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Organization of Cytoplasmic Microtubules during Maturation of Goldfish Oocytes

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ABSTRACT—The organization of cytoplasmic microtubules during hormone-induced meiotic maturation of goldfish oocytes *in vitro* was examined by confocal immunofluorescence microscopy using an anti-tubulin antibody. The microtubule network was well distributed in fully grown immature oocytes. Once goldfish oocytes resumed meiotic maturation by a proposed maturation-inducing hormone of this species (17α, 20β-dihydroxy-4-pregnen-3-one, 17α, 20β-DP), cytoplasmic microtubules serially re-organized. Soon after the onset of the germinal vesicle (GV) migration towards the animal pole, the former microtubule network disappeared, followed by the appearance of a long perinuclear tail with high ordered microtubules extending from the vegetal surface of the GV. Incubation of fully grown immature follilces in colcemid, an inhibitor of tubulin polymerization, caused the disappearance of microtubules. However, this treatment did not prevent either the 17α, 20β-DP-induced migration of the GV or GVBD. Coincident with the breakdown of the GV (GVBD), numerous microtubules intruded into the GV from its vegetal surface. Soon after GVBD, a disk-shaped ring consisting of microtubule asters and a small ring with a radial array of microtubules in its center was observed at the animal pole region. In mature oocytes with meiotic spindles at the animal pole surface, cytoplasmic microtubules were concentrated in a small region around the animal pole showing complicated microtubule arrays. The results presented define distinct changes in microtubule organization during the 17α, 20β-DP-induced meiotic maturation of goldfish oocytes.

INTRODUCTION

During oogenesis, oocytes of many animal species are arrested at the first meiosis prophase and undergo tremendous growth. Fully grown immature oocytes then resume meiosis under the influence of maturation-inducing hormone (MIH) and become fertilizable. A number of studies on oocyte maturation have been perfomed in fish oocytes because their maturation can be easily induced *in vitro*. Furthermore, individual females generally contain a large number of fully grown immature oocytes. Under the influence of MIH, then mediated by maturation-promoting factor (MPF), fully grown immature oocytes will progress through a series of events of meiotic maturation (Nagahama and Adachi, 1985; Nagahama, 1987a, b; Nagahama and Yamashita, 1989). The first event is the migration of a centrally located germinal vesicle (GV) towards the animal pole, followed by the breakdown of the GV (GVBD), condensation of chromosomes and the formation of spindles. When oocytes are immersed in clearing solution, the GV position in oocytes during migration can be analyzed under the dissection microscope (Lessman and Kavumpurath, 1984). Therefore, fish oocytes provide a useful experimental system with which to study the mechanisms of hormonally induced meiotic maturation in animal oocytes. Recently, MPF has been purified and characterized from carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*) oocytes (Yamashita *et al.*, 1992), and the basic mechanisms involved in the activation of MPF have been investigated extensively in the latter (Katsu *et al.*, 1993).

Despite rapid progress in studies of fish oocyte maturation, very little is known about the organization of the cytoskeletal components such as microtubules and microfilaments and their possible roles in the meiotic maturation of fish oocytes. Habibi and Lessman (1985, 1986) have examined the influence of cytoskeleton inhibitor drugs on goldfish oocyte maturation. Microfilament bundles have been briefly identified in zebrafish (*Zebradanio rerio*) oocytes using electron microscopy but without knowing the oocyte size (Kessel *et al.*, 1984). However, there have been no detailed morphological studies on the cytoskeletal components in fully grown oocytes or maturing oocytes of fish. The main obstacle for cytoskeleton observations may arise from the large size and amount of yolk in fully grown oocytes, which make serial paraffin sections difficult to prepare.

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In the present study, we examined the organization of microtubules in fully grown immature and maturing oocytes of goldfish using confocal immunofluorescence microscopy. The results indicated that microtubules spread in yolk-free regions of cytoplasm throughout fully grown immature oocytes, forming a well-distributed network. As oocytes underwent maturation induced by MIH *in vitro*, the significant reorganization of cytoplasmic microtubule distribution coincided with GV migration and GVBD. These novel results provide a detailed description of microtubule organization during hormonally induced oocyte maturation in fish *in vitro*.

MATERIAL AND METHODS

Hormone-induced oocyte maturation in vitro

Goldfish were obtained commercially and housed in freshwater aquaria at 15°C until use. Female goldfish with fully grown oocytes were killed by decapitation and the ovaries were immediately placed in goldfish Ringer's solution (Kagawa *et al.*, 1984). Oocytes were separated from ovary by fine forceps and incubated at room temperature in goldfish Ringer's solution containing 1 μg/ml 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DP, a proposed MIH of goldfish). Oocytes were collected every hour after the administration of 17α, 20β-DP for fixation. At the same time, some oocytes were immersed in clearing solution (5% acetic acid) to examine GV migration and GVBD.

Detergent extraction and fixation

Triton X-100 (0.5%) extraction solution containing 0.5 μM taxol was prepared with microtubule assembly buffer (BRB) (Gard, 1991). Oocytes were incubated in extraction solution for 5-10 min before fixation. During extraction the follicle cells formed a loose collar around the oocyte, which was easily removed by forceps under dissection microscope.

The fixatives were 3.7% formaldehyde prepared with BRB containing 0.2% Triton X-100 and 0.5 μM taxol, as well as 3.7% formaldehyde plus 0.25% glutaraldehyde in BRB containing 0.2% Triton X-100 and 0.5 μM taxol. Immediately after extraction, oocytes were transferred to either fixative for 3-4 hr at room temperature.

