Microtubule Patterning during Meiotic Maturation in Mouse Oocytes Is Determined by Cell Cycle-Specific Sorting and Redistribution of γ-Tubulin

Catherine M. H. Combelles and David F. Albertini
Department of Anatomy and Cellular Biology, Tufts University School of Medicine, Boston, Massachusetts 02111

The topography of microtubule assembly events during meiotic maturation of animal oocytes demands tight spatial control and temporal precision. To better understand what regulates the timing and location of microtubule assembly, synchronously maturing mouse oocytes were evaluated with respect to γ-tubulin, pericentrin, and total tubulin polymer fractions at specific stages of meiotic progression. γ-Tubulin remained associated with cytoplasmic centrosomes through diakinesis of meiosis-1. Following chromatin condensation and perinuclear centrosome aggregation, γ-tubulin relocated to a nuclear lamina-bounded compartment in which meiosis-1 spindle assembly occurred. γ-Tubulin was stably associated with the meiotic spindle from prometaphase-1 through to anaphase-2, but also exhibited cell cycle-specific relocalization to cytoplasmic centrosomes. Specifically, anaphase onset of both meiosis-1 and -2 was characterized by the concomitant appearance of γ-tubulin and microtubule nucleation in subcortical centrosomes. Brief pulses of taxol applied at specific cell cycle stages enhanced detection of γ-tubulin compartmentalization, consistent with a γ-tubulin localization-dependent spatial restriction of microtubule assembly during meiotic progression. In addition, a taxol pulse during meiotic resumption impaired subsequent γ-tubulin sorting, resulting in monopolar spindle formation and cell cycle arrest in meiosis-1; despite cell cycle arrest, polar body extrusion occurred roughly on schedule. Therefore, sorting of γ-tubulin is involved in both the timing of location of meiotic spindle assembly as well as the coordination of karyokinesis and cytokinesis in mouse oocytes. © 2001 Academic Press

Key Words: γ-tubulin; centrosome; spindle; nuclear lamina; oocyte; meiosis; nuclear; cytoplasmic maturation.

INTRODUCTION

The final stages of oogenesis are critical to the establishment of normal embryonic development. Meiosis is reinitiated in prophase-1 arrested oocytes; during ovulation and upon fertilization, the completion of two reductive divisions results from the coordination of meiotic spindle assembly and function with cell cycle progression (Albertini, 1992a,b; Albertini et al., 1993). Thus, changes in oocyte cytoplasmic organization are executed with great temporal and spatial precision to ensure that peri- and postfertilization events of embryogenesis proceed on schedule and without error. How animal oocytes achieve this terminally differentiated state is a fundamental problem in development that has impacted practical applications ranging from production of cloned mammals to treatment of infertility by assisted reproductive technologies.

Oocyte maturation encompasses many critical events that culminate in the production of “mature” and developmentally competent oocytes in vertebrates. The focus of work in this area has been on cell cycle control and the attendant structural alterations in the oocyte cytoplasm that underlie both nuclear or chromosomal maturation of the oocyte as well as maturation of cytoplasm (Eppig et al., 1994). Cytoplasmic maturation refers to the expression and organization of factors deemed essential for egg activation, pronuclear development, axis specification, and maternal inheritance (Eppig et al., 1994; Antczak and Van Blerkom, 1997; Gardner, 1999; Cheung et al., 2000). Nuclear maturation usually refers to the processes underlying the segregation of oocyte chromosomes during the reductive meiotic divisions (Albertini, 1992a,b; Albertini et al., 1993). Whether the coordination of nuclear and cytoplasmic matu-
rature relies on spatial patterning of important cell cycle factors remains to be fully established but represents an active area of current inquiry.

Compartmentalization of regulatory proteins into the nucleus or cytoplasm is now recognized as a major determinant of cell cycle progression and cell polarity (Pines, 1999; Kemphues, 2000). Partitioning of cell cycle components such as cdc2 and cyclins has been implicated as a meiotic cell cycle control mechanism (Mitra and Schultz, 1996; reviewed by Albertini and Carabatsos, 1998). In addition, factors that regulate genomic imprinting during oogenesis or calcium signaling during oocyte maturation have also been shown to be spatially regulated in the mouse (Avazeri et al., 2000; Howell et al., 2001). Manifestations of cell polarity reported in vertebrates include the asymmetric localization of cytoskeletal elements, organelles, maternal mRNAs, and proteins (Gard et al., 1997; Zhang et al., 1998; Bowerman and Shelton, 1999; Gardner 1999; Kemphues, 2000; Nakaya et al., 2000). The establishment and maintenance of cellular asymmetry during oogenesis, oocyte maturation, and/or early embryogenesis relies on microtubule arrays (Gard et al., 1997). Regulation of microtubule patterning during maturation of mammalian oocytes has been implicated in both the assembly of the meiotic spindle and positioning of cytoplasmic organelles (Albertini, 1987; Van Blerkom, 1991; reviewed by Albertini, 1992b). Thus, microtubule-organizing centers (MTOCs) or centrosomes have been studied with respect to the regulation of microtubule patterning during oogenesis. γ-Tubulin is a constitutive centrosomal protein known to play a key role in microtubule patterning in a variety of cell systems (Joshi et al., 1992; Zheng et al., 1995; reviewed by Murphy and Stearns, 1996; Pereira and Schiebel, 1997; Wiese and Zheng, 1999). Carabatsos et al. (2000) suggested that γ-tubulin sorting might explain the temporal and spatial cell cycle regulation of microtubule nucleation in mouse oocytes. γ-Tubulin distribution has been studied only at select stages of meiotic progression and in relatively few vertebrate species. For example, γ-tubulin was demonstrated in the spindle and cytoplasm of metaphase-2 oocytes and during early embryonic development in the mouse (Palacios et al., 1993; Gueth-Hallonet et al., 1993; Lee et al., 2000). In Xenopus and pig oocytes, changes in γ-tubulin distribution were reported during meiotic maturation, and γ-tubulin was shown to be associated with the cortex and spindle of mature frog and pig oocytes (Gard, 1994; Lee et al., 2000). In metaphase-2 bovine oocytes, a protein pool of γ-tubulin was reported with no details provided on its localization during meiosis (Simerly et al., 1999). Thus, interrelationships between MTOC, γ-tubulin, and microtubule patterning have not yet been unraveled in mammalian oocytes.

