# A Molecular Marker for Centriole Maturation in the Mammalian Cell Cycle

**Bodo M. H. Lange and Keith Gull**

School of Biological Sciences, University of Manchester, Oxford Road, Manchester, M13 9PT, United Kingdom

***Abstract.*** The centriole pair in animals shows duplication and structural maturation at specific cell cycle points. In $G_1$, a cell has two centrioles. One of the centrioles is mature and was generated at least two cell cycles ago. The other centriole was produced in the previous cell cycle and is immature. Both centrioles then nucleate one procentriole each which subsequently elongate to full-length centrioles, usually in S or $G_2$ phase. However, the point in the cell cycle at which maturation of the immature centriole occurs is open to question. Furthermore, the molecular events underlying this process are entirely unknown. Here, using monoclonal and polyclonal antibody approaches, we describe for the first time a molecular marker which localizes exclusively to one centriole of the centriolar pair and provides biochemical evidence that the two centrioles are different. Moreover, this 96-kD protein, which we name Cenexin (derived from the Latin, *senex* for "old man," and Cenexin for centriole) defines very precisely the mature centriole of a pair and is acquired by the immature centriole at the $G_2$/M transition in prophase. Thus the acquisition of Cenexin marks the functional maturation of the centriole and may indicate a change in centriolar potential such as its ability to act as a basal body for axoneme development or as a congregating site for microtubule-organizing material.

Centrioles are a feature of eukaryotic cells that construct cilia or flagella at some time in their life cycle. They are composed of nine microtubule triplets which are interconnected by a complex pattern of radial spokes and fibers (Wheatley, 1982). Thus the centriole represents one of the most beautiful and highly ordered of cellular organelles with an almost complete conservation of structure throughout evolution. The centriole is widely believed to function in both cell division and the formation of cilia or flagella. There is good evidence to support the role of centrioles in the formation of cilia and flagella. Basal bodies of both cilia and flagella and centrioles are structurally analogous and are often interchangeable. However, the role of centrioles in cell division and other cellular events is still a matter of debate. Animal cells have two centrioles which are cylindrical structures surrounded by an amorphous pericentriolar material that acts as the microtubule organizing center of the cell.

The centriole pair is an unusual organelle in that, apart from the chromosomes, it is one of the few cell organelles which exist in a single copy. Hence, there is a precise control over centriole number, position and coordination with other cell cycle events. Recently, it has become clear that the presence of centrioles can be modulated in development as both of the meiotic reduction divisions can take place without centrioles and the poles of these spindles are less focused and more barrel shaped (Schatten et al., 1985). Centrioles are also absent from the early cleavage mitoses of the mouse and possibly other rodents and only form later in development (Calarco et al., 1972; Szöllösi et al., 1972). This suggests that although centrioles are formally not a prerequisite for mitosis/meiosis, they may play a fundamental and essential role in congression of the pericentriolar, microtubule nucleating material (Calarco-Gillam et al., 1983). Their reappearance in the embryo might therefore be linked to a developmental function of the interphase microtubule array.

The centrioles need to be duplicated in the cell cycle to provide each daughter cell with a complete pair of centrioles. The duplication of the centrioles is semiconservative with each daughter cell receiving (via the mitotic spindle poles) an old centriole which was generated in a previous cell cycle and a new centriole which was generated in the current cell cycle (Kochanski and Borisy, 1990). The centriolar duplication cycle has been studied intensively at an ultrastructural level in tissue culture cells (for a review see Vorobjev and Nadezhdina, 1987). In $G_1$, a cell has two centrioles. One of the centrioles is termed the mature centriole and was generated at least two cell cycles ago. The other centriole was produced in the previous cell cycle and is immature, as judged by a variety of structural features. Both centrioles then nucleate one procentriole each and these procentrioles subsequently elongate to full-length centrioles, usually in S or $G_2$ phase. Thus, the centriole duplication cycle must involve (within one cell cycle) the production of the two procentrioles and the maturation of the original immature centriole resulting in two pairs of centri-

Please address all correspondence to K. Gull, School of Biological Sciences, 2.205, Stopford Building, University of Manchester, Oxford Road, Manchester, M13 9PT, United Kingdom. Tel.: 44161 275 5108. Fax: 44161 275 5082.

oles, one for each daughter cell (Fig. 1). Only the mature centriole is capable of nucleating a primary cilium at its distal end. (Sorokin, 1962; Vorobjev and Chentsov, 1982; Rieder and Borisy, 1982) and carries fibrous appendages and satellites. The primary cilium is an immotile 9 + 0 cilium formed by many $G_1$ or quiescent cells in culture and is common to most cell types within the human body (Wheatley, 1982).

Beyond this classical description of centriolar cell biology it is remarkable that we have no knowledge of proteins that are uniquely localized only to this cell organelle. A characterization of the centriolar proteins has proven to be difficult because cells usually carry only one pair of centrioles, hence an extremely high cell number is required to isolate a sufficient amount of centriolar proteins for molecular analysis. A number of proteins of the pericentriolar material have been identified (for reviews see Kalt and Schliwa, 1993; Kuriyama, 1992) using autoimmune antibodies (Calarco-Gillman et al., 1983; Doxsey et al., 1994) or nonimmune sera (Gosti-Testu et al., 1986), or antibodies raised against mitotic extracts (Rao et al., 1989). Recently methods have been devised (Mitchison and Kirschner, 1984; Bornens et al., 1987; Komesli et al., 1989) which produce a biochemically significant amount of centriolar preparations and which can now be used as antigens for the direct production of monoclonal and polyclonal antibodies.

