The Localization of Inner Centromeric Protein (INCENP) at the Cleavage Furrow Is Dependent on Kif12 and Involves Interactions of the N Terminus of INCENP with the Actin Cytoskeleton[□] [▽]

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The inner centromeric protein (INCENP) and other chromosomal passenger proteins are known to localize on the cleavage furrow and to play a role in cytokinesis. However, it is not known how INCENP localizes on the furrow or whether this localization is separable from that at the midbody. Here, we show that the association of *Dictyostelium* INCENP (DdINCENP) with the cortex of the cleavage furrow involves interactions with the actin cytoskeleton and depends on the presence of the kinesin-6–related protein Kif12. We found that Kif12 is found on the central spindle and the cleavage furrow during cytokinesis. Kif12 is not required for the redistribution of DdINCENP from centromeres to the central spindle. However, in the absence of Kif12, DdINCENP fails to localize on the cleavage furrow. Domain analysis indicates that the N terminus of DdINCENP is necessary and sufficient for furrow localization and that it binds directly to the actin cytoskeleton. Our data suggest that INCENP moves from the central spindle to the furrow of a dividing cell by a Kif12-dependent pathway. Once INCENP reaches the equatorial cortex, it associates with the actin cytoskeleton where it then concentrates toward the end of cytokinesis.

INTRODUCTION

The chromosomal passenger proteins are an interesting group of proteins that play many important roles in cell division. The inner centromeric protein (INCENP), Aurora B, and Survivin associate with each other to form a chromosomal passenger-protein complex (CPC) known to exist in unicellular and multicellular organisms (Kang *et al.*, 2001; Bolton *et al.*, 2002; Cheeseman *et al.*, 2002). In addition, the chromosomal passenger proteins TD60 and Borealin can associate with the CPC and regulate its activity (Mollinari *et al.*, 2003; Gassmann *et al.*, 2004; Sampath *et al.*, 2004; Klein *et al.*, 2006). It has been well established that the CPC is essential for the regulation of chromosome condensation, chromosome congression, spindle formation, chromosome segregation, and cytokinesis (Mackay *et al.*, 1998; Kim *et al.*, 1999; Adams *et al.*, 2001a; Giet and Glover, 2001; Cheeseman *et al.*, 2002, 2004; Tanaka *et al.*, 2002; Sampath *et al.*, 2004).

A hallmark property of chromosomal passenger proteins is their dynamic localization during cell division. The CPC is localized on chromosomes and centromeres before meta-

phase and then is found on the spindle midzone during anaphase and at the cleavage furrow and midbody during cytokinesis (Cooke *et al.*, 1987; Earnshaw and Cooke, 1991). The correct localization of the CPC at each stage of the cell cycle is crucial for its proper function. For example, an INCENP mutant that remains tethered to centromeres throughout mitosis inhibits cytokinesis (Eckley *et al.*, 1997). Interestingly, the chromosomal passenger proteins are interdependent on their localization (Adams *et al.*, 2000; Wheatley *et al.*, 2001; Honda *et al.*, 2003; Gassmann *et al.*, 2004). In particular, the kinase activity of Aurora B is important for the proper distribution of the CPC during cell division (Honda *et al.*, 2003). The dynamic localization of the CPC must be regulated by specific mechanisms acting at distinct locations of the cell at different stages of the cell cycle. Some of these mechanisms have been discovered in recent years. For example, the redistribution of the CPC from the centromeres to the spindle midzone is controlled by dephosphorylation of INCENP by the phosphatase CDC14 (Pereira and Schiebel, 2003). In some systems, the mitotic kinesin-like protein (MKLP)-2 also seems to be essential for the redistribution of CPC from centromeres to the central spindle (Gruneberg *et al.*, 2004).

In contrast to these events in mitosis, the association of INCENP and other chromosomal passenger proteins with the cortex of the cleavage furrow has not been explored in detail. Immunofluorescence and electron microscopy studies have shown that INCENP associates with the cortex of the cleavage furrow during cytokinesis (Earnshaw and Cooke, 1991; Eckley *et al.*, 1997). However, in most animal

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systems, it is difficult to distinguish the localization of the CPC at the spindle midzone from that at the cleavage furrow because they are often closely juxtaposed. The presence of the microtubule-rich midbody, derived from the spindle midzone makes this distinction even more difficult. In animal cells, all manipulations that abrogate localization of the CPC at the spindle midzone also result in lack of localization at the midbody (Ainsztein *et al.*, 1998; Mackay *et al.*, 1998; Honda *et al.*, 2003). By contrast, this distinction can be made more readily in *Dictyostelium* cells. The *Dictyostelium* spindle is slender compared with that of metazoans, and it is easily distinguished from the ingressing cleavage furrow. Furthermore, the *Dictyostelium* spindle is dismantled before the cleavage furrow severs the cell, and no midbody is formed (Roos and Camenzind, 1981; Roos *et al.*, 1984; McIntosh *et al.*, 1985).

