Centrosome replication in hydroxyurea-arrested CHO cells expressing GFP-tagged centrin2

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Summary
Centrosome duplication is tightly coupled with the cell cycle and neither too many nor too few centrosomes are induced in a normal cell. To study how centrosome assembly is regulated, we analyzed the abnormal process of multiple centrosome replications in Chinese hamster ovary (CHO) cells induced by hydroxyurea (HU), which is known to uncouple the centrosome cycle from the cell cycle. Green fluorescent protein (GFP)-tagged centrin2 expressed in CHO cells labels both centrioles and the pericentriolar material (PCM). Counting fluorescent spots of GFP-centrin in synchronized cells showed that in G1/S-arrested cells, centrioles are initially duplicated in a template manner. Further treatment with HU overrides the suppression of excess centriole/centrosome replication in a cell where the full complement of centrioles/centrosomes already exists. Time-lapse fluorescence microscopy revealed that small centrin-containing foci emerged in the cytoplasm during HU treatment. These foci are surrounded by a PCM cloud and their number continuously increases as cells are exposed to HU for longer periods of time. Both the centrosome and cytoplasmic foci are highly mobile, continuously changing their position in a manner dependent on microtubules/microtubule dynamics. The centrosome number increases as small foci grow in size and resolve into recognizable centrosomes. As this occurs in a random fashion, the cells arrested longer with HU induced highly heterogeneous numbers of centrosomes.

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Key words: Centrosomes, Centrioles, Pericentriolar material, GFP-centrin, CHO cells, Time-lapse fluorescence microscopy, Hydroxyurea

Introduction
A primary microtubule-organizing center in animal cells is the centrosome, which is composed of a pair of centrioles and a surrounding amorphous cloud of pericentriolar material (PCM). The centrosome is associated with a number of molecules: some are permanent core components, whereas others are temporarily recruited to the centrosome/centrosomal region in a cell cycle- and/or developmental stage-specific manner. Structural proteins, such as pericentrin (Dictenberg et al., 1998) and Cep135 (Ohta et al., 2002), have been shown to be important for maintenance of the overall shape of the PCM/centrosome structure by serving as centrosomal scaffolds. γ-tubulin/γ-tubulin ring complex (γ-TuRC) components (Zheng et al., 1995; Moritz et al., 1995) and ninein (Mogensen et al., 2000) are required for the centrosomal activity of microtubule nucleation and anchorage. Recent evidence has indicated that one of the kinases associated with the centrosome (Plk4/Sak) plays an important role in controlling the formation of new centrioles (Habedanck et al., 2005; Bettencourt-Dias et al., 2005). However, the vast majority of molecules located at and/or around the centrosomes appear to have no functional relationships with microtubules and microtubule organization. It is, therefore, tempting to speculate that the centrosome is functioning not only as a microtubule-organizing center, but also as a site for the localization of molecules with various biological activities. To study the centrosome, it is important to understand how the centrosome is assembled and how it recruits such a wide variety of molecules.

One of the characteristic features of the centrosome is the tight regulation of its numbers. The centrosome is normally duplicated once, and only once, during each cell cycle, which is prerequisite for the equal segregation of the chromosomes, thereby maintaining genomic stability in normal cells. Morphological changes in the centriole have been documented by electron microscopy (Kuriyama and Borisy, 1981). A daughter cell formed after cell division receives a pair of centrioles oriented perpendicular to one another. During G1, the two centrioles become disoriented and lose their characteristic orthogonal configuration. A new daughter centriole appears at the proximal end of each mother centriole in late G1 to early S and grows slowly, attaining almost full length at the onset of M phase. A pair of centrioles is positioned at each spindle pole during M phase, and then segregated into daughter cells by cell division. In contrast to the centriole, almost nothing is known about the change in the PCM during the cell cycle.

To study the mechanism of normal centrosome duplication and its regulation, it is useful to analyze cells that produce abnormal numbers of centrosomes. In 1995, Balcacz et al. first reported that hydroxyurea (HU) caused uncoupling of the centrosome cycle from the cell cycle in Chinese hamster ovary...
Multiple centrosome replications in CHO cells

Consequently, cells arrested at G1/S and S phase produce multiple centrosomes by undergoing repeated cycles of centriole/centrosome replication. This provides a convenient assay system for identification of the molecular components, such as E2F transcription factors and Cdk2/cyclin A (Meraldi et al., 1999), that are involved in the initiation of centrosome duplication in cycling cells. Although direct quantification of numbers of centrosomes has been done in non-synchronized cell populations (Balczon et al., 1995), no detailed analysis has yet been made of how new centrosomes emerge in the cell.

