Centrosomes competent for parthenogenesis in *Xenopus* eggs support procentriole budding in cell-free extracts

(cell cycle/protein synthesis)

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ABSTRACT Heterologous centrosomes from diverse species including humans promote egg cleavage when injected into metaphase-arrested *Xenopus* eggs. We have recently isolated centrosomes from calf thymocytes and shown that they were unable to induce egg cleavage, an inability that was apparently correlated with the peculiar structure of these centrosomes rather than with a lack of microtubule-nucleating activity: the two centrioles were associated in a collinear orientation by their proximal ends. To promote cleavage, a heterologous centrosome probably is required to duplicate, although this has not yet been demonstrated. Therefore, we designed an *in vitro* assay that would enable us to directly observe the duplication process. We show that competent centrosomes from KE37 cells synchronized in G1 phase initiate procentriole budding in interphasic extracts from *Xenopus* eggs in the absence of protein synthesis, whereas calf thymocyte centrosomes do not. Since calf thymocyte centrosomes do not support parthenogenesis, the present results suggest that duplication of the foreign centrosome is required for centrosome-induced parthenogenesis. Furthermore, procentriole budding takes place in the absence of protein synthesis in egg extracts arrested in S phase. This *in vitro* assay should contribute to the identification of molecular mechanisms involved in the initiation of centrosome duplication.

In somatic cells, centrosome duplication encompasses the whole cell cycle and progression in the centrosome cycle appears to be a marker of progression in the cell cycle. The importance of centrosomes in establishing the spatial organization of the mitotic apparatus mandates that the cell tightly controls the reproduction of its centrosome, which must occur only once in each cell cycle, prior to mitosis. The morphological events of the centrosome duplication cycle in somatic cells have been described and involve budding of daughter centrioles off the wall of the parent structures in late G1 phase, elongation of the daughter centrioles during S and G2 phases, redistribution of pericentriolar material, and separation of duplicated centrosomes in prophase (1–3). However, the molecular mechanisms that govern centrosome duplication are not known.

As amphibian eggs apparently lack a functional centrosome, which can be replaced by the injection of foreign centrosomes (parthenogenetic development), they represent a favorable system to study the duplication of centrosomes. In this system, no species specificity is apparently required for the centrosomes: heterologous centrosomes isolated from sea urchin (4), rodent (5, 6), or human (7–9) cells can induce cleavage of *Xenopus* eggs. In this parthenogenetic test, the heterologous centrosomes are believed to duplicate, but direct experimental evidence is lacking. The parthenogenetic activity of the centrosome is independent of the stage of the cell cycle from which they have been isolated: centrosomes isolated from G1 or G2 human lymphoid cells (KE37 cell line) or from quiescent cells (peripheral human lymphocytes in G0) were shown to possess a similar parthenogenetic activity (8). We have isolated (10) centrosomes from calf thymocytes (CTs) and shown that the two centrioles were linearly associated by their proximal ends through a mass of dense material. Furthermore, they were unable to induce egg cleavage, although centrosomes isolated from other bovine cells and from thymocytes of other species could induce egg cleavage (6). These results suggest that the centrosome cycle can be blocked when the centrioles are prevented from separating into a nonlinear configuration, a step that might be critical for the initiation of procentriole budding.

Therefore, we have prepared cell-free extracts from *Xenopus* eggs in the mitotic or interphasic stage, in which we could directly compare the duplicative capacity of centrosomes that are competent and incompetent (such as CT centrosomes) to induce parthenogenesis. We show that competent centrosomes from human lymphoblasts (KE37 cell line), synchronized in G1 phase, initiate procentriole budding in interphasic extracts from *Xenopus* eggs in the absence of protein synthesis, whereas incompetent (CT) centrosomes do not. Since CT centrosomes do not support parthenogenesis, the present results strongly suggest that duplication of the foreign centrosome is required for centrosome-induced parthenogenesis.

