Mode of Centriole Duplication and Distribution

Rex S. Kochanski and Gary G. Borisy

Laboratory of Molecular Biology, University of Wisconsin, Madison, Wisconsin 53706

Abstract. Centriole stability and distribution during the mammalian cell cycle was studied by microinjecting biotinylated tubulin into early G1 cells and analyzing the pattern of incorporation into centrioles. Cells were extracted and cold treated to depolymerize labile microtubules, allowing the fluorescent microscopic visualization of the stable centrioles. The ability to detect single centrioles was confirmed by use of correlative electron microscopy. Indirect hapten and immunofluorescent labeling of biotinylated and total tubulin permitted us to distinguish newly formed from preexisting centrioles. Daughter centrioles incorporated biotinylated tubulin, and at mitosis each cell received a centrosome containing one new and one old centriole. We conclude that in each cell cycle tubulin incorporation into centrioles is conservative, and centriole distribution is semiconservative.

How are centrioles duplicated and then distributed to daughter cells? Previously, the centriole cycle had been studied by electron microscopy of cells fixed at successive stages of the cell cycle (Robbins et al., 1968; Rieder and Borisy, 1982; Vorobyev and Chentsov, 1982), or from analysis of centrioles isolated from populations of synchronized cells (Kuriyama and Borisy, 1981). Centrioles were identified by their characteristic array of nine triplet microtubules.

The centriole cycle was inferred to consist of steps of disorientation, elongation, and separation (Fig. 1). A pair of centrioles begins the cycle in orthogonal configuration. They lose their orthogonal relationship (disorientation) in early G1 phase. The resultant single centrioles are then termed parental for the next generation. Procentrioles are nucleated orthogonal to the parental centrioles near S phase, and then elongate to full-length (daughter centrioles), normally by late G2 or early G1 of the next cell cycle. The centriole pairs migrate in prophase to opposite sides of the nucleus as the spindle forms. During mitosis, the centriole pairs are distributed to the daughter cells and the cycle is completed.

The morphological relationships of the centriole cycle strongly suggest that the parent is conserved and that the daughter is newly formed, but micrographs of fixed cells provide only static images of a dynamic process. Recently, the concept of a rigid cytoskeleton, derived from such micrographs, has been dramatically modified. Continuous turnover of tubulin has been shown to occur in the microtubule "cytoskeletal" network in living cells (Saxton et al., 1984; Sollys and Borisy, 1985; Schulze and Kirschner, 1986). Did the concept of the stable centriole need to be similarly revised?

Centrioles double in number each typical animal cell cycle and so their number remains stable. However, they sometimes arise de novo, without a visible precursor (reviewed by Wheatley, 1982; Vorobyev and Nadezhdina, 1987), indicating that their formation does not require a preexisting mature centriole. Nevertheless, as expressed in a recent review (Goodenough, 1989) "the notion has persisted that centrioles have something autonomous about them." This notion has received support from the recent report that the centrioles (basal bodies) of the algae Chlamydomonas are in close association with a unique chromosome encoding genes affecting basal body structure and function (Hall et al., 1989; see Goodenough, 1989, for review).

Whereas the mode of DNA replication has long been understood, no similar molecular understanding is available for the centriole. Centriole doubling and DNA replication are superficially similar. Both centrioles and DNA strands come in pairs, are doubled precisely, and are distributed equally to daughter cells.

However, centrioles are complex and are not complementary in structure as are the strands of a DNA duplex. The members of a centriole pair split apart before duplication, so the unit of duplication is the individual centriole. In contrast, the unit involved in distribution is the centriole pair.

The replication of the DNA duplex could, a priori, have been conservative, semiconservative, or nonconservative. The pattern of labeled subunit (nucleotide) incorporation during replication established the semiconservative mode (Meselson and Stahl, 1958).

