CELL STRUCTURE AND FUNCTION 27: 457–467 (2002)

Molecular Dynamics of Aurora-A Kinase in Living Mitotic Cells Simultaneously Visualized with Histone H3 and Nuclear Membrane Protein Importinα

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ABSTRACT. **Aurora-A is known to be a mitotic kinase required for spindle assembly. We constructed a human stable cell-line in which Aurora-A, histone H3 and importinα were differentially expressed as fusions to green, cyan, and red fluorescent proteins (GFP, CFP and DsRed). In interphase cells, GFP-Aurora-A was localized in the centrosome. Its molecular behavior in living mitotic cells was extensively analyzed by an advanced timelapse image analyzing system. In G2 phase, duplicated centrosomal dots of Aurora-A separated and moved to the opposite poles, a process requiring 18 min. In prophase, the Aurora-A dots approached closer and the nuclear membrane of DsRed-importinα beneath them became thick and invaginated, resulting in a "dumb-bell" shaped nucleus with condensed chromatin. As the importinα membrane further shrank and disappeared, the condensed chromatin was excluded from the nucleus and the Aurora-A dots grew rapidly into a spindle-like structure. Congression of mitotic chromosomes continued for 20–50 min until they were properly aligned at the spindle equator and then the sister chromatids started to segregate, taking 4–6 min for them to reach the poles. An importinα membrane reappeared around the surface of chromatin 10 min after anaphase onset. Aurora-A gradually decreased in size in telophase and returned to the surface of the newly formed small sister nuclei. These observations showed that the morphological change of Aurora-A was cooperated with the breakdown and reformation of nuclear membrane. Immunostaining with anti-α or γ-tubulin further indicated that Aurora-A was involved in the formation of mitotic spindle in metaphase as well as the subsequent chromosome movement in anaphase.**

Key words: Aurora-A/centrosome/GFP/histone H3/importinα/timelapse

In mitotic cells, we observe drastic morphological changes of subcellular structures, such as nuclear membrane, centrosome, mitotic spindle and chromosome. Many studies have shown that centrosomes and kinetochores play an important role in mitosis. Centrosomes locate on the surface of nuclear membrane in interphase, duplicate in late S, and migrate to the opposite poles in G2/M (for a review see Goepfert and Brinkley, 2000). Upon entry into prophase, they act as microtubule-organizing centers (MTOC) and microtubules nucleate from the duplicated centrosomes and form a bipolar spindle. Kinetochores, on the other hand, are morphologically defined as the microtubule attachment sites of mitotic chromosomes. Interestingly, prekinetochore structures are present in interphase nuclei, duplicate in G2 and maturate into the fully differentiated kinetochores that are visible in metaphase chromosomes (Brenner *et al.*, 1981; Sugimoto *et al.*, 2000). Although these two representative organelles are physically separated by nuclear

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Abbreviations: CENP, centromere protein; CFP, cyan fluorescent protein; GFP, green fluorescent protein; DsRed, *Discosoma* red fluorescent protein.

membrane in interphase, they are connected with each other by kinetochore microtubules in the subsequent mitotic processes. This physical association is considered to ensure the proper alignment and accurate segregation of mitotic chromosomes in mitosis.

Although a number of reports have described the identification and characterization of molecular components of these organelles, the molecular mechanisms coordinating their morphological changes have yet to be fully understood. Recent development of fluorescent proteins such as GFP, CFP, YFP and DsRed enable us to analyze the molecular dynamics of the subcellular components in living cells (Chalfie *et al*., 1994; Finley *et al*., 2001). In previous studies, we constructed several mammalian stable cell-lines in which kinetochore and centromere heterochromatin were simultaneously visualized. The molecular behaviors of GFP-CENP-A and DsRed-HP1α in living mitotic cells were characterized and these fluorescent proteins were shown to be useful for live image analyses of mammalian cells (Sugimoto *et al*., 2001).

Protein phosphorylation plays a crucial role in centrosome separation and chromosome segregation. So far, distinct families of serine/threonine kinases are shown to be associated with centrosomes (for a review see Nigg, 2001). Aurora, a protein kinase of one family, was first identified in *Drosophila* as a protein that regulates the structure and function of the mitotic spindle (Glover *et al*., 1995). In mammals, at least three homologs have been identified in this family (Schumacher *et al*., 1998; Bischoff and Plowman, 1999; Giet and Prigent, 1999). Interestingly, each homolog is required only for a particular event of mitosis and displays a distinct morphological change by itself. Aurora-A localizes to interphase centrosomes and then to mitotic spindles and poles (Kimura *et al*., 1997; Zhou *et al*., 1998), while Aurora-B first appears in centromere heterochromatin in late G2, then localizes to the spindle equator in metaphase and is finally discarded in midbody in telophase (Bischoff and Plowman, 1999). In contrast, Aurora-C localizes to anaphase centrosomes.

To clarify the function of Aurora kinases, two approaches have been made with different organisms, genetically and biochemically. Mutant analyses with *Drosophila* and RNAi experiments with Nematoda (*C. elegans*) indicated the role of Aurora-A in centrosome separation and/or spindle assembly and the role of Aurora-B in chromosome segregation and cytokinesis (Hannak *et al*., 2001; Berdnik and Knoblich, 2002; Rogers *et al*., 2002). *In vitro* experiments with *Xenopus* showed that its Aurora-A ortholog pEg2 is associated with and phosphorylates histone H3 or kinesin-related protein pEg5 (Scrittori *et al*., 2001). Interestingly, histone H3 has also been identified as the target for Aurora-B kinase *in vitro* (Zeitlin *et al*., 2001; Sugiyama *et al*., 2002), although its significance *in vivo* has yet to be fully characterized.

Here, to clarify the molecular dynamics of Aurora-A kinase in mammalian living cells, we expressed it as a fusion to green fluorescent protein (GFP) in human MDA435 cells (Vig *et al*., 1996; Sugimoto *et al*., 2000, 2001). To precisely compare its position relative to other subcellular components, histone H3 and importinα were additionally visualized by expressing them as fusions to cyan and red fluorescent proteins (CFP and DsRed), respectively. Live images of these three subcellular components throughout mitosis were successfully captured by an advanced computer-assisted image analyzing system equipped with a highly sensitive CCD camera.