To analyze the process of centriole/centrosome formation in living cells, we established stable CHO cell lines expressing green fluorescent protein (GFP)-tagged human centrin2. Here, we report that HU treatment of cells caused the induction of highly mobile, centrin-containing cytoplasmic foci, which increase in size, developing into recognizable centrosomes. Time-course analysis of synchronized cell populations showed that initial centriole/centrosome formation is under the control of pre-existing centrosomes. However, HU treatment overrides the suppression of the production of excess centrioles/centrosomes in cells where the full complement of centrosomes already exists. Uncoupling of the centrosome cycle from the cell cycle induces an asynchronous manner of centrosome replication, which results in the formation of highly heterogeneous numbers of centrosomes in cells treated for extended periods with HU.

Results

Centrosomes are labeled with GFP-tagged centrin in CHO cells

Centrioles are present in both the centriole and PCM of the centrosome

To visualize centrosomes in living cells, we established stable CHO cell lines constitutively expressing GFP-tagged human centrin2. Fig. 1 shows fluorescence images of centrosomes in exponentially growing, non-synchronized cells after brief fixation with cold methanol. The cells include predominantly two (Fig. 1A-C) or occasionally four fluorescent sites of GFP-centrin (Fig. 1D-F), with varying distances between them. As centrin is a widely used centriolar marker (White et al., 2000; Piel et al., 2001), they probably correspond to the centrioles of the centrosomes. The majority of cells contain dots of more or less equal size. However, we sometimes detected fluorescent sites of smaller sizes, each of which was next to one of a larger size (Fig. 1G-I,I'): these pairs of foci represent mother and daughter centrioles in cells at early S phase.

In addition to labeling the centriole, GFP-centrin localizes to indistinct masses around the centrioles (Fig. 1J-N): some are faint and subtle, whereas others are more prominent (arrows). The amorphous mass corresponds to the PCM, indicating that centrin localizes not only to centrioles but also to the PCM in CHO cells. The PCM labeled by GFP-centrin comprises various shapes, from irregular amorphous clouds (Fig. 1J-J') to discrete dots and/or lines (Fig. 1K-N). The identical distribution of GFP fluorescence was noted in cells before and after fixation, and cells derived from a single established cell line showed fundamentally the same expression pattern of GFP-centrin at the centrosome.

Centrosomal components distinctively localize at the centrosome

To examine the centrin distribution in more detail, we stained the cells with other centrosomal antibodies for high-resolution analysis. Fig. 2A-D illustrates centrin-expressing cells labeled with pericentrin (Fig. 2A'), Cep135 (Fig. 2B'), PCM1 (Fig. 2C') and hCenexin antibodies (Fig. 2D'). The centrosome shown in Fig. 2A was resolved as two pairs, each consisting of one

Fig. 1. Localization of GFP-centrin to centrosomes. Exponentially growing, non-synchronized CHO cells possess centrosomes revealed as two (A-C) or sometimes four (D-F) fluorescent dots with varying distance between them. Arrowheads indicate a small centrosome next to the mother centrosomes (G-I,I'). In addition to the centriole, GFP-tagged centrin labels the PCM, which appears morphologically varied, from a fuzzy indistinct mass (J) to discrete dots or lines (arrows, K-N). Areas outlined in I' and J' are seen in I and J at high magnification. Bars, 2 μm (A-N); 10 μm (I'-J').
large and one small dot, which are surrounded by a pericentrin cloud (Fig. 2A'). Pericentrin also forms numerous particles of different sizes. Cep135 is a scaffolding protein (Ohta et al., 2002), which we detected in a lattice-like organization around the centrioles (Fig. 2B-B''). PCM1 is known to be a component of centriolar satellites (Kubo et al., 1999), and many PCM1-containing particles are seen around the centrioles as shown in Fig. 2C-C''.

