Cell, Vol. 114, 585–598, September 5, 2003, Copyright ©2003 by Cell Press

Aurora-A and an Interacting Activator, the LIM Protein Ajuba, Are Required for Mitotic Commitment in Human Cells

Toru Hirota,[1,2,3] Naoko Kunitoku,[1] Takashi Sasayama,[1] Tomotoshi Marumoto,[1] Dongwei Zhang,[1] Masayuki Nitta,[1] Katsuyoshi Hatakeyama,[2] and Hideyuki Saya[1,*]

[1]Department of Tumor Genetics and Biology
Graduate School of Medical Sciences
Kumamoto University
1-1-1 Honjo
Kumamoto, 860-8556
Japan

[2]Department of Surgery
Graduate School of Medical and Dental Sciences
Niigata University
1-757 Asahimachi-dori
Niigata, 951-8510
Japan

Summary

Aurora family kinases contribute to regulation of mitosis. Using RNA interference in synchronized HeLa cells, we now show that Aurora-A is required for mitotic entry. We found that initial activation of Aurora-A in late G2 phase of the cell cycle is essential for recruitment of the cyclin B1-Cdk1 complex to centrosomes, where it becomes activated and commits cells to mitosis. A two-hybrid screen identified the LIM protein Ajuba as an Aurora-A binding protein. Ajuba and Aurora-A interact in mitotic cells and become phosphorylated as they do so. In vitro analyses revealed that Ajuba induces the autophosphorylation and consequent activation of Aurora-A. Depletion of Ajuba prevented activation of Aurora-A at centrosomes in late G2 phase and inhibited mitotic entry. Overall, our data suggest that Ajuba is an essential activator of Aurora-A in mitotic commitment.

Introduction

Mitosis is conventionally divided into five phases—prophase, prometaphase, metaphase, anaphase, and telophase—on the basis of changes in the structure and behavior of the spindle and chromosomes. Prophase is characterized by the apparent onset and progression of chromosome condensation in the nucleus and by centrosome enlargement and an increase in microtubule nucleation activity, referred to as centrosome maturation, in the cytoplasm.

Centrosome maturation is associated with the recruitment of multiple proteins, including not only γ-tubulin and pericentriolar components required for microtubule nucleation, but also cell cycle regulators such as cyclin B1, cyclin-dependent kinase 1 (Cdk1), and Polo-like kinase 1 (Plk1) (Bailly et al., 1989; Nigg, 2001). The active cyclin B1-Cdk1 complex appears first at the centrosome, suggesting that this organelle, as it matures, may facilitate the activation of mitotic regulators required for the commitment of cells into mitosis (Jackman et al., 2003; Hannak et al., 2001). The signaling pathways that initiate centrosome maturation are thus considered to be activated during late G2 phase before this event becomes morphologically discernible at M phase, but those signals remain largely unknown, however (Pines and Rieder, 2001).

Genetic studies in yeast and flies have identified several kinases, collectively known as mitotic kinases, that are associated with the mitotic machinery and whose activities peak during late G2 to M phases. Like Cdk1, these mitotic kinases are also thought to play important and specific roles in both morphological and biochemical mitotic transitions (Nigg, 2001). These enzymes include Polo kinase, NIMA-related kinase, WARTS-related kinases, and Aurora kinases, and their structures and functions have been conserved, with slight deviations, through evolution. It remains to be determined, however, how and where these mitotic kinases are activated in the cell as well as how their activation is coordinated with that of Cdk1 (Nigg, 2001).

The Aurora family comprises a large number of kinases whose structure and function are evolutionarily conserved. Whereas budding yeast contains only one Aurora kinase, termed Ipl1, metazoans contain at least two subfamilies of these enzymes, designated Aurora-A and Aurora-B, that appear to play distinct roles. In mammalian cells, the contributions of Aurora-B to chromosome alignment and cytokinesis are relatively well characterized. Aurora-B localizes to condensing chromatin and then gradually accumulates at the centromere by prometaphase in order to control kinetochore-microtubule connections (Hauf et al., 2003; Ditchfield et al., 2003). In anaphase, Aurora-B moves to the central spindle or midbody to regulate cytokinesis (Terada, 2001; Adams et al., 2001). The activity of Aurora-B is regulated through its association with the inner centromere protein (INCENP) in a manner similar to that by which the activity of Ipl1 is regulated by its association with Sli15, the budding yeast ortholog of INCENP (Kim et al., 1999; Adams et al., 2001).

In contrast, relatively little is known about Aurora-A. Although phenotypic analyses of hypomorphic mutants or of the effects of RNA interference (RNAi) in various organisms and cell types have yielded slightly different results, Aurora-A has been implicated in regulation of the centrosome and mitotic microtubules. Mutations in the Aurora-A gene of *Drosophila melanogaster* have been associated with centrosomal abnormalities, including a characteristic monopolar organization (Glover et al., 1995). A weak hypomorphic Aurora-A mutation in this organism resulted in short astral microtubules (Giet et al., 2002). The Aurora-A protein Eg2 in *Xenopus* directly associates with microtubules, and its kinase activity is essential for bipolar spindle assembly in vitro (Roghi et al., 1998). Analysis of Aurora-A function in *Caenorhabditis elegans* embryos by RNAi revealed an

*Correspondence: hsaya@gpo.kumamoto-u.ac.jp
[3]Present address: Research Institute of Molecular Pathology (IMP), Dr. Bohrgasse 7, A-1030, Vienna, Austria.

important role for this protein in centrosome maturation (Hannak et al., 2001): Aurora-A does not contribute to centrosome separation before nuclear envelope breakdown (NEBD) but, rather, is important for maintenance of centrosome separation. The molecular pathways by which Aurora-A controls the mitotic machinery and by which it is itself regulated remain largely unknown, however.

We now provide molecular insight into the regulation of mitotic entry in human cells. With the use of antibodies specific for the active form of Aurora-A, we found that Aurora-A is first activated in late G2 phase at the centrosome of HeLa cells. Application of RNAi to synchronized HeLa cells revealed that this activation of Aurora-A is required for the initial activation of cyclin B1-Cdk1 at the centrosome and, therefore, for entry into mitosis. A two-hybrid screen identified Ajuba as a protein that binds to Aurora-A, and this interaction was shown to result in the autophosphorylation and consequent activation of Aurora-A. The autophosphorylation of Aurora-A facilitated by interaction with Ajuba underlies the activation of Aurora-A at the centrosome that is required for the commitment of cells to mitosis.

Results

Temporal and Spatial Control of Aurora-A Activation

To study the temporal regulation of Aurora-A activation, we synchronized HeLa cells at the G1-S boundary by the double thymidine-block protocol and then monitored the abundance and kinase activity of the protein through mitosis. Consistent with previous observations (Marumoto et al., 2002), the amounts of both Aurora-A protein and kinase activity increased during late G2 to M phase, beginning ~8.5 hr after release of the block (Figure 1A). Given that phosphorylation of Aurora-A on Thr288, which is located in the activation T loop of the kinase, results in a marked increase in enzymatic activity (Walter et al., 2000), we examined the phosphorylation state of Thr288 by immunoblot analysis with antibodies (T288P) specific for Aurora-A phosphorylated on this residue. The phosphorylation of Aurora-A on Thr288 occurred during G2-M phases, coincident with the enzymatic activation (Figure 1A). The phosphorylation of Thr288 thus provides an index of the enzymatic activation of Aurora-A.

We next examined the subcellular distribution of activated Aurora-A by immunofluorescence analysis with the T288P antibodies (Figure 1B). The activation of a small proportion of Aurora-A was first evident at centrosomes before chromatin condensation (Figure 1Bb). Activated Aurora-A was subsequently detected in the nucleus, coincident with visible chromatin condensation, at prophase. Finally, increased amounts of activated Aurora-A were observed at the spindle poles, after NEBD, at prometaphase.

To clarify the temporal relation between the enzymatic activation and subcellular localization of Aurora-A, we categorized the pattern of T288P staining of synchronized cells during cell cycle progression as exemplified by the six panels, a–f, in Figure 1B. Activation of Aurora-A was first detected 8.5 hr after release from G1-S arrest by both immunoblot analysis and in vitro kinase assay (Figure 1A). In contrast, immunofluorescence analysis revealed that activated Aurora-A was present at centrosomes in more than 40% of cells at 8.5 hr after the release, and a substantial population (~20%) of cells was already positive for active Aurora-A 1 hr earlier, when most cells were still in G2 phase (Figure 1C). To determine more precisely when Aurora-A is first activated, we stained cells with both T288P and antibodies specific for histone H3 phosphorylated on Ser10, a marker of M phase. Phosphorylation of Aurora-A was apparent before that of histone H3 (Figure 1D), demonstrating that Aurora-A is first activated during G2 phase. These observations reveal that active Aurora-A initially appears at centrosomes during late G2 phase and that this event precedes the robust activation detected biochemically during early mitosis.

Aurora-A Kinase Is Essential for G2-M Progression

To investigate the importance of human Aurora kinases in G2-M transition, we combined cell synchronization with RNAi. HeLa cells were transfected with small interfering RNAs (siRNAs) in the interval between the two thymidine-blocks and were examined for expression of Aurora-A and Aurora-B between 6–9 hr after release into the cell cycle (Figure 2A). Transfection of cells with siRNAs specific for Aurora-A or Aurora-B resulted in specific reduction in levels of the corresponding protein (Figure 2B). Depletion of Aurora-A markedly impaired the ability of cells to enter mitosis, whereas transfection of cells with the siRNA specific for Aurora-B or with a control siRNA had no such effect (Figures 2C and 2D). Similar results were obtained with U2OS human osteosarcoma cells (data not shown).

To characterize the impairment in mitotic entry in Aurora-A-deficient cells, we examined cell cycle progression by flow cytometry. Monitoring of DNA content revealed that the kinetics of DNA replication (S phase progression) was not affected by Aurora-A depletion (see Supplemental Figure S1A online at http://www.cell.com/cgi/content/full/114/5/585/DC1). Rather, the Aurora-A-deficient cells appeared to be delayed in G2 phase, as indicated by the marked cytoplasmic accumulation of cyclin B1 (Figure 2E). This G2 delay induced by Aurora-A depletion was not clearly overridden by treatment of the cells with caffeine (Supplemental Figure S1B), which inhibits the checkpoint pathway activated by DNA damage. Moreover, there were no obvious differences in the synthesis of mitotic cyclins between Aurora-A-deficient cells and those transfected with the control siRNA (Supplemental Figure S1C), excluding the possibility that the G2 delay was due to inhibition of protein synthesis triggered by double-stranded RNA. Consistent with the delayed mitotic entry, Aurora-A-depleted cells lacked substantial cyclin B1-associated kinase activity toward histone H1 in vitro, despite the marked accumulation of cyclin B1 (Figure 2F). This lack of kinase activity was associated with the phosphorylation of Cdk1 on Tyr15 (Supplemental Figure S1D). Together, these observations indicate that Aurora-A, but not Aurora-B, is essential for mitotic entry in cultured human cells.

