Dictyostelium Aurora Kinase Has Properties of both Aurora A and Aurora B Kinases

Hui Li,1 Qian Chen,1‡ Markus Kaller,2 Wolfgang Nellen,2 Ralph Gräu,3 and Arturo De Lozanne1*

Section of Molecular Cell & Developmental Biology and Institute for Cellular and Molecular Biology, University of Texas at Austin, Austin, Texas 78712; Abt. Genetik, Universität Kassel, Heinrich-Plett-Str. 40, 34132 Kassel, Germany; and Universität Potsdam, Institut für Biochemie und Biologie, Zellbiologie, Karl-Liebknecht-Str. 24-25, Haus 26, 14476 Potsdam-Golm, Germany

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Aurora kinases are highly conserved proteins with important roles in mitosis. Metazoans contain two kinases, Aurora A and B, which contribute distinct functions at the spindle poles and the equatorial region respectively. It is not currently known whether the specialized functions of the two kinases arose after their duplication in animal cells or were already present in their ancestral kinase. We show that Dictyostelium discoideum contains a single Aurora kinase, DdAurora, that displays characteristics of both Aurora A and B. Like Aurora A, DdAurora has an extended N-terminal domain with an A-box sequence and localizes at the spindle poles during early mitosis. Like Aurora B, DdAurora binds to its partner DdINCENP and localizes on centromeres at metaphase, the central spindle during anaphase, and the cleavage furrow at the end of cytokinesis. DdAurora also has several unusual properties. DdAurora remains associated with centromeres in anaphase, and this association does not require an interaction with DdINCENP. DdAurora then localizes at the cleavage furrow, but only at the end of cytokinesis. This localization is dependent on DdINCENP and the motor proteins Kif12 and myosin II. Thus, DdAurora may represent the ancestral kinase that gave rise to the different Aurora kinases in animals and also those in other organisms.

Universal to all eukaryotes, Aurora kinases are known to function during multiple stages of cell division. The activity of Aurora kinases regulates almost every crucial stage of M phase, from chromosome condensation and separation to the very end of cytokinesis (10). Most animal cells contain two related kinases, Aurora A and B, which have distinct localizations and specialized roles during mitosis (1). Aurora A is localized primarily at the spindle poles and is implicated in the regulation of entry into mitosis, centrosome maturation, and spindle assembly (3, 28, 52). Aurora B forms the well-known chromosome passenger complex together with INCENP, survivin, and borealin (60). This complex associates with centromeres early in mitosis and then redistributes to the central spindle at the metaphase/anaphase transition (2, 53, 57). Accordingly, Aurora B is required for correct chromosome segregation and cytokinesis (31). Vertebrates contain an additional Aurora C kinase that is expressed exclusively in the testis, where it may play a role similar to that of Aurora B (35, 40). In contrast to animals, yeast cells contain only one Aurora kinase (Ipl1 in budding yeast and Ark1 in fission yeast) (12, 48). The yeast kinases have been shown to be mainly involved in chromosome segregation and cytokinesis (22, 39, 45, 47), processes that are usually regulated by animal Aurora B kinase. Similarly to animal Aurora B, yeast Aurora also associates with homologues of INCENP and survivin and localize to the centromeres and spindle (9, 34, 51). Unlike animal Aurora A, Ark1 mutant cells exhibited only a minor defect in spindle formation and Ark1 kinase does not seem to be localized on the spindle pole body (48).

To determine the separate roles of Aurora A and B kinases, it is important to consider their evolutionary origin. The high sequence similarity between these proteins suggests that they arose by gene duplication. Phylogenetic analysis suggests that the duplication of Aurora genes has occurred independently in vertebrates, invertebrates, and plants (8). However, it is not clear whether the original ancestral kinase had the properties and functions of Aurora A, Aurora B, or both. Given that, by most criteria, the yeast Aurora kinases resemble Aurora B, it would appear that the specialized features of Aurora A may have been a subsequent invention in animal cells after the duplication of the ancestral Aurora gene. Since fungi have a closed mitosis and a reduced set of spindle and cytoplasmic microtubules, it is possible that yeasts may have dispensed with the specialized activity of an Aurora A kinase. On the other hand, only the kinase domain of human Aurora A, but not of Aurora B, can partially complement the function of Saccharomyces cerevisiae Ipl1 when fused to the N terminus of Ipl1 (5). To better understand the similarities and differences among different Aurora kinases and to better appreciate what parallels can be drawn among them, it is important to analyze the function of Aurora kinases in other eukaryotes.

Other eukaryotes in which Aurora kinases have been identified include plants and the parasite trypanosomes, but the function of these kinases has not yet been characterized in detail. In higher plants, Aurora kinases have diversified into two major groups, alpha and beta, that are not related to the animal Aurora A and B kinase groups (18). Both types of
plant Aurora proteins localize on centromeres and spindle fibers and do not appear to be required for spindle formation (18). Given that plant mitotic spindles do not have centromeres, it would seem reasonable to postulate that plants may not need an Aurora A-like kinase. In trypanosomes, the Aurora kinases have diversified into three closely related proteins that seem most similar in sequence and function to animal Aurora B (58). However, no homologues of INCENP, survivin, or borealin seem to be present in the genomes of any plants or trypanosomes. Therefore, it is not clear whether the mechanism of regulation of Aurora kinases in these organisms shares any similarity to that in animal cells.

