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Abstract

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Beta- and Gamma-Cytoplasmic Actins Are Required for Meiosis in Mouse Oocytes

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ABSTRACT

In mammals, female meiosis consists of two asymmetric cell divisions, which generate a large haploid oocyte and two small polar bodies. Asymmetric partitioning of the cytoplasm results from migration of the meiotic spindle toward the cortex and requires actin filaments. However, the subcellular localization and the role of the existing two cytoplasmic actin (CYA) isoforms, beta and gamma, have not been characterized. We show that beta- and gamma-CYA are differentially distributed in the maturing oocyte from late metaphase I as well as in preimplantation embryos. Gamma-CYA is preferentially enriched in oocyte cortices and is absent from all cell-cell contact areas from metaphase II until the blastocyst stage. Beta-CYA is enriched in contractile structures, at cytokinesis, at cell-cell contacts, and around the forming blastocoel. Alteration of beta- or gamma-CYA function by isoform-specific antibody microinjection suggests that gamma-CYA holds a major and specific role in the establishment and/or maintenance of asymmetry in meiosis I and in the maintenance of overall cortical integrity. In contrast, beta- and gamma-CYA, together, appear to participate in the formation and the cortical anchorage of the second meiotic spindle in waiting for fertilization. Finally, differences in gamma-CYA expression are amongst the earliest markers of cell fate determination in development.

INTRODUCTION

Female meiosis in humans is highly error prone. Current risk figures suggest that by the fourth decade of life approximately half of the oocytes a woman ovulates may be chromosomally abnormal [1–3]. In addition, more than 90% of cases of trisomy 21 result from errors in maternal meiosis [4]. The causes of these age-related aneuploidies are most likely multi-factorial. Several aspects of meiosis have been shown to contribute to these chromosomal anomalies, such as aberrant meiotic chromosome recombination [5] or age-related decline in cytoskeletal functions [6–8]. Mammalian female meiosis begins and arrests in the embryonic ovary, resumes around ovulation, and is complete only after fertilization; in humans, this time span can represent 30 to 40 yr.

Many of the molecular mechanisms controlling meiotic maturation, i.e., the completion of meiosis I, are the same in mice and man. Though mice do not display age-related aneuploidy, presumably because of their short lifespan, and because their fully grown oocytes can be obtained in large numbers and spontaneously resume meiosis in vitro, they represent a good model to study fundamental biological processes driving oocyte maturation.

Cell polarization and asymmetrical divisions are crucial for the extrusion of small polar bodies during female meiosis and for the establishment of trophoblast and inner cell mass (ICM) cell lineages in the blastocyst [9]. The establishment of asymmetry in mouse oocytes relies on the positioning of the first meiotic spindle under the cortex and its anchorage in the cortex while waiting for fertilization [10]. Sperm entry triggers the completion of a second asymmetric division, meiosis II, which, after spindle rotation, results in the extrusion of the second polar body. The actin cytoskeleton is central to polarity in many cell types. It is required for the peripheral movement of the first meiotic spindle in mammalian oocytes and for compaction of the 8-cell embryo; this process leads to formation of the first two separate cell lineages in the blastocyst, the ICM and the trophectoderm, which give rise to the embryo and the placental tissues, respectively [9].

Vertebrates synthesize six different isoforms of actin, encoded by a set of structurally related genes, in a tissue-specific fashion [11]. They are divided into two classes, muscle (alpha-skeletal actin [SKA], alpha-cardiac actin, alpha- and gamma-smooth muscle actin [SMA]) and nonmuscle actins (cytoplasmic), and are designated according to their mobilities in isoelectric focusing gels as alpha, beta, or gamma, in order of increasing basicity. Beta- and gamma-cytoplasmic actin (CYA), the two major actin isoforms in nonmuscle cells (genes: ACTB and ACTG1, respectively), are ubiquitous and present in different proportions depending on the cell type and the state of differentiation. They are at least 93% identical to the muscle actins and differ from each other by only four amino acids, located at positions 1, 2, 3, and 9 of their amino terminus: beta-CYA contains a repetition of three aspartic acid residues and one valine (AcDDDIAALV), and gamma-CYA contains a repetition of three glutamic acid residues and one isoleucine (AcEEEAALALVI). These N-terminal differences are hypothesized to modulate isoform-specific functions, including actin-actin, actin-myosin, and actin-actin-binding protein interactions.

Monoclonal antibodies against beta-CYA (IgG1) and gamma-CYA (IgG2b) have been recently obtained and thoroughly characterized using ELISA techniques, Western blots after one-dimensional and two-dimensional SDS-PAGE transfers on membranes, and peptide-blocking assays [12]. In addition, highly specific goat-labeled secondary antibodies against mouse isotype immunoglobulin (anti-IgG1 and anti-
IgG2b) allowed double staining. In that study, beta- and gamma-CYA were shown to be differentially segregated in all cells studied. For example, during cytokinesis, beta-CYA was highly enriched in the contractile ring during cytokinesis, whereas gamma-CYA was present in a subcortical network throughout the dividing cell’s periphery [12]. Silencing RNA experiments support the hypothesis that beta- and gamma-CYA isoforms are involved in distinct cellular functions, with beta-CYA playing a preferential role in contractile activities, whereas gamma-CYA was important for the formation of the compliant filamentous networks necessary for cell-shape flexibility and motile activity [12]. In polarized cells, beta- and gamma-CYA segregated to basolateral and apical domains, respectively.

To date, no information is available on the presence, distribution, and role of these isoforms in mammalian oocytes. Taking advantage of these highly specific beta-CYA and gamma-CYA monoclonal antibodies [12], we assessed both the subcellular localization and function of these actin isoforms in mouse oocytes and early embryos. Our results demonstrate different localization patterns and distinct roles for beta- and gamma-CYA during meiosis; during preimplantation development, striking differences in localization of these two actin isoforms was also observed, which suggests that differences in gamma-CYA expression may represent one of the earliest markers of cell differentiation in development.