Immunofluorescence

Fixed oocytes were washed in PBS (128 mM NaCl, 8 mM NaH_2PO_4, 2 mM KH_2PO_4, pH 7.2), then in TBS (155 mM NaCl, 10 mM Tris-Cl, pH 7.4, 0.1% NP-40) for 6 hr at room temperature or overnight at 4°C. Oocytes were cut laterally or equatorially using fine blades under the dissection microscope before the incubation in primary antibody. For oocytes fixed with the combination of formaldehyde and glutaraldehyde, one more incubation in 100 mM $NaBH_4$ overnight at 4°C was necessary before the TBS wash.

Dissected oocytes were incubated in primary antibody DM1A, a monoclonal anti-α-tubulin antibody (Blose *et al.*, 1984) (Oncogene Science, INC.), diluted 1:200 in TBS containing 2% BSA for 24 hr, followed by a TBS wash for 24 hr with 5 changes of fresh TBS. The oocytes were transferred to a 100-fold dilution of FITC-conjugated goat anti-mouse IgG (MBL, Japan) for 24 hr and washed for 24 hr. All antibody incubations and washes proceeded at 4°C with gentle rotary agitation.

Oocytes were dehydrated in several changes of absolute methanol, then mounted in benzyl benzoate: benzyl alcohol (1 : 2). Confocal laser scanning microscopy was performed using a BioRad MRC-500 (Bio-Rad Microsciences; Cambridge, MA), fitted to a Nikon Optiphot equipped with 10 × (NA 0.45), 20 × (NA 0.75), 40 × (NA 1.0) and 60 × (NA 1.4) objectives and a BHS filter set. Samples were examined first at low magnification (10 × or 20 × objectives), then at high magnification (60 × objective) for detailed observation.

RESULTS

Fully grown oocytes

Unstimulated, fully grown immature oocytes of goldfish contain an extensive array of microtubules throughout the cytoplasm. The anti-tubulin antibody staining image at low magnification appeared as a bright, well-distributed network throughout the cytoplasm in which dark yolk granules were surrounded by bright yolk-free regions. Centrally located GV was not stained by DM1A (Fig. 1A). At high magnification, microtubules could be discerned in yolk-free regions of cytoplasm in oocytes (Fig. 1B). From a single optical section, numerous microtubules always appeared as short fibrous arrays spreading along the yolk granules. Among tightly contacted yolk granules, microtubules were located immediately around the surface of the yolk granules. In some areas where yolk-free regions were relatively large, linked microtubules were obvious. Serial optical sections also revealed microtubules surrounding yolk granules and continuously in yolk-free regions. A well-distributed microtubule network was present in fully grown oocytes except for the GV. A closer examination of the GV revealed a band of brightly stained microtubules, hereinafter referred to as the bright band, surrounding the surface of the GV. From the bright band, matrix material extended into clefts formed by the convoluted surface of the GV. No microtubules were discerned in this matrix material (Fig. 1C).

Folliculated oocytes maintained their micropylar cells. At high magnification, the micropylar cell was revealed as a bright tunnel-like structure that penetrated the zona radiata and attached to the animal pole surface of oocytes. Numerous microtubules were present in the micropylar cell (Fig. 1D).

Oocytes induced to mature by 17α, 20β-DP treatment in vitro

Fully grown immature oocytes of goldfish were induced to resume meiotic maturation (GV migration, GVBD, and spindle formation) by 17α, 20β-DP *in vitro*. In general, oocytes undergo GV migration 3-4 hr after incubation with 17α, 20β-DP and complete GVBD by 8 hr.

The onset of meiosis resumption in goldfish oocytes triggered by 17α, 20β-DP was characterized by migration of the GV. As soon as the GV began to migrate to the animal pole, the cytoplasmic microtubules exhibited marked reorganization. The former well-distributed fluorescence disappeared in oocytes at this stage. Instead, intense fluorescence was concentrated mainly in the animal hemisphere. The most conspicuous feature of oocytes at this stage was a long, brightly stained region of yolk-free cytoplasm, hereinafter referred to as the perinuclear tail, directly extending from the vegetal surface of the GV to the vegetal hemisphere of oocytes. At low magnification, this perinuclear tail was around 100 μm long (Fig. 2A). Closer examination of the perinuclear tail revealed numerous microtubules extending from the bright band at the vegetal surface of the GV toward the vegetal