We showed previously that *Dictyostelium* INCENP (DdINCENP) localizes to the cortex of the cleavage furrow and that it is required for the abscission of the daughter cells (Chen *et al.*, 2006). Importantly, the distribution of DdINCENP at the cleavage furrow is altered by the loss of myosin II. Instead of the broad cleavage furrow distribution observed in wild-type cells, DdINCENP localizes as a narrow band at the equatorial plane of myosin II null cells. These observations suggested that DdINCENP may be able to interact with the actomyosin cytoskeleton (Chen *et al.*, 2006).

MKLPs, a subgroup of the kinesin-6 family of proteins, have been suggested to play an important role in regulating the localization of chromosomal passenger proteins during cell cycle (Gruneberg *et al.*, 2004). *Dictyostelium* has a single kinesin-6-related protein called Kif12. Kif12 localizes to the spindle midzone, and it is important for mitosis and cytokinesis (Lakshmikanth *et al.*, 2004). Normal localization of myosin II to the cleavage furrow requires Kif12, suggesting a possible interaction between Kif12 and the actomyosin cytoskeleton. Interestingly, Kif12 null mutants frequently fail in the abscission of daughter cells in a manner similar to DdINCENP null mutants (Lakshmikanth *et al.*, 2004). The similarity of localization and mutant phenotype of these two proteins prompted us to explore possible interactions between them. We show here that, in fact, Kif12 is required for the furrow localization of DdINCENP. We also determined that an amino-terminal portion of DdINCENP is required for furrow localization and that it is able to interact with the actin cytoskeleton.

MATERIALS AND METHODS

Cell Culture, Transformation, and Constructs

The cells were cultured with HL-5 medium in Petri dishes at 19°C. Green fluorescent protein (GFP)-DdINCENP and GFP-Kif12 were described previously (Lakshmikanth *et al.*, 2004; Chen *et al.*, 2006). Both plasmids carry a Geneticin (G418)-resistance cassette. The constructs were introduced into cells with electroporation, and the transformants were selected in HL-5 medium with 10 µg/ml G418 (Invitrogen, Carlsbad, CA).

Microscopy of Live Cells

For live microscopy of mitotic cells, cells in log phase growth were plated on a small Petri dish with a coverslip on the bottom (MatTek, Ashland, MA). After the cells attached to the plate, the medium was removed, and it was replaced with low-fluorescence (LF) medium for at least 30 min before observations (Bretschneider *et al.*, 2002). The live imaging of the cells was conducted using a Nikon Eclipse TE200 microscope (Nikon Instruments, Dallas, TX) equipped with a 100× 1.4 numerical aperture PlanFluor Objective, shuttered illumination, and a Quantix 57 camera (Roper Scientific, Tucson, AZ) controlled by MetaMorph (Molecular Devices, Sunnyvale, CA). The exposure time for the GFP fluorescence was 50 ms with the interval time being at least 10 s.

Immunostaining and Microscopy of Fixed Cells

The cells were allowed to attach to acid-cleaned coverslips overnight in a 8.5-cm Petri dish, and then they were fixed according to published protocols (Koonce and McIntosh, 1990). Briefly, the cells were fixed at room temperature with 2.5% formaldehyde in a 1,4-piperazinediethanesulfonic acid (PIPES)-EGTA buffer for 3 min followed by 1% formaldehyde in dehydrated methanol at -10°C for 5 min. The anti- α -tubulin antibody is a monoclonal antibody from Sigma-Aldrich (St. Louis, MO). The secondary antibody is a Texas Red-conjugated goat-anti-mouse antibody (Invitrogen). The protocol for 4,6-diamidino-2-phenylindole staining was described previously (Gerald *et al.*, 2001).

The protocol for staining F-actin was modified from the protocol described previously by Gerald *et al.* (1998). Briefly, 2×10^5 cells were plated on a coverslip. Then, the cells were fixed with 3.7% formaldehyde in PDF (2 mM KCl, 1.1 mM K₂HPO₄, 1.32 mM KH₂PO₄, 0.1 mM CaCl₂, and 0.25 mM MgSO₄, pH 6.7) for 20 min at room temperature before being permeabilized by 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 5 min. The coverslip was washed three times in a fixing chamber with PBS buffer before the cells were stained with Texas Red-conjugated phalloidin (Invitrogen) in PBS for 30 min in a covered humid chamber at room temperature. The coverslip was washed three times in PBS, and then it was rinsed briefly in distilled H₂O before being mounted onto a glass slide.

Construction of Truncated DdINCENP Mutants

The C-terminal truncated mutants of DdINCENP were made by using internal restriction endonuclease sites in the DdINCENP gene. DdINCENP₁₋₂₇₃ was made by cutting one of the internal EcoRI sites closest to the N terminus of the DdINCENP sequence. DdINCENP₁₋₅₀₀ was made by cutting the only PvuII site in the gene. DdINCENP₁₋₁₀₁₃ was made by cutting the only BamHI site in the gene. In contrast, DdINCENP₄₈₈₋₁₃₂₁ was amplified with the primers AO386 (5'-GAGCTCGGTATTGCAA AGCCAACACCACCTTAC-3') and AO387 (5'-CTCGAGTTATTTTATTAACAATG ATTGGATTAGTAAAA-CCC-3'). All four truncated mutants were cloned downstream of the GFP sequence in the pTXGFP expression vector.

