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Domains Required for CENP-C Assembly at the Kinetochore

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Chromosomes segregate at mitosis along microtubules attached to the kinetochore, an organelle that assembles at the centromere. Despite major advances in defining molecular components of the yeast segregation apparatus, including discrete centromere sequences and proteins of the kinetochore, relatively little is known of corresponding elements in more complex eukaryotes. We show here that human CENP-C, a human autoantigen previously localized to the kinetochore, assembles at centromeres of divergent species, and that the specificity of this targeting is maintained by an inherent destruction mechanism that prevents the accumulation of CENP-C and toxicity of mistargeted CENP-C. The N-terminus of CENP-C is not only required for CENP-C destruction but renders unstable proteins that otherwise possess long half-lives. The conserved targeting of CENP-C is underscored by the discovery of significant homology between regions of CENP-C and Mif2, a protein of *Saccharomyces cerevisiae* required for the correct segregation of chromosomes. Mutations in the Mif2 homology domain of CENP-C impair the ability of CENP-C to assemble at the kinetochore. Together, these data indicate that essential elements of the chromosome segregation apparatus are conserved in eukaryotes.

INTRODUCTION

Chromosome segregation during cell division is brought about by discrete interactions between microtubules and molecular motors. Microtubules emanating from centrosomes dock at organelles on each sister chromatid known as kinetochores (Mitchison, 1988; McIntosh and Pfarr, 1991; Brinkley *et al.*, 1992; Earnshaw and Tomkiel, 1992). After docking at the kinetochore, the alignment of chromosomes on a metaphase plate is achieved by the addition or loss of tubulin subunits at the ends of these attached microtubules and the simultaneous actions of microtubule motors within the kinetochore (Desai and Mitchison, 1995). After chromosome alignment, sister chromatids are pulled to opposite poles of the dividing cell by the concerted disassembly of microtubules at the kinetochore and the tracking of microtubule motors toward the poles. Although studies of mammalian kinetochore have yielded exquisite data concerning the ultrastructure and biophysical forces of chromosome segregation (Ris and Witt, 1981; Rieder, 1982; Koshland *et al.*, 1988), less is known of the molecular components of higher kinetochores. In contrast, genetic approaches in the budding yeast *S. cerevisiae* have defined the minimal DNA sequence within the centromere required to assemble a kinetochore, which binds a single microtubule (Peterson and Ris, 1976; Clarke and Carbon, 1980; Carbon, 1984). Plasmids or linear DNA elements bearing these centromeric sequences display remarkable stability during both mitosis and meiosis in yeast (Fitzgerald-Hayes *et al.*, 1982; Hieter *et al.*, 1985). The functional *S. cerevisiae* centromere spans approximately 125 bp and shows sequence homologies that define three domains: CDEI, CDEII, and CDEIII. Mutations affecting CDEIII inactivate the centromere in vivo and prevent microtubule binding in vitro (McGrew *et al.*, 1986; Hegemann *et al.*, 1988; Kingsbury and Koshland, 1991). More recently, the CDEIII sequence was used to affinity purify a 240-kDa complex of proteins from yeast extracts known as centromere binding factor 3 (CBF3), composed of three proteins of 110, 64, and 58 kDa (Lechner and Carbon, 1991). Subsequently, analysis of

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temperature-sensitive mutants affecting chromosome segregation in yeast has identified two genes, *NDC10/CTF14* and *CTF13*, which encode, respectively, the 110- and 58-kDa proteins found within the CBF3 complex (Doheny *et al.*, 1993; Goh and Kilmartin, 1993; Jiang *et al.*, 1993; Lechner, 1994). These convergent observations indicate that CBF3 is a complex at the yeast kinetochore essential for chromosome segregation. However, yeast kinetochore function in vivo requires CDEI and CDEII, and microtubule attachment to centromeric sequences in vitro requires factors in addition to CBF3 (Sorger *et al.*, 1994). It is likely therefore that further biochemical and genetic studies will uncover an array of motors and assembly factors required for yeast kinetochore function.

The mammalian kinetochore has proved more intractable at a molecular level than that of budding yeast, in part due to its increased complexity. It is known, for instance, that mammalian kinetochores bind 15–25 microtubules, and therefore must contain elements that regulate and coordinate force generation across the organelle (Ris and Witt, 1981). Moreover, cytogenetic analysis indicates that the underlying centromeric DNA required for kinetochore assembly may span upwards of 1 megabase (Mb) of DNA (Vollrath *et al.*, 1992; Tyler-Smith *et al.*, 1993). Although some of the DNA elements within this 1 Mb sequence, such as the alphoid satellite repeats, appear to be common to all human centromeres (Willard and Waye, 1987), their role in kinetochore assembly or chromosome segregation has not been determined. Despite this complexity at the DNA level, autoantibodies from patients with the CREST syndrome of scleroderma have revealed a set of centromeric proteins, or CENPs (Moroi *et al.*, 1980). CENP-A, a 17-kDa protein, shares significant homology with histone H3 (Palmer *et al.*, 1991; Sullivan *et al.*, 1994). CENP-B, an 80-kDa protein, specifically binds alphoid DNA repeats (Earnshaw *et al.*, 1987; Masumoto *et al.*, 1989; Muro *et al.*, 1992). High resolution immunolocalization experiments demonstrate that CENP-B resides on the chromatin beneath the kinetochore rather than within the complex itself (Cooke *et al.*, 1990). Therefore it is unlikely that CENP-B plays a direct role in kinetochore assembly or function, although an exact conclusion here must await gene deletion experiments. CENP-C, a 110-kDa autoantigen, may play a more central role in kinetochore assembly because immunoelectromicroscopy has localized it to the base of the kinetochore complex itself (Saitoh *et al.*, 1992). More recently, monoclonal antibodies have identified a 312-kDa protein termed CENP-E, which localizes at the kinetochore during mitosis (Yen *et al.*, 1991). A similar transient association with kinetochores is also shown by CENP-F, an autoantigen of approximately 400 kDa (Rattner *et al.*, 1993). Significantly, the N-terminus of CENP-E shares extensive homology with the motor domain of kinesin-like proteins which, coupled with its unusual association with spindle microtubules at anaphase, implicates CENP-E in critical interactions with microtubules during both metaphase and anaphase (Yen *et al.*, 1992; Liao *et al.*, 1994).

