CENP-B Interacts with CENP-C Domains Containing Mif2 Regions Responsible for Centromere Localization*

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Recently, human artificial chromosomes featuring functional centromeres have been generated efficiently from naked synthetic alphoid DNA containing CENP-B boxes as a de novo mechanism in a human cultured cell line, but not from the synthetic alphoid DNA only containing mutations within CENP-B boxes, indicating that CENP-B has some functions in assembling centromere/kinetochore components on alphoid DNA. To investigate whether any interactions exist between CENP-B and the other centromere proteins, we screened a cDNA library by yeast two-hybrid analysis. An interaction between CENP-B and CENP-C was detected, and the CENP-C domains required were determined to overlap with three Mif2 homologous regions, which were also revealed to be involved in the CENP-C assembly of centromeres by expression of truncated polypeptides in cultured cells. Overproduction of truncated CENP-B containing no CENP-C interaction domains caused abnormal duplication of CENP-C domains at G2 and cell cycle delay at metaphase. These results suggest that the interaction between CENP-B and CENP-C may be involved in the correct assembly of CENP-C on alphoid DNA. In other words, a possible molecular linkage may exist between one of the kinetochore components and human centromere DNA through CENP-B/CENP-B box interaction.

The centromere is an essential region responsible for proper segregation of chromosomes. A pair of trilaminar structures called kinetochores are formed on the sides of centromeres in mitotic chromosomes, attach to spindle microtubules, and control the timing of sister chromatid dissociation. On human chromosomes, centromere/kinetochore-specific proteins, CENP-A, CENP-B, and CENP-C, were first identified with anti-centromere antibodies existing in autoimmune patient sera (1). These proteins exist at the centromere constitutively throughout the cell cycle. Recently, other constitutive centromere proteins, CENP-H, hMis6(CENP-I), and hMis12, have been identified (2–4). CENP-A has a histone fold domain similar to histone H3 at its carboxyl terminus and a unique amino-terminal region (5). It is co-purified with nucleosomes, and an in vitro assembly analysis indicated that histone H3 could be replaced with CENP-A in these structures (6, 7). Thus, CENP-A is a centromere-specific histone H3 found to be conserved in S. cerevisiae, Schizosaccharomyces pombe, Caenorhabditis elegans, Drosophila melanogaster, mice, and humans (8–13). CENP-A knockout mouse embryos die before day 6.5. In CENP-A-depleted mouse cells, CENP-C can be detected on whole nuclei and CENP-B localization becomes diffuse (14). CENP-A is present on active centromeres and neocentromere regions, but not on inactive centromeres or on dicentric chromosomes (15, 16), and it is therefore thought to be a marker for functional centromeres/kinetochores.

Electron microscopy has demonstrated that CENP-C exists at the inner plate of the kinetochore (17). Microinjection of an anti-CENP-C antibody into cells resulted in cell cycle arrest at the G2/M phase and a reduction in the kinetochore size (18). CENP-C, conserved in C. elegans, chickens, mice, and humans, has similarity with Mif2, a homologue in budding yeast, in its central and carboxyl-terminal domains (19–24). CENP-C has been reported to have DNA binding activity in its central region, but this has no sequence specificity (25, 26). Two centromere-targeting regions have been reported, one overlapping with the DNA binding domain and the other with the COOH-terminal region (26–28). CENP-C knockout mouse embryos lived only 3.5 days and suffered mortality as a result of mis-segregation of chromosomes (29). CENP-C-depleted nuclei from chicken DT-40 cells showed cell cycle arrest at the metaphase/anaphase transition and death by apoptosis, further indicating that CENP-C is essential for chromosome segregation and cell viability (21). In DT-40 cells, both CENP-H and the Mis6 homologue CENP-I were demonstrated to be necessary for the localization of CENP-C (30, 31). However, in HeLa cells, CENP-C was demonstrated to be localized at the kinetochore independent of the hMis12 and hMis6(CENP-I) assemblies (3). Thus, it is still unclear why these centromere-specific constitutive components assemble at α satellite regions of human chromosomes, except for CENP-B.

CENP-B binds to a specific 17-bp sequence called the CENP-B box in centromere-specific α satellite (alphoid) DNA in humans and in minor satellite DNA in mice, via its amino-terminal DNA binding domain and forms a homodimer with another carboxyl terminus (32–36). CENP-B exists at both active and inactive centromeres on dicentric chromosomes but does not exist at centromeres on the Y chromosome or neocentromeres (15, 37). CENP-B is highly conserved between mice...
The PCR product was cloned into pGADGH. To construct pGADCC728–943, pGADCC728–943 was digested with BalI and SacI, and cloned into BalI and SacI-treated pDsRED-C1 (Clontech). To construct pRFCC1–429 and pRFCC283–429, pGADCC1–429 and pGADCC283–429 were treated with EcoRI and ApaI and cloned into pDsRED-C1. To construct pRFCC1–727, pRFPCEN-C was treated with SpeI, ApaI, and T4 DNA polymerase, and the resultant fragment was self-ligated with T4 DNA ligase. To construct pRFPC400–727, pGADCC400–727 was treated with EcoRI and XhoI and cloned into pDsRED-C1. To construct pRFCC727–943, the PCR product of pCEN-C 728–943 was cloned into pDsRED-C1. To construct pRFCC1–283, pGADCCEN-P was treated with SpeI, T4 DNA polymerase, and cloned into pDsRED-C1.