Tandem Affinity Purification (TAP) Purification

DdINCENP₁₋₅₀₀ was cloned downstream of the TAP-GFP sequence in the pTX-TAPGFP plasmid constructed by Joe Mireles in our laboratory. The procedure for purifying the TAP-tagged protein in *Dictyostelium* was modified from the procedure described by Rigaut *et al.* (1999) and that by Koch *et al.* (2006). For large-scale purification, 500 ml of cells expressing either TAP-GFP or TAP-GFP-DdINCENP₁₋₅₀₀ were cultured in a 2-liter flask on a shaker at 200 rpm. The cells were harvested when the density reached 5×10^6 cells/ml and washed twice with PDF buffer. The cell pellet was resuspended in 40 ml of lysis buffer (50 mM Tris-Cl, 50 mM KCl, 2 mM MgCl₂, 0.5 mM dithiothreitol [DTT], and 150 mM NaCl, pH 8.0) with protease inhibitors (5 µg/ml leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM tosyl-arginine-methyl ester 1 µg/ml trypsin inhibitor, 0.8 µM aprotinin, 1 mM benzamide, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 µM E-64, 1 µg/ml pepstatin, and 40 µM bestatin) (Sigma-Aldrich). The suspension was passed three to four times through a nebulizer (BioNEB cell disrupter; Glas-Col, Terre Haute, IN) on ice. The cell lysate was centrifuged at $14,000 \times g$ for 30 min. At the same time, 200 µl of immunoglobulin (Ig)G agarose beads (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) were pre-equilibrated and washed with 15 ml of IPP150 buffer (10 mM Tris-Cl, 150 mM NaCl, and 0.1% NP-40, pH 8.0). The IgG beads were added to the supernatant of the cell lysate and incubated on a rotator for 2 h at 4°C in a 50-ml tube to allow the TAP-tagged proteins to bind to the beads. The suspension was packed into a 15-ml column (Bio-Rad, Hercules, CA). The packed beads were washed with 30 ml of IPP150 buffer followed by 10 ml of TEV cleavage buffer (10 mM Tris-Cl, 150 mM NaCl, 0.1% NP-40, 0.5 mM EDTA, and 1 mM DTT, pH 8.0). Then, 150 U of AcTEV protease (Invitrogen) was added with 1 ml of TEV cleavage buffer to the column. The suspension was incubated at 4°C in the column on a rocker overnight to allow the thorough digestion of the proteins from the beads.

The flow-through (1 ml) from the column was collected, and a small aliquot was examined by SDS-polyacrylamide gel electrophoresis (PAGE) gel. As reported previously (Koch *et al.*, 2006), we found that this one step purification of our tagged proteins was sufficient to yield a highly pure preparation. Subsequent purification by binding to a calmodulin column (Rigaut *et al.*, 1999) did not improve the purity of our protein preparations.

Individual protein bands from the SDS-PAGE gel were excised and subjected to in-gel tryptic digestion (Shevchenko *et al.*, 1996). They were then identified through peptide mass fingerprinting (MASCOT; Matrix Sciences, Boston, MA). Tandem mass spectra were searched against the database of National Center for Biotechnology Information. Identification of the proteins was confirmed through manual evaluation of spectra. Mass spectrometry analysis was performed by the core facilities of the Institute for Cell and Molecular Biology at the University of Texas at Austin (Austin, TX).

Figure 1. Kif12 localizes on the spindle during mitosis and at the cleavage furrow during cytokinesis. Shown are two time-lapse fluorescence images of wild-type or Kif12 null cells expressing GFP-Kif12 during cytokinesis. GFP-Kif12 was found on the mitotic spindle. Moreover, it localized to the cortex of the cleavage furrow (white arrow) during cytokinesis and was clearly seen at the remnant of the intercellular bridge after the daughter cells separated (see Supplemental Movie 1). Times are indicated in minutes:seconds. Bar, 5 μ m.

RESULTS

The MKLP-related Protein Kif12 Localizes at the Cleavage Furrow and Is Required for the Localization of DdINCENP at the Cleavage Furrow

We showed previously that Kif12, like MKLP proteins in metazoans, is found on the *Dictyostelium* spindle during mitosis and that is important for mitosis and cytokinesis (Lakshmikanth *et al.*, 2004). Surprisingly, further detailed observations of cells expressing GFP-Kif12 revealed that a small but significant amount also localized at the ingressing cleavage furrow (Figure 1 and Supplemental Movie 1). Kif12 was never observed near the plasma membrane of interphase cells or in the polar cortical region of dividing cells, suggesting that its furrow localization may be tightly regulated and important for cytokinesis.