*Dictyostelium* is an excellent model system to study mitosis and cytokinesis in eukaryotic cells. In addition to the many molecular tools available to study these cells, the morphology of their mitotic apparatus is amenable to detailed microscopy analysis (43, 44, 50). *Dictyostelium* cells contain relatively few interphase microtubules that are rearranged into a well-defined intranuclear spindle during mitosis. A large effort to identify and characterize the function of centrosomal proteins is well under way (25, 49), and a large collection of mutants that affect mitosis or cytokinesis have been gathered over the years (16, 20, 36, 38, 46). Most importantly, *Dictyostelium discoideum* contains a protein homologous to animal INCENP, the activating partner of Aurora B (15). This protein, DiDINCENP, displays the dynamic behavior of chromosomal passenger proteins and is important for mitosis and cytokinesis. Since analysis of the *Dictyostelium* genome suggests that this organism has retained many of the ancestral properties of the last common ancestor of plants, animals, and fungi (19), it is an ideal system to dissect the original properties of Aurora kinases. Our findings presented here demonstrate that the single *Dictyostelium* Aurora kinase displays properties found in both Aurora A and B kinases and thus suggest that the ancestral Aurora kinase played both roles in primitive cells.

**MATERIALS AND METHODS**

Cloning of DiDaurora and construction of GFP-DiDaurora. A 1.6-kb sequence corresponding to the full length of DiDaurora gene was cloned from *Dictyostelium* genomic DNA by using the 5′ and 3′ primers AO-499 (5′-CGAGGCTCATGAGTTATCCAAATAATAAAGAAAATAGTAACAATATTGGTG-3′) and AO-500 (5′-CGAGGCTC TTTAATATGCTTGGTTAGTTAATGGGAAAGGCC-3′), respectively. The PCR product was further cloned into the pMal-c2X vector (New England Biolabs, Ipswich, MA). A maltose-binding protein (MBP)-DiDaurora fusion protein was expressed in *Escherichia coli* and purified according to the provided protocol. Five milliliters of amylase resin (New England Biolabs, Ipswich, MA) was used for 1 liter of *Escherichia coli* culture. The purified fusion protein was injected into rabbits to raise polyclonal anti-DiDaurora antibodies (Cocalico Biologicals, Reamstown, PA). To purify polyclonal anti-DiDaurora antibodies by affinity binding, purified MBP-DiDaurora was further cross-linked to AminoLink coupling gel (Pierce, Rockford, IL) according to the provided protocol. Tandem affinity purification (TAP)-tagged GFP was purified from *Dictyostelium* cell culture and used to generate polyclonal anti-GFP antibodies by the same procedure.

Affinity purification of polyclonal anti-DiDaurora antibodies. One milliliter of AminoLink coupling gel (Pierce, Rockford, IL) cross-linked with more than 10 mg of purified anti-DiDaurora antibody was used to purify polyclonal anti-DiDaurora antibody by affinity binding. 1 mg of affinity-purified anti-DiDaurora or monoclonal anti-DiDcp224. The second antibody was Texas red-conjugated goat anti-rabbit or fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Molecular Probes, Eugene, OR). For DAPI (4′,6-diamidino-2-phenylindole) staining of DNA, cover slides were immersed in 0.1 M glycine (pH 2.5) and equilibrated to pH 7.4 immediately with 1 M Tris-HCl (pH 8.0).

**Commmunoprecipitation with DiDaurora antibodies**. Affinity-purified polyclonal anti-DiDaurora antibodies were used in all immunoprecipitation experiments. Cells expressing target GFP-fusion protein were cultured in suspension in a 5- to 7-L bioreactor. When the cell population reached desired confluency, the cultures were sonicated six times for 15 s each with a 15-s rest in between. The lysate was then centrifuged at 13,000 rpm for 15 min at 4°C to remove cell debris. Forty microliters of purified anti-DiDaurora antibodies was added to 1 ml of supernatant after centrifugation. Either unspecific rabbit immunoglobulin G or no addition of antibody was used as a negative control. The mixture was rotated at 4°C for 1 h, and then 20 ml of prewashed protein A beads (Amersham Pharmacia, Piscataway, NJ) was added to the suspension. After another 30-min incubation, the antibody-protein A complex was spun down at 3,000 rpm for 1 min and washed by 6 × 1 ml 0.1 M sodium phosphate buffer. Finally, bound proteins were eluted by boiling the protein-bead complex in 100 ml SDS-sample buffer at 95°C for 10 min. The elutions were resolved on a 10% polyacrylamide gel and examined by Western blot analysis with anti-GFP antibodies.