Despite the significant progress in defining components of the yeast and higher eukaryotic kinetochores, elements common to the apparently conserved mechanism of microtubule-based chromosome segregation have yet to emerge. To test the possibility that the human kinetochore protein CENP-C could target in a conserved fashion to kinetochores, we expressed an epitope-tagged version in mammalian and amphibian cells. Human CENP-C assembles at centromeres of divergent species. The specificity of this targeting appears to be maintained by a highly efficient mechanism that destroys nonassembled CENP-C. This conserved targeting-destruction mechanism suggests that CENP-C plays an important role in kinetochore assembly and function. This view is supported by the recent discovery that CENP-C contains domains similar to those of the protein encoded by *MIF2*, a gene involved in chromosome segregation in budding yeast (Brown *et al.*, 1993; Meluh and Koshland, 1995).

MATERIALS AND METHODS

Cloning of cDNAs Encoding Human and Mouse CENP-C

The cDNA encoding human CENP-C was obtained from a HeLa lambda ZAPII cDNA library by hybridization using a 60 mer oligonucleotide corresponding to the N-terminal nucleotide sequence (Saitoh *et al.*, 1992). CENP-C was cloned into the mammalian expression vector pCMV1 (Pharmacia, Piscataway, NJ) containing a 200-bp 5′-untranslated sequence from human lamin A and an N-terminal c-Myc epitope tag (Munro and Pelham, 1984) using oligonucleotides and polymerase chain reaction techniques. The cDNA encoding mouse CENP-C was obtained by screening a mouse T-cell library in lambda ZAPII using the human cDNA as a hybridization probe. The conditions for hybridization were 50% formamide, 5× SSC at 40°C with a low stringency wash in 1.5× SSC at 40°C. The murine CENP-C sequence was obtained from a 3-kb clone at the Microchemistry facility at the University of California, San Francisco, CA.

Construction of N-Terminal, C-Terminal, and Internal Deletions in Human CENP-C

N-terminal and C-terminal deletions of CENP-C were generated by introducing restriction sites in the CENP-C cDNA using the double primer method (Zoller and Smith, 1984). Single-stranded M13 mp18 containing the wild-type CENP-C cDNA was used as a template for second strand synthesis using both the M13 universal primer (New England Biolabs, Beverly, MA) and mutagenic primers containing appropriate restriction sites for cloning the truncated cDNA into the pCMV1-c-Myc construct described above. The internal deletion of codons 922–926 was achieved using an oligonucleotide to loop out this sequence.

Construction of Fusions between Portions of CENP-C and β-Galactosidase

The β-galactosidase fusions were constructed by first introducing appropriate restriction sites at the 5′-end and 3′-end of the β-galac-

tosidase encoding sequence using polymerase chain reaction techniques and then cloning the resulting product in-frame to various N-terminal portions of CENP-C in the pCMV1-c-Myc vector described above.

Expression of Human CENP-C in Mammalian and Xenopus Cell Lines

COS and BHK cells were maintained in a 5% CO_2 atmosphere at 37°C in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (HyClone, Logan, CT), 2 mM glutamine, and 10 μg/ml of penicillin-streptomycin (complete medium). A6 cells were maintained in a 5% CO_2 atmosphere at 25°C in NCTC-109 medium (Life Technologies) supplemented with 10% fetal bovine serum (HyClone), 2 mM glutamine, and diluted with 15% H_2O (complete medium). DNA transfections were performed as described previously (Heald *et al.*, 1993). Cells were plated onto coverslips in 400 μl complete medium on day 1. On day 2, a total of 2 μg of cesium chloride-purified plasmid DNA was added to 30 μl of 0.2 M $CaCl_2$ and precipitated by adding 30 μl of 280 mM NaCl, 1.5 mM Na_2HPO_4, and 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.1, over 15 s with stirring. After 20 min, 350 μl of complete medium was added to the DNA precipitate, and the mixture was applied to the cells. Each coverslip was allowed to incubate in a tissue culture incubator for 3 h for COS and BHK cells, and 6 h for A6 cells. The coverslips were then washed twice with complete medium and returned to the incubator for an additional 14 h. A6 cells were incubated for 2 min in complete medium supplemented with 20% glycerol before washing and returned to the incubator for 24 h.

Immunofluorescence

For immunofluorescence staining, transfected cells on coverslips were fixed in 3% formaldehyde in phosphate-buffered saline (PBS) for 10 min and then washed three times with 0.1% Nonidet P40 (NP40) in PBS (PBS-NP40). Primary antibodies were applied to the cells on coverslips for 30 min, followed by four rapid rinses with PBS-NP40. Cy3-conjugated goat anti-mouse (Jackson Immunologicals, West Grove, PA) antibodies were also diluted in PBS-NP40 and applied to the coverslips for an additional 30 min. DNA was stained using Hoechst dye 33258 (Sigma, St. Louis, MO) at 1 μg/ml PBS-NP40 for 1 min. Coverslips were mounted on glass slides in 90% glycerol and 20 mM Tris-HCl, pH 8.0. The 9E10 mouse monoclonal antibody against the c-Myc epitope was obtained from the Cell Culture Facility at Harvard University (Cambridge, MA) and the mouse monoclonal antibodies against β-galactosidase were obtained from Promega (Madison, WI).

