A Calsequestrin-like Protein in the Endoplasmic Reticulum of the Sea Urchin: Localization and Dynamics in the Egg and First Cell Cycle Embryo

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Abstract. Using an antiserum produced against a purified calsequestrin-like (CSL) protein from a microsomal fraction of sea urchin eggs, we performed light and electron microscopic immunocytochemical localizations on sea urchin eggs and embryos in the first cell cycle. The sea urchin CSL protein has been found to bind Ca^{++} similarly to calsequestrin, the well-characterized Ca^{++} storage protein in the sarcoplasmic reticulum of muscle cells. In semi-thin frozen sections of unfertilized eggs, immunofluorescent staining revealed a tubuloreticular network throughout the cytoplasm. Staining of isolated egg cortices with the CSL protein antiserum showed the presence of a submembranous polygonal, tubular network similar to ER network patterns seen in other cells and in egg cortices treated with the membrane staining dye $DiIC_{16}[3]$. In frozen sections of embryos during interphase of the first cell cycle, a cytoplasmic network similar to that of the unfertilized egg was present. During mitosis, we observed a dramatic concentration of the antibody staining within the asters of the mitotic apparatus where ER is known to aggregate. Electron microscopic localization on unfertilized eggs using peroxidase-labeled secondary antibody demonstrated the presence of the CSL protein within the luminal compartment of ER-like tubules. Finally, in frozen sections of centrifugally stratified eggs, the immunofluorescent staining concentrated in the clear zone: a layer highly enriched in ER and thought to be the site of calcium release upon fertilization. This localization of a CSL protein within the ER of the egg provides evidence for the ability of this organelle to serve a Ca^{++} storage role in the regulation of intracellular Ca^{++} in nonmuscle cells in general, and in the regulation of fertilization and cell division in sea urchin eggs in particular.

INTRACELLULAR calcium as a second messenger regulates a wide spectrum of cellular processes (54). In the extensively studied case of muscle cells, calcium uptake, storage, and release are controlled by a system of Ca^{++} binding and transport proteins located in the sarcoplasmic reticulum (13, 42, 64), a specialized smooth ER. Calsequestrin, a well characterized moderate affinity and high capacity Ca^{++} binding protein, is the principle Ca^{++} storage protein in the sarcoplasmic reticulum (16, 41, 43). Recently, a calsequestrin-like protein from a microsomal fraction of sea urchin eggs has been purified and partially characterized (45). This egg protein has been shown to have a number of characteristics similar to muscle calsequestrin and cross reacts with cardiac muscle calsequestrin antibodies (45).

In the sea urchin egg, transient elevations in intracellular calcium levels regulate fertilization, pronuclear migration, chromatin condensation, nuclear envelope breakdown, and cell division (51, 62, 63, 71, 74). The ER has been implicated as the storage site and regulator of Ca^{++} within the egg (28). In centrifugally stratified eggs, the ER-rich clear zone has been shown to be the site of Ca^{++} release upon fertilization (14). In ultrastructural localization studies, Ca^{++} precipitates have been found within the ER of the egg (50). Active uptake of Ca^{++} has been shown to occur in microsomal fractions and ER-derived vesicles from egg homogenates (26, 44), in vesicles associated with the mitotic apparatus (61), and in isolated egg cortices (44, 46). In addition, inositol triphosphate (IP_3),[1] known to trigger Ca^{++} release from ER in other cell types (3), has been shown to cause Ca^{++} release from egg ER derived vesicles (8), egg cortices (44, 46) and whole eggs (73, 77). IP_3 has also been shown

1. *Abbreviations used in this paper*: ASW, artificial seawater; AV, acidic vesicles; CFSW, calcium-free seawater; CG, cortical granules; CSL, calsequestrin-like; CZ, clear zone; IP_3, inositol triphosphate; MA, mitotic apparatus; NC, nitrocellulose; RT, room temperature.

to increase in the egg after fertilization (72). However, little is known concerning the proteins involved in Ca^{++} storage and transport in the ER of the egg, or nonmuscle cells in general.

In this paper, the sea urchin calsequestrin-like (CSL) protein was immunocytochemically localized in sea urchin eggs and embryos in the first cell cycle. The CSL protein is present within the luminal compartment of a tubuloreticular network that is distributed throughout the egg cytoplasm and is associated with the cytoplasmic face of the plasma membrane. This network resembles the ER in its morphological organization, staining with carbocyanine dyes, capacity for dynamic reorganizations, concentration in the mitotic apparatus (MA), and segregation to the ER-rich clear zone of centrifugally stratified eggs. These results indicate that the CSL protein containing ER may function as a Ca^{++} sequestration organelle involved in the regulation of fertilization and cell division of sea urchin eggs.

Materials and Methods

Materials

The following purchases were made: sea urchins of the species *Strongylocentrotus purpuratus* from Marinus Inc. (Westchester, CA); fluorophore-, peroxidase-, and alkaline phosphatase-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies from Hyclone Laboratories (Logan, UT); mouse monoclonal antibody to tubulin from Sigma Chemical Co. (St. Louis, MO); the fluorescent membrane carbocyanine dye 1,1-dihexadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate [$DiIC_{16}(3)$] from Molecular Probes Inc. (Eugene, OR); glutaraldehyde from Electron Microscopy Sciences (Ft. Washington, PA); Spurr embedding resin from Polysciences Inc. (Warrenton, PA); and all other chemicals from Sigma Chemical Co.

A previously characterized antiserum against the sea urchin CSL protein purified from the smooth ER microsomal fraction of *S. droebachiensis* eggs (45) was used in this study.

Preparations

S. purpuratus gametes were obtained by intracoelomic injection of 0.5 M KCl, and eggs were dejellied and maintained in artificial seawater (ASW) according to standard methods (21). Fertilized eggs had their fertilization envelopes stripped mechanically in urea or by aminotriazole treatment (59) and embryos were cultured in calcium-free seawater (CFSW) (5).

