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Human CENP-H multimers colocalize with CENP-A and CENP-C at active centromere–kinetochore complexes

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Centromere and kinetochore proteins have a pivotal role in centromere structure, kinetochore formation and sister chromatid separation. However, the molecular architecture and the precise dynamic function of the centromere–kinetochore complex during mitosis remain poorly understood. Here we report the isolation and characterization of human CENP-H. Confocal microscopic analyses of HeLa cells with anti-human CENP-H-specific antibody demonstrated that CENP-H colocalizes with inner kinetochore plate proteins CENP-A and CENP-C in both interphase and metaphase. CENP-H was present outside centromeric heterochromatin, where CENP-B is localized, and inside the kinetochore corona, where CENP-E is localized during prometaphase. Furthermore, CENP-H was detected at neocentromeres, but not at inactive centromeres in stable dicentric chromosomes. *In vitro* binding assays of human CENP-H with centromere–kinetochore proteins suggest that the CENP-H binds to itself and MCAK, but not to CENP-A, CENP-B or CENP-C. CENP-H multimers were observed in cells in which both FLAG-tagged CENP-H and hemagglutinin-tagged CENP-H were expressed. These results suggest that CENP-H multimers localize constitutively to the inner kinetochore plate and play an important fundamental role in organization and function of the active human centromere–kinetochore complex.

INTRODUCTION

Recent studies have identified an increasing number of kinetochore proteins, many of which play a pivotal role in centromere structure, kinetochore formation and sister chromatid separation (1–6). Immunoelectron microscopic analyses revealed that the centromere–kinetochore complex consists of up to five structurally differentiated domains, starting with the centromeric heterochromatin between kinetochores, the inner plate, interzone, outer plate and, finally (in cells with depolymerized microtubules), the fibrous corona. However, the molecular architecture and the precise dynamic function of the various proteins that make up the centromere–kinetochore complex during mitosis remain poorly understood (7–9).

Kinetochore proteins can be divided into two groups: facultative kinetochore proteins, which are localized in kinetochores transiently only during mitosis, and constitutive kinetochore proteins, which are found at centromeric foci throughout the cell cycle. Facultative kinetochore proteins include the regulatory factors of the spindle assembly checkpoint, such as the MAD and BUB families, which are localized preferentially at kinetochores that have not yet achieved a bipolar orientation (6). These factors were found to have a conserved primary structure and physiological function from yeast to human. Mitotic centromere-associated kinesin (MCAK) is present in the centromeric heterochromatin from prophase to telophase, possibly overlapping with the inner kinetochore (10). Functional deficiency of MCAK results in abnormal chromosome segregation (11). Another microtubule motor protein, CENP-E, was recently proved to play a critical role in the spindle assembly checkpoint (12,13). Cytoplasmic dynein and the dynactin complex may be involved in microtubule attachment and/or chromosome segregation (14). CENP-E (15,16), CENP-F (17), possibly ZW10 (18), cytoplasmic dynein and its associated dynactin complex (19) are localized mainly in the fibrous corona. The functions of CENP-F and ZW10 during mitosis and chromosome segregation have not been clarified. Recently, human ZW10-interacting protein, Hzwint, was isolated and found to localize at active centromeres (20). A phosphorylated 3F3/2 antigen, which has been proposed to regulate the spindle assembly checkpoint, is transiently

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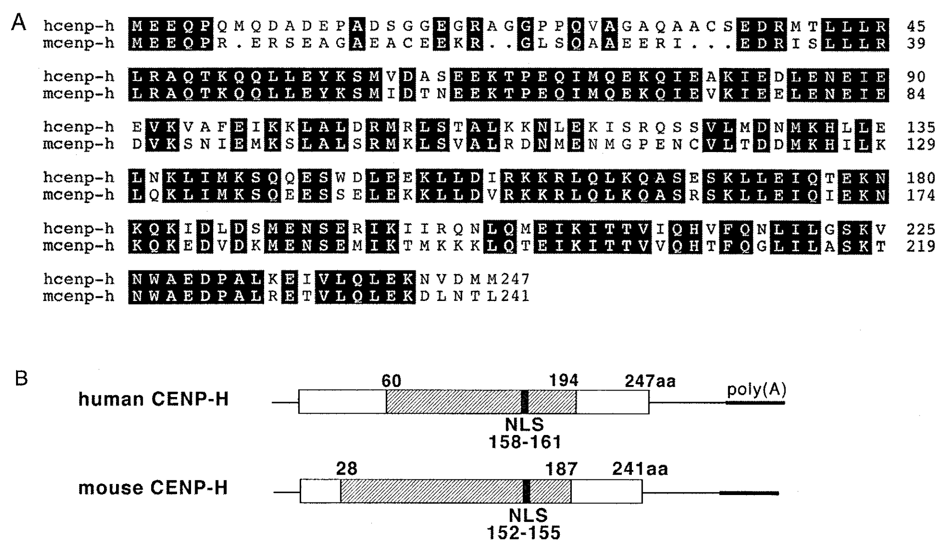

Figure 1. Primary structure of human CENP-H. (**A**) Amino acid sequence alignment of human CENP-H and mouse CENP-H. (**B**) Schematic presentation of human and mouse CENP-H. The shaded box indicates the predicted coiled-coil structure and the black box shows the putative NLS.

detected at the interzone between the inner and outer plates (21).