Materials and Methods

Construction of pECFP-H3, pEGFP-Aurora-A and pDsRed-importinα

pEGFP-Aurora-A was constructed by inserting a 1.2 kb fragment of human Aurora-A cDNA into the *Bgl*II and *Sal*I site of pEGFP-C1 (Clontech, Palo Alto, CA, USA). pZErO-hisotoneH3 (Tachibana *et al*., 2001) was digested with *Eco*RI and *Xho*I and the 430 bp fragment of mouse histone H3 cDNA was recloned into the same sites of pECFP-C1 (Clontech), resulting in pECFP-H3. Plasmid pDsRed-importinα was constructed by inserting a 1 kb fragment of human importinα cDNA (will be described elsewhere) into pDsRed-C1 (Clontech).

Construction of a human stable cell line MDA-Auro-imp-H3

Human MDA-435 cells were grown in DMEM (Nissui Pharmaceutical, Tokyo, Japan) containing 10% FCS at 37°C in 5% CO_2 atmosphere. Plasmid DNAs (16 μg of pEGFP-Aurora-A, pECFP-H3 or pDsRed-importinα) were mixed with a selection plasmid (7 μg for pTK-Hyg or pPuro) and introduced into cells by electroporation, as described elsewhere (Sugimoto *et al*., 2000). Stable transformants were selected in the presence of hygromycin B (Roche Molecular Biochemicals, Mannheim, Germany) or puromycin (Sigma Chemical Co., St. Louis, MO, USA).

Immunofluorescence

Aliquot of cells ($1–3\times10^5$) were grown on glass coverslips in 35 mm dishes for 48 hr, fixed with 4% paraformaldehyde for 20 min and treated with 0.1% Triton X-100 for 5 min. The fixed cells were incubated with mouse anti-Aurora-B (Sugiyama *et al*., 2002), anti-α-tubulin or anti-γ-tubulin monoclonal antibodies (1:1500; Sigma Chemical) and probed with Cy3-conjugated goat anti-mouse antibodies (1:1500; Jackson Immunoresearch Laboratories, West Grove, PA, USA). After counterstaining with DAPI (1 μg/ml), they were observed under an Eclipse E600 fluorescent microscope (Nikon, Tokyo, Japan) equipped with a PlanApo 60x objective (NA 1.40, Nikon) and a MicroMAX 1300Y cooled CCD camera (Princeton Instruments, Princeton, NJ, USA). Images were obtained by a MetaMorph software (Universal Imaging Corp., West Chester, PA, USA).

Live image analysis

Cells were grown in a 35 mm dish equipped with a glass coverslip on the bottom (Iwaki glass base dish, Asahi Techno Glass, Funabashi, Chiba, Japan) on the stage of an Eclipse TE300 fluorescent microscope (Nikon) with a PlanApo 60x objective (NA 1.40, Nikon), controlling the temperature and CO_2 concentration in a humid chamber using a thermostat and a CO_2 inject control timer (Kokensha Engineering Co. Ltd., Tokyo, Japan) as described previously (Sugimoto *et al*., 2001). Live images were obtained by an advanced version of LuminaVision software (Version 1.40, Mitani Corp., Tokyo, Japan), controlling a highly sensitive CCD camera (ORCA-ER, Hamamatsu Photonics, Shizuoka, Japan) and a BioPoint MAC5000 controller system for excitation and emission filter wheels with a filter set for CFP/YFP/RFP (No. 86006, Chroma Technology Corp., Brattleboro, VT, USA) and a Z-axis motor (Ludl Electronic Products Ltd., Hawthorne, NY, USA), for collecting Z-series optical sections (1–2 μm intervals). Distances between the duplicated centrosomes were measured by a MacScope software (Version 1.64, Mitani Corp).

Results

Ectopical expression of GFP-Aurora-A in human MDA-435 cells

The expression level of Aurora-A kinase has been reported to peak at G2/M phase (Bischoff *et al*., 1998). To visualize its molecular behavior during the cell cycle, pEGFP-Aurora-A was constructed and introduced into a human breast cancer cell line, MDA435 (Vig *et al*., 1996; Sugimoto, *et al*., 2000, 2001). As shown in Figure 1, GFP-fused Aurora-A, ectopically expressed, localized to mitotic spindles and poles (Fig. 1A–D), consistent with the localization of endogenous Aurora-A (Kimura *et al*., 1997; Bischoff *et al*., 1998). Its subcellular localization was quite distinct from that of Aurora-B, since the latter was restricted to centromere heterochromatin from late G2 to metaphase (Fig. 1A–C) and the spindle equator in anaphase (Fig. 1D), as described by others (Adams *et al*., 2000; Zeitlin *et al*., 2001; Crosio *et al*., 2002). Although both kinases are classified in the same family, they did not colocalize with each other in the cell cycle.

In interphase, GFP-Aurora-A existed as small discrete centrosome-like dots near or on the surface of nuclear membrane, as previously indicated by Zhou *et al*. (Zhou *et al*., 1998). When compared with the endogenous γ-tubulin, one of the components of centrosome, GFP-Aurora-A was colocalized with it throughout the cell cycle (Fig. 1E–L), except for telophase, where γ-tubulin rather stayed on the inner side of condensed chromatin (Fig. 1M). Recently, centrosomal localization of GFP-Aurora-A has been reported in *Caenorhabditis elegans* as well (Hannak *et al*., 2001). These results clearly showed that GFP-Aurora-A has the ability to localize to interphase centrosomes as well as mitotic spindles and poles, drastically changing its morphology depending on the cell cycle.

Construction of a multicolor cell line with Aurora-A, histone H3 and importinα differentially visualized

To correlate the molecular behavior of Aurora-A with each mitotic event in the cell cycle, we further visualized the whole chromatin and nuclear membrane by expressing histone H3 and importinα cDNAs as fusions to cyan and red fluorescent proteins (CFP and DsRed), respectively. Figures 1N–1Q show the representative images of a multicolor transformant cell line, MDA-Auro-imp-H3. The simultaneous visualization of these subcellular structures was essential for us to identify the exact phase of the target cells in the subsequent analyses.