We deemed it important to obtain similarly direct molecular evidence for the stability and distribution of centrioles. Since tubulin is the principal structural component of the centriole, our approach was to microinject biotinylated tubulin into living cells before duplication and to determine the pattern of incorporation into centrioles. Centrioles or centriole pairs were identified at the light microscopic level as fluorescent dots and the identification was confirmed by electron microscopy of the same cells.
The locater pattern permitted the relocation of the injected cells at a later phase cells. Early G1 cells were identified as two small cells connected by microinjection continued in culture for 1 h to obtain a population enriched in early G1.

**Materials and Methods**

**Cell Culture**

Porcine kidney epithelial cells of the line LLC-PK (American Type Culture Collection, Rockville, MD), were cultured in DME (Gibco, Grand Island, NY) with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 20 mM HEPES, and antibiotics. Cells were grown to 25% confluence on cover-slips prepared with a carbon locater pattern, generated by evaporating carbon through an electron microscope grid. New centrioles incorporated biotinylated tubulin and there was no detectable labeling of old centrioles, which remained stable for at least one cell cycle. Each pole received one new and one old centriole. We conclude that tubulin incorporation into centrioles is conservative, and centriole pair distribution is semiconservative, as previous interpretations based on electron microscopy had suggested.

**Preparation of Biotinylated Tubulin**

Microtubule protein was purified from porcine brain by cycles of assembly and disassembly (Borisov et al., 1975), separated from microtubule associated protein by DEAE Sephadex chromatography (Vallee and Borisy, 1978), and then conjugated by the addition of N-hydroxysuccinimidy biotin (Kristofferson et al., 1986). After two assembly-disassembly cycles using 5% (vol/vol) dimethyl sulfoxide (Himes et al., 1976), the protein was distributed into 10-μl aliquots at 4 mg/ml, and drop-frozen and stored in liquid nitrogen.

**Microinjection**

Cells were first accumulated in mitosis by addition of 0.1 μM nocodazole for 4 h. After rinsing three times in nocodazole-free medium, they were continued in culture for 1 h to obtain a population enriched in early G1 phase cells. Early G1 cells were identified as two small cells connected by a midbody.

Microinjection was carried out according to general protocols previously reviewed (Kreis and Birchmeier, 1982). One member of each pair of early G1 cells was injected at positions recorded on a map of the locater pattern. The locater pattern permitted the relocation of the injected cells at a later time. The average LLC-PK cell cycle time was determined to be ~16 h by measuring the peak mitotic index of cultures blocked with nocodazole and then released (data not shown). This time was then chosen as the point of analysis of the pattern of incorporation after duplication and distribution. A point 3 h after release (at least 1 h after injection), was chosen as a control for any incorporation before duplication. In some 16-h experiments, cells were again accumulated in mitosis by nocodazole for 4 h. A population enriched in second generation early G1 cells was obtained by releasing the block and fixing 1 h later (21 h after the first release). This procedure had no detectable effect on the observed pattern of incorporation.

**Lysis and Fixation**

For analysis, cells were treated to depolymerize labile cytoplasmic microtubules by lysis and extraction for 10 min in an ice-cold solution of 100 mM Pipes buffer, pH 6.94, containing 1% Triton-X 100 detergent, and 50 μM CaCl2. Cells were then fixed for 10 min in 100 mM Pipes buffer (pH 6.94), containing 0.7% glutaraldehyde, and reduced by two treatments with 2 mg/ml sodium borohydride for 15 min each. Cells prepared for anticientosome staining were fixed at room temperature with 2% formaldehyde.