Only a limited number of kinetochore proteins have been isolated. The best characterized constitutive protein is CENP-B (22), which binds to a 17 bp motif found in human centromeric α-satellite DNA (23). CENP-B is not found at human neocentromeres, which do not contain α-satellite DNA, but is found at the inactive centromere in stable dicentric chromosomes, which contain α-satellite DNA (24). These findings, along with the normal viability of *CENP-B* null mice (25), suggests that CENP-B is not essential for centromere function but instead fulfills an as-yet unknown function on α-satellite DNA (26).

CENP-A is a centromere-specific histone-H3 homolog, which has been identified in all species examined except *Aspergillus thaliana* (27,28). CENP-A is concentrated in the inner kinetochore plate, whereas at normal human centromeres it was shown to be associated with α-satellite DNA-containing nucleosomes (29). However, its association with neocentromeres but not with inactive centromeres in dicentric chromosomes, suggests that localization of CENP-A to centromeres is not due simply to sequence specificity, but may represent an epigenetic phenomenon (30). CENP-C and its putative yeast homolog Mif2 are essential for proper chromosome segregation and maintenance of functional kinetochores (31,32) and deficiency of *CENP-C* leads to cell death (33). CENP-C is constitutively present in the inner kinetochore plate, which is closely associated with the centromeric heterochromatin (34). CENP-G is also localized at the inner plate and its cDNA has not yet been isolated (35).

Recently we reported the isolation of a mouse centromere–kinetochore protein CENP-H (36). However, the exact localization of CENP-H in the centromere–kinetochore complex and the identity of CENP-H-interacting proteins have not yet been determined. We therefore isolated human CENP-H cDNA in order to determine the precise localization and the binding partners of human CENP-H in the centromere–kinetochore complex. Confocal microscopic analyses and *in vitro* and *in vivo* binding assays revealed that CENP-H may form multimers that are localized to the inner plate of kinetochores. Furthermore, we found that CENP-H is present constitutively at normal centromeres and is found at neocentromeres but not at inactive centromeres in stable dicentric chromosomes. These results indicate that CENP-H is one of the fundamental components of the human active centromere–kinetochore complex.

RESULTS

Isolation of human CENP-H

We isolated several independent human *CENP-H* cDNAs from a HeLa cell cDNA library. All clones had identical coding sequences with various lengths of 3′ non-coding regions. The 5′ end of human *CENP-H* cDNA was confirmed by the 5′ rapid amplification of cDNA ends (RACE) method and the translational initiation site was consistent with the Kozak consensus sequence. Figure 1 shows the confirmed amino acid sequence of human CENP-H, aligned with that of mouse, which we reported previously (36). The full-length human *CENP-H* cDNA encoded a polypeptide of 247 amino acids with a predicted molecular mass of 28 135 Da. Human CENP-H contains a predicted coiled-coil structure (amino acids 60–194) and a putative nuclear localization signal RKKR (amino acids 158–161). Human CENP-H has only 67% amino acid sequence identity and 75% amino acid similarity with mouse CENP-H (Fig. 1A). The cDNA of human CENP-H encoded a polypeptide 6 amino acids longer than that of mouse. The differences between these two species, including deletions and amino acid substitutions, were most prevalent in the N-terminal 40 amino acids (Fig. 1A). The predicted coiled-coil structure of mouse CENP-H was 25 amino acids longer than that of human CENP-H (Fig. 1B).

Colocalization of CENP-H with CENP-A and CENP-C

We prepared an affinity-purified anti-human CENP-H-specific rat antibody. In addition, the previously described anti-mouse CENP-H rabbit antibody could also specifically stain the centromere–kinetochore regions of human chromosomes in HeLa cells (data not shown). Immunofluorescence staining of HeLa cells with the affinity purified rat anti-human CENP-H antibody clearly showed that human CENP-H was localized in the centromere–kinetochore regions throughout the cell cycle and formed paired CENP-H signals at each centromere during mitosis (data not shown).

To determine more detailed localization of human CENP-H in the centromere–kinetochore complex, we performed confocal microscopic analyses with the anti-human CENP-H-specific antibody together with antibodies to human CENP-A, CENP-B, CENP-C or CENP-E in HeLa cells.

Double staining of the HeLa cells with anti-CENP-H antibody together with anti-CENP-B antibody demonstrated that the paired dots of CENP-H staining flanked the centromeric heterochromatin region where CENP-B was clearly seen during prometaphase/metaphase (Fig. 2A). During interphase, CENP-H did not completely colocalize with CENP-B, but was closely associated with it (Fig. 2B). In chromosome spreads, CENP-H was also seen as a pair of dots flanking the CENP-B-containing region of the centromere–kinetochore complex.