Isolated egg cortices were obtained by the method of Vaquier (75) as modified by Henson and Begg (21). Eggs were settled onto poly-L-lysine-coated coverslips and adherent eggs were sheared with cortex isolation buffer (0.6 M mannitol, 50 mM Hepes, 50 mM Pipes, 2.5 mM $MgCl_2$, 20 mM EGTA, pH 6.8).

Isolated MAs were obtained from embryos undergoing the first cell cycle mitosis using methods adapted from Silver et al. (61). Embryos during mitosis in CFSW were washed with MA isolation buffer (20 mM MES, 10 mM EGTA, 1 mM $MgCl_2$ and 0.6 M mannitol). The embryos were then passed through a 53 μm nitex mesh, and isolated MAs were collected by a series of low speed centrifugations.

Unfertilized eggs were stratified by a modification of the method of Harvey (20). A 4% suspension of eggs in ASW was layered over a solution of 18% Ficoll in ASW, pH 8.0, and centrifuged at 12,000 *g* for 8 min in a microfuge (No. 12; Beckman Instruments Inc., Palo Alto, CA). Eggs were collected from the ASW/Ficoll interface and washed twice by low speed centrifugation in ASW.

Gel Electrophoresis and Immunoblotting

Gel samples of *S. purpuratus* unfertilized eggs, isolated unfertilized egg cortices, and isolated egg MAs were obtained by direct lysis and homogenization in boiling SDS sample buffer. Purified CSL protein was obtained from *S. droebachiensis* as described previously (45) with the addition of a final electrophoretic purification step. Samples were run on 5–20% SDS polyacrylamide gradient gels according to Laemmli (35). Proteins from the gel were transferred onto 0.45 μm nitrocellulose (NC) using the methods of Towbin et al. (69). The NC was blocked with BLOTTO (29) and incubated in diluted (1:1,000) antiserum raised against the purified CSL protein. The NC was then treated with an alkaline phosphatase conjugated goat anti-rabbit secondary antibody diluted 1:2,000. Visualization of alkaline phosphatase bound to the NC was performed according to the methods of Byers et al. (6, 12).

Immunofluorescent Staining

S. purpuratus unfertilized and centrifugally stratified unfertilized eggs were fixed in 1–3.7% formaldehyde (or 1–3% paraformaldehyde) plus 0.1% glutaraldehyde in buffer AC320 (75 mM KCl, 2 mM $MgCl_2$, 10 mM EGTA, 100 mM lysine, 320 mM sucrose, and 25 mM Pipes, pH 6.8, according to Bonder et al. [4]) for 1 h on ice. Fertilized eggs were fixed in 1–3.7% formaldehyde, 0.1% glutaraldehyde in CFSW containing 50 mM EGTA for 1 h on ice. Fixed eggs were processed for frozen sectioning according to the methods of Tokuyasu (70) as adapted by Bonder et al. (4). Briefly, eggs were embedded in gelatin, equilibrated in a graded sucrose series (0.8, 1.6, and 2.3 M sucrose in PBS), and then frozen in liquid freon cooled with liquid nitrogen. Semi-thin frozen sections (0.5–1.5 μm) were cut on a ultramicrotome (Ultracut E, Riechert Jung, Vienna) equipped with a cryo stage. Sections were collected on gelatin-coated slides and stored at −20°C. For antibody localizations, sections were incubated for 1 h in aldehyde quench solution (buffer AC320 with 150 mM glycine in place of 100 mM lysine) at 4°C, then 1 h in blocking buffer (0.3–1% BSA, 1–2% normal goat serum and 0.1% Triton X-100 in PBS, pH 7.5) at 4°C, and then placed in a 1:250 to 1:500 dilution of the CSL protein antiserum in blocking buffer for 14–16 h (overnight) at 4°C. Sections were washed with multiple changes of PBS and blocking buffer over a 30-min period and then incubated for 2 h at room temperature (RT) in rhodamine conjugated goat anti-rabbit secondary antibody diluted 1:200 in blocking buffer. Sections were washed extensively in PBS and mounted in an antibleaching glycerol *p*-phenylenediamine solution (49). To identify chromosomes in dividing embryos, 1 μg/ml of the DNA specific fluorescent Hoechst dye (33258) was included in the secondary antibody incubation mixture.

Isolated egg cortices were fixed in 3% formaldehyde, 0.1–0.5% glutaraldehyde in cortex isolation buffer for 30 min at RT. Cortices were then processed for indirect immunofluorescence staining with the sea urchin CSL protein antiserum following the same protocol as described above for frozen sections.

For staining with the membrane carbocyanine dye $DiIC_{16}[3]$, unfixed isolated cortices from unfertilized eggs were incubated for 1 min at RT in cortex isolation buffer containing a 1:300 dilution of a $DiIC_{16}[3]$ stock solution (2.5 mg/ml in 100% ethanol) (67).

Isolated MAs were settled onto poly-L-lysine-coated coverslips and fixed in 3% formaldehyde, 0.5% glutaraldehyde in MA isolation buffer for 1 h at RT. MAs were then processed for immunofluorescence as described for frozen sections, but in addition they were double labeled with a monoclonal-tubulin antibody (diluted 1:200). Fluorescein-conjugated goat anti-mouse and rhodamine-conjugated goat anti-rabbit secondary antibodies were used to visualize tubulin and the CSL protein respectively.

For staining of embryos with antitubulin antibody for confocal microscopy, embryos attached to coverslips were fixed in 100% methanol at −20°C for 2 min and washed in PBS. Samples were then processed for immunofluorescence using a monoclonal-tubulin antibody followed by a fluorescein-conjugated goat anti-mouse secondary antibody.