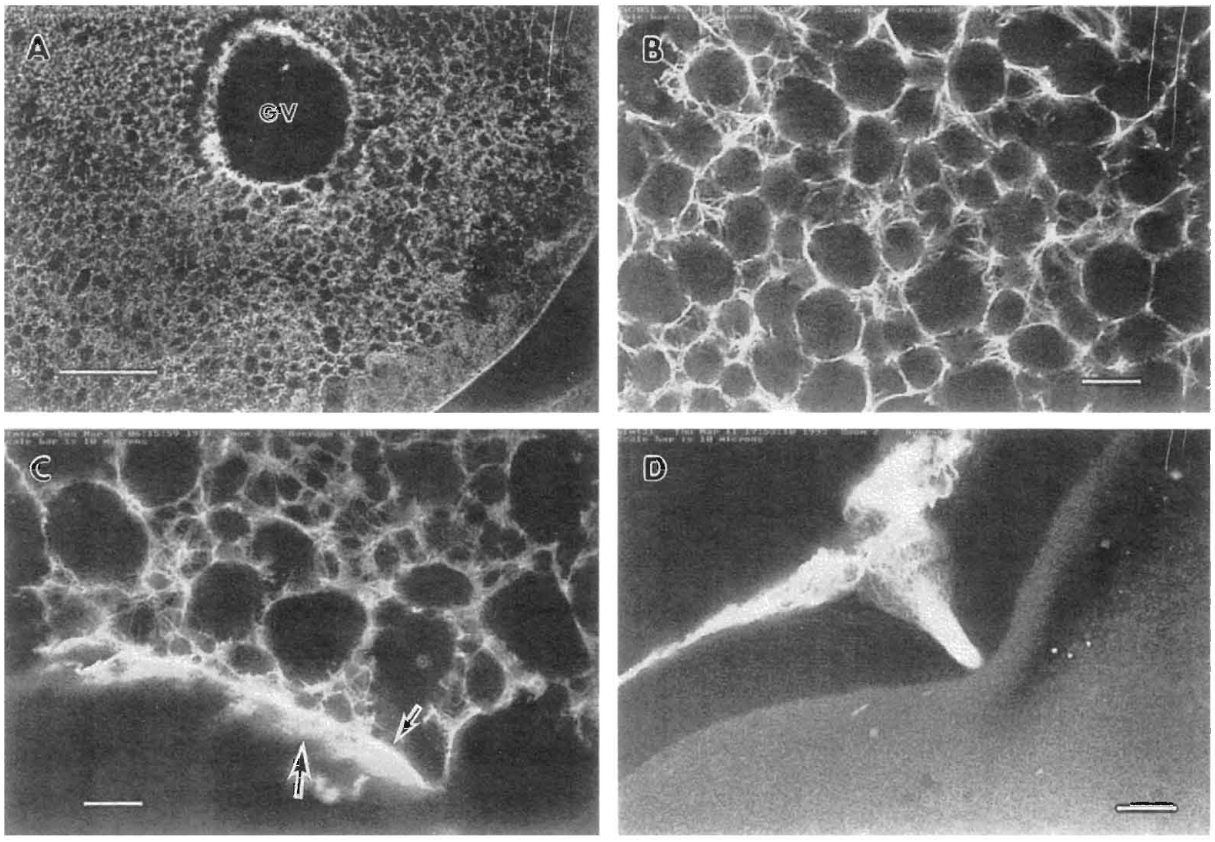

Fig. 1. Microtubule network in fully grown oocytes. A: Well-distributed anti-tubulin staining of cytoplasm at low magnification (10 X obj; bar is 100 μm). B: Microtubules in yolk-free regions in fully grown oocytes at high magnification (60 X obj; bar is 10 μm). C: Bright band of microtubules (small arrow) surrounding the GV surface and bright matrix material (large arrow) in clefts (60 X obj; bar is 10 μm). D: A micropylar cell at the animal pole surface (60 X obj; bar is 10 μm).

hemisphere (Fig. 2B). Continuous microtubules were traced as far as 30 μm from a single optical section. Microtubules in the bright band were continuous with microtubules in the perinuclear tail. Meanwhile, microtubules appearing in the matrix material in clefts, emanated from the bright band. Except for the perinuclear tail, microtubules disappeared from most areas of the vegetal hemisphere (Fig. 2C). In the animal hemisphere, microtubules remained in the yolk-free regions but with a different configuration. They appeared not as immediately around yolk granules. Instead, since the yolk-free regions in the animal hemisphere became large at this stage, microtubules formed complex networks in the yolk-free regions (Fig. 2D).

As the GV reached the animal pole of oocytes, the animal surface became flattened due to pressure against the animal pole surface. A semi-circular GV with a bright vegetal surface was observed at low magnification (Fig. 3A). Closer examination of the vegetal surface revealed leaf-like structures extending along the bright band into the GV. Parallel microtubules extended from the bright band and spread throughout each "leaf" (Fig. 3B). Numerous microtubules were also be observed in the region near the vegetal surface of half-round GV at high magnification, with an apparent connection to those in the bright band.

As the GV continually moved forward, the GV shape seen at low magnification was in fact rectangular with its vegetal surface becoming large and brightly stained (Fig. 3C). At high magnification, both the bright band at the vegetal surface of the rectangle GV and the individual leaf-like structure disappeared. Instead, numerous microtubules spread in the large brightly stained region (Fig. 3D).

In oocytes that had undergone GVBD (GVBD oocytes), cytoplasmic microtubules disappeared from most areas except the animal pole region, where they underwent a final reorganization coinciding with the assembly of meiotic spindles. Shortly after GVBD, a bright disk-shaped structure surrounded by a bright region appeared. There was a narrow, dark, microtubule-free region between the disk-shaped and the bright region (Fig. 4A). High magnification resolved the disk into a complex structure in which the microtubule asters were most remarkable (Fig. 4B).

The organization of microtubules in the surrounding bright region was very complex at high magnification. Usually, microtubules appeared to spread in amorphous cytoplasm with complicated associations. In some areas, microtubule asters were embedded in numerous non aster-like microtubules (Fig. 4C).