The inner kinetochore plate is closely associated with centromeric heterochromatin and has been reported to contain CENP-A and CENP-C (30,34). Thus, we next performed immunofluorescence staining of the cells with anti-CENP-H antibody together with anti-CENP-A or anti-CENP-C. The paired dots of CENP-H and CENP-A staining showed a complete overlap (Fig. 2A) on the sister chromatids during prometaphase/metaphase and throughout mitosis (data not shown). During interphase, CENP-H was also completely colocalized with both CENP-A and CENP-C (Fig. 2B).

To confirm the location of CENP-H, we performed an indirect immunofluorescence staining of HeLa cells with anti-CENP-H together with anti-CENP-E at various stages of mitosis. CENP-E has been reported to be present in the outer plate and/or fibrous corona (15,16). We confirmed the previous findings that CENP-E is not associated with chromosomes during G_1 and early S phase and is localized at kinetochores during prometaphase and metaphase and at mitotic spindles and the midbody during anaphase and telophase (Fig. 3). We found that CENP-H localized at kinetochores throughout the cell cycle, whereas it was slightly displaced from CENP-E during prometaphase and metaphase (Fig. 3).

These results indicate that throughout the cell cycle CENP-H is localized outside centromeric heterochromatin regions where CENP-B is present and inside the fibrous corona where CENP-E is localized. Thus, CENP-H is most probably localized in the inner plate together with CENP-A and CENP-C.

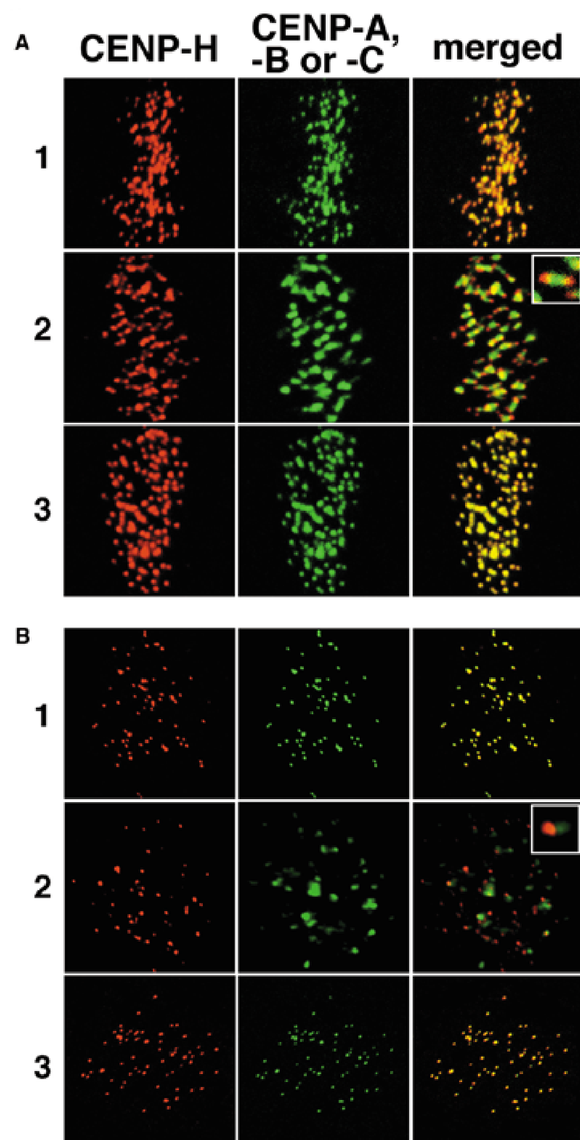

Figure 2. CENP-H colocalizes with CENP-A and CENP-C. HeLa cells in prometaphase/metaphase (**A**) or interphase (**B**) were stained with the purified anti-human CENP-H-specific rat antibody (left and right panels; shown in red), together with anti-human CENP-A (row 1, middle and right panels; shown in green), anti-human CENP-B (row 2, middle and right panels; shown in green) or anti-human CENP-C (row 3, middle and right panels; shown in green) antibody. The right panels show merged images. Enlargements are shown in the inset. Cy3-conjugated anti-rat or FITC-conjugated anti-mouse or rabbit IgG was used as a secondary antibody.

CENP-H associates specifically with active kinetochores

In order to address the functional significance of CENP-H at centromeres, its presence was assayed on two different types of human centromere variant, neocentromeres and stable dicentric chromosomes. Two independent neocentromere-containing derivatives of chromosome 13, previously described by Warburton *et al.* (37), were assayed for the presence of CENP-H (Fig. 4). Fluorescence *in situ* hybridization (FISH) with a chromosome 13 painting probe identified the neocentromere-containing chromosomes, as well as the normal and/or derivative chromosomes 13, in each cell. Simultaneous immunofluorescence using rat anti-human CENP-H antibodies demonstrated the presence of CENP-H on the invdup 13 (q21-qter) chromosome containing a neocentromere in 13q21 (Fig. 4A) and on the invdup 13 (q31-qter) chromosome containing a neocentromere in 13q31 (Fig. 4B). These results confirm that CENP-H binds to active centromeres even if they lack canonical centromeric DNA sequences.

