Distribution of 5-chloromethylfluorescein diacetate staining during meiotic maturation and fertilization in vitro of mouse oocytes

J. J. Tarín1,2* and A. Cano2

1Department of Molecular, Cellular, and Developmental Biology, University of Colorado at Boulder, USA; and 2Department of Paediatrics, Obstetrics, and Gynaecology, Faculty of Medicine, University of Valencia, Valencia 46010, Spain

The aim of this confocal microscopy study was to determine whether the pattern of CellTracker Green 5-chloromethylfluorescein diacetate (CMFDA) staining changes during meiotic maturation and fertilization in vitro of mouse oocytes. At different times during meiotic maturation and fertilization, oocytes, zygotes and two-cell embryos were stained with CMFDA to demonstrate intracellular glutathione S-transferase activity. After washing in CMFDA-free medium, most oocytes, zygotes and embryos were stained with dihydroethidium (HE) to visualize DNA structures. Meiotic maturation and fertilization in vitro of mouse oocytes were associated with changes in the pattern of intracellular CMFDA staining. In particular, accumulations of CMFDA-positive membranes were observed around the nucleus of germinal vesicle (GV) oocytes, overlaying the sperm nucleus as well as overlaying the first mitotic spindle if this approached the plasma membrane. Staining of oocytes and zygotes with the probes 3,3'-dihexyloxacarbocyanine iodine [DiOC₆(3)], which stains all the intracellular membranes, and rhodamine 123, which stains active mitochondria, demonstrated that the intracellular structures evidenced by CMFDA staining did not correspond to accumulations of mitochondria. Exposure of oocytes and zygotes to the microtubule-disrupting agent nocodazole or the actin-depolymerizing drug cytochalasin D revealed an autonomous microfilament-dependent transport and relocation of CMFDA-positive membranes during meiotic maturation and fertilization. Such a transport of CMFDA-positive membranes may be envisaged as a protective shield built to prevent damage to DNA from endogenous and exogenous mutagen metabolites.

Introduction

Like other cells and tissues, oocytes and embryos are potential targets for the toxic effects of hydrophobic electrophilic xenobiotics. Cells protect themselves against these xenobiotics by direct glutathione conjugation of the xenobiotic or one of the reactive intermediates formed by the metabolism of xenobiotics via the cytochrome P-450 system. The conjugation reaction between reduced glutathione (GSH) and a compound with an electrophilic centre is catalysed by a family of enzymes generically called glutathione S-transferases (GSTs). Not only xenobiotic electrophiles or their metabolic derivatives are substrates for GSTs, there are a number of endogenous electrophiles that can also be metabolized by these enzymes. Examples of such endogenous substrates are (i) reactive intermediates of oestradiol and 17β-2-hydroxyoestradiol; (ii) quinones generated by oestradiol and benzo(a)pyrene derivatives, and reactions involving menadione; (iii) leukotrienes, prostanoids, prostaglandins, epoxides and sulphate esters; and (iv) by-products of lipid peroxidation (see Mannervik (1985) for a review).

One of the probes used to measure concentrations of GSTs and thiols (including GSH) in single living cells by flow cytometry and imaging is the CellTracker Green 5-chloromethylfluorescein diacetate (CMFDA). This dye is a membrane permeant probe that freely passes through cell membranes. Within the cell, the dye undergoes conjugation with GSH, which is catalysed by GSTs, producing a cell-impermeant S-conjugate. This conjugate is nonfluorescent until cytotoxic esterases cleave off the acetate groups. In this study the intracellular distribution of CMFDA staining during meiotic maturation and fertilization in vitro of mouse oocytes was analysed by confocal laser scanning microscopy. The aim of this analysis was to determine whether the pattern of CMFDA staining changes during meiotic maturation and fertilization of mouse oocytes. Differences among stages in intracellular distribution of CMFDA staining may indicate functional or adaptive responses of cells against intracellular or environmental hydrophobic electrophilic toxicants.
Materials and Methods