The disk-shaped structure soon disappeared and a small

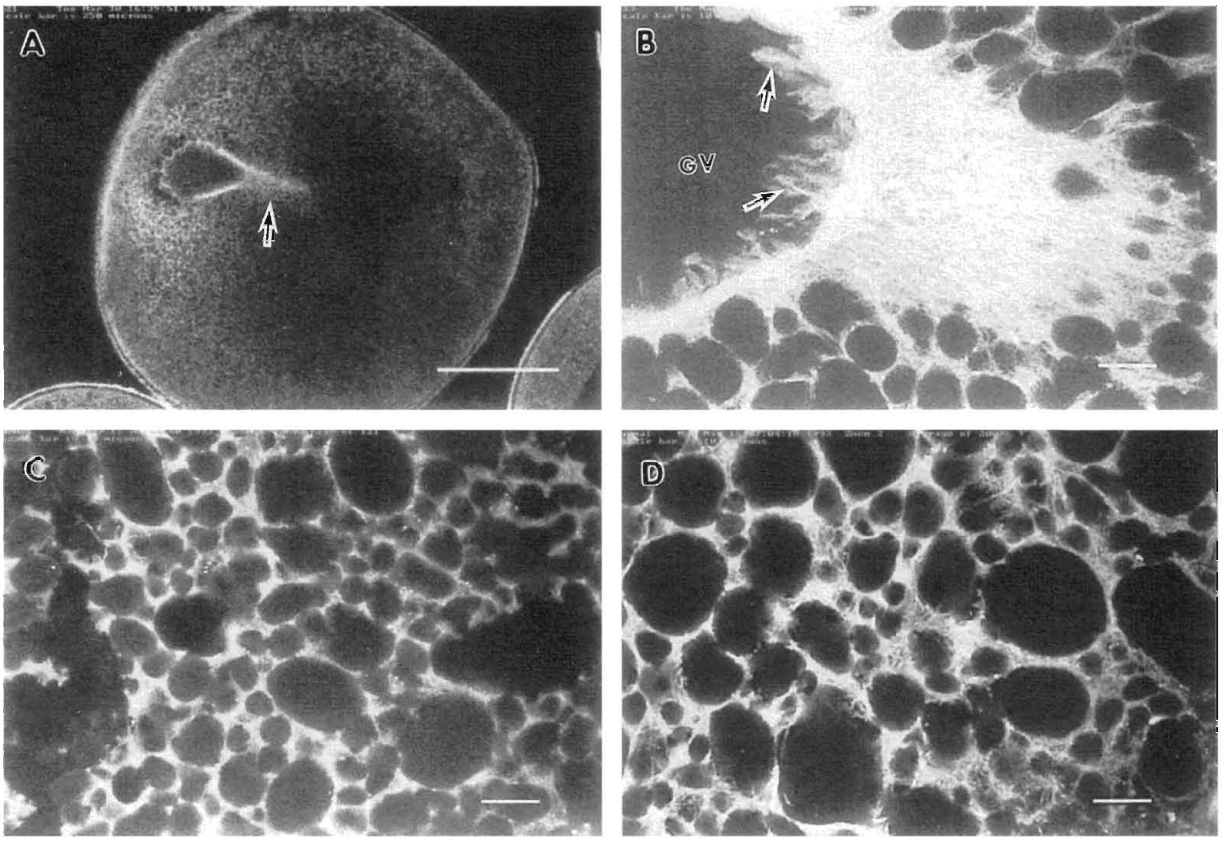

Fig. 2. Microtubule reorganization during GV migration to the animal pole. A: Migrating GV with a bright long perinuclear tail (arrow) (10 X obj; bar is 250 μm). B: Numerous long microtubule arrays in the perinuclear tail and short microtubules in clefts (arrow) (60 X obj; bar is 10 μm). C: Vegetal hemisphere cytoplasm with no microtubules in oocytes with migrated GV (60 X obj; bar is 10 μm). D: Microtubules in the yolk-free region in the animal hemisphere of oocytes with migrated GV (60 X obj; bar is 10 μm).

ring appeared (Fig. 4D). Closer examination of this small ring revealed that it was also formed by microtubules but without asters. Serial optical sections often revealed that the center of the ring consisted of radial array of microtubules that resembles a partial spindle (Fig. 5A). This small ring disappeared, then there were no more big or special microtubule structures, and only a small bright region remained around the animal pole at low magnification (Fig. 5B). Closer examination of this region resolved the complex cytoplasmic microtubule arrays including microtubule asters. At the animal surface a barrel-shaped meiosis spindle was evident at high magnification (Fig. 5C).

Effect of colcemid upon 17α,20β-DP-induced oocyte maturation

Colcemid did not prevent either 17α, 20β-DP-induced GV migration or GVBD in fully grown goldfish oocytes. One hour after incubation with 17α, 20β-DP and colcemid (100 μg/ml), microtubules disappeared in the yolk-free region. As the GV migrated toward the animal pole, the perinuclear tail appeared (Fig. 6A). However, no microtubules were discerned even upon closer examination (Fig. 6B). Shortly after GVBD, a bright region also appeared around the animal pole (Fig. 6C), but there was no microtubular structure in this region (Fig. 6D). Spindles and microtubule asters in the bright region were not found in oocytes after GVBD.

DISCUSSION

Confocal immunofluorescence microscopy has become a useful means of microtubule observation that has been applied to animal oocytes (Gard, 1991, 1992; Schroeder and Gard, 1992). The optical sectioning capabilities of confocal microscopy allow microtubule observation in large oocytes without using traditional paraffin sections. In this study, using formaldehyde-taxol or formaldehyde-glutaraldehyde-taxol fixation after Triton X-100 extraction, microtubules in both fully grown and maturing oocytes of goldfish *in vitro* were observed under confocal microscope. Triton X-100 extraction was necessary before fixation to remove soluble cytoplasmic tubulins, which allowed the removal of background immunofluorescence (Balczon and Schatten, 1983). In addition, Triton X-100 damaged follicle layers, which helped the penetration of fixatives throughout the oocytes. There were no differences between oocytes with or without follicle cells after extraction.