Western Blot Analysis

Transfected cells were directly extracted on coverslips with 200 μl of 2 × SDS-sample buffer (0.1 M Tris, pH 6.8, 20% glycerol, 2% SDS, and 10% β-mercaptoethanol). The extract was transferred to a microfuge tube, boiled for 5 min, and centrifuged at 10,000 × *g* for 5 min. Twenty microliters of each sample was fractionated on a 8.5% SDS-polyacrylamide gel and transferred to Immobilon membranes (Millipore, Bedford, MA). The membranes were blocked for 1 h in PBS containing 5% dry milk and incubated in primary antibody diluted in the same solution for 1 h. After several 5-min washes in blocking solution, membranes were incubated with goat anti-mouse antibodies conjugated to horseradish peroxidase (Tago, Burlingame, CA) for 1 h. Membranes were then washed and developed using chemoluminescence substrates according to manufacturer's instructions (ECL, Amersham, Arlington Heights, IL).

Northern Blot Analysis

BHK cells plated on 10 cm-dishes were transfected with pCMV1-CENP-C and pCMV1-Δ373N as described above. Eighteen hours after transfection total RNA was isolated using RNAzol B (Tel-Test, Friends Wood, TX) according to manufacturer's instructions. Northern blots to detect CENP-C and Δ373N transcripts were performed at high stringency as described (Sambrook *et al.*, 1989) using the cDNA encoding human CENP-C as a hybridization probe.

RESULTS

Human CENP-C Targets Centromeres of Diverse Species

The 110-kDa human CENP-C protein localizes to the inner plate of the mitotic kinetochore complex as well as at the less organized, prekinetochore complex of interphase cells (Saitoh *et al.*, 1992). Despite the apparent conservation of kinetochore structure and function in eukaryotes, CENP-C homologues have not been described in nonmammalian species. To test the possibility that human CENP-C could assemble into kinetochores of other species, we expressed an epitope-tagged version of human CENP-C in monkey (COS), hamster (baby hamster kidney, BHK), and *Xenopus* (A6) cell lines (Figure 1). Immunofluorescence detection of epitope-tagged human CENP-C using antibodies to the c-Myc tag revealed a pattern of discrete dots in interphase cells (Figure 1, left panels) and an organized array of dots at the centromeres of chromosomes in metaphase cells (Figure 1, right panels). These patterns of transfected CENP-C localization are identical to those observed upon labeling endogenous CENP-C with anti-CENP-C antibodies (Saitoh *et al.*, 1992). That human CENP-C assembles at prekinetochores was supported by co-staining with anti-centromere antibodies from CREST sera (Moroi *et al.*, 1980), which we observed to colocalize with foci positive for the anti-c-Myc epitope antibodies in BHK cells. Additionally, direct counts of anti-epitope positive foci in transfected cells yielded values in agreement with the number of chromosomes of the particular cell lines (60 chromosomes for COS, 44 for BHK, and 36 for A6 cells).

The remarkably precise targeting of human CENP-C in divergent species suggests that it assembles at the kinetochore through either conserved DNA binding sites or through conserved interactions with other kinetochore proteins.

CENP-C Instability May Prevent Toxic Overexpression and Mistargeting

A surprising feature of these transfection experiments was that, in spite of driving CENP-C expression from the strong cytomegalovirus (CMV) promoter, excessive accumulation of CENP-C in the nucleus was rarely observed (Figure 1). We considered several possibilities, including low expression

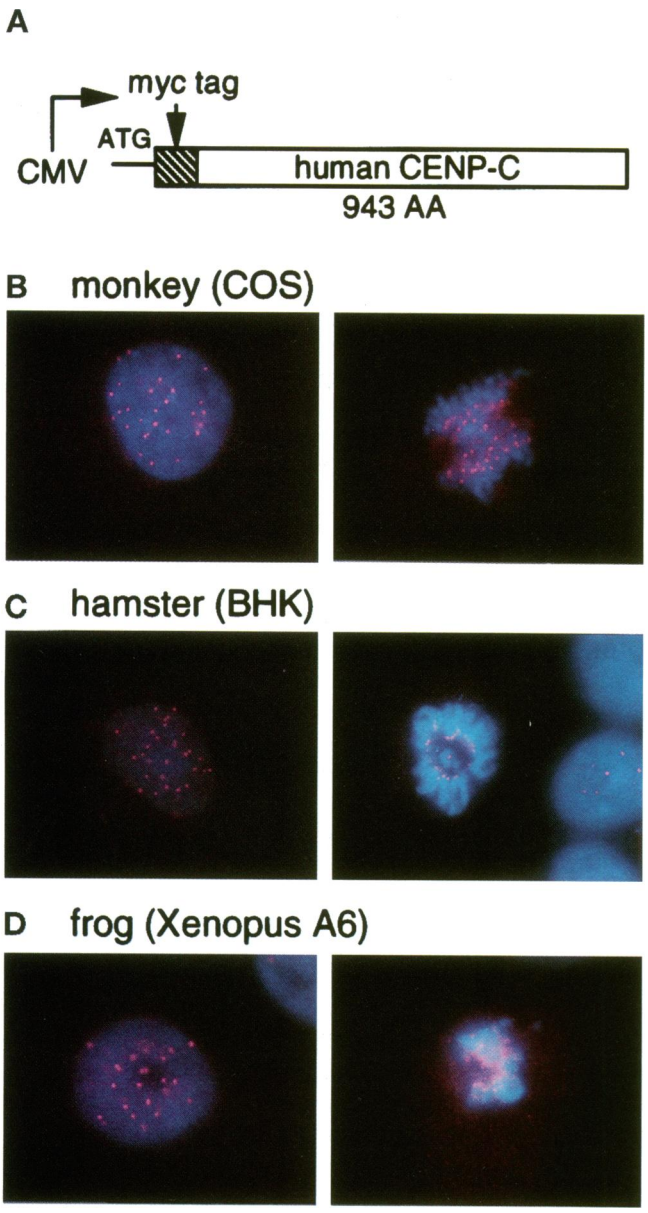

Figure 1. Human CENP-C targets the centromere of divergent species. (A) Human CENP-C was modified at the N-terminus with the c-Myc epitope tag and placed under the control of the CMV promoter. (B–D) Immunofluorescent localization of transfected human CENP-C in COS (African green monkey, B), BHK (baby hamster kidney, C), and A6 (*Xenopus laevis*, D) cells. Interphase cells (left panels) show a dotted nuclear pattern of anti-Myc staining, while mitotic cells (right panels) show an array of dots at specific regions of the chromosomes.