Meiotic maturation of oocytes in vitro

Fully grown germinal vesicle (GV)-stage oocytes were obtained from unstimulated 8-week-old ICR female mice (The Jackson Laboratory, Bar Harbor, Maine) by tearing the ovaries apart in medium M2 (Quinn et al., 1982) supplemented with 4 mg bovine serum albumin ml⁻¹ (BSA; Fraction V, Sigma Chemical Co., St. Louis, MO). GV oocytes were denuded of granulosa cells (if present) by repeated passage through a micropipette. Oocytes were cultured for 9 h (metaphase I (MI) oocytes) or 16 h (metaphase II (MII) oocytes) in medium M16 (Whittingham, 1971) supplemented with 4 mg BSA ml⁻¹. Oocyte culture was carried out in 200 µl well dishes at 37°C in an humidified atmosphere of 5% CO₂ in air.

In vitro fertilization

ICR females were superovulated at 8 weeks of age by intraperitoneal injection of 5 IU pregnant mares’ serum gonadotropin (PMSG) (Sigma Chemical Co.) followed 48 h later by 5 IU human chorionic gonadotropin (hCG) (Profasi; Serono Laboratories Inc., Randolph, MA.). At 14 h after hCG injection, females were killed by cervical dislocation and oviducts excised and placed into 2 ml of medium M2. Oocytes enclosed in cumulus masses were released from the ampullae, washed, and transferred directly to insemination droplets. In some experiments, cumulus cells and zona pellucidae were removed from oocytes by gentle agitation in medium M2 containing 80 IU hyaluronidase (Sigma Chemical Co.) and Tyrode’s acid (pH 2.4), respectively. Oocytes were then washed and allowed to recover in medium KSOM (Lawitts and Biggers, 1993) for 30 min before insemination.

In each experiment, one 12- to 14-week-old ICR male was killed by cervical dislocation 2 h before insemination. Both cauda epididymides were excised and placed into 0.5 ml medium T6 (Quinn et al., 1982) supplemented with 30 mg BSA ml⁻¹. Ten minutes later, the caudae were discarded and the sperm solution adjusted to a concentration of 10 x 10⁹ spermatozoa ml⁻¹. Spermatozoa were then incubated until insemination in 20 µl droplets overlaid with mineral oil (light white oil; Sigma Chemical Co.) in an atmosphere of 5% CO₂ in air at 37°C. A maximum of two cumulus masses was transferred to each sperm droplet. Zona-free oocytes were incubated in the presence of 1-2 x 10⁶ spermatozoa for 10 min. Thereafter, oocytes were washed and cultured for a maximum of 8 h in groups of 10 in 20 µl droplets of medium KSOM. Zona-enclosed oocytes were kept in the insemination droplets for 8 h. Afterwards, they were washed, transferred, and incubated in groups of 10 in 20 µl droplets of medium KSOM. Oocytes were considered to be fertilized if they extruded a second polar body and had two pronuclei at 8 h after insemination.

Fluorescent probes

At different times during meiotic maturation and fertilization, oocytes, zygotes and two-cell embryos were incubated for 15 min in a loading solution of 5 µmol CMFDA 1⁺(Molecular Probes, Inc., Eugene, OR) in medium M2. After washing for 15 min in CMFDA-free medium, most oocytes, zygotes and embryos were incubated for a further 15 min in 175 µmol dihydroethidium 1⁺(HE) (Molecular Probes, Inc.). This dye is the reduced, non-fluorescent precursor of ethidium and binds to DNA when oxidized by either superoxide radicals (O₂⁻) or hydrogen peroxide (H₂O₂). As oocytes from the GV to the MI stage, zygotes and two-cell embryos showed a bright red DNA florescence, HE was used to visualize DNA structures when performing double labelling with fluorescein probes such as CMFDA.