CENP-B N-terminal polypeptide (CB1–180)-expressing plasmids were constructed with pMT161a (50) and pETCBN-160 (32). To construct pMTCENP-B, CB1672–1800 was amplified by pRFPCEN-P with 1672XK (5’-GGAGTACCTGACCTGATTCG-3’) and 1800B (5’-GGTGATCGCTTGGGTTGATC-3’) were annealed and the fragments were cloned into enzyme-treated pMID135. To construct pMTCB1–160c, pETCBN-C (32) was treated with XhoI and KpnI. For the KpnI-BamHI linker KB21 (5’-GTAGG-3’) and KB13 (5’-CTAGCCCTCCTC-3’) were annealed and cloned into pMID135 treated with XbaI and KpnI. pGADCC429 and pGADCC728 were treated with EcoRI and BamHI. For the T7 tag, two oligonucleotides (5’-GATTGACATGCGACTGCTGCTGCGCGATC-3’) and 5’-CTAGCCCTCCTCCTC-3’) were annealed and cloned into enzyme-treated pMID135.

**EXPERIMENTAL PROCEDURES**

**Cloning of CENP-α and CENP-C.** The CENP-α gene was cloned by PCR with two sets of primers for amplifying base pairs 1–159 and 160–2847. CENP-C was amplified from adult monkey (32), and genomic DNA of CENP-C was digested with SpeI and cloned into pGADGH. The PCR product was digested with SpeI and KpnI. For the KpnI-BamHI linker KB21 (5’-GTAGG-3’) and KB13 (5’-CTAGCCCTCCTC-3’) were annealed and cloned into pMID135 treated with XbaI and KpnI. pGADCC429 and pGADCC728 were treated with EcoRI and BamHI. For the T7 tag, two oligonucleotides (5’-GATTGACATGCGACTGCTGCTGCGCGATC-3’) and 5’-CTAGCCCTCCTCCTC-3’) were annealed and cloned into enzyme-treated pMID135.

**Construction of Plasmids.** pLexACB132–599, pLexACB132–556, pLexACB132–366, and pGADCB132–599 and pGADCB368–599 was described previously (32). To construct pLexACENP-C and pGAD-CENP-B, full-length CENP-B was cloned into NcoI site-generated pBTM116 (49) or NcoI site-generated pGADGH (Clontech). To construct pLexACB132–405 and pLexACB132–405, pLexACB132–399–405 was treated with SacI or Earl, T4 DNA polymerase, and EcoRI, and cloned into pBTM16 treated with BamHI, the Kloen fragment, and EcoRI. To construct pLexACB132–399–405, pLexACB132–405 was treated with T4 DNA polymerase, and EcoRI, and cloned into pCTGC1–277, pGADCCEN-P was treated with SpeI and cloned into pGADGH. To construct pGADCC1–605, pGADCC1–553, pGADCC1–429, and pGADCC1–283, pGADCCEN-P was treated with BglII, NsiI, PvuII, or SspI, and cloned into pCTGC1–277, pGADCC1–277, and pGADCC1–283. To construct pGADCC1–605, pGADCC1–553, pGADCC1–429, and pGADCC1–283, pGADCCEN-P was treated with BglII, NsiI, PvuII, or SspI, and cloned into pCTGC1–277, pGADCC1–277, and pGADCC1–283. To construct pGADCC1–283, pGADCC1–283 was amplified by pRFPCEN-P with CNPC10 against pGADCC728 and CNPC6 against pGADCC728. The PCR product was cloned into pGADGH. To construct pGADCC728–836, pGADCC728–836 was digested with BalI and SacI, and cloned into pDsRED-C1 (Clontech). To construct pRFCC1–429 and pRFCC283–429, pGADCC1–429 and pGADCC283–429 were treated with EcoRI and ApaI and cloned into pDsRED-C1. To construct pRFCC1–727, pRFPCEN-C was treated with SpeI, ApaI, and T4 DNA polymerase, and the resultant fragment was self-ligated with T4 DNA ligase. To construct pRFPC400–727, pGADCC400–727 was treated with EcoRI and XhoI and cloned into pDsRED-C1.

**Examination of Yeast Transformations.** Yeast cells corresponding to 5 × 10⁶ transformants were plated onto Yc/UTL plates (Tryp/-His/-Ura/-Lys/-Leu) containing 50 mg/mL 5-aminotriazole as screen for transformation activation of the HIS3 gene at 30°C, yeasts were isolated and streaked on Yc/UTL plates (Tryp/-His/-Ura/-Leu) to obtain single colonies. Three colonies were analyzed using a β-galactosidase filter assay.

**Isolation of HeLa Nuclei and Preparation of Soluble Chromatin.** 90% confluent HeLa cells were washed twice with PBS and once with isolation buffer (3.75 mM Tris·HCl, pH 8.0, 0.05 mM spermine, 0.125 mM spermidine, 0.5 mM EDTA, 20 mM KCl, 0.1 mg/mL phenylmethylsulfonyl fluoride, 0.5 mM DTT, 40 μM z-Leu-Leu-Leu-H aldehyde (Peptide Institute, Japan)). Isolation buffer containing 0.1% digitonin (Wako) was added with incubation on ice for 10 min. Cells were collected with a cell scraper, centrifuged at 4 °C for 5 min at 1,000 rpm, and resuspended in wash buffer (20 mM HEPES, pH 8.0, 0.5 mM EDTA, 20 mM KCl, 0.1 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT, 40 μM z-Leu-Leu-Leu-H aldehyde) at 1 × 10⁶ nuclei/mL and lysed by 3 cycles of 5°C for 5 min at 1,000 rpm with an RL-131 microcentrifuge. Cells were pelleted and suspended on Yc/UTL plates (Tryp/-His/-Ura/-Leu) to obtain single colonies. Three colonies were analyzed using a β-galactosidase filter assay.