of CENP-C or high instability of the CENP-C protein, to explain the lack of accumulation and the resulting accurate targeting of CENP-C to the centromere in transfected cells. In an effort to identify putative destabilizing sequences in CENP-C, we tested the stability of various deletion mutants in BHK cells (Figure 2). Although wild-type CENP-C, as well as an N-terminal deletion mutant lacking 249 amino acids showed precise kinetochore labeling and failed to accumulate in cells (Figure 1), mutants lacking the N-terminal 323 and 373 amino acids (Δ323N and Δ373N, respectively) dramatically accumulated in the nucleus (Figure 2A). Significantly, cells that accumulated these deletion mutants in their nuclei showed highly distorted patterns of chromatin organization compared with their non-transfected neighbors and many appeared dead by 24 h (Figure 2A). The accumulation of the CENP-C deletion mutants was accompanied by homogenous labeling of noncentromeric regions of interphase chromatin and mitotic chromosomes (Figure 2, A and B). Although this loss of specificity might have been due to improper folding of the deletion mutant, we observed occasional cells expressing low levels of the deletion mutants Δ323N and Δ373N in which proper targeting to the centromere was apparent (Figure 2C). Immunoblots of cells transfected with the various CENP-C mutants showed that wild-type CENP-C, Δ100N, and Δ249N did not accumulate to detectable levels in BHK cells (Figure 2D), despite the intense labeling of kinetochores by these proteins observed by immunofluorescence. In contrast, Δ323N and Δ373N, which appeared by immunofluorescence to grossly accumulate in the nuclei of transfected cells, yielded strong signals on immunoblots (Figure 2D).

Attempts at pulse-chase analysis of transfected cells using [^{35}S]methionine and short pulses failed to incorporate detectable label into wild-type CENP-C, whereas the Δ373N mutant yielded consistently strong signals. Our inability to incorporate sufficient label into wild-type CENP-C precluded a determination of its stability and half-life upon translation in mammalian cells. However, to exclude the possibility that the mutant transcripts were more stable than that encoding wild-type CENP-C we performed Northern blots on transfected cells. We did not detect significant differences in the steady state levels of transcripts in cells transfected with CENP-C and Δ373N cDNAs (Figure 2E), an indication that post-transcriptional mechanisms regulate the accumulation of CENP-C.

To further analyze the role of N-terminal sequences in the stability of CENP-C, we tested the possibility that these sequences can mark a heterologous protein (β-galactosidase) to degradation. Fusions between portions of CENP-C and β-galactosidase were tested for stability in vivo (Figure 3A). Immunofluorescence and immunoblot analysis of cells transfected with the fusion constructs revealed that β-galactosidase, which is otherwise stable in BHK cells, failed to accumulate to high levels when fused to either the first 323 and 373 amino acids of CENP-C or full-length CENP-C