**MATERIALS AND METHODS**

**Antibodies and Peptides**

Primary monoclonal antibodies against beta-CYA (4C2, IgG1 or 27B2, IgG2b), gamma-CYA (2A3, IgG2b), or alpha-SMA (I4. IgG2a) [12, 13] were used at 1:50, 1:100, or 1:500, respectively, for Western blotting. For double immunofluorescence, primary antibodies of different isoatypes (anti-beta-CYA [4C2, IgG1] and anti-gamma-CYA [2A3, IgG2b]) were used. Monoclonal anti-beta tubulin IgG1-Cy3 conjugate (C4585; Sigma-Aldrich, Buchs, Switzerland) was used at 1:1000, and a monoclonal pan-actin antibody (anti-actin clone C4, IgG1-Cy3) was conjugate (C4585; Sigma-Aldrich, Buchs, Switzerland) was used at 1:10000 in Western blotting of two-dimensional gels to reveal beta-CYA and gamma-CYA simultaneously. Monoclonal antibodies used for microinjections—anti-beta-CYA (27B2), anti-gamma-CYA (2A3) and anti-alpha-SMA—were affinity purified and dialyzed in 10 mM Tris, 10 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4 buffer (Tris-EDTA buffer), or PBS, concentrated to 0.9–1 mg/ml, and stored at –20°C in single-use aliquots. Tris-EDTA buffer and PBS were shown not to induce any anomalies in oocyte morphology or maturation rates when injected alone (data not shown).

Secondary antibodies goat anti-mouse IgG1-fluorescein isothiocyanate (FITC) conjugate (Southern Biotechnology Associates Inc., Birmingham, AL) and goat anti-mouse IgG2b-rhodamine - and Cy5 conjugates (Southern Biotechnology) were all used at 1:50 for immunofluorescence. For Western blotting, horseradish peroxidase-conjugated rabbit anti-mouse antibody (Bio-Rad Laboratories Inc., Reinach, Switzerland) was used at 1:10000.

**Oocyte and Embryo Collection and Culture**

Protocols for the use of animals were approved by the Commission d’Ethique de l’Expe rimentation Animale of the University of Geneva Medical School and the Geneva Veterinarian Office. Oocytes were isolated by follicular puncture from ovaries of 3- to 8-week-old B6D2F1 mice (Elevage Janvier) in M2 medium supplemented with 50 μg/ml of dBCAMP (Sigma-Aldrich) to prevent germlinal vesicle breakdown (GVBD). Cumulus cells were removed by pipetting with a fine-bore glass-pulled pipette. Oocytes included in our study were selected visually by size, not by chromatin configuration. Based on this selective criteria, the baseline GVBD rate for all control experiments was about 60%–70%. Oocytes were either used immediately (germlinal vesicle [GV] stage) or washed free from dBCAMP in M2 medium and cultured in M16 medium (Sigma-Aldrich) in 5% CO2 at 37°C in a humidified incubator for 7 h (metaphase I [MI] stage) or 20 h (metaphase II stage [MII]). For retrieval of MII oocytes and preimplantation embryos, 3- to 8-week-old B6D2F1 mice were superovulated using a standard protocol [14]. Fifteen hours after human chorionic gonadotropin (hCG) injection, MII oocytes or 1-cell embryos were collected from the ampullae and cumulus cells were removed by hyaluronidase treatment (150 IU/ml; Sigma-Aldrich). Two- to eight-cell embryos were retrieved by Fallopian tube flushing on Day 1.5 to 2.5 after hCG, and blastocysts were retrieved on Day 3.5 by uterine flushing.

For in vitro fertilization, a sperm suspension was prepared from the cauda epididymis of male B6D2F1 mice, as described [10]. At time 0, MII oocytes were incubated with a final sperm concentration of 1–2 × 10^6/ml. For sperm cone visualization and cytokinesis II, zygotes were collected and fixed at 0, 1, 2, and 3 h after insemination.

**Western Blot Analysis**

**One-dimensional gel electrophoresis.** Oocytes, collected as described above, were washed in PBS at the GV, MI, and MII stages. Oocytes were then deposited in a microfuge tube under the binocular microscope and were counted visually at the bottom of the tube. Excess media was removed under the microscope. The oocytes were counted again, and 5 μl of Laemml buffer (80 mM Tris/HCl [pH 6.8], 5% 2-mercaptoethanol, 2% SDS, 10% Glycerol, and Bromophenol Blue) was added. The totality of the lysate was heated for 3 min at 90°C. Samples were then concentrated in a stacking gel and separated at a 10% polyacrylamide gel. Electrophoresis was run at 110V in running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3). Proteins were transferred to nitrocellulose membranes (Millipore) using transfer buffer (25 mM Tris base, 192 mM glycine, 20% v/v methanol, and 0.037% SDS) for 30 min at room temperature at 60V. Transferred proteins on the membrane were visualized using Coomassie blue (0.1% Coomassie blue in 10% acetic acid, 40% MeOH) for 10 sec, followed by two washes in destain solution (10% acetic acid, 40% MeOH). Membranes were then blocked in 5% nonfat milk in TBS (10 mM Tris, 0.154M NaCl; pH 7.4) for 1 h at room temperature to avoid nonspecific binding. Primary antibodies against beta-CYA (4C2) or gamma-CYA (2A3) were used at 1:50 and 1:500, respectively, in TBS-TX (TBS, 0.1% Triton X-100) containing 3% bovine serum albumin (BSA; Sigma-Aldrich); horseradish peroxidase-conjugated rabbit anti-mouse (Bio-Rad) secondary antibody was used at a 1:10,000 dilution in TBS-TX containing 0.1% BSA. The membrane was then washed three times for 20 min in TBS-TX, and the signal was revealed using ECL Western Blotting Detection Reagents (as per manufacturer’s instructions; GE Healthcare, Glattbrugg, Switzerland) and exposure to Hyperfilm ECL (GE Healthcare).

