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Non-plasmalemmal localisation of the major ganglioside in sea urchin eggs

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Summary

M5 ganglioside (NeuGcα2-6Glcβ1-1'Cer) is the predominant glycosphingolipid in sea urchin eggs. Distribution of M5 ganglioside was studied in unfertilised and fertilised eggs of the sea urchin *Hemicentrotus pulcherrimus* by indirect immunofluorescence microscopy. In the cortices of unfertilised eggs, anti-M5 antibody strongly stained the submembranous, polygonal and tubular network of endoplasmic reticulum that was revealed by a membrane-staining dye, DiIC18(3). In addition to the cortical network of endoplasmic reticulum, at least two morphologically distinct vesicles were positive to the antibody. In the cortices isolated from fertilised eggs 30 min after insemination, the antibody stained only a similar network of endoplasmic reticulum, presumably the one reconstructed 5–10 min after fertilisation. During mitosis the endoplasmic reticulum is known to aggregate within the asters of the mitotic apparatus. Indeed, the antibody stained the asters and (more strongly) the vesicular components attaching to the periphery of the mitotic apparatus.

Keywords: Anti-ganglioside antibody, Endoplasmic reticulum, Ganglioside, Mitotic apparatus, Sea urchin egg

Introduction

Glycosphingolipids are generally believed to exist primarily at the outer leaflet of the plasma membrane in most cells. It is suggested that they play important roles as targets for pathogenic microbes and toxins, mediators of cell–cell recognition and adhesion, regulators of cell physiology, and modulators of development (Hakomori, 1981). In early embryogenesis the expression of cell-surface glycosphingolipids is regulated both quantitatively and qualitatively. For example, some glycosphingolipids are identified as stage-specific embryonic antigens: the spatiotemporally regulated cell surface antigens of mammalian embryos (Fenderson, 1990). They are expressed only at a specific stage(s) of embryonic development in the cells of a certain lineage(s). The functional significance of most saccharide changes at the cell surface is not yet clear. However, evidence is accumulating to suggest that glycosphingolipids, particularly gangliosides, play an important role(s) in cell–cell recognition and adhesion during embryonic development.

Embryonic development is well documented in the echinoderms, particularly in sea urchins. Sea urchin gametes are very rich in gangliosides (Nagai & Hoshi, 1975) and the chemical structures of invertebrate gangliosides were first determined in sea urchin spermatozoa (Hoshi & Nagai, 1975). Two major gangliosides in sea urchin eggs have been identified as NeuGcα2-6Glcβ1-1'Cer (M5) and HSO_3-8NeuGcα2-6Glcβ1-1'Cer (T1) (Kubo *et al.*, 1990). M5 ganglioside is by a long way the predominant ganglioside in sea urchin eggs. For example, in *Anthocidaris crassispina* it constitutes more than 90% of the egg gangliosides and 0.8% or more of egg dry weight (Kubo *et al.*, 1990). It is therefore suggested that most, if not all, M5 ganglioside exists outside the plasma membrane. Indeed, indirect

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immunofluorescence microscopy of egg cryo-sections has shown that the distribution of M5 ganglioside is not restricted to the egg plasma membrane (Kubo & Hoshi, 1990). The distribution changes dramatically upon fertilisation. Before fertilisation, the egg cortex stains strongly with anti-M5 antibody, while in the rest of the cytoplasm staining was uniform but much weaker. After fertilisation, the staining rapidly decreases in the cortex and is restricted to a very thin peripheral layer and cytoplasmic patches. However, it is unclear which organelles are positive to the antibody.

In this study we have investigated further the subcellular localisation of M5 ganglioside in unfertilised and fertilised eggs by comparing images of immunofluorescence staining and membrane-dye staining. In unfertilised eggs, the network of cortical endoplasmic reticulum (ER) and unidentified vesicles were strongly stained with anti-M5 antibody. In the cortex of fertilised eggs 30 min after insemination, only a similar network of ER, presumably the one reconstructed 5–10 min after fertilisation, was positive to the antibody. During mitosis, the antibody strongly stained the vesicular components attaching to the periphery of the mitotic apparatus and, to a lesser but still appreciable extent, the asters.