**In vitro pull-down with MBP-DiDaurora**. Because different DiDINCP truncations have very different expression levels in DiDINCP-null cells, an in vitro assay was used instead of communoprecipitation to keep the amount of DiDaurora input at the same level. Cells were cultured and harvested in the same way as that of the communoprecipitation experiment. After sonication, GFP-DiDINCENP-4C lysate was diluted 10 times before centrifugation. By this dilution, the input levels of GFP-fusion proteins were adjusted to the same level too. After centrifugation, 200 ml of MBP-DiDaurora-saturated amylase beads was added to the supernatant and incubated at 4°C for 2 h. After extensive wash, the bound for the GFP fluorescence was 100 ms or less, with the interval time being at least 10 s.

**Immunostaining and microscopy of mitotic cells**. For immunostaining of mitotic cells, cells in active log-phase growth were harvested from stationary culture and resuspended to a concentration of 2 × 10⁶ cells/ml. Cell suspension (200 ml) was put on each coverslip and allowed to sit still for at least 20 min. For fixation, cells were first fixed in 1× PDF buffer (20 mM KCl, 11 mM K₂HPO₄, 13.2 mM KH₂PO₄, 1 mM CaCl₂, 2.5 mM MgSO₄, [pH 6.4]) with 2% formaldehyde and 0.01% Triton X-100 for 15 min at room temperature and then in dehydrated methanol with 1% formaldehyde at –20°C for 5 min. The primary antibody was affinity-purified anti-DiDaurora or monoclonal anti-DiDcp224. The second antibody was Texas red-conjugated goat anti-rabbit or fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Molecular Probes, Eugene, OR). For DAPI (4′,6-diamidino-2-phenylindole) staining of DNA, cover slides were immersed in 0.1 M glycine (pH 2.5) and equilibrated to pH 7.4 immediately with 1 M Tris-HCl (pH 8.0).
proteins were boiled off from the beads by 100% SDS-sample buffer. The elutions were also resolved on a 10% polyacrylamide gel and examined by Western blot analysis with anti-GFP antibodies.

RESULTS

Dictyostelium has a single protein with similarities to both Aurora A and Aurora B kinases. A search of the Dictyostelium genome database with Aurora kinase sequences from other organisms yielded only one related gene, which has been named aurK (entry DDB0216254 in http://dictybase.org; GenBank accession no. XP_641803). The sequence of the aurK gene encodes a serine-threonine protein kinase of 43 kDa that has a high degree of sequence similarity to Aurora kinases from other organisms, including both Aurora A and Aurora B kinases (Fig. 1). Therefore, we will refer to this protein simply as Dictyostelium Aurora, or DdAurora.

DdAurora contains the signature motifs found in all Aurora kinases, including a KEN box at its N terminus, the activation loop in the catalytic domain (DFGWSXXXXXXXRXTXCGTXDYLPPE), and a D2-type destruction box (LLXXXPXXRXXLXXXXXHPW), near its C terminus (11, 13, 54). In addition, DdAurora has a potential A box (PXXXQRVXXQ) near its N terminus, which is a specific feature found in vertebrate Aurora A kinases. The A box has been shown to be important to regulate the Cdh1-dependent destruction of Aurora A. Phosphorylation of the consensus serine site (in boldface) in this motif is able to block destruction of Aurora A (41).

To explore the relationship between DdAurora and Aurora kinases from other organisms, we constructed a phylogenetic tree with protein sequences from many organisms (Fig. 2).

FIG. 1. Alignment of DdAurora with Aurora kinases from other organisms. DdAurora has high sequence similarity to both Aurora A and Aurora B kinases. DdAurora has the KEN sequence commonly found at the N terminus of Aurora kinases. DdAurora also has an N-terminal A box (PXXXQRVXXQ; framed in dashed box), which is important for Aurora A destruction. Within the A box, the conserved phosphorylation site, serine 22 (boldface in the A-box sequence [asterisk on the figure]) is known to be phosphorylated in vertebrates to block Cdh1-dependent destruction of Aurora A (41). DdAurora also contains the Aurora signature motif (DFGWSXXXXXXXRXTXCGTXDYLPPE) within the activation loop (framed) and a D box (LLXXXPXXRXXLXXXXXHPW) near its C terminus (underlined in the figure). All Aurora kinases have these two motifs to regulate their activation and degradation. H.s., Homo sapiens; X.l., Xenopus laevis; S.c., Saccharomyces cerevisiae.

Vertebrate proteins group into three distinct clades: the known Aurora A, B, and C kinases (8). Plant proteins form two clades, the Aurora alpha and beta kinase groups. The Aurora proteins from different fungi also form a clearly defined group. The Dictyostelium kinase branches off near the base of the animal and plant groups as it is about equally similar to the animal, plant, and fungus kinases (57.5% to human Aurora A, 56% to human Aurora B, and 61.5% to Arabidopsis Ipl1). The protein most similar to DdAurora is an uncharacterized Aurora protein from the cycad Cycas rumphii (71.2%). Since it is generally thought that Dictyostelium diverged early in the evolution of eukaryotes, our analysis suggests that the archetype Aurora kinase in the common ancestor of plants, fungi, and animals already had characteristics of both Aurora A and Aurora B kinases. Importantly, this kinase had the A-box signature of Aurora A kinases.
phase plate, probably in association with centromeres (Fig. 3c).