All fluorescent samples were viewed on an inverted microscope (ICM 405; Carl Zeiss Inc., Thornwood, NY) using a 63× planapo NA 1.4 lens or a 63× neofluor NA 1.25 lens and photographed on 35 mm film (Tri-X 400 ASA; Eastman Kodak Co., Rochester, NY). For confocal fluorescence microscopy, a confocal laser scanning system (Bio-Rad Laboratories, Cambridge, MA) attached to an axioscope (Carl Zeiss Inc.) was used. Samples were viewed using a 63 × planapo N.A. 1.4 lens and photographs of frame averaged- and contrast-enhanced images were taken directly from a video screen (TMAX 100 ASA Film; Eastman Kodak Co.). All film was developed in Edwal FG7, Acufine, or Diafine developer.

Immunoperoxidase Ultrastructural Localization

Ultrastructural localization of sea urchin CSL protein was performed using the immunoperoxidase method of Graham and Karnovsky (17) as modified by Louvard et al. (38). Briefly, unfertilized *S. purpuratus* eggs were fixed with 0.5% glutaraldehyde, 0.2% formaldehyde in ASW for 30 min on ice. Eggs were washed with PBS, placed in a modified blocking buffer (with 0.075% Triton X-100 in place of 0.1% Triton X-100) for 1 h at RT and then incubated overnight at 4°C in the CSL protein antiserum diluted 1:500 in

modified blocking buffer. After multiple washes in PBS, eggs were incubated for 1 h at RT in peroxidase-conjugated goat anti-rabbit antibody diluted 1:100 in modified blocking buffer. Eggs were washed with PBS and fixed with 1% glutaraldehyde in 100 mM cacodylate buffer, pH 7.2, for 30 min at RT. After washing with 100 mM Tris-HCl, pH 7.5, the eggs were incubated in the same buffer plus 0.3 mg/ml diaminobenzidine and 0.03% H_2O_2 for 2 h at RT in the dark. The eggs were then washed in PBS, postfixed with 0.5% OsO_4 in 100 mM cacodylate buffer, pH 7.2, for 30 min at RT, dehydrated in a graded ethanol series and embedded in Spurrs epoxy resin. Thin sections (60–75 nm) were cut on an ultramicrotome (MT-2; Ivan Sorvall Inc., Norwalk, CT), stained with uranyl acetate and viewed on a transmission electron microscope (300; Philips Electronic Instruments, Inc., Mahwah, NJ) operating at an accelerating voltage of 60 kv.

Results

Immunoblotting Using the Sea Urchin CSL Protein Antiserum

The antiserum against the *S. droebachiensus* CSL protein (45) cross reacted specifically with extracts from *S. purpuratus* whole eggs, isolated egg cortices, and isolated MAs, producing a single immunoreactive band that comigrates with the purified CSL protein band (Fig. 1, *A* and *B*). The CSL protein, originally reported to have a molecular mass of 58 kD (45), migrates at ~63 kD in our gel system. Immunoblotting with preimmune serum at the same dilution did not generate any significant immunoreactive bands (Fig. 1, *C*).

Immunofluorescent Localization of the Sea Urchin CSL Protein

Unfertilized Eggs. Immunofluorescent staining of frozen sections of unfertilized eggs (1.0–1.5 μm) with the antiserum against the CSL protein reveals a complex reticular network present throughout the cytoplasm (Fig. 2 *b*). The network appears attenuated in the cortical granule layer that appears as a circumferential layer in the phase-contrast image of the same section (Fig. 2 *a*, *arrows*). Punctate stained elements are evident directly beneath the plasma membrane (Fig. 2 *b*, *arrows*), and some immunofluorescent elements can be seen extending through the cortical granule layer from the plasma membrane to the inner cytoplasm. In grazing sections (Fig. 2 *c*), the staining pattern forms three distinct regions arranged in concentric circles. The inner region (Fig. 2 *c*, *1*) is characterized by the reticular staining of the inner cytoplasm, while the intermediate region (Fig. 2 *c*, *2*) has diminished staining corresponding to the cortical granule layer. The outer region (Fig. 2 *c*, *3*), located at the level of the plasma membrane, contains a polygonal network of stained tubules (Fig. 2 *c*, *arrow*). Control staining of sections with preimmune serum (Fig. 2 *d*), secondary antibody alone, or antiserum preabsorbed with purified CSL protein showed no specific staining.

Isolated Egg Cortices. To examine better the submembranous stained network apparent in grazing frozen sections, isolated cortices from unfertilized eggs and embryos in the first cell cycle were stained with antiserum against the CSL protein. In isolated cortices of unfertilized *S. purpuratus* eggs, immunofluorescence reveals a striking polygonal, tubuloreticular network in association with the plasma membrane (Fig. 3 *a*). This staining pattern closely resembles the network seen in grazing frozen sections (Fig. 2 *c*, *3*). Tubular elements of the network frequently meet in three-way junctions with equal spacing between intersecting elements (Fig. 3 *a*, *inset*). This junctional arrangement has been shown to be characteristic of ER networks in other cell types (36, 65) and ER networks generated in vitro (9). Images from grazing sections and isolated cortices suggest that the punctate stained sources directly beneath the plasma membrane of unfertilized eggs (Fig. 2 *b*) represent the cross-sectional profiles of tubular elements of the submembranous network.