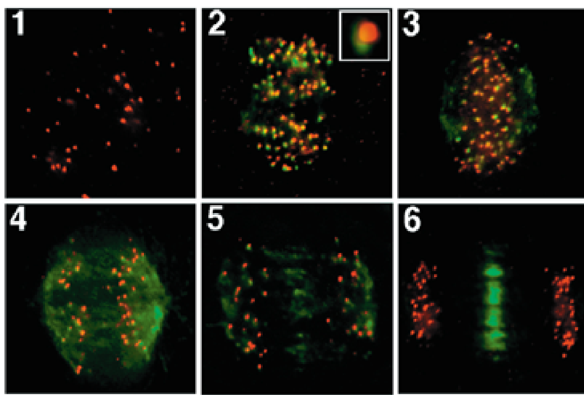

Figure 3. Localization of CENP-H and CENP-E during mitosis. HeLa cells were stained with anti-human CENP-H antibody (red) and with anti-human CENP-E antibody (green) at various stages of mitosis. (**1**) interphase; (**2**) prometaphase; (**3**) metaphase; (**4**) anaphase A; (**5**) anaphase B and (**6**) telophase. An enlargement of the centromere is shown in the inset.

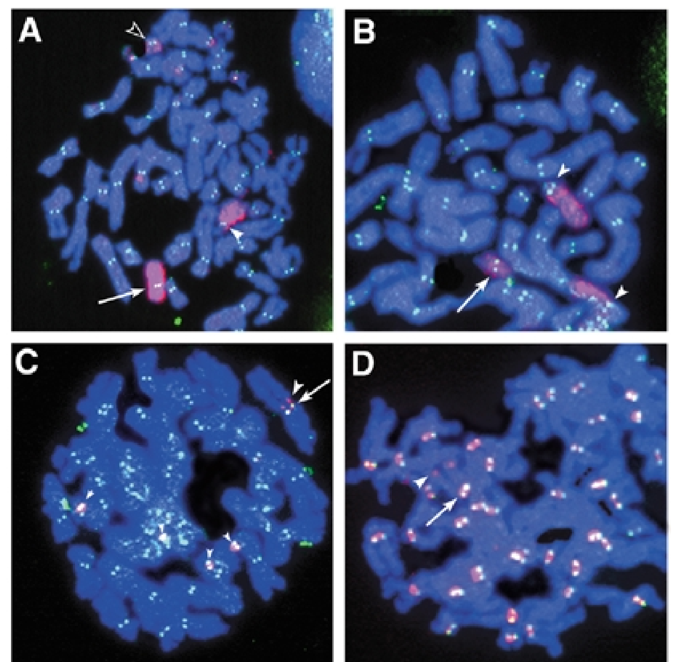

Figure 4. CENP-H is associated with neocentromeres and with the active centromere in dicentric chromosomes. (**A**) CENP-H (green) is present on invdup 13 (q21-qter), which contains a neocentromere in 13q21 (white arrow). Chromosome 13 paint (red) identifies the neocentromere-containing chromosome (white arrow), the normal chromosome 13 (white arrowhead) and the derivative chromosome 13 ring (13pter-q21) (open arrowhead). (**B**) CENP-H is present on invdup 13 (q31-qter), which contains a neocentromere in 13q31 (white arrow). Chromosome 13 paint (red) identifies the neocentromere-containing chromosome (white arrow), and the two normal chromosomes 13 (white arrowheads). (**C**) CENP-H (green) is present specifically on the active centromere (white arrow) of a dicentric RT14q15q chromosome. Chromosome 14 and 15 α-satellite DNA probes (red) hybridize to the active (white arrow) and inactive (white arrowhead) centromeres on the RT14q15q chromosome, as well as normal chromosomes 14, 15 and 22 (smaller white arrowheads). (**D**) CENP-H (green) is present specifically at the active centromere (white arrow) of a dicentric (18q) chromosome. CENP-B (red) is seen at both the paired active (white arrow) and separated inactive (white arrowhead) centromeres. Chromosomes are counterstained with DAPI (blue).

Two independent stable dicentric chromosomes were assayed for the presence of CENP-H at their active and inactive centromeres (Fig. 4). The first was a stable dicentric Robertsonian Translocation chromosome (RT14q15q) that contains an active centromere from chromosome 14 and an inactive centromere from chromosome 15 (30,38). FISH using similarly labeled α-satellite DNA probes from both chromosomes 14 and 15 identified the two closely opposed centromeres on the RT14q15q chromosome (Fig. 4C). Simultaneous immunofluorescence with rat anti-human CENP-H antibodies shows the presence of CENP-H specifically on the active but not on the inactive centromere in this chromosome (Fig. 4C). Similar analysis with either the chromosome 14 or chromosome 15 α-satellite DNA probe confirmed the specific colocalization of CENP-H with the chromosome 14-derived active centromere (data not shown). Note in Figure 4C that CENP-H colocalized with the four additional α-satellite DNA FISH signals in the cell, which includes the normal chromosome 15 and chromosome 14 centromeres, as well as the two chromosome 22 centromeres (which cross-hybridize with the chromosome 14 probe). Thus, CENP-H does colocalize with the α-satellite DNA on the normal chromosome 15 centromere, but does not colocalize with the α-satellite DNA at the inactive chromosome 15 centromere in the dicentric chromosome.