We wished to study the synthesis of centriolar components and subsequent centriolar ontogeny in the cell cycle and so have focused our attention on obtaining immunological probes that are very specific to the centriole. This direct approach has allowed us for the first time to define a centriolar protein that is located to only one of the two centrioles in a mammalian cell and whose addition to a centriole provides the first molecular evidence of a defined centriolar maturation point in the cell cycle. Moreover, this result emphasizes the important difference between the centriole duplication cycle and the event of functional centriole maturation in the cell cycle.

## Materials and Methods

### Reagents

Chemicals were obtained from Sigma Chemical Ltd. (Dorset, UK) unless otherwise stated.

### Isolation of Centrioles

Centrioles were isolated from lamb thymus tissue as described for calf thymus by Komesli et al. (1989). Essentially, this procedure isolates a centrosomal complex. However, in thymocytes there is only a small amount of pericentriolar material surrounding the centriole pair.

### Production of Monoclonal and Polyclonal Antibodies

Monoclonal antibodies were raised according to Galfre et al. (1977) and Birkett et al. (1985) using centriolar preparations as the immunogen and screening of centriolar preparations. Rat polyclonal antibodies were raised by separating centriolar proteins by SDS-PAGE and Western blotting. Nitrocellulose strips with centriolar proteins of different molecular weight ranges were generated, powdered, and injected into six rats. Four different rat polyclonal antibodies were produced. The antibody RAC2 which is used here was raised against centriolar proteins of a molecular weight range between 70–100 kD. The other monoclonal antibodies used were an antibody against acetylated α-tubulin (C3B9) and a general anti–α-tubulin antibody (TAT1) described by Woods et al. (1989).

### Immunofluorescence Screening Assay

A novel screening assay was developed which allows the screening of more than one hundred hybridoma supernatants per day on isolated centrioles. Hybridoma tissue culture supernatants were screened by immunofluorescence microscopy on crude centriolar preparations which had been settled onto poly-L-lysine–coated multiwell slides (ICN Flow, Irvine, UK). The assay facilitated the detection of antibodies staining centriolar or subcentriolar structures because large centriolar aggregates enhanced the staining of these tiny structures.

### Immunofluorescence Microscopy

Indirect immunofluorescence microscopy was performed essentially as described by MacRae et al. (1990). Care was taken to avoid staining artefacts as described by Melan and Sluder (1992). Staining controls were performed with no first, no secondary, and a nonrelevant monoclonal antibody. The staining was performed on a wide range of mammalian cell types using primary and established cell lines (human, mouse, rat, monkey, rat kangaroo, sheep, i.e., HeLa, 3T3, rat primary fibroblasts, Vero, PtK2, and fetal lamb muscle cells). A higher incidence of primary cilia were induced in 3T3 cells by serum deprivation according to Tucker et al. (1979). Vero cells were incubated for 1 h on ice to depolymerize all cytoplasmic microtubules, and then cells were briefly extracted with 0.75% Triton X-100, 5 mM Pipes, 2 mM EGTA (pH 6.7) to remove all soluble tubulin from the cytoplasm. This method reveals the centriolar pair stained with an anti-tubulin antibody.

A double-labeling "sandwich"-technique was developed to facilitate the labeling with two murine monoclonal antibodies: double labeling was performed using either two mouse derived monoclonal antibodies or a mouse derived monoclonal and a rat polyclonal. Cells were fixed in –20°C methanol, rehydrated, incubated with the first antibody for 45 min, washed, incubated with a secondary anti–mouse FITC-conjugated antibody (DAKO, Bucks, UK), washed. Free-binding sites of the secondary antibody were then blocked for 45 min with a nonrelevant monoclonal antibody washed, incubated with a mouse monoclonal antibody against tubulin, washed, incubated with an anti–mouse Texas red–conjugated antibody (Jackson Immunochemicals, Stratech, Luton, UK), and mounted. The samples were observed with an Zeiss Axioskop microscope equipped with filter sets for fluorescein, Texas red, and DAPI. All incubation steps were performed at 30°C. Photographs were taken on Ilford XP2-400 film at a camera setting of ASA 800.

### High Resolution Video Microscopy and Confocal Microscopy

A high resolution immunofluorescence microscopy technique was used to investigate the precise relationship of the structure stained by the CDIB4 antibody and the centriolar pair or primary cilium. Images were recorded using a SIT camera (Hamamatsu, Enfield, UK) and a 4 × TV tube using wedge-free filter sets (Zeiss) allowing image merging without an optical shift. Images were recorded and merged using the Fluovision imaging system (Improvision, Coventry, UK) running on a Macintosh Quadra 950 computer. An MRC600 (Biorad, Cambridge, UK) microscope was initially used to analyze the results of the double-labeling experiments equipped for the fluorescent stains fluorescein and Texas red.

### Electron Microscopy

Immunogold labeling and negative staining was performed using a Philips 400 microscope according to Sherwin and Gull (1989*a*, *b*) after centrioles were spun down onto Formvar and carbon-coated EM grids in an EM-90 rotor in a Beckman airfuge at 7.5 psi for 15 min (Mitchison and Kirschner, 1984). A secondary anti-mouse antibody was used coupled to 10-nm gold particles (Sigma). Grids were negatively stained with aurothioglucose. We tested many different stains and have found this to be especially suitable for staining centrioles.

In situ immunogold labeling with the CDIB4 antibody was performed in solution on isolated thymocyte cells. Lamb thymocytes were produced as described by Komesli et al. (1989). Cells then were fixed and lysed in a 0.1 M cacodylate buffer (pH 7.2) containing 4% paraformaldehyde and 0.3 % Triton X-100 (Sigma) for 10 min at room temperature. Free-binding

sites were blocked with 20 mM Glycine. Incubation with the first and secondary antibody was both performed separately overnight at 4°C. Washing steps in between were carried out with PBS (phosphate buffered saline), per litre double distilled water: 0.2 g KCl, 0.114 g $KH_2PO_4$, 8.0 g NaCl, 2.27 g $Na_2HPO_4 \times 12\ H_2O$, pH 7.2). Fixation and subsequent embedding was performed according to Tooze (1985).