A polygonal network pattern essentially identical to that seen with the immunofluorescent staining is observed in isolated cortices of unfertilized eggs stained with the fluorescent membrane dye $DiIC_{16}[3]$ (Fig. 3 *e*). This lipophilic molecule is one of a family of carbocyanine dyes recently shown to stain ER in fixed and living cells (65). The close resemblance between the $DiIC_{16}[3]$ stained network in unfixed

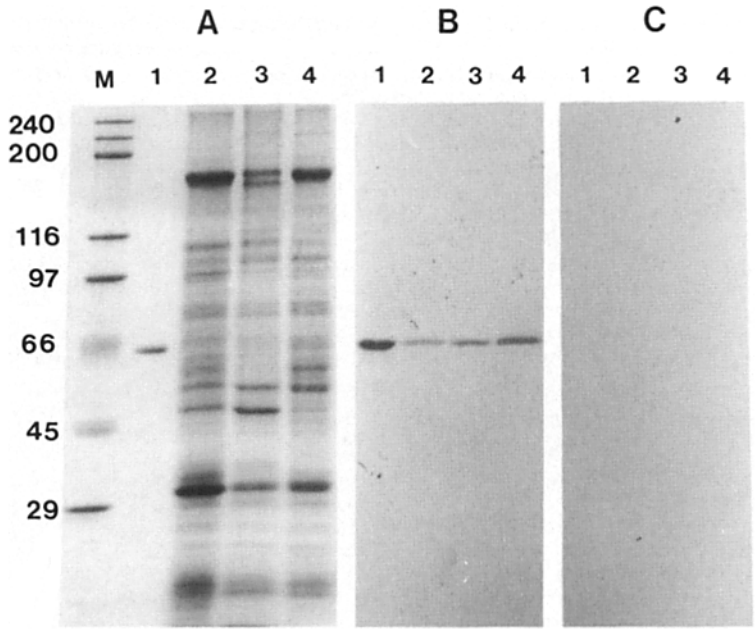


Figure 1. Immunoblot analysis of the sea urchin CSL protein antiserum. The antiserum was immunoblotted against purified sea urchin CSL protein from *S. droebachiensis* (lane *1*), and samples of *S. purpuratus* unfertilized eggs (lane *2*), isolated unfertilized egg cortices (lane *3*), and isolated MAs (lane *4*). *A* is an SDS-PAGE gel (5–20% gradient) of the samples plus a lane of molecular mass markers (*M*). *B* is a nitrocellulose replica of a companion gel probed with the CSL protein antiserum. *C* is a nitrocellulose replica of a companion gel probed with preimmune serum. The immune serum reacts specifically with a single band in all samples that comigrates with the purified CSL protein (*B*). No reaction is observed in the preimmune serum blot (*C*).