With the onset of anaphase, GFP-DdAurora localized at the central spindle and remained there during telophase and early cytokinesis (Fig. 3d to f). The Dictyostelium spindle is known to dismantle during late telophase or early cytokinesis (50). Accordingly, we observed that GFP-DdAurora disappeared from the region of the central spindle during early cytokinesis (Fig. 3f). At the end of cytokinesis, GFP-DdAurora localized at the cytoplasmic bridge that connects the two daughter cells (Fig. 3g). When this bridge finally severed, GFP-Aurora persisted at the breaking point for a short time (Fig. 3i and see Movies S1 and S2 in the supplemental material). From anaphase to the end of cytokinesis, GFP-DdAurora could also be constantly observed near the spindle pole regions (Fig. 3d to f; not shown in panels g to i because of the focus plane).

Our observations indicate that DdAurora displays the distribution of both Aurora A (polar localization) and Aurora B (central spindle localization). DdAurora also has novel properties not previously reported for any other Aurora kinase (localization at the cytoplasmic bridge in the absence of midbody microtubules). As expected for a protein participating in multiple steps during mitosis, we were not able to generate a knockout of the DdAurora gene (data not shown).

**Polar localization of DdAurora.** To investigate the localization of DdAurora in greater detail, we raised polyclonal antibodies against DdAurora. The anti-DdAurora antibodies specifically recognized DdAurora as a single band in Western blot analysis of whole-cell lysates (see Fig. S1 in the supplemental material). Immunofluorescence microscopy with affinity-purified anti-DdAurora antibodies revealed a distribution of endogenous DdAurora similar to that observed with GFP-DdAurora (Fig. 4). The only difference between GFP-DdAurora and endogenous DdAurora distribution was their relative intensity on centromeres during prometaphase and metaphase plate. Endogenous DdAurora appeared brighter on centromeres than at the poles (Fig. 3a to b), whereas GFP-DdAurora was brighter at the poles than on centromeres (Fig. 4b and c). This might indicate a weaker association of GFP-DdAurora with the centromeres at these stages or a stronger association with the poles.

We then used double-immunofluorescence microscopy to determine the localization of DdAurora in relation to that of the centrosomal protein DdCP224 (24). In Dictyostelium, the nuclear envelope is not dismantled during mitosis (44). Instead, the centromere duplicates and becomes embedded in the nuclear envelope early in mitosis (59). The embedded

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**FIG. 2.** Phylogenetic tree of Aurora kinases from different species. The core kinase domains of Aurora sequences from the indicated species were aligned by ClustalW, and the corresponding phylogenic tree was generated using MegAlign. Aurora kinases in vertebrates are divided into three groups of Aurora A, B, and C subfamilies. Plants have two groups of Aurora kinases: the alpha and beta subfamilies. Most fungi have a single Aurora kinase, similar to other members within this clade. Caenorhabditis elegans and Drosophila contain divergent Aurora A and B kinases that probably arose through an independent duplication from that giving rise to vertebrate kinases (8). The Dictyostelium DdAurora kinase (asterisk) is about equally similar to the kinases of animals, plants, and fungi.
centrosomes subsequently nucleate an intranuclear spindle. DdCP224, an ortholog of XMAP215, regulates microtubule dynamics (26) and is colocalized with \(H\)-tubulin exactly at the mitotic spindle poles (24). Close observation of the double-stained wild-type cells revealed that, rather than displaying an overlapping colocalization with DdCP224, DdAurora localized in close proximity but either distal to DdCP224 during prometaphase and metaphase (d to f). Notice that GFP-DdAurora did not accumulate at the cleavage furrow early in cytokinesis (e) or even when the furrow was very advanced (f). In contrast, DdINCENP has been shown to localize at the cleavage furrow early in cytokinesis (15). Near the end of cytokinesis, GFP-DdAurora accumulated at the cytoplasmic bridge formed between the two daughter cells (g and h). GFP-DdAurora persisted at the breaking point of the cytoplasm bridge for a time after cytokinesis (f). Bar, 5 \(\mu\)m. (See Movies S1 and S2 in the supplemental material.)

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DdCP224, an ortholog of XMAP215, regulates microtubule dynamics (26) and is colocalized with \(\gamma\)-tubulin exactly at the mitotic spindle poles (24).