### *Western Blotting of Centriolar Preparations*

Western blotting was performed according to Birkett et al. (1985) with the modification that the more sensitive enhanced chemiluminescence reagent (Amersham, Little Chalfont, UK) was used for the detection of centriolar proteins. Reprobing of Western blots was performed according to the manufacturer (Amersham). Controls were performed with no first antibody and with a nonrelevant monoclonal antibody.

## *Results*

### *Polyclonal and Monoclonal Antibodies to Centriolar Components*

We isolated centrioles with a small amount of pericentriolar material from lamb thymus cells (Komesli et al., 1989) and used these as an immunogen to produce a number of monoclonal and polyclonal antibodies with specificity for centriolar components. At this initial light microscope level we differentiated between antibodies staining very discrete dots (anti-centriolar) or a larger round structure (anti-centrosomal). We raised a panel of polyclonals against a series of centriolar proteins separated into different molecular weight ranges by SDS-PAGE. Such gels were blotted to nitrocellulose and distinct molecular weight ranges cut from the nitrocellulose and used as immunogens. These polyclonal antisera showed remarkable specificity for the centrioles and located them in immunofluorescence microscopy at the center of the microtubule-organizing center region (MTOC) (Fig. 2, *a* and *b*). Monoclonal antibodies were raised by injecting denatured centriole preparations and screened using a novel immunofluorescence assay (see Materials and Methods) on isolated centrioles. Among this panel of hybridomas we identified one cell line (CDIB4) producing antibodies staining a single structure which was smaller than the centriolar pair. The single dot was always located in the region of the cell's MTOC (Fig. 2, *c* and *d*) and was obviously intimately associated with the centrioles, since we also detected a "one dot" structure (Fig. 2, *e* and *f*) when we probed our purified preparations of isolated centrioles. This single dot at the center of the MTOC was detected by the CDIB4 monoclonal at very high fidelity in a wide range of mammalian cell lines (human, mouse, rat, monkey, sheep; HeLa, 3T3, rat primary fibroblasts, Vero, PtK2, and fetal lamb muscle cells). These results raised the intriguing question as to the precise identity of the structure stained by this antibody; is it the connection between the two centrioles, a novel noncentriolar single structure or one of the two centrioles?

### *The CDIB4 Monoclonal Identifies One Centriole of the Centriolar Pair*

To define unequivocally the nature of this "single dot" structure we used laser scanning confocal microscopy and a "sandwich" double-labeling technique (see Materials and Methods) using two mouse monoclonal antibodies.

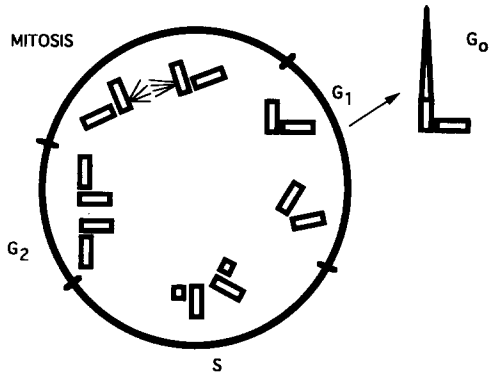


*Figure 1*. The centriolar and cell cycle. In $G_1$ the centriolar pair disorientates and loses its orthogonal configuration, in S-phase procentrioles are nucleated and during the $G_2$ period until mitosis the procentrioles elongate. In mitosis each daughter cell receives a pair of centrioles via the mitotic spindle. The mature centriole often nucleates a primary cilium in interphase cells and with increased frequency in $G_0$-cells.

Vero cells were cold treated to depolymerize all cytoplasmic microtubules after which the soluble tubulin was removed with a brief detergent wash, thereby leaving the two centrioles as the only cold stable microtubules in the cell. These cells were then double labeled with the CDIB4 antibody (FITC channel) and a monoclonal antibody (TAT1) recognizing α-tubulin (Texas red channel). The images from the two channels were recorded and then merged revealing that the CDIB4 antibody specifically detects only one centriole of the centriolar pair (Fig. 3). We confirmed that the antigen detected by the CDIB4 monoclonal is located to only one of the two centrioles by immunogold-labeling of whole mount isolated centriole pairs (Fig. 4 *b*) and labeling of fixed and permeabilized thymocyte cells using transmission electron microscopy and thin section techniques (Fig. 4, *c–f*). The whole mount technique allows reasonable access of gold labelled antibodies to the three-dimensional structure of the centriolar pair. The labeling is always very specific but there are few gold particles reflecting the unabundant nature of the antigen. The thin section technique produces two-dimensional sections hence the limited number of gold particles seen. Again this very different labeling technique shows the high specificity of antibody localization. Moreover it appears that the protein detected by the CDIB4 antibody localizes close to the centriolar cylinder and is concentrated towards the distal end of one centriole.