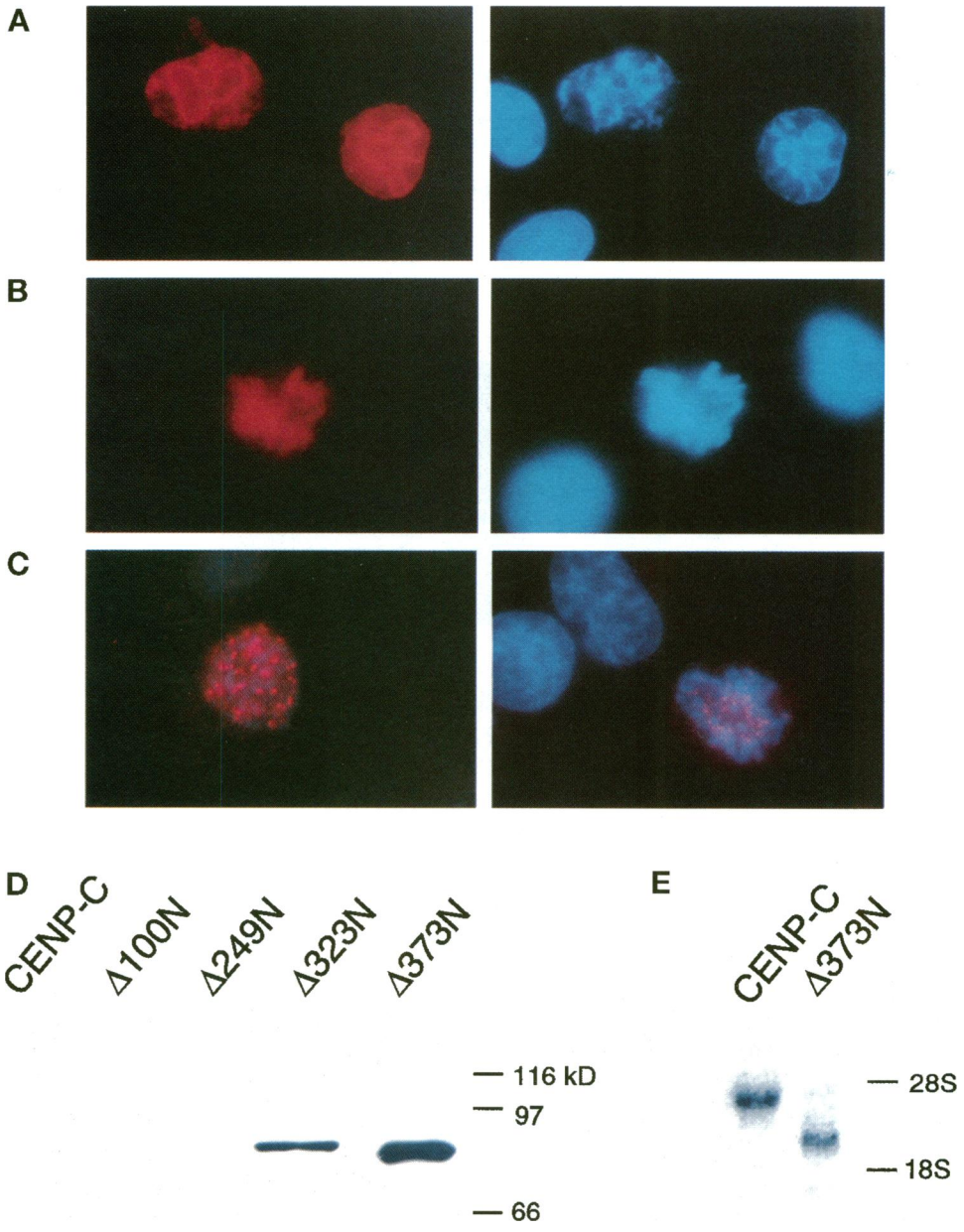

Figure 2. The deletion of 323 and 373 N-terminal residues of human CENP-C results in the massive accumulation of the deletion mutant in the nucleus. (A and B) Immunofluorescent localization of the N-terminal deletion mutant Δ373N upon transfection in BHK cells. (Left panels) Anti-Myc staining. (Right panels) Hoechst staining of the DNA. The accumulation of the deletion mutant results in an abnormal pattern of chromatin organization (A) and in the binding of noncentromeric regions (B). (C) Low levels of expression show that the deletion of N-terminal sequences does not significantly affect the association with centromeres (left Δ323N, right Δ373N). (D) Western blot of BHK cells transfected with CENP-C and N-terminal deletion mutants. The blot was probed with anti-Myc antibodies. Δ323N and Δ373N migrate higher than predicted from their calculated molecular weight, similarly to CENP-C (Saitoh *et al.*, 1992). (E) Northern blot with total RNA isolated from BHK cells transfected with CENP-C and Δ373N. The blot was probed with the cDNA encoding human CENP-C. The transcripts for transfected CENP-C and Δ373N run at 3 kb and 1.9 kb, respectively.

(Figure 3, B and C), presumably due to the instability of the fusion proteins. Based on Western blot analysis, full-length CENP-C appeared more effective in destabilizing β-galactosidase (Figure 3C). Interestingly, the full length CENP-C-β-galactosidase fusion targeted the centromere in a manner remarkably similar to that of CENP-C (Figure 3B, right panel), leaving a very low background of protein in the cytoplasm. The fusions between the N-terminus of β-galactosidase showed a cellular distribution similar to that of β-galactosidase (Figure 3B, left panel).

Taken together, these data support the possibility that the N-terminus of CENP-C is dispensable for centromere targeting and may function exclusively in destabilizing CENP-C that fails to assemble at the kinetochore. We suggest that the inherent instability

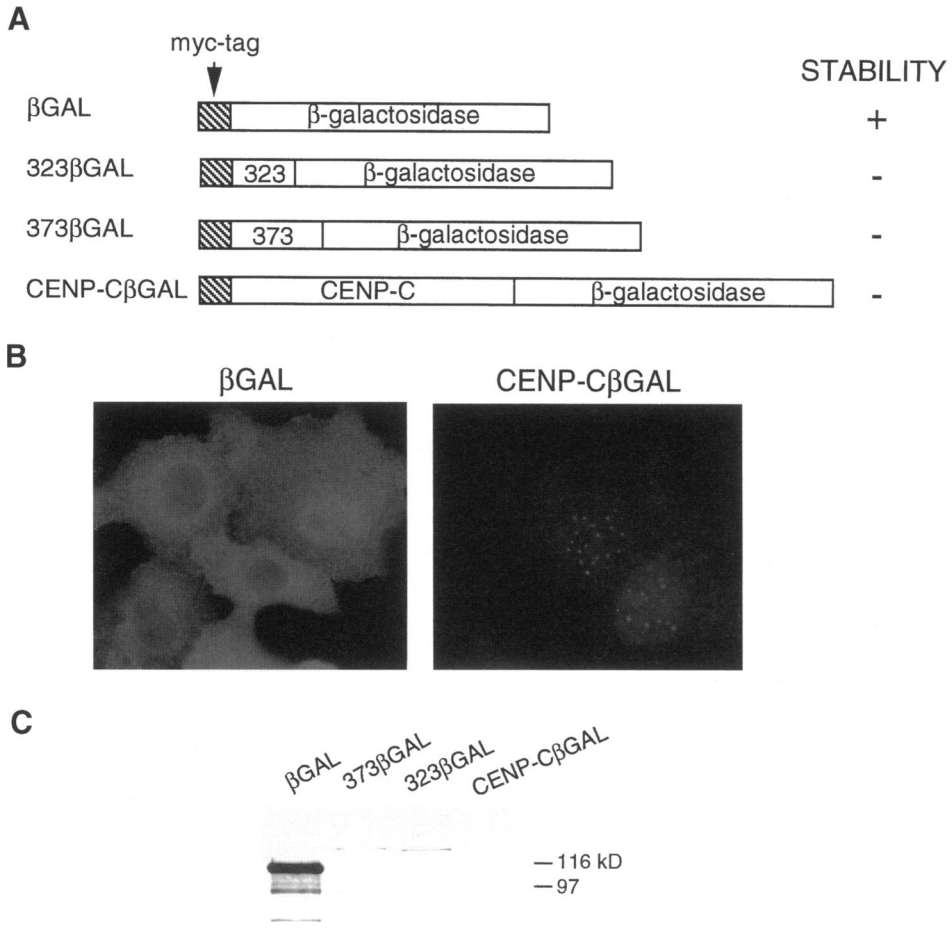

Figure 3. The N-terminus of CENP-C directs a heterologous protein (β-galactosidase) to degradation. (A) Schematic of the fusion proteins obtained by fusing N-terminal sequences of CENP-C or full-length CENP-C to β-galactosidase. (B) Immunofluorescence of BHK cells transfected with β-galactosidase (left) and the fusion CENP-C-β-galactosidase (right). Anti-β-galactosidase antibodies were used. Note that the fusion protein correctly associates with centromeres. (C) Western blot of BHK cells transfected with the fusion constructs. Blots were probed with anti-β-galactosidase antibodies. β-galactosidase is unstable and fails to accumulate at high levels when fused to N-terminal sequences of CENP-C or to full-length CENP-C.