Close observation of the double-stained wild-type cells revealed that, rather than displaying an overlapping colocalization with DdCP224, DdAurora localized in close proximity but either distal to DdCP224 during prometaphase and metaphase (Fig. 4a to c) or proximal to DdCP224 during anaphase and telophase (Fig. 4d and e). Therefore, our images suggested that DdAurora was localized on the outer (cytosolic) side of the spindle poles during prometaphase and metaphase (Fig. 4a to c). Upon entry into anaphase, the outer polar DdAurora localization disappeared, to be replaced by DdAurora on the inner (intranuclear) side of the spindle poles (Fig. 4d and e).

**DdAurora remains bound to centromeres after anaphase.**

*Dictyostelium* centromeres migrate in close proximity to spindle poles in anaphase and remain associated with spindle poles through the rest of cell division (44). Thus, the presence of DdAurora on the inner side of the spindle poles, in close apposition with the segregated chromosomes (Fig. 4d), suggested the possibility that DdAurora remains associated with centromeres after metaphase. To test this possibility, we compared the localization of endogenous DdAurora with that of HcpA, a centromeric protein homologous to mammalian HP1 (32). Examination of cells expressing HcpA-GFP and immunostained with anti-DdAurora antibodies revealed that DdAurora colocalized with HcpA-GFP at centromeres in metaphase (Fig. 5a). During metaphase, *Dictyostelium* cells contain all centromeres congregated as a single dot at the center of the metaphase spindle and the chromosome arms spread outward perpendicularly to the spindle (50). At this stage, DdAurora was also present on the outer (cytosolic) side of the spindle poles (Fig. 4b).

We confirmed the observation that the localization of HcpA-GFP changes early in anaphase (33). HcpA-GFP was bound to centromeres at metaphase but became diffuse inside the nucleus of cells in early anaphase (Fig. 5b). This suggests that...
HcpA-GFP dissociates from the centromeres at this time. HP1, the mammalian homologue of HcpA, is known to be displaced from mitotic chromosomes by Aurora B kinase during mitosis (21, 29). Later in telophase, HcpA-GFP was again localized at centromeres (Fig. 5c), which are closely apposed to the inner side of the spindle poles (32). At this stage, DdAurora clearly colocalized with HcpA-GFP at the centromeres of the telophase cells (Fig. 5c). The spindle poles of these cells, as marked by DdCP224 or γ-tubulin, are located farther away from the center of the spindle (Fig. 4e). These observations indicate that DdAurora remained bound to the centromeres of the separated chromatids during anaphase and telophase. HcpA is known to remain associated with the centromeres through interphase (33). The retention of Aurora kinase on centromeres after chromosomes have separated is unusual and has not been observed in any other species.

The Aurora B-like properties of DdAurora are dependent on its interaction with the IN-box domain of DdINCENP. We have shown that DdAurora displays the distribution characteristic of both Aurora A and Aurora B kinases. Interestingly, the chromosomal passenger protein DdINCENP was found at some, but not all, of these locations in a mitotic cell (15). DdINCENP localized on centromeres during prometaphase and metaphase and on the central spindle during anaphase and telophase. These observations suggest the possibility that the DdAurora protein that colocalizes with DdINCENP may be part of a chromosomal passenger complex that has the properties of the similar complex formed by animal Aurora B kinase. On the other hand, the DdAurora found at the spindle poles would not be part of the chromosomal passenger complex and would behave as an Aurora A kinase. If this scenario is correct, then DdINCENP should be important only for the Aurora B-like localization of DdAurora. To test this hypothesis, we examined the localization of DdAurora in DdINCENP-null cells expressing the centromeric marker HcpA-GFP (32).

The localization of DdAurora on the spindle poles of metaphase cells was not influenced by the absence of DdINCENP (Fig. 6a and see Movie S3 in the supplemental material). This observation supports the idea that the polar localization of DdAurora is analogous to the polar localization of Aurora A and therefore does not require DdINCENP. In contrast, DdAurora failed to localize to the central spindle of DdINCENP-null cells during anaphase and telophase (Fig. 6b and c). The requirement of DdINCENP for the central spindle localization of DdAurora strengthens the model that these two proteins form a complex at the central spindle. However, DdINCENP was not required for the centromeric localization of DdAurora. DdAurora colocalized with HcpA-GFP on the centromeres of DdINCENP-null cells from metaphase to telophase (Fig. 6a, b, and c). Remarkably, HcpA-GFP was not displaced from the centromeres of DdINCENP-null cells during early anaphase (Fig. 6b) as it was displaced in wild-type cells (Fig. 5b). We interpret this observation as indicative that the loss of DdINCENP renders the centromeric DdAurora inactive and unable to displace HcpA from the centromere at
the onset of anaphase. This is also consistent with the observation that DdINCENP-null cells have defects in chromosome segregation (15), which may be caused by failure to activate DdAurora at the centromeres.

