP-H was initially identified as a component of the mouse centromere and human CENP-H protein was recently isolated and shown to localize in the inner plate together with CENP-A and CENP-C (18, 19). Careful observation of a conditional loss-of-function mutant of CENP-H in the chicken cell line DT40 revealed that CENP-H is a fundamental component of the active centromere complex (20).

Moreover, extensive analysis of the interactions between centromeric DNA and kinetochore complexes in budding yeast showed that a molecular core consisting of CENP-A, CENP-C, CENP-H, and Ndc80/HEC plays a central role in linking centromeres to spindle microtubules (21). We have previously reported that CENP-A was overexpressed and mistargeted in primary human colorectal cancer (22). In this report, we showed that CENP-H was markedly up-regulated in most colorectal cancers. Furthermore, overexpression of CENP-H induced chromosome missegregation and aneuploidy in a diploid cell lines. This aneuploidy is presumably caused by the mislocalization of the CENP-H. We propose that stoichiometric expression of core kinetochore components is essential to prevent chromosomal instability and carcinogenesis.

Materials and Methods

Human tissue samples. Tissues from 15 cases of primary colorectal cancer were surgically resected. Written informed consent was obtained from each patient before surgery. The excised samples were obtained within one hour after the operation from tumor tissues and corresponding nontumor tissues 5 to 10 cm from the tumor. All excised tissues were immediately placed in liquid nitrogen and stored at −80°C until further analysis.
Plasmid DNA, cell culture. Full-length CENP-H and CENP-A were amplified by PCR and cloned into the pcDNA3.1 (Invitrogen, Carlsbad, CA) or pTRE2hyg vector plasmid (BD Biosciences Clontech, Palo Alto, CA). Plasmids were purified with an Endofree Plasmid Maxi Kit (Qiagen, Tokyo, Japan) and the DNA sequences were verified. CACO-2, LoVo, HCT116, SW48, and RKO colorectal cancer cell lines were purchased from American Type Culture Collection (Manassas, VA). The HT29 cell line was kindly provided by Dr. Takenaga (Division of Chemotherapy, Chiba Cancer Center Research Institute, Chiba, Japan). Cells were grown at 37°C, in 5% CO₂ in Iscove’s Modified Dulbecco’s Medium (IMDM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin-streptomycin (Invitrogen).

Transient and stable transfection. Transient and stable transfections were done using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions. Briefly, HCT116 cells or MEF/3T3 Tet-Off cells were plated in 6-well plates in IMDM containing 10% FBS [+100 μg/mL of geneticin (Invitrogen) for MEF/3T3 Tet-Off cells] without antibiotics 1 day before transfection so that they will be 70% to 90% confluent at the time of transfection. On the day of transfection, dilute 4 μg of plasmids and 10 μL of LipofectAMINE 2000 in 250 μL of Opti-MEM 1 Reduced Serum Medium (Invitrogen), respectively. After 5 minutes of incubation at room temperature, diluted plasmids and LipofectAMINE 2000 were combined and were further incubated for 20 minutes at room temperature. Then, the DNA-LipofectAMINE 2000 complexes were added to each well and cells were incubated for 48 hours at 37°C in CO₂ incubator. For transient transfection, cells were processed for immunostaining and fluorescence in situ hybridization (FISH) analysis.

For stable transfection, 5 × 10⁴ MEF/3T3 Tet-Off cells transfected with pTRE2hyg/CENP-H plasmid were transferred to 10-cm dishes after 48 hours after transfection; 400 μg/mL of hygromycin and 1 μg/mL of doxycycline were added to the complete medium containing IMDM, 10% FBS, 1% penicillin-streptomycin and 100 μg/mL of geneticin at that time. The complete medium with hygromycin was replaced every 4 days and fresh doxycycline was added every 2 days until hygromycin-resistant colonies begin to appear. At least 30 clones were screened by immunoblotting with anti-CENP-H antibody to find the clone with the highest induction in the absence of doxycycline and lowest background.