of CENP-C could be one of the mechanisms for ensuring correct targeting to centromeres and for preventing toxic accumulation of CENP-C.

The C-Terminus of CENP-C, Including the Mif2 Homology Domain, Is Involved in CENP-C Assembly at Centromeres

The ability of CENP-C to correctly target the centromere of divergent species (Figure 1) suggests not only the conservation of interactions at the centromere-kinetochore complex, but also the existence of CENP-C homologues outside of mammals. This concept was strengthened by the discovery that the product of the *MIF2* gene of *S. cerevisiae*, which plays an essential role in chromosome segregation, shows significant homology to the C-terminus (residues 696–943) of human and mouse CENP-C (Figure 4A; Brown *et al.*, 1993; Meluh and Koshland, 1995). Because deletions of the N-terminal sequences of CENP-C did not significantly affect CENP-C targeting to centromeres, we asked whether the Mif2 homology domain of CENP-C functions in the assembly of this molecule into the kinetochore. Initially, we tested whether CENP-C lacking the entire Mif2 homology domain (residues 728–943, ΔMif2-CENP-C) could target to centromeres when expressed in mammalian cells. Although ΔMif2-CENP-C did retain some ability to assemble at the centromere, the overall signal appeared consistently uneven and decreased in intensity and selectivity (Figure 4B). A similar decrease in affinity for centromeres was observed with a mutant lacking the highly conserved residues 922–926 within the Mif2 homology domain (not shown). In contrast, a deletion of an additional 150 amino acids beyond the Mif2 homology domain (Δ584C) abolished assembly of CENP-C at the kinetochore, resulting in a protein that