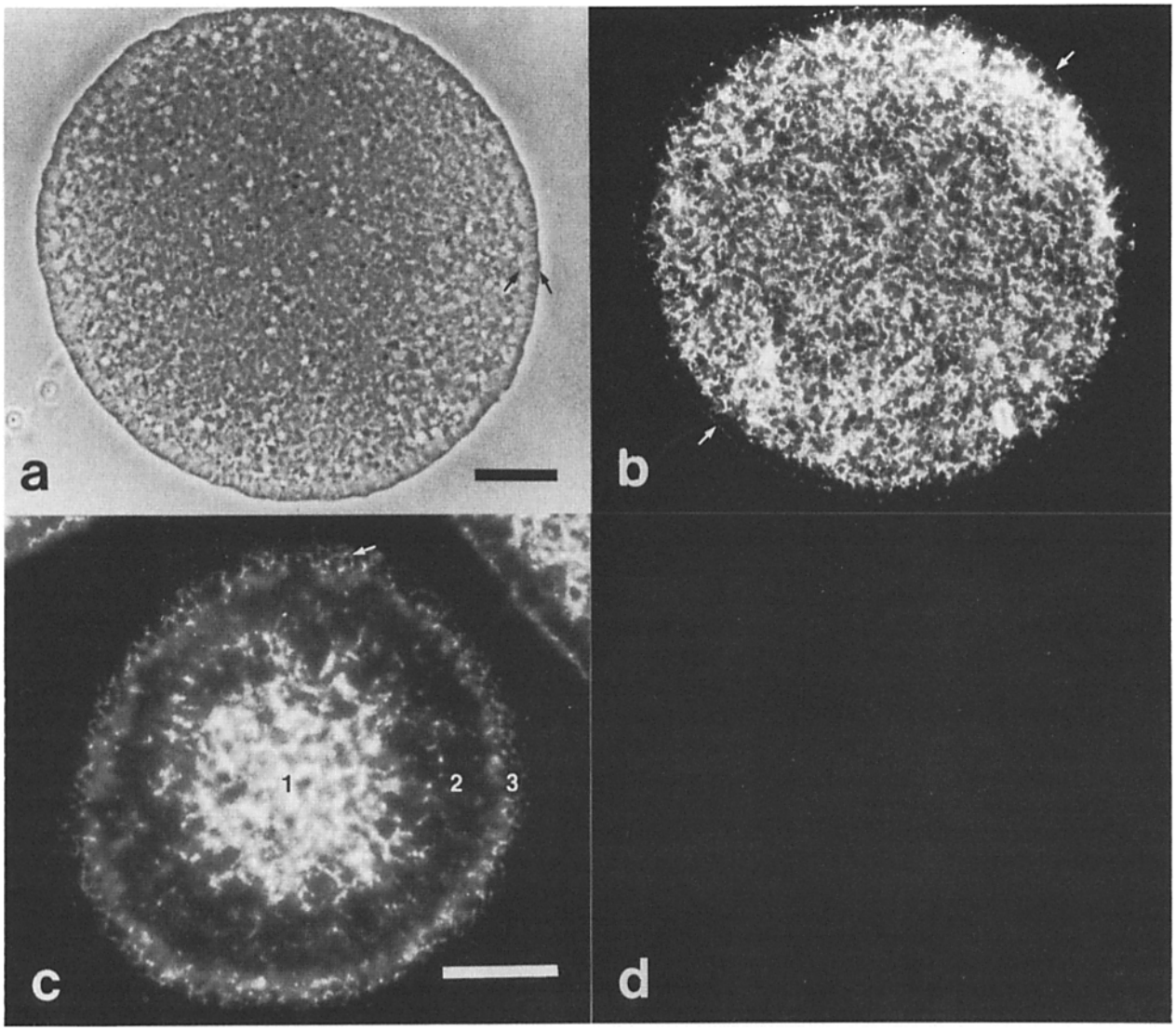


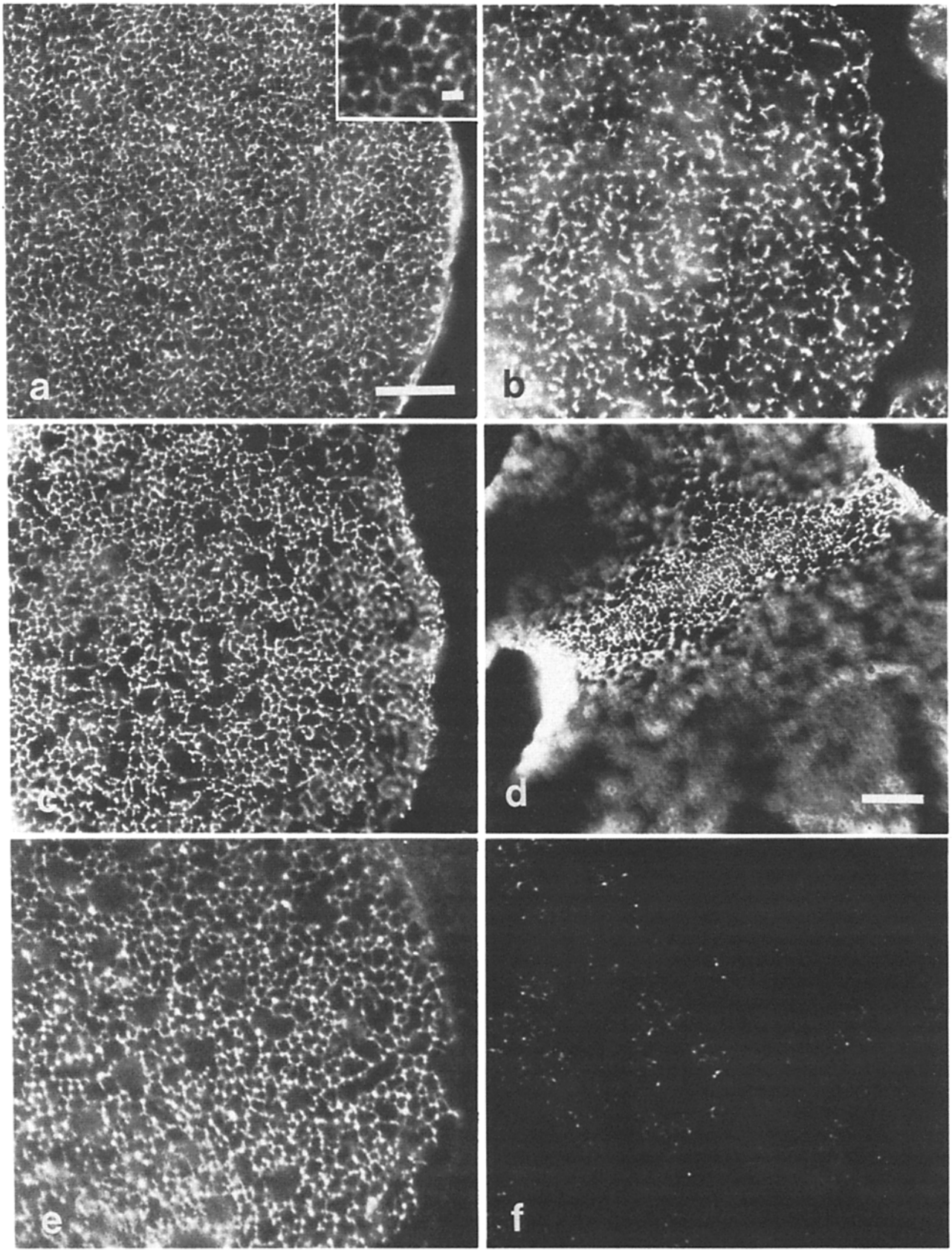
Figure 2. Immunofluorescent localization of the CSL protein in unfertilized eggs. (*a*) Phase-contrast image of frozen section showing the peripherally located CG layer (*arrows*). (*b*) Antiserum staining of this section reveals a cytoplasmic tubuloreticular network, with diminished staining in the CG layer and punctate staining just beneath the plasma membrane (*arrows*). (*c*) In a grazing frozen section the staining has three zones: (*1*) the inner cytoplasmic network, (*2*) the dimished staining of the the CG region, and (*3*) a polygonal network (*arrow*) associated with the plasma membrane. (*d*) Control preimmune serum staining of frozen section of egg. Magnifications of *a*, *b*, and *d* are equal. Bars, (*a* and *c*) 10 μm.

cortices and the CSL stained network in fixed cortices argues against the existence of fixation-dependent network reorganizations, although minor effects are still possible.

To determine if the CSL protein is within a membrane bound compartment, and not on the membrane surface, unfertilized egg cortices were processed for immunofluorescence without postfixation detergent extraction. This resulted in negligible staining of the network (Fig. 3 *f*), suggesting that the CSL protein is contained in the lumen of the elements comprising the network.