Molecular behavior of GFP-Aurora-A in prophase

We first focused on the transition from late G2 to prophase in which these subcellular structures were expected to change dramatically. Timelapse images were captured by an advanced computer-assisted image analyzing system. Figure 2A shows a living cell with two duplicated centrosomes located in the opposite poles. This cell should be in late G2 or early prophase, since its chromatin, visualized by CFP-histone H3, is under condensation (Fig. 2B) but the nuclear membrane, visualized by DsRed-importinα, is still present (Fig. 2C). In fact, this cell soon entered into prophase; the nuclear membrane beneath centrosomes became thick and invaginated (2 min). The two centrosomal dots of Aurora-A located in the opposite poles approached closer to each other and the nucleus was transformed into a “dumbbell”-like structure (8 min). By this period, the whole chromatin has been condensed along the nuclear membrane and compressed from the pole sides (Fig. 2B). DsRed-importinα, on the other hand, has been concentrated to the invaginated region (Fig. 2C). Ten minutes after observation, nuclear membrane breakdown occurred at the periphery of the “dumbbell” and the two centrosomal dots of GFP-Aurora-A grew rapidly in size. Interestingly, the membrane structure of DsRed-importinα remained in the invaginated region so as to keep the maturated centrosomes separated for several minutes, and then disappeared (16 min, Fig. 2C).

To carefully observe the morphological change of the membrane surface, live images of another G2 cell were captured at 1 min intervals from a different angle. As shown in Figure 2D, duplicated centrosomal dots were first located in a groove of the nucleus. They separated and moved to the opposite sides of the nucleus (0–2 min), and quickly returned to come closer (3–7 min). Interestingly, the membrane between the two centrosomes looked to be “pinched up” during this course. This movement seemed to be responsible for the above morphological change of the nucleus shown in Figure 2A. Again, DsRed-importinα was

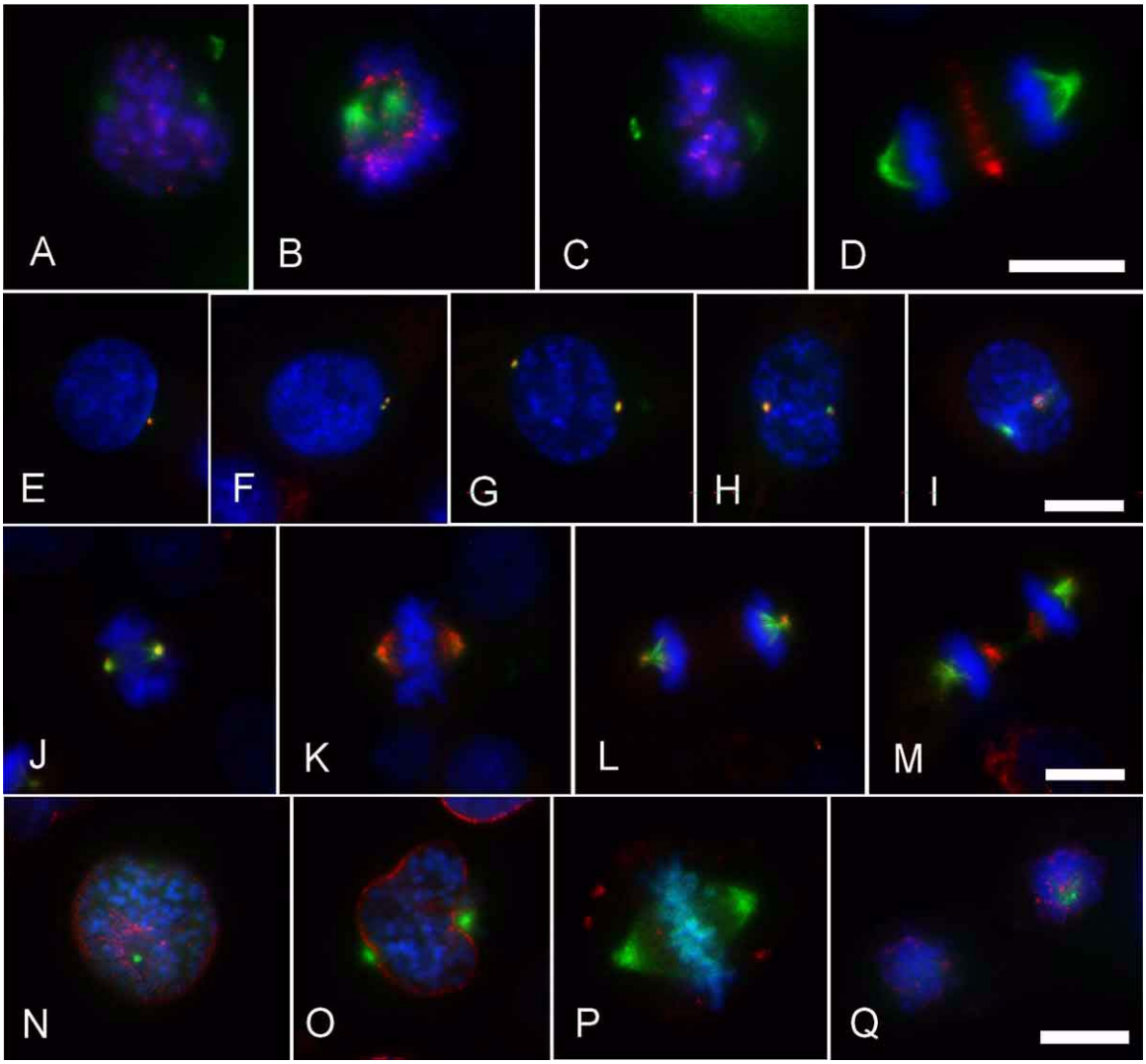

Fig. 1. Subcellular localization of GFP-fused Aurora-A in human MDA435 cells. Human MDA435 was transfected with pEGFP-Aurora-A. One of the stable transformants, MDA-GFP-Aurora-A, ectopically expressing GFP-Aurora-A was observed under a fluorescent microscope. A random culture of the cells were grown on coverslips, fixed with paraformaldehyde, stained with mouse anti-Aurora-B (A–D) or anti-γ-tubulin monoclonal antibodies (E–M), probed with Cy3-conjugated goat anti-mouse antibodies (red) and counterstained with DAPI (blue). A three-color stable transformant, MDA-Auro-imp-H3, simultaneously expressing GFP-Aurora-A, DsRed-importinα and CFP-histoneH3, was also obtained as described in *Materials and Methods*. A random culture of the cells was fixed with paraformaldehyde but directly observed without staining (N–Q). Interphase (E, F); G2 phase (G, N), prophase (A, H, I, O), prometaphase (B, J), metaphase (C, K, P), anaphase (D, L), telophase (M) and early G1 phase (Q). Bar: 10 μm.

concentrated in the invaginated region (5–8 min). As the Ds-Red-importinα gradually disappeared, GFP-Aurora-A dots maturated into a spindle-like structure (15 min).