We also observed that centrosomes remained immature in the Aurora-A-depleted cells, whereas those in

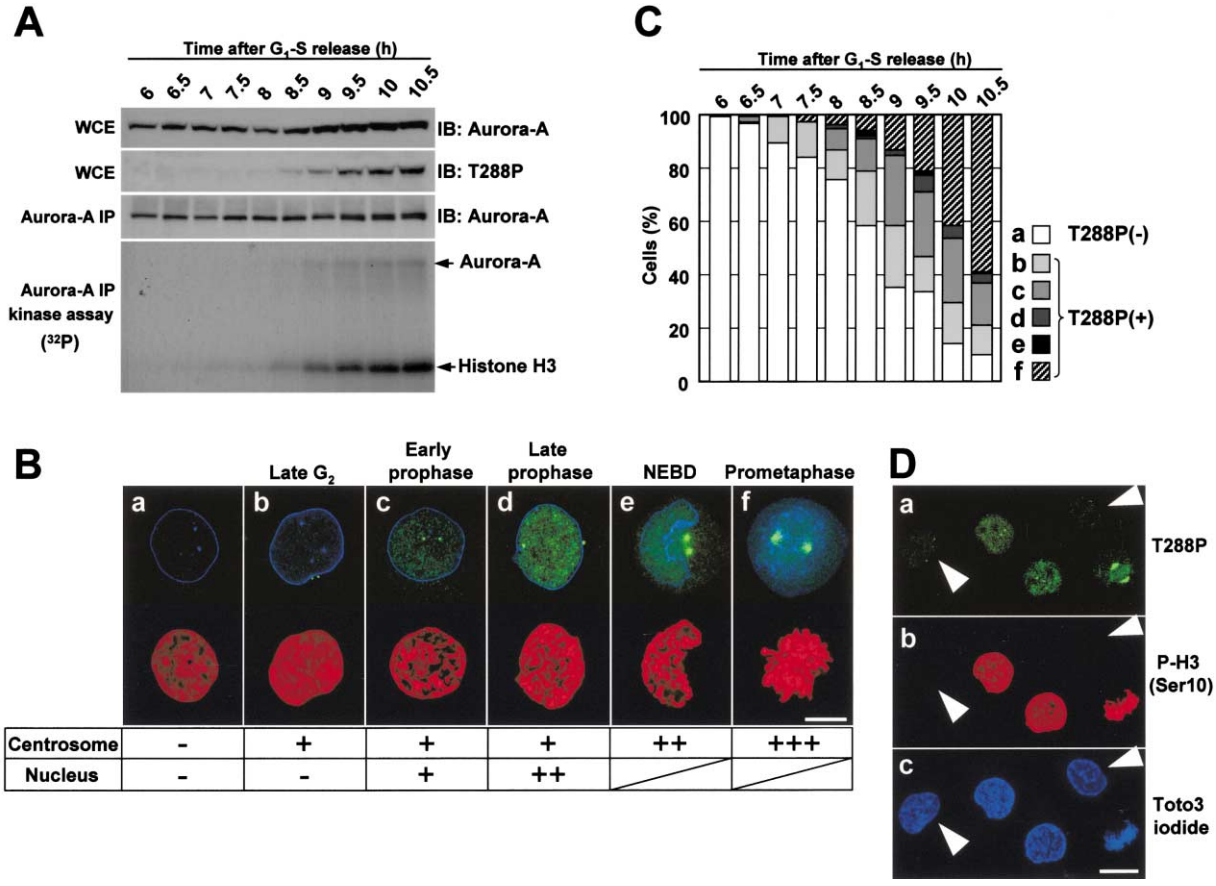

Figure 1. Initial Activation of Aurora-A in Late G2 Phase

(A) Regulation of Aurora-A during the cell cycle. Synchronized HeLa cells were released from the G1-S boundary and, 5.5 hr after release, nocodazole was added to the medium at a final concentration of 50 ng/ml. At the indicated times after release, whole-cell extracts (WCE) were subjected to immunoblot analysis (IB) with antibodies to Aurora-A (top) or with T288P (second panel). Equal amounts of cell extracts were also subjected to immunoprecipitation (IP) with antibodies to Aurora-A, and the resulting precipitates were subjected either to immunoblot analysis with the same antibodies (third panel) or to an in vitro kinase assay with [γ-^{32}P]ATP and histone H3 as exogenous substrate (bottom panel).

(B) Distribution of activated Aurora-A during the cell cycle. HeLa cells treated as in (A) were subjected to indirect immunofluorescence staining with the T288P antibodies (FITC, green) and antibodies to lamins A and C (Cy5, blue). DNA was visualized by staining with propidium iodide (red). Cells were staged on the basis of the distribution of activated Aurora-A as indicated: cells negative for T288P staining (a), cells in late G2 with T288P staining at centrosomes (b), cells in early prophase with staining at centrosomes and weak nuclear staining (c), cells in late prophase with staining at centrosomes and robust nuclear staining (d), cells at the time of NEBD (e), and cells in prometaphase with staining at the spindle poles (f).

(C) Spatiotemporal analysis of Aurora-A activation during G2-M phases. At the indicated times after release from G1-S block, ~200 cells were counted and classified according to the pattern of T288P staining as described in (B).

(D) Initial appearance of active Aurora-A at centrosomes during late G2 phase. At 8.5 hr after release from G1-S block, HeLa cells were subjected to immunofluorescence staining with T288P (FITC, green) and antibodies specific for histone H3 phosphorylated on Ser10 (Texas red, red). DNA was visualized with Toto3 iodide (blue). Arrowheads indicate cells with active Aurora-A at centrosomes without phosphorylation of histone H3.

control cells recruited γ-tubulin and nucleated mitotic microtubules (Figure 2E). Aurora-A thus appears to contribute to the regulation of centrosome maturation in human cells, consistent with its function in *C. elegans* and *D. melanogaster*, and raises the possibility that centrosome maturation mediated by Aurora-A might play a role in mitotic entry.

Aurora-A Is Required for Initial Activation of Cyclin B1-Cdk1 at Centrosomes

To study further the nature of the G2 delay associated with Aurora-A deficiency, we examined the relation between the initial activation of Aurora-A and that of the cyclin B1-Cdk1 complex, which is a key initiator of mitosis. Like Aurora-A, the active cyclin B1-Cdk1 complex appears at centrosomes before the substantial activation characteristic of cell cycle progression through mitosis (Jackman et al., 2003). We therefore examined whether cyclin B1 is recruited to centrosomes in cells depleted of Aurora-A. Immunofluorescence analysis of cells transfected with the control siRNA revealed that the number of cells in which cyclin B1 was localized to centrosomes increased between 7.5–9 hr after release from G1-S arrest (Figures 3A and 3B). In contrast, such

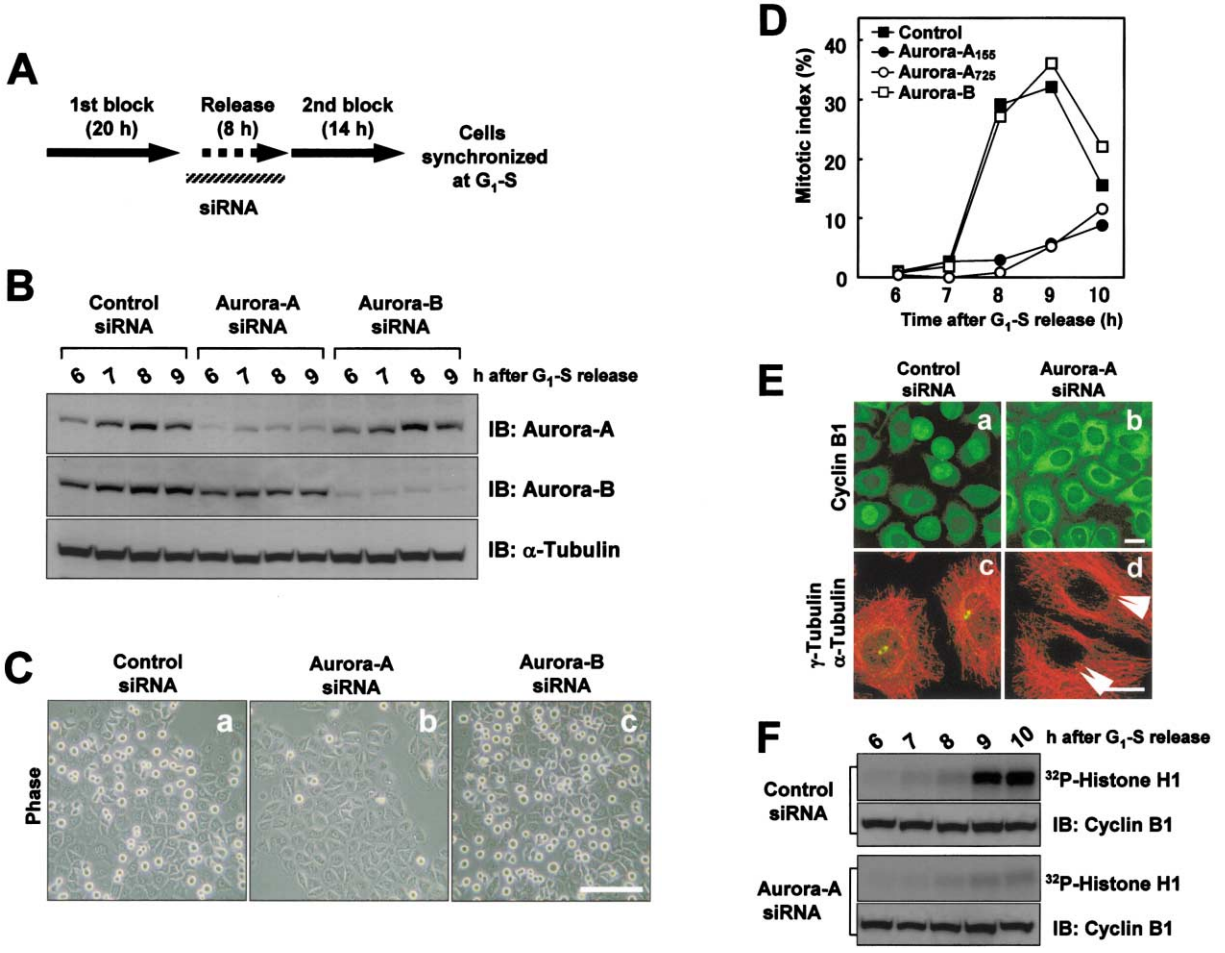

Figure 2. Aurora-A Is Essential for Mitotic Entry in HeLa Cells

(A) Experimental protocol for cell cycle synchronization by double thymidine-block and for transfection with siRNA.