Materials and methods

Gametes and zygotes

The sea urchin *Hemicentrotus pulcherrimus* was collected on the Pacific coast of Chiba Prefecture, Japan, in the breeding season and maintained in a circulating sea water aquarium. Spawning was induced by intracoelomic injection of 10 mM acetylcholine. Pooled eggs were washed three times in artificial sea water (423 mM NaCl, 9 mM KCl, 9.27 mM $CaCl_2$, 22.91 mM $MgCl_2$, 25.5 mM $MgSO_4$, 10 mM Hepes, pH 8.0) and dejellied by multiple washes in a calcium-free isotonic buffer (500 mM NaCl, 10 mM $MgCl_2$, 10 mM EGTA, 10 mM Pipes, pH 6.7). Sperm were collected 'dry', stored at 4 °C, and diluted in artificial sea water before insemination. Egg batches with at least 95% fertilisation were used. Fertilised eggs had their envelopes stripped mechanically in 1 M urea containing 1 mM $CaCl_2$ (Sardet, 1984) and were cultured in calcium-free sea water (423 mM NaCl, 9 mM KCl, 22.91 mM $MgCl_2$, 25.5 mM $MgSO_4$, 10 mM Hepes, pH 8.0).

Isolation of cortex and mitotic apparatus

It is known that hypotonic buffers disrupt the ER network (Terasaki *et al.*, 1991) and change the structure of the cortical granule membrane even after fixation of the isolated cortices. As ganglioside is a membrane component, we used isotonic buffers for isolation of cortices and avoided using any surface-active agents throughout the staining process. Initially we employed the method of Vacquier (1975) as modified by Henson & Begg (1988), using an isotonic buffer called CIB (0.8 M mannitol, 50 mM Hepes, 50 mM Pipes, 2.5 mM $MgCl_2$, 20 mM EGTA, pH 6.8; Henson & Begg, 1988). It was found that, at such a high concentration, mannitol as well as sucrose caused some artefacts (e.g. exocytosis of cortical granules) before and sometimes even after fixation. It was also found that potassium ions improved the preservation of the continuity of the ER network and the integrity of the cortical granules. Therefore, a modified CIB (0.2 M KCl, 0.4 M mannitol, 50 mM Hepes, 50 mM Pipes, 2.5 mM $MgCl_2$, 20 mM EGTA, pH 6.8) was used as the medium for isolation and fixation of cortices.

Eggs were allowed to settle on poly-L-lysine-coated coverslips. Adherent eggs were sheared by a gentle stream of the modified CIB, with microscopic examination of cortical lawns. Cortical lawns were washed in the same buffer and processed immediately for immunofluorescence microscopy.

Mitotic apparatuses (MAs) were obtained from fertilised eggs undergoing the first mitosis, using method of Silver *et al.* (1980) as modified by Henson *et al.* (1989). Shortly, fertilised eggs during mitosis in calcium-free sea water were washed with the MA isolation buffer (20 mM MES, 10 mM EGTA, 1 mM $MgCl_2$, 0.6 M mannitol, pH 6.5), and passed through a 60 μm nitex mesh. The MAs were collected by centrifugation, washed with the same buffer and processed for immunofluorescence staining.

Staining and immunofluorescence microscopy

Rabbit anti-M5 antibody was prepared and purified as described previously (Kubo & Hoshi, 1990). The specificity of the antibody has been verified by enzyme-linked immunosorbent assay and TLC immunostaining. In *H. pulcherrimus* eggs, M5 ganglioside is the only component positive to the antibody, which allows it to be used as a monospecific probe for M5 ganglioside (Kubo & Hoshi, 1990).

Isolated cortices were fixed at room temperature for 30 min in 3% formaldehyde/0.5% glutaraldehyde in the modified CIB. The fixed cortices were washed for 10 min in the medium termed ISB (300 mM glycine, 300 mM potassium gluconate, 10 mM NaCl, 10 mM Pipes, 10 mM EGTA, pH 6.7; Sardet, 1984). After washing three times to block unreacted glutaraldehyde completely, the specimens were further incubated at room temperature for 1 h in the blocking buffer, 10 mg/ml Block Ace Powder (Yukijirushi) in CIB.

For labelling with the anti-M5 antibody, the specimens were placed in a 1:70 dilution of the antibody in the blocking buffer for 1–1.5 h at room temperature. They were washed with multiple changes of the modified CIB for 30 min and then incubated for 1–1.5 h at room temperature with the secondary antibody, fluorescein-conjugated goat anti-rabbit IgG (Cappel) diluted 1:100 in the blocking buffer. They were then washed three times with the modified CIB and mounted. For delipidated controls, the cortices were extracted before blocking, first with methanol and then with chloroform–methanol, 1:1 (v/v), for 10 min each (Suzuki & Yamakawa, 1981).

For double labelling with the anti-M5 antibody and the carbocyanine dye DiIC18(3) (Molecular Probes Inc.), the cortices stained with the antibody were incubated for 1–2 min at room temperature in the modified CIB containing 7.5 μg/ml DiIC18(3). The dye solution was made by diluting 3 μl of a stock solution (2.5 mg/ml in ethanol) with 1 ml of the modified CIB (Terasaki *et al.*, 1991).