MATERIALS AND METHODS

**Animals.** *Xenopus laevis* females were obtained from the Service d'Élevage d'Amphibiens du Centre National de la Recherche Scientifique (France). Eggs were obtained from females injected 3–8 days before use with 100 international units of pregnant-mare serum gonadotropin (Intervet, Angers, France) and 1 day before with 1000 international units of human chorionic gonadotropin (Sigma). The eggs were collected in 100 mM NaCl to prevent activation.

**Preparation of Extracts.** Egg extracts were prepared according to Felix *et al.* (11). Egg jelly was removed in 2% (wt/vol) cysteine hydrochloride (pH 7.8), and eggs were washed four times in 0.25× MMR (1× MMR = 0.1 M NaCl/2 mM KCl/1 mM MgSO₄/2 mM CaCl₂/5 mM Hepes/0.1 mM EDTA, pH 7.4) containing cycloheximide (150 µg/ml; Sigma), and then activated by an electric shock (11) in the same buffer for 90 min at 20°C. Activated eggs were transferred to Beckman SW 50.1 rotor tubes filled with ice-cold acetate buffer [AB = 100 mM potassium acetate/2.5 mM magnesium acetate/cytosolalanin D (10 µg/ml; Sigma)]/1 mM diethyfriol/250 mM sucrose/leupeptin (10 µg/ml)/pepstatin (10 µg/ml)/aprotinin (10 µg/ml), pH 7.2]. Excess acetate buffer was removed from the tubes containing packed eggs prior to centrifugation. The eggs were crushed by centrifugation at 9929

*Abbreviation: CT, calf thymocyte.

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10,000 × g (with maximum acceleration rate) for 10 min at 4°C in a L5-65 Beckman centrifuge. The cytoplasmic material between the upper lipid layer and the yolk pellet was collected (10,000 × g supernatant) and an ATP regenerating system [10 mM creatine phosphate, creatine phosphokinase (80 μg/ml), and 1 mM ATP (Boehringer Mannheim)] and protease inhibitors as above were added. A sample was kept in ice during further steps. The 100,000 × g extracts were obtained by further centrifugation of the 10,000 × g supernatant at 100,000 × g for 60 min at 4°C in the SW 50.1 rotor with adaptors for 0.6-ml tubes. The yellow cytoplasmic layer (100,000 × g supernatant) was collected and kept at 4°C, because freezing the extracts gave inconsistent results in the centrosome duplication assay.

**Cell Synchronization and Isolation of Centrosomes.** KE37 is a human T-lymphoblastic cell line. KE37 cells, cultured in RPMI 1640 medium (Eurobio, Les Ulis, France) containing 7% (vol/vol) calf serum, were first synchronized at the G1/S-phase border with 2.5 mM thymidine (Sigma) for 20 hr (12) and then separated in a cell-cycle-dependent manner using centrifugal elutriation according to Tournier et al. (8). Centrifugal elutriation led to an enriched fraction containing >80% G1 phase cells. Centrosomes were prepared from KE37 cells in G1 phase according to Tournier et al. (8) and from CTs according to Komesli et al. (10).

**Duplication Assay.** Typically, 10 μl of KE37 G1 centrosomes (1 × 10⁶ centrosomes per μl) was mixed with 100 μl of extract (either 10,000 × g or 100,000 × g supernatant) at 4°C. The mixture was equally distributed into four tubes and incubated at room temperature for 0–3 hr. After incubation, nocodazole was added to 30 μg/ml and the tubes were kept at 4°C for 30 min. Then 0.8 ml of lysis buffer [1 mM Tris-HCl, pH 7.5/0.1% 2-mercaptoethanol/0.5% Nonidet P-40/0.5 mM MgCl₂/1 mM phenylmethylsulfonyl fluoride/aprotinin (100 μg/ml)/leupeptin (1 μg/ml)/pepsstatin (1 μg/ml)] at 4°C was added to each tube and the material in each tube was layered onto 25% (vol/vol) glycerol in RG2 buffer (80 mM Pipes-KOH/1 mM MgCl₂/1 mM EGTA, pH 6.8) in a 15-ml modified Corex tube with a special adaptor containing a 12-mm round coverslip. The material was centrifuged at 12,000 rpm (20,000 × g) in a JS-13.1 rotor (Beckman) at 4°C for 15 min to apply the centrosomes to the coverslips. The coverslips were removed, fixed in methanol at −20°C for 5 min, and processed for immunofluorescence staining.