Because both Kif12 and DdINCENP are found at the central spindle and cleavage furrow (Supplemental Movies 1 and 2) and because the loss of either protein leads to a similar late cytokinesis defect (Lakshmikanth *et al.*, 2004; Chen *et al.*, 2006), we explored the possibility of an interaction between these two proteins during cytokinesis. We first determined whether Kif12 was required for the localization of DdINCENP during mitosis or cytokinesis by comparing the localization of GFP-DdINCENP in wild-type and Kif12 null cells. We found that GFP-DdINCENP localized normally to the spindle poles and central spindle during anaphase and telophase in both cells types. In contrast, GFP-DdINCENP did not localize at the cortex of the cleavage furrow in Kif12 null cells (Figure 2 and Supplemental Movie 3). The failure of DdINCENP to localize on the cleavage furrow was not due to a defective central spindle in these

Figure 2. Kif12 is essential for the redistribution of DdINCENP from the central spindle to the cortex of the cleavage furrow. Fluorescence microscopy images of cells expressing GFP-DdINCENP during cytokinesis. (A and B) GFP-DdINCENP is normally found at the spindle poles and central spindle as seen in these dividing wild-type cells. During cytokinesis, GFP-DdINCENP then localizes on the cortex of the cleavage furrow (arrows) (see Supplemental Movie 2). (C and D) GFP-DdINCENP is still localized on the central spindle and spindle poles in Kif12 null cells. However, GFP-DdINCENP failed to localize at the cleavage furrow in the absence of Kif12 (arrows). (E) A time-lapse series of a DdKif12 null cell expressing GFP-DdINCENP shows that GFP-DdINCENP was never found at the cleavage furrow during cytokinesis. Arrows point to the cleavage furrow. Noticeably, GFP-DdINCENP was also absent from the cytoplasmic bridge at the end of cytokinesis, whereas it highly concentrated on the bridge in wild-type cells (see Supplemental Movie 3). Times are indicated in minutes:seconds. Bar, 5 μ m.

cells. We examined the morphology of the central spindle in Kif12 null cells, and we found that it was indistinguishable from that in wild-type cells (Supplemental Figure 1). In addition, we found that GFP-DdINCENP was retained on the central spindle of Kif12 null cells throughout cytokinesis. Even after the central spindle fractured into two halves, GFP-DdINCENP was still associated with the broken end of the dismantling spindle in each daughter cell (Figure 2 and Supplemental Movie 3). We observed a total of 14 Kif12 null cells undergoing mitosis and cytokinesis. Of these, five cells were able to initiate cytokinesis, and all of them failed to localize GFP-DdINCENP at the cortex of the cleavage furrow retaining it, instead, on their central spindle. The remaining nine cells were not able to initiate cytokinesis, although GFP-DdINCENP still localized on their central spindle (data not shown). We also examined the cleavage furrow of fixed Kif12 null cells, and we did not detect any GFP-DdINCENP at the cortex of the cleavage furrow (data not shown). Thus, these results suggest that Kif12 is required for the redistribution of DdINCENP from the central spindle to the cleavage furrow during cytokinesis.

We then investigated the possibility that the localization of Kif12 and DdINCENP at the cleavage furrow is interdependent by determining the localization of GFP-Kif12 in DdINCENP null cells. We found that the localization of Kif12 did not change in the absence of DdINCENP. GFP-Kif12 still localized to the spindle midzone and cleavage furrow in DdINCENP null cells (Figure 3 and Supplemental

Figure 3. The localization of Kif12 during mitosis and cytokinesis does not depend on DdINCENP. Time-lapse fluorescence micrographs of DdINCENP null cells expressing GFP-DdKif12. (A) GFP-Kif12 still localized on the mitotic spindle (arrowheads) in an DdINCENP null cell. The cell was entering cytokinesis when the central spindle dismantled and GFP-Kif12 began to accumulate at the cortex of the cleavage furrow (arrows). (B) In DdINCENP null cells, GFP-Kif12 was still found at the cortex of the cleavage furrow (arrows) at the early stage of cytokinesis and continued to build up at the furrow area. At the end of the cytokinesis, GFP-Kif12 was highly enriched at the cytoplasmic bridge (see Supplemental Movie 4). Note that the central spindle was out of focus in most frames in this movie. This focal plane helps highlight the association of GFP-Kif12 with the ingressing cleavage furrow. Times are indicated in minutes:seconds. Bar, 5 μ m.

tal Movie 4; $n = 6$). Therefore, Kif12 does not depend on DdINCENP to target to the region of the cleavage furrow.

The N Terminus of DdINCENP Is Necessary for Cleavage Furrow Localization

Although the functions of INCENPs from different species are highly conserved, their sequences are not. Besides the small IN-box domain at their C termini and coiled-coil segments, no other sequence conservation is discernible among them (Adams *et al.*, 2000). Hence, it is not known what portion of INCENP proteins mediates their localization at the cleavage furrow. To address this question, we made a series of DdINCENP mutants tagged with GFP, and we expressed them in DdINCENP null cells.