The dynamics of the plasma membrane associated network was examined by isolating cortices of embryos at intervals during the first cell cycle. In cortices isolated 2 min after fertilization, cortical granule discharge has occurred and the stained network (Fig. 3 *b*) appeared disrupted with an opening up of the polygons and a coalescence of the network elements into thicker, more intensely stained structures. By 30 min after fertilization, the stained network (Fig. 3 *c*) has reorganized and the original pattern present in the unfertilized cortex has been regenerated. The submembranous polygonal

Figure 3. Immunofluorescent localization of the CSL protein in isolated egg cortices. (*a*) Staining of unfertilized egg cortex reveals a polygonal, tubular network that has primarily three way junctions (*inset*). (*b*) In a cortex isolated 2 min after fertilization this network is opened up and distrupted in response to CG exocytosis. (*c*) A stained network resembling that of the unfertilized egg is once again present in a cortex isolated from an embryo 30 min after fertilization. (*d*) Staining of a cortex from an embryo undergoing cytokinesis shows the network



to be present in the focal plane of the invaginating cleavage furrow. (*e*) $DiIC_{16}[3]$ dye staining of an unfixed unfertilized egg cortex showing a network similar to the CSL protein network. (*f*) An egg cortex not subjected to postfixation detergent extraction shows negligible antiserum staining indicating that the CSL protein is contained within the lumen of the membranous reticulum. Magnifications of *a*, *b*, *c*, *e*, and *f* are equal. Bars, (*a* and *d*) 10 μm; (*inset*) 1 μm.

network remained in cortices of embryos undergoing division, even at the membrane of the invaginating cleavage furrow (Fig. 3 *d*). Control cortices stained with preimmune serum or secondary antibody alone showed no significant staining.

Embryos during the First Cell Cycle. The immunofluorescent staining of frozen sections of eggs and isolated egg cortices so far reveals a tubuloreticular network similar to the ER in eggs (7, 39, 57) and other cells (36, 65). To further assess whether this network is indeed the ER, we studied the distribution of the CSL protein during the first cell cycle, where ER is known to aggregate in the MA (19, 24). Sections were double labeled with Hoechst DNA-specific dye to visualize chromosomes for accurate determination of the mitotic stages of samples. In addition, confocal microscopy was performed on antitubulin stained whole embryos to compare microtubule organization with the CSL protein patterns during mitosis.

In interphase embryos fixed 30 min after fertilization, the CSL protein antiserum staining reveals a widespread tubuloreticular pattern (Fig. 4 *a*) similar to that seen in the unfertilized egg (Fig. 2 *b*), except for the cortical region. In contrast to the unfertilized egg, the cortical region now has a dense stained network. This network occupies the space previously held by the cortical granules, which were exocytosed at fertilization.

During prophase, there is a concentration of the stained reticular network at two distinct sites on opposite sides of the nucleus, and stained linear elements appear to radiate from these sites towards the cortex (Fig. 4 *b*). This distribution corresponds to the location of the microtubules forming the early asters of the assembling MA, as seen in prophase embryos stained with antitubulin antibody (Fig. 5 *a*) (19). Chromosome condensation appears to have begun (Fig. 4 *b*, *inset*), and the nucleus is swollen in preparation for nuclear envelope breakdown.

During metaphase, chromosomes are aligned on the metaphase plate, and the CSL protein antiserum staining is clearly concentrated in the mitotic apparatus (Fig. 4 *c*). The staining is apparently denser in the astral regions than in the body of the spindle and appears to be absent in the centriolar region (Fig. 4 *c*, *arrow*). Within the astral centers and the spindle the staining appears more punctate and vesicular, while in the peripheral cytoplasm a predominantly tubular network is observed. Antitubulin staining of metaphase embryos shows that microtubules are primarily restricted to the region of the MA and do not extend far out in the peripheral cytoplasm (Fig. 5 *b*) (2, 19).

During anaphase, staining remains concentrated in the more punctate elements in the astral centers (Fig. 4 *d*), and is reduced in the interzone between the two groups of migrating chromosomes (Fig. 4 *d*, *inset*). At the edges of the asters (Fig. 4 *d*, *arrows*), stained elements appear linear and extend from the asters towards the cortex. These linear arrays of tubular elements are most apparent in polar views of anaphase embryos where the plane of section cuts through a single aster (Fig. 4 *e*). In these images, the chromosomes are seen embedded in the concentration of stain in the astral center. The peripheral cytoplasm is filled with linear tubular elements radiating from the aster to the cortex (Fig. 4 *e*, *arrow*). This structural organization is similar to the distribution of astral ray microtubules that can be seen in antitubulin stained anaphase embryos to extend from the astral centers out to the submembranous region (Fig. 5 *c*) (2, 19).

During telophase, the staining concentrates in the remnants of the asters associated with each forming blastomere (Fig. 4 *f*). The peripheral network appears unaltered in the region of the cleavage furrow, consistent with the findings in isolated cortices from cleaving embryos (Fig. 3 *d*), and no real concentration is apparent in the microtubule-rich midbody (Fig. 5 *d*).