(B) Inhibition of Aurora-A and Aurora-B expression by transfection of cells with siRNAs. HeLa cells were subjected to the protocol shown in (A) with control, Aurora-A, or Aurora-B siRNAs. At the indicated times, whole-cell extracts were subjected to immunoblot analysis with antibodies to the indicated proteins.

(C) Phase-contrast images of cells transfected with control (a), Aurora-A (b), or Aurora-B (c) siRNAs. Cells were observed 9 hr after release. Scale bar, 200 μm.

(D) Time course of the mitotic index of cells transfected with control, Aurora-A_{155} (nucleotides 155–173), Aurora-A_{725} (nucleotides 725–743), or Aurora-B siRNAs. Cells were fixed and stained with DAPI at the indicated times, and the number of mitotic cells out of a total of ~500 cells examined was determined.

(E) Distribution of cyclin B1 and tubulin in Aurora-A-depleted cells. Cells transfected with control (a and c) or Aurora-A (b and d) siRNAs were subjected to immunofluorescence analysis 9 hr after release. The distribution of cyclin B1 was revealed by FITC fluorescence (a and b), and those of γ-tubulin and α-tubulin were revealed by FITC and Texas red, respectively (c and d). Arrowheads indicate γ-tubulin-containing centrosomes. Scale bar, 20 μm.

(F) Lack of cyclin B1-associated kinase activity in Aurora-A-deficient cells. At the indicated times after release, cells transfected with control or Aurora-A siRNAs were subjected to immunoprecipitation with antibodies to cyclin B1, and the resulting precipitates were both assayed for kinase activity with histone H1 as substrate and subjected to immunoblot analysis with antibodies to cyclin B1.

an effect was not observed in Aurora-A-deficient cells. These results suggest that Aurora-A is required for the initial recruitment of cyclin B1-Cdk1 at the centrosome, an event that correlates with centrosome maturation.

Cells lacking Cdk1 activity were also analyzed for initial Aurora-A activation at the centrosome. Activation of Cdk1 was inhibited either by exposure of G2 cells to the potent Cdk1-specific inhibitor butyrolactone I (at a concentration [50 μM] that does not inhibit the kinase activity of Aurora-A in vitro [Marumoto et al., 2002]) or by transfection with a cyclin B1 siRNA (Figures 3C and 3D). Although the entry of cells into mitosis was greatly inhibited by each of these treatments (data not shown), the percentage of cells showing Aurora-A activation at centrosomes was largely unaffected (Figure 3D). However, the intensity of the T288P signal at centrosomes of cells transfected with cyclin B1 siRNA was weaker than that apparent in control cells, and the cyclin B1-depleted cells did not manifest the pronounced nuclear activation of Aurora-A as observed in control cells (Fig-

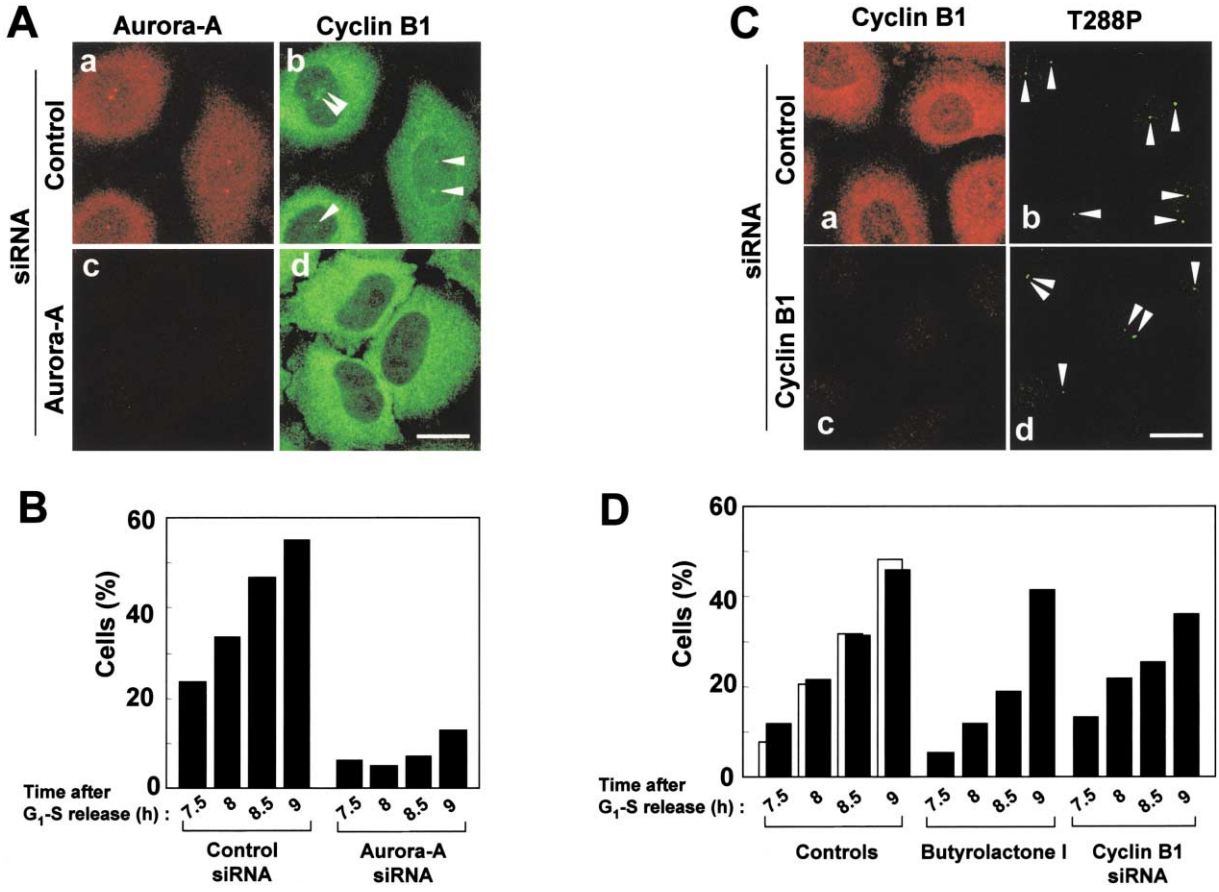

Figure 3. Relation between the Activation of Aurora-A and that of Cyclin B1-Cdk1 at the Centrosome

(A) Localization of cyclin B1 in Aurora-A-depleted cells. HeLa cells transfected with control (a and b) or Aurora-A (c and d) siRNAs were subjected to immunofluorescence analysis 9 hr after release from G1-S arrest. Aurora-A and cyclin B1 were revealed by Rhodamine (a and c) and FITC (b and d) fluorescence, respectively. Arrowheads indicate cyclin B1-positive centrosomes. Scale bar, 20 μm.

(B) Role of Aurora-A in recruitment of cyclin B1 to the centrosome. At the indicated times after release from G1-S arrest, ~100 cells transfected with control or Aurora-A siRNAs were scored by immunofluorescence analysis for the presence of cyclin B1 at centrosomes, which were detected with antibodies to pericentrin.

(C) Localization of active Aurora-A in cyclin B1-depleted cells. HeLa cells transfected with control (a and b) or cyclin B1 (c and d) siRNAs were subjected to immunofluorescence analysis 9 hr after release from G1-S arrest. The distribution of cyclin B1 was revealed by rhodamine (a and c) and that of T288P immunoreactivity by FITC (b and d). Arrowheads indicate T288P-positive centrosomes. Scale bar, 20 μm.

(D) Centrosomal localization of active Aurora-A independent of Cdk1 activity. At the indicated times after release from G1-S arrest, ~100 HeLa cells that had been transfected with cyclin B1 siRNA or treated with butyrolactone I were scored for centrosomal staining with T288P; centrosomes were detected by staining with antibodies to γ-tubulin. Control cells were either transfected with control siRNA (open bars) or not treated with the Cdk1 inhibitor (solid bars), as appropriate. Butyrolactone I was added to the medium 7 hr after release to a final concentration of 50 μM.

ure 3C). These observations indicate that the activation of both Aurora-A and Cdk1 at centrosomes constitutes an essential early event in the progression of cells toward mitosis, and they suggested the existence of a mechanism for Aurora-A activation that is independent of cyclin B1-Cdk1.

Identification of Ajuba As an Aurora-A Binding Protein

To obtain molecular insight into Aurora-A function and its regulation at the G2-M transition, we performed a yeast two-hybrid screen to identify proteins that interact with Aurora-A. The region of human Aurora-A comprising amino acids 1–129 was used as the bait to screen a HeLa cDNA library. From a total of 2.4×10^7 transformants, 135 positive clones were obtained. These clones were then subjected to secondary screening to eliminate false positives. Nucleotide sequencing revealed that five of the ten confirmed positive clones contained an open reading frame, including a stop codon, that encoded a protein with substantial homology to murine Ajuba (Figure 4A), a LIM domain-containing protein that has been suggested to promote meiotic maturation of *Xenopus* oocytes (Goyal et al., 1999). Isolation of a full-length cDNA for this human homolog of Ajuba from a HeLa cDNA library revealed that the predicted protein shares 92.9% amino acid identity with mouse Ajuba.

