Molecular Analysis of the INCENPs (Inner Centromere Proteins): Separate Domains Are Required for Association with Microtubules During Interphase and with the Central Spindle During Anaphase

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Abstract. It has recently been proposed that mitotic chromosomes transport certain cytoskeletal proteins to the metaphase plate so that these proteins are able to subsequently participate in the assembly of the anaphase spindle and the cleavage furrow. To understand how such proteins accomplish their dual chromosomal:cytoskeletal role, we have begun a molecular and functional analysis of the inner centromere proteins (INCENPs), founder members of the class of "chromosome passenger proteins." cDNA clones encoding the open reading frames of the two chicken INCENPs were recovered. The predicted proteins, class I INCENP (96,357 D) and class II INCENP (100,931 D) are novel, and differ from each other by the inclusion of a 38-codon insert within the class II coding region. Transient expression of the chicken INCENPs in mammalian cells confirms that the signals and structures required for the transfer of these proteins from chromosomes to cytoskeleton are evolutionarily conserved. Furthermore, these studies reveal that INCENP association with the cytoskeleton is complex. The amino-terminal 42-amino acid residues are required for transfer of the INCENPs from the chromosomes to the mitotic spindle at anaphase, but not for binding of INCENPs to cytoplasmic microtubules. In contrast, an internal 200 amino acid coiled-coil domain was required for association with microtubules, but dispensable for spindle association. These experiments suggest that proteins required for assembly of specialized cytoskeletal structures during mitosis from anaphase onwards might be sequestered in the nucleus throughout interphase to keep them from disrupting the interphase cytoskeleton, and to ensure their correct positioning during mitosis.

The onset of anaphase is a crucial time both for the distribution of chromosomes to the daughter cells and for the partitioning of the cytoplasm. Recent experiments have revealed the existence of a class of chromosomal proteins that may play important roles in both chromosomal and cytoplasmic events at this time. We have termed these components "chromosome passenger proteins," and suggested that they may be transiently associated with chromosomes as a means of transport to the spindle midzone, where they may perform essential roles in the later stages of mitosis (8).

The first chromosome passenger proteins to be described, the INCENPs (inner centromere proteins), were originally identified as components of the mitotic chromosome scaffold (6). The INCENPs are restricted to the nucleus in interphase, and tightly bound to the chromosomes until early metaphase. However, during late metaphase, they leave the chromosomes to become concentrated in linear arrays that transect the metaphase plate between the chromosomes (9). As anaphase begins and the sister chromatids move towards the spindle poles, the INCENPs remain behind at the spindle midzone, where they are intimately associated with the bundled microtubules at the region of overlap between the two half spindles. Later in anaphase, a subpopulation of the INCENPs becomes closely associated with the cell cortex at the point where the cleavage furrow will subsequently form. By telophase, the INCENPs are concentrated at each side of the midbody in the intercellular bridge, with which they are discarded after cytokinesis.

Since the identification of the INCENPs (6), five other chromosomal passenger proteins have been identified by the use of experimental or autoimmune antibodies. The JB autoantigen (38 kD) (20) and antigen TD-60 (60 kD) (2) both have distributions through mitosis that are strikingly similar to that of the INCENPs. Two other proteins, CENP-E (312 kD) (37) and the 37A5 antigens (140 and 155 kD) (29) resemble the INCENPs in that these proteins associate with the centromeric region of chromosomes in early mitosis, and the spindle midzone and stem body from anaphase onwards. However, neither is associated with the cell cortex at the cleavage furrow, and neither is detected in interphase cells. Finally, MSA-36 (36 kD) is detected at centromeres in unblocked metaphase chromosomes, then migrates to the spindle at anaphase (32).