Isolated MAs were settled onto coverslips coated with poly-L-lysine and fixed in 3% formaldehyde/0.5% glutaraldehyde in the MA isolation buffer for 1 h at room temperature. They were then processed for immunofluorescence staining as described above and, in addition, labelled with a monoclonal anti-β-tubulin mouse antibody (diluted 1:30). Fluorescein-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-rabbit IgG (Cappel) were used as the secondary antibody to visualise tubulin and M5 ganglioside, respectively. To identify chromosomes, 1 μg/ml of a DNA-specific fluorescent dye (Hoechst 33342) was added to the secondary antibody incubation mixture.

All fluorescent samples were observed with a Microphot-FX microscope equipped for epifluorescence (Nikon) and photographed on 35 mm film (Fuji, Presto 400).

Results

Immunofluorescence staining of isolated cortices

When cryostat-sections of unfertilised *H. pulcherrimus* eggs were examined by indirect immunofluorescence microscopy with the anti-M5 antibody, the cortical region was distinctively stained (Kubo & Hoshi, 1990). To investigate the details of the localisation of M5 ganglioside in the cortical region, isolated cortices were examined. Immunofluorescence staining of the cortices isolated from unfertilised eggs revealed a striking polygonal, tubuloreticular network (Fig. 1*a*). Every polygon of the two-dimensional network encircled at least one cortical granule (Fig. 1*a*, *c*). The overall impression of the network was quite similar to the images of polygonal ER networks found at the inner face of the plasma membrane of isolated cortices (Sardet, 1984). It was often observed that the tubular network extended to the more central (or inner) region of cytoplasm (data not shown).