**Hapten and Immunolabeling**

Biotinylated and total tubulin were localized by combined hapten and indirect immunofluorescent labeling. Fixed cells on coverslips were reacted with reagents for 30 min at 37°C, followed by three 5-min rinses in PBS. Biotinylated tubulin was localized after injection with Texas Red-strepavidin (Jackson Immuno Research, Avondale, PA), at a 1:10 dilution in PBS; total tubulin was localized by a mouse monoclonal beta-tubulin antibody (Amersham Corp., Arlington Heights, IL), at a 1:100 dilution, and a fluorescein-conjugated goat anti-mouse IgG secondary antibody (Hyclone Laboratories) at a 1:25 dilution. Human anticientrosomal serum 3051, used at a 1:100 dilution, and a fluorescein-conjugated goat anti-human secondary antibody, used at a 1:20 dilution, was provided by Dr. Gerald Schatten (University of Wisconsin). All dilutions were made with PBS containing 5% goat serum. Coverslips were mounted on microscope slides in a polyvinyl alcohol-based medium containing the anti-bleaching agent para-phenylenediamine (Sammak et al., 1987). Injected cells were relocated by use of the carbon locater pattern, observed with a Zeiss Universal microscope equipped with epifluorescence optics, photographed with hypersensitized Tech Pan 2415 film (Smith, 1986), and developed in Kodak D19 for 6 min.

**Correlated Light and Electron Microscopy**

Coverslips containing selected cells were demounted (using mountant minus polyvinyl alcohol), dehydrated in ethanol, and flat-embedded in Epon blocks several mm thick. At this thickness, minimally distorted phase micrographs could still be taken to relocate the cells of interest. The coverslip was separated from the block by rapid freezing in liquid nitrogen, and the position of the cells was carefully monitored as the blocks were trimmed and mounted for serial thin sectioning. A Reichert ultramicrotome was used to cut 0.1-μm sections, which were then stained with uranyl acetate and lead chloride, and examined with a Phillips 300 transmission electron microscope. The centrioles were often located within the first few sections from the bottom of the cell.

**Results**

**Means of Analysis**

Isolated centriole pairs have been detected as paired dots by antitubulin immunofluorescence (Bornens et al., 1987). However, in situ, the numerous microtubules of the cytoskeleton pose a major obstacle to the visualization of the centrioles. The focus of the microtubule array is generally taken to indicate the location of the centrosome, and occasionally a bright dot can be seen at the focus (Aubin et al., 1980). Yet, in many cells, no obvious focus is present, and the location of the centrioles is effectively obscured.

Visualization of the centrioles could be routinely enhanced by selectively removing the cytoplasmic microtubules (Fig. 2). Centriole microtubules remained stable when subjected to conditions that caused most or all cytoplasmic microtubules...
bules to depolymerize, including the presence of drugs like nocodazole, the presence of calcium ions, or cold treatment. We decided to use a combination of cold and CaCl₂ to reveal centrioles without physically isolating them from the cell.

Bright dots, near the nucleus or at spindle poles, were revealed by antitubulin staining after cold treatment of uninjected cells. By position, size, and number the dots were candidates for centriole pairs. For example, in late prophase cells two widely separated perinuclear dots were frequently observed (Fig. 2 b). When Texas Red-streptavidin was applied to the same uninjected cells, a faint nonspecific signal corresponding to the location of the perinuclear dots was detectable but was judged to be acceptably low (Fig. 2 c).

Biotinylated tubulin was injected into early G₁ cells, identified by the presence of a midbody connecting the two newly formed daughter cells. The number of these cells was enriched by mitotic block followed by release. The procedure had no detectable effect upon the results, as judged by control injection of unsynchronized early G₁ cells (data not shown). After injection, cells were given time, up to one cell cycle, to incorporate the biotinylated tubulin. The labeled tubulin was eventually localized by staining with Texas Red-streptavidin, and compared with the total tubulin signal. A short period, 1–2 h, during which the cells were still in G₁, served as a negative control for our ability to detect specific incorporation of biotinylated tubulin into centrioles.

Cytoplasmic microtubules visualized 1 or 2 h after microinjection in cells not subjected to cold treatment were largely biotinylated (data not shown), indicating that most had turned over. This result was consistent with reports in other cell types (Schulze and Kirschner, 1986; Sammak et al., 1987; Webster et al., 1987). However, there was no significant incorporation of biotinylated tubulin into the perinuclear dots observed in cold treated cells (compare Fig. 2, d and e). This result is consistent with studies (Kuriyama and Borisy, 1981; Vorobyev and Chentsov, 1982) that show that nucleation of the daughter centriole does not occur until near the beginning of S phase. The low background level signal from the