Molecular dynamics of GFP-Aurora-A from G2 through to early G1

We next focused on the molecular behavior in the processes of centrosome separation in G2 and nuclear membrane reformation in telophase. Live images of 4 mitotic cells, Cell A to Cell D, were successfully captured throughout mitosis at 2 min intervals. Figure 3 shows the complete images of Cell A. Fourteen minutes after observation, the duplicated centrosomes started to separate and move toward opposite poles, taking 18 min (late G2, 12–30 min). Without interruption, the nuclear membrane beneath centrosomes became thick and invaginated (prophase). It took 14 min from the initial invagination of the nuclear membrane (30 min) to its disappearance at the end (44 min). Again, the centrosomal dots of Aurora-A grew rapidly during the nuclear membrane breakdown (38 min) and then transformed into a typical spindle-like structure (44 min). In

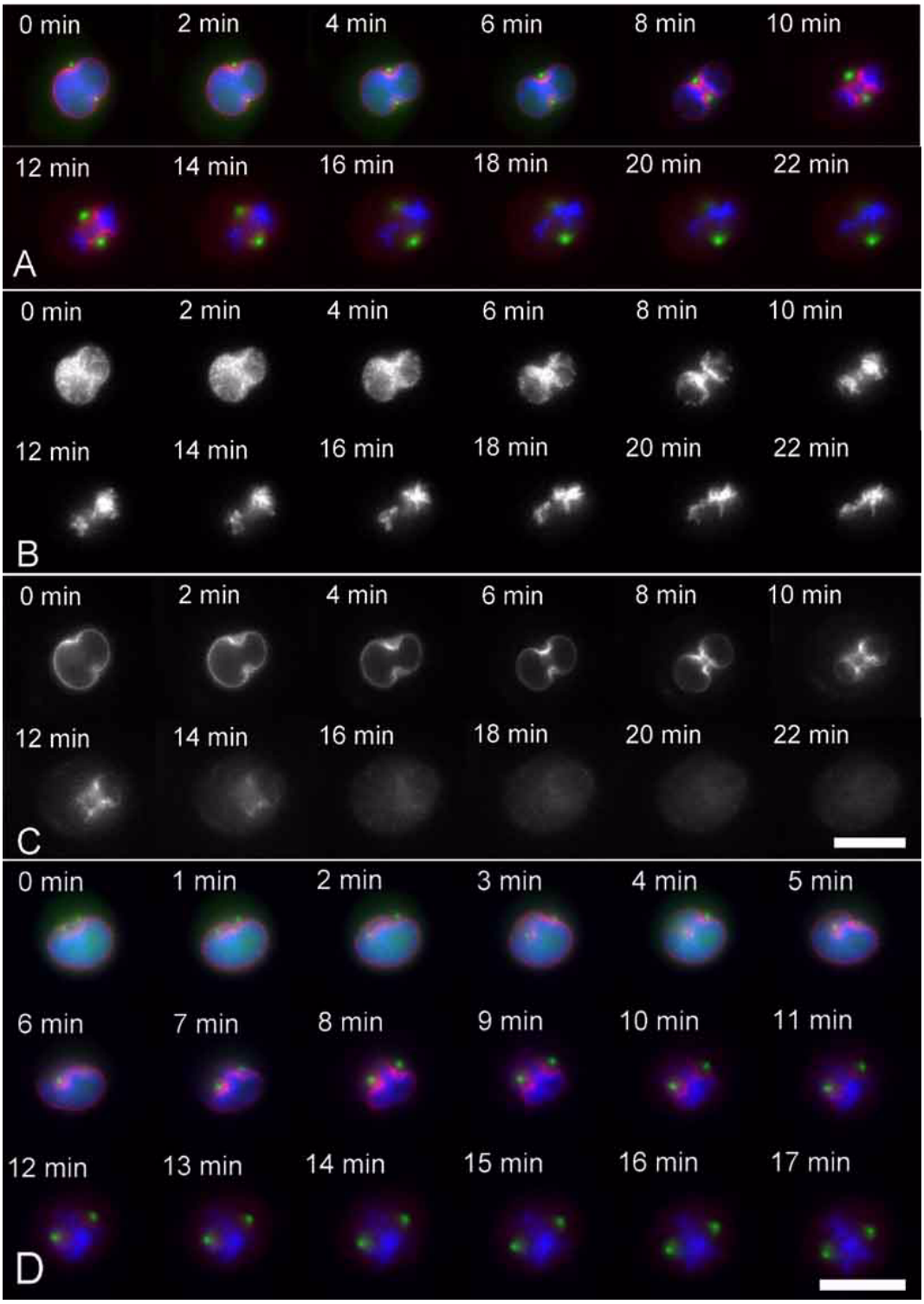

Fig. 2. Molecular behavior of GFP-Aurora-A in late G2/prophase. Fluorescent live images of MDA-Auro-imp-H3 in late G2/prophase were captured by an advanced computer-assisted image analyzing system equipped with a highly sensitive CCD camera (http://www.biochem.osakafu-u.ac.jp/ AMB/MAIN-J.html, to view the system). Three-colored timelapse images of two independent cells were shown in panels A and D with the elapsed time in the upper left corner of each image. Timelapse images of CFP-histone H3 and DsRed-importinα in panel A are separately shown in panels B and C in black and white. Note that the nuclear membrane breakdown occurred at 10 min (panels A–C) and 9 min (panel D) after observation. Bar: 10 μm.