### *A 96-kD Protein ("Cenexin") Is Identified on Western Blots in Purified Centriolar Preparations*

We used the highly purified centriolar preparations to define the nature of the antigen detected by the CDIB4 monoclonal. We banded the centrioles in a sucrose density gradient and confirmed their position by assaying gradient fractions using immunofluorescence and electron microscopy and by Western blotting of protein fractions with the monoclonal antibody TAT1 which is specific for the major centriolar protein component, tubulin. These three assays gave unequivocal evidence for the presence of centrioles in fractions 6 to 8 (Fig. 5, *right hand panel*). In the same

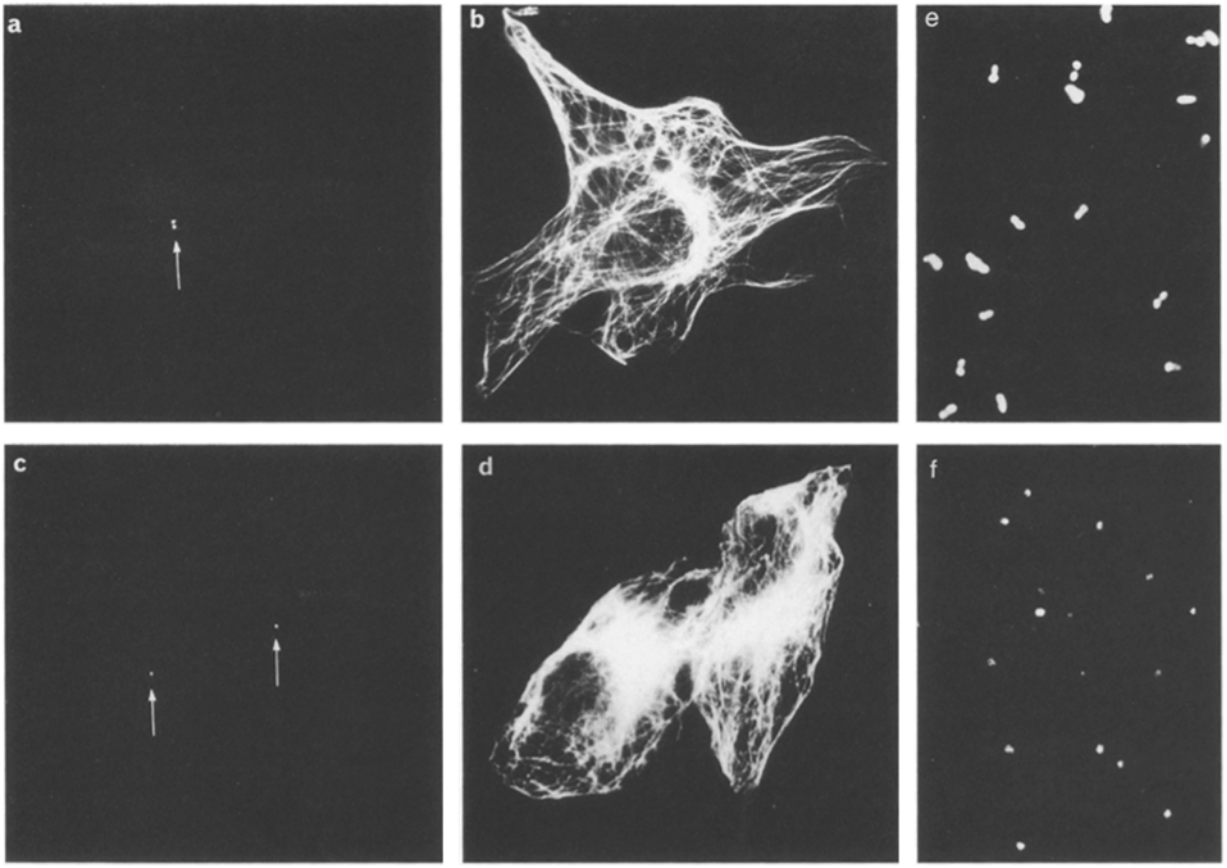


*Figure 2.* (*a* and *c*) Immunofluorescence double labeling showing cells stained with anti-centriolar antibodies (RAC2 and CDIB4) and an antibody against tubulin (TAT1) ( *b* and *d*). (*a*) Vero cell stained with the rat polyclonal antibody RAC2 showing the centriolar pair; (*b*) the same cell stained with an anti-tubulin antibody revealing the array of interphase microtubules; (*c*) two Vero cells stained with the mouse monoclonal antibody CDIB4 revealing a structure smaller than two centrioles; (*d*) the same cells stained with an anti-tubulin antibody; (*e*) a purified centriolar preparation stained with the anti-tubulin antibody showing centriolar pairs; (*f*) centriolar preparation stained with the CDIB4 antibody showing the staining of single centrioles.

centriole fractions the CDIB4 monoclonal detected a single 96-kD protein (Fig. 5, *left hand panel*). We have termed this protein "Cenexin" (derived from the Latin, senex for old man, and Cenexin for centriole, given that the protein is only located to the old "mature" centriole). Western blots of whole cell extracts did not allow detection of cenexin, confirming the high specificity of the CDIB4 monoclonal seen in immunofluorescence. Detection of the cenexin protein only in the purified centriole preparations correlates with the observed low abundance, centriole specific location.

### *A Marker for the Mature Centriole of the Pair*

Having established that the epitope identified by the CDIB4 antibody is located to only one centriole by immunofluorescence microscopy and electron microscopy, we next addressed whether this marker could be ascribed to a particular centriole of the pair. In many mammalian cells the mature centriole of the pair can be recognized at certain points in the cell cycle since it carries an extension, the primary cilium (Sorokin, 1962) (Fig. 1). This is an immotile cilium which lacks the normal central microtubule pair usually found in an axoneme. One of our antibodies, C3B9 (Woods et al., 1989), specifically recognizes acetylated tubulin and in Swiss 3T3 cells this posttranslationally modified form of α-tubulin is located in a small subset of cytoplasmic microtubules and in the centriole pair and primary cilium. We have used this primary cilium as a marker of the mature centriole in double-label immunofluorescence microscopy with the CDIB4 monoclonal to ask whether cenexin is located to the mature or immature centriole of the pair. Using laser scanning microscopy and high resolution fluorescence video microscopy we were able to show that cenexin is located at the base of the primary cilium, so defining its association with only the mature centriole of the pair (Fig. 6, *d–i*). This specific detection of the single mature centriole contrasts with other antibodies in our panel of polyclonals, which allow detection of both the mature and immature centrioles. The staining with one of the polyclonals (RAC2) is shown in Fig. 6 , *a–c*.