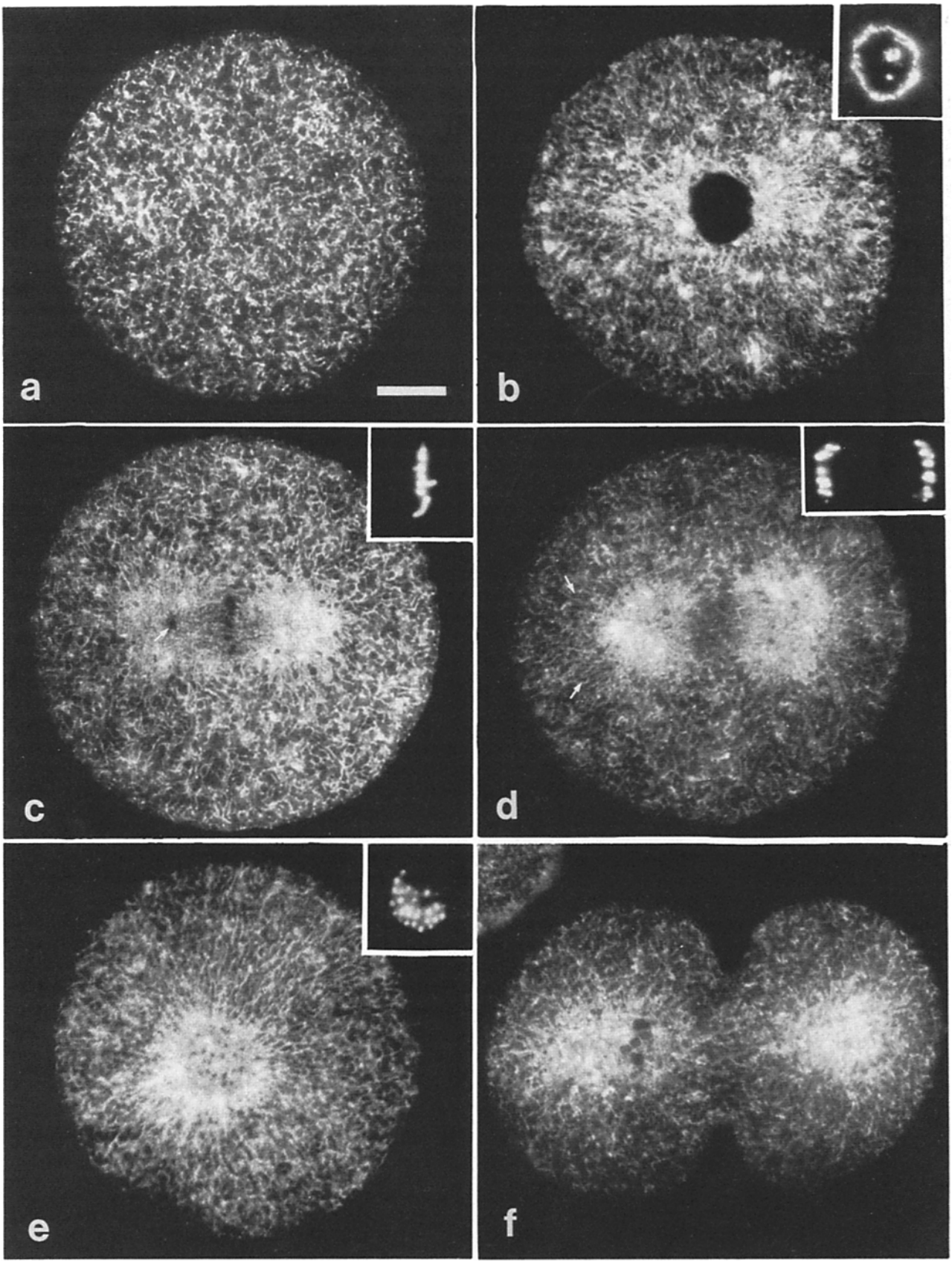
Isolated Mitotic Apparatus. The localization of the sea urchin CSL protein in the MA was further investigated by double labeling isolated *S. purpuratus* MAs with sea urchin CSL protein antiserum and a monoclonal antibody to tubulin. MAs isolated by the method of Silver et al. (61) retain many membranous vesicles as seen in phase-contrast (Fig. 6 *a*). The anti-CSL protein stain is concentrated in the asters and greatly diminished in the spindle region (Fig. 6 *c*), in contrast to the anti-tubulin staining that outlines both the asters and the spindle (Fig. 6 *b*). In certain areas of the asters, CSL protein containing tubular elements (Fig. 6 *c*, *arrow*) appear to align with microtubules (Fig. 6 *b*, *arrow*). The CSL protein antibody staining pattern of the isolated MAs corresponds to the staining of the in situ MAs observed in the frozen sections (Fig. 4).

Centrifugally Stratified Unfertilized Eggs. In unfertilized sea urchin eggs subjected to centrifugation the cytoplasm stratifies into layers whose components have been ultrastructurally identified (1). The whole egg seen in Fig. 7 *a* shows the lipid droplet at the centripetal pole, the yolk platelet rich granular zone at the centrifugal pole and the ER rich and nucleus containing clear zone (CZ) in between. To determine the distribution of the CSL protein in these cytoplasmic strata, frozen sections of stratified eggs were stained with antiserum against the CSL protein. A phase-contrast image of a frozen section (Fig. 7 *b*) shows the CZ containing the nucleus. Antiserum staining of this section (Fig. 7 *c*) reveals a dramatic concentration of immunofluorescent elements in the CZ, where there is an enrichment of the ER. The staining also reveals a sparsely distributed network remaining in the rest of the cytoplasm.

Electron Microscopic Localization of Sea Urchin CSL Protein in Unfertilized Eggs

Immunoperoxidase methods (38) were used to investigate the ultrastructural localization of the CSL protein in unfertilized

Figure 4. Immunofluorescent localization of the CSL protein in first cell cycle embryos. (*a*) Interphase embryo (30 min after fertilization) has an immunofluorescent tubuloreticulum extending throughout the cytoplasm resembling the network present in the unfertilized egg. (*b*) Prophase embryo showing concentration of CSL protein containing elements on opposite sides of the nucleus and linear stained elements radiating from these sites towards the cortex. Inset shows Hoechst-stained condensed chromosomes. (*c*) Metaphase embryo showing concentration of punctate and vesicular stained elements within the MA, particularly in the asters. Staining is absent from the centriolar region (*arrow*). Inset shows chromosomes aligned on the metaphase plate. (*d*) Anaphase embryo has staining still concentrated in the asters and



diminished in the interzone. Stained linear elements are evident at the edges of the asters (*arrows*). Inset shows the migrating chromosomes. (*e*) Polar view of an embryo in anaphase shows staining to be concentrated in the astral center and to extend from the aster to the cortex in the form of linear elements. Inset shows cross-sectional profiles of chromomsomes. (*f*) Telophase embryo showing that the CSL protein staining remains concentrated in the residual asters of the two forming blastomeres. Magnifications of *a–f* are equal. Bar, 10 μm.