**Two-dimensional gel electrophoresis.** Fifty oocytes at GV, MI, and MII stages were washed in 0.9% NaCl and flash frozen in approximately 2 to 5 μl of 0.9% NaCl containing a protease inhibitor cocktail (“complete mini”; Roche Pharmaceuticals, Mannheim, Germany). Total proteins were extracted in lio buffer for two-dimensional PAGE [15]. Cell lysates were run using 2% Phamalyte (pH 4–6.5; GE Healthcare) for the first dimension, run using 10% SDS-PAGE for the second dimension, and electroblotted to nitrocellulose membranes. Membranes were probed with a pan-actin antibody (clone C), allowing for the detection of both isoforms in the same extract, thus permitting relative quantitation. Quantitative evaluation of the scanned spots was performed using ImageQuant TL (Amersham Biosciences). The relative contribution of each isoform to total actin was plotted at each meiotic stage. Three replicates were obtained for each maturation stage.

**Colchicine Treatment**

MII oocytes were incubated for 5 h in 25–ml drops of M16 medium containing 20 μM colchicine at 37°C, under oil, in 5% CO2 in a humidified chamber. They were then fixed in collagen lattices for immunofluorescence for beta- and gamma-CYA.

**Immunofluorescence for Beta- and Gamma-CYA**

Oocytes and embryos were fixed for 1–2 h in 1% paraformaldehyde in M2 medium at 37°C and washed in PBS for 1 h at 37°C. A drop of 4% of ice-cold collagen type I solution (0.75 mg/ml) was laid on a Nunclon dish (a cell culture surface; Nunc) into which the oocytes were carefully deposited by mouth pipetting. After 1 h at 37°C, the oocytes in the polymerized collagen lattice were incubated 5 min in ice-cold methanol on ice and washed three times in PBS.

The collagen lattices containing the fixed oocytes were then sequentially incubated with primary antibody (anti-beta-CYA 4C2 at 1:50 in PBS and anti-gamma-CYA 2A3 diluted at 1:100 in PBS), PBS, and secondary antibodies (FITC-conjugated goat-anti-mouse IgG1 and Rhodamin-conjugated anti-mouse IgG2b) were both used at 1:50 in PBS, each incubation lasting 1 h at 37°C. Mounting medium (50 ng/ml Hoechst 33342 [Sigma-Aldrich] and 5 μM Draq5 [Biotostatus Limited] in 50% glycerol in PBS) was added to the lattices, and a
RESULTS

Beta-CYA and Gamma-CYA Are Differentially Localized During Female Meiosis

Using beta- and gamma-CYA-specific monoclonal antibodies [12], we investigated by Western blot the presence of cytoplasmic actin isoforms in mouse oocytes at the GV, MI, and MII stages and in 2- to 4-cell embryos. A single band at the molecular weight of actin (45 kDa) was revealed in all samples, with both antibodies (Fig. 1A). In order to evaluate the relative abundance of the two isoforms, which have similar molecular weights but different isoelectric points, we used two-dimensional PAGE followed by Western blotting with a polyclonal pan-actin antibody that recognizes all actin isoforms (Fig. 1B). The use of a pan actin antibody allowed us to detect both isoforms in a single extract; this way, we can measure relative amounts of both isoforms without the interference of variable affinities of monoclonal antibodies. We found that the relative expression of the two isoforms varied significantly during maturation (Fig. 1C). In GV oocytes, beta-CYA (67.5%) was twice as abundant as gamma-CYA (32.5%); in MI oocytes, both were equivalent; after polar body extrusion (MII), gamma-CYA (72.8%) was significantly more abundant than beta-CYA (27.2%).

The subcellular localization of actin isoforms was then determined by immunofluorescence on maturing oocytes (Fig. 2) and zygotes (Fig. 3). The results illustrated here were obtained in several independent experiments and represent consistent findings for 15–40 oocytes from each stage. Beta-CYA and gamma-CYA were both present at all stages of oocyte maturation and preimplantation development. In contrast, both alpha-SKA and alpha-SMA, two muscular actins, were absent from all oocytes and embryos studied (up to the blastocyst stage, data not shown). In GV oocytes, cytoplasmic actins localized as a dense microfilament network in the oocyte’s cortex (Fig. 2A), although they did not always colocalize in a homogeneous manner throughout the cortex’s periphery. At about 8 h after GVBD, when the chromatin/spindle apparatus has migrated in close proximity to the cortex, both beta-CYA and gamma-CYA were enriched in the actin cap, a dense cortical microfilament network overlying the spindle (Fig. 2B).