A double staining procedure was used to compare immunostaining images directly with the patterns revealed by DiIC18(3), a fluorescent dye for the ER network (Henson *et al.*, 1989). Images obtained by the two staining methods were essentially identical (Fig. 1*a*, *b*; and 1*d*, *e* for high magnification). Controls with rabbit non-specific IgG (Fig. 1*g*, *h*) and without the primary antibody (data not shown) did not show significant staining. Delipidated cortices were not positive to the antibody (Fig. 1*i*, *j*). These results indicate that M5 ganglioside exists in the ER.

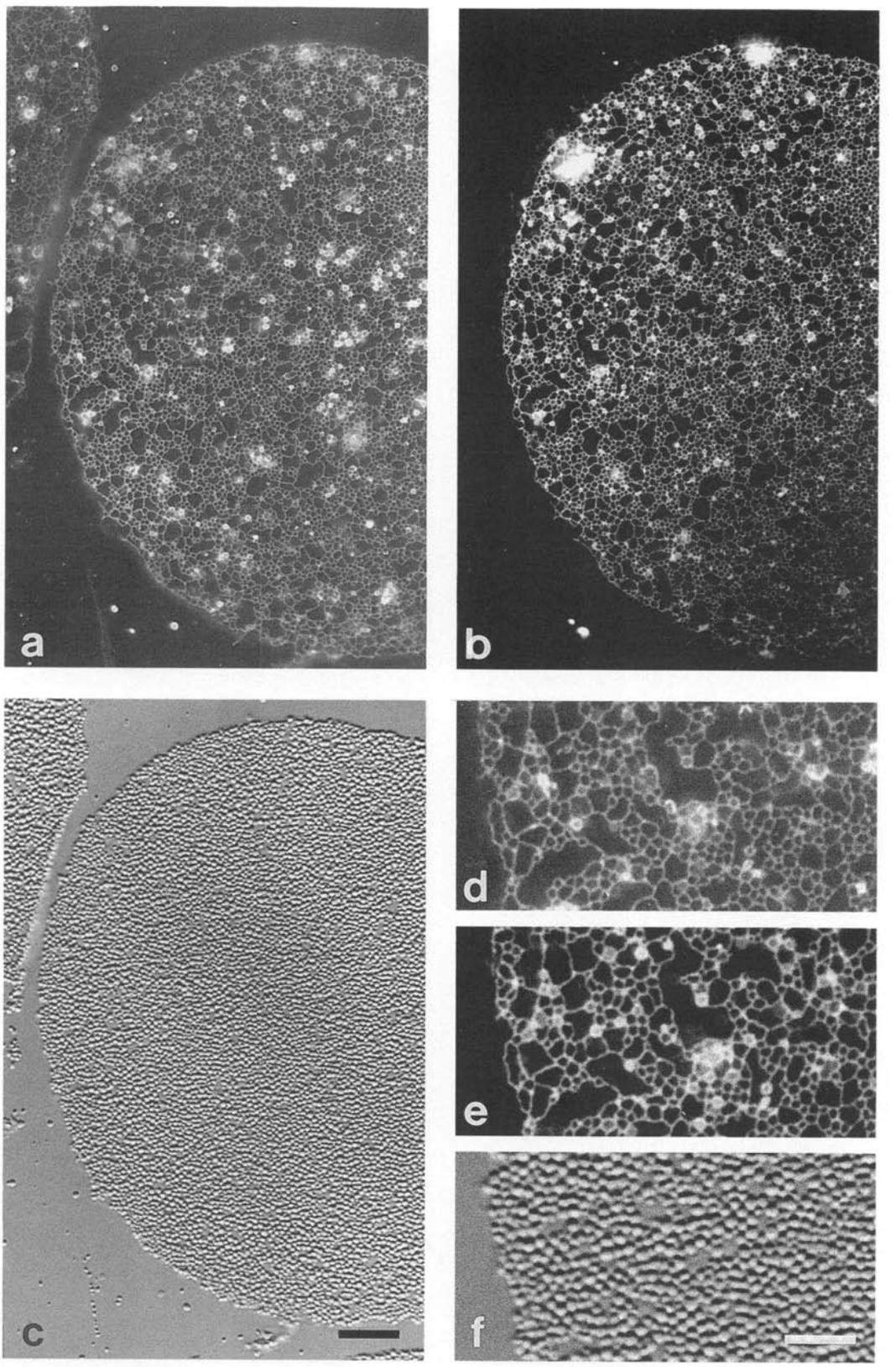
The tubular network of cortical ER is disrupted upon fertilisation and a similar structure is reconstructed within 5–10 min after fertilisation (Henson *et al.*, 1989; Terasaki & Jaffe, 1991). To confirm further that the tubular network observed in isolated cortices is the cortical ER, the cortices of fertilised eggs were prepared at intervals during the first cell cycle and examined with anti-M5 antibody. The immunofluorescence staining showed a tubuloreticular network very similar to the one found in the cortices of unfertilised eggs except that the meshes appeared larger and less regular after fertilisation (Fig. 2*a*). Naturally, the cortices of fertilised eggs lacked the cortical granules stuck to the plasma membrane (Fig. 2*c*). Two images revealed by immunofluorescence staining and by DiIC18(3) staining were identical in the double-labelled cortices of fertilised eggs (Fig. 2*a*, *b*).