1. Abbreviations used in this paper: AMV, avian myoblastosis virus; INCENP, inner centromere protein; RT, reverse transcription.
Morphological studies have served to make the biological function of these proteins a tantalizing puzzle. The distributions of the chromosome passenger proteins during mitosis has led to suggestions that they could be important for such disparate events as regulation of sister chromatid pairing, in

Materials and Methods

Library Screening. mAb 3D3 (6) was used to screen a λgt11 chicken embryo fibroblast cDNA library (gift of B. Vennstrom, Karolinska Institure, Stockholm, Sweden) by standard techniques (3). The insert from one positive plaque was recloned in the pUC18 vector to generate clone I4-14/pUC18. This sequence was used for further screening by nucleic acid hybridization as well as for antibody production. As 5' extensions of I4-14/pUC18 were not recovered from the λgt11 library, we constructed a λZAP II library. Poly(A) RNA was recovered from log-phase MSB-1 cells by oligo-dT batch chromatography (4). Reverse transcription with avian myeloblastosis virus (AMV) reverse transcriptase and library construction were according to the manufacturer's instructions (Stratagene, La Jolla, CA). Multiple rounds of screening yielded overlapping INCENP clones (Fig. 1). However, no candidate translational start site was identified.

Reverse Transcription–PCR. Reverse transcription (RT)–PCR primers were chosen from the 5'-most sequence of INCENP clones. RNA was denatured by exposure to 10 mM methylmercuric hydroxide (Aldrich Chemical Co., Milwaukee, WI) followed by 10 mM /3-mercaptoethanol, then annealed to primer Anti-3 (5'TGTAGACACGTAATCC3') (3). 77th DNA polymerase (Pharmacia Fine Chemicals, Piscataway, NJ) under standard conditions was used to prepare cDNA. Polymerase (Perkin-Elmer Corp., Norwalk, CT) under standard conditions (13), but with 1 mM tetramethyl ammonium hydroxide added to suppress mispriming. Between steps, Sephadex S-300 (Pharmacia) spin-columns were used to separate unincorporated primers from reaction products. 5' extensions were cloned on by PCR (30 cycles: 30 s at 94°C, 30 s at 50°C, 30 s at 72°C; final incubation at 72°C for 7 min) with RACE-1 (5' GATGGATCCTCGAGAAGGTCTCCAGCAATGTGTGTTGCTAGTACCGAATGC 3'; Hind III site underlined) and accomplished with Taq polymerase (Perkin-Elmer Corp., Norwalk, CT) under standard conditions (13), but with 1 mM tetramethyl ammonium hydroxide added to suppress mispriming. Between steps, Sephadex S-300 (Pharmacia) spin-columns were used to separate unincorporated primers from reaction products. 5' extensions were amplified by PCR and then cloned. The resulting clones were sequenced by the method of Sanger et al.

Construction of Deletion Clones

Two deletion mutants of INCENP/pECE were created to dissect INCENP domain structure. The sequence upstream from the unique SalI site at position 82 was eliminated by cutting INCENP/pECE with BamHI and SalI, then religating the blunt-ended fragments. Translation was initiated at the start codon enciphered after this point, methionine as. Accordingly, this construct was identified as INCENP Al-42/pECE. In the second deletion construct, most of the putative coiled-coil region was removed by partial digestion of CI/BS with PstI. The fragment that was cut was used in reverse genetics to disrupt the pECE gene.

Production of Antibodies to INCENPs

Clone 114-1/pUC18 encodes the COOH-terminal 22-kD of the Class I INCENP open reading frame. It was digested with EcoRI and placed into the pATH1 vector (21), and a pLPE-INCENP fusion protein was produced in E. coli. Fusion protein was immunoprecipitated with antibody 3D3 and run on a preparative SDS-polyacrylamide gel. The band migrating at 59 kD (37-kD trpE + 22-kD INCENP) was excised, ground into a powder under liquid nitrogen, and injected into the footpads of two New Zealand White rabbits, using acrylamide as adjuvant (17). Rabbits were boosted three times at 5-wk intervals with the same antigen. The third bleed from each rabbit (rab1INCENP and ra2-INCENP) were used as immune sera, with preimmune sera serving as controls.