At anaphase, CYA isoforms began to display distinct distributions. Oocytes in anaphase I (Fig. 2C) displayed a striking staining pattern, with beta-CYA and gamma-CYA appearing differentially enriched within the first actin cap. At telophase I (Fig. 2D) and cytokinesis I (Fig. 2E), the two actin isoforms were present in the remnants of the first actin cap; gamma-CYA was again more abundant. During telophase II (Fig. 2F), alpha-SMA was present in the second actin cap, whereas beta-CYA and gamma-CYA were still present in the remnants of the first actin cap. By the time of cytokinesis II (Fig. 2G), all three actin isoforms were again present in the second actin cap. In 2- to 4-cell embryos, beta-CYA and gamma-CYA were present in the cytoplasm and in the cortical microfilament network. Alpha-SMA was only present in the cortical microfilament network, whereas alpha-SKA was absent from all stages of oocyte maturation and preimplantation development.
FIG. 2. Subcellular localization of beta-CYA and gamma-CYA isoforms in maturing oocytes. Double immunolabeling for beta-CYA (green) and gamma-CYA (red) in oocytes at various stages of meiotic maturation: GV (A), MI (B), anaphase I (C), and telophase I (E). For each stage, Draq5 DNA counterstaining is shown in blue. A merged image of blue, red, and green is shown in the right panels. Images are single Z-stack, taken from three different channels with the Zeiss Lsm510 confocal microscope. Both CYA isoforms are recruited to the thick actin cap overlaying the MI spindle. Beta-CYA is enriched in the cleavage furrow, the contractile ring, and at the PB/zygote contact region. Gamma-CYA is enriched throughout the oocytes/zygotes cortex and is specifically excluded from the contact region between the polar body and zygote. Arrows point to areas where both isoforms display distinct sorting (see text for more details). Bar = 10 μm for all panels.
FIG. 3. Subcellular localization of beta-CYA and gamma-CYA isoforms during fertilization. Double immunolabeling for beta-CYA (green) and gamma-CYA (red) on in vitro-fertilized oocytes/zygotes were collected after about 1 (A), 2 (B), and 3 h (C-E) after insemination. For each stage, Draq5 DNA counterstaining is shown in blue. A merged image of blue, red, and green is shown on the right panels. Both CYA isoforms are present in the sperm cone, although gamma-CYA appears to be located distally and beta-CYA under gamma-CYA. Beta-CYA is otherwise enriched in the cleavage furrow and contractile ring and at the polar body-zygote contact region (arrows). Gamma-CYA is enriched throughout the oocytes/zygotes cortex and is specifically excluded from the contact region between the polar body and zygote. Bar = 10 μm.
isofoms were present in the distal and lateral cortex of the extruding first polar body, whereas beta-CYA alone appeared enriched in the region of the cleavage furrow (arrow) and in the region of contact between the first polar body and the MI oocyte. Gamma-CYA remained present throughout the oocyte and polar body cortices. In MII oocytes, the main cortical actin isofom was gamma-CYA, except in the cell contact region with the polar body.

At fertilization, both isofoms were enriched in a dense subcortical network in the fertilization cone (Fig. 3A). During cytokinesis II and shortly thereafter, beta-CYA was mostly enriched around the contractile ring and cleavage furrow (Fig. 3, B and C, arrows), whereas gamma-CYA was more widely distributed in the entire embryo cortex and was also abundant in the distal cortex of the second polar body. In zygotes, after polar body extrusion and pronuclear formation, gamma-CYA localized to an outer network at the periphery of the oocyte and polar body; remarkably, it was absent or highly reduced at the cell-cell contacts between the second polar body and the embryo and between the first and second polar bodies (Fig. 3, C–E). Beta-CYA, by contrast, was enriched in these particular areas (Fig. 3, C–E, arrows) and around the second polar body. Both isofoms colocalized in the vicinity of chromatin in the polar body cortex (Fig. 3E), as shown above in the actin cap and the first polar body (Fig. 2E). Overall, we found that in mouse MI and MII oocytes and zygotes, cortical beta-CYA and gamma-CYA localize distinctly from anaphase and thereafter, suggesting distinct roles for these two related proteins in these specialized asymmetric cell divisions.

**Alteration of Gamma-CYA Function Causes Severe Defects in Fully Grown Oocytes**

As an initial attempt to study the function of these two proteins, we used siRNAs designed specifically against each isofom, but found that siRNA silencing was not applicable due to the long (2–3 days) half-life of actin [18](data not shown). To investigate the role of actin isofoms during oocyte maturation, we thus used microinjection of antibodies, a technique known to interfere with protein function in mouse oocytes [19–23]. The morphology of oocytes was assessed after microinjection of different isofom-specific monoclonal antibodies at concentrations ranging from 0.1 to 1 mg/ml (Fig. 4A). Injection of anti-beta-CYA (n = 67) and anti-alpha-SMA (n = 77) antibodies did not induce gross morphological alterations. In sharp contrast, oocytes injected with anti-gamma-CYA antibody (n = 120) displayed severe, dose-dependent anomalies (Fig. 4A) such as cortical blebbing and nuclear anchoring/shifting to the periphery of the oocyte; many died in the hours following injection. Co-injection of the anti-gamma-CYA antibody with the gamma-CYA N-terminal-specific peptide used to raise the antibody prevented the morphological effects caused by the antibody alone (n = 91; Fig. 4B).

We next assessed the effect of antibodies on overall microfilament organization, using Alexa 488-phaloidin staining (Fig. 4C). Whereas injection of anti-alpha-SMA antibody at 0.9 mg/ml (n = 30) did not cause an alteration to the microfilament networks as compared to noninjected oocytes (not shown), injection of anti-beta-CYA (n = 38) and anti-gamma-CYA (n = 15) antibodies, at the same concentration, increased microfilament polymerization in GV oocytes. This was most dramatic in anti-gamma-CYA-injected oocytes, although the lethality of injections at this concentration precluded a quantitative analysis; in such oocytes, nuclei appeared displaced to the periphery and anchored to the cortex as well as engaged in a network of dense microfilaments.

Results from these preliminary studies were useful in choosing an appropriate antibody concentration for quantitative analysis of the effects of injections on meiotic maturation, but suggested differential isofom-specific effects between beta-CYA and gamma-CYA and in comparison to controls.

