Chromosoma (2001) 110:65–74
DOI 10.1007/s004120100130

ORIGINAL ARTICLE

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Human *INCENP* colocalizes with the Aurora-B/AIRK2 kinase on chromosomes and is overexpressed in tumour cells

Received: 15 October 2000 / In revised form: 23 December 2000 / Accepted: 23 December 2000 / Published online: 30 March 2001

Abstract The inner centromere protein (INCENP), which has previously been described in chicken, frog and mouse, is required for correct chromosome segregation and cytokinesis. We have identified the human *INCENP* gene by library screening and reverse transcription-polymerase chain reaction (RT-PCR) and localized it to chromosomal region 11q12. *HsINCENP* is a single-copy gene that consists of 17 exons and covers 25 kb of genomic DNA. The gene is expressed at highest levels in the colon, testis and prostate, consistent with its likely role in cell proliferation. *HsINCENP* encodes a highly basic protein of 915 amino acids that localizes to metaphase chromosomes and to the mitotic spindle and equatorial cortex at anaphase. Recently we showed that INCENP is stockpiled in a complex with the Aurora-B/XAIRK2 kinase in *Xenopus* eggs. Here we demonstrate that, consistent with such an interaction, the two proteins colocalize on human metaphase chromosomes. Levels of Aurora-B are increased in several human cancers, and we show here that HsINCENP protein levels are also significantly increased in several colorectal cancer cell lines.

Abbreviations *aa:* amino acid(s) · *ACA:* anti-centromere antibodies · *AIRK:* Aurora/Ipl1-related kinase · *bp:* base pair(s) · *cAMP-PKA:* cyclic AMP-dependent protein kinase A · *DAPI*: 4′,6-diamidino-2-phenylindole · *EST:* expressed sequence tag · *INCENP:* inner centromere protein · *ORF*: open reading frame · *MAP kinase:* mitogen-activated protein kinase · *PBS:* phosphate-buffered saline · *RT-PCR:* reverse transcription-polymerase chain reaction

Edited by: W. Hennig

Accession number: *HsINCENP*, AF282265

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Introduction

During mitosis, the replicated chromosomes must segregate equally to the two new daughter cells. In order for this to succeed, it is essential that cell cleavage (cytokinesis) does not occur until sister chromatids have segregated to opposite poles of the cell during anaphase. The reorganization of the cytoskeleton that occurs during cytokinesis therefore needs to be tightly coupled to the movement of the chromosomes. One class of molecules that has been proposed to integrate chromosomal and cytoskeletal behaviour during mitosis is the "chromosomal passenger proteins" such as the inner centromere protein (INCENP) (Cooke et al. 1987), and the unidentified antigen TD-60 (Andreassen et al. 1991) (for reviews see Earnshaw and Bernat 1990; Earnshaw and Mackay 1994; Adams et al. 2001). These proteins are associated with chromosomes during the early stages of mitosis and become concentrated at the centromeres by metaphase. However, as the sister chromatids separate at anaphase, the passengers transfer to the antiparallel microtubule array of the central spindle and to the nascent cleavage furrow at the equatorial cortex. Indeed, the concentration of INCENP at the equatorial cortex precedes that of myosin (Eckley et al. 1997), suggesting that INCENP could be involved in early events during the assembly of the

cleavage furrow. The serine/threonine kinase Aurora-B also appears closely associated with INCENP and TD-60 during ana/telophase (Martineau-Thuillier et al. 1998). Ultimately INCENP and the other passenger proteins become focused at the midbody before their elimination from the cell following cytokinesis.

INCENP was first isolated as a component of the chicken mitotic chromosome scaffold (Cooke et al. 1987). Subsequent molecular cloning analysis revealed that INCENP is an M_r 130,000 basic protein containing an extensive predicted coiled-coil domain and multiple predicted phosphorylation sites for CDK1, cyclic AMP-dependent protein kinase A (cAMP-PKA) and mitogenactivated protein kinase (Mackay et al. 1993). An INCENP mutant lacking its C-terminal half is unable to transfer to the central spindle microtubules during metaphase. In transient transfections, this dominant-negative mutant disrupts prometaphase chromosome congression, anaphase chromosome segregation and the completion of cytokinesis (Mackay et al. 1998). The phenotype of embryos from knockout mice homozygous for a disruption of the murine INCENP gene suggested that the gene is required at multiple stages of mitosis (Cutts et al. 1999). Mutant embryos die at the 32–64 cell stage with multinucleated cells, multipolar mitotic spindles, microtubule abnormalities, and chromatin bridges. Unfortunately, the early death of the embryos precluded the observation of significant numbers of anaphase cells, and the role of INCENP during ana/telophase could not be directly determined in these experiments. Furthermore, the possibility of dominant-negative effects could not be entirely excluded as the knockout allele had the potential to encode the N-terminal portion of INCENP.

In a recent study, we have shown that INCENP is stockpiled in *Xenopus* eggs in a soluble 11S complex with an Aurora/Ipl1-related kinase, Aurora-B/XAIRK2 (Adams et al. 2000). For a discussion of Aurora kinase nomenclature, see Adams et al. (2001). We also demonstrated that INCENP is required for the correct mitotic localization of this kinase in cultured human cells. The Aurora family of kinases is present from yeast to human and may regulate several stages of mitosis such as centrosome separation, chromosome segregation and cytokinesis (Bischoff and Plowman 1999; Giet and Prigent 1999). Functional analysis of the Aurora-B/AIR-2 kinase by RNA interference (RNAi) in *Caenorhabditis elegans* and overexpression of dominant mutants in human tissue culture cells have revealed that this protein is necessary for faithful chromosome segregation and for the successful completion of cytokinesis (Schumacher et al. 1998; Tatsuka et al. 1998). Recent work has shown that *C. elegans* Aurora-B and *Saccharomyces cerevisiae* Ipl1p may regulate chromosome behaviour via phosphorylation of histone H3 on serine-10 (Hsu et al. 2000). Defects in the regulation of this phosphorylation lead to aberrant chromosome condensation and segregation (Speliotes et al. 2000). We speculate that INCENP may act to target Aurora-B to histone H3 and to other substrates. Additionally, INCENP itself may also be a substrate or regulator of kinase activity.

