Characterization of a Novel Kinetochore Protein, CENP-H*

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Naoko Sugata‡, Eisuke Munekata‡, and Kazuo Todokoro‡

From the ¥TuskuLife Science Center, The Institute of Physical and Chemical Research (RIKEN), 3-1, Kovadai, Tsukuba, Ibaraki 305-0074, Japan and the ¥Institute of Applied Biochemistry, Tsukuba University, 1-1, Tennoudai, Tsukuba, Ibaraki 305-8572, Japan.

Macromolecular centromere–kinetochore complex plays a critical role in sister chromatid separation, but its complete protein composition as well as its precise dynamic function during mitosis has not yet been clearly determined. Here we report the isolation of a novel mouse kinetochore protein, CENP-H. The CENP-H, with an apparent molecular mass of 33 kDa, was found to contain a coiled-coil structure and a nucleolar localization signal. The CENP-H transcripts were relatively scarce but were detectable in most tissues and embryos at various stages of development. Immunofluorescence stainings of mouse fibroblast cells with anti-CENP-H-specific antibody demonstrated that the CENP-H is specifically and constitutively localized in kinetochores throughout the cell cycle; this was also confirmed by stainings with anti-centromere-specific antibody. Thus the newly isolated CENP-H may play a role in kinetochore organization and function throughout the cell cycle.

A replicated chromosome possesses two discrete complex macromolecular assemblies, kinetochores, which are positioned on opposite sides of the centromere region of replicated chromosomes. Recent studies have identified several kinetochore proteins that have a pivotal role in centromere structure, kinetochore formation, and sister chromatid separation (1–5). However, the complete protein composition of the kinetochore and the precise dynamic function of centromere–kinetochore complex during mitosis have not yet been clearly determined.

The centromeric heterochromatin between kinetochores contain a number of α-satellite DNA, CENP-B (6), and an inner centromere protein INCENP (7) that might be involved in maintaining sister-chromatid cohesion. This region also appears to contain mitotic centromere–associated kinesin (MCAK)1 (8), which has been shown in Xenopus extracts to be required for spindle formation and maintenance (9). Immunoelectron microscopic analyses revealed that the kinetochores consist of four structurally differentiated domains: inner plate, interzone, outer plate, and fibrous corona. The inner plate is the region associated with the centromeric heterochromatin and contains CENP-C (10), which is required for the maintenance of a functional kinetochore, and CENP-G (11). The zone between the inner and outer plates (the interzone) contains a phosphorylated protein, of which phosphopeptide–epitope can be recognized by 5F3/2 antibody and which has been proposed to regulate metaphase–anaphase transition through the spindle checkpoint (12). MCAK might also be localized in this region. Kinetochore microtubule plus-ends attach to the outer plate, which has been reported to contain CENP-F (13), CENP-E (14–16), ZW10 (17), possibly cytoplasmic dynein, and its associated dynactin complex (18). The fibrous corona extends from the outer plate, which exists only on unattached kinetochores, and contains CENP-E (18), ZW10 (17) and cytoplasmic dynein (18). The cytoplasmic dynein may be involved in microtubule attachment and poleward force production (18). Unattached but not fully attached kinetochores also contain Mad family and Bub family, which are known to play important roles in regulating the spindle assembly checkpoint (19–27). We report here the isolation of an additional kinetochore protein called CENP-H, which is constitutively localized in kinetochores and thus may function in kinetochore organization and function.

EXPERIMENTAL PROCEDURES

Cloning of CENP-H cDNA—Poly(A)+ mRNA was purified from erythrocytopenic-responsive mouse erythroleukemia SKT6 cells (28), and the cDNA library was constructed in AzipLor™ phage (Life Technologies, Inc.). The digoxigenin-labeled 330 bp CENP-H cDNA, which was incidentally obtained by differential display, was used for screening the library. Four positive clones were isolated from 5 × 108 plaques, and their sequences were determined. The 5' end of the CENP-H transcript was determined by 5' rapid amplification of cDNA ends (RACE) methods, following the manufacturer's instructions (Life Technologies, Inc.). The cDNAs for 5'-RACE were synthesized by SuperScript™ II (Life Technologies, Inc.) at 50 °C for 30 min or by ThermoScript™ (Life Technologies, Inc.) for 60 min at 55 °C with a primer CCGAAGTGCAACTGAAA. The primers used for PCR were GCACAGCCCGCTTC-TCT or TCCATTTGCAAGGCCCGCTAGGTT, both of which resulted in the isolation of the identical cDNA.