**REFERENCES**

1 The abbreviations used are: PBS, phosphate-buffered saline; DTT, dithiothreitol; RFP, red fluorescent protein; DAPI, 4',6-diamidino-2-phenylindole; BSA, bovine serum albumin.
at 37 °C. The enzyme reaction was stopped by adding EDTA to 2 mM, and the supernatant suspension was recovered by centrifugation at 4 °C for 5 min at 8,000 rpm. After precipitation in buffer A containing 1 mM EDTA and sonication with a Sonifier 450 (Branson), the supernatant was recovered by centrifugation at 4 °C for 5 min at 8,000 rpm.

**Immunoprecipitation with Anti-CENP Antibodies**—Aliquots of 150 μl of soluble chromatin were transferred to siliconized tubes and mixed with 150 μl of IP buffer (55 mM Hepes, pH 8.0, 600 mM NaCl, 4 mM MgCl₂, 4 mM ATP, 1.5 mM aprotinin, 10 mM leupeptin, 1 mM DTT, 40 mM z-Leu-Leu-Leu-H aldehyde, 0.2% Nonidet P-40). Antibodies against CENP-A, -B, and -C were added, and samples were rotated at 4 °C for 16 h. Protein G-Sepharose (Amersham Biosciences) or Dynabeads M-280 sheep anti-mouse IgG (Dynal) were washed twice with IP buffer (50 mM Hepes, pH 8.0, 300 mM NaCl, 2 mM MgCl₂, 2 mM ATP, 1.5 mM aprotinin, 10 mM leupeptin, 1 mM DTT, 40 mM z-Leu-Leu-Leu-H aldehyde, 0.1% Nonidet P-40), blocked with IP buffer containing 5% BSA at 4 °C for 30 min, added to samples, and rotated for 1 h. Sepharose was collected by centrifugation at 2,000 rpm for 1 min and washed five times with IP buffer. The magnetic Dynabeads were collected with Dynal MPC-M (Dynal) for 1 min and washed five times with IP buffer. Beads were suspended in SDS sample buffer and boiled for 5 min before electrophoresis.

**In Vitro Transcription and Translation of CENP-B and CENP-C Polypeptides**—Polypeptides of full-size CENP-B (CB1–599), CB1–160, or CENP-C were generated in vitro transcription and translation using pMTCEB-B, pMTCEB-CB1–160 or pMTCEC-C, respectively, in a Tnt-coupled wheat germ extract system (Promega) according to the instructions from the manufacturer. To generate and solubilize polypeptides of CENP-B and CB1–160, linearized plasmid DNAs (0.25 μg) and annealed 29-bp oligonucleotide DNA containing a CENP-B box (CB29) (54) were added to 25 μl of the reaction lysate. Ten μl of each polypeptide mixture was mixed with IP (150 mM Hepes, pH 8.0, 150 mM NaCl, 2 mM MgCl₂, 2 mM ATP, 1.5 mM aprotinin, 10 mM leupeptin, 1 mM DTT, 40 mM z-Leu-Leu-Leu-H aldehyde, 0.1% Nonidet P-40), blocked with IP buffer containing 5% BSA at 4 °C for 30 min, added to samples, and rotated for 1 h. Sepharose was collected by centrifugation at 2,000 rpm for 1 min and washed five times with IP buffer. The magnetic Dynabeads were collected with Dynal MPC-M (Dynal) for 1 min and washed five times with IP buffer. Beads were suspended in SDS sample buffer and boiled for 5 min before electrophoresis.

**Isolation of Cell Lines Expressing CENP-B Polypeptides**—To establish cell lines expressing the CENP-B N-terminal polypeptide (CB1–160), CB1–160–expressing cell lines were transfected with pMTCEB-CB1–160 and the supernatant suspension was recovered by centrifugation at 8,000 rpm.

**Cell Cycle Analysis of Cells Expressing CENP-B Polypeptides**—For synchronized, CB1–160-expressing cells were treated with 2.5 mM thymidine for 20 h, washed twice, and incubated for 10 h in the absence of the drug. Cells were then incubated in the presence of 5 μg/ml aphidicolin for 20 h. After its removal, 0.6 μM cadmium chloride was added to the medium. Cells were harvested every 1 and 4 h, fixed onto coverslips, and stained with DAPI, and then the mitotic cells were counted. To analyze the cell cycle of CB1–581-expressing cell lines, 293S cells were transfected with pMTCEB-CB1–581 and pMTCEB-B polypeptides by co-preparation with calcium phosphate, and resistant colonies were selected with 400 μg/ml Geneticin.

**Transfection of Expression Plasmids and Indirect Immunofluorescent Staining**—To express RFP-CENP-C deletion mutants, tetON HeLa (Clontech) cells were grown on poly-l-lysine-coated coverslips and transfected with Polyfect transfection reagent (Qiagen) using the protocol from the manufacturer for HeLa cells. Cells were fixed with 75% acetone/methanol at −20 °C for 30 min. The cells were then fixed again with peridate-lysine-paraformaldehyde at 4 °C for 30 min, and finally washed three times in PBS for 5 min. Monoclonal anti-CENP-B (2D8D8), monoclonal anti-T7 tag (Novagen) or guinea pig polyclonal anti-CENP-C antibodies were applied to the coverslips for 1 h at 37 °C, followed by washing three times in PBS for 5 min each. Fluorescein isothiocyanate- or rhodamine-conjugated anti-mouse IgG or anti-guinea pig IgG antibodies in PBS containing 0.2% BSA and 0.2% skim milk were then applied to the coverslips for 1 h at 37 °C. After washing three times in PBS for 5 min each, the coverslips were stained with 1 μg/ml DAPI in PBS for 5 min and rinsed again in PBS. Samples were finally mounted and analyzed using a Zeiss microscope equipped with a Photon TCSPC cooled CCD camera (DX1, 1400) and a time image analysis system (IPLab, Signal Analytics Corp.). In Figs. 8–10, images were taken at 0.2-μm z-steps using Zeiss microscope equipped with a cooled CCD camera (MicroMax, Princeton Instruments) coupled to MetaMorph (Universal Imaging) and used to produce deconvolved reconstructions of the image stacks with AutoDeblur (AutoQuant Imaging). Images are presented as maximum intensity projections. In Figs. 7B and 9B, z-stack images were processed with HazeBuster (VayTek).