Two probes (i) 3,3'-dihexyloxacarbocyanine iodine [DiOC₆(3)] (Eastman Kodak Co., Rochester, NY), which stains all the intracellular membranes, and (ii) rhodamine 123 (Eastman Kodak Co.), which stains active mitochondria, were used to ascertain whether the CMFDA-positive patches observed in oocytes, zygotes and embryos corresponded to aggregates of mitochondria. Oocytes and zygotes were incubated for 10 min in 2.5 µg DiOC₆(3) or 10 µg rhodamine 123 ml⁻¹ medium M2, followed in most cases by 15 min in 175 µmol HE 1⁺. After washing several times, specimens were transferred to a 10 µl droplet of M2 under mineral oil and examined immediately at intervals of 5 µm with a confocal laser scanning microscope (Molecular Dynamics, Sunnyvale, CA) fitted to a Nikon Diaphot microscope using a x 60 objective. All sections were of the same total thickness. Owing to signal bleaching after several exposures, only the first sections, which showed a strong and clear signal, were analysed. A 488 laser wavelength filter, 510 primary beam splitter and 510 barrier filter were used for CMFDA, DiOC₆(3) and rhodamine 123 staining. The optical setting used for double-labelling with rhodamine 123 and HE was a 488 laser wavelength filter, 535 primary beam splitter and 535 barrier filter. Double-labelling with CMFDA and HE or DiOC₆(3) and HE was visualized with a 488 laser wavelength filter, 510 primary beam splitter, 565 DRLP secondary beam splitter, 600 EFLP detector 1 filter and 530 DF 30 detector 2 filter. GV oocytes were always stained and examined in the presence of 0.1 mmol dibutylryl cyclic AMP 1⁺(dbcAMP) to prevent spontaneous resumption of meiosis I during manipulations in vitro.

Cytochalasin D and nocodazole treatment

The mechanism by which intracellular membranes are translocated during oocyte maturation was determined by incubating fully grown GV oocytes for 6 h and MII oocytes for 3 h in the presence of 10 µmol methyl [5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl]carbamate 1⁺(nocodozale) (Sigma Chemical Co.) or 0.5 µg cytochalasin D ml⁻¹(Sigma Chemical Co.) in M16. Thereafter, oocytes were stained with DiOC₆(3), rhodamine 123 or CMFDA and observed through the confocal microscope. GV oocytes were recovered from ovaries in medium M2 supplemented with 0.1 mmol dbcAMP 1⁺. The mechanism of membrane transport at fertilization was evidenced by incubating zona-free oocytes for 1 h before insemination, 10 min during insemination, and 8 h after insemination in the presence of
10 μmol nocodazole l⁻¹ or 0.5 μg cytochalasin D ml⁻¹. Oocytes and zygotes were cultured in 200 μl well dishes at 37°C in a humidified atmosphere of 5% CO₂ in air.

Results

Distribution of CMFDA fluorescence in mouse oocytes and zygotes

CMFDA fluorescence at the GV stage (n = 43) was distributed in many bright patches of different size located in the cortex beneath the plasma membrane and throughout the deeper cytoplasm of the oocyte with larger accumulations around the nucleus (Fig. 1a). At MI stage (n = 20), no large accumulations of CMFDA-positive patches were observed around the MI spindle. CMFDA staining was widespread throughout the oocyte, although the biggest patches tended to be located in the cortex of oocytes (Fig. 1b). The first meiotic spindle was encircled by a ring-like area devoid of CMFDA fluorescence. This area corresponded to the mitochondrial ring that typically surrounds the first meiotic spindle.