Protein extraction and immunoblotting. Frozen tissue samples were solubilized in lysis buffer [7 mol/L urea, 2 mol/L thiourea, 2% CHAPS, 0.1 mol/L DTT, 2% IGP buffer (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom), 40 mmol/L Tris] using a Polytron homogenizer (Kinematica, Littau, Switzerland) following centrifugation (100,000 × g) for 1 hour at 4°C. Cultured cells were solubilized in 2x SDS sample buffer [125 mmol/L Tris-HCl (pH 6.8), 2% SDS, 20% glycerol, 0.2% bromophenol blue, 0.025% [mercaptoethanol] and heated for 5 minutes at 100°C. After passing through 26-gauge needles, the samples were centrifuged for 15 minutes at 15,000 × g. The supernatant proteins were separated by electrophoresis on 10% to 20% gradient gels (Bio-Rad, Hercules, CA). Proteins were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA) in a tank transfer apparatus (Bio-Rad) and the membranes were blocked with 5% skim milk in PBS. Rabbit anti–CENP-H antibody diluted 1:5,000 (23), rabbit anti-hMis12 antibody (ref. 24; from Drs. Goshima and Yanagida, Department of Gene Mechanisms, Graduate School of Biostudies, Kyoto University, Kyoto, Japan) diluted 1:100 and goat anti–β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:500 in blocking buffer were used as primary antibodies. Goat anti-rabbit immunoglobulin G horsedens peroxidase (HRP; Bio-Rad) diluted 1:3,000 and rabbit anti-goat immunoglobulin G HRP (Cappel, West Chester, PA) diluted 1:500 in blocking buffer were used as secondary antibodies. Antigens on the membrane were detected with enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech). The intensity of each band was measured using NIH Image.

Reverse transcription-PCR and real-time quantitative PCR. Total RNA was extracted from tumor and nontumor tissues with RNeasy mini kit (Qiagen). cDNA was synthesized from total RNA with the first strand cDNA Synthesis Kit for reverse transcription-PCR (RT-PCR, Roche, Mannheim, Germany). Using the cDNA as a template, CENP-H cDNA was amplified with suitable primers: forward 5'-CAGCTAGTGTGCTAGGAT-3' and reverse 5'-TCCATCGTTAGTTTGCTG-3'. For the control, glyceraldehyde-3-phosphate dehydrogenase or β-actin cDNA was amplified. Serial dilutions of the template cDNA were made for reactions to optimize the PCR products within the linear range. Real-time quantitative PCR of CENP-H cDNA using the LightCycler instrument (Roche) was carried out in 20 μL of reaction mixture containing LightCycler-DNA Master SYBR Green I (FastStart Tag DNA polymerase, deoxynucleotide triphosphate, buffer, SYBR Green I), 3.0 mmol/L MgCl₂, and 0.5 μmol/L each of the forward and reverse primer described above in a LightCycler capillary. LightCycler software version 3.3 (Roche) was used for analysis of quantitative PCR. The optimization of primers was done at the Nihon Gene Research Laboratories, Inc. (Sendai, Japan).

Immunocytochemistry. Tissues or cultured cells were fixed on slide glasses with acetone for 10 minutes at 4°C. Mitotic chromosome spreads were prepared from growing HCT116 cells treated with 0.1 μg/mL of Colcemid (Invitrogen) for 3 hours, harvested by mitotic shake off, and hypotonsionally swollen for 30 minutes at room temperature in 75 mmol/L KCl. Cytospin-spread chromosomes were fixed with acetone for 10 minutes at 4°C. After three washes with PBS, the nonspecific binding of antibodies was blocked with blocking buffer (10% fetal bovine serum/PBS) for 1 hour. Samples were incubated for 1 hour with rabbit anti–CENP-H antibody diluted 1:2,000, mouse anti-human CENP-A monoclonal antibody diluted 1:1,000, and/or nuclear centromere autoantibody positive control (ANA serum; The Binding Site, Birmingham, United Kingdom) diluted 1:3,000 in 3% bovine serum albumin/PBS. After a wash with PBS, samples were incubated with 1:1,000 diluted Alexa Fluor 488 or 594-conjugated goat anti-rabbit, anti-mouse, anti-human immunoglobulin G secondary antibody (Molecular Probes, Eugene, OR) for 1 hour. DNA was counterstained with 4',6-diamidino-2-phenylindole III Counterstain (Vysis, Abbott Park, IL). Samples were observed with a fluorescence microscope (Leica QFISH; Leica Micro-systems, Tokyo, Japan). For H&E staining, tissue sections stained with hematoxylin for 30 seconds followed with eosin for 15 seconds were dehydrated with 100% ethanol and xylene, and coverslips were mounted with Permount.