**Antibodies**—The anti-CENP-A antibody was generated as described previously (47, 51). To prepare anti-CENP-C antibodies, CENP-C 630–943 polypeptides were purified by the inclusion body method, and then fractionated in a 13% polyacrylamide gel including 0.1% SDS with the Prep Cell System (Bio-Rad). They were then used to immunize rabbits, guinea pigs, and mice. Immunoglobulin G fractions were obtained using protein A-Sepharose (Amersham Biosciences) for the rabbit serum (CRa1) and the guinea pig serum (CGP2), and protein G-Sepharose (Amersham Biosciences) for the mouse monoclonal antibody. To prepare monoclonal anti-CENP-B antibodies, mice were immunized with purified CENP-B polypeptides and then the immunoglobulin G fractions were obtained with protein G-Sepharose. To prepare anti-CENP-B antibody against CENP-B COOH-terminal region (BC1), rabbits were immunized with purified CENP-B541–599 polypeptide. The CENP-B polyclonal antibody, BN1, was generated as described previously (32)

**RESULTS**

**Detection of CENP-B and CENP-C Interactions Using a Yeast Two-hybrid System**—Using a mammalian artificial chromosome assay, de novo centromere assembly occurred with the alpheid DNA depending on the existence of functional CENP-B boxes. Therefore, we analyzed possible interactions between CENP-B and centromere proteins using a yeast two-hybrid system. We introduced bait plasmids encoding a LexA DNA binding domain fused with the CENP-B central and COOH-terminal domains (LexAB132–599) into yeast L40 cells. By two-hybrid screening from a prey plasmid encoding a GAL4 activation domain fused with a mouse cDNA library, a truncated mouse CENP-C containing amino acids 671–907 was obtained (Fig. 1A). We confirmed that the yeast cells expressing the GAL4 activation domain fused with a human CENP-C (GADCENP-C) could grow as well as positive controls express-
CENP-B Interacts with CENP-C Mif2 Regions

Fig. 2. Two-hybrid mapping of the CENP-C interacting domains in CENP-B. Figure is a schematic representation of the CENP-B constructs and the results of β-galactosidase assays and colony growth on the selective media. Numbers indicate the amino acid residues of CENP-B encoded by the bait or prey plasmids.

CENP-B binds to CENP-B boxes in alphoid DNA via an N-terminal 125 amino acid domain, and therefore the CB1–160 peptide also specifically binds to chromosomal alphoid DNA containing CENP-B boxes (32, 34, 36; see Fig. 8). If the complex formation including CENP-B and CENP-C were mediated only by a presumptive independent interaction of CENP-C to alphoid DNA, then CB1–160, which also bound to the CENP-B box, must have been co-immunoprecipitated with CENP-C as well as endogenous CENP-B or tagged full-length CENP-B (CB1–599). CB1–599 also co-immunoprecipitated with CENP-C as well as endogenous CENP-B, whereas CB1–160 did not (Fig. 3B). Moreover, the amount of co-immunoprecipitated endogenous CENP-B was decreased when CB1–160 was overproduced. Thus, CB1–160 may compete with endogenous CENP-B for binding to the CENP-B box. These results indicate that the interaction between CENP-B and CENP-C does indeed exist and was lost without the CENP-C interaction domain in CENP-B in cells.

Direct interaction between CENP-B and CENP-C was then confirmed with polypeptides generated by in vitro transcription and translation (Fig. 3C). CENP-C and CB1–160 (lane 1) or CB1–599 (lane 2) were, respectively, mixed and immunoprecipitated with anti-CENP-C antibody (lanes 5 and 6) or control IgG (lanes 3 and 4). CB1–599 co-immunoprecipitated with CENP-C, whereas CB1–160 did not (Fig. 3C). All these results as well as the results from the two-hybrid analyses indicate the possible molecular interaction between one of the kinetochore components, CENP-C, and the human centromere DNA-binding protein CENP-B.

CENP-C Mif2 Homologous Regions Are Involved in CENP-B Interactions—To determine the interaction domain of CENP-C, we generated deletion mutants for the two-hybrid analysis. Interaction between CB132–599 and CENP-C (CC728–943) was identified (Figs. 1A and 4). The latter domain contains two regions that are highly conserved with S. cerevisiae Mif2 (20). We therefore generated truncated mutants deleting either region of Mif2. None of the resultant polypeptides, CC728–886,
CC728–786, CC886–943, or CC787–943, interacted with CENP-B on two-hybrid analysis (Fig. 4). Unexpectedly, however, CC1–727 lacking both of the two COOH-terminal Mif2 blocks did show some interaction activity. Analysis of deletion constructs from the COOH-terminal region showed that CC1–429 and CC283–429, but not CC1–283, could interact (Fig. 4). Therefore, CC283–429 containing another Mif2 region (block1) also had the capacity to interact with CENP-B. Weak β-galactosidase activity units and growth on selective plates (Fig. 4). These results indicate that CENP-C interacts with CENP-B through two regions, 283–429 and 727–943, both of which contain Mif2 homologous regions.