We provide several lines of evidence that the antibody stains the mature centriole even when it is not forming a cilium and it also stains a single centriole in cells which do not possess primary cilia. First, we show that cenexin is present in established cell lines which do not possess primary cilia, e.g., HeLa cells (Wheatley, 1982). Second, we also show staining of the poles of mitotic cultured cells which never have cilia (Fig. 7 *g*).

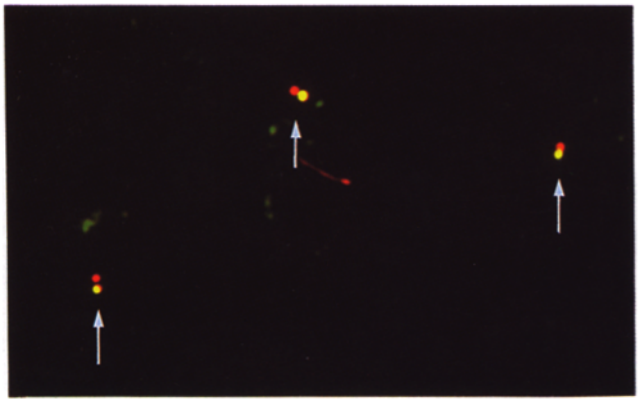

*Figure 3.* Location of the structure stained with the CDIB4 antibody in relationship to the centriolar pair. Laser scanning confocal immunofluorescence microscopy was used to identify the exact position of the structure stained with CDIB4 to the centriolar pair because it allows the merging of the fluorescein and Texas red channel without mechanical or optical shift. Laser scanning immunofluorescence microscopy showing a merged picture of Vero cells double stained with CDIB4, in the fluorescein channel (*green*, pseudocolor) and an antibody against α-tubulin, TAT1, in the Texas red channel, (*red*, pseudocolor). The cytoplasmic microtubules have been depolymerized to visualize the centriolar pairs more clearly. Pairs of centrioles in three cells are visible. The anti-tubulin antibody labels both centrioles of a pair red, the CDIB4 antibody labels one centriole of the pair green. Hence, these double-labeled centrioles appear yellow in this merged micrograph (*red* and *green*).

### *Cenexin Defines Centriolar Maturation in the Cell Cycle*

Since cenexin provides the first molecular marker for the mature centriole we asked when in the cell cycle does a centriole acquire this protein? Using the configuration of the nuclear DNA stained with the DNA intercalating dye DAPI (4,6-Diamidino-2-phenylindole) as a marker for the cell cycle, we investigated the appearance of cenexin with the immature centriole by immunofluorescence. This study revealed that all interphase cells which have an homogeneous DAPI stained nucleus have the "one-dot" pattern (Fig. 7, *a* and *b*), i.e., they exhibit a single cenexin positive mature centriole. Cells only start to express "two dots" (Fig. 7 *c*) in early prophase when chromosomes begin to condense (Fig. 7 *d*). At this early prophase stage the second cenexin positive centriole appears less intense than the other. The staining of both centrioles becomes equally strong (Fig. 7 *e*) when cells progress further into prophase (Fig. 7 *f*). At this stage both centriole pairs have migrated quite a distance apart as the cells prepare for mitotic spindle formation. Finally, in late anaphase chromosomes have separated (Fig. 7 *h*) and cells display only a single dot at each spindle pole (Fig. 7 *g*). Thus each centriole pair at a mitotic spindle pole possesses one cenexin positive mature centriole. Although centriole duplication and other events occur during interphase it is not until the $G_2$/M transition that a second cenexin positive centriole appears (see Fig. 8). Thus we have identified for the first time a centriolar protein that is located to only one of the two centrioles in a mammalian cell and whose addition to a centriole provides the first molecular evidence for a specific centriolar maturation point in the cell cycle.

## *Discussion*

Recent electron microscopy has established a centriole cycle that is characteristic of mammalian cells (for reviews see Vorobjev and Nadezhdina, 1987; Vandre and Borisy, 1989*a*). The centriole cycle is intriguing since its completion requires more than one and a half cell cycles (Rieder and Borisy, 1982; Vorobjev and Chentsov, 1982). This phasing across two cell cycles comes about because there are two centrioles within a mammalian cell of different ages which have different structural and functional properties. There are therefore two major events that occur at different points of the cell cycle to produce the centriole pair, duplication and maturation (Kuriyama and Borisy, 1981). Here we describe a molecular event which is specific for centriolar maturation in the cell cycle.

### *The Centriole Cycle*

In early $G_1$, mammalian cells possess a pair of centrioles with an orthogonal configuration. There are defined structural and functional differences between the two centrioles of the pair. One centriole possesses appendages and satellites (Rieder and Borisy, 1982; Vorobjev and Nadezhdina, 1987; Paintrand et al., 1992) and is termed "mature" in that it is capable of nucleating a primary cilium (Sorokin, 1962). The "immature" centriole neither carries these structures nor can nucleate a primary cilium. As the cell proceeds through its cell cycle, the right angle configuration is lost and centriole duplication occurs such that two new procentrioles appear, one procentriole associated with each of the original centrioles. This centriole duplication event usually occurs during S phase (Robbins et al., 1968; Vorobjev and Chentsov, 1982; Rieder and Borisy, 1982). In the latter part of the cell cycle ($G_2$) the procentrioles elongate and the two centriole pairs partition to the mitotic spindle poles. Thus each centriole pair at the mitotic pole contains a centriole that was produced in this immediately previous cell cycle and a second one that was produced at least one cell cycle earlier. This process ensures that each daughter cell receives an equal number of centrioles. However, there must be a second process of centriole maturation that ensures that both pairs are of equal potential, i.e., that each cell receives one mature and one immature centriole. This maturation of the immature centriole of the $G_1$ centriole pair could presumably take place in either S, $G_2$, M or every $G_1$-phase of the subsequent cell cycle. However, this debate is made more difficult because the morphological markers that the mature centrioles possess in interphase are either missing or change their morphology during mitosis.