To verify that the loss of DdAurora localization at the central spindle was caused by the absence of DdINCENP, we expressed GFP-DdINCENP in DdINCENP-null cells. We have shown previously that GFP-DdINCENP can fully rescue the mitosis and cytokinesis defects observed in DdINCENP-null cells (15). Similarly, we found that the localization of DdAurora to the central spindle was rescued by GFP-DdINCENP and both proteins colocalized extensively at the central spindle (Fig. 7). This result strongly suggests that DdINCENP and DdAurora formed a chromosomal passenger complex that localized at the central spindle at the metaphase/anaphase transition. To confirm the interaction between these two proteins we precipitated endogenous DdAurora or TAP-tagged DdAurora and found that endogenous DdINCENP or GFP-DdINCENP coprecipitated with DdAurora (see Fig. S2 in the supplemental material).

The conserved C-terminal IN-box domain of INCENP is known to be essential for its interaction with Aurora B and activation of the kinase (6, 30). In addition, the N-terminal domain of DdINCENP is necessary and sufficient to localize DdINCENP to the cleavage furrow (14). To investigate the contribution of these domains to the localization of DdAurora, we determined the distribution of endogenous DdAurora in DdINCENP-null cells expressing two different DdINCENP truncation mutants (Fig. 8A). The localization of DdAurora at the central spindle of DdINCENP-null cells was rescued by expression of DdINCENP-ΔN (arrowheads), but not in those expressing GFP-DdINCENP-ΔC. As shown in Fig. 6, the centromeric localization of DdAurora near the spindle poles (arrows) was independent of DdINCENP.

To test whether the localization results described above are mediated by the interaction between various DdINCENP truncations and DdAurora, we performed pull-down experiments on all different cell lines. We found that the proteins that contained the IN-box domain (full-length DdINCENP and DdINCENP-ΔN) coprecipitated with DdAurora (see Fig. S3 in
In mammalian cells, the DdAurora at the central spindle.
The recruitment of DdAurora to the central spindle.
Critical for its interaction with DdAurora and is required for
Localization of DdAurora at the cleavage furrow.
Aurora B and INCENP are known to localize at the cleavage furrow and midbody of dividing animal cells but not at the mother-bud neck of yeast cells (9, 42, 57). Accordingly, Aurora B is required for cytokinesis in animal cells but the requirement is not so stringent in yeast cells (4, 48, 57). In Dictyostelium, DdINCENP localizes at the cleavage furrow early in cytokinesis and concentrates at the cytoplasmic bridge connecting the two daughter cells (15). DdINCENP is also required for the scission of the cytoplasmic bridge at the end of cytokinesis (15). Live imaging of GFP-DdAurora and immunofluorescence microscopy of endogenous DdAurora demonstrated that DdAurora localized at the region of the cleavage furrow, but only late in cytokinesis (Fig. 4h). DdAurora was visible at the cleavage furrow only near the end of cytokinesis when the furrow becomes a thin cytoplasmic bridge between the two daughter cells (Fig. 4h). When this bridge finally severed, GFP-Aurora persisted at the breaking point for a short time (Fig. 4i).

Since little is known about how Aurora B localizes at the cleavage furrow, we determined the ability of GFP-DdAurora to localize at the furrow of different mutant cell lines. We found that GFP-DdAurora failed to localize at the cleavage furrow of DdINCENP-null cells (see Movie S3 in the supplemental material). Given that DdINCENP is required for the localization of DdAurora at the central spindle, it is reasonable to expect a similar requirement for DdAurora localization at

The motor protein Kif12 is important for the localization of DdAurora at the central spindle. In mammalian cells, the kinesin 6-like protein MKLP2 is essential for the localization of the chromosomal passenger complex to the central spindle (27). In Dictyostelium, Kif12 was identified as a kinesin 6-like protein required for myosin localization to the furrow (37). In Kif12-null cells, GFP-DdINCENP can still localize to the central spindle but fails to localize at the cleavage furrow (14). To test whether Kif12 influences the distribution of DdAurora, we determined the localization of endogenous DdAurora in Kif12-null cells expressing HcpA-GFP. We found that DdAurora localized normally at the spindle poles and colocalized with HcpA-GFP at the centromeres of Kif12 mutant cells (Fig. 9a). However, the localization of DdAurora at the central spindle was abrogated in the Kif12 mutant cells (Fig. 9b and c and see Movie S4 in the supplemental material). Similar to our observations in wild-type cells (Fig. 5b), HcpA-GFP was diffusely distributed in the nucleus of Kif12-null cells during early anaphase (Fig. 9b). This finding suggests that Kif12 is not required for the activation of DdAurora at the centromeres in metaphase/anaphase.

Intriguingly, while we found that DdAurora failed to localize at the central spindle of Kif12-null cells, GFP-DdINCENP did localize at the central spindle in these mutant cells (14). This discrepancy could reflect different mechanisms of localization of these two proteins at the central spindle or may be due to the overexpression of GFP-DdINCENP. To distinguish between these possibilities, we stained Kif12-null cells expressing GFP-DdINCENP for endogenous DdAurora localization (Fig. 10). We found that DdAurora was restored at the central spindle of these cells indicating that overexpression of GFP-DdINCENP overcomes the defect caused by loss of Kif12. Immunoprecipitation assays also showed that GFP-DdINCENP interacted with DdAurora in Kif12-null cells (see Fig. S4 in the supplemental material).
the cleavage furrow. It seems likely that the absence of DdAurora at the cleavage furrow is the basis of the cytokinesis defect observed in DdINCENP-null cells (15).