Mi2 Regions of CENP-C Are Involved in Its Localization to Centromeres—In previous reports, centromere targeting of CENP-C was indicated to be the result of the central region (amino acids 478–537) by Yang et al. (26) and the carboxy-terminal region (amino acids 584–943) by Lanini and McKeon (27). Full-length and truncated CENP-C polypeptides fused with RFP were transiently expressed in HeLa cells to analyze their localization (Fig. 5 and Table I). RFP-CENP-C (full-length) was detected at the centromere (97.4% of cells), and abnormal localization was only observed at a very low frequency (2.6% of cells) (Fig. 5 and Table I). Truncated CENP-C, RFPCC1–727, RFPCC728–943, and RFPCC1–429 containing the CENP-B interaction domains were detected at the centromere like full-length CENP-C, although abnormal localizations of RFPCC1–429 to nucleoli was more frequent at 5.8% or to whole nuclei at 2.3%. RFPCC1–283 containing no CENP-B interaction domain did not show any signal (Table I), whereas without fusion (RFP only) showed whole cell staining of 100% of cells. RFPCC283–429 was observed at centromeres and nu-
cleared together (Fig. 5 and Table I). RFPCC430–727 was localized at centromeres with a very low frequency (0.5%) and in the whole nucleus in 95% cells, consistent with an earlier report for CENP-C 421–591 (28). The localization signals of truncated CENP-C polypeptides at the centromere drastically decreased with replacement of the RFP tag with a FLAG tag (data not shown). Thus, the stability of truncated CENP-C polypeptides may be influenced by the tagged sequences. RFP without the fusion protein showed no specific affinity for the centromere, and thus the RFP tag is very effective for CENP-C detection.

These results indicate that the localization of CENP-C at the centromere mainly depends on the domains containing the three Mif2 homologous regions, although a weak centromere localization activity exists in 430–727.

Overproduction of a Truncated CENP-B Polypeptide Lacking the CENP-C Interaction Domain Affects Cell Cycle Progression at Mitosis—To examine whether the existence of CENP-B lacking the CENP-C interaction domain on the centromeric alphoid locus affected the assembly of the centromere structure or cell cycle progression, we induced overproduction of the CENP-B N-terminal polypeptide (CB1–160) in a stable HeLa cell line (HBN160-E cells). Within 24 h after induction, a 5–10-fold excess of the CB1–160 polypeptide was detected with the anti-CENP-B antibody, as compared with the amount of endogenous CENP-B (Fig. 6A). When the cells were synchronized in the G0/S phase using drugs, and then the number of mitotic cells was counted with every 4 h (Fig. 6B) or 1 h (data not shown) after simultaneous release from the G0/S block and induction of CB1–160 expression, the majority of tested cells, both normal HeLa and HBN160-E cells, entered mitosis within 12 h–13 h. After 16 h, the mitotic index decreased in control cells, but HBN160-E cells still showed a high rate (Fig. 6B). Similar results were obtained when the induction of CB1–160 expression was started before the G0/S block release, indicating that CB1–160-overproducing cells progressed through the cell cycle normally from the G0/S phase to the beginning of mitosis, but then took longer to exit.

To further analyze details of the mitotic state of the CB1–160-overproducing cells, we observed random cultured HBN160-E cells at 2-h intervals. Five of the HBN160-E cells in the uninduced state (arrowheads) in this microscopic field changed their shape to round mitotic cells and 2 h later, all finished cell division and became flat (Fig. 6C). However, many of the HBN160-E cells in the induced state for CB1–160 overproduction took over 2 h to complete cell division (Fig. 6C, small arrowheads) and two cells stayed in mitosis for 8–10 h (Fig. 6C, arrows). The chromosome DNA alignments of these two cells indicated that one cell was arrested at prometaphase (Fig. 6C, inset, lower cell) and the other was arrested just before metaphase because of the existence of a few chromosomes apart from the metaphase plate (Fig. 6C, inset, large arrowheads). Indeed, the mean mitotic period of individual dividing cells was prolonged to 71.7 min (n = 22) in the CB1–160-overproducing case, compared with 55.2 min (n = 24) for cells without induction, as estimated by time lapse analysis, and 9.1% of the cells were arrested for longer than 6 h (Fig. 7A). These results indicate that overproduction of CB1–160 causes cell cycle arrest or at least a delay between prometaphase and metaphase.

Microtubule and Kinetochore Interactions become More Unstable in the CB1–160-overproducing Cells—In metaphase-arrested HBN160-E cells, lagging or mal-oriented chromosomes was affected by the expression of CB1–160, we analyzed the sensitivity of the cells to the microtubule-depolymerizing drug, colcemid (Fig. 7A). Even without colcemid treatment, HBN160-E cells took a longer time (71.7 min) to complete mitosis than normal HeLa cells (47.0 min). On treatment with colcemid at a low concentration, 5 ng/ml, the average mitotic length of dividing HBN160-E cells was increased by almost 30 min (101.1 min) compared with cells without colcemid treatment, whereas that of normal HeLa cells increased by only 6.6 min (53.6 min). Moreover, ~10% of cells did not complete mitosis within 6 h of observation and this number increased in HBN160-E cells under the above conditions. Mono-oriented (mal-oriented) or lagging chromosomes were detected at a 4-fold higher rate (27% of cells) in CB1–160-overproducing cells than that in parental HeLa cells (7%) (Fig. 7B). Microtubule and kinetochore interactions thus presumably become unstable in the CB1–160-expressing state.