Tissue Culture and Electroporation

DU-249 chicken cells (23), primary chick embryo fibroblasts, human HeLa cells, and porcine LLCPK cells were grown as monolayers in plastic flasks (Corning Inc., Corning, NY) in DME (GIBCO-BRL) with 10% FCS (Hy-Cone Laboratories, Logan, UT). MSB-1 chicken lymphoblasts were grown in spinner culture as previously described (10).
**Results**

The INCENPs Are Novel Multidomain Proteins with a Predicted Central Coiled-Coil Motif

We have recovered cDNA clones that encode the bulk of the INCENP transcript. Restriction digests of 26 independent isolates revealed the presence of two classes of clones that could be distinguished by the absence (class I) or presence (class II) of an internal EcoRI site. Clone X24/BS extended farthest in the 5' direction, but did not include an initiation codon. The 5' portion of the cDNA was ultimately recovered by RT-PCR (see Materials and Methods). Using this method, we recovered an additional 49 base pairs of 5' cDNA sequence. This contains a single candidate methionine codon in a context consistent with the consensus proposed for translational initiation (22). Four codons upstream is a TAA stop codon in the same reading frame (Fig. 2). Clone PCR-1/BS and six other clones containing both the ATG and upstream TAA were recovered from independent RT-PCR reactions.

The primary sequences of both classes of INCENP cDNAs were determined by the dideoxy method. Both contained single, long open reading frames that were virtually identical to one another, with the notable exception of an insert of 114 bp in the class II clones (see large box in Fig. 2). In addition, two single-base polymorphisms were observed: ACT to GCT led to a thr to ala substitution at residue 257, while an A to G change in the wobble position left residue 281 unaltered (Fig. 2).

The predicted INCENP polypeptides have molecular weights of 96,357 D (class I) and 100,931 D (class II). They are hydrophilic along their lengths and highly basic (pl ≈ 9.5; ~22% lys + arg + his). Searches of the GenBank database with the TFASTA (31) and BLAST (1) search strategies indicated that the INCENPs do not closely resemble other known proteins.

The initial analysis of the predicted open reading frames of the INCENP cDNAs indicated that residues 503–717 (class I) and 503–752 (class II) contain heptad repeats that might permit the formation of an α-helical secondary structure (Fig. 3 A). Further analysis of the INCENP deduced amino acid sequences with the "coilcoil" computer program showed that the INCENP sequences in these regions have primary sequence similarities to the coiled-coil domains of myosins, tropomyosins, and keratins (24). Based on this comparison, the coilcoil program estimates that there is a high probability that these INCENP residues can form a coiled-coil structure. Interestingly, a single frame for the predicted heptad repeat is not maintained for the length of the putative coiled coil. Instead, "stutters" appear to break up the domain into eight (class I) or nine (class II) blocks (Fig. 3 A).

One possible mechanism for bringing about the changes in INCENP intracellular localization during mitosis would be posttranslational modification, as is the case for the nuclear lamins (16) and numerous other proteins. Examination of the deduced sequences for consensus phosphorylation motifs revealed many potential sites for modification shared by the two classes. These include possible sites for pS/pT (X24/BS) at residues 57 (KTPS), 193 (KSPK), and 448 (KTPS), and a tyrosine kinase site at residue 425 (KRAVDQRY) (Fig. 2). In addition, INCENPs may be substrates of casein kinase (12...
bands on immunoblots. These affinity-purified antibodies recognize a human homologue of the INCENPs by indirect immunofluorescence, both rabbit sera recognized a set of epitopes defined by antibody 3D3 (6) and used to immunize two rabbits.

To generate polyclonal antisera against the INCENPs, polyclonal antisera were produced in rabbits using the amino acid sequence spanning residues 68, 303, 532, 592, and 614 of RaMNCENP and ra2-INCENP. Each antisera recognized both bands on fresh immunoblots, and gave a pattern indistinguishable from whole serum by immunofluorescence of chicken cells (data not shown).