A bright accumulation of CMFDA-positive patches was noticed in the cortical area adjacent to the spindle during the anaphase I–telophase I transition (n = 25) (Fig. 1c). However, this accumulation of CMFDA-positive patches was not observed after the extrusion of the first polar body (n = 40) (Fig. 1d). Ninety minutes after insemination of zona-free oocytes, CMFDA-positive patches were more or less uniformly distributed throughout the cytoplasm, although there was still a slight tendency for the biggest patches to be in the cortex beneath the plasma membrane (n = 10) (Fig. 1e). CMFDA staining was detected all around the telophase II spindle with marked fluorescence in the equator of the spindle (the membranous and vacuolar midbody). At this time, a bright focus of CMFDA staining was noticed close to the decondensing spermatozoon, in the cortex between the plasma membrane and the sperm head (Fig. 1f). This CMFDA-positive region increased in size over time until pronucleus formation (n = 20) (Fig. 1g). The female pronucleus (which was closer to the second polar body) was never associated with a CMFDA-positive structure (arrow in Fig. 1g). During and after migration of both male and female pronuclei to the centre of the oocyte, CMFDA-positive patches were not observed close to either of the pronuclei (n = 9) (Fig. 1h). Relatively small CMFDA-positive patches were located in the cortex of the zygote. Such a peripheral distribution of CMFDA fluorescence was maintained during karyogamy (Fig. 1h), metaphase, anaphase and telophase of the first mitotic division (n = 24) as well as in two-cell embryos (n = 10). In some zygotes, the mitotic metaphase plate rather than occupying a central position was located in the periphery of the cell. In these zygotes, bright accumulations of CMFDA fluorescence were observed in the cortical area between the chromosomes and the plasma membrane (n = 8) (Fig. 1i). No differences in the pattern of CMFDA staining were observed between oocytes, zygotes and embryos labelled only with the CMFDA probe and those double-labelled with the CMFDA and HE probes.

Intracellular localization of CMFDA fluorescence

GV, prometaphase I, MII and fertilized oocytes were stained with either DiOC₆(3) or rhodamine 123 to determine whether the CMFDA-positive patches observed in oocytes, zygotes and embryos corresponded to aggregates of mitochondria. At the GV stage, DiOC₆(3) staining (n = 15) (Fig. 2b) revealed membrane accumulations distributed throughout the cytoplasm. The nucleus was always surrounded by DiOC₆(3)-positive accumulations similar to those visualized with the CMFDA probe (Fig. 2a). However, rhodamine 123 staining (n = 10) showed a uniform distribution of mitochondria throughout the oocyte (Fig. 2c). After 7 h of maturation, oocytes stained with DiOC₆(3) (n = 9) exhibited both the typical mitochondrial ring around the meiosis I spindle and several cortical and intracellular membrane accumulations (arrows in Fig. 2e). These membrane accumulations had similar size, morphology and distribution to those seen in CMFDA-stained oocytes (Fig. 2d). Rhodamine 123 staining (n = 10) showed an aggregation of mitochondria around the meiosis I spindle with no accumulations in the cortical area of the oocytes (Fig. 2f). After 16 h of in vitro maturation, DiOC₆(3) staining of MII oocytes (n = 20) (Fig. 2h) revealed fluorescent patches of similar size, morphology and distribution to those observed in CMFDA-treated oocytes (Fig. 2g). The main difference in the distribution of DiOC₆(3) and CMFDA staining was the presence of a bright accumulation of DiOC₆(3)-positive membranes within the first polar body. Rhodamine 123 showed a polarization of mitochondria in the hemisphere containing the MI spindle (n = 10), as reported by Calarco (1995) (Fig. 2i). At 8 h after insemination of zona-free oocytes, a bright accumulation of DiOC₆(3) staining was observed in the cortex between the plasma membrane and male pronuclei (n = 20) (arrows in Fig. 2k). The female pronucleus was never associated with accumulations of DiOC₆(3) staining. This pattern of DiOC₆(3) staining was similar to that obtained in CMFDA-stained oocytes (Fig. 2l). Rhodamine 123 staining (n = 15) did not show any cortical accumulation of mitochondria in the cortex between the plasma membrane and male pronuclei (Fig. 2l).