The aim of the present study was to characterize human INCENP, map its genomic structure and determine its mRNA and protein expression patterns in normal and transformed cells. HsINCENP and HsAurora-B/AIM-1 colocalize not only at the cleavage furrow and spindle midzone but also at centromeres during metaphase. Furthermore, like HsAurora-B, INCENP expression is elevated in a number of colorectal cell lines.

Materials and methods

Cloning of *HsINCENP*

Standard molecular biology methods were used throughout (Sambrook et al. 1989). To clone *HsINCENP*, a HeLa cDNA expression library was screened with a cross-reactive antibody, Rb1005. Two clones were obtained, the largest containing a 1671 bp insert with extensive homology at the amino acid level to chicken *INCENP* between residues 311–738. Following the release of the sequence of a 104 kb bacterial artificial chromosome clone of human genomic DNA (Genbank AA003023) consisting of 22 unordered contigs, and expressed sequence tags (ESTs) of the mouse INCENP gene, we were able to assemble the full *HsINCENP* sequence by searching for exons with homology to mouse INCENP. The predicted *HsINCENP* sequence was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) using the Enhanced Avian system (Sigma). Reverse transcription of 1 µg of HeLa mRNA was performed at 60°C using oligonucleotides 5′-CATGAGCTGGGTGACATGC-3′ for the 5′ end of the gene and 5′-AGACATGGACAGGAGGCGAGG-3′ for the 3′ end. The resulting cDNAs were amplified using 5′-TCACCAGACAGAGCACCATGG-3′ and 5′-AGCTGCAGAGGGAACTGGAGG-3′, respectively, cloned into pGEM-T (Promega) and sequenced on both strands. The sequence is available from GenBank, Accession number AF282265.

RNA and Southern blotting

Total human genomic DNA (8 µg, Stratagene) was digested with SspI or SphI, electrophoresed in a 0.6% agarose gel and transferred to nitrocellulose. The blot was probed with a ^{32}P-labelled 500 bp fragment derived from the 3′ end of the *HsINCENP* cDNA. For analysis of INCENP expression in different tissues, a commercially prepared human multiple tissue RNA blot (Clontech) was probed according to the manufacturer's instructions using the same probe as for Southern blotting.

Cell culture, immunofluorescence staining and immunoblotting

Adherent HeLa cells grown on polylysine-coated coverslips were fixed for 5 min using 4% formaldehyde, then permeabilized with 0.2% Triton X-100 in cytoskeleton buffer CB [137 mM NaCl, 5 mM KCl, 1.1 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 2 mM $MgCl_2$, 2 mM EGTA, 5 mM piperazine-N,N′-bis(2-ethanesulphonic acid), 5.5 mM glucose, pH 6.1] (Small 1981). Fixed cells were blocked in PBS, 1% bovine serum albumin and immunostained with antibodies to INCENP (RbD, 1:500) and to tubulin (Tu27B, undiluted; a gift from Lester Binder). Secondary antibodies (Vector) were applied at a 1:100 dilution; DNA was stained with 1 µg/ml 4′,6-diamidino-2-phenylindole (DAPI) simultaneously with the secondary antibodies. For immunoblotting of HeLa cell extracts, chromosomes were isolated from 10^6 cells, run on 7.5% SDS-polyacrylamide gels and blotted to nitrocellulose. HsINCENP was detected with the cross-reactive antibodies Rb1005, Rb1186 (Eckley et al. 1997), and ra2-INCENP (Mackay et al. 1993), all at a dilution of 1:500.

Mitotic chromosome spreads were prepared from exponentially growing HeLa cells treated with 30 ng/ml Colcemid for 2 h. Cells were harvested by mitotic shake-off, collected by centrifugation at 1200 rpm for 10 min and then gently resuspended in 75 mM KCl. After 10 min at 25°C, the hypotonically treated cells were sedimented onto slides by cytocentrifugation. Slides were washed for 10 min in buffer A (10 mM TRIS-HCl, 120 mM NaCl, 0.5 mM EDTA, 0.1% Triton X-100, pH 8.0; Jeppesen et al. 1992). Slides were then incubated for 30 min at 37°C with primary antibodies [ACA, 1:500; anti-INCENP 1186, 1:500; anti-Aurora-B/AIM-1 (Transduction Labs), 1:50], washed three times for 10 min in buffer A and then incubated with fluorescent secondary antibodies (Jackson). After washing three times in buffer A, cells were fixed using 4% paraformaldehyde in buffer A for 15 min. After washing in PBS the DNA was stained with DAPI (Sigma) at 1 µg/ml for 8 min.