Duplication and Assembly of CENP-C and CENP-E at the Kinetochore Were Affected by Overproduction of CB1–160—To analyze whether the proper kinetochore structure was formed...
in CB1–160-overproducing cells, we observed the localization of the kinetochore components, CENP-C and CENP-E. In normal cells, CENP-B and CENP-C were found to co-localize at centromeres in nuclei through the G1 to S phases, the patterns for CENP-B and CENP-C overlapped each other and the sizes of each staining spot varied among the centromeres in a nucleus (Fig. 8A). The smaller (small arrows) or the larger (large arrows) sizes of CENP-C signals correlated with those of CENP-B signals (Fig. 8A). However, during the G2 phase, each centromere spot is duplicated (52). Duplication of the CENP-C spots was also observed at the G2 phase, and the compact and paired round dots of CENP-C became uniform among each centromere and located within the CENP-B-stained areas (Fig. 8A, arrowheads and insets). In the CB1–160-overproducing cells, although endogenous CENP-B detected with anti-CENP-B COOH-terminal region still existed at the centromeres overlapped with the signals of CB1–160, the signals of endogenous CENP-B were decreased and expanded to the broader areas as compared with those of CENP-B in non-CB1–160-producing cells (Fig. 8B). Thus, both the immunoprecipitation (Fig. 3B) and the cytological analyses indicated that CB1–160 competes well with endogenous CENP-B for binding to the CENP-B box. CENP-C existed at these centromeres as compact dots through the G1 to S phases (data not shown). However, in the G2 phase, CENP-C-positive structures were not compact or uniform but abnormally stretched and varied in size, and sometimes multiple dots appeared on a single centromere (Fig. 8C, insets). Such abnormal duplication of CENP-C was observed during

| RFP-fused CENP-C | Centromere | Centromere + nucleoli | Nucleoli | Whole nuclei | Whole cell |
|------------------|------------|-----------------------|----------|--------------|-----------|
| 1-943            | 97.4% (480/493) | 2.0% (10/493) | 0% (0/493) | 0.6% (3/493) | 0% (0/493) |
| 1-727            | 97.9% (549/560) | 0.4% (2/560) | 0% (0/560) | 1.6% (9/560) | 0% (0/560) |
| 1-429            | 89.3% (278/311) | 1.0% (3/311) | 5.8% (18/311) | 2.3% (7/311) | 1.6% (53/311) |
| 1-283            | ND         | ND                  | ND       | ND           | ND        |
| 283-429          | 0.5% (2/434) | 50.9% (221/434) | 42.0% (182/434) | 5.1% (22/434) | 1.6% (7/434) |
| 430-727          | 0.5% (2/388) | 0% (0/388) | 0% (0/388) | 98.0% (380/388) | 1.6% (6/388) |
| 728-943          | 95.4% (418/438) | 2.1% (9/438) | 0.2% (1/438) | 1.3% (6/438) | 0.9% (4/438) |
| RFP only         | 0% (0/102)   | 0% (0/102)   | 0% (0/102)   | 0% (0/102)   | 100% (102/102) |

**Table I**

Summary of the localization patterns of RFP-fused CENP-C

The number of cells with each specific localization pattern is shown, with the total number of analyzed cells in parentheses. ND means that no RFP signal was detected in the analyzed cells.

**Fig. 6.** HeLa transformants stably expressing CB1–160 show mitotic arrest. A, stable HeLa transformant HBN160-E cells were induced to express CB1–160. An antibody against the CENP-B N-terminal region, BN1 (left panel), and an anti-T7 tag antibody (right panel) were used to stain SDS-PAGE-separated whole cell lysates of HBN160-E cells, and normal HeLa cells for comparison. The expression of the CB1–160 polypeptide was induced for 0, 24, or 48 h. B, cell cycle progression of synchronized HBN160-E cells. Cells were synchronized at the G1/S phase at time 0, and then the inhibiting drug was removed and the expression of CB1–160 was induced. Numbers of mitotic cells were counted every 4 h. The black columns show the results for normal HeLa cells treated under the same conditions for the induction, and the open and shaded columns show those for HBN160-E cells not expressing and expressing the CB1–160 polypeptide, respectively. C, sequential observation of random cultured HBN160-E cells under expressing and non-expressing conditions by phase contrast microscopy at 2-h intervals from 12 h after induction of CB1–160 expression. Insets, phase contrast (left) and chromosome DNA (right) images of the two arrested HBN160-E cells indicated by arrows at 22 h after the induction of CB1–160 expression. In one cell, chromosomes apart from the metaphase plate are shown (arrowheads). Chromosome DNA in living cells was stained with Hoechst 33342.
the centromere duplication period in 70% of G2 phase cells overproducing CB1–160.

In the CB1–160-overproducing cells at prometaphase, CENP-C signals were also abnormal on mono-oriented or mal-oriented chromosomes (no CENP-C signal on lagging chromosome in Fig. 9A), in some cases being stretched and only one side of the paired centromere regions being detected by the anti-CENP-C antibody (data not shown). In normal HeLa cells at prometaphase, strong and compact dotlike CENP-E signals almost co-localized with, but were slightly peripheral to, the CENP-B signals. In mitosis-arrested HBN160-E cells, the majority of CENP-E signals on lagging (or mal-oriented) chromosomes were also abnormal. In some cases, a pair of CENP-E signals was both localized to one side of paired CENP-B signals and in other cases, CENP-E signals were enlarged or diffused (Fig. 9B, inset). In normal HeLa cells at late prometaphase, the intensity of CENP-E signals was much stronger than that of metaphase cells. In HBN160-E prometaphase cells containing lagging chromosomes, CENP-E signals on the lagging chromosomes were strong and abnormally large, whereas those on the chromosomes at the metaphase plates were almost undetectable (Fig. 9B). In normal HeLa cells, lagging chromosomes appeared at a lower rate (less than 5%), but we did not observe such abnormal localization of CENP-C and CENP-E on these lagging chromosomes. These results demonstrate that the duplication and assembly of CENP-C and CENP-E at the kinetochore were affected by overproduction of CB1–160.