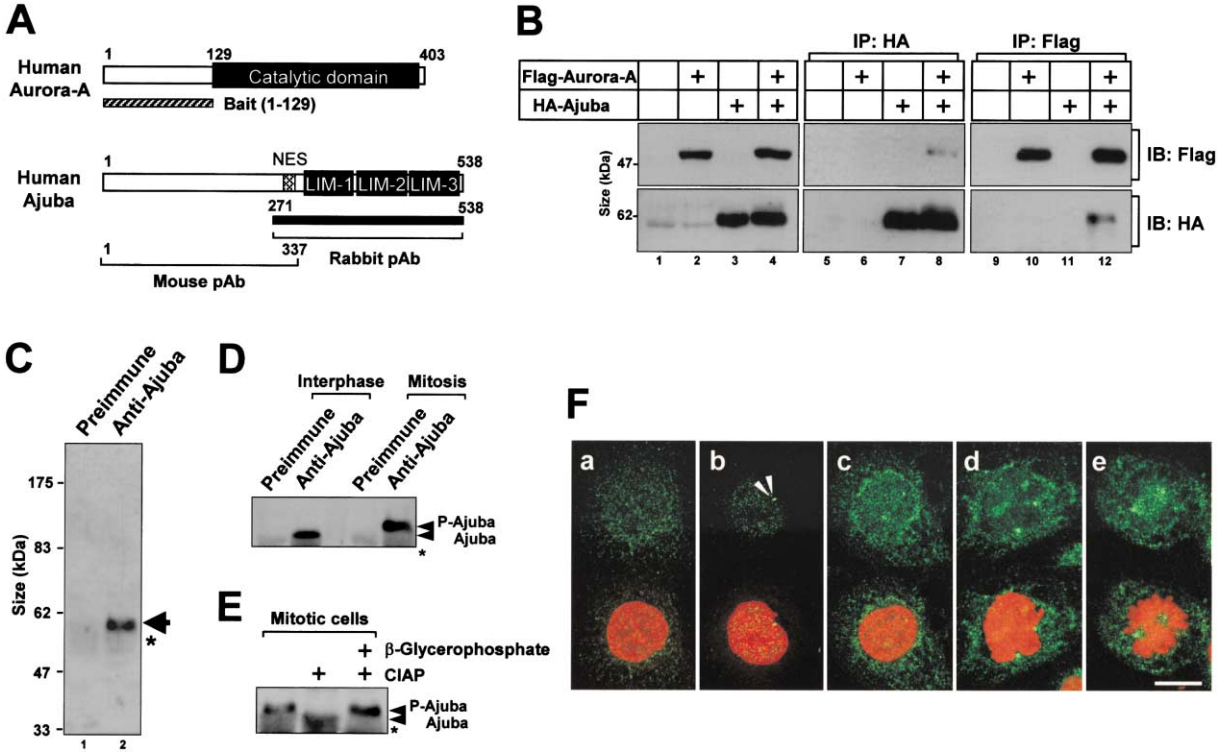

Figure 4. Identification of Ajuba As an Aurora-A Binding Protein

(A) Domain organization of human Aurora-A and human Ajuba. The hatched bar below the Aurora-A structure represents the portion of the protein used as the bait for two-hybrid screening. The solid bar below the Ajuba structure indicates the portion encoded by the positive clones recovered in the screen. NES, nuclear export sequence. Polyclonal antibodies to human Ajuba were generated in rabbits and mice by injecting recombinant proteins corresponding to the indicated sequences.

(B) Interaction of ectopically expressed Ajuba and Aurora-A in COS-7 cells. Lysates of cells expressing Flag-tagged Aurora-A, HA-tagged Ajuba, or both proteins were subjected to immunoblot analysis with antibodies to Flag or to HA as indicated (lanes 1–4). The same cell lysates were also subjected to immunoprecipitation with antibodies to HA (lanes 5–8) or to Flag (lanes 9–12), and the resulting precipitates were subjected to immunoblot analysis with antibodies to Flag or to HA.

(C) Detection of endogenous Ajuba. Lysates of exponentially growing HeLa cells were subjected to immunoprecipitation with either preimmune rabbit serum (lane 1) or rabbit antiserum to human Ajuba (lane 2), and the resulting precipitates were subjected to immunoblot analysis with mouse antibodies to Ajuba. Arrow indicates Ajuba, and asterisk indicates rabbit immunoglobulin.

(D) Comparison of the electrophoretic mobility of Ajuba between interphase and mitotic cells. Lysates of interphase or mitotic HeLa cells were subjected to immunoprecipitation and immunoblot analysis as in (C).

(E) Phosphorylation of Ajuba during mitosis. Ajuba immunoprecipitates prepared from mitotic HeLa cells were incubated for 30 min at 37°C with or without 50 U of calf intestinal alkaline phosphatase (CIAP) (200 U/ml) or 50 mM β-glycerophosphate, as indicated. The precipitates were then subjected to immunoblot analysis with antibodies to Ajuba.

(F) Subcellular localization of endogenous Ajuba. Synchronized HeLa cells were subjected to immunofluorescence staining with purified rabbit antibodies to Ajuba and FITC-labeled secondary antibodies (top). Cells were also stained with propidium iodide (red) and the signal was merged with that for FITC (bottom). Representative cells in G2-M phase are shown in chronological order (a–e). Note that Ajuba appears on centrosomes that are located next to each other (b, arrowheads). Scale bar, 20 μm.

To examine whether Ajuba interacts with Aurora-A in mammalian cells, we transfected COS-7 cells with expression vectors for the full-length proteins tagged at their NH_2 termini with the hemagglutinin (HA) and Flag epitopes, respectively. Lysates of cells expressing both HA-Ajuba and Flag-Aurora-A were then subjected to immunoprecipitation with antibodies to Flag. Immunoblot analysis of the resulting precipitates revealed the presence of HA-Ajuba (Figure 4B). Conversely, Flag-Aurora-A was detected in immunoprecipitates prepared with antibodies to HA. These results thus indicate that Ajuba and Aurora-A interact in mammalian cells.

To characterize human Ajuba, we prepared mouse and rabbit polyclonal antibodies to the protein (Figure 4A). Because of its low abundance in HeLa cells, it was barely possible to detect Ajuba directly by immunoblot analysis of whole-cell extracts. We therefore immunoprecipitated Ajuba with the rabbit antibodies and subjected the resulting precipitates to immunoblot analysis with the mouse antibodies. Endogenous Ajuba was detected as a broad band corresponding to a molecular size of 55–60 kDa (Figure 4C), consistent with the calculated molecular mass of 56.9 kDa. Furthermore, Ajuba derived from mitotic cells exhibited a lower electrophoretic mobility than did that from interphase cells (Figure 4D). This lower-mobility form of Ajuba was converted to the higher-mobility form by phosphatase treatment in a manner that was sensitive to β-glycerophosphate

(Figure 4E), indicating that the lower-mobility form corresponded to phosphorylated Ajuba. Ajuba thus undergoes phosphorylation during mitosis.

We examined the subcellular distribution of Ajuba in HeLa cells by immunofluorescence analysis with the purified rabbit antibodies to human Ajuba. Consistent with previous observations with mouse Ajuba (Goyal et al., 1999), faint staining of endogenous Ajuba was apparent in the cytoplasm. We noticed, however, that Ajuba is localized to the centrosome in a fraction of cells, and those cells with centrosome staining increased when cells were synchronized at G2-M. Closer observation showed that Ajuba first appears at the centrosome earlier than obvious chromosome condensation and becomes stronger as cells proceed through mitosis (Figure 4F). This centrosomal staining was not apparent in cells transfected with Ajuba siRNA (data not shown), eliminating the possibility that it was due to a nonspecific reaction of the antibodies. The observation that Ajuba concentrates on centrosome during G2-M phase suggests that the interaction with Aurora-A could take place in this specific phase and place.

Delineation of the Ajuba Domain Responsible for Interaction with Aurora-A

To delineate the region of Ajuba responsible for its association with Aurora-A, we generated various deletion mutants of Ajuba fused with glutathione S-transferase (GST) (Figure 5A). GST-Ajuba fusion proteins were incubated with a purified hexahistidine (His_6)-tagged fragment of Aurora-A (amino acids 1–129), the same fragment that was used as the bait in the two-hybrid screen, and were then precipitated with glutathione-Sepharose beads. Whereas the Aurora-A fragment coprecipitated with GST-AjubaΔ1 (containing all three LIM domains) and GST-AjubaΔ3 (containing LIM-2 and LIM-3), it did not associate with the Δ2, Δ4, Δ5, or Δ6 GST-Ajuba mutants (Figure 5B). We confirmed that the full-length recombinant proteins, GST-Ajuba and His_6-tagged Aurora-A, interacted directly (Figure 5C). However, GST-AjubaΔ7, which lacks the LIM-2 and LIM-3 domains, did not interact with Aurora-A. The interaction between Ajuba and Aurora-A was suggested to depend on the conformation of the LIM domain by the observation that it was blocked by TPEN, a Zn^{2+}-specific chelator that disrupts this conformation. These in vitro binding experiments thus demonstrated that both LIM-2 and LIM-3 domains of Ajuba mediate the interaction with the NH_2-terminal region of Aurora-A.

In Vivo Interaction between Aurora-A and Ajuba

To determine whether Ajuba is a substrate for the kinase activity of Aurora-A, we performed an in vitro kinase assay with recombinant His_6-tagged wild-type Aurora-A or a kinase-inactive mutant thereof (K162M, in which Lys162 in the ATP binding site is replaced with Met) and with GST-Ajuba. Ajuba underwent marked phosphorylation on incubation with wild-type Aurora-A, but not when incubated with the kinase-inactive mutant (Figure 5D). Moreover, when either COOH-terminal half-truncated (pre-LIM domain) or NH2-terminal half-truncated (LIM domain) of GST-Ajuba was used as substrates in the assay, phosphorylation site(s) in Ajuba appeared to reside in the pre-LIM domain, but not in the LIM domain (Supplemental Figure S2A). These observations thus raised the possibility that Ajuba might be a target of the kinase activity of Aurora-A.

We next examined whether the kinase activity of Aurora-A affects the interaction between the two proteins. COS-7 cells were transfected with expression vectors for both HA-Ajuba and either Flag-tagged wild-type Aurora-A or kinase-inactive mutants thereof (K162M and T288A, in which Thr288 and Thr287 are both replaced with alanines to prevent T loop activation). Immunoprecipitation followed by immunoblot analysis revealed the presence of HA-Ajuba in the precipitates prepared from cells expressing wild-type Aurora-A, but not in those prepared from cells expressing either of the kinase-inactive mutants (Figure 5E). Furthermore, phosphorylated Ajuba, which appeared as a lower-mobility band in cells coexpressing wild-type Aurora-A, was specifically coprecipitated with Aurora-A.

We also noticed that a lower-mobility form of Aurora-A appeared in cells expressing both wild-type Aurora-A and Ajuba, suggesting that both proteins are phosphorylated during complex formation. To examine this possibility further in vivo, we subjected immunoprecipitates of endogenous Ajuba prepared from mitotic HeLa cells to immunoblot analysis with antibodies to Aurora-A. Although both the phosphorylated and nonphosphorylated forms of endogenous Aurora-A were detected in the input lysate, the phosphorylated form was specifically coprecipitated with Ajuba (Figure 5F). These data thus demonstrate that Aurora-A interacts with Ajuba during mitosis and that both proteins are phosphorylated as they form a complex.

Activation of Aurora-A by Ajuba

Based on our immunoprecipitation and immunoblot data, we speculated that phosphorylation of Aurora-A is induced by interaction of the kinase with Ajuba. We examined this possibility first in a cell-free system. Purified His_6-tagged Aurora-A was incubated with various concentrations of GST-Ajuba in a kinase reaction buffer. The extent of phosphorylation of Aurora-A on Thr288, as revealed by immunoblot analysis with T288P antibodies, increased in a manner dependent on the amount of GST-Ajuba (Figure 6A). Ajuba did not induce phosphorylation of the kinase-inactive Aurora-A mutant K162M on Thr288 (Supplemental Figure S3A). These data thus indicated that Ajuba facilitates the phosphorylation of Aurora-A on Thr288 and that this phosphorylation is mediated by an autocatalytic mechanism.