Quantitative immunoblotting of INCENP levels in colorectal carcinomas

Human WI38 fibroblasts and colon cancer cell lines were cultured in the media recommended by the supplier (American Type Culture Collection, Manassas, Va.) until they were 50% confluent. Cells were then harvested by trypsinization, washed twice with serum-free RPMI 1640 medium containing 10 mM HEPES (pH 7.4 at 4°C), and solubilized at a concentration of 3×10^7 cells/ml in SDS sample buffer consisting of 62.5 mM TRIS-HCl (pH 6.8 at 21°C), 4 M urea, 2% (w/v) SDS, 1 mM EDTA and 5% (v/v) β-mercaptoethanol. Alternatively, cells were washed twice with RPMI-HEPES and lysed by incubation for 15 min at 4°C with buffer A [50 mM TRIS-HCl (pH 7.4 at 4°C), 250 mM sucrose, 5 mM $MgSO_4$, 1 mM freshly added phenylmethylsulphonyl fluoride, 100 U/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin and 1% (v/v) β-mercaptoethanol] containing 0.5% (w/v) Nonidet P-40. The nuclei were sedimented at 800 *g* for 10 min, washed once with buffer A, and solubilized at a concentration of 1×10^8 cells/ml in SDS sample buffer. Sonicated samples containing protein from 3×10^5 cells or 1.5×10^6 nuclei were separated on SDS-polyacrylamide gels containing a 5%–15% acrylamide gradient, transferred to nitrocellulose, and probed with anti-INCENP serum or anti-Aurora-B/AIM-1 antibodies followed by antibody to histone H1 (kindly provided by James Sorace, Veteran's Affairs Medical Center, Baltimore, Md.) as a loading control. Blots were scanned on a Kodak Umax Supervista S-12 scanner, and data were expressed as the INCENP:histone H1 ratio in various cell lines.

Results

Identification of *HsINCENP*

Human INCENP is very poorly represented in EST databases, and the complete open reading frame (ORF) of human *INCENP* (*HsINCENP*) was assembled from a number of sources. Initially we screened a HeLa cDNA expression library using an antibody raised against chicken INCENP. This antiserum cross-reacted with a similar-sized protein on immunoblots of human chromosomal protein. This screen yielded a partial cDNA encoding a protein with homology to the central part of chicken INCENP. Efforts to obtain further INCENP cDNA clones by screening a number of other cDNA libraries were unsuccessful. Over time, a number of ESTs corresponding to the murine INCENP were released into the databases. These, together with the sequence of our cDNA enabled us to use the release of an unordered human genomic sequence (AA003023) to deduce the organization of the human INCENP gene. This deduction was confirmed by RT-PCR of the 5′ and 3′ ends of the gene using primers based upon the genomic sequence and by sequence comparison with mouse *INCENP*. In addition, an EST from a human fetal cDNA library covering the 3′ end of the *INCENP* gene (AA993703) was found to contain a polyadenylation sequence 310 nucleotides downstream of the stop codon, consistent with the genomic sequence.

Southern blotting of human genomic DNA using a 3′ cDNA probe confirmed the existence of a single *HsINCENP* gene (Fig. 1B). The human INCENP gene covers approximately 25 kb of DNA in chromosomal region 11q12–13 and contains 17 exons (Fig. 1A). All the major inherited disease genes in this region have previously been identified and so it is unlikely that defects in *HsINCENP* are responsible for inherited disease.

The deduced protein sequence of HsINCENP is shown in Fig.2. We believe we have located the correct translation initiation site for three reasons. First, the ATG is contained within an exact match of the Kozak translation initiation consensus sequence (Kozak 1987). Second, the predicted first seven amino acids of HsINCENP are identical to the first seven amino acids of mouse INCENP. Third, there is no other candidate upstream ORF that is not interrupted by a stop codon before reaching the known INCENP sequence.

HsINCENP is deduced to be a polypeptide of 915 amino acids with a predicted molecular weight of M_r 106,000. The protein has extensive homology to the INCENPs of mouse (65% identity), *Xenopus* (39.9% identity) and chicken (44% identity). Like INCENPs in other organisms, HsINCENP is extremely basic, with a calculated isoelectric point of pH 9.91. HsINCENP contains multiple consensus bipartite nuclear localization signals, and predicted phosphorylation sites for CDK1 (KTPS at 58–61 and 475–478), PKA (8 sites), casein kinase II (10 sites) and protein kinase C (11 sites). A coiled-coil-forming region is predicted between residues 520–780 (grey box in Fig. 2).

Three peptide motifs are absolutely conserved in all vertebrate INCENP sequences reported to date: PELPMPTKPSQK (54–64), YGMDLNSDDSTDDE (819–832) and KRTSSAVW (887–894). We have previously shown that the first of these sequences is necessary for the timely transfer of INCENP from the centromere to the spindle at anaphase (Ainsztein et al. 1998) but the significance of the latter two domains remains unknown. However, both are found in the conserved IN-Box (blue box in Fig. 2), which defines the INCENP family from yeasts to mammals, and are therefore likely to be functionally significant.

Analysis of tissue expression of *HsINCENP*

To determine the expression pattern of HsINCENP mRNA in different cell types, we probed a human multiple tissue RNA blot with a 5′ and 3′ cDNA probe