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**Figure 2. Controls: microtubule depolymerization reveals perinuclear dots.** The effect of treatment to depolymerize cytoplasmic microtubules is illustrated by comparing two uninjected cells, (a) untreated, (b and c) treated. One injected cell (d and e), treated and fixed before centriole duplication, controls for incorporation unlinked to duplication. One uninjected cell (f), treated and then indirectly immunostained for human anticentrosomal serum controls for the extent of the pericentriolar material. (a) This prophase cell was fixed in glutaraldehyde after extraction at 37°C for 60 s in 0.15% Triton-X 100, then visualized by antitubulin indirect immunofluorescence. A complete cytoplasmic microtubule network, with two centrosomal foci, is present. Centrioles cannot be identified due to the density of microtubules at the foci. (b and c) This cell was glutaraldehyde-fixed after extraction at 0°C for 10 min in 1% Triton-X 100 in buffer containing 50 μM CaCl₂. (b) Antitubulin immunofluorescence shows that a few cytoplasmic microtubules still remain, principally over the nuclear region. The two bright perinuclear dots (arrows) correspond to two centriole pairs. (c) Same uninjected cell reacted with Texas Red-streptavidin to determine the background level. The perinuclear dots (arrows) are barely detectable. (d and e) Injected G₁ phase cell fixed 2 h postinjection. Two perinuclear dots are clearly present (arrows) in the total tubulin channel (d) that are not detectable (arrows) over background in (e) the biotinylated tubulin channel. No cytoplasmic microtubules remained in this cell. (f) Anticentrosomal staining of a prophase cell shows that the size of the centrosomes (arrowheads) is much greater than the size of the perinuclear tubulin dots (compare with b and d). The nucleolar and chromatin fluorescence observed in the cells is an artifact of glutaraldehyde fixation. Bar, 5 μm.
The centrosome of cells injected and fixed before duplication, which we estimated to be less than the brightness of a single biotinylated microtubule, indicated that little or no soluble tubulin remained bound to pericentriolar material after the CaCl2/cold treatment. As additional evidence, the size of the pericentriolar cloud as visualized with human ant centrosomal serum 5051 (Calcaro-Gillam et al., 1983) was much greater than the size of the tubulin dots (Fig. 2f, compare with b).

Through use of a locater pattern (see Materials and Methods), injected cells were readily relocated after a generation time in culture. At 15 h after injection (16 h after release from nocodazole) about half the cells had divided (data not shown). This result demonstrated that incorporation of the biotinylated tubulin did not inhibit progress through the cell cycle. Thus, we were now in a position to evaluate specific incorporation of biotinylated tubulin into centrioles.

Pattern of Incorporation

Cells injected in G1 and fixed 15 h later were caught in various stages of mitosis or near mitosis. The pattern of tubulin incorporation and centriole distribution was analyzed in all cases where separation of centrosomes (centriole pairs) had occurred. This included stages from late G2 of the first cell cycle after injection to early G1 of the next cell cycle. Cells were classified according to the number of total tubulin dots observed at the centrosome (Fig. 3).

In most instances (167/201), the two centrioles of a pair were not resolved and a single total tubulin dot was seen for each centrosome (Fig. 4a). In all of these cases, each centrosome also contained a single biotinylated dot (Fig. 4b). This result clearly demonstrates incorporation of biotinylated tubulin into centrioles.

In the remaining cells analyzed (34/201), individual centrioles were resolved in at least one of the centrosomes. Commonly (26/201), this was due to the splitting apart of centrioles (disorientation) which characteristically occurs in early G1. The centrioles of each pair are then frequently separated by several micrometers, and are easily resolved in the light microscope (Fig. 5a, also see Bré et al., 1987). Only one biotinylated tubulin dot per centrosome was observed (compare Fig. 5a and b). We estimated that the signal from a biotinylated dot was over an order of magnitude brighter than that of a single biotinylated microtubule, consistent with a structure containing nine triplet microtubules.