Rodent oocytes possess multiple MTOCs or cytoplasmic centrosomes that lack centrioles but possess pericentriolar material with microtubule-nucleating activity (Szélösi et al., 1972). Messinger and Albertini (1991) demonstrated cell cycle stage-specific changes in cytoplasmic centrosome numbers and microtubule nucleation capacity during oocyte maturation in the mouse. Given that mouse oocytes possess two populations of acentriolar centrosomes associated with the meiotic spindle or the cortex depending on cell cycle stage (Maro et al., 1985; Schatten et al., 1986; Messinger and Albertini, 1991), divergence of function due to the altered positioning and/or nucleation capacity of centrosomes is likely to participate in segregating and coordinating localized microtubule assembly in the spindle or cortex of the maturing oocyte.

The goal of this work was to determine the role of γ-tubulin in microtubule patterning during meiotic progression in mouse oocytes. Our results document the dynamic nature of the spatial and temporal sorting of γ-tubulin and provide a basis for understanding how spindle morphogenesis, cytokinesis, and cytoplasmic reorganization are integrated during the process of meiotic maturation.

MATERIALS AND METHODS

Collection and Culture of Mouse Oocytes

For all in vitro maturation experiments, oocytes were obtained from 20- to 25-day-old CF-1 mice injected 44–48 h earlier with 5 IU of equine chorionic gonadotropin (Sigma Biosciences, St. Louis, MO). All culture reagents were obtained from Life Technologies Gibco/BRL (Gaithersburg, MD). Cumulus-enclosed oocytes were expressed in collection medium consisting of Hapes-buffered Eagle's MEM with Hanks' salts supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.3% bovine serum albumin. Cumulus-enclosed oocytes were matured in vitro in Eagle's MEM supplemented with Earle's salts, 2 mM glutamine, 0.23 mM pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.3% bovine serum albumin (in vitro maturation or IVM medium) in a humidified atmosphere of 5% CO₂ at 37°C (Schroeder and Eppig, 1984). In some experiments, 0.2 mM 3-isobutyl-1-methyl xanthine (IBMX; Sigma Biosciences; Downs et al., 1989) was added to collection medium to prevent meiotic resumption and to synchronize oocytes at early stages of meiotic progression. Maturation was initiated after washing oocytes three times in IBMX-free collection medium and subsequently oocytes were cultured for 0–3.5 h in IVM-medium. In most cases, however, spontaneous maturation was allowed to occur without IBMX pretreatment and oocytes were fixed at specific time points between 0 and 14 h of culture (see below).

In vivo and in vitro matured metaphase-2 oocytes were also analyzed before and after spontaneous aging or artificial activation. In vivo matured metaphase-2 oocytes were obtained from 6-week-old CF-1 mice injected with 5 IU equine chorionic gonadotropin followed 44–48 h later by 5 IU human chorionic gonadotropin (Sigma Biosciences). Ovulated oocytes were flushed from oviducts at 13–14 or 20–22 h post-hCG representing fresh or aged oocytes, respectively. Cumulus cells were removed with 0.01% hyaluronidase (Sigma Biosciences) for 3–5 min, and oocytes were immediately fixed for later analysis. In vitro matured metaphase-2 oocytes cultured for an additional 6–8 h in IVM-medium (20–22 h total culture time) were designated as in vitro aged oocytes.

To evaluate microtubule patterning during anaphase of meiosis-2, metaphase-2 arrested oocytes were activated by fertilization or chemical stimuli. For in vitro fertilization, zona were
removed with Acid Tyrode's, and zona-free oocytes were allowed to recover for 90 min before sperm addition. Metaphase-2 oocytes recovered 13-14 h post-hCG were fertilized with capacitated caudae epididymal sperm at a concentration of 100,000 sperm/ml (Hogan et al., 1986). Oocytes were fixed approximately 20 min after insemination. For artificial activation, in vivo matured metaphase-2 oocytes (14 h post-hCG) were treated with 10 μM ionomycin (Sigma Biosciences) for 2 min in Earle's/Hepes supplemented with 0.4% PVP. The calcium ionophore, ionomycin, induces the release of intracellular stores of calcium, thereby mimicking the intracellular calcium transients that occur at fertilization in mammalian oocytes. Ionomycin treatment was followed by washes in drug-free IVM-medium and cultured for 15-30 min, at which time anaphase onset was apparent and samples were fixed immediately.