Fig. 2. Immunofluorescence staining of GFP-centrin-expressing CHO cells (A-C,D1-D5,E1-E3,F1-F3,G1-G3) with antibodies specific to pericentrin (A'), Cep135 (B'), PCM1 (C'), hCenexin (D1'-D5'), Nek2 (E1'-E3'), ninein (F1'-F3') and γ-tubulin (G1'-G3'). Merged images are shown in A''-C'',D1''-D5'',E1''-E3'',F1''-F3'',G1''-G3''. Centrin is distributed differently than these molecules in the centrosome. Bars, 5 μm.
Multiple centrosome replications in CHO cells

hCenexin, a variant of the centrosomal scaffolding protein of Odf2, has been shown to selectively associate with a subset of centrioles (Soung et al., 2006) (Fig. 2D). In cells with unequal sizes of centrioles, hCenexin is recognized only at the mother centriole (Fig. 2D1-D1” and Fig. D2-D2”). As it enlarges, the daughter centriole starts to recruit the protein...
(Fig. 2D3'-D4'); as a result, eventually two centrioles of equal size become labeled by the antibody. Although preferentially localized to the centrioles, we also detected hCenexin in the amorphous PCM mass to varying degrees (Fig. 2D5-D5"). It is interesting to note that the PCM labeled by hCenexin does not colocalize exactly with the PCM labeled with GFP-tagged centrin. Fig. 2E-G summarizes the results of double staining of cells with GFP-centrin and Nek2 (Fig. 2E1'-E3') or ninein (Fig. 2F1'-F3') and γ-tubulin (Fig. 2G1'-G3'). Nek2 appeared to preferentially localize at one end, possibly the proximal end, of both mother and daughter centrioles. By contrast, the entire mother and daughter centrioles were positive by ninein staining (Fig. 2F1''-F2''), which is consistent with previous observations (Bornens, 2002). We frequently observed centrioles that were entirely and extensively covered with ninein proteins (Fig. 2F2-F2''). γ-tubulin, responsible for microtubule nucleation onto the centrosome, surrounded the centrioles, and in particular the mother centrioles (Fig. 2G1'-G3'). Like Nek2 (Fig. 2E3-E3'') and ninein (Fig. 2F3-F3''), γ-tubulin is a PCM component, and thus it identifies amorphous masses of various sizes and shapes around the centriole (Fig. 2G3-G3').

Centrosome replication analyzed in fixed cells

To determine the time course of the replication pattern in detail, we performed experiments using synchronized mitotic cell populations. After plating harvested M-phase cells in a HU-containing medium (0 hours), a sample was taken at different time points during incubation. To facilitate the visualization of fluorescent dots (Fig. 3A-O), the cells were lysed in a detergent-containing medium, and the number of centrosomes on or at the periphery of each nucleus (Kuriyama and Borisy, 1981) was counted to determine the frequency of cells with one to two, three to four and more than five centrosomal dots (Fig. 3P). At 13 hours after cell division, ~20% of cells already contain three to four centrosomes (Fig. 3D-F,P), however the majority of cells still have one to two centrosomes (Fig. 3A-C). Because one generation time of CHO cells is approximately 14 hours, these results are consistent with the previous report of Balczon et al. (Balczon et al., 1995) that the timing of centrosome replication in HU-arrested cells lagged considerably behind the cell cycle time of control CHO cells. As the proportion of cells with one to two centrosomes decreased to 60% by 16.25 hours, the proportion of cells containing three to four fluorescence sites of GFP-centrin increased to 40% (Fig. 3P). This suggests that the cells at early stages of HU treatment already contain one to two centrosomes, and do not initially generate more than two additional centrosomes. However, during prolonged HU treatment, the cell population with one to two centrosomes continuously decreased as cells with three to four dots became more abundant. These results suggest that initial formation of centrioles/centrosomes in G1/S-arrested cells is controlled by the presence of pre-existing centrosomes.

After the initial assembly of daughter centrioles/centrosomes, the cells continued to replicate additional centrosomes, indicating that HU overrides the suppression of excess centrosome formation in cells where the full complement of centrioles/centrosomes already exists. Previous observations showed that centrosome doubling does not progress from two to four to eight during HU arrest (Balczon et al., 1995), suggesting that the mechanism controlling the formation of a precise number of new centrioles/centrosomes becomes deregulated when centrosome duplication is uncoupled from the cell cycle. To confirm this, we examined centriole/centrosome profiles revealed by centrin fluorescence in cells treated for extended periods with HU. Fluorescent foci of various sizes and shapes embedded in the PCM were commonly observed (Fig. 3G). Fluorescence analysis also revealed the presence of smaller daughter centrosomes emerging next to the larger mother centrosomes asynchronously (arrows in Fig. 3H,J). Because this happened in a highly asynchronous manner, cells treated with HU for increasing lengths of time induce variable numbers of centrosomes from less than four to more than several dozen (Fig. 3L,M). It was also noted that one mother centrosome was associated with more than two smaller-sized centrosomes (Fig. 3I,K), suggesting that multiple daughter centrioles are simultaneously induced next to the mother centriole.