Mechanism of intracellular membrane transport

GV oocytes were allowed to mature in vitro for 6 h in the presence of nocodazole or cytochalasin D, agents that inhibit polymerization or turnover of tubulin microtubules and actin filaments, respectively, to ascertain the mechanism by which intracellular membranes relocate during oocyte maturation. Light microscopy and rhodamine 123 staining showed that nocodazole treatment prevented mitochondria from forming a ring around prometaphase I chromosomes. However, it did not prevent intracellular membranes, as evidenced by DiOC₆(3) staining, from moving towards the cortex of the oocyte. Chromosomes were clumped and only a very light DiOC₆(3) fluorescence was observed around the compact DNA mass (n = 15) (Fig. 3a). In contrast, oocytes treated with cytochalasin D showed a bright DiOC₆(3) fluorescence around the normally distributed prometaphase I chromosomes (n = 15) (Fig. 3b).
Fig. 1. Selected sections (5 µm thick) obtained by confocal laser scanning microscopy of mouse oocytes and zygotes show the distribution of CellTracker Green 5-chloromethylfluorescein diacetate (CMFDA) staining during meiotic maturation, fertilization and first mitotic division in vitro. Oocytes were double-labelled with dihydroethidium (HE) to visualize DNA structures. (a) Oocyte at the germinal vesicle (GV) stage. (b) Metaphase I (MI) oocyte after 9 h of culture. (c) Oocyte at the anaphase I–telophase I transition. In all anaphase I–telophase I oocytes analysed, a bright accumulation of CMFDA-positive patches in the cortical area adjacent to the spindle was observed. (d) Metaphase II (MII) oocyte after 16 h of culture. (e) Activated MII oocyte cut at the level of the telophase II spindle. This photograph was taken 90 min after insemination of zona-free oocytes. At this time, the cortical area between the plasma membrane and the decondensing sperm head showed a bright CMFDA fluorescence (f). (g) Early male and female (arrow) pronuclei at 2.5 h after insemination. The female pronucleus was closer to the second polar body, which is out of focus in this picture. Note the accumulation of CMFDA-positive patches in the subcortical area overlying the male pronucleus. (h) Zygote during karyogamy at 19 h after insemination. (i) Chromosomal plate of the first mitotic division at 23 h after insemination. Note the presence of bright accumulations of CMFDA-positive patches in the cortical region between the plasma membrane and the chromosomal plate. Scale bars represent 10 µm.

The role of microtubules and microfilaments in the transport of intracellular membranes to the proximity of the sperm nucleus was also determined. Zona-free oocytes were incubated before, during, and after insemination in the presence of nocodazole or cytochalasin D. Oocyte incubation in the presence of nocodazole for 8 h after insemination did not prevent intracellular-membrane transport, as evidenced by DiOC₆(3) staining, to the cortical area overlaying the sperm nucleus (n = 8) (Fig. 3c). However, cytochalasin D treatment blocked the movement and accumulation of DiOC₆(3)-positive membranes in the proximity of the sperm nuclei (n = 15) (Fig. 3d).

The property of MII chromosomes to attract intracellular membranes when located under the plasma membrane was
Fig. 2. Selected sections (5 μm thick) obtained by confocal laser scanning microscopy of mouse germinal vesicle (GV) (a–c), metaphase I (MI) (d–f) and metaphase II (MII) (g–i) oocytes and pronuclear stage zygotes (j–l) showing the pattern of distribution of CellTracker Green 5-chloromethylfluorescein diacetate (CMFDA) (a,d,g,j), 3,3'-dihexyloxacarbocyanine iodine [DiOC₆(3)] (b,e,h,k) and rhodamine 123 (c,f,i,l) staining. Some oocytes and zygotes (g,h,j,l) were double-labelled with dihydroethidium (HE) to visualize DNA structures. MI chromosomes in section (g) and one of the two male pronuclei in section (k) (the female pronucleus is closer to the second polar body) are out of focus. Note the similar size, morphology, and distribution of some membrane structures (arrows in e) evidenced by DiOC₆(3) staining compared with CMFDA staining (d). Scale bars represent 10 μm.
changes in the pattern of intracellular CMFDA staining, demonstrated by exposing MII oocytes for 3 h to 10 μmol nocodazole 1°. This treatment caused dispersion of chromosomes in several discrete clusters around the cortex as reported by Maro et al. (1986). DiOC₅(3) staining of these oocytes revealed accumulations of intracellular membranes in the proximity of the dispersed clusters of MII chromosomes (n = 20) (Fig. 3c).