Polyclonal Antibodies Validate the cDNA Clones

To confirm that the INCENPs are chromosome scaffold components, cDNA clones were expressed in human (HeLa) cells using the pECE expression vector. The coding regions of the class I and II INCENP cDNAs were amplified from cDNA of colcemid-arrested cells by RT-PCR, cloned and expressed in human cells. The constructs were termed INCENPAS11-696/pECE (see below).

Comparison of the reactivities of mAb 3D3, ral-INCENP, and ra2-INCENP reveals at least three epitopes shared by the cloned and bona fide INCENPs. First, mAb 3D3 cross-reacts in immunoblots with an 80-kD nuclear protein expressed in chicken cells (data not shown).

Because the INCENPs are nuclear proteins during interphase, we searched for the presence of these proteins using indirect immunofluorescence beginning at residues 68, 303, 532, 592, and 614 of RaMNCENP and ra2-INCENP. Each antisera recognized both bands on fresh immunoblots, and gave a pattern indistinguishable from whole serum by immunofluorescence of chicken cells (data not shown).

Figure 2. DNA and deduced amino acid sequence of the class II INCENP cDNA. This sequence was compiled from clones PCR-1/BS, X24/BS, II/73/BS, and I-75/BS. The in-frame stop codon at position 12 is underlined. Codons including polymorphisms (positions 769 and 843) are boxed; amino acid residue 257 is altered to thr in class I. The 114-bp (38 residues) that are omitted from class I (bases 2146 to 2259) are also boxed, and the class II internal EcoRI site (2259) is italicized. These sequences are available from EMBL and GenBank under accession numbers Z25419 (class I), and Z25420 (class II).
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have expressed using the pECE vector, we could find many transfected with INCENP eDNA expression constructs. We examined the ability of class I and class II INCENPs to localize in mammalian cells when cells were transiently transfected mammalian cells migrate close to native chicken INCENPs from metaphase chromosomes in SDS-PAGE. Whole cell lysates of LLCPK cells were prepared 24 h after electroporation with INCENP expression constructs. Left panel (lanes 1–6): Immunoblot was probed with a mixture of raI-INCENP (1:1,000) and adsorbed ra2-INCENP (1:1,000). LLCPK cells were transfected with INCENPpECE (lane 2), INCENPpECE (lane 3), INCENPpECE (lane 5), or pECE (lane 6). Chromosomes from colcemid-blocked chicken cells (lanes 1 and 4) served as controls. 18-h exposure. Right panel (lanes 7–9): Immunoblot was probed with unadsorbed ra2-INCENP (1:500) to maximize detection of INCENP, INCENP, and INCENP, respectively. Bands visible at 50, and 160 kD result from the recognition by ra2-INCENP of unidentified, non-INCENP cellular proteins. 36-h exposure. Or, origin; St, stacker.

**Cloned Chicken INCENPs Localize Correctly in Transfected Mammalian Cells**

We examined the ability of class I and class II INCENPs to localize in mammalian cells when cells were transiently transfected with INCENP cDNA expression constructs. With each of the full-length and deletion constructs that we have expressed using the pECE vector, we could find many cells in which INCENP products accumulated to near physiological levels, as judged by indirect immunofluorescence. This is a significant advantage when examining localization of the protein to intracellular compartments.

Within 24 h of electroporation of the constructs encoding INCENP, or INCENP into HeLa or LLCPK cells, 30 to 60% of the cells expressed recombinant INCENP antigen that was readily detectable by immunoblotting and by indirect immunofluorescence. We routinely detected the expressed proteins with raI-INCENP (Fig. 7) or mAb 3D3 (data not shown), since these antibodies do not recognize the endogenous INCENPs of mammalian cells. Thus, the transfected proteins could be detected selectively in the presence of a background of endogenous proteins without the necessity of attaching epitope tags.