Multiple fluorescent sites of GFP-centrin colocalize with other centrosomal proteins in HU-arrested cells. Although their overall distribution is similar, they are not identical as some centrosomes/cytoplasmic foci are stained by only a particular centrosomal probe (supplementary material Fig. S1). Presence or absence of γ-tubulin in the cytoplasmic foci of GFP-centrin well correlates to their microtubule-nucleating activity (supplementary material Fig. S2). In cycling cells, centrosomal components localize to different subregions of the centrosome (Fig. 2). This differential distribution is more prominent in cells

![Fig. 4](image_url)

Fig. 4. (A) Time-lapse fluorescence microscopy of centrosomes labeled with GFP-centrin in HU-arrested CHO cells. Fluorescence images were taken at indicated time points after incubation with 2 mM HU. (B) The centrosomes were frequently surrounded by the prominent PCM (arrows). Bar, 10 μm.
undergoing active centrosome replication. Therefore, it is highly probable that different molecules associate with centriolar/centrosomal precursors at different stages of assembly.

**Time-lapse analysis of centrosome replication in living cells**

To directly monitor the process of centrosome replication in living cells, we recorded GFP fluorescence in CHO cells by time-lapse fluorescence microscopy. Fig. 4A shows a series of fluorescence images of non-synchronized cells taken at different time points after HU addition. We detected one fluorescent dot at 0 hours and the centrosome number increased to four to five and six to seven by 34 hours and 47 hours, respectively. The fluorescent sites are sometimes so close to each other, which makes it difficult to determine the precise number. The size and fluorescent intensity of centrin-containing sites are not uniform: some sites are larger and more prominent than others, which may be because of the different stages of centrosomal assembly. The centrosomes are frequently surrounded by the prominent PCM mass (arrows in Fig. 4B). It was also noted that as the time of HU incubation increased, the GFP fluorescence became apparent inside the nucleus (Fig. 4A, frame 43:01 to 47:29). This is in good agreement with previous observations of fixed cell samples (Fig. 3M-O).

In the cell shown in Fig. 5, we observed two centrosomes at 19:46 hours, three at 25:52 hours, four at 28:59 hours and five at 31:38 hours. In addition to those distinct sites, many faint, dust-like particles were scattered in the cytoplasm (arrowheads). The small aggregates of GFP-centrin became large and eventually resolved to a quantifiable centrosomal dot, suggesting that the centrosomes originate from the smaller-sized of centrin-containing cytoplasmic foci. It is, however, unknown whether individual foci develop into each centrosome or whether smaller sizes of foci fuse to become a centrosome. The size of fluorescent foci increases in a highly asynchronous manner, resulting in the formation of a random number of centrosomes in cells arrested with HU for extended periods.

**Movement of the centrosome and centrin-containing cytoplasmic foci**

Live cell observations showed that centrosomes constantly changed their position relative to one another. The cell in Fig.
6 induced multiple centrosomes, forming a cluster during early stages of HU treatment. The fluorescent sites became dispersed to a certain degree by 8-9 hours, thereafter repeating continuous cycles of coalescence and dispersion. During dispersion, the centrosomes were separated over 20-30 μm in distance (frame 45:34 to 47:29), which made it easy to count the centrosomal number. It was also noted that the centrosomal cluster continuously changed its position relative to the cell nucleus.