The second dicentric chromosome analyzed consisted of a fusion of the long arms of chromosome 18, resulting in a dicentric (18q) chromosome with two well spaced centromeres, both containing chromosome 18 α-satellite DNA. In Figure 4D, this dicentric chromosome was visualized by immunofluorescence with rabbit anti-human CENP-B antibodies, which are known to be found at both active and inactive centromeres (24). CENP-H was seen to colocalize with only the active centromere on the dicentric chromosome. Similar results were obtained using a chromosome 18-specific α-satellite DNA FISH probe (data not shown). Note that the sister chromatids have separated at the inactive centromere but remain attached at the active centromere (Fig. 4D). The localization of CENP-H to neocentromeres but not to inactive centromeres in dicentric chromosomes demonstrates the association of CENP-H specifically with active centromere/kinetochore complexes.

CENP-H binds to itself *in vitro*

Since CENP-H was found to localize at or near the inner kinetochore plate, we examined the possible interactions of CENP-H with the kinetochore proteins that are present around inner plate, including CENP-A, CENP-B, CENP-C, MCAK and CENP-H itself, by using *in vitro* binding assays. An equal amount of glutathione *S*-transferase (GST)–CENP-H in the resin was mixed with *in vitro* translated ^{35}S-labeled CENP-A, CENP-B, the N-terminal half (amino acids 1–484) or the C-terminal half of CENP-C (amino acids 380–944), MCAK or CENP-H, and the proteins bound to GST–CENP-H were separated by SDS–PAGE. We could not produce *in vitro* translated full-length CENP-C due to its large size (107 kDa), and therefore, the possible interactions of CENP-H with the N-terminal half of CENP-C and C-terminal halves of CENP-C were examined here.

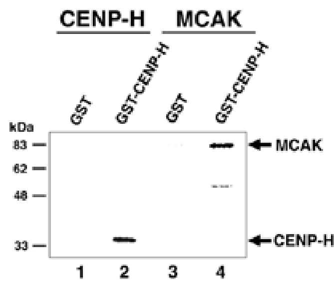

Figure 5. *In vitro* binding assay of GST–CENP-H with CENP-H and MCAK. GST alone (lanes 1 and 3) or GST–CENP-H (lanes 2 and 4) bound to the resin was mixed with *in vitro* translated [^{35}S]CENP-H (lanes 1 and 2) or [^{35}S]MCAK (lanes 2 and 4). The proteins bound to GST–CENP-H were separated by 10% SDS–PAGE and visualized by the imaging analyzer.

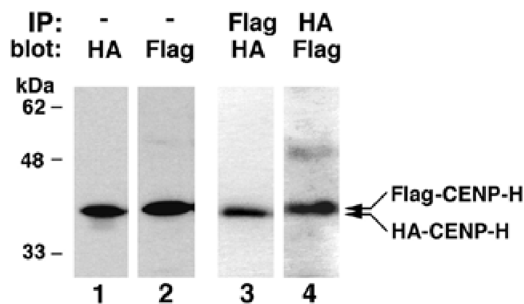

Figure 6. CENP-H forms a homo-oligomer *in vivo*. FLAG-tagged CENP-H and HA-tagged CENP-H were expressed in HeLa cells and the cell extracts were immunoblotted with anti-HA antibody (lane 1) or with anti-FLAG antibody (lane 2). The immunoprecipitates with anti-FLAG antibody was immunoblotted with anti-HA antibody (lane 3) and the immunoprecipitate with anti-HA antibody was blotted with anti-FLAG antibody (lane 4).

The labeled CENP-A, CENP-B and either fragment of CENP-C did not bind to GST–CENP-H (data not shown), but the labeled CENP-H and MCAK (Fig. 5) could clearly bind to the resin. Neither CENP-H nor MCAK bound to GST alone (Fig. 5) and thus the binding of CENP-H and MCAK to GST–CENP-H was specific at least *in vitro*. Both CENP-H and MCAK contain a predicted coiled-coil structure and thus they could bind each other through these motifs. These results indicate that CENP-H may complex with itself and/or MCAK at least *in vitro*.