A CENP-B Polypeptide Lacking Only the Homo-dimerization Domain Causes Cell Cycle Arrest in the G2 Period—CB1–160 includes neither the CENP-C interaction domain nor the homodimerization domain. Analyses of the effects of a CENP-B C-del polypeptide (CB1–561) lacking the homodimerization domain but containing that for CENP-C interaction were therefore performed. Despite repeated experiments, we could not obtain any stable transformants with inducible expression of CB1–561. Therefore, we analyzed cells transiently expressing CB1–561 in 293S cells. Compared with CB1–160, CB1–561 was localized in a larger region in nuclei forming aggregates. Strong or weak CENP-C multiple signals overlapped with and surrounded these abnormal assemblies of CB1–561 sites (Fig. 10A). However, non-overlapped CENP-C signals in variable sizes were also increased in far beyond the expected number of centromeres in whole nuclei in high level of CB1–561-expressing cells (Fig. 10A, lower). The excess of CB1–561 in the nuclei or the absence of the homodimerization domain at the alphoid sites caused the abnormal accumulation or the multiple nucleation of CENP-C sites. Next, we analyzed the cell cycle state of CB1–561-expressing cells by observation of samples stained with indirect immunofluorescence. At 1.5 days after the transfection, the proportion of late S-G2 cells was increased compared with normal or full-length CENP-B-expressing cells (data not shown). After 2.5 days, the numbers of late S-G2 cells were increased further and no metaphase cells were observed (Fig. 10B), indicating that expression of CB1–561 caused cell cycle arrest in the G2 period. Overproduction of both CB1–160 and CB1–561 affects the assembly or duplication of CENP-C in G2 and/or cell cycle progression in G2/M.

DISCUSSION

Assemblies of Kinetochore Proteins on Specific Alphoid DNA—CENP-A, -B, and -C exist on alphoid DNA at normal human centromeres throughout the cell cycle and were reported to be co-immunoprecipitable with the chromatin complex corresponding to the position of the tetra- to pentanucleosome units (51, 54). However, in CENP-B knockout mouse cells, functional structures of pre-existing kinetochores are maintained without CENP-B (41–43). No CENP-B was detected at the centromere on the Y chromosome or neocentromeres on fragmented chromosome arms lacking centromeric alphoid DNA (15, 37). It was also reported that mis-targeting CENP-A to non-centromeric regions by overexpression caused misassembly of CENP-C to the same ectopic loci, but without a function as centromeres (55). Thus, CENP-C is able to assem-
It is possible that the centromere is formed through other pathways than those involving interaction with CENP-B. Despite these discrepancies, CENP-B and CENP-B boxes still exist in centromeres of all normal human and mouse autosomes and X chromosomes. The mechanisms that specify the location of centromeres are not simple. Because, on the \textit{de novo} artificial chromosomes derived from the transfection of naked alphoid DNA into cells, not only CENP-B but also CENP-A, CENP-C, and CENP-E assemble (46, 47). Moreover, synthetic alphoid DNA repeats with only two nucleotides exchanged within the CENP-B boxes lose their ability to assemble mammalian artificial chromosomes and active centromere/kinetochore structures as well as the binding of CENP-B, whereas synthetic alphoid DNA repeats containing canonical CENP-B boxes still have these activities (48). Thus, CENP-B and CENP-B boxes are necessary for at least \textit{de novo} assembly of centromere components on naked alphoid DNA.

In the present study, among centromere proteins, we have demonstrated that CENP-B interacts with CENP-C using a yeast two-hybrid system. The interaction between CENP-B and CENP-C involves two domains on CENP-C at amino acid residues 283–429 and 727–943, both of which contain Mif2 homologous regions. This molecular interaction between CENP-B and CENP-C was confirmed by immunoprecipitation with solubilized chromatin prepared from HeLa cells expressing truncated CENP-B polypeptides and with \textit{in vitro} translated polypeptides, and by cytological observation of the truncated polypeptides.

Therefore, although CENP-C is able to assemble at the pre-existing centromere without CENP-B, the molecular interaction between CENP-C and CENP-B does indeed exist in human cells. Thus, we speculate that the interaction may specify and increase the probability of the assembly of CENP-C on alphoid DNA.

**FIG. 8.** CB1–160 polypeptide expression affects the centromere localization of CENP-C and cell cycle progression. **A**, CENP-C and CENP-B in HeLa cells were stained with the anti-CENP-C antibody, CGP2 (green), and BN1 (red), respectively, at the late S or late G2 phases. Weak signals (small arrows) and strong signals (large arrows) indicate the correlation of CENP-B and CENP-C signals. The insets show enlarged images captured at several foci as indicated by arrowheads in late G2 phase. **B**, endogenous CENP-B and expressed CB1–160 polypeptides in HeLa cells were stained with antibodies against the CENP-B COOH-terminal region, BC1 (green) and T7 tag (red), respectively. C, CENP-C and expressed CB1–160 polypeptides in HeLa cells were stained with CGP2 (green) and anti-T7 tag (red), respectively. The insets in B or C show the weak but overlapped endogenous CENP-B signals or abnormal CENP-C signals at centromeres indicated by arrowheads in the CB1–160-expressing cell. The cells were counterstained with DAPI (blue).
DNA containing CENP-B boxes, or alternatively provide a molecular linkage between one of the kinetochore components and human centromere DNA through the CENP-B/CENP-B box interaction.