**Injection of Gamma-CYA-Specific Antibodies Significantly Reduces Meiotic Maturation**

Injections of anti-beta-CYA, -gamma-CYA, and -alpha-SMA antibodies at 0.4 mg/ml did not completely block meiotic maturation, as witnessed by polar body extrusion, even in anti-gamma-CYA-injected oocytes, and did not result in highly increased mortality. We thus quantitatively evaluated potential isofom-specific effects on GVBD (4 h after injections) and polar body extrusion (20 h after injections) in three independent experiments. Microinjection of both anti-beta-CYA (n = 57) and anti-gamma-CYA (n = 75) antibodies significantly reduced GVBD rates as compared to anti-alpha-SMA antibodies (Fig. 4D).

Maturational status (cytokinesis, i.e., presence of a polar body) and spindle morphology were assessed in injected oocytes cultured for 20 h after GVBD. More than 80% of anti-beta-CYA- (n = 127) and anti-alpha-SMA- (n = 63) injected oocytes emitted a polar body (Fig. 4E). These results are comparable to sham-injected oocytes (data not shown). In sharp contrast, only approximately 50% of anti-gamma-CYA antibody-injected oocytes (n = 84; Fig. 4D) completed cytokinesis 20 h after injection, and 13% died before polar body extrusion. The analysis of spindle morphology, evaluated by tubulin immunostaining, revealed that anti-gamma-CYA antibody-injected oocytes, arrested in MI, displayed consistent spindle anomalies (Fig. 4F). The spindle was often misshapen and anchored in the cortex by either one or both poles, thereby creating a cortical dimple, and often included misaligned chromosomes. Thus, we found that modulations of gamma-CYA function, but not beta-CYA, significantly disturbed passage through meiosis and the integrity of the first meiotic spindle/chromosome complex, as well as its interaction with the overlying cortex.

**Injection of CYA Antibodies Perturbs Asymmetry of Cytokinesis and MI Spindle Assembly**

MII spindles and chromosome arrangement were assessed in antibody-injected oocytes having undergone polar body extrusion using tubulin immunostaining and DNA staining (Fig. 5). Control oocytes injected with anti-alpha-SMA antibody (Fig. 5C) were not perturbed. They contained a bipolar spindle under the oocyte’s cortex with a uniform distribution of tubulin. Chromosomes were aligned on the metaphase plate, and the other set of chromosomes was found in the first polar body. Strikingly, among the approximately 35% of anti-gamma-CYA-injected oocytes that completed meiosis I, about 30% of these underwent a symmetric division, yielding two cells of approximately equal size (Fig. 5, B and F) and containing a spindle severely abnormal in shape with misaligned chromosomes. This phenotype was never seen upon injection of anti-alpha-SMA or -beta-CYA antibodies.

In oocytes that had extruded a normal-sized polar body, comparable phenotypes were observed upon injection of anti-gamma-CYA or -beta-CYA antibodies: the MII spindles were very disorganized, small, and in some cases there was an almost complete loss of tubulin staining (Fig. 5, D and E); they
FIG. 4. Microinjection of anti-gamma-CYA and beta-CYA antibodies in GV oocytes. A) Photographs of representative GV oocytes injected with anti-alpha-SMA, anti-beta-CYA, and anti-gamma-CYA; anti-alpha-SMA and anti-beta-CYA do not cause any visible abnormalities, whereas anti-gamma-CYA induces anomalies in cortical integrity and germinal vesicle position. Original magnification ×400. B) Micrographs of GV oocytes microinjected with either anti-gamma-CYA (Ab) or preincubated with N-terminal specific-gamma-CYA pentapeptide (Ab + Pep) and left to mature over 20 h; the Ab-injected oocytes display severe abnormalities such as widespread cortical blebbing, which are reversed upon preincubation of Ab + Pep. Original magnification ×200. C) Micrographs of GV oocytes injected with either anti-alpha-SMA, anti-beta-CYA, or anti-gamma-CYA and stained for F-actin (FITC-phalloidin; green), beta-tubulin (anti-beta-tub-cy3; red), and DNA (Draq5). Injection of 0.9 mg/ml of anti-gamma- or anti-beta-CYA antibodies induced important microfilament accumulation, which was never observed in anti-alpha-SMA injections. Nuclear shifting to the periphery of the oocyte and cortical deformation was only observed upon anti-gamma-CYA injection. The photographs were taken at constant microscope settings to compare microfilament intensity. Original magnification ×400. Effects of anti-actin isoform antibodies on GVBD rates (D) and polar body extrusion rates (E). The GVBD rates are significantly affected by anti-beta-CYA (P = 0.0014, n = 57) and anti-gamma-CYA (P = 0.0007, n = 75) antibody injection relative to that of anti-alpha-SMA using a Fisher exact test. The proportion of oocytes with a polar body over total GVBD oocytes was scored after 20 h. The polar extrusion rate is significantly affected, using a Fisher exact test, by anti-beta-CYA (P = 0.022, n = 127) and anti-gamma-CYA (P = 0.0001, n = 84) antibody injections. F) Micrographs of oocytes, which have not undergone cytokinesis, injected with anti-gamma-CYA or alpha-SMA antibodies and stained for beta-tubulin; the spindles of MI-blocked gamma-CYA antibody-injected oocytes display anomalous morphology, cortical anchorage, and altered chromosome organization. Beta-tubulin is shown in red, F-actin in green (FITC-phalloidin), and chromatin stained with draq5 in blue. Original magnification ×400.
were often not bipolar, with misaligned chromosomes, sometimes appearing like a chromatin mass rather than individual chromosomes. The spindles were always close to or anchored in the cortex but seemed to display erratic positioning/interaction with it. Quantitative evaluation of anomalous spindles (Fig. 5A) confirmed the dramatic increase in MII spindle anomalies in oocytes injected with anti-beta- (n = 86, \( P = 0.0001 \)) and gamma-CYA (n = 22, \( P = 0.0001 \)) antibodies when compared to alpha-SMA (n = 45).