Given that the phosphorylation of Thr288 appears to be critical for the kinase activity of Aurora-A (Figure 5E), we next determined whether the Ajuba-induced autophosphorylation of Aurora-A increases the kinase activity of Aurora-A toward exogenous substrates. In the absence of GST-Ajuba, His_6-tagged Aurora-A exhibited little kinase activity toward histone H3 (Figure 6B). In the presence of GST-Ajuba, however, the phosphorylation of histone H3 (and of His_6-tagged Aurora-A itself) was greatly increased in a manner dependent on the concentration of Aurora-A, indicating that Ajuba stimulates the kinase activity of Aurora-A.

To examine further whether Ajuba induces the activa-

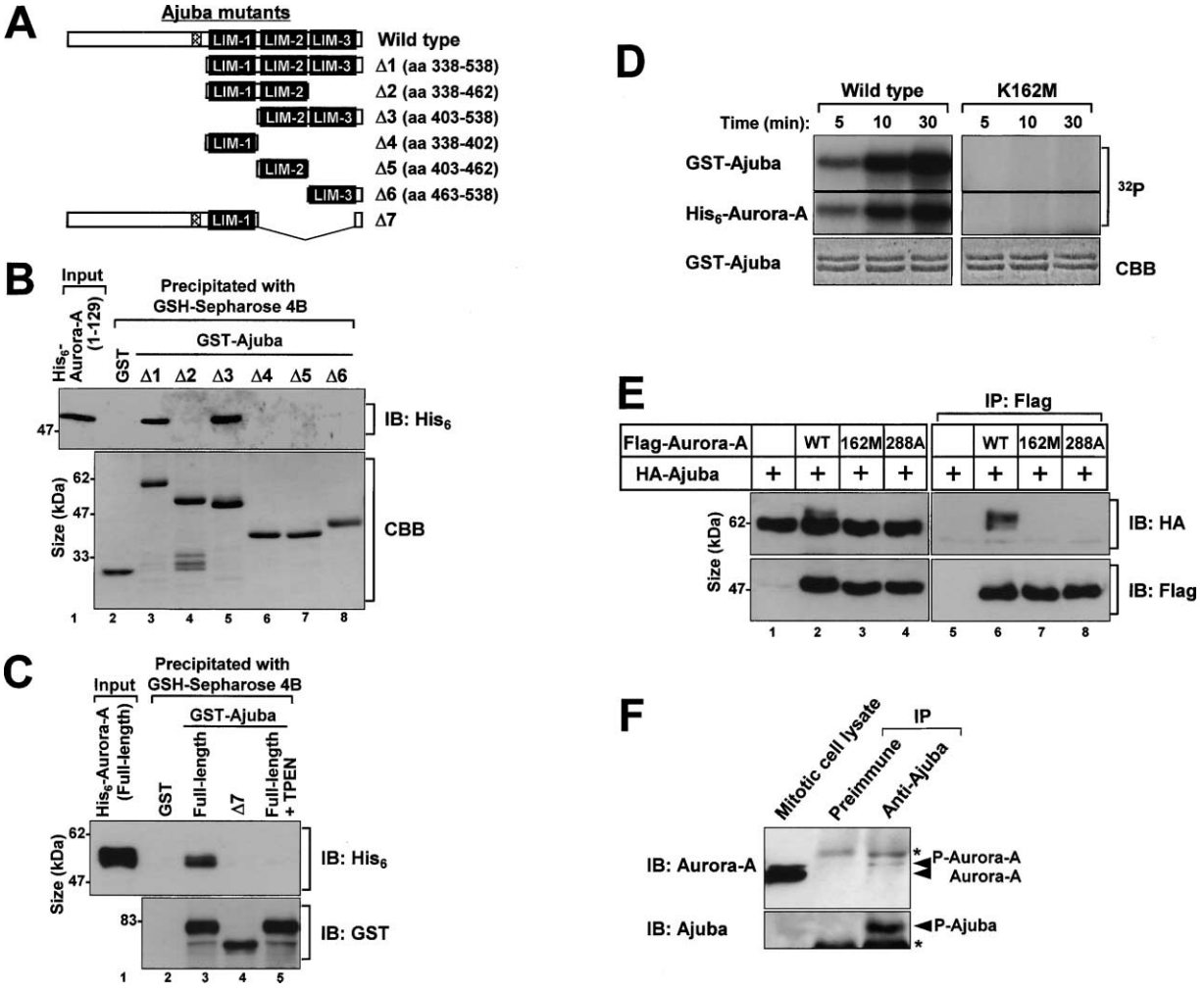

Figure 5. Interaction of Ajuba with Aurora-A

(A) Deletion mutants of human Ajuba.

(B) In vitro binding assay with a recombinant Aurora-A fragment and Ajuba deletion mutants. Purified GST-Ajuba fusion proteins (400 nM) or GST as a control were incubated with a purified His_6-tagged Aurora-A fragment (amino acids 1–129) (400 nM) and then precipitated with glutathione-Sepharose 4B beads. Bead bound proteins were subjected to immunoblot analysis with antibodies to the His_6 tag (yop); the gel was also stained with Coomassie brilliant blue (CBB) (bottom). The input of His_6-Aurora-A(1–129) into the binding reaction in shown in lane 1.

(C) Requirement for LIM-2 and LIM-3 of Ajuba for binding to Aurora-A. Purified His_6-tagged full-length Aurora-A was incubated with GST, GST-Ajuba (full length) in the absence or presence of 10 μM TPEN, or GST-AjubaΔ7, as indicated. Proteins that bound to glutathione-Sepharose 4B beads were then precipitated and subjected to immunoblot analysis with antibodies to the His_6 tag or to GST. The input of His_6-Aurora-A (full length) into the binding reaction is shown in lane 1.

(D) Phosphorylation of Ajuba by Aurora-A in vitro. Purified GST-Ajuba was incubated for the indicated times with purified His_6-tagged wild-type Aurora-A or the kinase-inactive mutant K162M in the presence of [γ-^{32}P]ATP. The kinase reaction mixtures were then analyzed by SDS-PAGE and autoradiography (top and middle); the gel was also stained with Coomassie brilliant blue (bottom).

(E) Aurora-A interacts with phosphorylated form of Ajuba. Lysates of cells expressing both HA-tagged Ajuba and either Flag-tagged wild-type (WT) Aurora-A or kinase-inactive mutants thereof (K162M or T288A) were subjected to immunoblot analysis with antibodies to Flag or to HA (lanes 1–4). The same cell lysates were also subjected to immunoprecipitation with antibodies to Flag, and the resulting precipitates were subjected to immunoblot analysis with antibodies to HA or to Flag, as indicated (lanes 5–8). The lower-mobility form of HA-tagged Ajuba was converted to the higher-mobility form by phosphatase treatment (data not shown).

(F) Interaction of endogenous Ajuba and Aurora-A in mitotic HeLa cells. Mitotic cell lysates were subjected to immunoprecipitation with preimmune rabbit serum or rabbit antiserum to Ajuba, and the resulting precipitates (as well as cell lysate) were subjected to immunoblot analysis with antibodies to Aurora-A or to Ajuba. Asterisk indicates rabbit immunoglobulin.

tion of Aurora-A in cells, we subjected COS-7 cells to transient transfection with an expression vector for Flag-tagged Aurora-A in the presence of various amounts of a vector for Myc epitope-tagged Ajuba. Immunoblot analysis revealed that coexpression of Ajuba with Aurora-A resulted in the appearance of the lower-mobility (phosphorylated) forms of both proteins in a manner dependent on the abundance of Myc-Ajuba (Figure 6C). This effect correlated with the kinase activity of Flag-Aurora-A immunoprecipitates toward histone H3 (Fig-

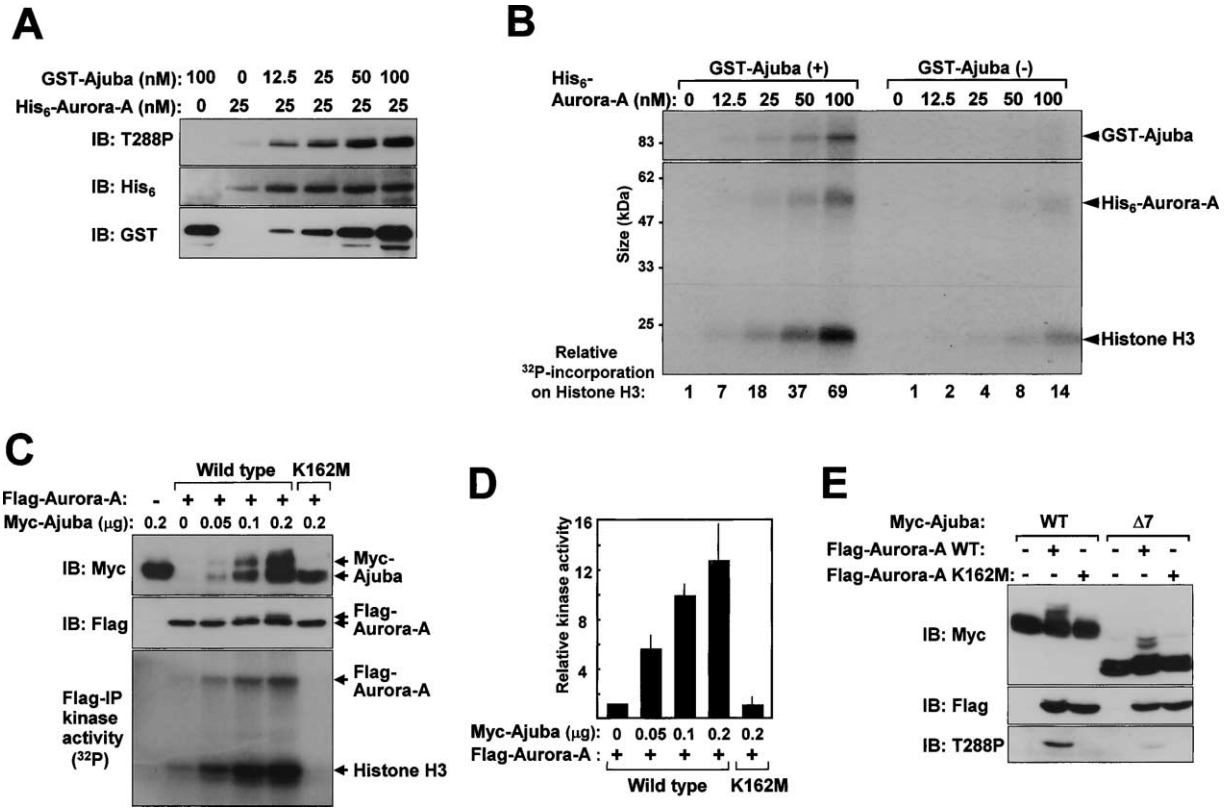

Figure 6. Ajuba-Induced Autophosphorylation and Activation of Aurora-A

(A) Ajuba-induced autophosphorylation of Aurora-A on Thr288 in vitro. Recombinant His_6-tagged Aurora-A (25 nM) was incubated with the indicated concentrations of GST-Ajuba for 30 min at 30°C in a kinase reaction buffer containing 20 μM ATP. The extent of Aurora-A phosphorylation was then determined by immunoblot analysis with T288P antibodies; the same blot was also probed with antibodies to the His_6-tag and to GST.