Overexpression of CENP-B Deletion Mutants Affects the Assembly of CENP-C and Cell Cycle Progression through G2/M—Overproduction of the truncated CENP-B polypeptide containing the domain sufficient for specific localization to the centromere (32, 35, 36) caused abnormal assembly and duplication of CENP-C at G2. An excess amount of the truncated CENP-B polypeptide in human cells might affect the centromere structure by competing with normal CENP-B for binding to the CENP-B box on alphoid DNA. Our results indicate that defects in the proper assembly of CENP-C during the prekinetochore duplication period, as a result of overproduction of the CENP-B truncated polypeptide, explain the prolonged mitotic periods observed and the increase in lagging or maloriented chromosomes. The hypersensitivity of the cells to the microtubule-depolymerizing drug and abnormal assembly of CENP-E also indicated that the same defects in kinetochore assembly might have occurred. These kinetochore abnormalities monitored by the mitotic checkpoint mechanism may result in the prolonged mitotic periods. In the CB1–561 polypeptide-expressing cells, both abnormal accumulation of overlapping CENP-C and non-overlapped CENP-C in variable sizes were also observed with no mitosis. Although some differences in phenotype were observed between the cells which overproduced the CB1–160 and CB1–561 polypeptides, defects occurred in the same G2 period when the CENP-C sites duplicate.

Microinjection of an anti-CENP-C antibody into cultured human cells induced cell cycle arrest at the G2/M phase and a reduction in kinetochore size (18). Interestingly, when Mif2 was overexpressed in budding yeast, chromosomes mis-segregated during mitosis and cells accumulated in the G2 and M phases of the cell cycle (19). In S. cerevisiae and chicken DT40 cells, temperature-sensitive Mif2 and CENP-C mutations in the domain homologous to human CENP-C (Mif2 block 2) showed similar phenotypes (19, 56). Thus, the abnormal duplica-
cation of CENP-C and cell cycle arrest at G2 caused by overproduction of truncated CENP-B polypeptides indicate a functional significance for the interaction between CENP-B and CENP-C at the kinetochore duplication period.

Molecular Interactions between CENP-B and CENP-C Domains Containing Mif2 Homologous Regions—The present study demonstrated an interaction between CENP-B and CENP-C involving two domains on CENP-C at amino acid residues 283–429 and 727–943, both of which contain Mif2 homologous regions. Overproduction of truncated CENP-B polypeptides showed similar phenotypes to temperature-sensitive Mif2 and CENP-C mutations in the domain homologous to human CENP-C (Mif2 block 2) in S. cerevisiae and chicken DT40 cells (19, 56).

Under the conditions in which we demonstrated the interaction between CENP-B and CENP-C, a truncated mouse CENP-C containing the COOH-terminal Mif2 regions highly conserved between humans and mice was also isolated from a mouse cDNA library by two-hybrid screening with CENP-B as the bait. However, negative results for an interaction have been described previously (57). We experienced that the expression of CENP-B caused a weak inhibitory effect on yeast cell
growth, so that some bias of $10^{-3}$–$10^{-4}$ arose for the transformation efficiency of the CENP-B cDNA clone when HeLa cDNA library and a certain amount of CENP-B cDNA were mixed and used for the transformation. The apparent anomaly might be caused by this bias or differences in the bait and reporter constructs.

CENP-C Domains and Multiple Assembly Pathways for Centromere Components—Our results indicate that CENP-C has two main domains for targeting centromeres, 283–429 and 728–943. CENP-C dimerization activity also exists in this region (283–429) (58) and allowed homotypic interaction, as detected by our two-hybrid analysis. The results suggest that not only the interaction between CENP-B or other assembly pathway components, but also the self-dimerization activity, must be involved in the centromere localization of CENP-C.

Several groups have reported that CENP-C has DNA binding activity but that this lacks sequence specificity (25, 26). Recently, Politi et al. (59) showed that CENP-C and CENP-B associate with the same types of alphoid arrays using a chromatin immunoprecipitation assay, but in distinct non-overlapping centromere domains by ultrastructural analysis. In their study, the alphoid DNA binding activity of CENP-C was only detected with polypeptides containing both the DNA binding domain and the CENP-B interaction domains described in our present study. CENP-C also exists at neocentromeres where no alphoid sequence was detected (15). In contrast, CENP-B has specific binding activity to CENP-B boxes in centromeric type I alphoid DNA, and therefore overlapped with type I alphoid DNA sites throughout the cell cycle (33, 34, 60). Multiple molecular interactions may influence the affinity of CENP-C for the specific sites.

Goshima et al. reported that both CENP-A and hMis12 are required for the localization of hMis6 to the kinetochore, although CENP-A and hMis12 assemble at the kinetochore independently. They suggested that there were different pathways for the assembly of kinetochore components by hMis12 and CENP-A (3). Liu et al. reported that the kinetochore localizations of hUB11, hUB1R, hROD, hZW10, CENP-E, and CENP-A were not affected but CENP-F, MAD1, and MAD2 were affected by RNAi of CENP-I (hMis6), and the authors suggested that CENP-I specifies a discrete branch of the kinetochore assembly pathway (4). Our previous studies including chromatin immunoprecipitation analyses showed that the information carried by the primary sequence of the c21 alphoid DNA and CENP-B box is important for de novo formation of functional centromere chromatin accompanied by binding of CENP-A, CENP-B, CENP-C and CENP-E (48, 61). Thus, there might be several pathways for the assembly of kinetochore components.

We demonstrated an interaction between CENP-B and CENP-C in the present study. These results suggest that CENP-B may be involved in the assembly of the centromere/kinetochore structure by mediating the linkage of the essential component CENP-C to specific alphoid DNA containing CENP-B boxes. De novo centromere assembly mechanisms on the naked centromere DNAs were observed not only with a human cultured cell line but also with the most characterized centromeres of two yeasts, S. cerevisiae, and S. pombe (62, 63). Complex components of the centromere/kinetochore structure may assemble to the specific centromere DNA in each species through multiple interactions and assembly pathways.

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