Like centrosomes, small centrin-containing foci in living cells show a wide range of distribution, as previously seen in fixed cells (Fig. 3N,O). To directly monitor the movement of fluorescent sites, we recorded images of GFP-centrin-expressing cells every 5 seconds. A cell shown in supplementary material Movie 1 contains one large fluorescent dot along with four to five smaller foci, all of which are mobile. Sometimes they rotate and repeatedly appear to and disappear from a focal plane. There seemed to be no regularity of motion as they often moved suddenly after having been immobile for a while. Nonetheless, all of the fluorescent sites appeared to maintain a certain degree of spatial relationship as they move back and forth, away from and towards each other. Fig. 7 includes a series of microscopic frames taken from supplementary material Movie 2, in which one bright centrosomal dot and several smaller sizes of spots are shown. One spot emerging to the focal plane (yellow arrows) moved away from the centrosome, and then made a sudden movement towards the centrosome. Meanwhile, another spot indicated by red arrows appears to the focal plane to approach the centrosome. Supplementary material Movie 3 shows a cell containing many fluorescent dots/foci that actively moved to change their position relative to each other by revealing the fundamentally identical motion pattern seen in other cells with fewer fluorescent sites (supplementary material Movies 1 and 2).

To determine whether the microtubule network that had originated from the interphase centrosome played a role in the movement of centrin-containing structures, we depolymerized microtubules using nocodazole. As shown in supplementary material Movie 4, the motion of fluorescent sites of GFP-centrin ceased soon after the drug was added to the culture medium. This inhibitory effect of microtubule...
depolymerization was reversible as the centrosomes/foci started to move again once the nocodazole was washed out (supplementary material Movie 4). In contrast to nocodazole, taxol does not depolymerize microtubules, but rather overstabilizes them. The movement of both centrosomes and cytoplasmic foci was effectively blocked in taxol-treated cells (data not shown), suggesting that the presence of dynamic microtubules is essential for the movement of centrin-containing sites.

Discussion
As already shown in other mammalian cells, including HeLa and Syrian hamster cells (White et al., 2000; Piel et al., 2001), we found that constitutive expression of exogenous centrin2 does not interfere with CHO cell growth. Consequently, we were able to establish stable cell lines expressing GFP-centrin that allowed us to directly monitor the process of centrosome replication during HU treatment.

Multiple centrosome formation in HU-arrested cells
Although HU treatment is a useful method to induce multiple centrosome formation (Balczont et al., 1995), it is effective only in certain types of cells, such as CHO and U2OS cells. The question remains as to whether the centrosomes induced under the abnormal conditions are functionally equivalent to those assembled during normal cell cycle progression. By comparing HU-responsive CHO cells with non-responsive HeLa cells, Balczon reported that cyclin A levels were depressed in HeLa cells relative to CHO cells after HU treatment (Balczon, 2001).

Because overexpression of cyclin A was able to convert HeLa cells to the CHO-type (Balczon, 2001), these results are consistent with a previous report that Cdk2/cyclin A is the key to initiation of centrosome formation in cycling cells (Meraldi et al., 1999). Maintenance of a high level of cyclin A may allow G1/S cells to continuously synthesize and stockpile molecules that are required for the construction of multiple centrosomes. Cyclin A is a nuclear protein shuttling between the nucleus and cytoplasm (Jackman et al., 2002). In our preliminary studies, we obtained evidence that the presence of nuclei is required for HU-arrested CHO cells to undergo multiple centrosome replications (R.K., unpublished). Therefore, it is possible that a cyclin A-mediated signal(s) derived from the nucleus may be essential for the initiation and continuation of centrosome assembly in HU-arrested cells.

Taking advantage of highly synchronized cell populations, we performed detailed time-course analyses of centrosome formation (Fig. 3). Synchronized M-phase cells plated at 0 hours each contained two centrioles inherited from previous cell cycle generation. During G1/S arrest by HU, the cell duplicates centrioles and the centrosome number generally does not exceed more than two, suggesting that the initial cycle of centriole/centrosome duplication is under the control of pre-existing centrosomes. This is in contrast with the de novo formation of centrosomes reported by Khodjakov et al. (Khodjakov et al., 2002), who showed that, after elimination of centrosomes by laser microsurgery, HU-arrested CHO cells first induced PCM clouds at 5-8 hours, and then a random number of two to 14 centrioles by 24 hours through the de novo pathway. The centrosomal reduplication normally occurs only after passing through cell division, and cells at G1/S contain centrosomes that possess the intrinsic capability of their reduplication (Wong and Stearns, 2003). Therefore, the prolonged HU treatment must override the suppression of excess centriole/centrosome formation in the cell where the full complement of centrioles/centrosomes already exists. After templated assembly, HU-treated cells continue to produce centrioles/centrosomes in a random fashion, which results in the formation of significantly heterogeneous numbers of centrosomes in G1/S cells arrested with HU for extended periods of time. Thus, the mechanism for controlling the precise number of centrioles and centrosomes becomes deregulated after uncoupling the centrosome cycle from the cell cycle. It would be interesting to examine whether the de novo pathway is involved in multiple centrosome replication in HU-arrested CHO cells as previously shown in the basal body formation in Chlamydomonas (Marshall et al., 2001).