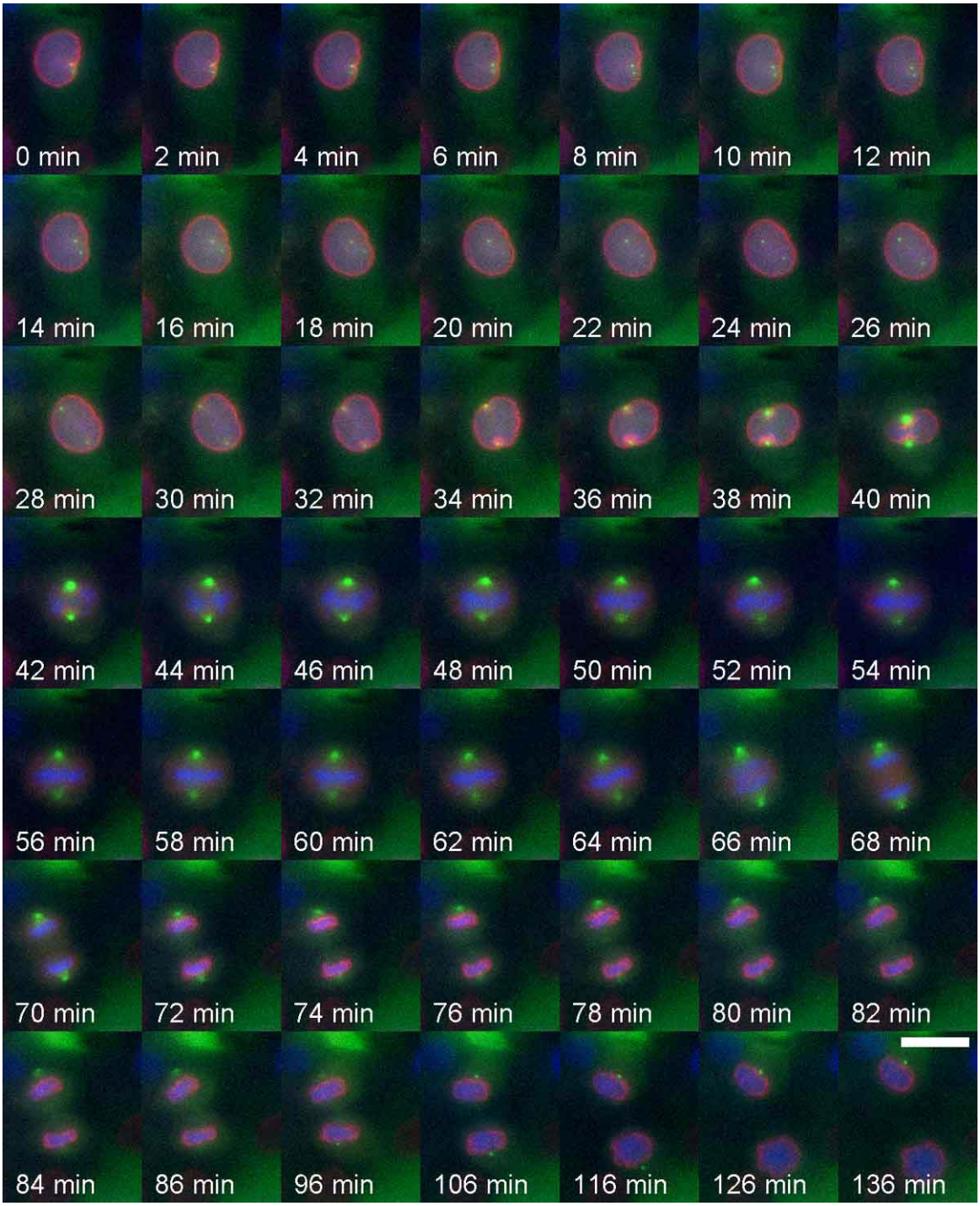

Fig. 3. Molecular dynamics of CFP-histone H3, GFP-Aurora-A and DsRed-importinα throughout mitosis in a human living cell. Timelapse images of MDA-Auro-imp-H3 in G2 were captured at 2 min intervals as described in the legend to Figure 2. Elapsed time is shown in the lower left corner of each image. Note that the nuclear membrane breakdown began 40 min after observation and the importinα membrane reformed at 74 min. Online supplemental video of this cell is available at http://csf.jstage.jst.go.jp. When you click "supplemental materials" under abstract, you can see the movie. Bar: 10 μm.

prometaphase, mitotic chromosomes were aligned at the spindle equator and the sister chromatids were suddenly separated 66 min after observation (anaphase onset). The chromatids reached the opposite poes in 6 min (70 min) and the nuclear membrane structure of importinα was reformed within another 4 min (74 min). In total, it took 34 min, start-

ing with the nuclear membrane breakdown and ending with its reformation. These mitotic events of Cell A are summarized in a diagram, correlating with the distance between the two centrosomal dots of Aurora-A (Fig. 4A).

Timelapse images of Cell B are also shown in Figure 5. The time required for prophase was relatively constant. The distances between two centrosomes in Cell B and Cell D were also calculated and summarized in Figure 4B. Anaphase onsets of Cell B and Cell D did not occur until 46 min and 56 min after the nuclear membrane breakdown, resulting in 20 min and 30 min longer time than Cell A, respectively. Thus, the time required for the chromosome alignment was apparently different from cell to cell in this cell-line. Nevertheless, the subsequent anaphase again proceeded straightforward. It took 6 min for sister chromatids to reach the opposite poles and another 4 min to form the nuclear membrane of importinα. The time required for these processes was calculated as 10 min (see diagrams shown in Fig. 4). Cytokinesis occurred during the chromosome segregation and cleavage furrow was observed on the cell surface at 4 min after anaphase onset (66 min, Fig. 5). Interestingly, some portion of GFP-Aurora-A was present in the spindle midzone and then accumulated in the midbody in the completion of cytokinesis (88 min). During this process, the Aurora-A spindle gradually decreased in size and returned to interphase centrosomes. These results clearly showed that Aurora-A kinase drastically changes its morphology at two transition stages in mitosis, G2 to prophase and anaphase to telophase, which are well correlated with its physical isolation from the whole chromatin by nuclear membrane.