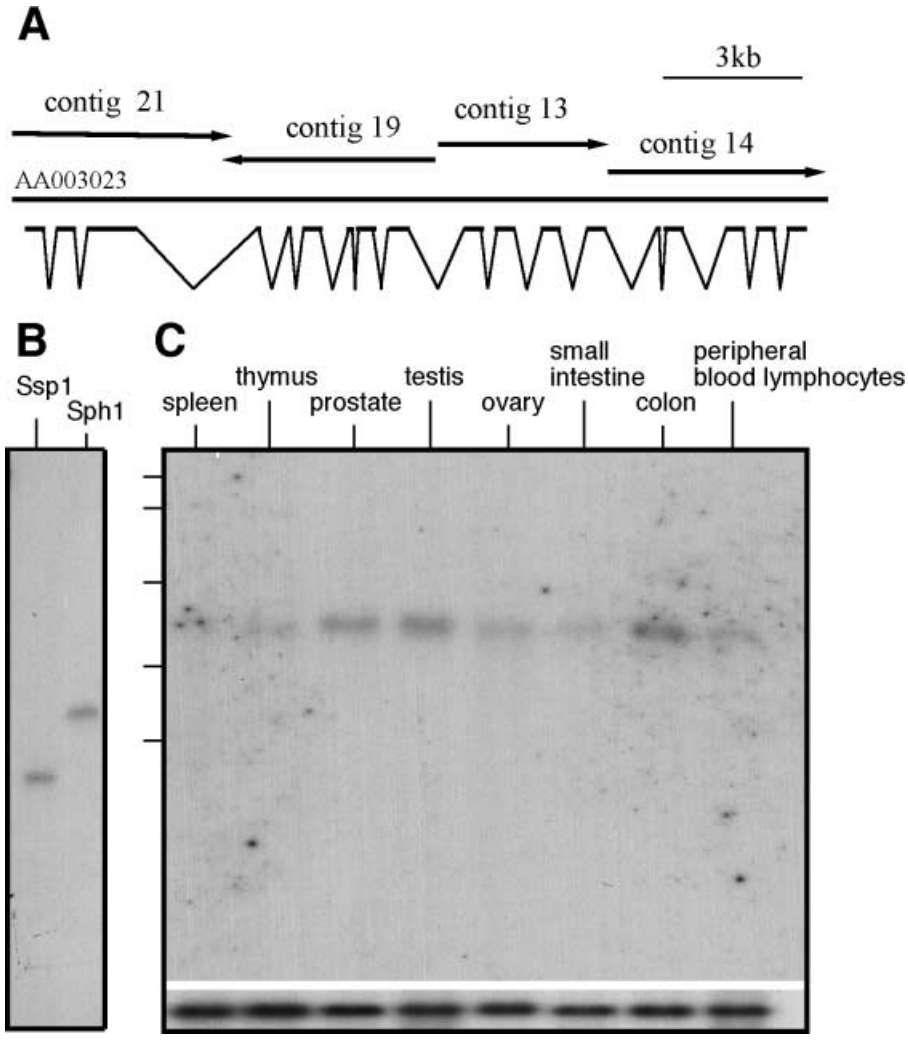

Fig. 1 **A** Genomic organization of HsINCENP. The INCENP coding sequence is present on four unordered contigs of bacterial artificial chromosome clone AA003023. The ordering of the contigs and the exon positions are shown. **B** *HsINCENP* is a single-copy gene. Southern blot of 8 µg human genomic DNA digested with SspI or SphI, and hybridized with a ^{32}P-labelled 500 bp 3′ probe to *HsINCENP*. **C** Multiple tissue RNA blot, containing 2 mg mRNA per lane, was hybridized with the same probe as in **A**, or with a control β-actin probe (*lower section*). Molecular weight markers (from top to bottom): 9.6 kb, 7.5 kb, 4.4 kb, 2.4 kb, 1.4 kb

(Fig. 1C). A single transcript of 3.2 kb was seen in all tissues examined except for spleen, suggesting that INCENP is expressed in most cell types. The greatest levels of expression were seen in testis, prostate and colon. This is consistent with a role for INCENP in cell proliferation.

HsINCENP is a chromosomal passenger protein

To examine the sub-cellular localization of HsINCENP, we performed immunofluorescence staining on mitotic HeLa cells with antibodies against chicken INCENP that cross-react with HsINCENP. These antibodies recognize a single M_r 135,000 band on immunoblots of HeLa chromosomal proteins, corresponding to the INCENP protein (Fig. 3A). All three antibodies gave a similar pattern of localization of INCENP. In prophase, HsINCENP is present along the length of condensing chromatin before concentrating at the centromere during metaphase (Fig. 3B, C). Upon anaphase entry, HsINCENP leaves the chromatin to associate with the central spindle microtubules (Fig. 3D). Later, during ana/telophase, an accumulation of HsINCENP can be seen at the equatorial cortex where the cleavage furrow will form (Fig. 3E, arrowhead). During cytokinesis HsINCENP becomes concentrated in the midbody (Fig. 3F). Thus, HsINCENP behaves like a classical chromosomal passenger protein.

INCENP colocalizes with the Aurora-B/AIM-1 kinase on chromosomes

Previously, the chromosomal passenger proteins INCENP, Aurora-B/AIM-1 and TD-60 were shown to colocalize at the spindle midzone and equatorial cortex in anaphase (Martineau-Thuillier et al. 1998). In a recent study, we showed that Aurora-B and INCENP exist in an 11S complex in *Xenopus* egg extracts, and that correct Aurora-B localization is dependent on INCENP (Adams et al. 2000). We wished to know whether Aurora-B was also a chromosomal protein, and if so, whether it colocalized with INCENP. Immunostaining of chromosomal spreads using an antibody raised against HsAurora-B revealed that the kinase was concentrated at centromeres of metaphase chromosomes, between the two kinetochores (Fig.4). Co-