Pharmacological Manipulation of Microtubule Patterning in in Vitro Matured Oocytes

In this study, we took advantage of the microtubule-stabilizing activity of taxol to manipulate microtubule dynamics at specific stages of meiotic progression (Albertini, 1987; Rime et al., 1987; Van Blerkom, 1991; Carabatsos et al., 2000). Our rationale was that taxol-induced stabilization of preexisting microtubules or enhanced microtubule assembly would delay or impair processes requiring microtubule dynamics. In all experiments, 1 μM taxol (Sigma Biosciences) was prepared in fresh IVM-medium from dimethylsulfoxide (DMSO) stocks stored at −20°C. Denuded oocytes were treated for 10 min at 37°C, washed three times in control medium, and were either fixed immediately or returned to taxol-free culture medium for various times before fixation. Controls for each experiment (replicates of 9 experiments, with 40–50 oocytes per group) consisted of oocytes matured in medium containing DMSO carrier (0.01% v/v) for identical culture times.

Processing of Oocytes for Fluorescence Microscopy

After removal of cumulus cells by gentle pipetting, oocytes were fixed and processed as previously described (Messinger and Albertini, 1991). Microtubules were labeled by using either a monoclonal anti-α-tubulin and anti-β-tubulin mixture (Sigma Biosciences) or a rat monoclonal antibody against α-tubulin (YOL 34; Kilmartin et al., 1982) at a 1:100 final dilution, and followed by an affinity-al., 1994) or a monoclonal mouse antibody against α-tubulin and always contained the epitope. As reported previously, centrosomes assume a strictly perinuclear position up to metaphase-1 (Messinger and Albertini, 1991; Carabatsos et al., 2000). Three-dimensional reconstruction and the spatial resolution of laser scanning confocal microscopy were used to define nuclear and cytoplasmic boundaries and monitor changes in γ-tubulin distribution during spindle morphogenesis. Oocytes were analyzed by using the Odyssey XL confocal system (Noran Instruments, Inc., Middleton, WI) mounted on a Nikon Diaphot inverted microscope equipped with a ×60 objective. The 488-, 528.7-, and 650-nm lines of a krypton-argon laser were used to excite fluorescein, Texas Red, and Cy5 markers, respectively. Triple-fluor images were collected by using Intervision acquisition module software (Noran Instruments) running on a Silicon Graphics Inc., Indy 500 workstation (Mountain View, CA). All images were collected by using slow scan, with a sample time of 400 ns, and real-time image processing using an integrated average of 32 frames. Signal gain settings were kept constant for each PMT detector to minimize differences in intensity both between flours and samples. A series of z-axis image stacks was acquired using 0.5-μm steps and three-dimensional reconstructions were performed by using 3-D Intervision software.

RESULTS

To understand the regulation of microtubule patterning during meiotic progression, greater than 2600 mouse oocytes were analyzed by conventional and confocal fluorescence microscopy. Specifically, changes in centrosome position, organization, γ-tubulin content, and microtubule nucleation capacity were analyzed relative to chromatin condensation and phosphorylation and nuclear lamina integrity.

γ-Tubulin Undergoes a Cytoplasmic to Nuclear Redistribution during Meiotic Resumption and Spindle Assembly

Three-dimensional confocal microscopy was used to analyze γ-tubulin distribution during meiosis-1 with respect to sequential modifications in nuclear lamina stability (nuclear lamina B, chromatin condensation (Hoechst 33258 or histone H1), histone phosphorylation (phospho-histone H3), and microtubule nucleation (α/β-tubulin). Germinal vesicle (GV)-stage oocytes, arrested at prophase of meiosis-1, were collected in IBMX and released from meiotic arrest as cohorts fixed after 0.5, 1.5, 2.5, or 3.5 h in culture. Samples processed for microtubule, γ-tubulin, histone H1, phospho-H3, and nuclear lamina (B-type) organization revealed four sequential patterns in nuclear lamina structure that were correlated to changes in microtubule organization and chromatin structure (Fig. 1.1). Unless otherwise noted (see below), centrosomes were identified with anti-pericentrin and always contained the γ-tubulin epitope. As reported previously, centrosomes assume a strictly perinuclear position up to metaphase-1 (Messinger and Albertini, 1991). The frequencies of each pattern were recorded at different times following release from IBMX (Fig. 1.2).