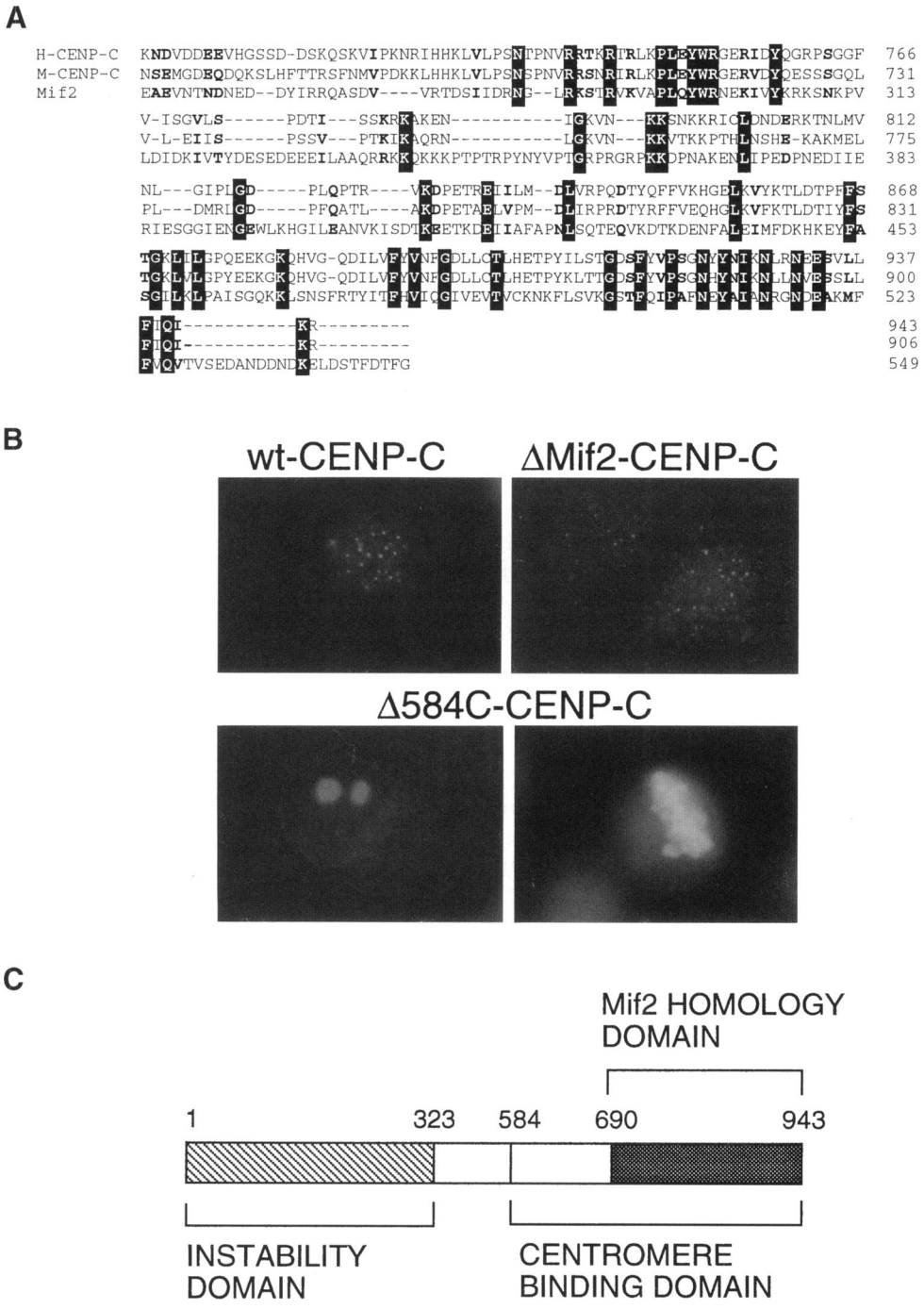

Figure 4. The C-terminus of CENP-C shows significant homology to the yeast protein Mif2 and is involved in the assembly of the protein to centromeres. (A) Alignment of the last 250 amino acids of human (Saitoh *et al.*, 1992) and mouse CENP-C with the sequence of the yeast protein Mif2 (Brown *et al.*, 1993). Identical residues are boxed and conserved changes are shown in bold. This alignment was generated with the GeneWorks 2.2 (IntelliGenetics). (B) Immunofluorescence with anti-Myc antibodies of BHK cells transfected with CENP-C (upper left panel), ΔMif2-CENP-C (upper right panel), and the C-terminal deletion Δ584C (both lower panels). The deletion of the Mif2 homology domain appears to affect the assembly at centromeres, whereas the deletion of the C-terminal one-half of CENP-C results in a protein that associates with nucleoli (left) and is excluded from mitotic chromosomes (right; chromosomes are stained with Hoechst, while the surrounding Δ584C-deletion mutant is visualized by anti-Myc staining). (C) Domain organization of CENP-C: the N-terminus is responsible for the instability of nonassembled CENP-C; the C-terminus, which includes the Mif2 homology domain, is essential for targeting CENP-C to centromeres.

localized both in the cytoplasm and the nucleus, in association with nucleoli (Figure 4B, left lower panel). The failure to interact with centromeres was particularly clear in mitotic cells, where the C-terminal mutant appeared dispersed around the condensed chromosomes (Figure 4B, right lower panel).

These data indicate that the C-terminus of CENP-C, including the Mif2 homology domain, participates in the significant interactions with DNA or proteins required for assembly of CENP-C at the centromere-kinetochore complex. The putative domain organization of CENP-C is summarized in

Figure 4C: the N-terminus of CENP-C, although not essential for centromere targeting, is responsible for signalling nonassembled CENP-C for degradation. The C-terminal one-half of CENP-C, including the Mif2 homology domain, appears to be essential for stable interactions at the centromere.

DISCUSSION

We present data here which indicate that human CENP-C can assemble into kinetochore-centromere complexes upon expression in diverse species, and that nonassembled CENP-C is unstable. A highly efficient means of destroying nonassembled CENP-C appears to be essential because mutants lacking the N-terminal instability sequence bind nonspecifically to chromatin and are highly toxic to the cell. Significantly, Mif2, the product of a yeast gene involved in chromosome segregation, possesses sequence homology to the C-terminus of CENP-C. The C-terminus of CENP-C, including the Mif2 homology domain, is involved in targeting CENP-C to centromeres. Together, these data suggest that CENP-C and related proteins play critical and conserved roles in kinetochore assembly and function.

CENP-C Is a Conserved Component of Kinetochores

Using transient transfection assays, we have shown that epitope-tagged human CENP-C efficiently assembles into the kinetochore-centromere complex in cells of monkey, hamster, and amphibian origins. Thus human CENP-C must either recognize conserved regions of centromeres in these various species or, more likely, associate at the kinetochore through interactions with other conserved kinetochore proteins. The existence of common centromeric DNA motifs recognized by CENP-C is not obvious at present. Cloning and functional characterization of the centromere of the yeasts *S. cerevisiae* and *S. pombe* have revealed surprisingly high levels of divergence (Clarke *et al.*, 1986; Nakaseko *et al.*, 1986; Fishel *et al.*, 1988; Chikashige *et al.*, 1989; Carbon and Clarke, 1990). Centromeres of mammalian chromosomes consist mainly of rapidly evolving classes of repetitive DNA (Willard and Waye, 1987; Joseph *et al.*, 1989), which can form Mb-arrays at the primary constriction. The ability of human CENP-C to interact with presumably divergent centromeres, along with its homology to the yeast protein Mif2, suggest that at least some of the processes involved in the organization of centromere-kinetochore complexes are considerably more conserved than a comparison of the DNA composition of such complexes might predict.

Recently, advances through cytogenetics and large-scale physical mapping have defined the functional centromere of the human Y chromosome to within 1 Mb of DNA (Vollrath *et al.*, 1992; Tyler-Smith *et al.*, 1993; Brown *et al.*, 1994b). The next challenge will be to construct a functional map of this region and it is likely that CENP-C, by its ability to interact in a conserved way with centromeres, will be an important marker for defining domains within the centromere of mammalian chromosomes involved in kinetochore assembly.

CENP-C Is Stabilized by Assembling at Kinetochores

Our transfection experiments with epitope-tagged CENP-C have also revealed that the levels of CENP-C appear to be strictly controlled in cells. We provide evidence here that this control is achieved, at least in part, by the conditional instability of CENP-C. Using deletion mutants, we have identified a discrete region of the N-terminus whose removal resulted in a gross overexpression of the CENP-C mutants in transfected cells, and localized the putative instability sequence to the first 323 amino acids. Significantly, this N-terminal domain acts to destabilize a stable protein such as β-galactosidase, when fused to it. In addition, we show that this putative destabilizing sequence is dispensable for CENP-C targeting to the centromere, suggesting a separation between functional domains in the CENP-C sequence.