### *Biochemical Evidence for the Difference between Individual Centrioles of the Pair*

Previously there has been only structural evidence for differences between the centrioles of the pair. Centriolar components have been identified, e.g., tubulin, but none of these have been rigorously identified as being unique to this organelle and none provides a marker to differentiate individual stages of the centriole cycle. This is, in the main, because the centriole is one of the least abundant organelles in the cell and does not occur in organisms such as

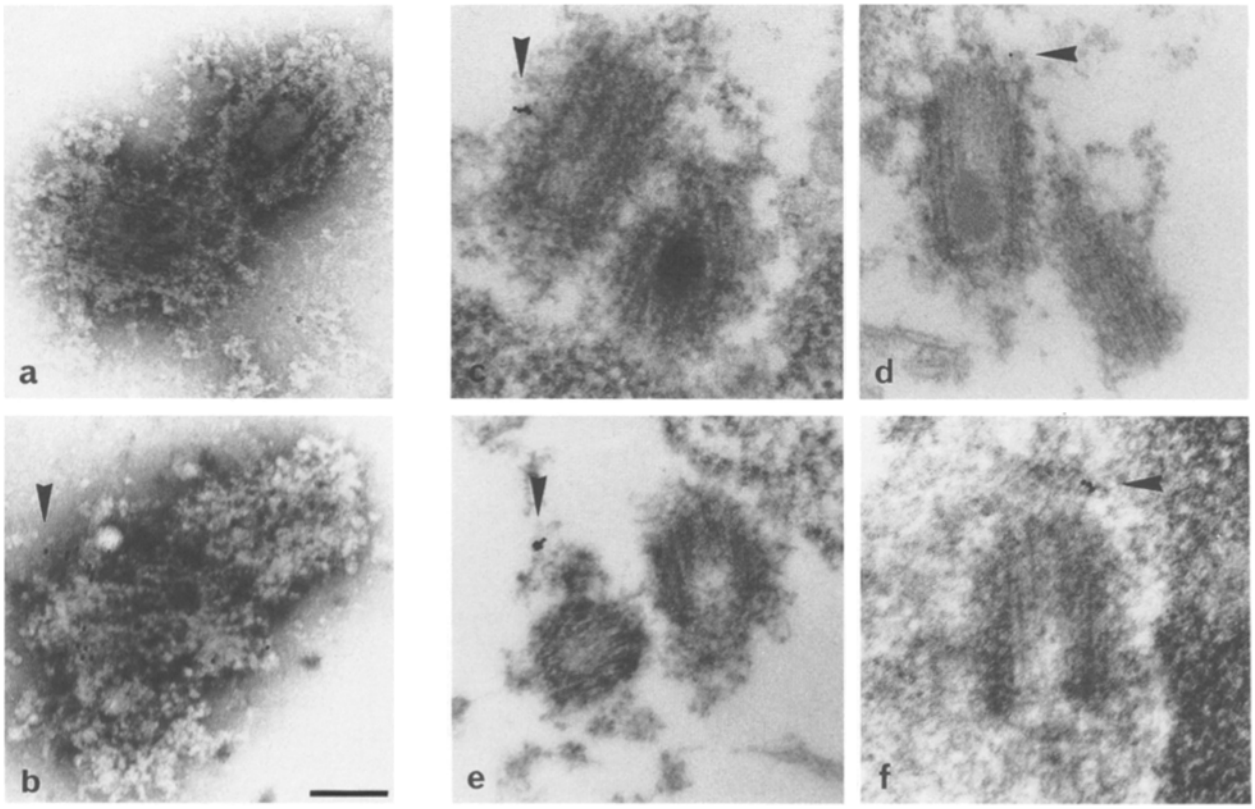


*Figure 4.* (*a*) An isolated centriolar pair negatively stained in a whole mount to show both centrioles. (*b*) Whole mount CDIB4 immunogold-labeling of an isolated negatively stained centriole pair showing the labeling of one end of one centriole. The fine ultrastructure of the isolated centriolar pairs is less defined in this micrograph than in *a* because of the inclusion of protein blocking agents for the immunolocalization. (*c–f*) Four examples of thin sections of thymocyte centrioles labeled in situ with the CDIB4 antibody and secondary 10-nm gold-labeled anti–mouse antibodies are shown . The labeling is located very specifically to only one of the two centrioles. Bar, 0.2 μm.

yeast which have good selectional genetics. Isolation and purification of organelles such as the yeast spindle pole body and their use as immunogens, have resulted in the production of specific monoclonals (Rout and Kilmartin, 1990). The use of interphase mammalian centrosomes, mitotic spindles and mitotic cell extracts has led to the production of some interesting probes (Kuriyama, 1992) but none have so far been defined as specific for the centriole.