As mammalian cells expressing chicken class I INCENPs traversed the mitotic cycle, the expressed proteins underwent the characteristic distribution pattern originally observed in chicken cells (Fig. 7). In interphase, INCENP staining was typically confined to the nucleus. In mitosis, the expressed proteins were concentrated on the chromosomes from prophase through metaphase. The expressed proteins separated from the chromosomes by earliest anaphase, becoming localized to streaks in the spindle midzone. These streaks were parallel to the long axis of the spindle. The cell cortex in the neighborhood of the nascent cleavage furrow was also stained. As anaphase proceeded, the INCENP polypeptide became progressively more concentrated at the spindle midzone. At the conclusion of telophase, the intercellular bridge stained intensely for INCENP, on each side of the midbody. In some cells with very high expression, additional staining could be seen in the reforming nucleus (data not shown). Expression of moderate levels of INCENP polypeptide did not appear to have deleterious effects on transiently transfected LLCPK cells. Most mitotic figures were normal in appearance (Fig. 7), and daughter cells separated by chicken INCENP-containing intercellular bridges were commonly seen.

These results provide conclusive proof that the cDNA clones described above encode INCENP polypeptides that contain all the signals needed for proper INCENP localization in the cell (Fig. 7). In addition, the results demonstrate that the cellular factors responsible for the various alterations in targeting of the INCENPs across the mitotic cycle are conserved between chicken and mammalian cells.

A small proportion of cells transfected with class I INCENP cDNA greatly overexpressed expressed the protein (“jackpot” cells). As expected, the nuclei of such cells stained intensely for chicken INCENP. Unexpectedly, however, significant levels of INCENP staining were also observed in the cytoplasm as a bright, fibrillar network. This cytoplasmic network was completely coincident with that...
Figure 7. Class I chicken INCENP distribution in transiently transfected mitotic LLCPK cells mimics the INCENP distribution in chicken cells. Cells were fixed 24 h after electroporation with INCENP/pECE. Interphase (A), prophase (B), metaphase (C), anaphase (D), and late telophase/early G1 (E). Cells were stained with ral-INCENP (left column), mouse monoclonal anti-tubulins (center column), and DAPI (right column). Bar, 10 μm.
INCENP and INCENPΔΔ-42 decorate cytoplasmic microtubules in interphase "jackpot" LLCPK cells, while INCENP-Δ511-696 does not. Cells were fixed 24 h after electroporation with INCENP/pECE (D), INCENPΔΔ-42/pECE (B), or INCENP-Δ511-696/pECE (C). Cells in A-C were "jackpot" cells, while the cell shown in D was expressing INCENP at a lower level. Cells were stained with m1-INCENP (left column, A, B, D) or adsorbed m2-INCENP (left column, C), mouse monoclonal anti-tubulins (center column), and DAPI (right column). Bar, 10 μm.

The appearance of INCENP in the cytoplasm seemed to alter the structure of cytoplasmic microtubules. The microtubules appeared to be bundled into coarser fibrils than those seen in control cells. While many microtubules in control cells ran in more or less straight lines from near the nucleus to the periphery of the cell, those in "jackpot" cells were usually curly. Rather than terminating at the periphery of the cell, fibrils seemed to curve back into the cell (Fig. 8 A). In addition, cytoplasmic INCENPs appeared to enhance mi-

visualized by staining with antibodies to tubulin (Fig. 8 A). The INCENP-decorated fibrils did not colocalize with either intermediate filaments (visualized with antibodies to keratins and vimentin), or with actin (visualized with phalloidin staining—data not shown). We do not yet know if the INCENP polypeptide binds directly to microtubules, or interacts indirectly via microtubule-associated proteins.

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Figure 8. INCENP and INCENPΔΔ-42 decorate cytoplasmic microtubules in interphase "jackpot" LLCPK cells, while INCENP-Δ511-696 does not. Cells were fixed 24 h after electroporation with INCENP/pECE (D), INCENPΔΔ-42/pECE (B), or INCENP-Δ511-696/pECE (C). Cells in A-C were "jackpot" cells, while the cell shown in D was expressing INCENP at a lower level. Cells were stained with m1-INCENP (left column, A, B, D) or adsorbed m2-INCENP (left column, C), mouse monoclonal anti-tubulins (center column), and DAPI (right column). Bar, 10 μm.