N-Terminally-Tagged GFP-Gamma-CYA Is Not Polarized in Transgenic Mice Oocytes

In an attempt to confirm the essential role of gamma-CYA in the maintenance of asymmetry and cortical integrity, we took advantage of an existing GFP-gamma-CYA transgenic mouse line [17] whose heterozygous females were found to be sterile (A. Matus, personal communication). Interestingly, the GFP reporter protein was fused in frame to the gamma-CYA coding region at its N-terminus [24], potentially affecting the function and/or localization of the gamma-isoform. We assessed the potential reasons for this sterility by studying the distribution of GFP-gamma-CYA, F-actin, and microtubules in transgenic oocytes, as well as their rate of polar body extrusion.

Oocytes from 3- to 10-wk-old transgenic and nontransgenic littermate females were retrieved, and meiotic maturation was assessed (n = 86; Fig. 6). Polar body emission rates were determined after 20 h in culture (Fig. 6, A–E); only 48% of transgenic oocytes that had undergone GVBD completed cytokinesis, as compared to 100% in age-matched controls (n = 30). Strikingly, among the oocytes having undergone cytokinesis, 35% divided symmetrically (Fig. 6C) or had a very large polar body (\( >30\% \) of the oocyte’s size) as compared to about 7% in age-matched controls (Fig. 6B).

To investigate GFP-gamma-CYA localization in transgenic oocytes, we determined the distribution of fluorescence either by live visualization (Fig. 6, F and G) or in oocytes fixed without permeabilization (Fig. 6, H and I). Using either method, GFP-gamma-CYA displayed a localization pattern different from that of endogenous gamma-CYA described above. GFP-gamma-CYA localized to the oocyte cortex in all oocytes, irrespective of meiotic status; it was enriched in both the first and second actin caps (Fig. 6, F and G) and in the polar body cortex. In contrast to endogenous gamma-CYA, GFP-gamma-CYA was found enriched at the zone of contact between the polar body and the oocyte, both in oocytes with a normal-sized polar body (Fig. 6H) or in “2-cell” embryos, like MII oocytes (Fig. 6I). Upon tubulin staining of transgenic oocytes with no polar body, spindles were observed either in the center of the oocyte or at the periphery, but the

![Image](https://www.biolreprod.org)
chromosomes had all progressed to varying degrees of anaphase/telophase (Fig. 6, J and K); no oocytes were found arrested at metaphase. Together, these results suggest that N-terminal-tagging of gamma-CYA affects its isoform-specific localization and that over-expression of this chimeric protein in oocytes affects the coordination of meiotic exit and cytokinesis.

Gamma-CYA Is Polarized in Early Embryos and Blastocysts

Double immunofluorescence for beta-CYA and gamma-CYA was also conducted on embryos up to the blastocyst stage. As shown above (Fig. 3, C–E), strong gamma-CYA staining was observed in the cortical regions of zygotes and polar bodies but was excluded from the contact regions of first polar body and oocyte, independent of polar body sizes. In addition, some oocytes were immunostained for tubulin (J, K). In both examples, anaphase has initiated and arrested in the center of the oocyte; cytokinesis has not occurred. Blue, chromatin; red, tubulin; green, GFP-gamma-CYA. Original magnification ×400.
between the two polar bodies and the polar bodies and the zygote. Beta-CYA was enriched in these contact regions and appeared concentrated at cytokinesis around the cleavage furrow and in the contractile ring. As shown in Figures 7 and 8, this differential staining was maintained during preimplantation embryogenesis.

In 2-cell embryos (Fig. 7A), both isoforms localized in the blastomere cortices. Strikingly, gamma-CYA staining was not detectable, or was highly reduced, in cell-cell contact regions (polar body-blastomere and blastomere-blastomere), whereas beta-CYA was enriched in these zones and at the cleavage plane of mitotic blastomeres. In 3- to 8-cell embryos (Fig. 7, B–D), before compaction, this differential staining was also observed. The cortex of 4-cell embryos (Fig. 7C) showed regions of enhanced gamma-CYA and beta-CYA staining overlying the nuclei; interestingly, this is reminiscent of what happens during meiosis upon microfilament accumulation in the cortical region overlying the spindle.

Within compacting 8-cell embryos (Fig. 7E), blastomeres displayed various morphologies: some were polarized and were flattening while some others are still rounded and presented little contact with their neighbours. In both groups, gamma-CYA was restricted to the cortex not in contact with other blastomeres. On the other hand, beta-CYA displayed a different distribution in these two groups: in the flattening blastomeres, beta-CYA was enriched in the outer cortex and around the apico-lateral area of contact between two adjacent cells the site of junction formation (Fig. 7E, arrows); in unpolarized, rounded blastomeres, beta-CYA was present throughout the cortex both outer and at cell-cell contact regions.

After compaction, in 16- to 64-cell embryos, again, beta-CYA was present in the cortex of all blastomeres (inner and outer), whereas gamma-CYA was found almost exclusively in the outer or apical cortex of the outer blastomeres. As the blastocoel formed, an enrichment of beta-CYA networks was observed at its inner periphery, and the protein was present throughout both the trophoblast and ICM (Fig. 8D). In comparison, gamma-CYA networks were almost exclusively detected in outer trophoblastic cells. It is interesting to note, as shown in Figure 8E (arrows), that gamma-CYA staining did not appear polarized in mural trophoblasts, whereas it appeared polarized in polar trophoblasts: gamma-CYA was enriched in the outer cortex of the latter cells when compared to the cortex in contact with the ICM.