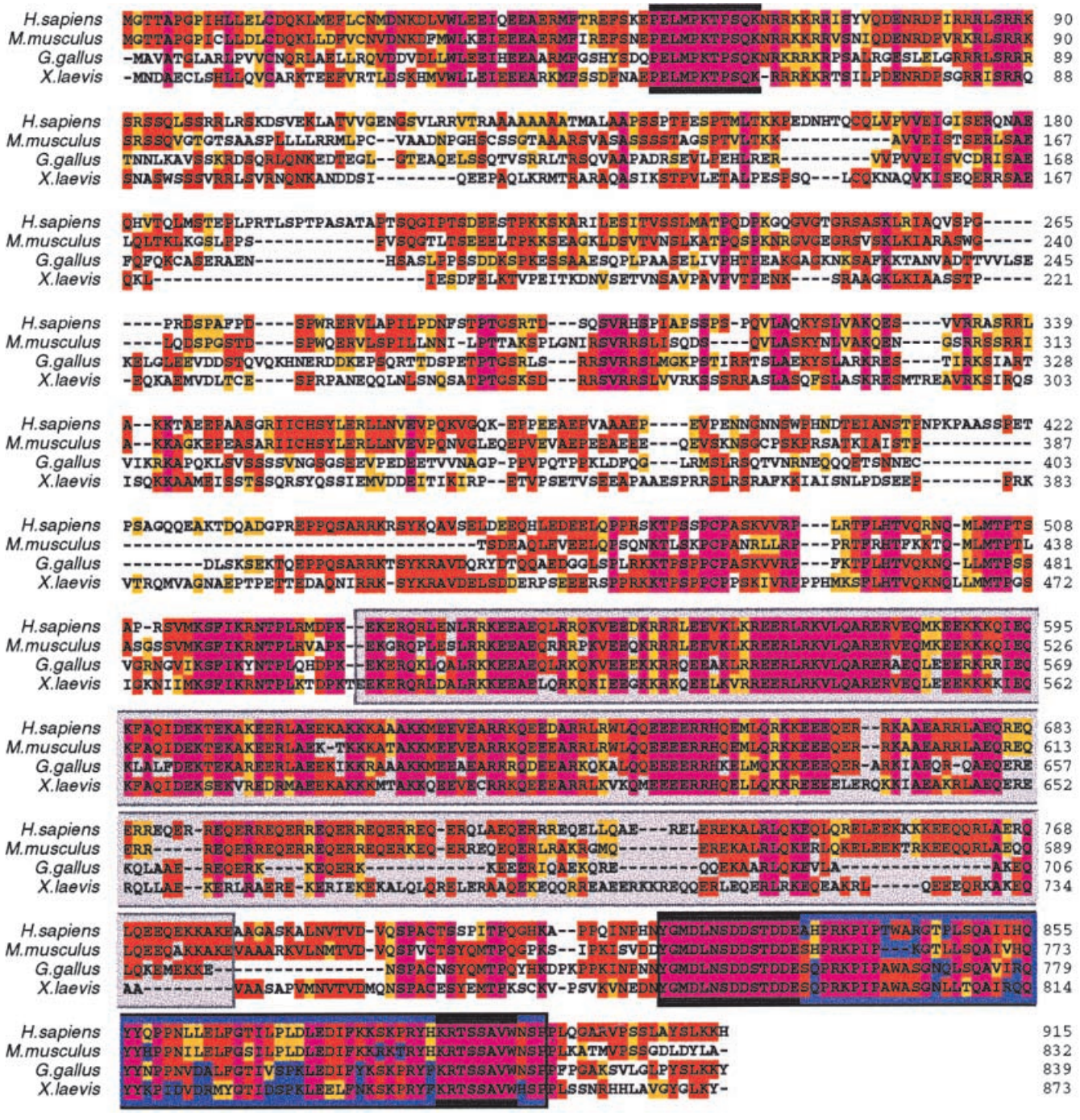

Fig. 2 Amino acid sequence alignment of human, mouse, chicken and *Xenopus* INCENPs. *Coloured* positions are: absolutely conserved (*magenta*), highly conserved (*orange*), or display conservative substitutions (*yellow*). *Boxed* regions are: *purple* the highly conserved C-terminal IN-box; *grey* sequence regions with coiled-coil prediction scores >0.4 by Multicoil. Three conserved sequence motifs (see text) are *outlined in black*. The figure shows a multiple sequence alignment generated through manual editing of an automated alignment by ClustalX and rendered with Box-shade

immunostaining of INCENP and Aurora-B revealed that they are tightly colocalized at the centromere (Fig. 5). Therefore, like INCENP, Aurora-B is a chromosomal passenger protein, and the two proteins are closely associated throughout mitosis.

HsINCENP protein levels are elevated in colorectal tumors

The human Aurora-B kinase, AIM-1, has been found to be expressed at high levels in a number of human colo-