Oocytes were inseminated with spermatozoa previously incubated in the presence of CMFDA to ascertain whether sperm membranes contributed to the accumulation of CMFDA fluorescence in the cortex between the plasma membrane and the sperm nucleus. No CMFDA fluorescence was noticed inside the oocyte and, in particular, around the point of sperm entry (Fig. 3f).

Whereas CMFDA fluorescence at the GV stage was distributed in many bright patches of different size located preferentially in the cortex beneath the plasma membrane and in the perinuclear area, no large accumulations of CMFDA-positive patches around MI and MII plates were observed. After sperm penetration, a bright focus of CMFDA fluorescence was detected in the cortical area between the plasma membrane and the sperm head. This CMFDA-positive region increased in size over time until pronucleus formation. The female pronucleus, however, was never associated with a CMFDA-positive structure. During and after migration of both male and female pronuclei to the centre of the oocyte and the following division steps leading to the formation of a two-cell embryo, relatively small CMFDA-positive patches were located in the cortex of the cells. Such changes in size and distribution of CMFDA-positive patches during meiotic maturation and fertilization resembled those observed after staining with the probe DiOC₅(3), which stains all the intracellular membranes including mitochondria. However, they were totally different from the pattern shown by the mitochondrial probe.

**Fig. 3.** Selected sections (5 μm thick) obtained by confocal laser scanning microscopy of mouse oocytes and zygotes showing either the distribution of 3,3′-dihexylxocarbovocyanine iodine [DiOC₅(3)] staining after treatment with 10 μmol nocodazole 1° or 0.5 μg cytochalasin D ml⁻¹ (a–e) or the distribution of CellTracker Green 5-chloromethylfluorescein diacetate (CMFDA) in oocytes inseminated with spermatozoa previously incubated in the presence of CMFDA (f). Oocytes were double-labelled with dihydrothidium (HE) to visualize DNA structures. (a,b) Metaphase I (MI) oocytes incubated in the presence of nocodazole and cytochalasin D, respectively, during the first 6 h of meiotic maturation. (c,d) Zygotes treated with nocodazole and cytochalasin D, respectively, for 1 h before, 10 min during, and 8 h after insemination. (e) Metaphase II (MII) oocyte incubated for 3 h in the presence of nocodazole. Note the accumulation of DiOC₅(3) staining in the proximity of a dispersed cluster of MII chromosomes. Other clusters of chromosomes were visualized in different sections throughout the oocyte. (f) Oocyte inseminated with spermatozoa previously incubated in the presence of CMFDA. Note that no CMFDA fluorescence is observed inside the oocyte and, in particular, around the point of sperm entry. Scale bars represent 10 μm.

**Discussion**

The study reported here shows that meiotic maturation and fertilization in vitro of mouse oocytes are associated with changes in the pattern of intracellular CMFDA staining.
rhodamine 123, suggesting that the intracellular structures evidenced by CMFDA staining did not correspond to accumulations of mitochondria.

It appears that transport of intracellular membranes in mouse oocytes occurs in a relatively short period. In fact, the aggregations of intracellular membranes around the nucleus at the GV stage were not seen when oocytes were stained and examined after GV breakdown. Accumulation of intracellular membranes in the cortical area from which the first polar body originates was observed in oocytes at the anaphase I – telophase I transition but not in MII oocytes. Ninety minutes after insemination, a bright DiOC(3) fluorescence was visible in the cortical area between the plasma membrane and the sperm head. However, this accumulation of intracellular membranes increased in size, when pronuclear zygotes were examined. Cytochalasin D and nocodazole treatment pointed out the actin microfilament system as the major mechanism used by mouse oocytes to reorganize and translocate intracellular membranes during oocyte maturation and fertilization. In fact, incubation of mouse oocytes in the presence of the microtubule-disrupting agent nocodazole did not preclude membrane transport towards the periphery of the cell after GV breakdown and towards the proximity of the sperm nucleus after sperm penetration. However, these movements and translocations of intracellular membranes were inhibited by the actin-depolymerizing drug cytochalasin D. These findings are in agreement with the actomyosin-based motility of endoplasmic reticulum and chloroplasts in mesophyll cells from the aquatic angiosperm Vallisneria (Lieber and Menzel, 1995). However, they contrast with other studies showing that (i) membrane and microtubule tip attachment complexes push and pull membranes into tubules and polygonal networks in interphase Xenopus oocyte extracts (Storer-White et al., 1995); and (ii) the development and organization of tubulovesicular membranous organelles such as the endoplasmic reticulum in an African green monkey epithelial cell line (CV-1 cells) uses microtubules but not actin fibres for movement of membranes (Lee et al., 1989).