The first pattern, typical of GV-stage oocytes cultured for
FIG. 1. Nuclear lamina integrity and γ-tubulin compartmentalization during meiotic resumption. (1.1) Representative three-dimensional confocal reconstructions (17 optical sections) of the perinuclear region of oocytes double-labeled for nuclear lamin B (red) and γ-tubulin (green) (A–D), or nuclear lamin B (red) and α/β-tubulin (green) (a–d). Images depict four sequential stages in meiotic progression from germinal vesicle (GV; A, a), diakinesis (B, b), to early (C, c) and late (D, d) prometaphase. Insets (A–D) represent phosphohistone 3 reactivity at each stage, and document the appearance of epitope on condensing bivalents during diakinesis that persist through to prometaphase.
0.5 h, was characterized by an ovoid, smooth surface nuclear lamina (Figs. 1.1A and 1.1a; red). γ-Tubulin was localized to one to several perinuclear centrosome(s) (Fig. 1.1A; green, arrow) closely apposed to the nucleolus (NO), where discontinuity of the nuclear lamina overlying the nucleolus was confirmed by optical reconstructions. At this stage, perinuclear centrosome-associated microtubules were external to the nuclear lamina (Fig. 1.1a; microtubules, green; nuclear lamina, red), and chromatin remained decondensed based on histone-1 and Hoechst staining (data not shown), consistent with the lack of phosphohistone H3 epitope (Wei et al., 1999; Fig. 1.1A, inset). This pattern of nuclear lamina, γ-tubulin, and microtubule organization was observed in 100% of oocytes at 0.5 h following release from IBMX (n = 132; Fig. 1.2).

At 1.5 h following release from IBMX, the nuclear lamina exhibited a collapsed configuration (Figs. 1.1B and 1.1b; red). Concomitantly, γ-tubulin distribution remained restricted to one to several pericentrin-containing centrosomes (data not shown) distributed along the outer surface of the nuclear lamina (Fig. 1.1B; γ-tubulin, green; nuclear lamina, red). At this stage, phosphohistone H3 reactivity was observed in 81.2% of oocytes that showed clear signs of chromatin condensation (n = 102) (Fig. 1.1B, inset). Microtubules were found penetrating the nuclear lamina at multiple invaginations (Fig. 1.1b; microtubules, green; nuclear lamina, red). This pattern was observed in the majority of oocytes (72.9%, n = 140; Fig. 1.2).

Following 2.5 h in culture, the nuclear lamina appeared distended (Figs. 1.1C and 1.1c; red), and pronounced gaps in lamin B staining coincided spatially with the location of γ-tubulin-positive centrosomal aggregates (Fig. 1.1C; green, arrowheads). γ-Tubulin was also present in perichromosomal nucleoplasm (Fig. 1.1C; green). The appearance of γ-tubulin within the boundaries of the nuclear lamina was associated with a distinct change in the location of microtubule assembly since microtubules were confined within a microenvironment delineated by the nuclear lamina (Fig. 1.1c; microtubules, green; nuclear lamina, red). The majority of oocytes fixed after 2.5 h in culture exhibited this pattern (61.5%, n = 156; Fig. 1.2). All oocytes (100%) in this category exhibited complete chromatin condensation and were positive for phosphohistone 3 reactivity (Fig. 1.1C, inset; n = 96).

By 3.5 h, disruption of the nuclear lamina and the exclusive detection of γ-tubulin staining associated with spindle microtubules were observed (Figs. 1.1D and 1.1d). Forming spindle structures were encased within a nuclear lamina that characteristically displayed an open end (Figs. 1.1D and 1.1d). This pattern was observed in 80.1% of oocytes at 3.5 h in culture (n = 151; Fig. 1.2). Therefore, a shift in γ-tubulin distribution from centrosomes to a nuclear microenvironment occurred concomitant with microtubule reorganization during early stages of meiotic progression.

### γ-Tubulin Exhibits Spindle Retention and Cytoplasmic Redistribution during Meiotic Progression

The distribution and organization of γ-tubulin was analyzed during the transition from metaphase-1 through telophase-2 (Fig. 2, also see Figs. 3 and 4). Although γ-tubulin was retained within spindles from metaphase-1 to telophase-2, its relationship to cytoplasmic centrosomes changed at specific cell cycle stages. At metaphase-1, γ-tubulin was concentrated at spindle poles (Fig. 2A; arrowheads) and organized as O- and C-shaped structures that colocalized with pericentrin (data not shown; Carabatsos et al., 2000). γ-Tubulin was associated with spindle microtubules and appeared less concentrated in regions of the spindle occupied by chromosomes (Fig. 2A). At anaphase of meiosis-1, γ-tubulin-containing aggregates were fragmented and located at the lateral margins of the spindle (Fig. 2B, arrowheads; see Carabatsos et al., 2000). γ-Tubulin was absent from spindle poles and the spindle midzone, but was present throughout spindles at telophase (Fig. 2C). At metaphase of meiosis-2, γ-tubulin was associated with both spindle poles, as a tight single focus at each end (arrowheads), and with spindle microtubules (Fig. 2D; Gueth-Hallonet et al., 1993). In summary, γ-tubulin remained associated with spindle microtubules between metaphase-1 and metaphase-2, and spindle pole-associated γ-tubulin aggregates undergo cell cycle-dependent reorganization. We next asked whether changes in γ-tubulin distribution occurred in oocyte cytoplasm between metaphase-1 and anaphase-2.

Cytoplasmic pericentrin-containing foci that lack microtubules are present through the oocyte cortex at metaphase-1 (Messinger and Albertini, 1991); these centro-
As described above, following artificial activation of metaphase-2 oocytes, spindle microtubules emanating from centrosomes were typically observed in association with the spindle in fertilized oocytes (84.4%; n = 42; Fig. 3d, arrow). As described for telophase-1 (see above), telophase-2-stage oocytes lacked cytoplasmic centrosomes (based on γ-tubulin and pericentrin staining) and no evidence of cytoplasmic microtubule nucleation was apparent (data not shown).