Isolated mitotic apparatus

A metaphase mitotic apparatus isolated by the method of Silver *et al.* (1980) retained many membranous components (Fig. 3*b*). The microtubules constituting the asters and spindle were stained by a monoclonal antibody against β-tubulin (Fig. 3*c*). Chromosomes aligning on the metaphase plate were revealed by Hoechst 33342 staining (Fig. 3*d*). The mitotic apparatus was positive to the anti-M5 antibody (Fig. 3a). Vesicular structures clustering along the perimeter of the mitotic spindle stained more strongly with the antibody than did any other substructures (Fig. 3*a*, *b*; arrow).

M5-positive vesicles

Cytoplasmic components blown off from unfertilised eggs were often found on the leeward side of the shearing stream. They were mostly ER and intracellular vesicles. The anti-M5 antibody stained a tubular network and many, but not all, vesicular components (Fig. 4*a*, *d*). The images by immunofluorescence staining (Fig. 4*a*) and by DiIC18(3) staining (Fig. 4*b*) of the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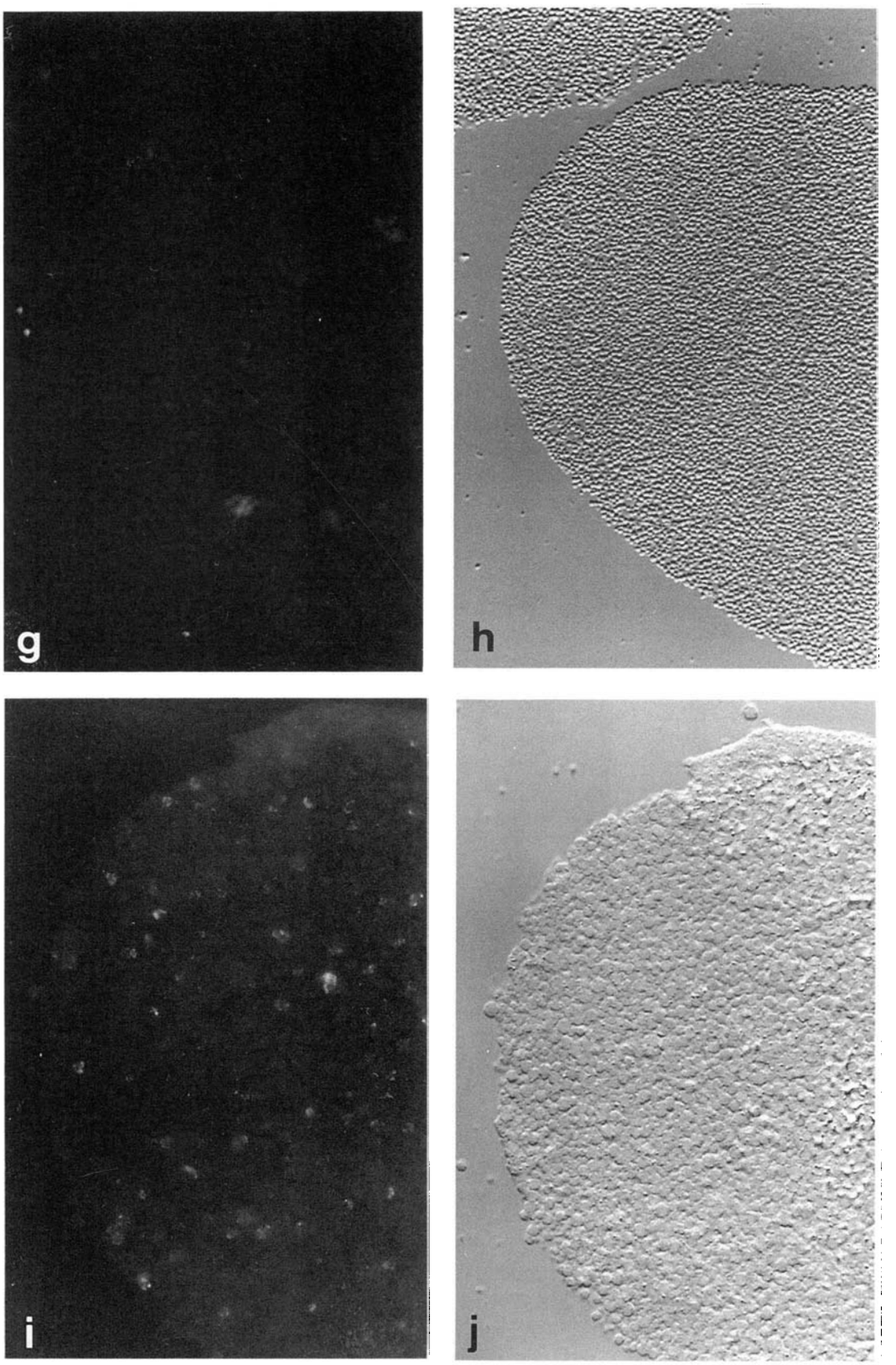

Figure 1 Localisation of M5 ganglioside in the cortex of unfertilised eggs. Isolated egg cortices were fixed and probed with anti-M5 antibody (*a*, *d*). Differential interference contrast images (*c*, *f*) show cortical granules adhering to the plasma membrane. Immunofluorescence staining shows a polygonal, tubuloreticular network that has primarily three-way junctions and encircles cortical granules. Images revealed by DiIC18(3) (*b*, *e*) are essentially identical to the immunofluorescence images of the same field (*a*, *d*). Controls with non-specific rabbit IgG (*g*, *h*) and those of delipidated specimens with anti-M5 antibody (*i*, *j*) are negative. Magnifications of (*a*, *b*, *c*, *g*, *h*, *i* and *j*) and of (*d*, *e* and *f*) are the same, respectively. Scale bars represent: (*c*) 10 μm; (*f*) 5 μm.