The first truncation mutant lacking the N-terminal 487-amino acids DdINCENP₄₈₈₋₁₃₂₀ still contained the IN-box domain, a coiled-coil domain, and a nuclear localization signal (Figure 4). This protein was found in the nucleus of interphase cells (data not shown), and it was still able to localize on the mitotic spindle poles and central spindle (Figure 6). However, this mutant protein did not show any enrichment either at the cortex of the cleavage furrow or the cytoplasmic bridge connecting the two daughter cells (Figure 5 and Supplemental Movie 5).

In contrast, our second mutant, DdINCENP₁₋₁₀₁₃, which lacks the C-terminal IN-box domain, retained the ability to

localize at the cleavage furrow of dividing cells (Figure 5 and Supplemental Movie 6). This protein also localized on the central spindle and poles (Figure 6). Thus, it seems that localization at the cleavage furrow is dependent on the amino-terminal portion of DdINCENP. Not surprisingly, neither of these two mutant proteins was able to rescue the cytokinesis defect of DdINCENP null cells (data not shown). Clearly, both localization at the furrow and the ability to interact with Aurora B kinase are important for its function during cytokinesis.

To further define the portion of DdINCENP involved in cleavage furrow localization, we constructed a mutant protein containing only the first 500 amino acids of DdINCENP. This protein lacks any nuclear localization signal, and it was thus found in the cytoplasm of interphase cells (see below). Interestingly, DdINCENP₁₋₅₀₀ was able to enter the nucleus in mitosis and localized at the central spindle and spindle poles (Figure 6). Most importantly, DdINCENP₁₋₅₀₀ was then able to localize at the cleavage furrow during cytokinesis and was clearly visible in the cytoplasmic bridge connecting the two daughter cells (Figure 5 and Supplemental Movie 7).

Because DdINCENP₁₋₅₀₀ still contained one of the two coiled-coil portions found in DdINCENP, we decided to examine the requirement of this region for cleavage furrow localization of DdINCENP. We found that a smaller truncation protein, DdINCENP₁₋₂₇₃, was still able to localize to the central spindle and the cleavage furrow during cytokinesis (Figures 5 and 6 and Supplemental Movie 8).

Logically, the requirement of Kif12 for the localization of DdINCENP at the cleavage furrow should also apply to these truncated mutants. Therefore, we investigated the localization of DdINCENP₁₋₅₀₀ in the Kif12 null cells. Similar to full-length DdINCENP, DdINCENP₁₋₅₀₀ failed to localize at the cleavage furrow in the absence of Kif12 (Supplemental Figure 2 and Supplemental Movies 9 and 10). This further confirmed our finding that Kif12 is required to target DdINCENP to the cortex of the cleavage furrow.

DdINCENP₁₋₅₀₀ Interacts with the Actin Cytoskeleton

We showed previously that the distribution of DdINCENP at the cleavage furrow is modulated by myosin II, suggesting the ability of DdINCENP to interact with the actin-myosin cytoskeleton (Chen *et al.*, 2006). Presumably, this ability is found at the amino terminus of DdINCENP be-

Figure 4. Domain analysis of DdINCENP. DdINCENP contains an IN-box domain near its C terminus, two coiled coil domains, and a single nuclear localization signal (*) at position 728–755. Shown are the four truncated mutants of DdINCENP used in this study. The IN-box is the only region with recognizable similarities among different INCENPs. The IN-box domain of DdINCENP is 44% identical to that of fission yeast INCENP and 21% identical to that of chicken INCENP (Chen *et al.*, 2006).

Figure 5. The N terminus of DdINCENP is essential for its localization at the cleavage furrow during cytokinesis. The four different truncated mutants of DdINCENP were tagged with GFP and expressed in DdINCENP null cells. Time-lapse fluorescence micrographs of these cells during cytokinesis are shown. The mutant protein that lacks the N terminus, DdINCENP₄₈₈₋₁₃₂₀, was absent from the cortex of the cleavage furrow (arrows), and it was homogenously distributed in the cytoplasm during cytokinesis. In contrast, the three proteins that contain the N terminus, DdINCENP₁₋₁₀₁₃, DdINCENP₁₋₅₀₀, and DdINCENP₁₋₂₇₃, were all found at the cortex of the cleavage furrow during cytokinesis (arrows) (see Supplemental Movies 5–8). The focal planes shown here do not display the association of these proteins with the spindle poles and central spindles, but they are clearly shown in Figure 6. Times are indicated in minutes:seconds. Bar, 5 μ m.

cause this region was able to localize at the cleavage furrow (Figure 5). Thus, we decided to explore in more detail the behavior of this portion of DdINCENP.