**Immunofluorescence Study.** Centrosome duplication was monitored by measuring the numbers of centrioles and centrosomes by double immunofluorescence (7). A monoclonal antibody against α-tubulin (Amersham) stained centrioles and a human autoimmune serum stained the pericentriolar material of human centrosomes (J. E. Dominguez, E.K., and J. Avila, unpublished data).

**Negative Staining.** For negative staining of electron microscopy preparations, 15 μl of G1 centrosomes (1 × 10⁶ centrosomes per μl) was mixed at 4°C with 300 μl of extract. Further steps were identical to the immunofluorescence protocol until the lysis step. For each point (at 0, 2, and 3 hr of incubation), 100 μl of extract was mixed with 200 μl of lysis buffer and incubated 2 min at 4°C. The material was loaded

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**Fig. 1.** Double immunofluorescence staining of G1 centrosomes (KE37) in *Xenopus* extracts arrested in S phase at 0 time (a and a') and after 3 hr of incubation (b and b'). Centrosomes stained with the human autoimmune serum (a) appear to be doublets of tubulin-containing dots corresponding to doublet centrioles (a'). In this experiment, the two centrioles in a centrosome do not split (see Table 1) and after 3 hr of incubation in the extract, the centrosomes (b) contain three or four tubulin-containing dots as judged by the anti-tubulin staining (b'). In the lower parts of each panel, other examples at 0 time (a and a') and after 3 hr of incubation (b and b') are shown. The variable intensities of the centrioles probably depend on their orientation on the coverslip. (×16,900.)
onto 200 µl of 25% glycerol in RG2 buffer in a 0.6-ml tube containing a Teflon adaptor supporting a carbon grid. The material was centrifuged at 14,000 rpm (25,000 × g) in a SW 50.1 rotor (Beckman) for 20 min (4°C) to apply centrosomes to the grid. Grids were then removed, rinsed in RG2 buffer, stained with 2% (wt/vol) phosphotungstate (pH 7 with KOH), and dried.

RESULTS

Duplication of G1 Centrosomes in Cell-Free Extracts: Immunofluorescence Study. Competent centrosomes were isolated from KE37 cells synchronized in G1 phase. We have shown (8) that these centrosomes were capable of inducing parthenogenesis when injected into unfertilized X. tropicalis eggs. The use of cell-free extracts and immunofluorescence to study microtubule polymerization nucleated by isolated centrioles has been described (13–15). In the present study, visualization of the centrosomes by immunofluorescent staining was done after nocodazole-induced disassembly of the microtubules, which had been nucleated in the extract by the added centrosomes. Double staining was used to identify both the pericentriolar domain and the centrioles. This allowed us to identify the centrosomes unambiguously by distinguishing them from tubulin aggregates. More reproducible results were obtained from cytoplasmic extracts of X. tropicalis eggs prepared from activated oocytes in the presence of cycloheximide (interphase extracts).

G1 centrosomes from KE37 cells were incubated in 100,000 × g Xenopus egg extracts for 0–3 hr. At 0 time, centrosomes were observed as pairs of centrioles (Fig. 1 a and a'), and after 3 hr of incubation, centrosomes contained three or four centrioles (Fig. 1 b and b'). We interpreted these dots as duplicative forms of centrosomes in which the centrioles did not separate. The numbers of centrosomes and centrioles were recorded at various incubation times by double immunofluorescence. After 3 hr of incubation, the number of centrosomes remained constant but the number of centrioles increased from 1 (reference value at incubation time) to 1.95 (Fig. 2a) and the number of centrosomes containing 3 or 4 identifiable centrioles increased from 1% to 48% (Fig. 2b). Typically, the number of KE37 centrosomes doubled after 2–3 hr of incubation. We noted, however, that the efficiency of centrosome duplication depended largely on the batch of extract used.