Preparation of Anti-CENP-H Antibody—The CENP-H cDNA encoding amino acid residues 27 to 241 was cloned into pGEX-2T (Amersham Pharmacia Biotech). The GST-fused CENP-H was expressed in XL1-Blue MRF* with 0.1 mM isopropyl β-D-thiogalactopyranoside and isolated by affinity chromatography on glutathione-Sepharose beads according to the manufacturer's protocol (Amersham Pharmacia Biotech). The purified GST fusion protein was used to immunize a rabbit. Antibody was affinity-purified with GST-CENP-H fusion protein coupled to HiTrap NHS-activated Sepharose (Amersham Pharmacia Biotech).

Quantification of CENP-H Transcripts in Various Tissues—Semi-quantitative reverse transcriptase PCR of various mouse tissues and embryos at various stages of development was conducted using Mouse Rapid-Scan™ Panel (OriGene) containing cDNAs of 4-log ranged diluted cDNAs. The primers used were: CACGTTGACCTCCTGGGATAACA and TAGCGTGTTGAGGTCCTTCT corresponding to 405–425 and 800–820 bp of CENP-H cDNA. The PCR (30 cycles) was carried at 94 °C for 30 s, 62 °C for 1 min, and 72 °C for 2 min using 0.4 μM primer per reaction.

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† To whom correspondence should be addressed. Tel.: +81 298 36 9075; Fax: +81 298 36 9090; E-mail: todokoro@rtc.riken.go.jp.
‡ The abbreviations used are: MCAK, mitotic centromere-associated kinesin; bp, base pair(s); 5'-RACE, 5' rapid amplification of cDNA ends; DAPI, 4,6-diamidino-2-phenylindole.

PCR, polymerase chain reaction; PIPES, 1,4-piperazinediethanesulfonic acid; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; DAPI, 4,6-diamidino-2-phenylindole.
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RESULTS

Structure of CENP-H—During the course of isolation of erythropoietin-inducible methods by differential display method in erythropoietin-responsive mouse SKT6 cells (28), a cDNA fragment encoding a novel kinetochore protein was incidentally isolated. We called it CENP-H because of its constitutive subcellular localization in kinetochores (see below). The full-length CENP-H cDNA was isolated from SKT6 cDNA library constructed in ZipLox™ phage using 330-bp cDNA fragment as a probe, and the translational initiation site was confirmed by 5′-RACE method using SuperScript II at 50 °C or ThermoScript™ at 60 °C. The sequence analysis of the isolated cDNA revealed that it encodes a novel protein. Fig. 1A shows the confirmed full-length mouse CENP-H sequence. The sequence surrounding the first ATG is in agreement with that of the translational initiation site (so-called Kozak consensus sequence) (30). The cDNA encodes a polypeptide of 241 amino acids, and its predicted molecular mass is 28,135 daltons. The predicted isoelectric point is 5.35. The encoded protein contains a coiled-coil structure (amino acids 28 to 187) and a nuclear localization signal RKKR (amino acids 152 to 155) (Fig. 1A, lane 3), indicating that it might be located in nucleus and bind to another protein through this coiled-coil structure.