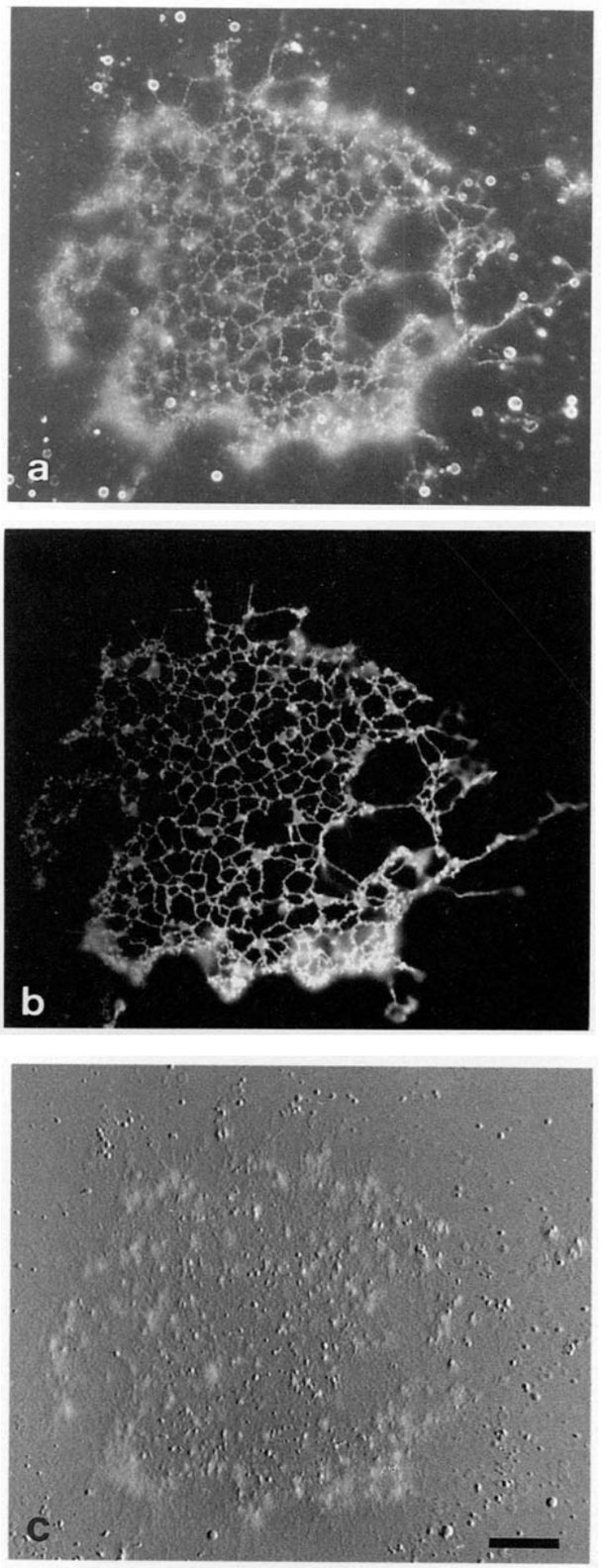

Figure 2 Localisation of M5 ganglioside in the cortex of a fertilised egg 30 min after insemination. Images of the same field by immunofluorescence staining (*a*), DiIC18(3) staining (*b*) and differential interference contrast optics (*c*) are compared. A submembranous, polygonal network of tubular elements shown in (*a*) and (*b*) appears more open and less regular than the similar structure observed in the cortex of unfertilised eggs (Fig. 1*a*). Magnifications are all the same. Scale bar represents 10 μm.

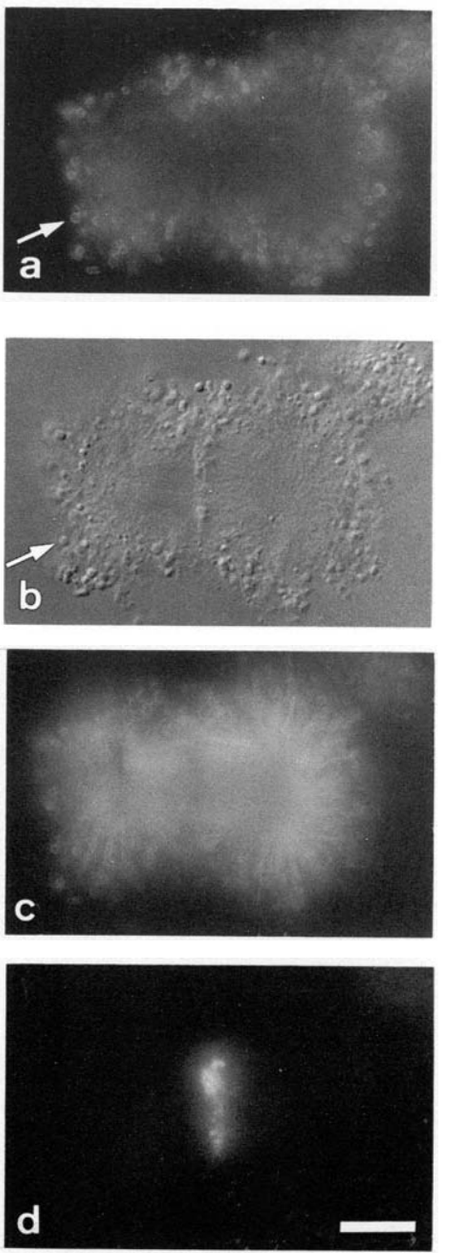

Figure 3 Localisation of M5 ganglioside in the mitotic apparatus. Images of a metaphase mitotic apparatus by differential interference contrast optics (*b*) and anti-β-tubulin immunostaining (*c*) are compared with the anti-M5 immunostaining image (*a*). Anti-M5 antibody stains the asters and spindle faintly, and the vesicular components (arrows) associated with the mitotic apparatus strongly. Hoechst 33342 stains the chromosomes (*d*). Control staining with non-specific rabbit IgG was negative (data not shown). Magnifications are all the same. Scale bar represents 10 μm.