In other experiments, the two centrioles in a given centrosome often separated from each other over large distances (Table 1) during the first hour of incubation as judged by the increase in the proportion of single centrioles counted at this time. Duplicated forms observed after 3 hr of incubation were identified as a pair of tubulin-containing dots associated with centrosome labeling.

When the same kinetic experiments were done using low-speed extracts (10,000 × g), no increase in centriole number was detected after 2 or 3 hr of incubation (Fig. 2c) or up to 6 hr of incubation (data not shown), suggesting that the 10,000 × g supernatant contained an activity that inhibited centrosome duplication.

Duplicative Forms of Centrosomes Observed by Negative Staining. To validate our scoring method by an independent approach (electron microscopy), we examined negatively stained centrosomes that had been incubated in the extract.

Table 1. Centriole splitting in interphase extracts during the first hour of incubation

<table>
<thead>
<tr>
<th>Cells</th>
<th>Exp.</th>
<th>n</th>
<th>Time, hr</th>
<th>Single</th>
<th>Double</th>
</tr>
</thead>
<tbody>
<tr>
<td>KE37</td>
<td>I</td>
<td>5</td>
<td>0</td>
<td>388 (44)</td>
<td>488 (66)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>2</td>
<td>1</td>
<td>952 (84)</td>
<td>189 (16)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0</td>
<td>49 (25)</td>
<td>148 (75)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>1</td>
<td>67 (27)</td>
<td>180 (73)</td>
</tr>
<tr>
<td>CT</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>42 (19)</td>
<td>183 (81)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>45 (23)</td>
<td>152 (77)</td>
<td></td>
</tr>
</tbody>
</table>

n represents the number of experiments. Centrosomes were recorded as pairs of tubulin-containing dots at 0 time and after 1 hr of incubation. For KE37 centrioles (experiments I), a splitting of the two centrioles in a given centrosome was observed (44% of single centrioles at 0 time and 84% after 1 hr of incubation). In five experiments (I), duplicated forms observed after 3 hr of incubation in the extract were identified as pairs of tubulin-containing dots associated with centrosome labeling. In two experiments (II), a splitting of the two centrioles was not observed (25% of single centrioles at 0 time and 27% after 1 hr of incubation). The duplicated forms observed after 3 hr in the latter case (II) were recorded as three or four tubulin-containing dots. For CT centrosomes, in all cases, we did not observe any significant splitting of the centrioles after 1 hr of incubation. Numbers in parentheses are percent of total centrioles.
Centrioles were scored either as single centrioles or as pairs and with or without an associated orthogonal procentriole (Fig. 3). A representative experiment is shown in Table 2. At 0 time, 46% of the centrioles were pairs and 52% were single. After 2 hr of incubation, 12% of centrioles were pairs and 82% of centrioles were single. These results were in agreement with immunofluorescence observations and indicated extensive splitting of the centrioles during incubation in the extracts. Duplicated forms were only 4% of the total centrioles at 0 time (Table 2), which is consistent with the fact that centrosomes were prepared from G1 cells. After 2 hr and 3 hr

| Table 2. Centriole splitting and duplicated forms by electron microscopy after negative staining |
|---|---|---|---|
| Time, hr | n | Centriole form, no. | Duplicated forms, no. |
| | | Single | Double | Triple |
| 0 | 97 | 34 (52) | 30 (46) | 1 (2) | 4 (4) |
| 2 | 21 | 14 (82) | 2 (12) | 1 (6) | 13 (62) |
| 3 | 36 | 20 (74) | 5 (19) | 2 (7) | 21 (58) |

n represents the number of centrioles. Note that, at 0 time, 52% of centrioles were observed as single centrioles and 46% were observed as centriole pairs. After 2 hr of incubation, single centrioles represented 82% of the total centrioles observed. Centrioles were sometimes observed (2-7%) as triplets. Duplicated forms correspond to centrioles observed with a centriole bud. For duplicated forms, 4% were observed at 0 time, 62% were observed after 2 hr of incubation, and 58% were observed after 3 hr of incubation. Numbers in parentheses are percent of total centrioles.