Because DdINCENP₁₋₅₀₀ lacks a nuclear localization signal (encoded by amino acids 728–755; Figure 4), it resided in the cytoplasm of interphase cells. We were intrigued to find

that DdINCENP₁₋₅₀₀ was associated with the cortex of the cell during interphase. In particular, we found that DdINCENP₁₋₅₀₀ was prominently found at the base of pinocytic cups, actin-rich structures (Figure 7 and Supplemental Movies 11 and 12). Although we do not interpret this localization as a normal function of DdINCENP (full-length DdINCENP is never found on these structures), we see it as indicative of an intrinsic ability of the N terminus of DdINCENP to interact with the actin cytoskeleton. Indeed, we found that

Figure 6. Localization of DdINCENP at the central spindle does not depend on either the IN-box or its N-terminal domain. The GFP-tagged DdINCENP mutants were expressed in DdINCENP null cells. Fluorescence microscopy images of these cells during anaphase are shown. (A–E) The GFP fluorescence signal is shown. (A'–E') Merged fluorescence micrographs to show DNA (blue) and GFP fluorescence (green). All four DdINCENP mutants localized to the spindle pole bodies and the central spindles (arrows) during anaphase. GFP–DdINCENP₁₋₁₀₁₃ had the brightest signal on the central spindle compared with the proteins (C, C', D, and D'). Additionally, GFP–DdINCENP₁₋₁₀₁₃ localized to the nuclear envelope (arrow heads) during telophase (D). Bar, 5 μ m.

Figure 7. DdINCENP₁₋₅₀₀ associates with the actin cytoskeleton during interphase. (A) Time-lapse fluorescence micrographs of wild-type cells (WT) expressing GFP–DdINCENP₁₋₅₀₀ are shown. The protein was enriched at the bottom portion of pinocytic cups (arrows) during endocytosis (see Supplemental Movie 9). (B, B', and B'') Wild-type cells expressing GFP–DdINCENP₁₋₅₀₀ were fixed and immunostained with Texas Red-labeled phalloidin to visualize F-actin (B). The chimera protein was colocalized with F-actin on the pinocytic cups (arrowheads). Times are indicated in minutes:seconds. Bar, 5 μ m.

Figure 8. TAP-tagged GFP-DdINCENP₁₋₅₀₀ associates with components of the actin cytoskeleton. TAP-GFP-DdINCENP₁₋₅₀₀ and TAP-GFP were expressed separately in wild-type cells and purified by binding to IgG beads followed by cleavage elution with TEV protease. The purified protein samples were loaded on the SDS-PAGE gel, which was stained with Coomassie blue. Comparison of the protein profile identified four proteins that specifically copurified with TAP-GFP-DdINCENP₁₋₅₀₀. These four proteins were identified by mass spectrometry as actin (a), ABP-50 (b), Hsp-70 (c), and myosin II heavy chain (d).

DdINCENP₁₋₅₀₀ sedimented with the Triton X-100-insoluble cytoskeleton (data not shown). However, we should point out that we did not find DdINCENP₁₋₅₀₀ in all actin-rich regions of the cell. For example, we did not see this protein at the leading pseudopod of migrating cells, which is enriched in many other F-actin-binding proteins (data not shown). Thus, we hypothesized that DdINCENP₁₋₅₀₀ interacted with a particular portion of the actin cytoskeleton.

To explore the interaction of DdINCENP₁₋₅₀₀ with the actin cytoskeleton in more detail, we constructed a TAP-tagged GFP-DdINCENP₁₋₅₀₀, which had a similar localization as the untagged protein (data now shown). The tagged protein was purified from *Dictyostelium* extracts. As a control we also purified proteins associated with TAP-GFP. By comparing the profiles of these two purifications, we identified four proteins that copurified with TAP-DdINCENP₁₋₅₀₀ but not with TAP-GFP control (Figure 8). Mass spectrometry analysis revealed the identity of these proteins as myosin II, actin, heat-shock complex (HSC)70, and the actin-binding protein ABP-50. Western blot analysis of our fractions with an antibody against Kif12 failed to detect any Kif12 copurifying with GFP-DdINCENP₁₋₅₀₀. However, because our purifications are prepared from asynchronous cultures, it is unlikely that we would copurify proteins that interact with GFP-DdINCENP₁₋₅₀₀ during cytokinesis. Unfortunately, there are no currently available methods to synchronize *Dictyostelium* cultures to a satisfactory degree for these studies. Nonetheless, these results highlight the fact that the N terminus of DdINCENP has the ability to interact with a unique subset of the actin cytoskeleton.

DISCUSSION

Kif12 Is Required for the Localization of DdINCENP at the Cleavage Furrow

The CPC has a dynamic localization during mitosis and cytokinesis. First, it localizes on the chromosome arms concentrating on centromeres at the transition from prophase to prometaphase. Then, it relocates to the central spindle at the transition from metaphase to anaphase. Finally, it redistributes to the cortex of the cleavage furrow as cells enter cytokinesis (Cooke *et al.*, 1987; Honda *et al.*, 2003). Some

details about the molecular mechanisms controlling CPC movement have been recently described. In *Saccharomyces cerevisiae*, the action of CDC14 phosphatase regulates the localization of INCENP and Aurora B kinase at the central spindle (Pereira and Schiebel, 2003). More recently, it has been demonstrated that MKLP2 is required for the redistribution of Aurora B kinase from centromeres to the central spindle (Gruneberg *et al.*, 2004). However, little is known about how the CPC can change localization from the central spindle to the cortex of the cleavage furrow at the beginning of cytokinesis.