Although the monoclonal antibody DM1A is broadly reactive with α-tubulin from a variety of species (Blose *et al.*,

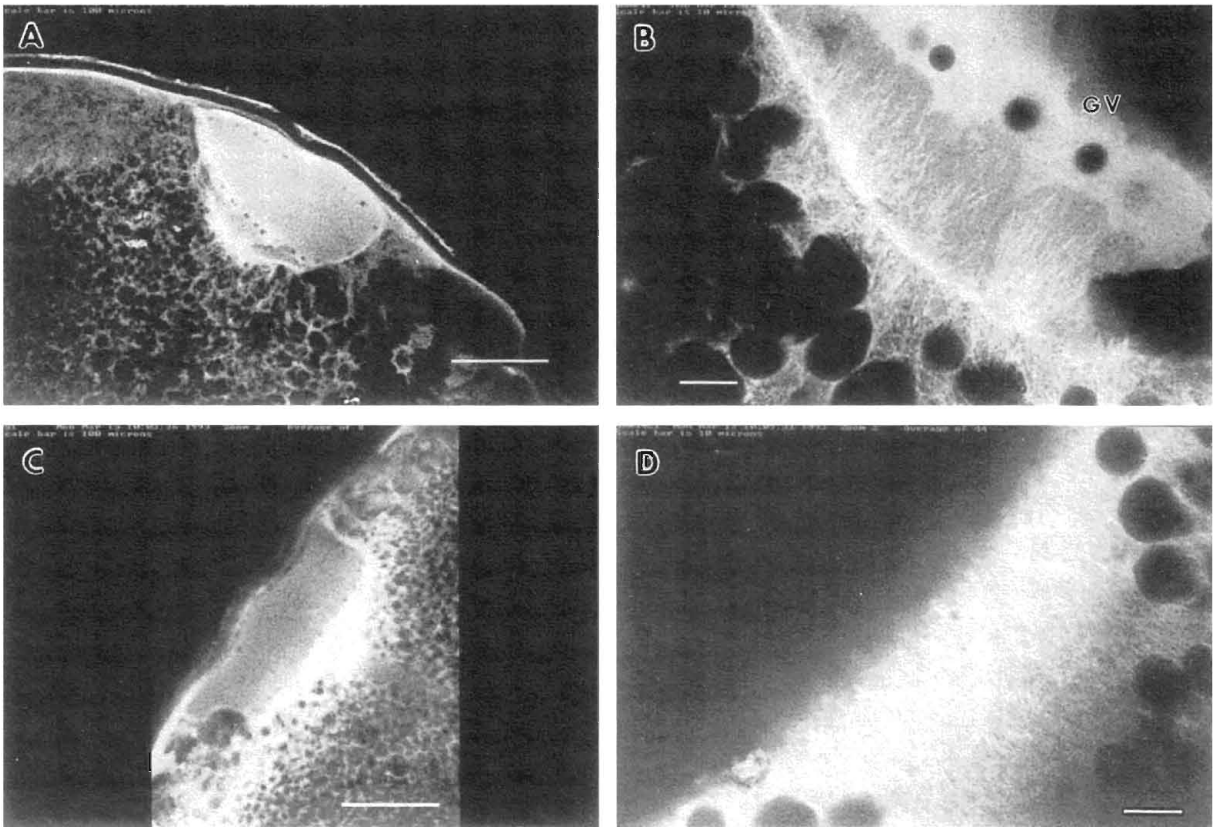

Fig. 3. The GV at the animal pole and microtubules at its vegetal surface. A: Half-round GV with a bright vegetal surface(10 X obj; bar is 100 μm). B: High magnification reveals leaf-like microtubule structures intruding into the GV from the bright band at the vegetal surface of GV (60 X obj; bar is 10 μm). C: Rectangle GV with its bright region along the vegetal surface (10 X obj; bar is 100 μm). D: Numerous microtubules are embedded in the vegetal surface (60 X obj; bar is 10 μm).

l984), control experiments have been conducted using either no primary antibody or an anti-tilapia prolactin anybody that has no immunoreaction in goldfish oocytes (Specker *et al.*, 1993). No microtubules were observed in control oocytes (data not shown). In addition, oocytes incubated in MIH and colcemid can be also considered controls. These results confirmed the positive staining of microtubules in goldfish oocytes.

Although microtubules or anti-tubulin staining has been demonstrated in oocytes of many animal species (Albertini, 1987; Gard, 1991, 1992; Maro *et al.*, 1985, 1988; Otto and Schroeder, 1984; Schroeder and Gard, 1992; Wassarman and Fujiwara, 1978; Zernicka-Goetz *et al.*, 1993), Gard (1991) was the first to describe microtubule networks in *Xenopus* oocytes by means of confocal immunofluorescence microscopy. In *Xenopus*, there is a complex network of microtubules in oocytes at all stages of oogenesis, and polarization of microtubule arrays is apparent as the GV migrates to the animal pole during the later stage of oogenesis. Fully grown immature oocytes of goldfish have a well-distributed complex network of microtubules. On a single optical section, microtubules in fully grown oocytes always appeared as short fragments along yolk granules. Serial optical sections showed that microtubules surrounded yolk granules and continued in the yolk-free region in oocytes. Therefore, a complex network of microtubules was distributed throughout the whole oocyte except for the GV region. The GV surrounded by the bright band is located at the center of this microtubule network.