We frequently observed that in each split centrosome, one

![Figure 4. Label incorporation into polar dots after duplication. Late anaphase cell treated to depolymerize cytoplasmic microtubules, fixed 15 h after injection, indirectly labeled for total and biotinylated tubulin, showing label incorporation. Two bright dots (arrows), one at each spindle pole, visible in a, the total tubulin channel, are also visible in b, the biotinylated tubulin channel. A single dot corresponds to a centriole pair. A few cytoplasmic microtubules are still detectable, in both channels. Chromatin fluorescence is a fixation artifact. Bar, 5 μm.](image)

![Figure 5. Selective label incorporation into dots assayed after centrosome (centriole pair) splitting. Early G1 cell pair, treated to depolymerize cytoplasmic microtubules and fixed 15 h post injection, indirectly labeled for total tubulin and biotinylated tubulin. (a) Two widely separated dots (arrow 1, arrow 2) are present in each cell when stained for total tubulin. (b) Only one dot (arrow 1) is detected in each cell when stained for biotinylated tubulin. The midbody remnant, with biotinylated microtubules, is indicated by M. Nucleolar fluorescence is a fixation artifact. Bar, 5 μm.](image)
total tubulin dot was brighter than the other (Fig. 5 a). The brighter dot corresponded to the one biotinylated tubulin dot (Fig. 5 b), presumably representing the newly formed daughter centriole. We interpret the dimmer dot to represent the parental centriole. The presence of pericentriolar material, primarily around the parental centriole (Rieder and Borisy, 1982; Vorobyev and Chentsov, 1982), may restrict immunolabeling, especially after fixation.

However, after centriole pair splitting, the orthogonal relationship between centrioles is lost and it is not possible to be certain which of the centrioles was the parent and which the daughter. The orthogonal relationship, by means of the axis-intercept rule (Rieder and Borisy, 1982), provides a simple way of identifying parent and daughter. According to the rule, the axis of the daughter intercepts the parent but the axis of the parent does not intercept the daughter (Fig. 1). The centrioles when in orthogonal relation are very close to each other, with a center-to-center spacing of ~0.25 μm as determined from electron micrographs. From most viewing angles, one centriole dot will overlap the other and not be resolved at the light microscopic level.

Nevertheless, two very closely spaced total tubulin dots were occasionally (8/201) observed, probably as a result of a favorable viewing angle. An example in a late G2 phase cell is shown (Fig. 6 a). The two dots per centrosome visible by antitubulin immunofluorescence strongly suggest that, in these images, each dot represents an individual centriole. Only one dot per centrosome had incorporated biotinylated tubulin (Fig. 6 b). Correlative electron microscopy of the same cell confirmed that each dot was due to a single centriole (Fig. 6 c). Moreover, it demonstrated that the centrioles were still orthogonally oriented (Fig. 6, inset). Applying the axis-intercept rule, we can see that the daughter centriole corresponds to the biotinylated tubulin dot. This result proves that the biotinylated dot is indeed the daughter centriole and that the parental centriole remained stable.

**Discussion**

Our experiments establish that nascent centrioles have the ability to incorporate biotinylated brain tubulin introduced by microinjection. We confirm the interpretations of previous studies based on electron microscopy. We conclude that new, daughter centrioles draw upon the cytoplasmic pool of tubulin subunits during their construction. The inability of old, parental centrioles to incorporate detectable amounts of biotinylated tubulin after many hours immersed in the same pool implies that they are very stable structures once formed. The stability of the parental centriole combined with a newly formed daughter leads to the conclusion that centriole pairs are distributed semiconservatively at each division.

Several qualifications to these conclusions should be discussed. These are the appropriateness of a brain tubulin to study formation of a constitutive organelle in cultured kidney cells, limits on detection of biotinylated tubulin incorporation imposed by the signal-to-noise ratio, and finally, whether incorporation of tubulin as a building block of the centriole can be taken to reflect the mode of duplication of the centriole as a whole.