Fluorescence in situ hybridization analysis. Following immunocytochemistry, slides were incubated in 75 mmol/L KCl for 10 minutes and again fixed in 3:1 methanol/acetic acid for 10 minutes at room temperature and treated with 0.1 mg/mL of RNase A in 2× SSC for 30 minutes at 37°C. After being washed in PBS, slides were dehydrated by passage through an ethanol series (70%, 85%, and 100%), incubated in 2× SSC/0.1% NP40 solution for 30 minutes at 37°C, and dehydrated again. Target DNA was denatured for 5 minutes at 100°C in 2× SSC/0.1% NP40 (pH 7.5). Probe dilutions (10 μL) to the pericentromeric regions of chromosome 8 (CEP8 Spectrum Orange) and 12 (CEP12 Spectrum Orange; Vysis, Downers Grove, IL) were also denatured for 5 minutes at 73°C and hybridized to the target DNA by incubation overnight at 37°C. Posthybridization washes were done thrice in 50% formamide/2× SSC (pH 7.0) for 10 minutes at 45°C, once in 2× SSC and in 2× SSC/0.1% NP40 solution for 5 minutes at 45°C. Hybridization signals were observed and analyzed with Leica QFISH (Leica). At least 100 nuclei of each sample were evaluated for chromosome counts.

Results

To extend our previous observation of CENP-A overexpression in colorectal cancer, the levels of several kintochore proteins were examined by Western blotting. CENP-H was greatly overexpressed in all 15 cases of primary colorectal cancer (Fig. 1A). The relative expression level of CENP-H protein in tumors compared with adjacent normal tissue varied from 1.7 to 9.6. This overexpression is not a general phenomenon of kintochore proteins because the expression of other kintochore proteins such as CENP-B (22) and hMis12 did not increase in colorectal cancer tissues (Fig. 1B). FISH analysis of several tumor tissues with elevated CENP-H expression showed that not all the tumor cells are highly polyplody (ref. 22; data not shown). These results suggest that CENP-H overexpression is not a consequence of polyplody.
To check if CENP-H was overexpressed in cancer cells and not in surrounding mesenchymal cells, tissue sections of colorectal cancers and adjacent normal tissues were stained with anti-human CENP-H polyclonal antibody. Although CENP-H was observed as small punctate dots, like other centromere proteins, in normal colon epithelial cells (Fig. 1a, arrow), the number and size of CENP-H dots increased in tumor cells (Fig. 1b, arrow) compared with normal epithelium (e, arrow). In addition, diffuse CENP-H staining was observed in the tumor epithelium (b, arrowhead) but not in the surrounding stroma cells. Magnification is ×400 (a and b) and ×630 (c–e).

To test if overexpression of CENP-H is the result of increased transcription, CENP-H mRNA levels of colorectal cancer tissue and normal colon epithelium were examined by RT-PCR and real-time quantitative PCR (Fig. 2A and B). Relative mRNA levels significantly increased in tumor tissues and the mRNA levels correlated well with relative protein levels shown in Fig. 1A. No CENP-H gene amplification was observed in tumors (data not shown) indicating that overexpression of CENP-H occurred at the transcriptional level.

Colorectal cancer cell lines show two distinct types of genetic instability; one type is observed at the nucleotide level termed microsatellite instability (MIN) and the other is CIN which shows an abnormal chromosome number. If CENP-H overexpression is a potential cause of CIN, the expression might be up-regulated in CIN cell lines. We carefully compared the CENP-H expression level by Western blotting in several CIN and MIN cell lines and found that it increased in CIN cell lines LoVo, HT29, and CACO-2, compared with MIN cell lines SW48, RKO, and HCT116 (Fig. 3A). Immunostaining of the CIN and MIN cell lines with anti-CENP-H antibody as well as anti-CENP-A antibody showed more intense and diffuse CENP-H signals relative to CENP-A in a CIN cell line CACO-2 compared with a MIN cell line HCT116 (Fig. 3B, arrow). These results indicate that CENP-H overexpression is characteristic of CIN colorectal cell lines.