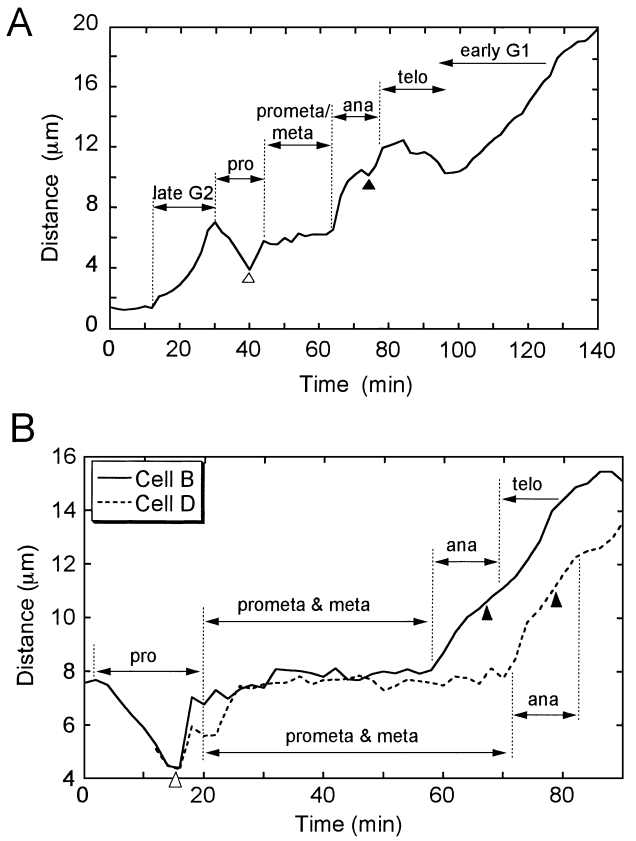

Fig. 4. Time course of the migration of GFP-Aurora-A in mitosis. The distances between the two centrosomal dots of GFP-Aurora-A were measured by MacScope software using the timelapse images of Cells A (Fig. 3) and Cells B (Fig. 5). Nuclear membrane breakdown (△) and reformation of nuclear membrane (▲). Note that the distance increases in late G2, decreases in prophase, is maintained in prometa/metaphase and increases again in anaphase.

Morphological changes of Aurora-A and microtubule nucleating activity of centrosome

Centrosomes are known to possess a microtubule nucleating activity. Aurora-A kinase colocalizes with γ-tubulin from G1 to metaphase (see Fig. 1E–1L) and is involved in the formation of mitotic spindle (Figs. 2, 3 and 5). To further correlate the molecular behavior of Aurora-A with the nucleating activity of centrosomes, MDA-GFP-Aurora-A cells were stained with anti-α-tubulin antibodies. In interphase, the microtubules identified by anti-α-tubulin antibodies were exclusively found in cytoplasm and a single centrosome did not indicate microtubule-nucleating activity (Fig. 6A). However, we noticed that some microtubules were projected from the duplicated and separated centrosomes (Fig. 6B and 6C). Interestingly, the microtubule nucleating activity further increased in prophase (Fig. 6D and 6E). After nuclear membrane breakdown, the microtubules of α-tubulin were projected from the poles and formed a mitotic spindle associated with Aurora-A in metaphase (Fig. 6F). In anaphase, α-tubulin still maintained the mitotic spindle structure, while the spindle halves of Aurora-A moved to the poles pulling sister chromatids along the rails of microtubules (Fig. 6G). As the cytokinesis begun, the mitotic spindle of α-tubulin was shrunk at the spindle equator in telophase (Fig. 6H). The result suggested that Aurora-A kinase associates with α-tubulin and changes its morphology in mitosis.

Discussion

Recent studies have shown that morphological features in mitosis are tightly regulated by protein modifications of subcellular components and that the modification enzymes themselves show restricted subcellular localizations within the cells. Well characterized are the mitotic kinases such as Aurora-A, PLK and NIMA that localize to centrosome (Nigg, 2001), histone H3 modification enzymes such as Suv39H1 methylase and Aurora-B kinase that localize to heterochromatin (Aagaard *et al*., 2000; Adams *et al*., 2000; Crosio *et al*., 2002), and the mitotic checkpoint kinases such as Bub1 and BubR1 that localize to metaphase kinetochore (Chan *et al*., 1998).