Soon after the onset of the GV migration toward the animal pole, the well-distributed microtubule network disappeared and cytoplasmic microtubules gradually concentrated in the animal hemisphere. Finally in mature oocytes, the cytoplasmic microtubules are present only in a small area around the animal pole. The role of microtubules in the migration of the GV and GVBD in goldfish oocytes has been studied by Lessman and his colleagues (Lessman and Kavumpurath, 1984; Habibi and Lessman, 1985, 1986; Lessman *et al.*, 1988). Microtubule destabilizing drugs including colcemid, cocodazole and vinblastine elicit the migration of the GV and enhance the induction of the GV migration by 17α, 20β-DP. By contrast, taxol, a microtubule stabilizing drug, inhibits steroid induced GV migration (Lessman *et al.*, 1988). We also observed a similar enhancing effect of colcemid on 17α, 20β-DP-induced GV migration in goldfish oocytes (Jiang and Nagahama, unpublished). In the present study, incubating fully grown goldfish oocytes in colcemid resulted in the disappearance of microtubules. However, colcemid did not prevent either the 17α, 20β-DP-induced migration of the GV or GVBD. These findings suggest that the microtubule network of fully grown

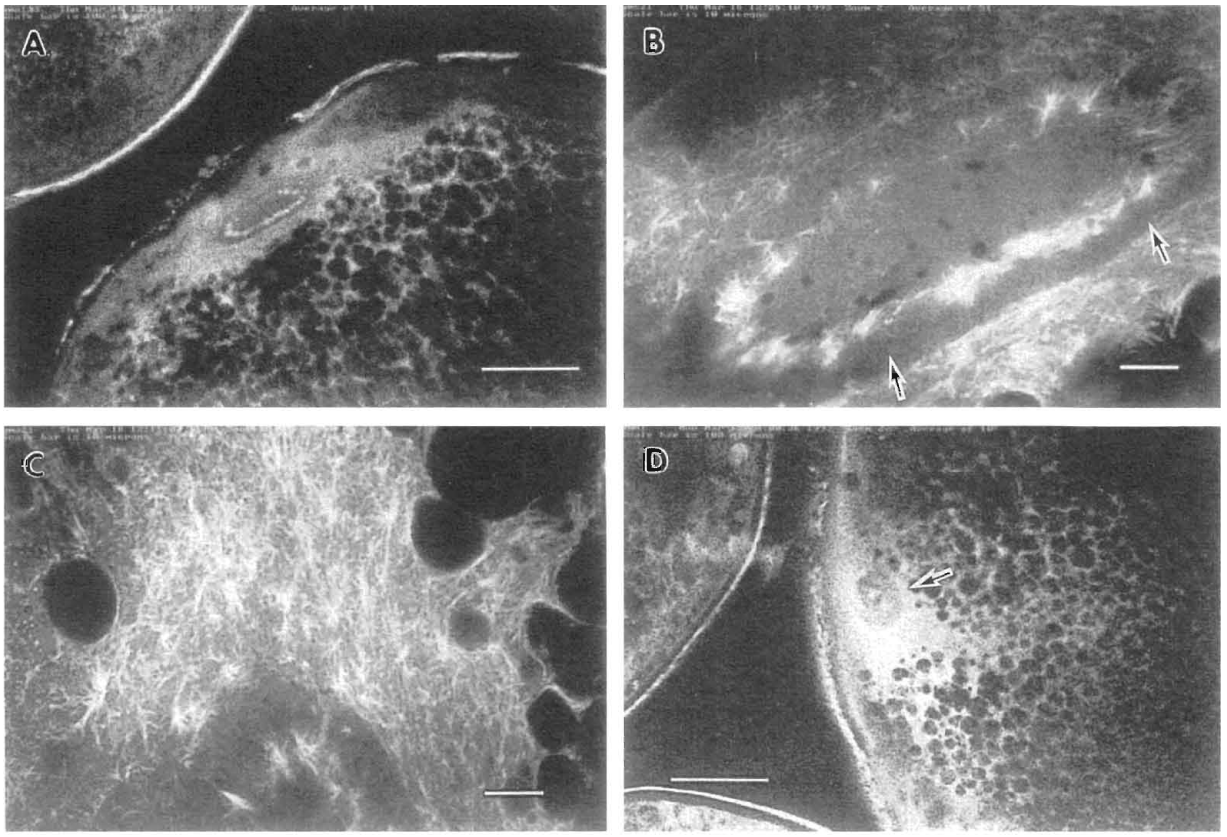

Fig. 4. Microtubule organization in the animal pole region after GVBD. A: Disk-shaped structure surrounded by a bright region (10 X obj; bar is 100 μm). B: Microtubule asters in the disk-shaped structure with a microtubule-free area (arrow) (60 X obj; bar is 10 μm). C: Microtubule asters embedded in non-aster microtubules in the bright region surrounding the disk-shaped structure (60 X obj; bar is 10 μm). D: The bright region surrounding a small ring (arrow) at low magnification (10 X obj; bar is 100 μm).

immature oocytes plays a role in stabilizing the GV in the center of oocytes, and that the disassembly of this network is necessary for GV migration to the animal pole. Gard (1991, 1993) also demonstrated a similar stabilizing role of microtubules in *Xenopus* oocytes. In his study, exposing stage VI oocytes to nocodazole or cold results in a variable displacement of the GV within the animal hemisphere. Habibi and Lessman (1985) reported that cytochalasin B, an inhibitor of microfilament-related contractile processes, prevented the 17α, 20β-DP-induced GV migration and GVBD. These results suggest the involvement of microfilaments in the mechanisms of GV migration and GVBD in goldfish oocytes.