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crotubule stability. Even when transfected cells were fixed under conditions that were not favorable for microtubule preservation, the microtubule network of "jackpot" cells could be visualized. No "jackpot" cells were ever observed in any stage of mitosis.

**Identification of an Amino Acid Motif Required for Targeting of the INCENPs to the Anaphase Spindle and Midbody**

The deduced amino acid sequence of the INCENPs suggested an obvious multidomain organization for these proteins, but gave no clues as to the biological function of any given domain. We therefore began a functional dissection of the INCENP polypeptide by creating chimeric forms of the protein to determine how these localized during the cell cycle. The properties of two of these chimeric INCENP polypeptides will be described here.

The first of these constructs identified a region of the INCENPs that is required for the transfer of class I INCENPs from the chromosomes to the spindle at the metaphase-anaphase transition. This region includes the amino-terminal 42 amino acids of class I INCENPs. These residues were deleted in the amino-terminal deletion mutant INCENP\(_\Delta\)-42/pECE. The resulting truncated INCENP was detected in transiently transfected mammalian cells. We will refer to this chimeric protein as INCENP\(_\Delta\)-42.

While INCENP\(_\Delta\)-42 localized normally to interphase cells and in mitosis through metaphase, a dramatic difference in localization occurred at the metaphase-anaphase transition. At this time, INCENP\(_\Delta\)-42 completely failed to transfer to the spindle midzone. Instead, it remained attached to the chromosomes as they moved towards the poles in anaphase (Fig. 9D).

Interestingly, the inability of the INCENP\(_\Delta\)-42 polypeptide to transfer from the chromosomes to the spindle does not appear to reflect an inability of the protein to bind to and bundle microtubules. As with full length INCENP\(_\alpha\), cytoplasmic INCENP\(_\Delta\)-42 polypeptide apparently attaches to microtubules and bundles them in "jackpot" cells (Fig. 8B).

**The INCENP Coiled-Coil Domain Is Apparently Required for Microtubule Colocalization and Bundling**

Perhaps the most obvious feature of the deduced INCENP polypeptide sequence is the predicted \(\alpha\)-helical coiled coil that spans residues 503–717 of the class I polypeptide. In addition to serving as rod-like "spacers," such coiled-coil domains are essential for the oligomerization of many structural proteins, including intermediate filament proteins (30) and myosin (14). We would thus predict that disruption of the coiled coil might have a profound effect on the INCENP structure and function.

To investigate the role of the predicted coiled-coil domain in INCENP function, we generated a construct in which most of this domain was deleted, reducing the predicted coiled-coil from 215– to 29–amino acid residues. We refer to this expressed protein as INCENP\(_\Delta\)-511–696 (Fig. 3B). INCENP\(_\Delta\)-511–696 was not recognized by antibodies 3D3 or ral-INCP. It was, however, recognized by ra2-INCP which had been pre-adsorbed against fixed LLCPK cells to remove its weak cross-reactivity to porcine INCENP homologues.

Most surprisingly, removal of the coiled-coil domain had no detectable effect on the ability of INCENP\(_\Delta\)-511–696 to undergo the normal pattern of INCENP rearrangements as transfected cells traversed the mitotic cycle. Thus, the protein entered the nucleus normally during interphase, was associated with the chromosomes through metaphase, and transferred to the central spindle, cleavage furrow and stem body during anaphase and telophase (data not shown).

Since INCENP\(_\Delta\)-511–696 apparently assembled and targeted correctly throughout mitosis, the behavior of this protein in "jackpot" cells was surprising. As was the case for all INCENP constructs, when grossly overexpressed, a substantial portion of INCENP\(_\Delta\)-511–696 was observed to accumulate in the cytoplasm. Unlike all other constructs examined to date, however, this altered protein did not associate with the cytoplasmic microtubules, which remained unbundled (Fig. 8C). However, some "jackpot" cells displayed disordered microtubule networks, and were rounded instead of flattened on the growth substrate (data not shown). It appears that the coiled-coil domain of INCENP\(_\alpha\) is required for this protein to associate with and bundle cytoplasmic microtubules.