The impetus for investigating CENP-C instability was the inability to overexpress the wild-type protein in mammalian cells, even though this expression was controlled by promoters and 5′- and 3′-untranslated regions that overexpress proteins of all other cDNAs tested, including nuclear lamins, cell cycle regulators, and signal transduction factors. In the case of CENP-C expression, the only staining in the transfected cells appeared at the centromere. When analyzed by anti-Myc antibodies on immunoblots, these transfected cells were found to express wild-type CENP-C at levels at least 100-fold lower than cells transfected with the identical vector expressing N-terminal CENP-C deletion mutants. Although our analysis of the underlying cause of the low accumulation of CENP-C supports a mechanism involving CENP-C protein instability, we cannot exclude a role for translational controls in this process. Northern analysis of cells transfected with wild-type CENP-C and the Δ373N mutant showed similar levels of transcripts, an observation that supports a post-transcriptional control of CENP-C accumulation.

The most obvious reason why the levels of CENP-C in cells need to be controlled comes from the transfection experiments with the deletion mutants lacking the instability domain. The increased stability of these mutants allows them to accumulate in the

nucleus and to associate with noncentromeric regions of interphase and mitotic chromatin, with toxic consequences for the cells. It is likely, therefore, that the inherent instability of CENP-C acts as a kinetic proofreading mechanism to ensure the correct assembly into the centromere-kinetochore complex while eliminating excess CENP-C molecules that bind noncentromeric regions and presumably other kinetochore components. Another important reason to have unstable kinetochore proteins is to allow high expression levels required to rapidly assemble kinetochores in the brief window between DNA replication and the onset of cell division. Similar mechanisms operate during ribosome production to coordinate the assembly of multiple ribosome components. Nonassembled ribosomal proteins are highly unstable, with a half-life of 1–5 min (Maicas *et al.*, 1988; Tsay *et al.*, 1988; Warner, 1989). However, other mechanisms, such as transcriptional and translational control, also appear to be involved in maintaining a balanced accumulation of ribosomal components (Warner, 1989). We suggest an analogous situation for the assembly of a centromere-kinetochore complex, with the high instability of nonassembled proteins being one of the mechanisms involved in this process. Whether the conditional instability of CENP-C described here plays a general role in kinetochore assembly must await similar analyses on other kinetochore proteins in yeast and mammalian cells. Interestingly, CENP-E, a kinetochore protein that assembles onto mitotic kinetochores, is suddenly degraded during anaphase (Brown *et al.*, 1994a). The fact that CENP-C is found at the centromere throughout the cell cycle suggests that its N-terminal sequence is subject to different surveillance mechanisms than those which function in CENP-E degradation.

At present it is unclear how the conditional instability of CENP-C operates. It is likely that the stability of correctly assembled CENP-C may involve the masking of the N-terminal domain. Also unclear is what sequences within the identified N-terminal domain are strictly required for signaling the degradation of CENP-C. The data obtained with the fusions between portions of CENP-C and β-galactosidase, which show that full-length CENP-C is more effective than N-terminal sequences in determining the degradation of β-galactosidase, suggest that additional factors, such as the localization of the protein (nucleus vs. cytoplasm) may be involved in determining the degradation of CENP-C.

The Mif2 Homology Domain in CENP-C Is Involved in Assembly at Kinetochores

The C-terminal domain of human CENP-C has significant homology to the protein encoded by *MIF2*, a gene involved in budding yeast chromosome segregation (Brown *et al.*, 1993; Meluh and Koshland, 1995). We tested the functional consequences of mutations in the Mif2 homology domain of CENP-C and found that either a deletion of the entire region or mutations affecting the region of high homology diminishes the ability of CENP-C to assemble at the kinetochore, presumably by affecting the interaction between CENP-C and conserved kinetochore components. The additional removal of the domain (residues 584–728) adjacent to the Mif2 homology domain abolishes all kinetochore association functions in CENP-C. To clearly establish the function of this highly conserved domain, more extensive mutagenesis analysis will be required. Mutations in the Mif2 homology domain that still allow CENP-C assembly at the centromere may result in dominant negative effects on chromosome segregation.

These observations on the involvement of the Mif2 homology domain in the assembly of CENP-C at centromeres also suggest that the loss-of-function or temperature-sensitive phenotypes resulting from mutations in the conserved domain in *MIF2* could be due to a decreased ability of Mif2 to associate with the yeast kinetochore (Meluh and Koshland, 1995). Meluh and Koshland provide significant genetic evidence that Mif2 interacts with proteins associated with both the CDEI and CDEIII domains of the yeast centromere. This evidence includes the observation that certain Mif2 mutations render both the CDEI domain of the centromere, as well the CDEI binding protein CBFI, essential for accurate chromosome segregation. Additional interactions have been suggested between the Mif2 protein and Ndc10/CTF14, a protein in the CBF3 complex that associates with the CDEIII domain of the yeast centromere (Doheny *et al.*, 1993; Goh and Kilmartin, 1993; Jiang *at al.*, 1993). The provisional assignment of Mif2 as a protein that interacts with the AT-rich CDEII domain is supported by the presence of a sequence within Mif2 (residues 352–364) similar to those seen in proteins that bind AT-rich DNA sequences (Brown *et al.*, 1993). The emerging picture of Mif2 then, is that it may play an important role in kinetochore function by mediating the interaction between kinetochore components. It is unclear at present how the data from Mif2 impact on concepts of CENP-C function in higher kinetochores. By analogy, however, CENP-C might establish a framework at the interphase kinetochore to which mitotic kinetochore proteins assemble during the formation of an active kinetochore at mitosis.

The question as to whether CENP-C directly binds to centromeric DNA remains open. Its primary structure does not contain characterized DNA-binding domains and thus its targeting to kinetochores might be dependent exclusively on protein-protein interactions. The DNA-binding motif present in Mif2 is not con-

served in CENP-C. Whether other DNA binding motifs have evolved in CENP-C or these functions are subsumed by separate proteins must be addressed by more detailed biochemical experiments. Regardless, the apparent structural and functional homology between mammalian CENP-C and yeast Mif2 suggests that important components of the kinetochore will be conserved in eukaryotes, and predicts that the functional analysis of these components in yeast and mammalian cells will be mutually revealing in understanding chromosome segregation.

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