DISCUSSION

The oocyte is one of the largest cells in mammals; it contains stores of macromolecules capable of sustaining preimplantation embryo growth and differentiation. In mouse oocytes, the first meiotic spindle forms centrally and progressively migrates to the cortex before first polar body extrusion, whereas the second meiotic spindle lies anchored under the cortex, allowing a second small polar body to be expelled after fertilization. The actin cytoskeleton has long been known to be the key player in establishing the asymmetry of the first meiotic division [10, 25]. However, no substantial data was available as to which actin isoforms were expressed in mouse oocytes and early embryos. The goal of the present work was to address, for the first time, the localization of beta- and gamma-CYA actins in oocytes and preimplantation embryos, as well as their function during meiotic maturation, using newly generated isof orm-specific antibodies [12]. We report that beta- and gamma-CYA are the only actin isoforms expressed in oocytes during meiosis I and II and in cleavage-stage embryos; they exhibit strikingly different distributions, from anaphase of meiosis I up until the blastocyst stage, and display functional diversity during meiosis I. Alpha-SMA and alpha-SKA were undetectable at all stages. Based on these results we have established a working model in which different networks of actin, differentially enriched in each cytoplasmic isoform, contribute in parallel to the uniquely complex cytoskeletal events driving female meiosis I, II, and early embryonic development (Fig. 9).

**Gamma-CYA Is Involved in the Establishment of Asymmetry During Meiosis I**

The specific and distinct sorting of CYAs in mouse oocytes (Figs. 2 and 3) during meiosis I and II suggests that beta- and gamma-CYA hold different as well as overlapping roles during spindle migration and polar body emission. We relied on microinjection of isoform-specific antibodies to evaluate their specific functions in mouse oocytes. Microinjections of high concentrations of anti-gamma-CYA antibodies, but not of anti-beta-CYA and anti-alpha-SMA, severely disrupted oocyte shape (cortical deformation, blebbing) and induced GV peripheral displacement and high mortality (see Fig. 4).

Upon functional disruption of gamma-CYA function using either lower antibody concentrations (at which oocytes did not present severe cortical phenotypes or high mortality) (Fig. 4), or by overexpression of N-terminal GFP-gamma-CYA in a transgenic mouse (Fig. 6), cytokinesis was reduced by about 50%. Among the oocytes that did undergo cytokinesis after gamma-CYA perturbations, approximately one-third divided symmetrically after antibody injection, and one fourth did so in the GFP-gamma-CYA transgenic mice. This result is novel and interesting, because perturbations of actin with cytochalasin B or D caused a complete metaphase block with no spindle migration and no cytokinesis [18]. The anti-gamma-CYA-injected oocytes that did not undergo cytokinesis were blocked in MI or anaphase I and often contained a disorganized spindle that was sometimes anchored in the cortex by one pole, thereby creating a small cortical dimple. Interestingly, this phenotype is similar to the spindle anomalies caused by perturbation of the small GTPase Rac1 in living mouse oocytes [26]. It has recently been shown that members of the Rho small GTPase family control actin isoforms specifically: Rac1 preferentially regulates networks enriched in gamma-CYA, whereas RhoA modulates beta-CYA-enriched networks [12]. The similarity in phenotypes observed in Rac1 and gamma-CYA perturbations suggests they may serve related functions at this meiotic stage. Our results suggest that gamma-CYA has a specific role, distinct from beta-CYA, in the asymmetry of the first meiotic division and MI spindle-cortex interactions.

Once meiotic cytokinesis I has been completed, we show that the localization of gamma-CYA remains polarized up until the blastocyst stage: it is undetectable in the submembranous networks of ICM cells but brightly stains the cortex of...
FIG. 8. Subcellular localization of beta-CYA and gamma-CYA isoforms in preimplantation embryos. Micrographs (single Z-stack, taken from three different channels with the Zeiss Lsm510 confocal microscope) of in vivo-fertilized embryos at the 16- to 32-cell stage (A), 32- to 46-cell stage (B, C), and at blastocyst stage (D, E) immunostained for beta-CYA (green) and gamma-CYA (red) isoforms. For each stage, Draq5 DNA counterstaining is shown in blue. A merged image of blue, red, and green is shown in the right panels. pTE, polar trophectoderm; mTE, mural trophectoderm. Bar = 10 μm for all panels.
trophectoderm cells, as if the role of gamma-CYA in establishing the asymmetry of the first meiotic division set the stage for its polarized localization thereafter. Interestingly, the localization of an N-terminal GFP-gamma-CYA fusion protein in transgenic mice (Fig. 6) did not recapitulate the localization pattern of endogenous gamma-CYA. GFP-gamma-CYA was cortical but was not polarized (i.e., not excluded from the cell-cell contact region) after cytokinesis I. We hypothesize that the acetylated N-terminal region of gamma-CYA, which contains the four amino acids that differentiate the two cytoplasmic isoforms, mediates specialized functions via binding to isoform-specific partners [27]. The GFP tag may thus prevent isoform-specific actin-binding-protein binding. The mislocalization of GFP-gamma-CYA may well be responsible for the female-specific sterility described in this transgenic strain.