Oocytes with a migrated GV exhibit a distinctive perinuclear tail with long fibrous microtubules within it. A similar structure, the perinuclear cap, is also attached to the vegetal surface of the GV in *Xenopus* stage VI oocytes (Gard, 1991). In contrast to the highly ordered microtubule spreading in the perinuclear tail of goldfish oocytes, microtubules in the perinuclear cap of *Xenopus* oocytes seem to spread randomly. Goldfish oocytes incubated in MIH and colcemid retained a bright perinuclear tail although no microtubules were observed even at high magnification. Tubulins seemed to accumulate in the perinuclear tail even though they could not be assembled into microtubules. It seems likely that the perinuclear tail represents one way by which cytoplasmic materials that are necessary for the following maturation processes accumulate and migrate.

In this study, the GVBD of goldfish oocytes was accompanied by a series of changes in microtubule organization at the vegetal surface of the GV. These observations suggest that the GVBD of goldfish oocytes begins at the vegetal surface of the GV and is accompanied by the invasion of microtubules. Studies on maturation in amphibian oocytes have indicated that GVBD begins at the basal or vegetal surface of the GV (Brachet *et al.*, 1970; Gard, 1992; Huchon *et al.*, 1981). Coincident with basal nuclear membrane breakdown, a transient microtubule array (TMA) appears, then rapidly moves toward the animal pole.

The disk-shaped structure at the animal pole region in GVBD oocytes of goldfish is unique with respect to its aster-like microtubule structures and its surrounding microtubule-free region. The exact function of this structure is not clear at present. However, the timing of its appearance and its position in oocytes suggests a role in spindle formation or meiotic chromosome organization. This notion is supported by the observation of a small ring that probably derived from the disk-shaped structure. A radial array of microtubules was located at its center, which resembled the microtubule array of a

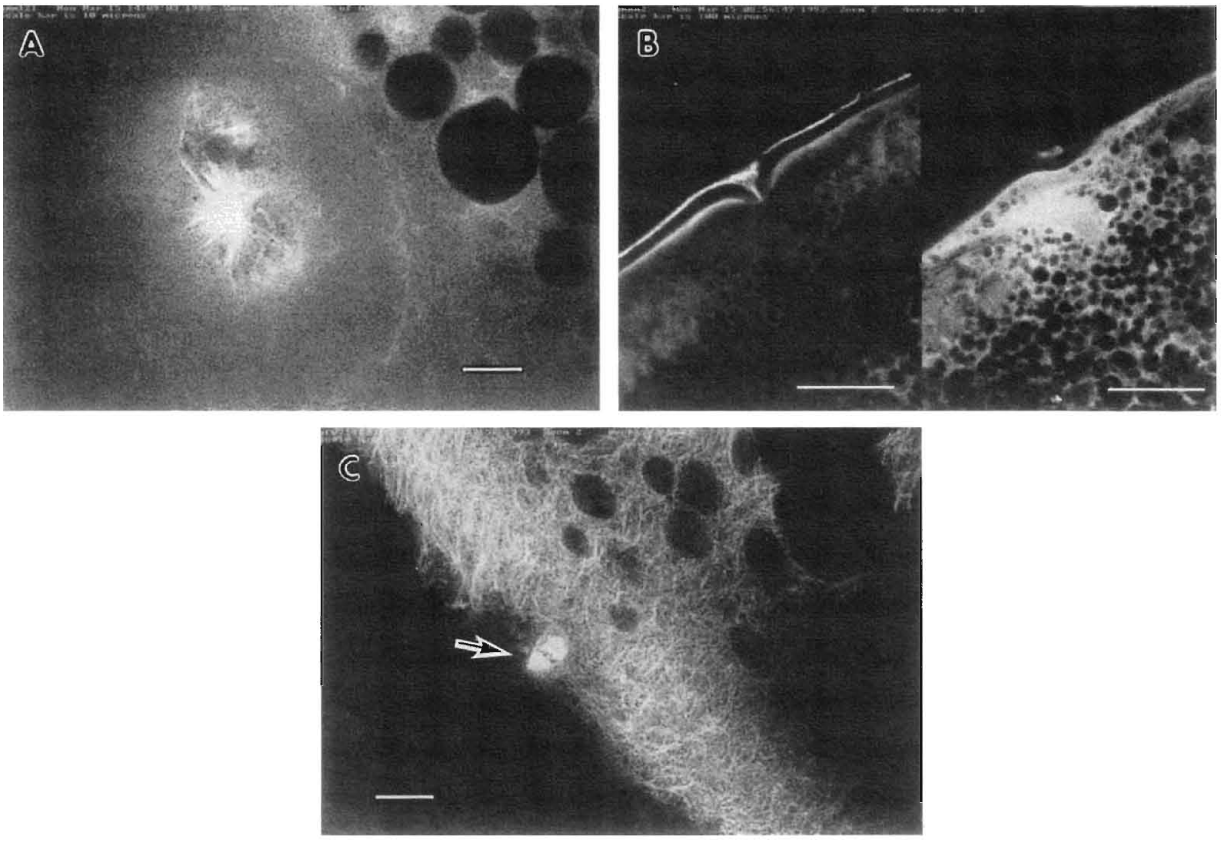

Fig. 5. A: Aster arrays of microtubules in the center of the small ring (60 X obj; bar is 10 μm). B: *Right*, Small bright region without a specific structure at the animal pole (10 X obj; bar is 100 μm). *Left*, The micropylar cell remained at the animal pole visualized at a different optical section. C: A spindle (arrow) at the animal pole surface surrounded by complex cytoplasmic microtubule arrays in the bright region in a mature oocyte (60 X obj; bar is 10 μm).

spindle. In amphibian oocytes, TMA is closely associated with meiotic chromosomes, suggesting that it gathers and transports oocyte chromosomes to the animal pole, and serves as an immediate precursor of the first meiosis spindle (Gard, 1992; Huchon *et al.*, l881; Jessus *et al.*, 1986). To investigate the function of the disk-shaped structure or small ring in goldfish oocytes, DNA and microtubule staining is necessary in the same oocyte.