In summary, while γ-tubulin was retained within meiotic spindles, spindle pole γ-tubulin exhibited aggregation, fragmentation, and disappearance sequentially through metaphase to telophase in both meiotic divisions. These changes in spindle-associated γ-tubulin are temporally correlated with the appearance of immunodetectable γ-tubulin in cytoplasmic centrosomes and a transient burst of cortical microtubule assembly at anaphase of both meiosis-1 and -2 and during aging of metaphase-2 oocytes.

Compartmental Patterning of Microtubule Assembly Is Enhanced by Taxol Pulses

Previous studies have shown that cell cycle-specific changes in centrosome positioning may underlie the preferential localization of microtubule assembly in nuclear (chromatin) or cytoplasmic compartments (Messinger and Albertini, 1991), but the extent to which these differences are due to changes in centrosome or γ-tubulin distribution have not been evaluated. Toward this end, mouse oocytes at specific stages of meiotic progression were subjected to 10-min taxol pulses to determine whether alterations in microtubule stability or dynamics would influence the localization of γ-tubulin. The initial perinuclear accumulation and later appearance of cytoplasmic centrosomes were unchanged in taxol-treated oocytes when compared to controls. When applied at diakinesis, taxol caused the appearance of parallel microtubule bundles in the oocyte cortex (Fig. 4A).

When applied to oocyte prior to chromatin condensation, a significant enlargement of the one to three perinuclear centrosomes bearing numerous radially oriented microtubules was observed (Figs. 4A and 4B). In contrast, following chromatin condensation, oocytes exhibited microtubule assembly solely in association with the nuclear lamina-bounded compartment, evidenced by multiple perinuclear plaques of γ-tubulin with radially oriented microtubules (Figs. 4C and 4D).

Taxol treatment at anaphase onset enhanced microtubule assembly at both nuclear and cytoplasmic sites (Fig. 4E).
The cytoplasm, expanded foci of γ-tubulin displayed radial microtubules, whereas the enlarged spindle retained γ-tubulin in association with spindle microtubules (Figs. 4E and 4F). Treatment at telophase-1 showed γ-tubulin retention in the spindle, and staining was excluded from the midbody as seen in control oocytes at this stage; again cytoplasmic γ-tubulin foci and microtubules were never observed at telophase despite the propensity of taxol to induce substantial new microtubule growth in the spindle (Figs. 4G and 4H). At all stages examined in this experiment, pericentrin staining patterns were identical to that observed for γ-tubulin centrosome aggregates. In summary, these experiments illustrate a cell cycle-dependent spatial restriction of microtubule assembly events consistent with changes in γ-tubulin localization during meiotic progression.

**Perturbations in Meiotic Progression and Microtubule Patterning Following Taxol Treatment of GV-Stage Oocytes**

To further explore the relationship between microtubule dynamics and partitioning of γ-tubulin, the consequences of brief taxol treatment of GV oocytes were evaluated with respect to their ability to mature in culture.

Following a brief pulse of taxol (10 min, 1 μM) and extensive washing in taxol-free medium, oocytes were analyzed for meiotic progression at various time points over a 20-h culture period. Over 80% of untreated control oocytes reached metaphase-2 by 20 h (n = 270), whereas taxol-exposed oocytes failed to complete meiosis-1 (100%; n = 224). That karyokinesis failed at meiosis-1 was evidenced by the persistence of unresolved chromosome bivalents at all time points examined. Moreover, while control oocytes extruded solitary polar bodies between 14 and 20 h of culture (77.9%, n = 146 at 14 h; 80.8%, n = 270 at 20 h), taxol-exposed oocytes retained the ability to undergo polar body emission, but exhibited aberrations in both the timing and characteristics of this process. Taxol-exposed oocytes extruded polar bodies in a delayed fashion: 8.0% at 14 h (n = 135); 43.6% at 17 h (n = 128); and 75.6% at 20 h (n = 224). By 20 h of culture, the majority of oocytes (75.6%, n = 224) exhibited multiple polar bodies. An average number of three polar bodies was observed in taxol-exposed oocytes (n = 98), which were comparable in size to polar bodies of control metaphase-2 oocytes. Most polar bodies contained unresolved bivalents and spindle remnants and, on occasion, polar bodies lacking chromatid were observed (8.0%, n = 224 at 20 h of culture). To better understand the underlying basis for cell cycle arrest and abnormal cytokinesis, oocytes receiving an initial taxol pulse were analyzed with respect to microtubule, centrosome, chromosome, and nuclear lamina organization as described above.