**Discussion**

**Cloning of Two Classes of INCENP cDNAs**

The INCENPs were first identified with a mAb elicited by immunization with chromosome scaffolds. The antibody recognized two polypeptide antigens (135 and 150 kD) that are chromosomal through metaphase, and associated with the cytoskeleton during the final stages of mitosis (6). The deduced amino acid sequences obtained from two classes of cDNA clones that span the entire open reading frames of the two INCENPs yield molecular masses of 96,357 D for class I INCENP (referred to here as INCENP\(_\alpha\)) and 100,931 D for class II INCENP (INCENP\(_\beta\)). This size difference arises from the insertion of an additional 38 residues in the INCENP\(_\beta\) polypeptide, apparently as the result of alternative splicing of a single primary transcript. The deduced INCENP polypeptides are very basic (pI \(\sim\) 9.5) polypeptides that contain long predicted coiled-coil domains. They do not share significant sequence relationships with other known proteins.

The cDNA clones described here seem to cover the entire INCENP coding region. All 5' cDNA clones contain an in-frame stop codon just upstream from the presumptive initiation codon. In addition, when the cloned cDNAs were transiently expressed in mammalian cells, they produced proteins that migrated close to the endogenous forms of these proteins in SDS polyacrylamide gels.

**The INCENPs and Their Intracellular Ligands Are Functionally Conserved between Birds and Mammals**

The characteristic pattern of movements undertaken by the INCENPs during the mitotic cycle suggests that these proteins interact specifically with at least four different cellular ligands. These include: (a) the nuclear import machinery (7, 15, 27); (b) chromatin, particularly in the region of the centromere; (c) the anti-parallel microtubules of the spindle overlap zone; and (d) the inner surface of the plasma membrane at the cleavage furrow. In addition, examination of
Figure 9. The first 42 amino acids of INCENP \textsubscript{p} are required for INCENP\textsubscript{p} transfer from chromosomes to the mitotic spindle. LLCPK cells were electroporated with INCENP\textsubscript{p},Δ1–42/pECE, fixed after 24 h, and stained with rat-INCENP (left column), mouse monoclonal anti-tubulins (center column), and DAPI (right column). Interphase (A), prophase (B), metaphase (C), anaphase (D), and telophase (E). Bar, 10 μm.
cells that grossly overexpress the INCENP polypeptide in transient transfection experiments ("jackpot" cells) reveals that the INCENPs are capable of associating with and bundling parallel microtubules.

The present studies indicate that the intracellular ligands with which the INCENPs interact and the regulatory signals that govern these interactions during mitosis are functionally conserved between birds and mammals. When chicken INCENP cDNAs were expressed in human (HeLa) and pig (LLCPK) cells, both INCENP I and INCENP II polypeptides showed a normal pattern of distribution across the cell cycle. In addition, antibody ra2-INCENP clearly recognizes proteins homologous to the chicken INCENPs in human and Indian muntjac cells. Antibody ra2-INCENP reactivity against porcine homologs in LLCPK cells is weaker, but still detectable.

At present, nothing is known about how INCENP affinities for their ligands change across the mitotic cycle.

**The INCENPs Are Cytoskeletal Proteins That Can Alter Microtubule Morphology**

Throughout most of the cell cycle, the INCENPs are located in the nucleus and on the chromosomes. In fact, when chromosomes are isolated from colcemid-blocked cells, the INCENPs are among the most tightly associated proteins known, being nearly quantitatively retained in the chromosome scaffold fraction.

The data obtained here demonstrate that the INCENPs may act as cytoskeletal proteins that are potentially capable of altering the morphology of the cellular microtubule network during interphase. We have shown here that when the INCENPs are sufficiently overexpressed, as in jackpot cells, the proteins appear to exceed the ability of the nucleus to sequester them, and accumulate in the cytoplasm. There, the cytoplasmic INCENPs associate with the microtubules and cause them to assemble into bundles, which curl around and terminate in the cellular interior rather than at the edge of the cell.