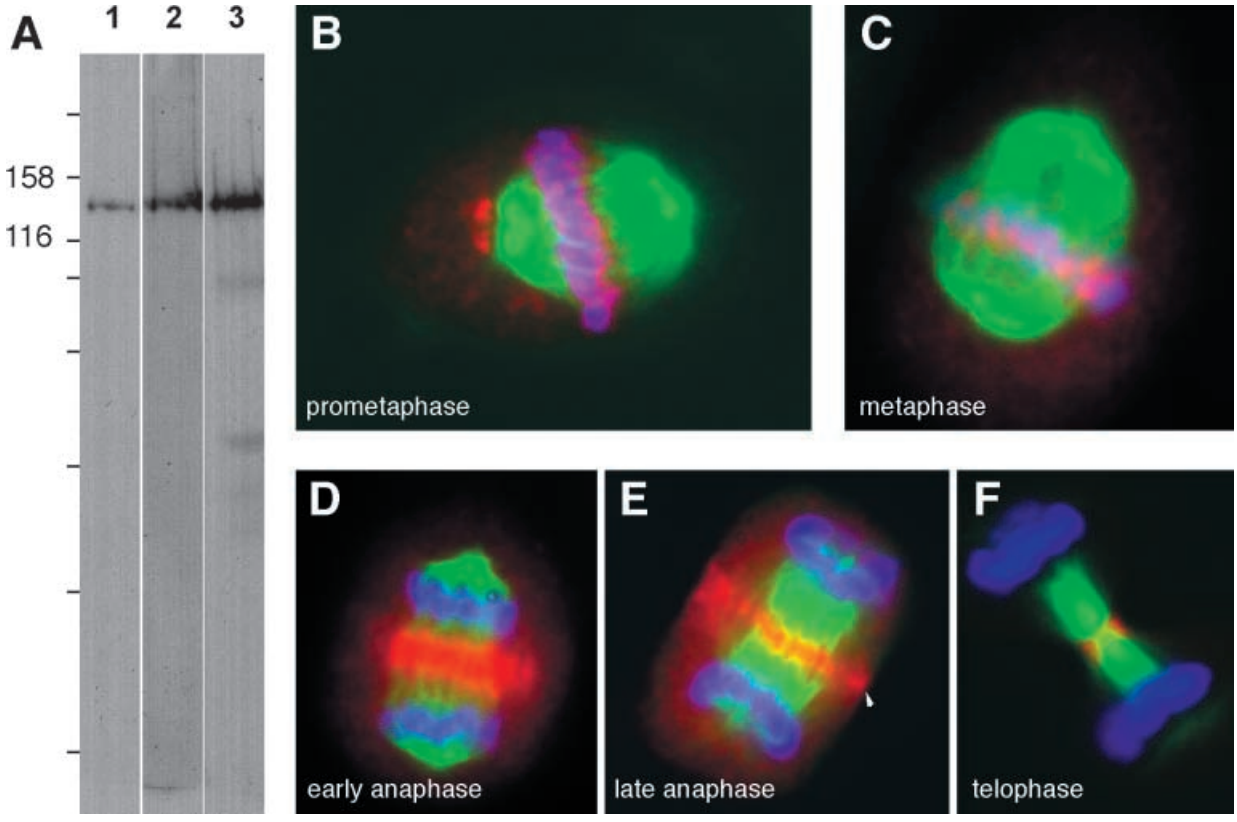

Fig. 3 **A** HsINCENP migrates as an M_r 135,000 band by SDS-polyacrylamide gel electrophoresis. Three antibodies, originally raised against chicken INCENP, cross-react with a single protein in whole nuclei. 10^7 nuclei were loaded per lane. The lanes were immunoblotted using the following antibodies: *lane 1* Rb1186, *lane 2* Rb1005, *lane 3* Ra2-INCENP. **B–F** Colour panels: HeLa cells at different mitotic stages stained for tubulin (*green*), DNA (*blue*) and INCENP (*red*). The *arrowhead* points to an accumulation of INCENP at the presumptive cleavage furrow

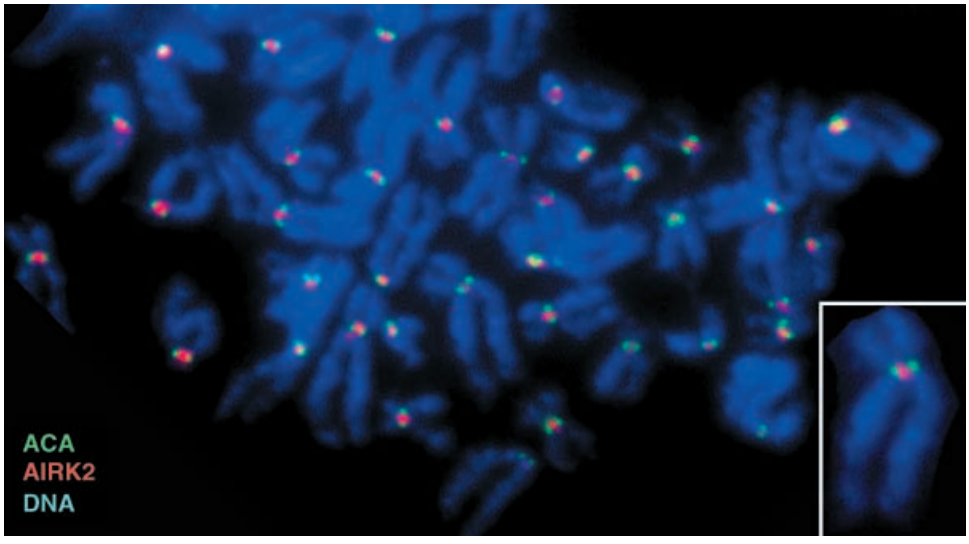

Fig. 4 HsAurora-B localizes to inner centromeres. Chromosome spreads from Colcemid-arrested HeLa cells were immunostained for Aurora-B/AIM1/AIRK2 and for kinetochores with autoimmune serum GS (Earnshaw et al. 1986). Aurora-B localized to the inner centromere, between the two spots of ACA staining, in a similar manner to INCENP (Mackay et al. 1998)

rectal cancer cell lines (Tatsuka et al. 1998). The fact that INCENP and Aurora-B kinase are stored in a complex in *Xenopus* eggs (Adams et al. 2000) raised the possibility that INCENP expression might also be altered in cancers where the expression of Aurora kinases is altered. To evaluate this possibility, we determined the levels of INCENP protein in a number of colorectal tumor cell lines compared with two non-transformed cell lines: WI38 fibroblasts and HMEC mammary epithelial cells (Fig.6). When INCENP levels were normalized with re-