Three hours after removal from taxol, the majority of oocytes (89.2%; n = 87) exhibited perinuclear punctate aggregates that contained both pericentrin and γ-tubulin (Figs. 5A and 5B). Confocal optical section analysis showed a distinct intranuclear subfraction of γ-tubulin not associated with pericentrin (Fig. 5B, dotted box). Microtubule assembly restricted to a nuclear compartment was observed under these conditions (data not shown). Following 8 h of culture when control oocytes have reached anaphase-1, the majority of taxol-treated oocytes (91.5%, n = 117) exhibited chromosome bivalents displaced to the outer edge of a monopolar spindle composed of radiating microtubule bundles oriented with chromosome-attached ends beneath the oocyte cortex (Fig. 5C). Analysis of confocal serial sections showed disorganized pericentrin foci disposed opposite to chromosomes in monopolar spindles (Fig. 5D). Also, cytoplasmic centrosomes devoid of γ-tubulin staining (data not shown) and microtubules were detected in the oocyte cortex at this and subsequent stages (Figs. 5C and 5D, n = 117). After 14 h of culture when control oocytes are arrested at metaphase-2, most oocytes (66.9%, n = 135) exhibited fragmentation of the monopolar spindle into variably sized monopolar spindles (Fig. 5E), over which prominent f-actin domains were observed based on rhodamine-phalloidin staining (data not shown). Chromosome bivalents were associated with monopolar spindles that exhibited a tapered end adjacent to the cortex and a broad end to which bivalents were attached. While multiple centrosome aggregates were observed in the cortex, a subpopulation of these were seen in association with the tapered end of monopolar spindles (Figs. 5E and 5F, arrowheads; 24.1%, n = 135). Again, all centrosome aggregates observed in oocytes between 14 and 20 h of culture were devoid of γ-tubulin and associated microtubules. Together, these observations show that taxol-treated oocytes are arrested in meiosis-1. Thus, although the initial γ-tubulin and centrosome relocalization during meiotic maturation occur on schedule in taxol-treated oocytes, the microtubule-stabilizing effect of this drug selectively disrupts later centrosome remodeling events required for bipolar spindle assembly, and the reassociation of γ-tubulin to centrosomes that accompanies anaphase onset.

**DISCUSSION**

The present study investigated the spatial and temporal parameters of microtubule dynamics during meiotic maturation of mouse oocytes. Since γ-tubulin is a known rate-limiting determinant of microtubule nucleation, it was postulated that redistribution of γ-tubulin could underlie maturation-associated changes in microtubule patterning. Our main findings include: (1) redistribution of γ-tubulin from predominantly cytoplasmic centrosomes to a nuclear compartment delimited by a persistent nuclear lamina, (2) sorting of γ-tubulin between cytoplasmic and nuclear (spindle) compartments during meiotic progression based on the reappearance of γ-tubulin in cytoplasmic centrosomes during anaphase of meiosis-1 and -2, and (3) functional uncoupling of karyokinesis and cytokinesis as a
FIG. 3. Changes in γ-tubulin content and microtubule nucleation capacity of cytoplasmic centrosomes at the metaphase-anaphase transition of meiosis-1, and during spontaneous aging and fertilization of metaphase-2 oocytes. Oocytes triple-labeled for pericentrin (A, B), γ-tubulin (a, b), and α/β-tubulin (a’, b’) were analyzed by confocal laser microscopy. Three-dimensional reconstructions (10 optical sections) of regions containing cytoplasmic centrosomes are shown at metaphase (A, a, a’) and anaphase (B, b, b’) of meiosis-1. Note the lack of immunodetectable γ-tubulin staining (a’) and absence of microtubules (a) in association with pericentrin-containing foci at metaphase-1.
result of impaired sorting of γ-tubulin after application of brief pulses of taxol. Together, these observations suggest that modifications in microtubule patterning during meiotic maturation are due to temporally and spatially regulated changes in γ-tubulin distribution.

**Cytoplasmic to Nuclear Redistribution of γ-Tubulin as an Early Determinant in Spindle Morphogenesis**

Our work has made evident the existence of repetitive sorting cycles of γ-tubulin between cytoplasmic and nuclear compartments of mouse oocytes, at times when demands for microtubule patterning shift from stabilization of the cortex to morphogenesis of the spindle. The present study demonstrates an accumulation of γ-tubulin around condensed chromosomes during spindle morphogenesis in mouse oocytes, in agreement with observations in pig oocytes (Lee et al., 2000). Concomitantly, a shift in microtubule assembly from outside to inside the nucleus occurs between diakinesis and prometaphase of meiosis-1 (see Fig. 1). This is consistent with earlier ultrastructural studies (Calarco et al., 1972; Szőlősi et al., 1972) and the work of Messenger and Albertini (1991), showing perinuclear accumulation of centrosomes prior to spindle morphogenesis. The events that intervene between perinuclear microtubule assembly and overt spindle morphogenesis remain unresolved. Our kinetic confocal microscopy analysis documents relocalization of γ-tubulin from a perinuclear to intranuclear sites (see Fig. 1). In combination with the demonstrated enhancement of localized microtubule assembly in response to taxol (Fig. 5), a mechanism for γ-tubulin delivery to chromatin at the onset of spindle formation that involves centrosome translocation from the oocyte cortex to the germinal vesicle seems plausible. Chromatin-mediated spindle assembly mechanisms have been implicated in cells containing acenitrofil centrosomes, including animal oocytes and plant cells (Heald et al., 1996; reviewed by Rieder et al., 1993; Tournebize et al., 1997). Although centrosomes normally harbor γ-tubulin, chromatin-mediated spindle assembly obviates a direct role for centrosomes in establishing spindle bipolarity. The recent demonstrations of γ-tubulin association with chromatin in plant cells and intranuclear γ-tubulin in protists where mitosis is intranuclear (Rotaru et al., 1999; Binarova et al., 2000) raise further questions about how γ-tubulin is delivered to and exchanged across the nuclear/cytoplasmic boundary as well as the need to partition γ-tubulin prior to cell cycle resumption. Together, these findings suggest a requirement for segregating γ-tubulin between nuclear and cytoplasmic compartments to check M-phase entry. Factors controlling centrosome position, and hence γ-tubulin availability, may thus underlie cytoplasmic to nuclear γ-tubulin exchange observed at this critical juncture in meiotic maturation.