Expression of CENP-H—Northern blot analysis showed CENP-H transcripts at 1.5 kilobases in SKT6 and many other hematopoietic cell lines (data not shown), but their expression levels were too low to exactly quantify by Northern blots. Therefore, to determine the transcriptional levels of mouse CENP-H gene in various tissues, a Mouse Rapid-Scan™ panel was used for the quantification (Fig. 2). The primers used were designed to amplify 415-bp fragment (405–820 bp), and the PCR was performed in a series of dilutions of cDNA, of which 1× contains 2.5 pg of cDNA (Fig. 2). Among 16 tissues and 4 embryos at different developmental stages, the most abundant transcript was found in embryos of day 9.5 (Fig. 2). The transcripts were detectable in various tissues except brain, heart, and adrenal gland. It was relatively abundantly expressed in thymus, spleen, uterus, ovary, testis, and muscle, but weakly expressed in small intestine, lung, and stomach. Expression in kidney, liver, skin, and prostate gland was barely detectable. In embryos, it was abundantly expressed between day 9.5 and day 12.5. One of the housekeeping enzymes glyceraldehyde-3-phosphate dehydrogenase was equally expressed in all tissues and embryos were examined (data not shown). The CENP-H transcripts could be detected in most tissues but they were relatively abundant in proliferating tissues, whereas the transcriptional levels might not reflect the protein level in a cell.

Subcellular Localization of CENP-H—To determine the subcellular localization of CENP-H, we performed indirect immunofluorescence microscopic analyses with the affinity purified anti-CENP-H antibody in NIH/3T3 cells. The CENP-H of 33 kDa was detected by the antibody in mouse NIH/3T3 fibroblast cells (data not shown). Because there exists a nuclear localization signal in CENP-H, it was expected to be localized in the nucleus. CENP-H was indeed found in the nucleus during interphase (Fig. 3, lane 1, top panel), and surprisingly, a number of paired CENP-H stainings on the chromosomes were clearly visible throughout the cell cycle (Fig. 3, lanes 1–6, top panels). The paired CENP-H stainings were always seen during mitosis: in prophase (Fig. 3, lane 2, top panel), prometaphase (Fig. 3, lane 3, top panel), metaphase (Fig. 3, lane 4, top panel), anaphase (Fig. 3, lane 5, top panel) and telophase (Fig. 3, lane 6, top panel). Double stainings of the cells with anti-α-tubulin antibody (Bioysa) and anti-CENP-H antibody (Fig. 4, lanes 1–5, top panels) together with anti-CENP-H antibody (Fig. 4, lanes 1–5, bottom panels) revealed that these paired dots on the chromosomes were centromeres/kinetochores of the sister chromatids (Fig. 4). In triple stainings (CENP-H, centromere, and DNA), the stainings of CENP-H completely overlapped with those of centromeres (Fig. 4, bottom panels). Therefore, we concluded that
the newly isolated cDNA encodes a novel kinetochore protein and thus designated it CENP-H.

**DISCUSSION**

In reporting this isolation of a novel kinetochore protein CENP-H, which is constitutively localized in kinetochores throughout the cell cycle, we have hypothesized that CENP-H may play a role in kinetochore organization and function. At least 5 proteins, CENP-A, CENP-B, CENP-C, CENP-D, and CENP-G, are known to be constitutively associated with kinetochores throughout the cell cycle. These proteins are thought to be involved in maintaining the structure of chromatides and kinetochores. In contrast, CENP-E, CENP-F, MCAK, Mad family, and Bub family, which are transiently associated with kinetochores during mitosis, are thought to have an important function in sister chromatid separation and/or spindle assembly checkpoint. Kalitsis et al. (31) recently reported, however, that mitotic chromosomes of CENP-C knockout mouse embryos displayed a scattered and highly condensed configuration and did not segregate in an ordered fashion, suggesting that some of these constitutively associated kinetochore proteins might also be indispensable not only for organizing kinetochores but also for acting in metaphase-anaphase transition.

The CENP-H was found to be a coiled-coil protein. Thus the possibility that CENP-H interacts with other kinetochore proteins is required study. The antibody we prepared could recognize human CENP-H in immunofluorescence stainings but not in immunoprecipitation or immunoblot. Thus we were unable to examine the interactions with the other known kinetochore proteins CENP-B and CENP-C, of which the available antibodies react only with human proteins. The identification of CENP-H binding proteins must await isolation of its human homologue and preparation of its specific antibody. Further studies are required to understand the detailed structure and dynamic biological functions of kinetochores during mitosis.

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