CENP-H forms multimers *in vivo*

To confirm the homotypic association of CENP-H *in vivo*, both FLAG-tagged CENP-H and hemagglutinin (HA)-tagged CENP-H were transiently expressed in HeLa cells and immunoprecipitation and immunoblot analysis were performed. Expression of approximately equal amounts of FLAG-tagged and HA-tagged CENP-H in the cells was confirmed by immunoblots with anti-FLAG and anti-HA antibodies (Fig. 6). FLAG-tagged CENP-H (39 kDa) migrated a little more slowly than HA-tagged CENP-H (38 kDa) and both migrated much more slowly than intact CENP-H (34 kDa). We could clearly detect HA-tagged CENP-H in the immunoprecipitates of anti-FLAG antibody (Fig. 6). Conversely, FLAG-tagged CENP-H was included in the immunoprecipitates with anti-HA antibody (Fig. 6), indicating that the tagged CENP-H molecules interact with each other *in vivo*.

We failed to express sufficient amounts of HA- or FLAG-tagged MCAK for detection by immunoprecipitation and by immunoblots in these cells, possibly due to a cell cycle arrest induced by MCAK expression (11) and, therefore, we could not examine the possible interaction of CENP-H with MCAK *in vivo*.

DISCUSSION

Here we report the isolation of the cDNA encoding the human homolog of mouse CENP-H, which we recently isolated (36). Surprisingly, human and mouse CENP-H share only 67% amino acid sequence identity. As demonstrated in this paper, CENP-H was found to be a component of active centromere–kinetochore complexes in mammals. However, we could not find any CENP-H homologs in the genomic sequence databases of *Caenorhabiditis elegans, Drosophilia melanogaster, A.thaliana, Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*. Thus, it is not clear whether CENP-H represents a mammalian-specific component of the active centromere–kinetochore complex, or whether functional homologs with limited sequence homology exist in other species. Some components of the centromere–kinetochore complex such as CENP-A and Bub1 were found to have conserved primary amino acid sequences as well as conserved function from yeast to human (39,40). On the other hand, CENP-C, of which homologs have been identified in budding yeast, mammals and plants, conserves only a limited sequence homology over a 23 amino acid long region (41). The homolog of the *Saccharomyces cerevisiae* CBF3 component, Cep3p, has not been identified in other species including mammals. ZW10 homologs have been found in *C.elegans*, *D.melanogaster*, *A.thaliana* and mammals, but not in the yeasts. Together, these observations may indicate that the functions of some centromere–kinetochore proteins are common in most eukaryotes, but that the primary sequences of these proteins are not always highly conserved.

Here we report evidence for both *in vitro* and *in vivo* multimerization of human CENP-H. The homodimerization of transient kinetochore proteins such as MCAK and HZwint had previously been suggested by size fractionation of intact proteins or yeast two-hybrid method (11,20). In addition, it has been described that both CENP-B and CENP-C also form homodimers (42,43). Heterochromatin protein 1 (HP-1), which is localized in heterochromatin regions of active centromeres, was recently proved to form homodimers through its chromo shadow domain (44). It was found that homodimerization of HP-1 was essential for forming complexes with other chromatin-binding proteins and presumably for its gene-silencing ability, although its effect on kinetochore function has not been clarified. The role of multimerization of CENP-H in the function of the centromere–kinetochore complex has yet to be determined.

We investigated the localization of CENP-H in the centromere–kinetochore complex by comparing it with several centromere proteins of known localization, using confocal

immunofluorescence microscopic analyses. Our observations showed that CENP-H was located at either end of the domain of α-satellite-rich centromeric heterochromatin where CENP-B was localized. CENP-H colocalized throughout the cell cycle with both CENP-A and CENP-C, which are found in the inner kinetochore plate. Furthermore, CENP-H was seen to be slightly displaced from CENP-E, which is present in the fibrous corona. Therefore, our light microscopy observations are most consistent with a localization of CENP-H at or near the inner kinetochore plate.

To date, four of the five known mammalian constitutive kinetochore proteins, CENP-A, CENP-C, CENP-G and CENP-H, have been localized to the inner kinetochore plate (30,34,35) whereas the fifth, CENP-B, is found in the underlying centromeric heterochromatin (45). Thus, this ‘prekinetochore’ structure (46) exists throughout the cell cycle, marking the location of the centromere on the chromosome. The prekinetochore contains CENP-A-containing nucleosomes, the additional DNA-binding protein CENP-C, and at least two other proteins, CENP-G and CENP-H. At mitosis, this structure condenses into the inner kinetochore plate, and the outer kinetochore plates, which appear to be composed of the facultative centromere proteins, are assembled onto it. Presumably, the inner plate and its DNA-binding proteins serve to anchor the outer plate mitotic machinery to the chromosome.

An *in vitro* binding assay also suggested that CENP-H interacts with another kinetochore protein, MCAK, which is one of the few facultative kinetochore proteins found in the centromeric heterochromatin and inner kinetochore (10). The interaction between CENP-H and MCAK observed here may serve during mitosis to strengthen the anchoring of the kinetochore inner plate to the chromosome. It seems likely that additional interactions between inner kinetochore plate proteins and the outer kinetochore plate will be identified.