Centrin distribution in the centrosome
Centrin has been used widely as a convenient marker for identifying the centrospheres of the centrosome. In HeLa cells it is almost exclusively seen in the lumen of the distal end of the centrioles (Paoletti et al., 1996). However, the specificity of centrin to the centriole varies among cell species, as the protein is also localized to the PCM (Paoletti et al., 1996; White et al., 2000) and to centriolar satellites (Baron and Salisbury, 1988) in other cell types. In CHO cells we detected GFP-centrin in both the centriole and PCM (Fig. 1). Because centrins show highly complicated gene organization and protein expression patterns (Le Dizet et al., 1998; Hart et al., 1999; Laoukili et al., 2000; Gavet et al., 2003), it is reasonable that the distribution and behavior of centrin proteins may vary among cell types under various conditions.

Centrosomal proteins localize to distinctive subdomains of the centriole and PCM (Bornens, 2002). Indeed, centrin does not colocalize exactly with other molecules at the centrosome (Fig. 2). This is particularly noticeable in cells actively undergoing centrosome replication (supplementary material Figs S1 and S2), suggesting that molecules become recruited to the centrosome through different pathways (Maekawa and Kuriyama, 1991). Depending upon the probe used for visualization of the centrosome, cells may reveal distinctive patterns of centrosome replication in HU-arrested CHO cells. Thus, it is interesting to compare the process of centrosome formation as monitored by GFP-centrin with other centrosomal markers.

Centrin-containing cytoplasmic foci and their movement
Centrin forms cytoplasmic foci that vary in size and number in HU-arrested CHO cells. Because the protein has been shown to be essential for duplication of mammalian centrioles (Salisbury et al., 2002), these cytoplasmic sites are likely to be involved in centriogenesis. Indeed, La Terra et al. have noted the presence of similar foci in HeLa cells that develop into individual centrioles during de novo centrosome assembly (La Terra et al., 2005). Unlike CHO cells where the centrin-containing foci continuously emerge in the cytoplasm, the number of ‘precentrioles’ identified in HU-arrested HeLa cells does not increase once they appear in the cytoplasm (La Terra et al., 2005). Thus, it is not known if the small, centrin-containing foci in CHO cells are identical to ‘precentrioles’ detected in HeLa cells during de novo assembly of the centrosome.
In cycling PtK2 cells, Baron and Salisbury provided unequivocal electron microscopy evidence that centrin is a component of the centriolar satellite (Baron and Salisbury, 1988). Like the centrin-containing foci analyzed in this report, the satellites are dynamic, actively changing their number and distribution during the cell cycle (Baron et al., 1994). Centriolar satellites are also associated with PCM1 (Kubo et al., 1999). During ciliogenesis, ciliated epithelial cells in the oviduct and respiratory organs induce numerous centrioles and/or basal bodies, which are preceded by the formation of fibrous electron-dense granules that are ~70-100 nm in diameter (Sorokin, 1968; Anderson and Brenner, 1971; Dirksen, 1971). Interestingly, these fibrous granules are associated with centrin and PCM1, and morphologically, closely resemble centriolar satellites (Kubo et al., 1999; Laoukili et al., 2000). These results strongly suggest that there may be a similarity between the centriolar satellites and centrin-containing cytoplasmic foci in centriole/centrosome biogenesis.

Time-lapse analysis showed that the centrin-containing foci are highly motile. Because other centrosome proteins, such as pericentrin (Young et al., 2000), Nek2 (Hames et al., 2005) and PCM1 (Kubo et al., 1999), move in a dynein-dependent manner, the movement seems to be universal among molecules constituting the centrosomal structure. The motion pattern is remarkably similar among different molecules; the proteins do not move in a uniform pattern, rather they make a sudden stop and resumption of their movement, and frequently change the speed and direction. Because it is sensitive to not only nocodazole but also a microtubule-stabilizing reagent of taxol, the movement is likely to be dependent on microtubule dynamics. PCM1 has been reported to be responsible for the trafficking of other centrosomal proteins, including centrin, Nek2, pericentrin, ninein and C-Nap1 (Dammermann and Merdes, 2002; Hames et al., 2005). Therefore, one possibility is that the components required for assembly of new centrioles/centrosomes are transported towards the cell center along microtubule tracks through the PCM1-containing structure. In cells undergoing the template type of centrosome duplication, a microtubule-organizing center of the centrosomes is already present at the center of the microtubule aster. In the case of the de novo pathway, the cells must first establish an initial site(s) for microtubule polymerization. Successive recruitment of additional molecules stimulates the microtubule-nucleating activity of the site, thus facilitating the accumulation of more building blocks necessary for new centrosome construction. Further analysis of centrin-containing foci will be important to advance our understanding of the mechanism and regulation of centriole/centrosome biogenesis.