Kif12 belongs to the KIF-6 family of kinesin-related proteins (Kollmar and Glockner, 2003; Lakshmikanth *et al.*, 2004). It is the only member of this kinesin family in *Dictyostelium*, whereas there are three KIF-6 kinesins in human, including MKLP1, MKLP-2, and M phase phosphoprotein 1 (Miki *et al.*, 2001; Kollmar and Glockner, 2003). All three mammalian MKLPs play important roles during cytokinesis (Hill *et al.*, 2000; Kuriyama *et al.*, 2002; Abaza *et al.*, 2003). Among the three, MKLP1 and MKLP2 have been well studied. Both of them localize to the central spindle at anaphase, and they highly concentrate in the midbody during cytokinesis. Additionally, they both can cross-link the antiparallel microtubules to help assemble the central spindle (Nislow *et al.*, 1992; Matulienė and Kuriyama, 2002; Neef *et al.*, 2003). However, they play different roles during cytokinesis. MKLP-1 interacts with MgcRacGAP to form a centralspindlin complex, which interacts with RhoA (Mishima *et al.*, 2002, 2004). In comparison, MKLP-2, also known as Rab6 kinesin (Echard *et al.*, 1998), is required for the redistribution of both Aurora B and Polo kinase from the centromeres to the central spindle (Neef *et al.*, 2003; Gruneberg *et al.*, 2004). ZEN-4 is the only kinesin-6 family protein found in *Caenorhabditis elegans*, and there are two kinesin-6 family proteins found in *Drosophila*, Pavarotti and KLP67A (Kollmar and Glockner, 2003). Whereas loss of Pavarotti led to a complete failure of cleavage furrow ingression, loss of Zen-4 led to a late cytokinesis defect where the cleavage furrow would initiate but eventually regressed (Adams *et al.*, 1998; Raich *et al.*, 1998; Dean *et al.*, 2005). We have shown that Kif12 null cells have similar cytokinesis defects; some cells fail to initiate cleavage furrow formation (Lakshmikanth *et al.*, 2004), whereas others initiate but fail to complete cytokinesis.

We demonstrated here that Kif12 associates with the cleavage furrow in addition to its association with the mitotic spindle. This is similar to MKLP2, a Kif12 homologue known to be localized on the cleavage furrow during cytokinesis (Hill *et al.*, 2000). Interestingly, MKLP2 was initially identified as a Rab-6 binding protein (Echard *et al.*, 1998). Our data suggested that MKLP proteins may have an interaction with membranes of the cleavage furrow during cytokinesis. How this interaction occurs is not currently known.

We showed that Kif12, unlike its mammalian homologue, is not required for either spindle assembly or the localization of DdINCENP at the central spindle. However, we found that Kif12 is necessary for the redistribution of DdINCENP from the central spindle to the cortex of the cleavage furrow. This conclusion is consistent with the observation that Kif12 null cells have a late cytokinesis defect, which is similar to the defect found in DdINCENP null cells. These observations raised the possibility that Kif12 may be involved in directly transporting DdINCENP to the furrow. Alternatively, Kif12 may also transport a third protein to the central spindle, which in turn is required for DdINCENP to localize at the furrow. Myosin II is a protein known to require Kif12 to assemble at the cleavage furrow (Lakshmikanth *et al.*, 2004). Because we have shown here that DdINCENP inter-

acts with the actin/myosin II cytoskeleton, it may seem plausible to postulate that Kif12 recruits myosin II, which in turn recruits DdINCENP to the cleavage furrow. However, we have shown previously that DdINCENP does not depend on myosin II to localize at the cleavage furrow (Chen *et al.*, 2006). Thus, we suggest that Kif12 is responsible for the localization of both DdINCENP and myosin II to the cleavage furrow.

We showed previously that DdINCENP localizes on equatorial furrows but not on ectopic (Rappaport) furrows (Chen *et al.*, 2006). Those observations suggested that DdINCENP may transfer directly from the central spindle to the cleavage furrow, because this will occur only at the equatorial plane. We would thus argue that the transport of DdINCENP to the cleavage furrow does not occur by astral microtubules, because such a mechanism would not distinguish between equatorial versus ectopic furrows. These conclusions would seem to be at odds with observations in mammalian cells showing INCENP on ectopic furrows (Savoian *et al.*, 1999). However, we would like to point out that in that paper cells also exhibited ectopic furrows that did not contain any INCENP, even though those furrows contained abundant microtubules. Only those ectopic furrows where the microtubules were organized into a midbody-like structure also contained INCENP. We reinterpret those data as suggesting that, like in *Dictyostelium*, metazoan INCENP also transfers equatorially from the central spindle to the cleavage furrow. A subsequent recruitment of INCENP to the metazoan midbody obscures this initial transfer to the furrow.