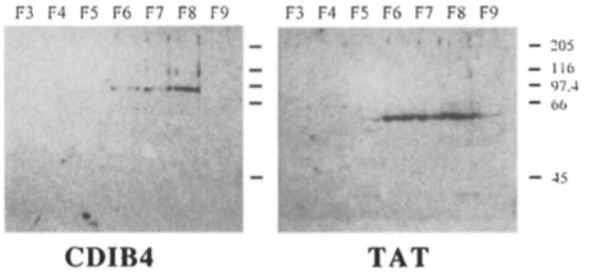


*Figure 5.* Purified centrioles were banded in a sucrose gradient, fractions prepared and protein separated by SDS-PAGE. Probing a Western blot with the CDIB4 monoclonal detects cenexin as a 96-kD molecular weight protein in fractions 6, 7, and 8 (*left hand panel*). Reprobing the same blot with the TAT1 anti-α-tubulin monoclonal confirms the presence of centrioles in only these fractions (*right hand panel*).

Our isolation of biochemically significant quantities of centriolar protein has facilitated the production of a number of immunological probes. This approach has allowed us to identify a 96-kD protein (cenexin) that, judged by many criteria, is localized specifically to only one centriole of the pair. The CDIB4 monoclonal therefore provides the first biochemical evidence for a molecular difference between the two centrioles in a mammalian cell. Although the cenexin protein is detected as a single band on a Western blot, there remains the formal possibility that the CDIB4 epitope includes a posttranslational modification. If so, this modification would need to be an extremely specific modification of a single centriolar protein and moreover, this modification would have to be nonreversible. This would contrast with all other known posttranslational modifications which occur at the centrosome; e.g., phosphorylation for example shows reversible cell cycle dependent modulation (Vandre and Borisy, 1989*b*). Notwithstanding this discussion point, the specific detection of a single protein and its unique cellular location serve to define this molecular marker of centriole maturation. The possibility that cenexin is masked on the immature centriole is ruled out by the use of many different fixation and staining methods at the light and electron microscopy level on isolated centrioles and on many different cell lines. All these techniques demonstrated unequivocally that cenexin localizes only to one centriole of the centriolar pair.

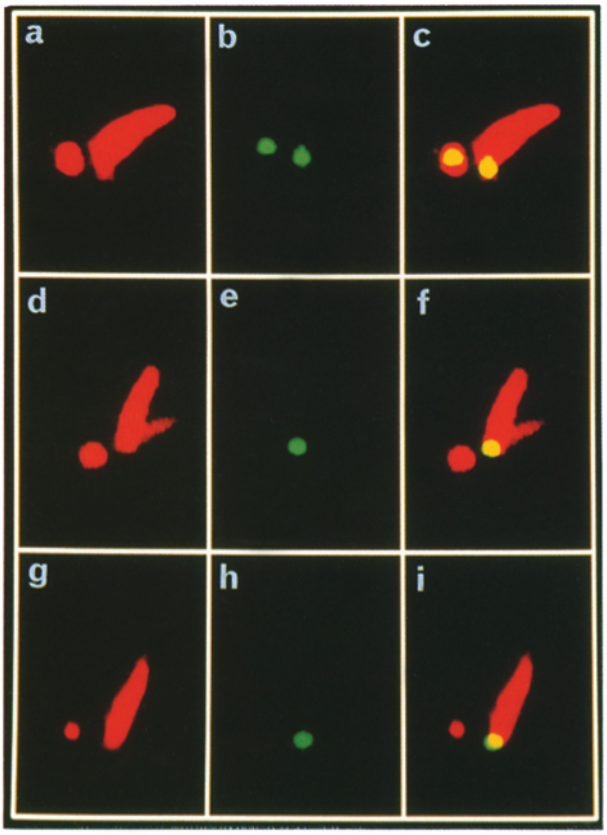


*Figure 6.* High resolution detection of centrioles and primary cilia in Swiss 3T3 cells. The anti-acetylated α-tubulin monoclonal C3B9 detects the immature centriole and the mature centriole subtending a primary cilium (Texas red channel, *red*, pseudocolor, *a*, *d*, and *g*). Cenexin and general centriolar proteins are detected by the CDIB4 monoclonal (*e* and *h*) and the RAC2 polyclonal (Fig. 6 *b*), respectively (fluorescein channel, *green*, pseudocolor). Cenexin can be ascribed to the mature centriole by image merging techniques (*f* and *i*). The cenexin one dot structure, detected by the CDIB4 monoclonal, is localized to the base of the primary cilium indicating that it is the mature centriole. The labeling of the RAC2 antibody is present on both centrioles (Fig. 6 *b*).