Here we report the association of CENP-H specifically with active kinetochores. CENP-H is present at two independent neocentromeres and absent from two independent inactive centromeres. Immunofluorescence analysis of these two complementary classes of human centromere variants permits evaluation of the centromeric activity and functional significance of centromere proteins as they are described (30,47). By far, the majority of normal kinetochore proteins are found at neocentromeres but not at inactive centromeres, leading to the suggestion that kinetochore assembly, once established, proceeds in a coordinated fashion (48). Interestingly, MCAK has been reported to weakly associate with inactive centromeres (49).

In contrast to the vast majority of facultative kinetochore proteins, only three of the five constitutive centromere proteins, CENP-A, CENP-C and CENP–H, have been associated specifically with active kinetochores (30,38). CENP-B binds to α-satellite DNA that contains its binding site, regardless of whether or not this DNA is located at active centromeres (26). Recently, CENP-G has been shown to localize to two independent neocentromeres, but remarkably was also found at one of two inactive centromeres (50). CENP-G may associate specifically with the α1 family of α-satellite DNA, even when it is present at inactive centromeres, yet may also localize to active kinetochores lacking these sequences through protein interactions. Identification and characterization of kinetochore proteins that are not specifically associated with active kinetochores, for example not found at neocentromeres (CENP-B) (24), or that persist at inactive centromeres (MCAK, CENP-B and CENP-G), should provide insight into DNA–protein and protein–protein interactions that are important for centromere formation. The role of the constitutive centromere proteins in establishing and propagating centromere location through multiple rounds of DNA replication and cell division remains one of the critical questions in understanding centromere formation and function.

In order to clarify the structure and function of the centromere–kinetochore completely, it will be necessary to identify all of the centromere–kinetochore proteins from a number of key species. Systematic large-scale studies using genomic DNA databases and DNA chips, which could determine the expression during the cell cycle, together with the detection of interacting proteins and immunofluorescence microscopic analyses, should eventually identify the all of the components of diverse centromere–kinetochore complexes in various species.

MATERIALS AND METHODS

Isolation of human *CENP-H* cDNA

Human *CENP-H* cDNAs were isolated from a HeLa cell cDNA library in λUni-ZapTM XR (Stratagene, La Jolla, CA) using mouse full-length *CENP-H* cDNA (36) as a probe. The membranes were hybridized with the ^{32}P-labeled probe in 6× SSC, 10× Denhardt’s solution, 0.5% SDS and 20 µg/ml denatured salmon sperm single strand DNA at 55°C for 19 h and washed twice with 0.2× SSC, 0.1% SDS at 53°C for 15 min. The sequences of the isolated cDNAs were determined by ABI310 sequence analyzer. (The GenBank accession number of full-length human CENP-H is AB035124.)

The cDNA for 5′ RACE was synthesized by SuperScriptTM II (Life Technologies, Rockville, MD) at 42°C for 30 min with a primer 5′-AAAGTCTCATCCTGTCT-3′, and 5′ RACE was performed with a primer 5′-CTTCACTTGCATCAACCA-3′.

Preparation of antibodies

The human *CENP-H* cDNA encoding amino acid residues 2–205 was cloned into pGEX-4T-2. GST-fused human CENP-H was expressed in *Escherichia coli* ER2566 cells (New England Biolabs, Beverly, MA) with 0.1 mM isopropyl β-D-thiogalactopyranoside for 2 h and solubilized with phosphate-buffered saline (PBS) containing 1% Triton X-100, then isolated by affinity chromatography on glutathione–Sepharose beads according to the manufacturer’s protocol (Amersham Pharmacia Biotech, Little Chalfont, UK). The purified GST–CENP-H was injected into lymph nodes of WKY/NCrj rats (Charles River Japan, Yokohama, Japan). The polyclonal rat antibody was affinity-purified with GST–CENP-H coupled to HiTrap NHS-activated Sepharose (Amersham Pharmacia Biotech). Human CENP-A peptide (amino acids 3–20) and human CENP-B (amino acids 160–234) were used as antigens for preparation of anti-human CENP-A monoclonal mouse antibody and anti-human CENP-B monoclonal mouse antibody (2D8D8), respectively. Anti-human CENP-C rabbit polyclonal antibody (R554) was prepared using essentially full-length

human CENP-C as antigen (51). Anti-human CENP-E mouse monoclonal antibody 177 was described as previously (52).