Similarly, GFP-DdAurora did not localize at the cleavage furrow of Kif12-null cells (see Movie S4 in the supplemental material). Since DdINCENP does not localize at the cleavage furrow in these mutant cells (14), it is likely that this is the cause for the failure to recruit DdAurora to the cleavage furrow.

We have shown previously that the distribution of DdINCENP at the cleavage furrow was disturbed by the absence of myosin II (15). In myosin II mutant cells, GFP-DdINCENP localized as a narrow band at the furrow instead of the broad cleavage furrow cortex localization observed in wild-type cells (15). To determine whether myosin II also influences the distribution of DdAurora, we determined the localization of endogenous DdAurora and of GFP-DdAurora in myosin II heavy-chain-null cells expressing GFP-Aurora during cytokinesis. The central spindle localization of GFP-DdAurora could still be observed at 01:19 (arrowhead). GFP-DdAurora failed to localize at the cytoplasmic bridge (arrows) formed between the two daughter cells. Bar, 5 μm.

**FIG. 11. Localization of GFP-DdAurora in myosin II-null cells.**

(A) Localization of endogenous DdAurora in myosin II-null cells was determined by immunostaining. In the merged images, DNA is shown in blue and endogenous DdAurora is shown in red. The polar and centromeric (arrow) localization during metaphase was normal in these cells. In addition, the central spindle localization of DdAurora (arrowheads) in anaphase was normal. Bar, 5 μm. (B) Live images of myosin II (myosin heavy chain)-null cells expressing GFP-Aurora during cytokinesis. Bar, 5 μm.

DdAurora has properties of Aurora A kinase. Aurora A kinases are distinct from Aurora B kinases in having a longer N-terminal domain that contains an A-box sequence important for the proteolytic regulation of these proteins. The *Dictyostelium* DdAurora kinase contains an A-box sequence within its N-terminal domain similar to that of Aurora A kinases. A second property characteristic of Aurora A kinases is their localization at the spindle poles, where they play an important role in the maturation of centrosomes and spindle assembly. We have shown that DdAurora is also localized at the spindle poles from the initiation of mitosis until the beginning of anaphase. Given its sequence similarity to Aurora A kinases and similar localization during mitosis, we conclude that DdAurora plays the role of Aurora A during *Dictyostelium* mitosis. It will be interesting to identify proteins that may regulate the localization and function of DdAurora at the spindle poles. Since the vertebrate Aurora A regulators Aip1 and TPX2 do not appear to have orthologs in invertebrates, fungi or protists, it is likely that other proteins yet to be discovered regulate the activity of this kinase at the spindle poles.

Colocalization of DdAurora with the centrosomal marker DdCP224 revealed that DdAurora is localized on the outer side of the spindle poles. In *Dictyostelium*, the centrosome is embedded in the nuclear envelope during mitosis (44). Thus, one centrosomal surface remains in the cytosol while the other resides inside the nucleus. Our observations suggest that there may be functional differences between these two surfaces and that DdAurora may play a role on the cytosolic side of the centrosome during prometaphase and metaphase. In mammalian cells, Aurora A kinase was found to localize to centrosomes and the adjacent spindle microtubules (55). It is possible that this pericentrosomal localization of Aurora A is equivalent to the localization of DdAurora on the outer portion of the spindle poles.

DdAurora has properties of Aurora B kinase. Aurora B kinases are characterized by their association with the proteins of the chromosomal passenger complex and dynamic sequential localization at centromeres, central spindle, and midbody. We have shown that DdAurora displays all these properties of Aurora B kinases. DdAurora is found on centromeres as early as prometaphase; it localizes at the central spindle during anaphase and at the cytoplasmic bridge at the end of cytokinesis. DdAurora also interacts with the chromosomal passenger protein DdINCENP and this interaction requires the conserved IN-box domain at the C terminus of DdINCENP. By all of these criteria, it is clear that DdAurora plays the role of Aurora B in *Dictyostelium* mitosis.
We demonstrated that DdAurora is found on centromeres by colocalization with the centromeric protein HcpA. In contrast with Aurora B from other organisms, we found that DdAurora remains associated with the centromeres even during anaphase and telophase. In *Dictyostelium*, the centromeres of the daughter chromatids congregate closely to the spindle poles during anaphase (44), and therefore, DdAurora and HcpA are visible as a single dot on the inner surface of the spindle poles. In contrast, animal Aurora B leaves the centromeres at the onset of anaphase and relocates to the central spindle (2, 53, 57). Interestingly, one of the Aurora isoforms (AtAuR-1) found in *Arabidopsis* appears to move with centromeres during anaphase (18). While this localization has not been proven to be centromeric, it opens the possibility that Aurora kinases play an additional centromeric function during anaphase, at least in plants and *Dictyostelium*.