In contrast to most mammalian species, including rabbits, cows and humans, a sperm aster with microtubules radially aligned from the sperm centrosome does not form after sperm penetration into the mouse oocyte (Pinto-Correia et al., 1994; see Schatten (1994) for review). In the mouse, the numerous centrosomal loci that characterize the unfertilized mouse oocyte are attracted to the surfaces of both the male and female pronuclei organizing a bipolar spindle at first mitosis. Thus, a microtubule-dependent system capable of transporting intracellular membranes to the proximity of the sperm nucleus cannot be envisaged in mouse oocytes. It is likely that intracellular membranes are relocated during oocyte maturation and fertilization by an autonomous mechanism dependent on the induction of actin polymerization by the proximity of non-enveloped chromatins. Maro et al. (1986) observed that when the sperm head decondenses and loses its nuclear membrane an extensive subcortical focus of actin microfilaments is formed in the oocyte. Likewise, after nocodazole-induced dispersion of the oocyte chromosomes, each cluster of chromosomes induces the formation of a subcortical domain of microfilaments (Maro et al., 1986). However, an apparent exception to this rule is that, in the present study, no CMFDA-positive nor DiOC(3)-positive accumulations were detected in the proximity of MII chromosomes. This paradox may be explained by the incorporation into the first polar body of most of the intracellular membranes located near the cortical area from which the first polar body is formed and that subsequently gives shelter to the MII spindle.

GSTs occur both in a microsomal form, which in rat hepatocytes is present only on the endoplasmic reticulum (80% of the total microsomal GST) and the outer mitochondrial membrane, and as many different cytosolic isozymes (see Ketterer et al. (1988); and Morgenstern and DePierre (1988) for reviews). The presence of a membrane-bound GST is important for detoxification of lipid-soluble substrates because it is expected that they accumulate in hydrophobic compartments such as intracellular membranes. In addition, since the microsomal GST demonstrates glutathione peroxidase activity, it may have an important role in protecting intracellular membranes from lipid peroxidation (see Morgenstern and DePierre (1988) for review). The presence of CMFDA fluorescence on intracellular membranes suggests, but does not demonstrate, that the microsomal form of GST is a component within the GST system of mouse oocytes, zygotes and two-cell embryos. Immunofluorescence localization and immunochemical quantitation studies with antibodies raised against microsomal and cytosolic forms of GSTs are needed to evaluate not only the presence and intracellular location of microsomal and cytosolic GSTs in mouse oocytes but also their relative concentrations and activities. It could be argued that a membrane location of GST activity may have a functional or adaptive role against intracellular or environmental hydrophobic electrophilic substrates. In particular, the accumulation of CMFDA-positive membranes around the nucleus of GV oocytes, overlaying the sperm nucleus as well as overlaying the first mitotic spindle if this approaches the plasma membrane could be envisaged as a protective shield built to prevent DNA damage from mutagen metabolites such as polycyclic aromatic and nitroaromatic hydrocarbons, aromatic amines, formamidines, nitrosamines and alkylating agents (see Hinson and Kladubar (1988) for review on role of GST in the modulation of DNA damage and carcinogenesis). The higher sensitivity of the maternal genome within the mouse zygote to the induction of point mutations by the alkylating agent N-ethyl-N-nitrosoureia when compared with the paternal genome (Russell and Bangham, 1991) may be explained by the lack of accumulations of CMFDA-positive membranes around the maternal genome shortly after sperm entry.

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