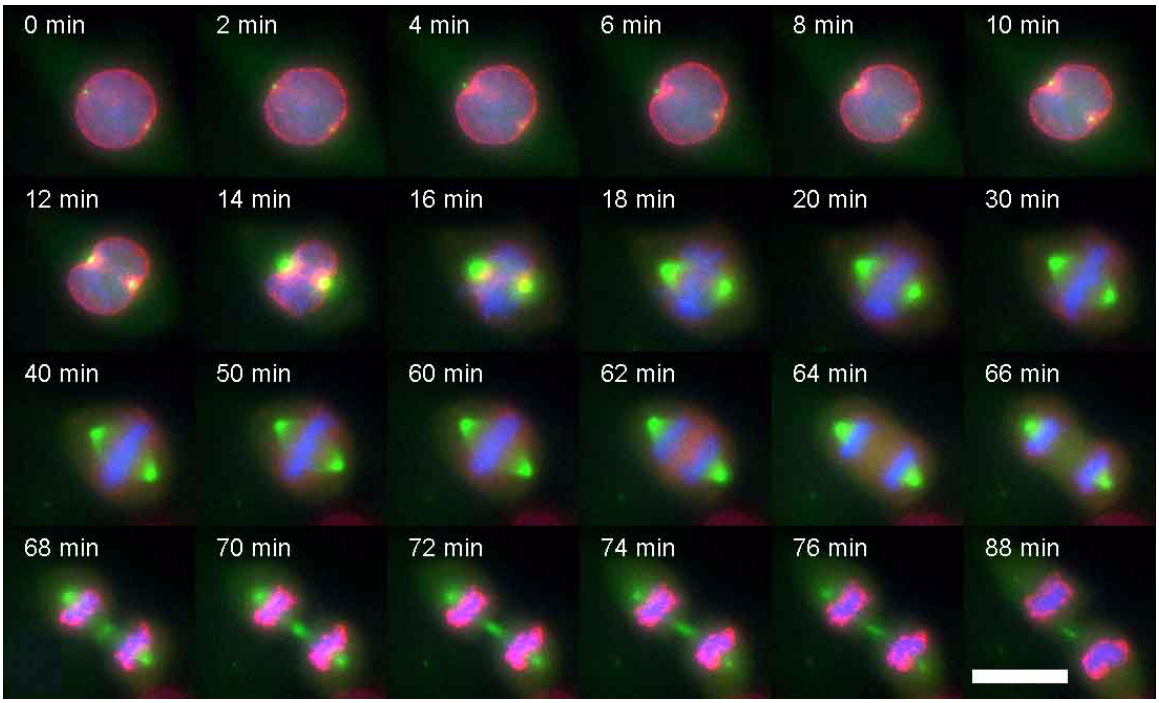

Fig. 5. Molecular behavior of GFP-Aurora-A in anaphase. Timelapse images of three more individual cells of MDA-Auro-imp-H3 were also captured at 2 min intervals. Shown here are the representative images of Cell B. Note that the nuclear membrane reformed 10 min after the chromosome segregation in these cells. Online supplemental video of Cell B is available at http://csf.jstage.jst.go.jp. When you click "supplemental materials" under abstract, you can see the movie. Bar: 10 μm.

The Aurora kinase family is evolutionary conserved among eukaryotes, from yeasts to mammals (Giet and Prigent, 1999). In mammalian cells, three related peptides, Aurora-A, B, and C, have been identified as "structural" homologs of *Drosophila* Aurora (for a review see Nigg, 2001). Interestingly, each has a distinct localization and function in the cell cycle. Aurora-A localizes to mitotic spindles and poles in mitosis (Kimura *et al*., 1997), while Aurora-B localizes to centromere heterochromatin from late G2 to metaphase and stay in spindle midzone in anaphase (Bischoff *et al*., 1998; Adams *et al*., 2001). Therefore, their enzyme activity should be further evaluated with the morphological changes during the cell cycle. This is the first report on the molecular dynamics of Aurora-A kinase in mammalian living cells.

In the cell cycle, centrosomes undergo a series of structural changes. It has been proposed that Aurora-A is involved in centrosome separation, spindle assembly or spindle maintenance (Goepfert and Brinkley, 2000). Recent studies with Nematoda and *Drosophila in vivo*, however, indicated that Aurora-A kinase has a significant role in centrosome maturation, rather than centrosome separation (Hannak *et al*., 2001; Berdnik and Knoblich 2002). In this study, simultaneous visualization of histone H3 and importinα greatly helped us to characterize the behavior of Aurora-A, compared to other subcellular structures such as chromosomes and nuclear membrane. As presented in Figure 3, Aurora-A is localized to centrosomes in interphase nuclei. Duplicated centrosomal dots separate and move to the opposite sides of nucleus in late G2. Prophase starts with the accumulation of importinα beneath the centrosomes. As the nuclear membrane of importinα thickens, the centrosomal dots of Aurora-A invaginate into the nucleus and approach closer to each other from the opposite poles. The prophase nucleus gradually transforms into a dumbbell-like structure and the condensed chromatin mass is pushed out from the nucleus. Similar observations of nuclear membrane breakdown have recently reported by others (Beaudouin *et al*., 2002; Salina *et al*., 2002). Membrane structure of importinα breakdowns at the periphery of the "dumbbell" but remains in the invaginated region between the centrosomes for a while. As it gradually disappears, the centrosomal dots of Aurora-A grow in size and display a typical spindle structure. It takes 14 min to enter into prometaphase. During prometaphase, chromosomes are aligned at the spindle midzone, although the length of this period varied from cell to cell in this cell line, possibly because of some defect in the checkpoint mechanism.

Although the exact point of prometaphase/metaphase transition could not be determined in this study, anaphase onset was easily identified, because of the success in the simultaneous visualization of sister chromatids. It takes 4–6