These observations have raised the question of whether overexpression of CENP-H is able to induce the CIN phenotype. To answer this question, CENP-H was transiently introduced into a MIN cell line HCT116 and FISH analysis following immunostaining was done. CENP-H localized to the entire nucleus of HCT116 48 hours after transfection of the CENP-H expression plasmid (Fig. 4A, a and d, arrowhead). FISH analysis with CEP8 and CEP12 centromere probes showed that many of the cells overexpressing CENP-H have abnormal chromosomal numbers (Fig. 4A, a and c, arrowhead), whereas untransfected cells are normal diploids (arrow). The number of centromeric signals for chromosomes 8 and 12 of at least 200 cells were counted and compared with about 6% of vector-transfected cells (Fig. 4B). We have previously reported that CENP-A is overexpressed in colorectal cancer tissues (22) and ectopic expression of CENP-A might also induce aneuploidy. Thus, the CENP-A expression plasmid was also introduced into HCT116 cells to examine if aneuploidy occurs. Although CENP-A localized to the entire nucleus of HCT116 as in the case of CENP-H 48 hours after transfection (data not shown), overexpression of CENP-A showed only a marginal effect for chromosomal number compared with CENP-H (Fig. 4B). These results indicate that the induction of aneuploidy is most prominent in CENP-H.
overexpressed cells. Note that the abnormal number of chromosomes is not due to an increase in apoptosis, because the viability of the CENP-H– or CENP-A–transfected and vector-transfected cells was identical (data not shown) and few fragmented nuclei were found in transfected cells (Fig. 4A).

What is the mechanism of the aneuploidy in cells overexpressing CENP-H? A recent report showed that overexpression of CENP-A in HeLa cells results in its incorporation into the entire chromosome, although mistargeting of CENP-A did not significantly alter the normal pattern of chromosome segregation (25). To investigate where the kinetochore proteins are localized in HCT116 cells overexpressing CENP-A or CENP-H, mitotic chromosomes of transfected HCT116 cells were prepared and stained with anti–CENP-A and CENP-H antibodies. Consistent with the previous report, CENP-A was localized to the entire chromosome, whereas CENP-H was localized to the centromere in CENP-A-overexpressing cells (Fig. 5D and E). In great contrast, CENP-H had disappeared from the centromere of metaphase chromosomes prepared from CENP-H-overexpressing cells, as if it were the mitotic chromosome of CENP-H–depleted cells (Fig. 5F). This disappearance of CENP-H was not refractory to poor quality of anti–CENP-H antibody or of experimental procedure because the centromeres of untransfected nuclei in the same field were clearly stained with the anti–CENP-H antibody (data not shown). In addition, failure of detecting CENP-H in its overexpressing cells was not refractory to the unavailability of the antibodies to the centromere of metaphase chromosomes because CENP-A was able to be detected on the centromere of the same chromosome (Fig. 5F). One explanation for the disappearance of CENP-H is the depletion of some limiting factors that recruit it to centromeres. CENP-A has been reported to associate with CENP-H (19, 20, 23), suggesting a candidate recruiting factor. Thus, CENP-A was cotransfected with CENP-H in HCT116 cells. In the cotransfected cells, CENP-H failed to localize to centromeres of mitotic chromosome indicating that CENP-A cannot recruit CENP-H to centromeres (data not shown).

To confirm the disappearance of CENP-H from the centromere in CENP-H–overexpressed cells, stable transfectant cell lines overexpressing CENP-H were generated using a Tet-Off gene expression system. MEF/3T3 Tet-Off cell line that expresses the tetracycline-controlled transactivator was stably transfected with the CENP-H expression plasmid under the control of the tetracycline-responsive promoter so that CENP-H was expressed only in the absence but not in the presence of doxycyclin (Fig. 6A, lanes 2 and 3). Then, localization of CENP-H was investigated in the stable transfectant using anti-centromere (ANA serum) and anti–CENP-H antibodies. In the presence of doxycyclin or untransfected control cells, CENP-H localized to the centromere either in interphase nuclei or in the mitotic chromosomes (Fig. 6B, b–d and C, a–c). In the absence of doxycyclin, however, CENP-H did not localize to the centromere in interphase nuclei and it disappeared from the centromere of metaphase chromosomes (Fig. 6B, f–h and C, d–f). Furthermore, aberrant micronuclei were

Figure 2. CENP-H gene expression is up-regulated in colorectal cancer. A, examples of mRNA levels in tumor (T) and adjacent normal tissue (N). The intensity of each band was measured with NIH Image and the relative mean CENP-H mRNA levels between tumor and normal tissue normalized with GAPDH mRNA levels were calculated. B, comparison of CENP-H mRNA levels between tumors and adjacent normal tissues of 15 cases by real-time quantitative RT-PCR.
more frequently observed in CENP-H overexpressed cells (Fig. 6B, e; 18.0%) than in control cells (Fig. 6B, a; 6.2%). These results suggest that overexpression of CENP-H induces aneuploidy probably due to chromosome missegregation caused by aberrant localization of CENP-H.