The question of how nuclear lamina stability functions during spindle morphogenesis in cells with a large cytoplasmic volume may be relevant to a role for segregating γ-tubulin. As shown in Fig. 1, a time-dependent collapse and reexpansion of the nuclear lamina coincides with both the transition from extranuclear to intranuclear microtubule assembly and the appearance of nuclear γ-tubulin. Our work extends previous reports establishing the persistence of the nuclear lamina well into meiotic M-phase with disassembly subsequent to the formation of a bipolar spindle (Albertini et al., 1993). Protracted nuclear lamina stability during M-phase has been viewed as a form of “intermediate” mitosis in syncytial Drosophila embryos when contrasted to the classical open or closed mitoses seen in many other cellular systems (Paddy et al., 1996). The syncytial mitotic divisions seen in Drosophila embryos also typify cells with a large cytoplasmic volume, and, interestingly, it has been shown in this system that microtubules penetrate the nuclear lamina at discrete sites (Paddy et al., 1996; also see Georgatos et al., 1997). The hypothesis that perinuclear microtubules provide selective delivery of factors across the nuclear lamina remains to be established in Drosophila. In mouse oocytes, we report the presence of a polarized centrosomal aggregate located at a large gap in the nuclear lamina seen during prometaphase of meiosis-1 (Fig. 1). Possibly, these localized changes in the structural integrity of the nuclear lamina allow selective access of spindle-assembly determinants, for example γ-tubulin, or cell cycle-regulatory components, like cyclins. Perinuclear aggregation of organelles is known to follow similar kinetics (Albertini, 1987; Van Blerkom, 1991), and recently a strikingly similar pattern of cytoplasmic to nuclear redistribution in phospholipase C-β1 was shown to occur during meiotic resumption in mouse oocytes (Avazier et al., 2000). As a result, both cell cycle events and microtubule patterning appear to be spatially and temporally integrated with bivalent chromosome interactions that direct spindle morphogenesis.

(A), as was also demonstrated in metaphase-2 oocytes. At anaphase onset, centrosomes concomitantly acquire γ-tubulin (b’) colocalized with pericentrin (b), and microtubule nucleation capacity (b). Correlative γ-tubulin (C, D) and α/β-tubulin (c, d) staining is shown in representative three-dimensional confocal reconstructions (10 optical sections) of metaphase-2-arrested oocytes following in vitro aging (20–22 h total culture time) (C, c) or 20 min postinsemination during in vitro fertilization (D, d). Aged metaphase-2 oocytes display short microtubules (c) that emanate from γ-tubulin-containing cytoplasmic foci (C). Anaphase onset is characterized by appearance of γ-tubulin in cytoplasmic foci, which exhibit extensive microtubule growth (D, d); note the interconnected microtubule pattern between cytoplasmic foci and the spindle (d, arrow). Scale bar, 10 μm.
Intrinsic and Cell Cycle-Regulated Changes in Meiotic Spindle γ-Tubulin

In the present work, taxol-induced disruptions in microtubule dynamics during meiotic resumption did not interfere with γ-tubulin relocalization to chromatin and, as a result, a spindle, albeit monopolar, assembled in association with chromatin (Fig. 5). Interestingly, monopolar spindles exhibited γ-tubulin staining but failed to reorganize into a bipolar spindle, possibly due to aberrant centrosome remodeling. The relative contribution of centrosomes to spindle assembly and function remains a disputed subject (Rieder et al., 1993; Tournebize et al., 1997; Karpen and Endow, 1998; Combelles et al., 2000), but the specific involvement of γ-tubulin in spindle function and meiotic progression has received some attention. The association of γ-tubulin with meiotic spindle microtubules has been reported previously in mouse and pig oocytes at metaphase-2 (Gueth-Hallonet et al., 1993; Lee et al., 2000). Our data clearly establish γ-tubulin persistence in the spindle from prometaphase through to the completion of meiosis (Fig. 2), suggesting that once integrated, chromosome-stabilizing forces may prevent its loss. We observed γ-tubulin staining with spindle microtubules over a consistently greater region than one restricted to spindle poles, the more typical pattern in somatic cells (Lajoie-Mazenc et al., 1994). Spindle microtubule polarity has not been determined in oocytes (Karpen and Endow, 1998). It is conceivable that the rather diffuse γ-tubulin spindle staining observed here would result from a staggered arrangement of microtubules within each half spindle consistent with the fact that γ-tubulin caps microtubule minus ends (Zheng et al., 1995). Thus, an unconventional arrangement of microtubules may exist in mammalian oocyte spindles that, as suggested, may involve unique changes in microtubule polarity during spindle elongation at the metaphase-1 to metaphase-2 transition in pig oocytes (Lee et al., 2000).