Materials and Methods

Cell culture and synchronization

GFP-centrin-expressing cells were prepared by transfection of CHO cells with a plasmid encoding GFP-tagged human centrin2 (White et al., 2000) (a gift of J. Salisbury, Mayo Clinic, Rochester, MN) using Lipofectamine transfection reagent (Invitrogen, Carlsbad, CA). Positive colonies screened by fluorescence microscopy were picked up with cloning rings (Labcor Products, Frederick, MD), and further subjected to multiple subcloning cycles. Cells derived from established cell lines resistant to G418 at a final concentration of 400 μg/ml revealed fundamentally identical expression patterns of GFP-centrin at the centrosome.

Cells were cultured in Ham’s F-10 medium containing 10% fetal bovine serum and synchronization was done according to the procedure reported previously (Ohta et al., 2002). Round mitotic cells were harvested by gentle shaking and washed once with fresh medium before plating on a dish. For time-lapse recording, cells were cultured in the medium lacking NaHCO3.

Immunofluorescence staining

Cells treated with or without 2 mM HU were fixed with methanol for 5 minutes at ~20°C. After rehydration with 0.05% Tween-20 containing phosphate-buffered saline (PBS), cells were immunostained using the following primary antibodies: monoclonal anti-γ-tubulin antibody (Sigma-Aldrich, St Louis, MO), rabbit polyclonal anti-Cep135 (Ohta et al., 2002), anti-pericentrin (Covance, Berkeley, CA), anti-hCenexin (Young et al., 2006), anti-ninein (a gift of J. B. Rattner, University of Calgary, Canada), anti-Nek2 (Abcam, Cambridge, MA) and anti-PCM1 (a gift of R. Balczon, University of South Alabama, Mobile, AL) antibodies. After incubation for 30 minutes at 37°C, cells were washed and further treated with secondary antibodies (fluorescein-conjugated anti-mouse IgG plus IgM and Texas Red-conjugated anti-rabbit IgG antibodies; Jackson ImmunoResearch, West Grove, PA). Microscopic observation was made on a Nikon Eclipse microscope with a 100× oil immersion objective (N.A. 1.4) using ImagePro Plus software. Projections of deconvoluted images were acquired using an Olympus microscope and a 100× 1.35 NA oil immersion objective with the DeltaVision program.

Live cell observations

CHO cells expressing GFP-centrin treated with 2 mM HU at 0 hours and placed on a microscopic stage prewarmed to 37°C. To monitor the process of centrosome replication, time-lapse fluorescence images were taken every 2-3 hours using an Olympus microscope and a 40× 1.0 NA oil immersion objective. A motorized XY stage allowed the concurrent filming of 20-30 fields of cells in the same dish, and projection images were obtained by acquiring centrosomal images in different focal planes. Movement of the centrosomes and centrin-containing foci was recorded by capturing images every 5 seconds. Acquired images were processed through the DeltaVision program to make Media Player or QuickTime movies.

Time-course analysis of centrosome replication in fixed cells

Harvested mitotic cells were placed in a six-well plate containing 2 mM HU at 0 hours and placed on a microscopic stage prewarmed to 37°C. To monitor the process of centrosome replication, time-lapse fluorescence images were taken every 2-3 hours using an Olympus microscope and a 40× 1.0 NA oil immersion objective. A motorized XY stage allowed the concurrent filming of 20-30 fields of cells in the same dish, and projection images were obtained by acquiring centrosomal images in different focal planes. Movement of the centrosomes and centrin-containing foci was recorded by capturing images every 5 seconds. Acquired images were processed through the DeltaVision program to make Media Player or QuickTime movies.

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