DISCUSSION

We have compared the capacity of centrosomes to initiate a centrosome duplication cycle in cell-free extracts by using an immunofluorescence-based assay. Centrosomes competent to induce parthenogenesis (G1 centrosomes from KE37 cells) initiate procentriole budding whereas incompetent centrosomes (CT centrosomes) do not. The electron microscopy study supported the conclusion that the increase in tubulin-containing dots observed by immunofluorescence during incubation of the centrosomes in extracts corresponds to an increase in centriole or procentriole number. But it also indicated that only procentrioles were formed. Indeed, we never observed pairs of centrioles tightly associated in an orthogonal configuration in which the two centrioles had the same length. Further incubation (up to 6 hr) did not increase the number of centrioles (data not shown). In particular, we never obtained evidence for successive rounds of centriolar duplication. This suggests that the duplication process is not completed under our in vitro conditions.

This may have several causes. First, the high-speed supernatant (100,000 x g) may lack components necessary for bud elongation. Such components may or may not be in the high-speed pellet, because the pellet apparently also contains an activity from the 10,000 x g supernatant that was shown to inhibit the initiation of centriole duplication (Fig. 2c). The most likely explanation is that the components necessary for assembling full-length centrioles are limiting in comparison to the number of centrosomes incubated in the extract. We added between 500 and 1500 centrosomes per μl of extract, which is the minimum number required to make the immunofluorescence study feasible. This figure is close to the number of centrosomes produced per embryo at the midblastula transition (16) (one embryo has a volume of about 1 μl). From such a figure, we may conclude that a single centrosome duplication cycle at most could take place under our

![Fig. 4. Centrosomes isolated from CTs do not duplicate in 100,000 x g supernatant, whereas KE37 centrosomes initiate procentriole budding, as shown by the increase of the number of centrioles. □, CT centrosomes; □, KE37 centrosomes.](image-url)
conditions. If this were the case, adding centrosomes at a lower concentration should allow multiple rounds of duplication in the extract, but we have not succeeded in designing an experiment to test this hypothesis.

Another implication of these results is that the first steps of centrosome duplication are independent of the cytoplasmic oscillator that drives the cell cycle, since procentriole budding takes place in cycloheximide-treated interphasic extracts. However, the incompleteness of the duplication cycle could suggest that a signal from the “mitotic clock” is necessary for the late steps of the process. This is unlikely since multiple cycles of centrosome duplication occurred in cycloheximide-treated *Xenopus* blastomeres (16) and a similar result was also reported for sea urchin eggs (17).

Centrosome duplication can be experimentally uncoupled from the synthesis of mitotic cyclins (refs. 16 and 17 and this report). Our experimental system should allow investigation of the coupling between the centrosome duplication cycle and the cytoplasmic oscillator. The asynchrony of centrosome reproduction in the absence of protein synthesis (16, 17) suggests indeed that phasing of the cytoplasmic oscillator and the centrosome cycle occurs in normal development. p34cdc2, a suc1 homologue (18), and cyclin B (E. Bailly, J. Pines, T. Hunter, and M.B., unpublished data), the major components of the cytoplasmic oscillator, are associated with the centrosome in G2 and M phases. It will be of interest to study centrosome duplication in cycling extracts and the role of protein phosphorylation controlled by various cyclins in synchronization of centrosome duplication with the mitotic cycle. Preliminary experiments in mitotic extracts suggest that procentriole budding cannot occur during mitosis (data not shown).

The inability of CT centrosomes to initiate a centriole bud in vitro strongly suggests that, in parthenogenetic Xenopus eggs, cleavage requires the duplication of the injected centrosome. This also indicates that the structural configuration of the centrosome is essential to initiate budding in a permissive cytoplasmic environment. Specific egg proteins are able to assemble on the two centrioles of the heterologous centrosome to form the two poles of the first mitotic spindle, in which each pole should contain a chimeric centrosome. The incompetence of CT centrosomes is not simply due to the species or the tissue origin, because the linear arrangement of the CT centrosomes apparently represents a locked configuration.

In conclusion, the present data demonstrate the potential of our in vitro assay to describe in molecular terms the orthogonal budding of procentrioles from the proximal end of parental centrioles.

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