In contrast to DdAurora, its binding partner, DdINCENP, does not remain associated with anaphase centromeres. Like its animal counterpart, DdINCENP redistributes to the central spindle during anaphase (15). Therefore, the association of DdAurora with the centromere appears to be independent of DdINCENP. We confirmed this possibility by showing that DdAurora is still found on centromeres in DdINCENP-null cells.

The localization of Aurora B at the centromeres has been shown to be important for the regulation of proper microtubule attachment to kinetochores (17, 56). This activity is dependent on the proteins of the chromosomal passenger complex (51). Aurora B is also known to be required for the dissociation of human HP1 proteins from chromosomes during mitosis (21, 29). We found that *Dictyostelium* HcpA, the ortholog of human HP1, also dissociates from centromeres, but only during early anaphase. Importantly, we observed that HcpA failed to dissociate from anaphase centromeres in the DdINCENP-null mutants. We interpret these observations as indicative that the activity of the DdAurora/DdINCENP complex at the centromeres dissociates HcpA at the onset of anaphase. Concomitantly, as in other organisms, DdINCENP dissociates from the centromeres and the DdAurora that remains associated with the anaphase centromeres would not have Aurora B-like activity. DdINCENP and a portion of DdAurora redistribute to the central spindle and eventually to the cleavage furrow.

**DdAurora forms a chromosomal passenger complex with DdINCENP.** We have shown that DdAurora interacts with DdINCENP and that this interaction requires the IN-box domain at the carboxyl terminus of DdINCENP. Therefore, it appears that *Dictyostelium* has a chromosomal passenger complex similar to that described in other organisms. However, the *Dictyostelium* genome does not encode any protein with similarity to the other chromosomal passenger complex proteins survivin/Bir1 and borealin. Elucidation of whether other proteins participate in the *Dictyostelium* chromosomal passenger complex will require the purification of this complex.

While it is clear that DdAurora and DdINCENP form a complex, we also provide evidence that their interaction must be transient and spatially regulated. The central spindle is a location where these two proteins clearly interact. DdAurora depends on DdINCENP to localize to the central spindle, although the reverse is not true. Similarly, both proteins are enriched at the cytoplasmic bridge connecting the two daughter cells at the end of cytokinesis. The localization of DdAurora at this location also requires the presence of DdINCENP. However, DdINCENP localizes to the invaginating cleavage furrow from the beginning of cytokinesis, whereas DdAurora does not. This would suggest that DdINCENP associates with the cleavage furrow and later recruits DdAurora. It seems likely that the late cytokinesis defect observed in DdINCENP-null cells is caused by a lack of DdAurora recruitment to the cytoplasmic bridge.

The differences in DdAurora and DdINCENP localization also suggest that, instead of being solely a DdAurora cofactor, DdINCENP may play additional roles in mitosis and cytokinesis by interacting with other proteins. For example, it has been shown recently that phosphorylated INCENP can bind to Polo-like kinase (23), another important mitotic kinase essential for mitotic entry, spindle formation, and cytokinesis (61). DdINCENP may also regulate mitosis by interacting with a Polo-like kinase protein in *Dictyostelium*.

The **cytoskeletal proteins Kif12 and myosin II modulate a subset of DdAurora localizations.** Similarly to the role of the animal motor protein MKLP2, *Dictyostelium* Kif12 is essential for the localization of DdAurora to the central spindle at the metaphase/anaphase transition. However, when GFP-DdINCENP is overexpressed in Kif12-null cells, it rescues the localization of DdAurora to the central spindle. It seems likely that Kif12 is also important for the localization of DdINCENP to the central spindle but that overexpression of GFP-DdINCENP overrides the loss of Kif12. Unfortunately, the antibodies raised against DdINCENP have not been useful for immunofluorescence studies to determine the distribution of endogenous DdINCENP. Nonetheless, while the overexpression of GFP-DdINCENP rescues the localization of DdAurora to the central spindle, it is not sufficient to recover the localization of either protein to the cleavage furrow of Kif12-null cells. This indicates that this motor protein is responsible, directly or indirectly, for the localization of the chromosomal passenger complex to the cleavage furrow.

The localization of proteins at the cleavage furrow is a poorly understood process. We showed previously that the distribution of DdINCENP at the cleavage furrow is abnormal in myosin II-null cells (15). We showed here that the localization of DdAurora was also affected by the absence of myosin II. Although GFP-DdAurora was still localized at the central spindle, it was absent from the cleavage furrows of myosin II-null mutant cells. This suggests that the absence of myosin II not only affects localization of DdINCENP to the contractile furrow, but also disrupts the furrow localization of DdAurora. Since Aurora kinase activity is important for the completion of cytokinesis, the cytokinesis defect of myosin II-null cells could be explained by both failure of contraction and the absence of Aurora kinase activity at the furrow during late cytokinesis.

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