same field show identical patterns of a network. Lamellar sheets of the ER indicated by asterisks in Fig. 4 imply that the cytoplasmic components were derived from more central (inner) cytoplasm (Terasaki & Jaffe, 1991). Therefore, the tubular network shown

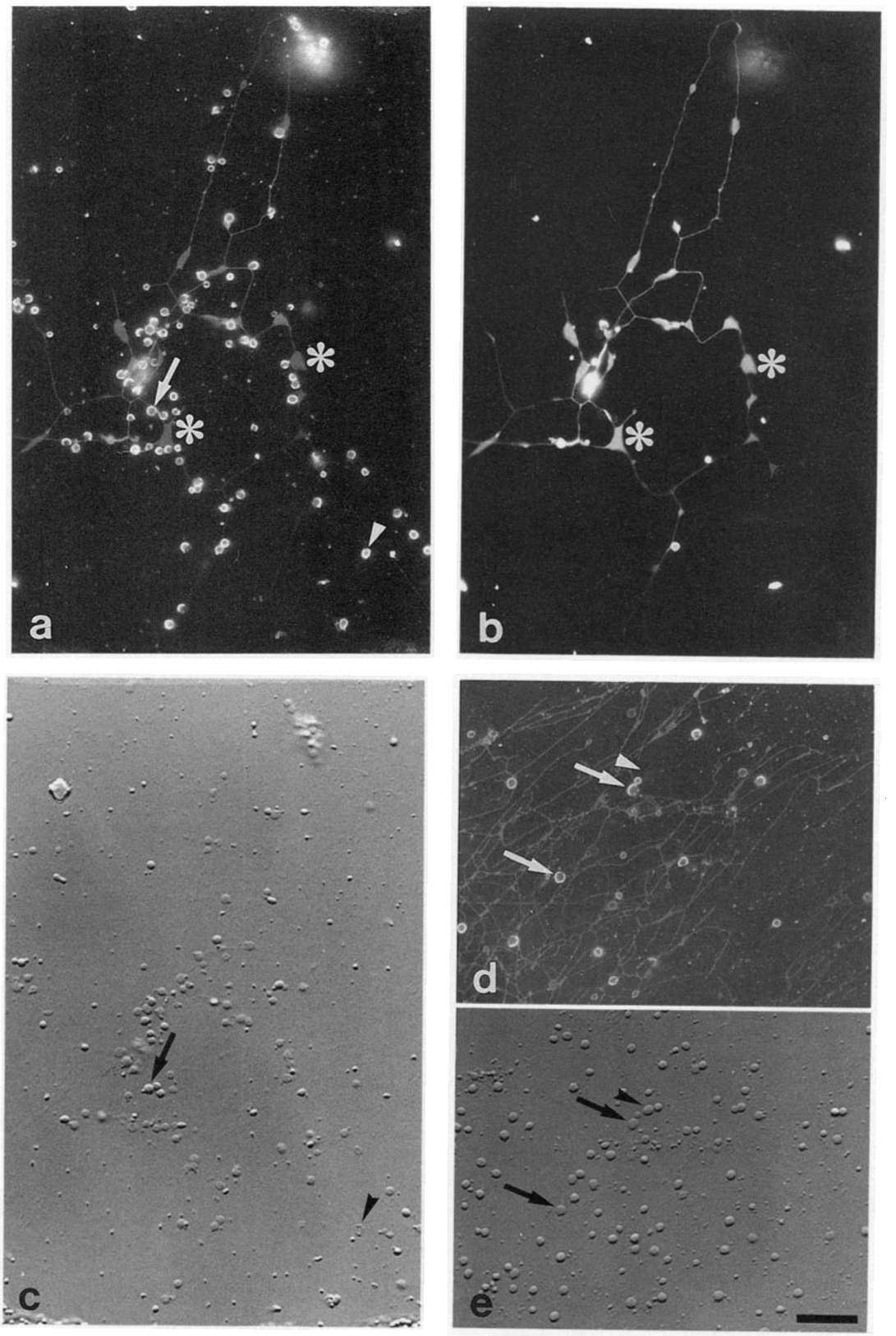

Figure 4 Detection of M5 in cytoplasmic components blown off from unfertilised eggs. Labelling by DiIC18(3) shows a network composed of tubular and cisternal elements (*b*). The presence of cisternal elements (asterisks) suggests that the network is the cisternal endoplasmic reticulum. This structure and some vesicles are strongly positive to anti-M5 antibody (*a*, *d*). At least two vesicles of different appearance are positive to the antibody; one appears clear and of high contrast (*a*, *c*; arrows), while the other is unclear (*a*, *c*; arrowheads). Among a population of vesicles with apparently the same morphology, M5-positive (*d*, *e*; arrow) and negative (*d*, *e*; arrowhead) vesicles are observed. Magnifications are all the same. Scale bar represents 10 μm.

in Fig. 4*a*, *b* and *d* presumably represents a subcortical and cisternal network of the ER described by Terasaki and Jaffe (1991).