Discussion

An abnormal or heterogeneous chromosome number in a cancer cell could result from catastrophic mitosis during tumor evolution. There are many potential mitotic targets (e.g., centrosome/microtubule formation, kinetochore function, chromosome condensation, sister-chromatid cohesion, and checkpoints; ref. 1), all of which are indispensable for the proper segregation of chromosomes. Although impaired kinetochore function is a potential causative factor for CIN, no direct evidence has been reported thus far. In this article, we provided the first evidence that overexpression of a kinetochore protein, frequently observed in primary colorectal cancers, induces aberrant mitosis in either diploid colorectal cancer cells or in normal mouse fibroblasts. In both cases, CENP-H disappeared from centromere, which might be the cause of the mitotic defect. These results provide new insight for understanding CIN in colorectal cancers. Our observation that CENP-H was overexpressed not only in polyploid tumors but in near diploid tumors (ref. 22; Fig. 1) indicates that overexpression of CENP-H was not fully associated with aneuploidy in vivo. Thus, some other factors might be needed to induce aneuploidy in primary colorectal cancer. Another possibility is that these diploid tumors might have chromosome structural changes that cannot be detected by FISH. Therefore, additional techniques such as comparative genomic hybridization or flow cytometric analysis might be useful to find small structural changes of chromosome (26).

The localization of centromere proteins in the cells overexpressing them is somewhat intriguing. CENP-A localized to the entire chromosome in the HCT116 cells overexpressing it. This result is consistent with the previous report by Van Hooser et al. that overexpression of CENP-A in HeLa cells results in its incorporation into the entire chromosome (25). They also showed that CENP-A overexpression causes a subset of centromere-kinetochore components to be recruited to noncentromeric regions of the chromatin, which creates a prekinetochore complex. In contrast, when CENP-H was overexpressed in HCT116 cells, it completely disappeared from the centromere of mitotic chromosomes as though CENP-H was depleted. One explanation for this disappearance is the depletion of some limiting factors that recruit CENP-H to centromeres. Thus, CENP-H itself is unable to localize to the centromeres and cause
aneuploidy. This is similar to the “squelching” which is a common mechanism to repress transcription by sequestering limiting components required for transcriptional activation away from promoters (27). Transcription factors generally activate transcription with coactivators. However, if excess of a transcription factor exist, it would sterically prevent coactivator recruitment to a promoter and thus transcription is repressed. Similarly, an excess of CENP-H would also sterically prevent a recruitment factor to deliver it to centromeres. It remains to be elucidated which factors recruit CENP-H to the centromere. These results suggest that the dosage of kinetochore proteins should be tightly regulated for their appropriate localization and the proper kinetochore function. This notion is also supported by the previous observation that when the yeast homologue of CENP-C, MIF2, is overexpressed, cells suffered increased missegregation of chromosomes during mitosis (28).

There is still much debate over the timing of CIN in the colorectal tumorigenic process. CIN has currently been postulated to be the first event for tumorigenesis. It creates loss of heterozygosity, which results in the activation of oncogenes and/or inactivation of
tumor suppressor genes (1). In fact, CIN was identified in the earliest stages of tumorigenesis (29). Thus, it is interesting to examine if overexpression of centromere proteins occurs in early stages of tumor development. Recent findings suggest that the mutation in a tumor suppressor gene APC, which is believed to be an initial event for colorectal tumorigenesis, is a potential initiator of CIN (11–13). This hypothesis came from the observation that APC localizes to the ends of microtubules embedded in kinetochores and a single truncating mutation in APC, similar to mutations found in tumor cells, was shown to have defect in microtubule plus-end attachments and to cause a dramatic increase in mitotic abnormalities. This inefficient microtubule attachment to kinetochore might be related to overproduction of some kinetochore proteins, such as CENP-H, which creates imbalanced kintochore and leads to aneuploidy. Further investigations are needed to understand the mechanism of abnormal expression of kinetochore proteins in colorectal cancers and how it contributes to tumor development.

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