Brain tubulin contains a mixture of tubulin isoforms different from those found in cultured cells (reviewed by Cleveland and Sullivan, 1985). Centrioles might have been largely constituted from isoforms not abundant in brain tubulin. Chemical modification of tubulin through biotinylation, though not interfering with the polymerization of cytoplasmic microtubules, might have interfered with some special feature of the centriole microtubules, such as triplet morphology. Thus, biotinylated brain tubulin might have been unable to incorporate into centrioles, or alternatively, it might have poisoned the construction process. We did not observe either effect, and we can thus reasonably conclude that the endogenous brain tubulin acted as a true marker for endogenous tubulin incorporation.

The relative signal-to-noise ratio of our results may be estimated from our result that a biotinylated centriole is over an order of magnitude brighter than a single biotinylated microtubule, as is consistent with a structure containing nine triplet microtubules. Thus relative brightness can be used as a measure to place an upper limit on incorporation of bi-
otinylated tubulin. Since the background noise from an un.injected centriole is similar to the brightness of a single microtubule, and the signal from a parental centriole after duplication is not detectably higher, we estimate parental centriole turnover, if it exists, can be no more than ~10% per generation.

A more fundamental limitation of these experiments is that, strictly speaking, they pertain only to tubulin incorporation and thus do not permit us to draw any definitive conclusions about the role of nontubulin components in the duplication of centrioles. Conceivably, centriole formation could be an assembly pathway wholly dictated by the interactions between its constituents. In this case, tubulin would truly act as a centriole duplication marker and it would be valid to conclude that centriole duplication was conservative. Alternatively, it is possible that the parental centriole contributes a template or seed that is essential for regulated daughter centriole formation.

Centriolar templates, if they exist, might play a functional role in "the generation of twoness in cell reproduction" (Mazia, 1978). Centriole number correlates exactly with the duplication capacity of spindle poles in most animal cells, although some cells divide without any centrioles (Debec et al., 1982; Keryer et al., 1984; Schatten et al., 1986). Centrioles can be destroyed without blocking the completion of mitosis after poles are formed (Borns and Richardson, 1977), so the functional link, if any, between centrioles and the centrosome is not direct.

Evidence suggestive of an indirect link does exist, in sea urchin eggs in which the cell and centrosome cycles have been put out of phase by drug-induced mitotic delay. Centrosomes then behave as if their duplication was actually from two to four, with two "mitotic centers" per normal pole that separate during the block to cause a tetrapolar mitosis after release (Mazia et al., 1960). In subsequent divisions the capacity to form spindle poles still correlates exactly with the number of centrioles found at each pole. This suggests that these centrioles might contain "polar organizer" information (Sluder and Rieder, 1986).

One candidate for a centriolar template and polar organizer would be a double-stranded nucleic acid. Persistent biochemical reports of nucleic acids associated with centrioles have remained controversial, lacking tests for specificity and biochemical purity (reviewed by Wheatley, 1982), but a recent study (Hall et al., 1989) has brought this possibility back to the fore (see Goodenough, 1989, for review).

Several mutants affecting basal body structure and function, the uni linkage group, have now been described in the algae Chlamydomonas (Dutcher, 1986; Ramanis and Luck, 1986). Probes recognizing uni DNA sequences have been localized to basal bodies, and are consistent with the presence of two copies per cell (Hall et al., 1989). As they note, one semiconservatively duplicated copy of uni per centriole would ensure the chromosome remained a homoygous diploid, given the semiconservative distribution of centrioles we have observed.

Whether or not the existence of centriolar DNA is confirmed, an important question remains. There is no solid evidence that centrioles are equipped with their own translation system, so any centriolar proteins, whether derived from a centriolar or nuclear genome, would presumably be translated in the cytoplasm. If so, what then would be the significance of the location of their encoded information?

Indeed, in the final analysis, a hypothetical centriolar template or seed, of whatever nature, need not encode structural components. A seed would serve a structural role or at least function as a "mailing address" with which structural components, wherever they were encoded, could interact. Further biochemical, cell biological and genetic studies will be needed to determine if centrioles possess any component that meets this requirement.

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