(B) Activation of Aurora-A by Ajuba in vitro. His_6-Aurora-A at the indicated concentrations was incubated for 30 min at 30°C in the absence or presence of 100 nM GST-Ajuba, after which His_6-Aurora-A was immunoprecipitated and incubated in kinase reaction buffer containing histone H3 and [γ-^{32}P]ATP. Phosphorylated proteins were then detected by SDS-PAGE and autoradiography. Aurora-A activity was quantitated by densitometric scanning of the histone H3 band and expressed relative to that without Ajuba.

(C) Autoactivation of Aurora-A induced by Ajuba in cells. COS-7 cells were transfected with an expression vector for Flag-Aurora-A (wild-type or K162M mutant) and the indicated amounts of a vector for Myc epitope-tagged Ajuba. Cell lysates were subsequently subjected to immunoblot analysis with antibodies to the Myc or Flag epitopes (top and middle, respectively). Cell lysates were also subjected to immunoprecipitation with antibodies to Flag, and the resulting precipitates were assayed for kinase activity with histone H3 as substrate (bottom).

(D) Aurora-A activity in experiments similar to that shown in (C) was quantitated by densitometric scanning of the histone H3 band and expressed relative to that of cells not transfected with the Ajuba vector. Data are means ±SD of values from three independent experiments.

(E) Requirement for physical interaction for Ajuba to induce autoactivation of Aurora-A. HeLa cells were transfected with the indicated combinations of expression vectors for Flag-Aurora-A (wild-type or K162 mutant) and Myc-Ajuba (wild-type or Δ7 mutant). Cell lysates were subsequently subjected to immunoblot analysis with antibodies to Myc or to Flag or with T288P antibodies, as indicated.

ures 6C and 6D). The kinase-inactive Aurora-A mutant K162M was not phosphorylated when coexpressed with Ajuba (Figure 6C), indicating that phosphorylation of wild-type Aurora-A is mediated autocatalytically.

We examined whether the Ajuba-induced activation of Aurora-A was dependent on the ability of Ajuba to bind to Aurora-A by coexpressing Aurora-A with the nonbinding mutant AjubaΔ7. Although the lower-mobility (phosphorylated) form of Ajuba was detected for AjubaΔ7 as well as for the wild-type protein, AjubaΔ7 did not induce the autophosphorylation of Aurora-A on Thr288 (Figure 6E). Collectively, both our in vitro and in vivo experiments thus demonstrated that Ajuba promotes the activation of Aurora-A and that this effect results from the physical interaction of the two proteins.

Ajuba-Induced Autoactivation of Aurora-A in Late G2 Is Required for Mitotic Entry

We have shown that the interaction between Aurora-A and Ajuba occurs during mitosis and that Ajuba promotes the autoactivation of Aurora-A. We therefore examined the possibility that Ajuba contributes to the activation of Aurora-A in late G2 phase. Synchronized HeLa cells were depleted of Ajuba by transfection with an Ajuba-specific siRNA (Figure 7A). Depletion of Ajuba greatly inhibited the activation of Aurora-A without substantially affecting the abundance of this protein (Figure 7B). Immunofluorescence analysis with T288P antibodies revealed that whereas the proportion of nontransfected cells or cells transfected with control siRNA that exhibited intense staining of centrosomes increased

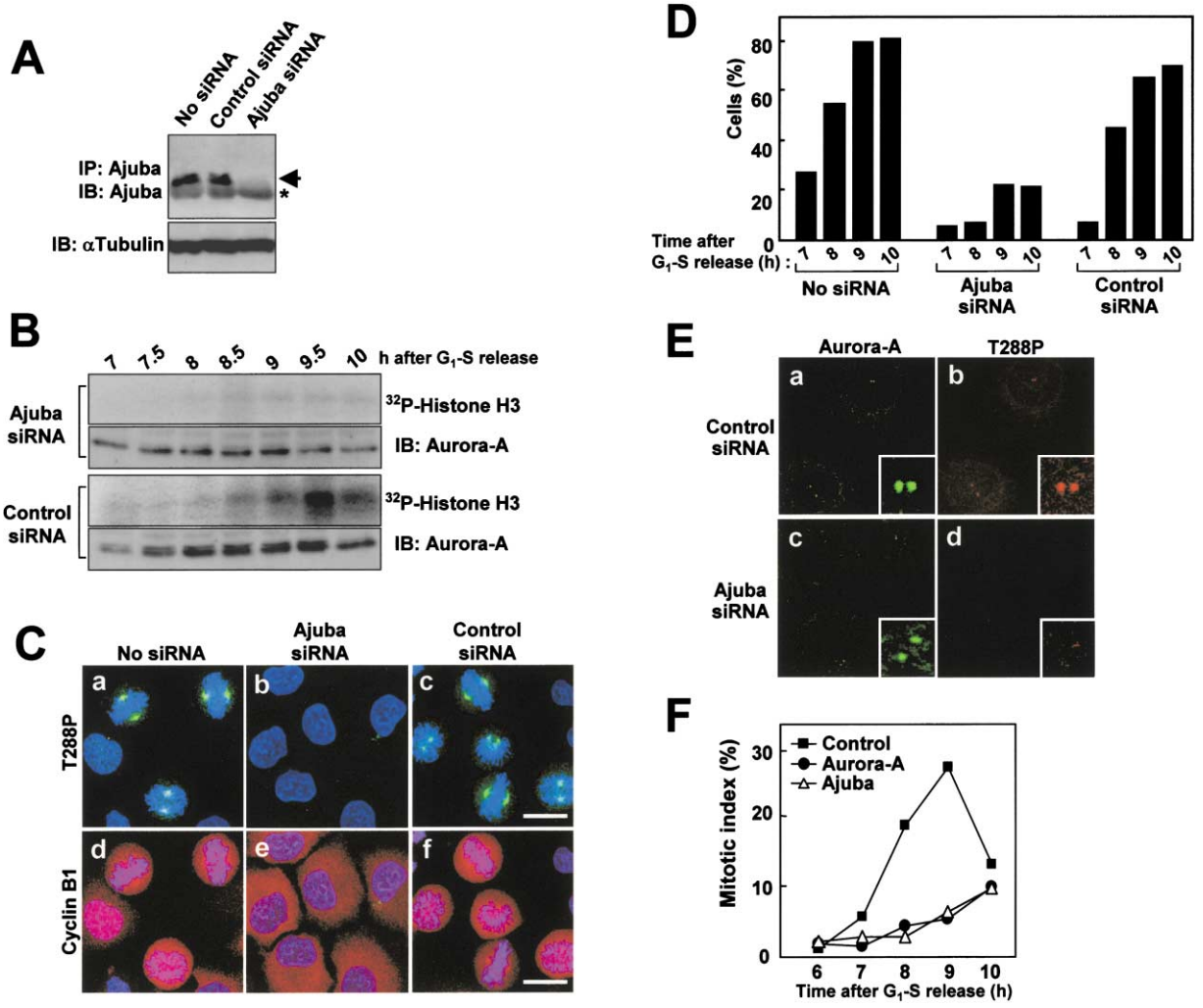

Figure 7. Ajuba Is Essential for the Initial Activation of Aurora-A in Late G2

(A) Inhibition of Ajuba expression in synchronized HeLa cells by RNAi. Cells were transfected (or not) with Ajuba or control siRNAs during the first thymidine-block of the cell synchronization protocol. 9 hr after the release from G1-S arrest, cells were subjected to immunoprecipitation and immunoblot analysis with antibodies to Ajuba (top) or to immunoblot analysis with antibodies to α-tubulin as a control (bottom). Arrow indicates Ajuba, and asterisk indicates rabbit immunoglobulin.

(B) Lack of Aurora-A activation in Ajuba-depleted cells. Cells transfected with Ajuba or control siRNAs were subjected to immunoprecipitation with antibodies to Aurora-A at the indicated times after release from G1-S arrest. The resulting precipitates were both assayed for kinase activity with histone H3 as substrate in the presence of [γ-^{32}P]ATP as well as subjected to immunoblot analysis with antibodies to Aurora-A, as indicated.

(C) Centrosomal activation of Aurora-A requires Ajuba. Ajuba-depleted or control cells were subjected 9 hr after release to staining with Toto3 iodide (blue) as well as to immunofluorescence analysis both with T288P antibodies (FITC) (a–c) and with antibodies to cyclin B1 (Texas red) (d–f). Scale bars, 20 μm.

(D) Quantitation of the centrosomal activation of Aurora-A during G2-M progression. Synchronized Ajuba-depleted or control HeLa cells were analyzed for centrosomal activation of Aurora-A at the indicated times after release as in (C). Approximately 200 cyclin B1-positive cells (G2 to metaphase) were scored for each sample.

(E) Aurora-A is recruited to centrosomes, but not activated, in Ajuba-depleted cells. Synchronized HeLa cells transfected with Ajuba or control siRNAs were subjected 9 hr after release to immunofluorescence analysis with antibodies to Aurora-A (FITC) and T288P (Texas red), as indicated. Insets represent higher-magnification images of centrosomes.

(F) Inhibition of mitotic entry in Ajuba-depleted cells. Synchronized HeLa cells transfected with control, Ajuba, or Aurora-A siRNAs were scored for the percentage of mitotic cells at the indicated times after release from G1-S arrest. Approximately 200 cells were scored at each time point.

markedly during progression to mitosis, only a small fraction of cells transfected with Ajuba siRNA exhibited centrosome staining, and such staining was of low intensity when present (Figures 7C and 7D). These observations thus indicate that Ajuba is required for the activation of Aurora-A at centrosomes in late G2 phase.

To examine further the mechanism of Aurora-A activation by Ajuba, we subjected cells transfected with Ajuba

siRNA to immunofluorescence analysis both with T288P antibodies and with rat antibodies that recognize both the phosphorylated (activated) and nonphosphorylated forms of Aurora-A. Aurora-A localized to centrosomes in the absence of Ajuba but remained in the inactive state (Figure 7E). The demonstration that Aurora-A is recruited to the centrosome regardless of its activation status supports the notion that interaction with Ajuba at this organelle promotes the activation of Aurora-A.