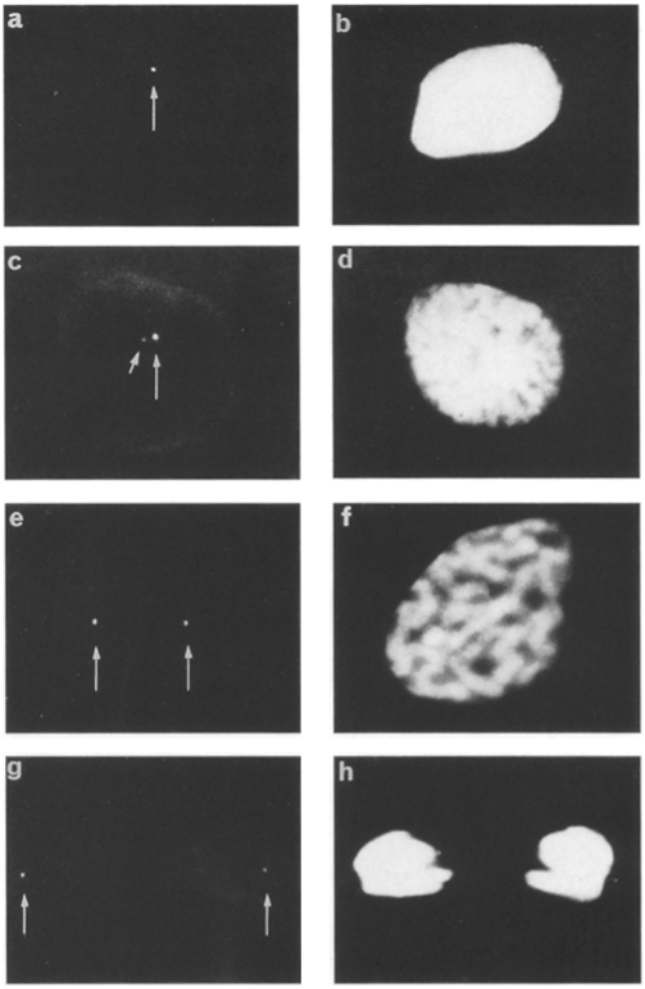


*Figure 7.* Detection of cenexin in PtK2 cells at different cell cycle stages. (*a, c, e,* and *g* anti-cenexin immunofluorescence; *b, d, f,* and *h* DAPI fluorescence). Interphase cell (*a* and *b*); early prophase cell (*c* and *d*); prophase cell (*e* and *f*); late anaphase cell (*g* and *h*). *c–f* show that cells acquire a second cenexin positive centriole as they progress into prophase. Cenexin first arises in proximity of the mature centriole in early prophase (7 *c, small arrow*). The cenexin positive centrioles are subsequently positioned at each pole of the mitotic spindle (7 *g*).

### *Cenexin Is a Marker for the Mature Centriole*

In the $G_1$ pair of centrioles the mature centriole is best defined by its ability to nucleate a primary cilium. Although this organelle occurs very widely its function is unknown (Wheatley, 1982). However, it provides for our purpose an excellent marker to define the mature centriole of the pair. We have shown that cenexin locates specifically to the centriole that is at the base of the primary cilium. The resolution of our microscopy is confirmed by the use of the RAC2 polyclonal which detects both centrioles. Although the primary cilium provides a reliable marker for the mature centriole in $G_1$ it is not present during the whole of the cell cycle. Thus, the question arises as to when the other (i.e., immature $G_1$ centriole) matures during the cell cycle? It could presumably be S, $G_2$, M, or even at the very beginning of the next $G_1$. The fact that cenexin correlates absolutely with the mature centriole allowed us to define the centriole maturation point. It is important in this respect that cenexin is also present in nonciliating cells such as HeLa and is also present in mitotic cells which do not have primary cilia (Wheatley, 1982). This implies that cenexin is unlikely to be a ciliary component but rather can be considered to be a centriolar component.

### *Centriole Maturation Occurs at the $G_2$/M Transition*

Interphase cells in the population all exhibit one mature, cenexin positive centriole. This argues strongly for maturation of the immature centriole at a very late stage of the cell cycle, during mitosis or very early in the next $G_1$ of that particular daughter cell receiving this centriole. If this latter model were to apply it would lead to an obvious asymmetry, in the two daughter cells. This is not, in fact, what is observed. Instead, careful study of the appearance of the second cenexin positive centriole in the cell shows that maturation occurs at the $G_2$/M transition: very early prophase cells show one cenexin positive centriole and

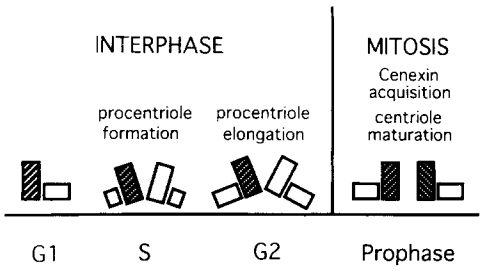


*Figure 8.* Diagram showing major centriolar events during the cell cycle. There is one cenexin positive mature centriole present during interphase. The centriole produced in the previous cell cycle acquires cenexin and matures only when the cell moves into prophase.

one that has started to acquire cenexin. Therefore, the centriole maturation event in the cell cycle of mammalian cells occurs at this $G_2$/M transition. Although we do not yet know how a centriole becomes cenexin positive, it is important to note that the centriole maturation event that we have defined is occurring coincidentally with other control events and biochemical and structural modulations within the cell cycle (Nurse, 1990; Norbury and Nurse, 1989). The maturation of the immature centriole at the $G_2$/M transition means that by the mitotic metaphase both spindle poles contain one mature and one immature centriole.

### *The Functional Consequences of Centriolar Maturation*

The definition of the mature centriole comes from the ability of such a centriole to nucleate the primary cilium. Centriole maturation is therefore important in that it defines a functional property of the centriole. Interestingly, our antibody location techniques show cenexin associated with the distal end of the mature centriole. This might be significant in two respects: First, the distal end of the centriole is the end which nucleates a primary cilium and in differentiating cells it is this distal part of the basal body which subtends the flagellum or the cilium. Second, the mature centriole carries arms at the distal end in interphase cells which might be involved in connecting the centrioles to the cytoskeleton or the plasma membrane (Wheatley, 1982). We have now been able to define a mature centriole for the first time as possessing a molecular marker, cenexin. The discovery of this transition time point in the mammalian cell cycle may be important in terms of centriole function as a component of the centrosomal MTOC of mammalian cells. There is evidence that the amorphous pericentriolar material is the material that nucleates assembly of the cytoplasmic and spindle microtubules (Gould and Borisy, 1977; Telzer and Rosenbaum, 1979). Also, it has often been suggested that a function of the centrioles might be to congregate such material to a single focus in the cell (Vorobjev and Nadezhdina, 1987). It is intriguing that the centriolar maturation point in the cell cycle that we have defined in this study correlates precisely with the time when a cell moves from a single interphase MTOC to the two MTOC required for bipolar spindle formation in mitosis. It may be, therefore, that there is a functional definition of a cenexin positive mature centriole as a unique congregation site for such organizing center material. Hence it might be of functional significance that only one of the two centrioles in interphase cells possesses cenexin and so influences cell architecture and cell geometry.

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