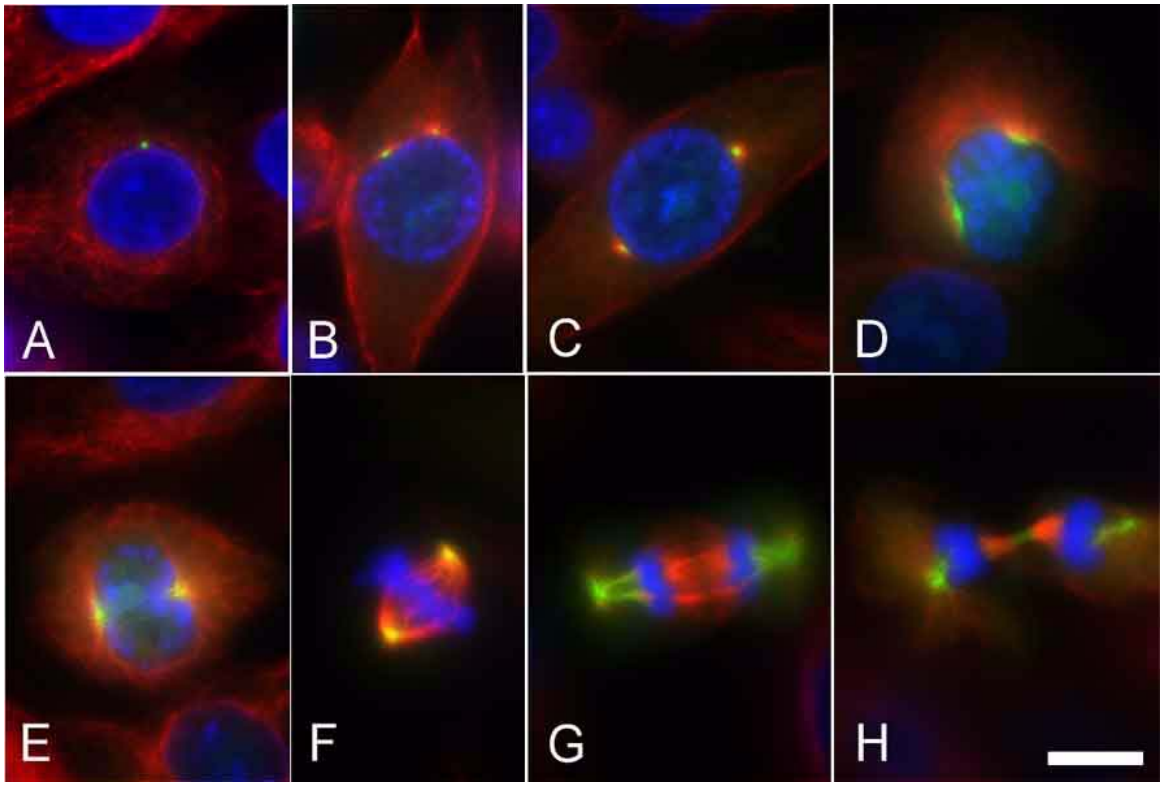

Fig. 6. Morphological change of Aurora-A and microtubule nucleating activity of centrosome in mitosis. A random cell culture of MDA-GFP-Aurora-A cells was fixed with paraformaldehyde, stained with mouse monoclonal anti-α-tubulin antibodies (red) and counterstained with DAPI (blue). Interphase cells with a single (A) or duplicated centrosomes (B), and mitotic cells in G2 phase (C), prophase (D, E), metaphase (F), anaphase (G) and telophase (F). Note that microtubules are partly nucleated from the duplicated centrosomes (B), form the typical mitotic spindle associated with Aurora-A in metaphase (F) and retain the spindle structure after the chromosome segregation in anaphase (G). Bar: 10 μm.

min for sister chromatids to reach the poles, which is consistent with our previous observation (Sugimoto *et al*., 2001). Telophase starts with reformation of nuclear membrane 10 min after anaphase onset and the transition from anaphase to telophase is straightforward. Interestingly, the cytokinesis begins in anaphase and ends in telophase. Cleavage furrow is observed on the cell surface 4 min after anaphase onset. Thus, the cytokinesis and the reformation of nuclear membrane are suggested to be the consequence of anaphase onset or chromosome segregation.

Aurora-A kinase is a candidate for regulators of centrosome maturation, modulating the activity of the components of mitotic apparatus. In addition to a kinesin-related motor protein, such as *Xenopus* Eg5 (Giet and Prigent, 1999), certain chromosomal components are known to be phosphorylated by Aurora-A. For example, phosphorylation at Ser-10 of histoneH3 has been shown to be a crucial event for the initiation of mitosis (Hooser *et al*., 1998; Lanlan *et al*., 1999). This modification appears early in the G2 phase within centromeric heterochromatin and spreads into the whole chromatin concurrent with chromatin condensation. Interestingly, both Aurora-A and Aurora-B have been shown to phosphorylate histone H3 at Ser-10 *in vivo* and *in vitro* (Crosio *et al*., 2002). In G2 phase, the centrosomal dots of Aurora-A are present outside of the nucleus, while Aurora-B is present in centromere heterochromatin and colocalizes with phosphorylated histone H3. Therefore, Aurora-B is likely to be responsible for the initiation of H3 phosphorylation. If so, how are these kinases involved in their phosphorylation of histone H3 during the cell cycle and how does this modification spread into the whole chromatin? It may be partly explained by the molecular behavior of Aurora-A clarified in this study. At the transition of G2/prophase, the condensed chromatin localized near to the inside wall of nuclear membrane, while Aurora-A invaginated into the nucleus from the outside of the membrane (see Fig. 2). After the nuclear membrane breaks down, the whole chromatin is exposed to the activated Aurora-A and then phosphorylated. As shown in Figure 1P, GFP-Aurora-A was able to associate with mitotic chromosomes. Recently, Scrittori *et al*. also reported that *Xenopus* Aurora-A, pEg2, phosphorylate specifically histone H3 at serine 10 and further incorporated into chromatin *in vitro* (Scrittori *et al*., 2001), although we could not observe such localization in living mitotic cells. Anyway, histone H3 tail may be a better substrate for

Aurora-A than Aurora-B *in vivo*.

Our data indicated that the morphological change of Aurora-A was regulated spatially and temporally at two critical stages in the cell cycle: G2/prophase and anaphase/telophase transitions, cooperated with the breakdown and the reformation of nuclear membrane. Our recent study with anti-phosphorylated Aurora-A monoclonal antibodies showed that the phosphorylated form Aurora-A was only detected in duplicated and separated centrosomes and greatly increased in the transition of prophase/prometaphase (Urano and Sugimoto, unpublished results). Since Aurora-A itself is phosphorylated, it may be possible that this modification causes its morphological change in mitosis. Alternatively, the active form of Aurora-A itself might provide a structural support for the mitotic spindle nucleating from centrosomes or a pulling force of chromatids to the poles along the mitotic spindles (see Fig. 6).

There are several reports on the artifacts by the fluorescent proteins driven by a strong promoter or the aggregation of DsRed-fused proteins (Baird *et al*., 2000). Although the localization of fused proteins should be carefully evaluated, *in vivo* labelling of cells with multiple fluorescent proteins have been offering very useful tools for modern biology (Finley *et al*., 2001). We previously reported fluorescent kinetochore and heterochromatin markers for live image analyses (Sugimoto *et al*., 2000, 2001). Here, we further constructed GFP-Aurora-A, CFP-histone H3 and DsRed-importinα and fully characterized their molecular behavior throughout mitosis. Because of its morphological change in the cell cycle, they would be excellent subcellular markers in mammalian living cells. For example, timelapse image analyses with these markers accurately dictate when the next coming event occurs in the cell cycle and how long it takes. In addition, the multicolor cell-line that we constructed may be used as a "cell sensor" for screening of mitotic inhibitors as well as for characterizing potent inhibitors of Aurora-A kinase in the future.

Acknowledgments. This work was supported by a grant for Specific Research Assistance Program from the Asahi Glass Foundation (2000–2001), Tokyo, Japan as well as a grant from the Nagase Science Foundation (2002), Osaka, Japan. We thank Kouichi Amami (Tsukasa Giken, Osaka, Japan) for technical assistance with the microscope.

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(*Received for publication, September 26, 2002*
and in revised form, October 30, 2002)