Immunofluorescence staining of cells

HeLa cells on coverslips were washed twice with PHEM (60 mM PIPES, 25 mM HEPES, pH 6.9 with KOH, 10 mM EGTA, 2 mM $MgCl_2$) at room temperature and lyzed with PHEM containing 0.1% Triton X-100 for 1 min at room temperature. The cells were fixed with methanol at –20°C for 5 min, immediately dried and washed briefly with PBS. The coverslips were then incubated with primary antibodies in (0.1 M PIPES–KOH pH 7.2, 1 mM $MgSO_4$, 1 mM EGTA, 1.83% L-lysine, 1% bovine serum albumin, 0.1% NaN_3) for 1 h at 37°C. Primary antibodies used were: rat anti-human CENP-H polyclonal antibody (1:150), anti-human CENP-A antibody (1:150), mouse anti-human CENP-B monoclonal antibody (1:30) and rabbit anti-human CENP-C polyclonal antibody (1:500). After washing with PBS, the coverslips were incubated with the secondary antibody; Cy3-conjugated anti-rat antibody (1:2500; Jackson ImmunoResearch, West Grove, PA), fluorescein isothiocyanate (FITC)-conjugated anti-rabbit (1:500; Jackson ImmunoResearch), or Alexa TM 488 anti-mouse antibody (1:500; Molecular Probes, Eugene, OR) and 1 µg/ml 4,6-diamidino-2-phenylindole (DAPI) for 45 min at 37°C. The coverslips were then washed with PBS for 3 min three times and mounted with PBS containing 90% glycerol and 1,4-para-phenylene diamine. Samples were observed with a confocal laser scan microscope (LMS 510; Carl Zeiss, Oberkochen, Germany).

FISH and immunofluorescence analysis of human variant centromere cell lines

The patient-derived immortalized lymphoblast neocentromere cell lines 13a [invdup 13 (q21-qter)] and 13e [inv dup 13 (q31-qter)] were described previously (37). The immortalized lymphoblast RT14q15q cell line 8828 was described previously (38). Lymphoblasts were cultured in RPMI 10% fetal calf serum (FCS). The primary fibroblast dicentric 18q cell line 0236JY, kindly provided by Dr Dorothy Warburton (Columbia-Presbyterian Medical Center, New York, NY), was cultured in Ham's F10 10% FCS. FISH and immunofluorescence shown in Figure 4 were performed on unfixed aqueous cytospin preparations using a Shandon Cytospin III, essentially as described (53). Rat anti-human CENP-H was detected with goat anti-rat–FITC (Sigma, St Louis, MO). Chromosome 13 paint was Alu-PCR amplified from genomic DNA from somatic cell hybrid RJ387.58Tl containing human chromosome 13 as its only human component (54) and directly labeled by nick translation with dUTP-SpectrumOrange (Vysis, Downers Grove, IL). Biotinylated chromosome 14 and 15 α-satellite DNA were detected with strepavidin–TRITC. Rabbit anti-human CENP-B was detected with goat anti-rabbit–TRITC (Sigma). Images were captured on a Nikon E-800 microscope equipped with a Sony DKC 5000 CCD camera and imported into Adobe Photoshop.

In vitro binding assay

The full-length human *CENP-A*, *CENP-C* and *MCAK* cDNAs were isolated by RT–PCR from human erythroleukemia K-562 cells or HeLa cells and subcloned into pBluescript KS(–). The *CENP-B* cDNA was inserted into pET3d. *In vitro* transcription by T7 RNA polymerase and translation in reticulocyte lysate were carried out in a 25 µl reaction mix containing 1 µg of a linearized human *CENP-A*, *CENP-B*, *CENP-C* (N- or C-terminal half), *CENP-H* or *MCAK* cDNA in each vector and [^{35}S]methionine (Amersham Pharmacia Biotech) following the manufacturer's instructions (Promega, Madison, WI). The GST–CENP-H was expressed in *E.coli* ER2566 cells with 0.1 mM isopropyl β-D-thiogalactopyranoside for 1 h at 37°C and lyzed with PBS containing 0.25% *N*-lauroyl sarcosine sodium salt. GST–CENP-H was purified by affinity chromatography with glutathione–Sepharose beads. The GST–CENP-H (10 µg) bound to the resin was incubated with ^{35}S-labeled proteins (10 µl) in the following binding buffer: 20 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40, 1 mM dithiothreitol, 1 µg/ml pepstatin A, 1 µg/ml aprotinin, 5 µg/ml leupeptin and 5 µg/ml Pefabloc SC (Roche Diagnostics, Indianapolis, IN) for 2 h at 4°C. The resins were then washed with the binding buffer five times at room temperature and bound proteins, which were separated by 10% SDS–PAGE, were visualized using an imaging analyzer (BAS 2500; Fujifilm, Tokyo, Japan).

In vivo binding assay

FLAG- and HA-tagged human *CENP-H* cDNAs in pcDNA3.1 were co-transfected into HeLa cells. Transfectants were harvested 24 h later and sonicated in lysis buffer (20 mM HEPES pH 7.4, 1 mM EDTA, 0.1 mM EGTA, 2 mM $MgCl_2$, 1 mM $NaVO_4$, 20 mM NaF, 150 mM NaCl, 5% glycerol, 1% NP-40, 1 µg/ml pepstatin A, 1 µg/ml aprotinin, 5 µg/ml leupeptin and 5 µg/ml Pefabloc SC). FLAG- and HA-tagged CENP-H were immunoprecipitated by anti-FLAG M2 monoclonal antibody (Sigma) and anti-HA rat monoclonal antibody (Roche Diagnostics), respectively, and immunoblotted with the antibodies.

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