**Beta-CYA and Gamma-CYA Both Have a Role in MII Spindle Organization?**

In view of our immunofluorescence data and the previously reported enrichment of beta-CYA in actomyosin bundles of contractile rings (Figs. 2 and 3 and [12]), its functional involvement in cytokinesis, and its importance for embryo survival [28], a more drastic defect in polar body extrusion upon functional perturbation of this isoform was expected. Injection of an antibody, as we have shown, drives actin polymerization in an isoform-specific manner (Fig. 4C and [29]); actomyosin bundles present in contractile rings [30] may be less sensitive to forced polymerization, when the epitope is not available due to its constant interaction with actin-binding proteins. Actomyosin bundles present in the actin cap of mouse oocytes have been shown elsewhere to be more stable and thus more resistant to functional perturbations [31]. We thus hypothesize that the relative stability of these networks makes them resistant to our interventions, causing only limited disruption of beta-CYA functions and no significant reduction in cytokinesis. However, 100% of anti-beta-CYA-injected oocytes that had managed to extrude a polar body displayed a severely disorganized MII spindle. Similarly, the majority (74%) of anti-gamma-CYA oocytes that extruded a polar body displayed the same severe MII phenotype. Both isoforms are thus important for the formation and/or maintenance of the MII spindle/DNA apparatus as well as its anchorage in the cortex. Our data support a common role for beta- and gamma-CYA at this stage of meiosis.

Beta-CYA’s localization in MII and embryogenesis, unlike gamma-CYA, is not polarized. Throughout meiosis and cleavage-stage embryogenesis, beta-CYA displays a dynamic and patchy distribution, with intense spots at specific areas along with a more faint staining throughout the cortex of all cells. The specific areas are the actin caps; the cleavage furrow/contractile rings during meiosis I, II and mitosis; cell-cell

FIG. 9. Working model of the involvement of beta-CYA and gamma-CYA in meiosis and early embryogenesis. Various types of actin networks, enriched in one or the other cytoplasmic actin isoform, may collaborate to drive meiotic maturation. Relocalization of bundles enriched in beta-CYA in the actin cap may prepare the cell for contractile ring formation. Cytoplasmic and cortical meshworks enriched in gamma-CYA may drive the spindle towards the cortex in MII oocytes. Restriction of gamma-CYA from the cleavage furrow and cell-cell contacts may be the first event marking an outer cortex in zygotes and blastomeres, which will remain the apical cortex of outer cells in compacted embryos and blastocysts.
contacts around compaction; and the forming blastocoele. Beta-CYA may thus be regulated to be part of networks that are required at a specific place and time. In agreement with that hypothesis, we observed that beta-CYA’s contribution to total actin decreases as maturation progresses (Fig. 1); whereas it is two times more abundant than gamma-CYA in GV oocytes, it is only half as abundant in MII oocytes 20 h after GVBD. One hundred percent of polar bodies from in vivo-matured mouse oocytes and 80% from in vitro-matured oocytes were shown to degenerate after 20 h in culture [32]. It is possible that a large proportion of beta-CYA-enriched networks are being recruited at a specific time to the actin cap, the contractile ring, and the extruding polar body; if most beta-CYA is restricted to the actin cap at MI and to the extruding polar body at MII, degeneration of the polar body could explain the observed diminution of the beta-CYA to gamma-CYA ratio during meiosis.

Do Beta-CYA and Gamma-CYA Integrate Differential Actin Networks in Meiosis I?

Recently, three elegant studies performed in live mouse oocytes have shed new light on the detailed actin dynamics responsible for spindle movement and cytokinesis in mouse MI oocytes [33–35]. They demonstrate that a highly dynamic cytoplasmic meshwork of short microfilaments, dependent on the nucleating activity of formin 2, controls spindle migration toward the cortex during MI. Once the spindle is close enough to the cortex in late meiosis I, the actin filaments organize around the spindle and are connected directly to the cortex via straight filaments. In fibroblastic and epithelial cells, gamma-CYA is the most prevalent actin isoform in branched networks (apical) regulated by Rac1, whereas beta-CYA is enriched in bundled networks (cytokinesis, adhesion) regulated by RhoA [12]. The fact that gamma-CYA causes such severe phenotypes during meiosis I when compared to beta-CYA could be due to a prevalent role for gamma-CYA at this meiotic stage, an increased sensitivity to perturbation of gamma-CYA networks, or both. Consequently, and in light of the new data on cytoplasmic actin networks in MI, we hypothesize that gamma-CYA may be the prevalent actin isoform in the cytoplasmic meshworks at this meiotic stage, since its perturbation affected asymmetric cytokinesis and the spindle’s interaction with the oocyte cortex at MI (Fig. 9).

The differential distribution of gamma- and beta-CYA in oocytes and embryos is in strong agreement with their distinct sorting in somatic cells. Many processes of oogenesis, oocyte maturation, and early embryonic development involve the actin cytoskeleton as well as the small GTPases [26, 36, 37]. For example, in mouse oocytes and embryos, RHOA [38] and RAC1 [39] have both been differentially implicated in the regulation of compaction. Actin polymerization and RhoA small GTPase are involved in the formation of the blastocoele cavity [40]. Future studies could take advantage of RAC1 and RHOA inhibitors as specific modulators of gamma- and beta-CYA networks, respectively, in order to investigate these isoforms functionally during meiosis and preimplantation development.

In conclusion, our study thus supports a working model (Fig. 9) in which a preexisting differential enrichment of beta-CYA or gamma-CYA in GVBD oocytes and the interaction of these isoforms with various proteins during spindle migration (e.g., PAR3 or PAR6A, PAR6B, Myosin IIA and IIB, formin-2, cofilin, spindle microtubules) establish their differential distribution in the first actin cap. In turn, this sets the stage for distinct sorting throughout meiosis II and preimplantation development up to formation of trophectoderm and ICM cells, the first two distinct cell lineages of the embryo. Furthermore, early polarization of the embryo by apical, cell-contact-dependent restriction of gamma-CYA distribution might be the first step in laying down apical/basal polarity, which itself may be required for tissue differentiation and function. Finally, although our study does not directly implicate beta-CYA or gamma-CYA in the etiology of age-related aneuploidies in human oocytes, it indicates that altered gamma-CYA function could result in reduced fertility.

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