While γ-tubulin is retained along spindle microtubules during meiotic progression, immunodetectable γ-tubulin and pericentrin-containing aggregates exhibit cell cycle-dependent fragmentation (anaphase-1), disappearance (telophase-1), and reappearance (metaphase-2) at spindle poles (Fig. 2; Carabatsos et al., 2000). Interestingly, γ-tubulin spindle pole aggregates fragment at anaphase onset, precisely when centrosome numbers increase in the oocyte cortex (Messerger and Albertini, 1991) and centrosomes acquire immunodetectable γ-tubulin and microtubule nucleation capacity (see Fig. 3). Also, drug treatments that cause fragmentation of spindle pole centrosomes have been shown to cause an increase in cytoplasmic centrosome number, suggesting that a dynamic relationship may exist between spindle-associated and cytoplasmic centrosomes (Maro et al., 1985; Combelles et al., 2000). Consistent with this idea is the observation that γ-tubulin is reincorporated into cytoplasmic centrosomes. The relationship between γ-tubulin release and anaphase onset, like many cell cycle control steps, may involve proteolysis of centrosomal com-

**FIG. 4.** Cell-cycle specific effects of taxol pulses on microtubule and γ-tubulin compartmentalization. Correlative α/β-tubulin (A, C, E, G) and γ-tubulin (B, D, F, H) staining patterns in oocytes following brief taxol exposures (1 μM, 10 min) at diakinesis (A, B), prometaphase-1 (C, D), anaphase-1 (E, F), and telophase-1 (G, H) using conventional immunofluorescence analysis. At diakinesis, enlarged perinuclear γ-tubulin-containing centrosomes with numerous microtubules and microtubule bundles parallel to the oocyte cortex are observed in response to taxol (A, B). At prometaphase, taxol enhances microtubule assembly restricted to the nuclear compartment (C), delimited by enlarged γ-tubulin aggregates (D). At anaphase-1, taxol enhances microtubule assembly at both the spindle and oocyte cortex (E, F). Surprisingly, little effect is observed at telophase-1 (G, H). Scale bar, 10 μm.
FIG. 5. Recovery of oocytes from taxol exposure during meiotic resumption alters cell cycle progression. Correlative pericentrin (A) and γ-tubulin (B), or α/β-tubulin (C, E) and pericentrin (D, F) staining patterns following 3 (A, B), 8 (C, D), and 14 (E, F) h of culture after removal from taxol. Confocal three-dimensional reconstructions are shown (24 optical sections), and insets (A, C, E) represent Hoechst chromatin patterns at each meiotic stage. Three hours following taxol exposure, perinuclear centrosome material is disposed as a solitary expanded lattice (A); note the location of γ-tubulin at both perinuclear and intranuclear sites (B; dotted white box highlighting the chromosomal region). After 8 h of culture, a solitary monopolar spindle is observed with chromosomes peripherally displaced (C); pericentrin is associated with both the subcortical monopolar spindle and cytoplasmic foci (D), which showed no evidence of microtubule nucleation (C). After 14 h of culture, multiple monopolar spindles are observed in the cortex distal from a larger monopolar spindle (E); centrosome foci are not associated with the larger monopolar spindle and infrequently associated with other monopolar spindles (F, arrows). Centrosome foci are disposed to the more tapered poles of monopolar spindles distal from the pole bearing attached bivalents. Cytoplasmic centrosomes fail to show associated microtubules (E), and lack γ-tubulin (see text). Scale bar, 10 μm.
ponents to liberate γ-tubulin stores for use in cortical microtubule assembly. The release of spindle γ-tubulin and concomitant burst of cortical microtubule assembly could account for local changes in actin-based motility needed for polar body extrusion (Longo and Chen, 1985; Maro et al., 1986; Van Blerkom and Bell, 1986). In this context, it is interesting to note that perturbation of γ-tubulin sorting during meiotic progression arrests karyokinesis but not cytokinesis (Fig. 5). Thus, the coordination of karyokinesis and cytokinesis during maturation of mammalian oocytes probably involves regulated exchange of molecules between the meiotic spindle and cortex to synchronize balanced segregation of genomic and cytoplasmic cargoes.

**Perspectives on γ-Tubulin Dynamics Across Animal Classes**

Modes of centrosome inheritance have been studied extensively in oocytes of several mammalian species at the time of fertilization (Schatten and Schatten, 1986; Schatten et al., 1986; Schatten, 1994). While it was first postulated that, in early embryos, centrosome material originated solely from paternal sources, except in the mouse, a new paradigm has emerged recently, highlighting the contribution from both maternal and paternal centrosome components to liberate γ-tubulin stores for use in cortical microtubule assembly. The release of spindle γ-tubulin and concomitant burst of cortical microtubule assembly could account for local changes in actin-based motility needed for polar body extrusion (Longo and Chen, 1985; Maro et al., 1986; Van Blerkom and Bell, 1986). In this context, it is interesting to note that perturbation of γ-tubulin sorting during meiotic progression arrests karyokinesis but not cytokinesis (Fig. 5). Thus, the coordination of karyokinesis and cytokinesis during maturation of mammalian oocytes probably involves regulated exchange of molecules between the meiotic spindle and cortex to synchronize balanced segregation of genomic and cytoplasmic cargoes.

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