The IN-Box Domain Is Not Required for the Localization of DdINCENP at the Central Spindle and at the Cleavage Furrow

INCENP interacts with Aurora B with its C-terminal IN-box domain to act as both a substrate and regulator of Aurora B kinase (Adams *et al.*, 2000; Kaitna *et al.*, 2000; Kang *et al.*, 2001; Bishop and Schumacher, 2002). Additionally, in some metazoans these proteins are interdependent for their localization during mitosis (Adams *et al.*, 2001b; Honda *et al.*, 2003; Romano *et al.*, 2003). However, our data suggest that an interaction with Aurora B kinase may not be necessary for DdINCENP to localize at the central spindle and the cleavage furrow. All DdINCENP constructs lacking the IN-box domain, including the smallest, DdINCENP₁₋₂₇₃, were found at the central spindle and the cleavage furrow. These results differ from those observed in mammalian cells where the N-terminal 405 amino acids of INCENP were able to localize on centromeres but failed to relocate to the central spindle during anaphase (Mackay *et al.*, 1998). However, it is important to note that the experiments in mammalian cells were carried out in the presence of endogenous INCENP, whereas we have expressed our constructs in INCENP null cells. Nonetheless, the interaction with Aurora kinase via the IN-box domain is clearly essential for function, because the expression of DdINCENP without this domain failed to rescue the cytokinesis defect of DdINCENP null cells.

The first 58 amino acids of mammalian INCENP can interact with Borealin and Survivin and localize to centromeres but not to the central spindle or cleavage furrow (Klein *et al.*, 2006). This portion of INCENP is not conserved between mammalian and *S. cerevisiae* INCENP proteins, even though both proteins bind to Survivin proteins. This suggests that a three-dimensional motif, not recognized in the primary sequence, is involved in the interaction between INCENP and Survivin. Because there are no discernible orthologues of Borealin or Survivin in the *Dictyostelium* ge-

nome, we surmise that the localization of DdINCENP may be regulated by a different mechanism.

DdINCENP Can Interact with the Actin Cytoskeleton by Its N-Terminal Region

The mechanism of INCENP localization at the cleavage furrow has not been explored in any detail. No previous study has determined whether INCENP is bound to the actin cytoskeleton or to the membrane of the furrow. We showed previously that the proper organization of DdINCENP at the cleavage furrow is dependent on myosin II suggesting the possibility of a direct interaction between DdINCENP and the actin-myosin cytoskeleton (Chen *et al.*, 2006). We have shown here that the N-terminal portion of DdINCENP is capable of interacting directly with the actin cytoskeleton. We found that the N-terminal domain of DdINCENP is able to localize to the cleavage furrow in a manner similar to the full-length protein. Interestingly, this portion of DdINCENP was also shown to interact with the actin cytoskeleton during interphase. DdINCENP₁₋₅₀₀ was visible on the cortex of interphase cells and concentrated particularly on pinocytotic cups. Macropinocytosis is an actin-dependent process that allows *Dictyostelium* cells to engulf the nutrient medium by forming a pinocytotic cup. This process is known to involve many actin-associated proteins such as coronin, talin, and profilin (Cardelli, 2001). Some of those proteins, which localize to the pinocytotic sites, are also found at the cleavage furrow and have important functions during both pinocytosis and cytokinesis (Konzok *et al.*, 1999; Cardelli, 2001; Rivero *et al.*, 2002).

Although the localization at the pinocytotic cup is clearly not a native function of full-length DdINCENP, it does demonstrate the ability of this protein to interact with the actin cytoskeleton. The localization of DdINCENP₁₋₅₀₀ at unique actin-rich structures, such as the pinocytotic cup, also suggests that this fragment is not binding to the actin cytoskeleton in general, but to a specific subset of the actin cytoskeleton. Indeed, purification of DdINCENP₁₋₅₀₀ identified a subset of actin cytoskeletal proteins that copurified with DdINCENP₁₋₅₀₀, including ABP-50, Hsc70, actin, and myosin II. The actin binding protein ABP-50 has been shown to cross-link actin filaments during chemotaxis and to localize to actin rich extensions (Demma *et al.*, 1990; Dharmawardhane *et al.*, 1991). Hsc70 is known as a regulator of the actin capping proteins CAP32/34 (Haus *et al.*, 1993). Neither ABP-50 nor Hsc70 is known to concentrate on the cleavage furrow. Notably absent from our DdINCENP₁₋₅₀₀ purification were the abundant actin cytoskeletal proteins ABP120, cortexillin, and α -actinin. Thus, we would suggest that DdINCENP has the intrinsic ability to bind directly to a specific network of actin cytoskeleton found at the cleavage furrow and that this ability is regulated in the context of the full-length protein, to occur only during cytokinesis. The DdINCENP₁₋₅₀₀ fragment behaves as an un-regulated protein that still binds to the actin network at the cleavage furrow, but in addition, to the pinocytotic cup. We hope that this protein can be used as a probe to define the structural elements at the cleavage furrow that are able to recruit INCENP and perhaps other chromosomal passenger proteins.

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