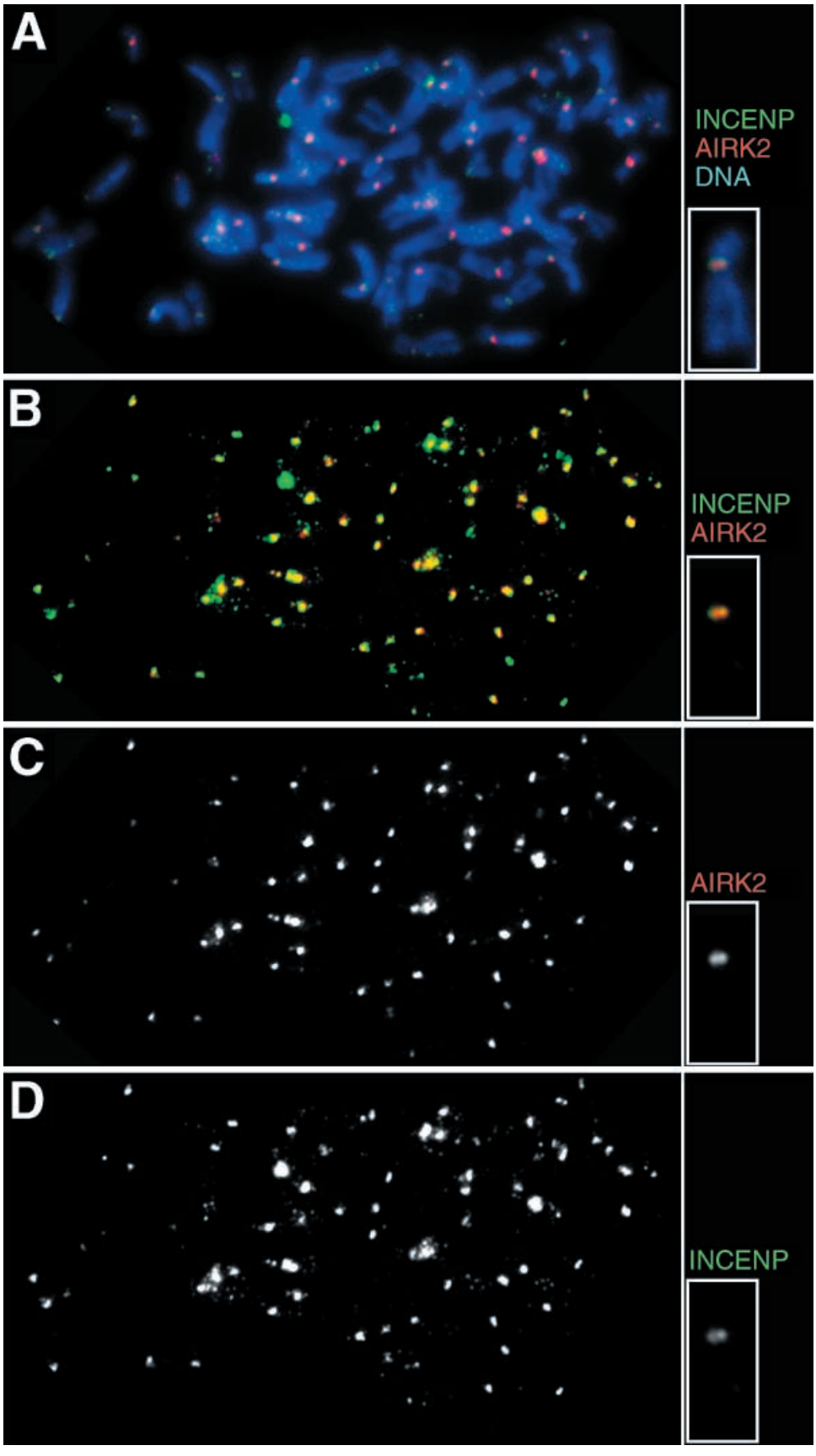

Fig. 5A–D HsAurora-B and INCENP colocalize on mitotic chromosomes. Mitotic chromosome spreads from HeLa cells were immunostained for Aurora-B/AIM1/AIRK2 and INCENP. A strong colocalization is seen in the inner centromeres. The images show **A** INCENP, Aurora-B and DNA triple labelling; **B** INCENP and Aurora-B double labelling; **C** Aurora-B labelling; and **D** INCENP labelling

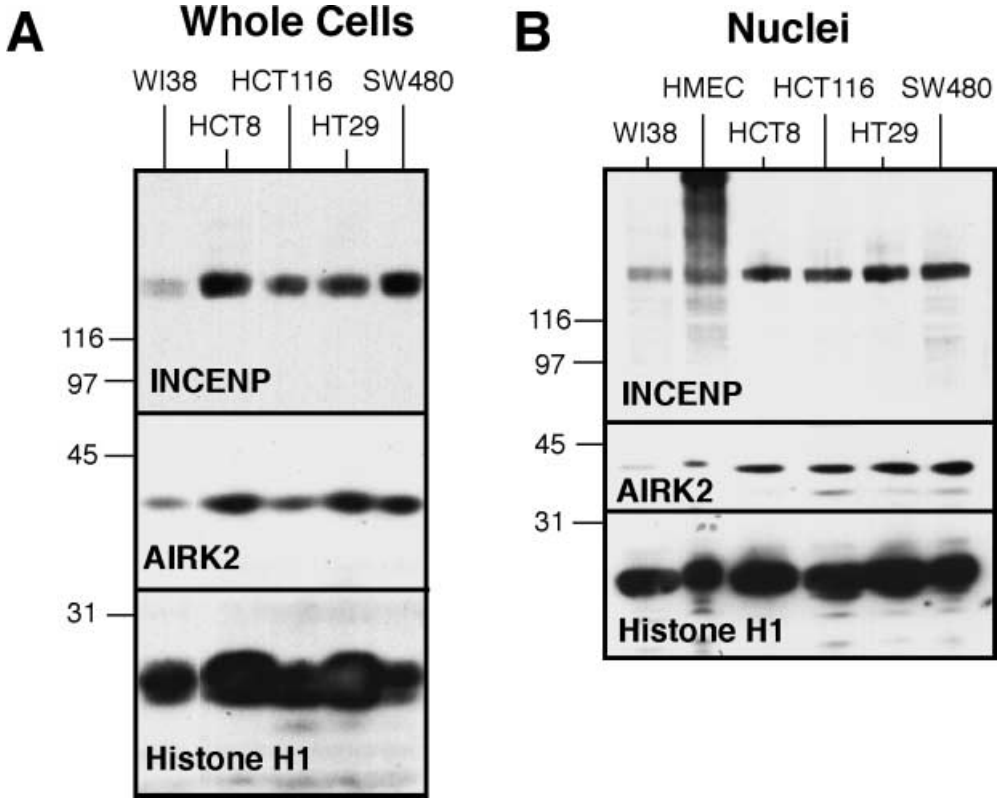

Fig. 6 HsINCENP is upregulated in colon carcinoma cell lines. Immunoblots of whole cells or purified nuclei from control non-transformed fibroblasts (W138), human mammary epithelium (HMEC) or colon carcinoma cells (HCT8, HCT116, HT29, SW480) were probed with anti-INCENP, anti-Aurora-B/AIM1/AIRK2, or anti-histone H1 antibodies. The amount of INCENP and Aurora-B in carcinoma cells relative to the non-transformed WI38 fibroblasts was determined as described in Table 1