As shown in Fig. 4*d* and *e*, among vesicular components of apparently the same morphology, there were M5-positive (arrows) and negative (arrowhead) populations. Furthermore, among M5-positive vesicles, at least two types were morphologically distinguished (Fig. 4*a*, *c*); one type gave clear and high-contrast images (arrow) while the other appeared of low contrast (arrowhead).

Discussion

The data presented here clearly demonstrate that M5 ganglioside, the predominant glycosphingolipid in sea urchin eggs, is localised mostly in the ER network and to a considerable extent in some unidentified vesicles (Figs. 1, 2 and 4). This conclusion accords well with the fact that M5 ganglioside constitutes 0.8% or more of egg dry weight (Kubo *et al.*, 1990). It also accords with the fact that the content of ganglioside per egg or embryo is rather constant, in spite of steady increases in the plasma membrane area, throughout early development before hatching (Hoshi & Nagai, 1970).

The ER network is distinctly dyed by DiIC18(3) in living as well as in fixed culture cells (Terasaki *et al.*, 1984). In sea urchin eggs this structure is well characterised (Henson *et al.*, 1989; Terasaki *et al.*, 1991) and is known to consist of two distinct structural (and possibly also functional) domains. One is the cortical ER that is associated with the plasma membrane and surrounds cortical granules. The other is a subcortical and cisternal network of the ER throughout the cell interior (Terasaki & Jaffe, 1991). Although M5 ganglioside appeared to be localised mostly in the cortical ER network, the cisternal ER network was appreciably positive to anti-M5 antibody (Fig. 4). Furthermore, the mitotic apparatus to and in which the ER aggregates during mitosis was positive to the antibody (Fig. 3). These results suggest that M5 ganglioside is localised in the ER throughout the cell interior.

It is generally accepted that the glycosylation of glycolipids takes place on the lumen side of the Golgi lamella and that they are transported to the plasma membrane only by carrier vesicles. No vesicular pathway between the Golgi apparatus and the ER has been described (Schwarzmann & Sandhoff, 1990; Wattenberg, 1990). Therefore, the presence of ganglioside in the ER membrane is rather unexpected. However, the presence of glycosphingolipids in the ER has already been suggested. Rat liver gangliosides are found in a purified ER fraction in an amount too high to be accounted for by contaminated plasma membranes (Matyas & Morré, 1987). The presence of glycosphingolipids in the ER is implied also by the immuno-electron microscopic detection of Forssman glycolipid in the nuclear envelope that is often interconnected with the ER (van Genderen *et al.*, 1991).

Morphological observations of the intracellular glycosphingolipids are mostly restricted to their metabolic processing and intracellular trafficking. Yet some reports suggest the functional importance of the intracelular glycosphingolipids. For example, galactocerebroside is present in the cytoplasm of cultured epithelial cell lines in close association with a colchicine-sensitive microtubule-like subcellular structure (Sakakibara *et al.*, 1981). In human umbilical vein endothelial cells, globoside and GM3 are reported to associate with intermediate filaments (Gillard *et al.*, 1991, 1992, 1993). These observations and our finding of M5 ganglioside in aster and spindle microtubules might reflect a role for glycosphingolipids in the architecture and/or function of the cytoskeleton.

The presence of M5 ganglioside in the cortical ER is a reminder that a calsequestrin-like protein in the cortical ER sequesters calcium ions (Henson *et al.*, 1989) and that calcium ions are released from the cortical ER upon fertilisation (Terasaki & Sardet, 1991). Possible functional involvement of gangliosides in the regulation of calcium ion levels is discussed in relation to the activity of calcium pump (Rahmann, 1992). Moreover, specific binding of gangliosides to calmodulin has recently been reported (Higashi & Yamagata, 1992; Higashi *et al.*, 1992). It would seem fruitful to investigate a possible role for gangliosides in the regulation of or by calcium.

Another clue to the function of M5 ganglioside may be found by the identification of at least two kinds of M5-positive vesicles (Fig. 4). Good candidates may be the yolk platelets, acidic vesicles and pigment granules, all of which are present in sea urchin eggs. These vesicles are suggested to function in the maintenance of the intracellular concentration of calcium ion and/or pH (Allemand *et al.*, 1987; Christen, 1983; Lee & Epel, 1983), and to be the potential storage site for future extracellular matrix components during early embryogenesis (Wessel *et al.*, 1984).

Electron microscopic observation of the distribution of M5 ganglioside is currently in progress in our laboratory. All the possibilities discussed above are to be tested in future experiments.

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