Having shown that the initial Aurora-A activation is required for commitment to mitosis (Figure 2), we tested whether depletion of Ajuba also inhibits mitotic entry. The kinetics of DNA replication were not affected by Ajuba depletion (Supplemental Figure S4A). However, the ability of Ajuba-depleted cells to enter mitosis was impaired to the same marked extent as was that of Aurora-A-depleted cells (Figure 7F). Also similar to Aurora-A-depleted cells, Ajuba-depleted cells appeared to arrest in G2 phase, as indicated by substantial accumulation of cyclin B1 in the cytoplasm. The accumulated cyclin B1 lacked associated kinase activity toward histone H1 (Supplemental Figure S4B). We conclude that Ajuba plays an important role in the activation of Aurora-A at centrosomes in late G2 phase and that this activation is essential for cells to initiate mitosis.

Discussion

We have investigated the role of Aurora-A in mitotic entry in human cells. We also identified Ajuba as a protein that binds to and activates Aurora-A. Our data reveal that Ajuba is required for the activation of Aurora-A at centrosomes in late G2 phase, which is important for activation of the cyclin B1-Cdk1 complex and commits cells to mitosis. We here propose that the signaling pathway for mitotic entry is activated in late G2 phase, before cells are morphologically defined as M phase.

Ajuba As a Mitotic Regulator

We have shown that Ajuba plays an important role in regulation of the kinase activity of Aurora-A and that the interaction of Ajuba with Aurora-A is mediated by the native conformation of the LIM-2 and LIM-3 domains of Ajuba. Ajuba appears to be a specific activator of Aurora-A, given that it does not activate Aurora-B (Supplemental Figure S5A) and that Aurora-A is not activated by zyxin (Supplemental Figure S5B), a protein that is structurally related to Ajuba. We previously showed that human WARTS, a mitotic kinase, interacts with zyxin via the LIM-1 and LIM-2 domains of the latter protein (Hirota et al., 2000). Phosphorylation of zyxin is also required for its association with WARTS during mitosis. Our present and previous observations thus indicate that LIM domain-containing proteins contribute to mitotic control by interacting with mitotic kinases and that these interactions require phosphorylation of the LIM proteins.

Overexpression of Eg2 (Aurora-A) in *Xenopus* oocytes resulted in a shortening of the time between release from G2 arrest and entry into meiosis after progesterone stimulation (Andresson and Ruderman, 1998). The effect of overexpression of Ajuba on *Xenopus* egg maturation is similar to that of Eg2 overexpression (Goyal et al., 1999). It is therefore possible that Ajuba and Eg2 also cooperate to regulate the G2-M transition during egg maturation and that this molecular mechanism is conserved through evolution and is shared between mitotic and meiotic regulation.

Activation of Aurora-A by the Interaction with Ajuba

One possibility for the mechanism by which Ajuba mediates the activation of Aurora-A is that the binding of Ajuba to Aurora-A induces an activating conformational change. We noticed that GST-Aurora-A, but not His_6-tagged Aurora-A, possessed marked kinase activity in the absence of Ajuba (data not shown). It is thus conceivable that the NH2-terminal region of Aurora-A interacts with the catalytic domain to inhibit kinase activity and that fusion of this NH2-terminal region with GST or its binding to Ajuba prevents this inhibitory interaction, leading to Aurora-A activation. Another possibility is that Ajuba stabilizes activated Aurora-A. Phosphorylation of Thr288 of Aurora-A is critical not only for catalytic activation but also for degradation of the protein (Walter et al., 2000), suggesting that activated Aurora-A is unstable. Consistent with this notion, we observed that recombinant Aurora-A was more stable in the presence of Ajuba than in its absence (Figure 6A).

We have shown that both Aurora-A and Ajuba are phosphorylated during their association and that the phosphorylation of both proteins is mediated by Aurora-A. A nonbinding mutant of Ajuba was not able to induce autophosphorylation of Aurora-A. Nevertheless, this mutant was phosphorylated by Aurora-A both in cells (Figure 6E) and in vitro, and the kinetics of the in vitro reaction were similar for both the wild-type and mutant Ajuba proteins (Supplemental Figures S2A and S2B). These observations better support the possibility that the Aurora-A-Ajuba interaction is facilitated by phosphorylation of Ajuba.

The activation of Aurora-A has also been shown to result from downregulation of protein phosphatase 1, which forms a complex with Aurora-A and inhibits its kinase activity (Walter et al., 2000; Katayama et al., 2001). Given that purified recombinant Aurora-A was activated in vitro by purified recombinant Ajuba and that this reaction was not affected by a phosphatase inhibitor (Supplemental Figure S3B), the activation of Aurora-A by Ajuba appears to be distinct from the phosphatase-mediated regulation of this kinase.

Requirement of Aurora-A for Mitotic Entry in Mammalian Cells

Our RNAi studies with HeLa cells have indicated that Aurora-A and Ajuba are required for mitotic entry. We noticed, however, that a small population ($\sim$10%) of cells transfected with Aurora-A or Ajuba siRNAs entered mitosis with or without a slight delay (Figures 2D and 7F); the cells that entered mitosis exhibited a variable level of T288P staining (data not shown), presumably due to inefficient transfection, whereas the vast majority of cells that remained in G2 phase were entirely negative for T288P staining (Figure 7C). These observations suggest that a minimal activation of Aurora-A allows cells to enter mitosis. Perturbation of Aurora-A function by injection of cells with antibodies specific for this protein

resulted in a substantial delay (~2 hr) in the initiation of mitosis (Marumoto et al., 2002). Although inhibition of mitotic entry was not complete because of the inherent limitation of antibody injection experiments, this observation also supports the notion of a requirement for only minimal Aurora-A activity.

Studies in flies and worms have indicated that Aurora-A controls centrosome maturation, but they did not reveal an obvious role for this protein in mitotic entry (Glover et al., 1995; Hannak et al., 2001; Giet et al., 2002). However, we have now shown that depletion of Aurora-A resulted in G2 delay in cultured mammalian cells. These apparently conflicting observations regarding the role of Aurora-A in mitotic entry might be due to species differences. Alternatively, they may be attributable to differences in the mode of cell cycle regulation between early embryonic cells and somatic cells. Early embryonic cells, such as the syncytium in *Drosophila*, undergo rapidly alternating S and M phases without intervening gap phases and without the checkpoints that are active in somatic cells.

The regulatory role of Aurora-A in centrosome maturation suggests that centrosome maturation might play an important role in the G2-M transition in mammalian cells. It is thus possible that immature centrosomes generate a signal that prevents cell cycle progression. Depletion of Aurora-A or Ajuba might therefore result in the activation of a checkpoint pathway that monitors centrosome maturation. A second, more likely possibility is that cell cycle progression from late G2 phase through prophase is positively regulated by centrosomes as they mature. Centrosomes might thus provide a site for the organization of macromolecular complexes that control the G2-M transition; the recruitment of key cell cycle regulators is important for efficient and rapid mutual coactivation. This hypothesis is supported by the observation that major mitotic regulators, including cyclin B1-Cdk1 (Figure 3; Bailly et al., 1989; Jackman et al., 2003), Plk1 (Lane and Nigg, 1996), as well as Aurora-A, accumulate at centrosomes during G2-M progression.

When Does Mitosis Begin?

Our data are consistent with the idea that Ajuba plays an essential role in activating Aurora-A for mitotic commitment. We observed that centrosome-deposited Aurora-A remains inactive without Ajuba (Figure 7E). Although the total protein amount of Ajuba does not largely fluctuate during cell cycle, Ajuba is present on centrosome specifically during G2-M phase (Figures 4D and 4F). Therefore, the simplest interpretation of these data would be that recruitment of Ajuba to centrosome is the major regulatory step for the activation of Aurora-A. However, this hypothesis does not exclude the possibility that Aurora-A and Ajuba come in contact in the cytoplasm, and then, the Aurora-A-Ajuba complex is recruited to the centrosome.

Pines and Rieder (2001) proposed the staging of mitosis on the basis of the status of cell cycle regulators that induce morphological changes rather than on the morphological changes themselves. Our observation that Aurora-A is activated during late G2 phase to promote G2-M transition provides insight into when mitosis actually begins. Although it remains unclear what ultimately triggers the onset of Aurora-A activation, we have demonstrated that activation of the Aurora-A-Ajuba complex, in addition to that of the cyclin B1-Cdk1 complex, is one of the earliest important molecular events in the initiation of mitosis.

Experimental Procedures

Two-Hybrid Screen

Two-hybrid screening was performed basically as described (Hirota et al., 2000). In brief, yeast strain L40 harboring pBTM116HA-KM/Aurora-A(1–129) was transformed with a HeLa cDNA library in pGAD-GH (Clontech). For efficient recovery of pGAD-GH plasmids, we changed the selection marker for prokaryotes in pBTM116HA from ampicillin resistance to kanamycin resistance.

Antibodies

Polyclonal antibodies to human Ajuba were generated in mice and rabbits by injection with a recombinant His_6-tagged NH_2-terminal fragment (amino acids 1–337) or a GST fusion protein of the COOH-terminal portion (amino acids 271–538) of the protein, respectively. The resulting rabbit antisera were subjected to affinity purification on a column of recombinant His_6-tagged Ajuba (271–538) for immunofluorescence analysis. Rabbit and rat polyclonal antibodies to Aurora-A were generated as previously described (Marumoto et al., 2002). The phosphoepitope-specific antibodies for Aurora-A (T288P) and Histone H3 (Ser10) were obtained from Cell Signaling Technology. Mouse monoclonal antibodies to Aurora-B, to cyclin B1, and to α-tubulin (B512) were from Transduction Laboratories, and those to γ-tubulin (GTU88) and Lamin A/C were from Sigma and Santa Cruz, respectively. Rabbit polyclonal antibodies for pericentrin were purchased from Convence. The experiment in Figure 3B was performed with monoclonal antibody GNS1 to cyclin B1 (Santa Cruz), and similar results were obtained with the monoclonal antibody from Transduction Laboratories.

Recombinant Proteins

For protein purification from Sf9 cells, Aurora-A cDNAs, wild-type, and K162M mutant were constructed into pFastBac-HT (Gibco BRL), thereby generating a baculovirus vector encoding a His_6-tagged Aurora-A. Optimized doses of baculoviruses were added in the growing medium 40 hr before the cell harvest. Recombinant proteins were isolated using TALON metal-affinity resin (Clonthech). Purified His_6-Aurora-A did not reveal kinase activity by itself, which was suitable to assess the autophorphorylation/activation of Aurora-A induced by Ajuba shown in Figure 6. For the preparation of activated His_6-Aurora-A, which was used in the in vitro kinase assays (Figure 5), 0.1 μM of okadaic acid was added to the culture medium for 2 hr, and also to the lysis buffer during the purification.

siRNA Experiments

The sequences of the siRNAs were as follows: human Aurora-A, 5′-AUUCUUCCCAGCGCGUUCC-3′ (corresponding to nucleotides 155–173 relative to the start codon) or 5′-AUGCCCUGUCUUACU GUCA-3′ (nucleotides 725–743) (Kufer et al., 2002), Aurora-B, 5′-GGUGAUGGAGAAUAGCAGU-3′ (nucleotides 168–186), Ajuba, 5′-GGGGCGCCUAAGUGGGUUG-3′ (nucleotides 105–123), and cyclin B1, 5′-GTCAGTGAACAACTGCAGG-3′ (nucleotides 331–349). A double-stranded RNA targeting luciferase (GL-2) was used as a control. For Aurora-A siRNA analysis, the oligonucleotide targeted to positions 725–743 was used unless indicated otherwise. The 21 nucleotide RNA-DNA chimeric duplexes were obtained from Japan Bioservice (Asaka, Japan). Annealing of the component strands of each siRNA and transfection were performed as described (Elbashir et al., 2001).