Cytoplasmic microtubule asters were obvious in the animal pole region of goldfish oocytes that had undergone GVBD. A similar structure was also found in the GVBD oocytes of several other fish species (data not shown). Thus, it seems likely that cytoplasmic microtubule asters are a common feature in fish oocytes that have undergone GVBD. Furthermore, the metaphase-II arrested oocytes of goldfish (unfertilized eggs, induced to mature and ovulate by HCG injection *in vivo*) also contained microtubule asters in the animal pole region (data not shown). These observations suggest that the cytoplasmic microtubule asters arise in oocytes during meiotic maturation subsequent to GVBD. Taxol induces the formation of microtubule asters in mature oocytes of mammals and *Xenopus*. However, this effect of taxol is stage-specific and limited only to oocytes that have undergone GVBD (Albertini, 1987; Heidemann and Gallas, 1980; Maro *et al.*, 1985, 1988; Zernicka-Goetz *et al.*, 1993). These results suggest that there is a critical change in the ability of the tubulin pool to polymerize in oocytes that had undergone GVBD. Further studies will be required to determine the role of the microtubule asters in goldfish GVBD oocytes during meiotic maturation, fertilization, and the later stages.

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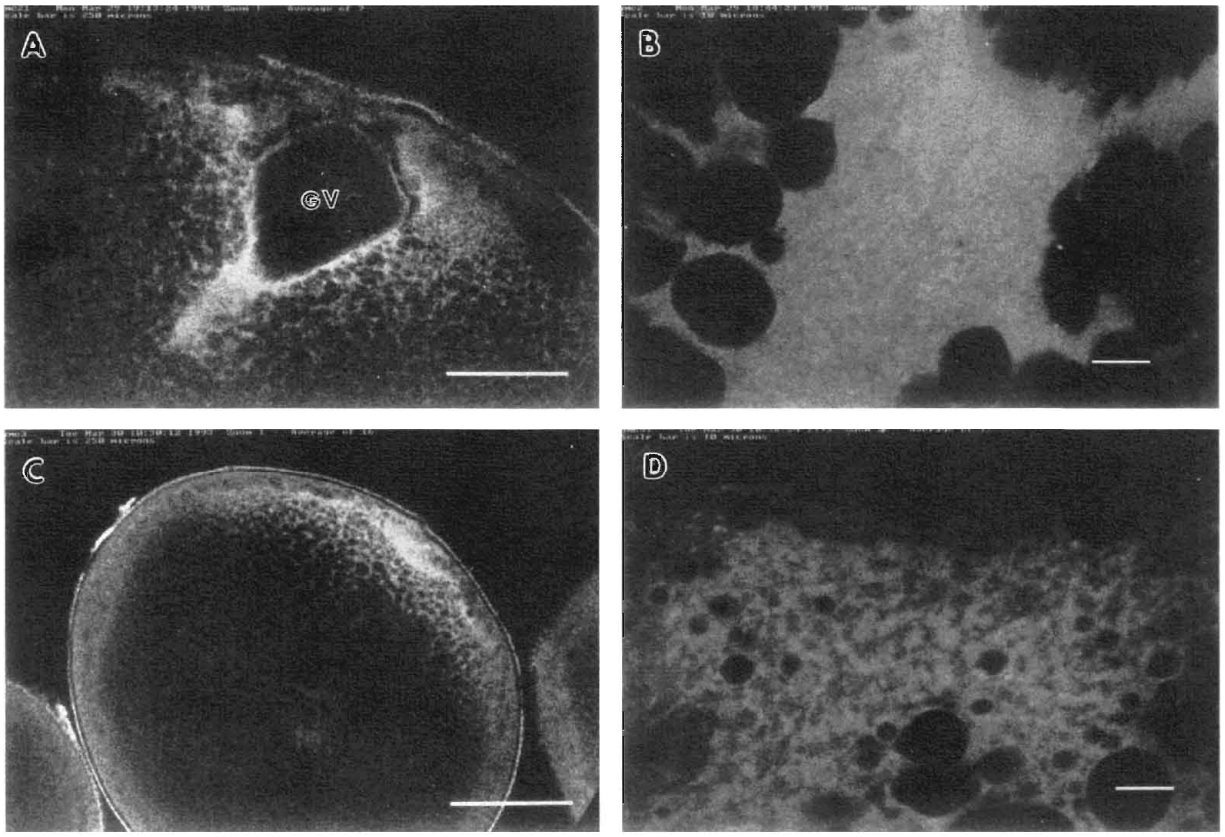

Fig. 6. Anti-tubulin fluorescence in oocytes incubated with 17α, 20β-DP and colcemid. A and C: The bright perinuclear tail at the vegetal surface of migrating GV and a small bright region around the animal pole region after GVBD (10 X obj; bar is 250 μm). B and D: High magnification showing no microtubules in the perinuclear tail and bright region (60 X obj; bar is 10 μm).

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