The apparent exclusion of INCENPs and INCENP homologs from the cytoplasm of wild-type, untransfected cells complicates the interpretation of jackpot cells. The association of INCENP I with microtubules could result from relatively unspecific, low-affinity interactions that are not reflective of any physiological role of the INCENPs. However, a number of lines of evidence suggest that INCENP I-microtubule interactions may be relevant to INCENP function. First, INCENPs appear to be closely associated with microtubules during the later stages of mitosis (8). Second, while few proteins that have been overexpressed by transient transfection of mammalian cells are known, being nearly quantitatively retained in the chromosome scaffold fraction.

Most of the Coiled-Coil Domain Is Dispensable for Localization of the INCENPs during Mitosis

Computer analysis of the deduced INCENP polypeptide sequence predicts the existence of an internal coiled-coil domain (residues 503 to 717 in class I, residues 503 to 733 in class II). Studies of proteins such as myosin have shown that such coiled-coil domains can be crucial for dimerization and higher-order assembly of structural proteins (14). Thus, the INCENPs are likely to form homodimers or heterodimers in vivo.

Given this prediction, together with the importance of coiled-coil interactions for intermediate filament structure
and organization (12) we anticipated that truncation of the coiled-coil domain should severely impair INCENP function. We were thus surprised to note that the distribution of INCENP-ΔD51-696 was apparently normal across the mitotic cycle. In this polypeptide, the coiled-coil has been reduced from 215 amino acid residues to 29.

This unexpected result could be explained if the remaining vestige of the coiled coil domain were sufficient to permit INCENP-ΔD51-696 to oligomerize with its normal binding partners. Alternatively, the coiled-coil might not be essential for the proper placement of INCENPs in mitotic cells. Thus, the subcellular localization of the INCENPs during the mitotic cycle could be primarily dictated by interactions of the amino and carboxy-terminal domains with ligands in the chromosome and spindle. If this is the case, then our results demonstrate that the spacing between these domains is not critical.

The Ability to Associate with Microtubules Is Insufficient to Target the INCENPs to the Mitotic Spindle

Comparison of the behavior of the mutant INCENP lacking the amino terminus with the one lacking the coiled-coil domain suggested an unexpected level of complexity in the associations of the INCENPs with the spindle midzone. On the one hand, the mutant that could not associate with the spindle midzone (INCENP-ΔA42) retained its ability to associate with and bundle cytoplasmic microtubules in jackpot cells. On the other hand, the mutant polypeptide lacking the coiled coil (INCENP-ΔD51-696) remained able to associate with the spindle and midbody throughout anaphase and telophase, but failed to associate with cytoplasmic microtubules in interphase jackpot cells. To our knowledge, this is the first instance where a putative coiled-coil domain has been implicated in association of a protein with microtubules.

These seemingly paradoxical observations could be reconciled if the coiled-coil regions of the INCENPs were required for interactions with parallel microtubules, while the amino-terminal 42 amino acids were required only for association with anti-parallel microtubules in the central spindle. Alternatively, INCENP localization in the central spindle might be due to interactions with spindle components other than microtubules, such as components of the stem body material. It will be important in future experiments to examine the binding of purified INCENPs to microtubules and isolated mitotic spindles in vitro.

Conclusions

The functional analysis of the INCENPs presented here provides support for the notion that interphase cells regulate the activity of cytoskeletal proteins that play an important role during the latter stages of mitosis. For example, the INCENPs are sequestered in the nucleus so that they cannot make contact with the interphase cytoskeleton. Thus, when chromosomes move to the metaphase plate during mitosis, they apparently bring with them not only the DNA in a form suitable for transmission to the daughter cells, but in addition, a class of cytoskeletal "passenger" proteins whose role is to participate in the construction of the mitotic spindle and cleavage apparatus. Further analysis of proteins such as the INCENPs should reveal the role this chromosomal:cytoskeletal "collaboration" plays in shaping events during the closing phases of mitosis.

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