Table 1 INCENP and Aurora-B levels in colon carcinoma cell lines relative to non-transformed WI38 cells

Protein	HCT8	HCT116	HT29	SW480
INCENP	2.8	3.1	2.4	4.7
Aurora-B	2.9	2.8	3.3	4.4

The signals from the anti-INCENP and Aurora-B antibodies in the immunoblots of whole cell extracts shown in Fig. 6 were normalized with respect to the anti-H1 signal using NIH image. Ratios were then calculated for the amount of INCENP and Aurora-B in the carcinoma cells relative to the amounts in non-transformed WI38 fibroblasts

spect to histone H1 levels, a 2.4- to 4.7-fold increase in total cellular INCENP protein was seen in the colon cancer lines compared with the control cell line (Table 1). In purified nuclei, a 1.8- to 3.0-fold increase in INCENP levels was apparent, confirming that the excess INCENP is localized in the nucleus rather than being sequestered in the cytoplasm. When the same blots were re-probed to look at levels of HsAurora-B in these cell lines, a similar result was obtained: Aurora-B levels were elevated 2.8- to 4.4-fold relative to the control cell line. Taken together, these results confirm the upregulation of Aurora-B/AIM-1 in colon cancer cell lines (Tatsuka et al. 1998), and indicate that INCENP is also upregulated in those tumour cell lines.

Discussion

The HsINCENP protein sequence is highly conserved throughout evolution

In this paper we describe the first characterization of the human *INCENP* gene. INCENP mRNA is expressed to some extent in all tissues examined, but is particularly elevated in proliferative tissues such as the prostrate, testis and colon. These results are consistent with a role for INCENP as an essential "housekeeping" gene required for mitosis.

HsINCENP is a single-copy gene encoding a protein that shows no significant homology outside the coiled-coil region with other predicted human proteins. HsINCENP is highly homologous to its chicken, mouse and frog counterparts, especially in the C-terminal domain containing the highly conserved IN-box (Fig. 2). While the roles of the N-terminal region of INCENP in centromere and microtubule binding are well defined (Ainsztein et al. 1998; Mackay et al. 1998), the function of the C-terminal domain and the IN-box in particular has not yet been determined. Interestingly, deletion of this region of the protein causes abnormalities in cytokinesis and plasma membrane stability, suggesting that it makes important functional contacts in vivo (A.M. and W.C.E., unpublished results). The IN-box domain is conserved throughout evolution, and INCENP homologues in yeasts, flies and worms have been identified on this basis (Adams et al. 2000). A budding yeast INCENP homologue, Sli15p, interacts with the Aurora-like kinase

Ipl1p (Kim et al. 1999) and this conservation of interaction between Aurora-B kinases and INCENP from yeasts to vertebrates may therefore take place via the IN-box.

The role of INCENP on chromosomes during mitosis

We have recently shown that vertebrate INCENP associates with the Aurora-B/XAIRK2 kinase (Adams et al. 2000). Overexpression of Aurora-B in human cells leads to cytokinesis defects and an increase in aneuploidy. Interestingly, the levels of Aurora-B are elevated several-fold in colorectal cancer lines (Tatsuka et al. 1998). Our experiments show that INCENP levels are also elevated to a similar extent in these cells. While mutations in the *HsINCENP* gene are unlikely to be the primary cause of tumorigenesis (the *INCENP* locus is not associated with any known genetically linked predisposition to disease), elevated levels of the protein could contribute to tumour progression either by stabilizing the Aurora-B kinase, or by targeting it to non-physiological, low affinity substrates. Alternatively, abnormalities of the INCENP/Aurora system could lead to aneuploidies that contribute to tumour progression.

We have shown here that INCENP and Aurora-B colocalize not only on the anaphase spindle and cleavage furrow but also at the inner centromere on metaphase chromosomes. One proposed role for Aurora-B is to phosphorylate histone H3 (Hsu et al. 2000; Speliotes et al. 2000), although other kinases such as nimA may also be involved in this modification (De Souza et al. 2000). When Aurora-B function is abrogated in *C. elegans* by RNAi, H3 remains unphosphorylated at serine 10 during mitosis with concomitant defects in chromosome condensation and segregation. A recent study has indicated that the *C. elegans* survivin-like molecule BIR-1 is required for correct targeting of *C. elegans* Aurora-B/AIR-2 to chromosomes and the spindle during mitosis (Speliotes et al. 2000). Our results raise the possibility that INCENP could act either together with or in parallel to survivin-like molecules to target Aurora-B to its H3 substrate on chromosomes. Interestingly, INCENP can also associate with a chromosomal adapter protein, heterochromatin protein 1 (HP-1) in vitro (Ainsztein et al. 1998). HP-1 has recently been shown to bind to core nucleosomes containing methylated histone H3 (Lachner et al. 2001; Bannister et al. 2001), and may therefore act as a "docking site" for INCENP on inactive chromatin.

Recent experiments have validated the chromosomal passenger hypothesis (Earnshaw and Bernat 1990), and have shown clearly that proteins such as INCENP are involved in coordinating the chromosomal and cytoskeletal events of mitosis (Mackay et al. 1998; Adams et al. 2000). The present results suggest that unravelling the molecular functions of INCENP and Aurora kinases in mitosis is of paramount interest, not only for basic studies of mitotic mechanisms, but to assess further and understand the link between overexpression of these proteins and the development of colorectal cancer.

Acknowledgements All experiments comply with the current laws of the UK. R.R.A is a postdoctoral training fellow of the Medical Research Council (UK). This work was supported by the Wellcome Trust, of which W.C.E. is a Principal Research Fellow.

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