Immunoprecipitation

Mitotic cells were collected by shaking of the culture dish 9 hr after the release from double thymidine block. For immunoprecipitation, cells were lysed on ice for 30 min with NP-40 lysis buffer, consisting of 0.5% NP-40, 20 mM Tris-HCl (pH 7.4), 500 mM NaCl, 0.5 mM

EGTA, 100 nM okadaic acid, 10 mM β-glycerophosphate, 2 mM AEBSF, 10 μg/ml leupeptin, 0.7 μg/ml pepstatin A, 1.5 μg/ml aprotinin, and 1 μM of zinc citrate. Lysates were centrifuged at 20,000 × g for 20 min. Supernatants were diluted with 4 volumes of lysis buffer without NaCl and were incubated for 3 hr on ice with rabbit anti-Ajuba antibodies, then 15 μl of Protein A-Sepharose beads were added and incubated for 2 hr. After washing five times with washing buffer, consisting of 0.05% NP-40, 20 mM Tris-HCl (pH7.4), and 100 mM NaCl, the bound proteins were separated by SDS-PAGE and were analyzed by immunoblot analysis as described (Marumoto et al., 2002). In the coimmunoprecipitation experiments after cotransfection, cell lysates were incubated for 2 hr at 4°C with antibodies to the Flag epitope (M2) (Sigma) or to HA (3F10) (Roche) conjugated with matrix beads.

Solution Binding Assays

GST fusion proteins (400 nM) were incubated for 1 hr on ice with 400 nM His_6-Aurora-A in 250 μl of a solution containing 50 mM HEPES NaOH (pH 7.2), 15 mM NaCl, 0.05% Triton X-100, 5% glycerol, 2 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Each mixture was then incubated with shaking for an additional 1 hr at 4°C with 15 μl of glutathione-Sepharose 4B beads (Amersham Pharmacia). After extensive washing with the binding buffer, proteins bound to the beads were subjected to immunoblot analysis.

Kinase Assays

Cells were lysed in a solution containing 20 mM Tris-HCl (pH 8.0), 0.4 M NaCl, 1% NP-40, 1 mM EGTA, 5 mM NaF, 1 mM AEBSF, 1 mM E-64, 0.5 mM Na_3VO_4, and 1 μM okadaic acid. Lysates were centrifuged at 20,000 × g for 20 min, and the resulting supernatants were incubated for 2 hr at 4°C either with Sepharose 4B beads conjugated with antibodies to Aurora-A or to cyclin B1 or with agarose beads conjugated with antibodies to Flag. After extensive washing, the beads were incubated for 20 min at 30°C with 2 μg of histone H3 or H1 (Roche) in 30 μl of a kinase buffer comprising 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 10 μCi of [γ-^{32}P]ATP. Alternatively, 0.4 μg of purified His_6-Aurora-A was used as the kinase source. The reaction was stopped by the addition of Laemmli sample buffer, and phosphorylated substrates were detected by SDS-PAGE and autoradiography.

Immunofluorescence Analysis

HeLa cells were grown in 35 mm petri dishes, fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 20 min at room temperature, and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline. After incubation for 60 min with 5% goat serum in phosphate-buffered saline, the cells were incubated at 4°C overnight with primary antibodies and then for 45 min at room temperature with fluorescein isothiocyanate (FITC)-, Texas red-, Cy5- or Rhodamine-conjugated goat secondary antibodies (Biosource, Molecular Probes, or Pharmacia). Chromatin was labeled with Toto3 iodide (Molecular Probes), 4′6-diamidino-2-phenylindole (DAPI), or propidium iodide. Mitotic index was determined by counting the number of mitotic cells (those with condensed chromosomes) as revealed by staining with DAPI. Cells were examined with a confocal microscopy (FV300, Olympus). Images were obtained by sequential excitation of 488/568 nm to minimize overlapping signals and were processed using Photoshop software.

Acknowledgments

We are grateful to M. Kimura and Y. Okano (Gifu University) for Aurora-A cDNA. We thank S. Honda, S. Iida, Y. Kawano (Kumamoto University), T. Iwanaga (Hokkaido University), J.-M. Peters, S. Hauf (Institute of Molecular Pathology, Vienna), J. Pines, and M. Jackman (University of Cambridge) for their critical discussions. We thank Y. Fukushima for secretarial assistance, and members of the Gene Technology Center in Kumamoto University for technical supports. This work was supported by grants from the Ministry of Education, Science, Sports, and Culture of Japan and the "Research for the Future" program of The Japan Society for the Promotion of Science (H.S.).

Received: November 11, 2002
Revised: July 18, 2003
Accepted: July 25, 2003
Published: September 4, 2003

References

Adams, R.R., Carmena, M., and Earnshaw, W.C. (2001). Chromosomal passengers and the (aurora) ABCs of mitosis. Trends Cell Biol. *11*, 49–54.

Andresson, T., and Ruderman, J.V. (1998). The kinase Eg2 is a component of the Xenopus oocyte progesterone-activated signaling pathway. EMBO J. *17*, 5627–5637.

Bailly, E., Doree, M., Nurse, P., and Bornens, M. (1989). p34cdc2 is located in both nucleus and cytoplasm; part is centrosomally associated at G2/M and enters vesicles at anaphase. EMBO J. *8*, 3985–3995.

Ditchfield, C., Johnson, V.L., Tighe, A., Ellston, R., Haworth, C., Johnson, T., Mortlock, A., Keen, N., and Taylor, S.S. (2003). Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores. J. Cell Biol. *161*, 267–280.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature *411*, 494–498.

Giet, R., McLean, D., Descamps, S., Lee, M.J., Raff, J.W., Prigent, C., and Glover, D.M. (2002). Drosophila Aurora A kinase is required to localize D-TACC to centrosomes and to regulate astral microtubules. J. Cell Biol. *156*, 437–451.

Glover, D.M., Leibowitz, M.H., McLean, D.A., and Parry, H. (1995). Mutations in aurora prevent centrosome separation leading to the formation of monopolar spindles. Cell *81*, 95–105.

Goyal, R.K., Lin, P., Kanungo, J., Payne, A.S., Muslin, A.J., and Longmore, G.D. (1999). Ajuba, a novel LIM protein, interacts with Grb2, augments mitogen-activated protein kinase activity in fibroblasts, and promotes meiotic maturation of Xenopus oocytes in a Grb2- and Ras-dependent manner. Mol. Cell. Biol. *19*, 4379–4389.

Hannak, E., Kirkham, M., Hyman, A.A., and Oegema, K. (2001). Aurora-A kinase is required for centrosome maturation in Caenorhabditis elegans. J. Cell Biol. *155*, 1109–1116.

Hauf, S., Cole, R.W., LaTerra, S., Zimmer, C., Schnapp, G., Walter, R., Heckel, A., Van Meel, J., Rieder, C.L., and Peters, J.M. (2003). The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. J. Cell Biol. *161*, 281–294.

Hirota, T., Morisaki, T., Nishiyama, Y., Marumoto, T., Tada, K., Hara, T., Masuko, N., Inagaki, M., Hatakeyama, K., and Saya, H. (2000). Zyxin, a regulator of actin filament assembly, targets the mitotic apparatus by interacting with h-warts/LATS1 tumor suppressor. J. Cell Biol. *149*, 1073–1086.

Jackman, M., Lindon, C., Nigg, E.A., and Pines, J. (2003). Active cyclin B1-Cdk1 first appears on centrosomes in prophase. Nat. Cell Biol. *5*, 143–148.

Katayama, H., Zhou, H., Li, Q., Tatsuka, M., and Sen, S. (2001). Interaction and feedback regulation between STK15/BTAK/Aurora-A kinase and protein phosphatase 1 through mitotic cell division cycle. J. Biol. Chem. *276*, 46219–46224.

Kim, J.H., Kang, J.S., and Chan, C.S. (1999). Sli15 associates with the Ipl1 protein kinase to promote proper chromosome segregation in Saccharomyces cerevisiae. J. Cell Biol. *145*, 1381–1394.

Kufer, T.A., Sillje, H.H., Korner, R., Gruss, O.J., Meraldi, P., and Nigg, E.A. (2002). Human TPX2 is required for targeting Aurora-A kinase to the spindle. J. Cell Biol. *158*, 617–623.

Lane, H.A., and Nigg, E.A. (1996). Antibody microinjection reveals an essential role for human polo-like kinase 1 (Plk1) in the functional maturation of mitotic centrosomes. J. Cell Biol. *135*, 1701–1713.

Marumoto, T., Hirota, T., Morisaki, T., Kunitoku, N., Zhang, D., Ichikawa, Y., Sasayama, T., Kuninaka, S., Mimori, T., Tamaki, N., et al. (2002). Roles of aurora-A kinase in mitotic entry and G2 checkpoint in mammalian cells. Genes Cells *7*, 1173–1182.

Nigg, E.A. (2001). Mitotic kinases as regulators of cell division and its checkpoints. Nat. Rev. Mol. Cell Biol. *2*, 21–32.

Pines, J., and Rieder, C.L. (2001). Re-staging mitosis: a contemporary view of mitotic progression. Nat. Cell Biol. *3*, E3–E6.

Roghi, C., Giet, R., Uzbekov, R., Morin, N., Chartrain, I., Le Guellec, R., Couturier, A., Doree, M., Philippe, M., and Prigent, C. (1998). The Xenopus protein kinase pEg2 associates with the centrosome in a cell cycle-dependent manner, binds to the spindle microtubules and is involved in bipolar mitotic spindle assembly. J. Cell Sci. *111*, 557–572.

Terada, Y. (2001). Role of chromosomal passenger complex in chromosome segregation and cytokinesis. Cell Struct. Funct. *26*, 653–657.

Walter, A.O., Seghezzi, W., Korver, W., Sheung, J., and Lees, E. (2000). The mitotic serine/threonine kinase Aurora2/AIK is regulated by phosphorylation and degradation. Oncogene *19*, 4906–4916.

Accession Numbers

The nucleotide sequence of the human Ajuba cDNA has been deposited in GenBank under the accession number AY169959.