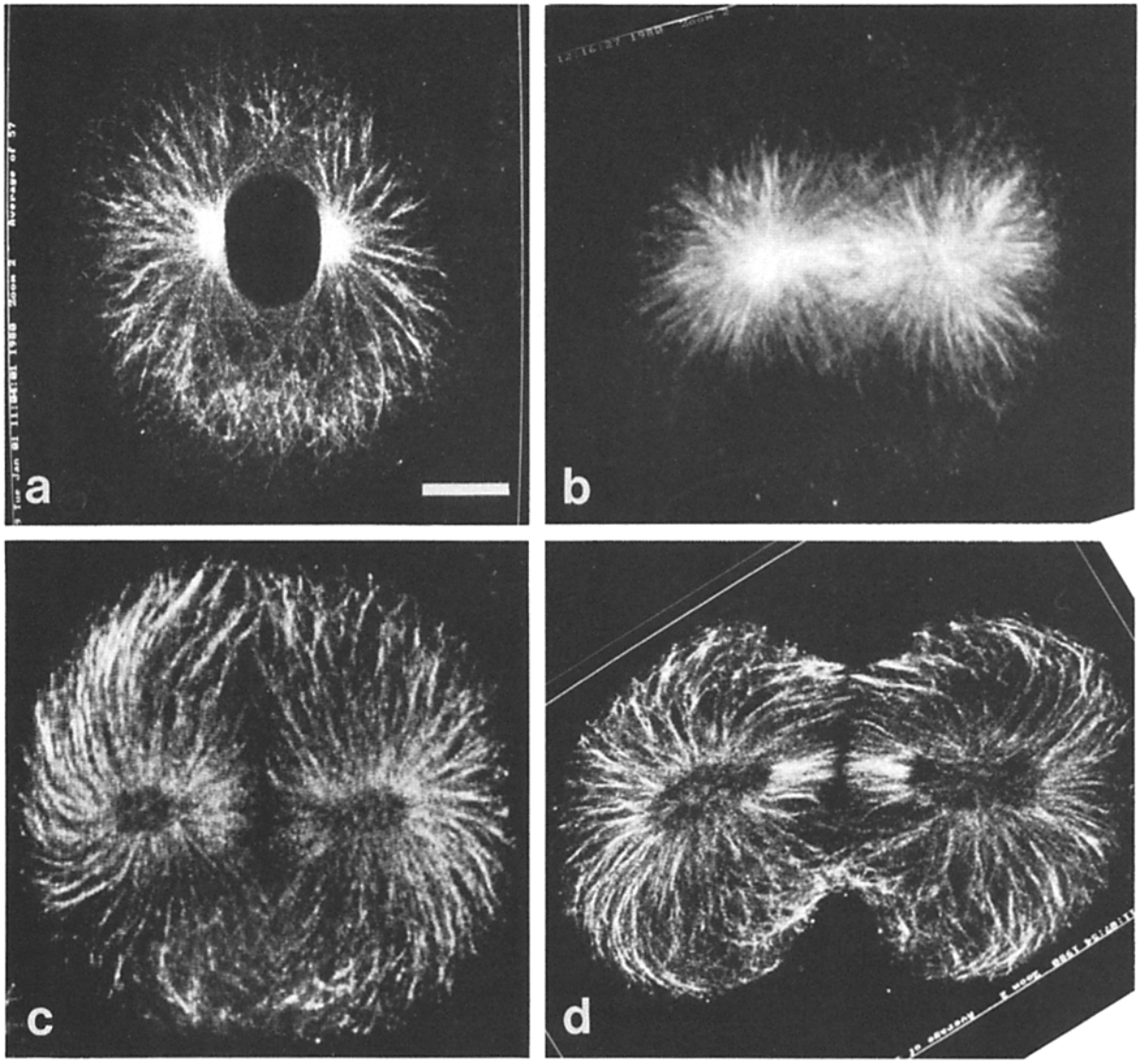


Figure 5. Confocal microscopy of antitubulin immunofluorescence of embryos undergoing mitosis. (*a*) Prophase embryo showing microtubules concentrated in the early astral centers and extending out towards the cortex. (*b*) Metaphase embryo showing microtubules in a compact MA. (*c*) Anaphase embryo reveals the extension of astral ray microtubules from the astral centers to the submembranous region. (*d*) Telophase embryo showing microtubules of the residual asters and the midbody. Magnifications of *a*, *b*, *c*, and *d* are equal. Bar, 10 μm.

eggs. Thin section EM of untreated eggs (Fig. 8 *a*) serves as a control for comparing the structure of detergent extracted, antibody-labeled eggs (Fig. 8 *b*). In eggs stained with the CSL protein antiserum, the labeling is primarily restricted to the lumen of tubular, ER-like elements cut both in longitudinal and cross section (Fig. 8 *b*, *arrows*). A higher magnification view (Fig. 8 *c*) shows the presence of label in the luminal compartment between the two opposing membranes of ER-like tubules (Fig. 8 *c*, *arrows*). Labeled tubules are prevalent in the inner cytoplasm, sparse in the cortical granule layer and appear primarily as cross-sectional profiles just beneath the plasma membrane (Fig. 8 *b*, *arrowheads*). This is consistent with the immunofluorescent staining pattern of frozen sections of unfertilized eggs (Fig. 2 *b*). Cortical granules (CGs), acidic vesicles (AVs), and mitochondria are all evident but do not stain with the CSL protein antiserum. Control staining of extracted eggs with preimmune serum, secondary antibody alone or the peroxidase reaction alone resulted in no specific staining.

Discussion

In this study, we have demonstrated by light and and electron microscopic immunocytochemical techniques the presence of a CSL protein in a tubuloreticular network in sea urchin eggs and early embryos. Supporting evidence for this network corresponding to the ER consists of the following: (*a*) Immunofluorescent staining of the network, particularly in isolated egg cortices (Fig. 3, *a* and *c*), is highly similar to ER networks fluorescently visualized by carbocyanine dyes in other cell types (36, 65) and in egg cortices (Fig. 3 *e*). The similarities include the network's polygonal geometry and

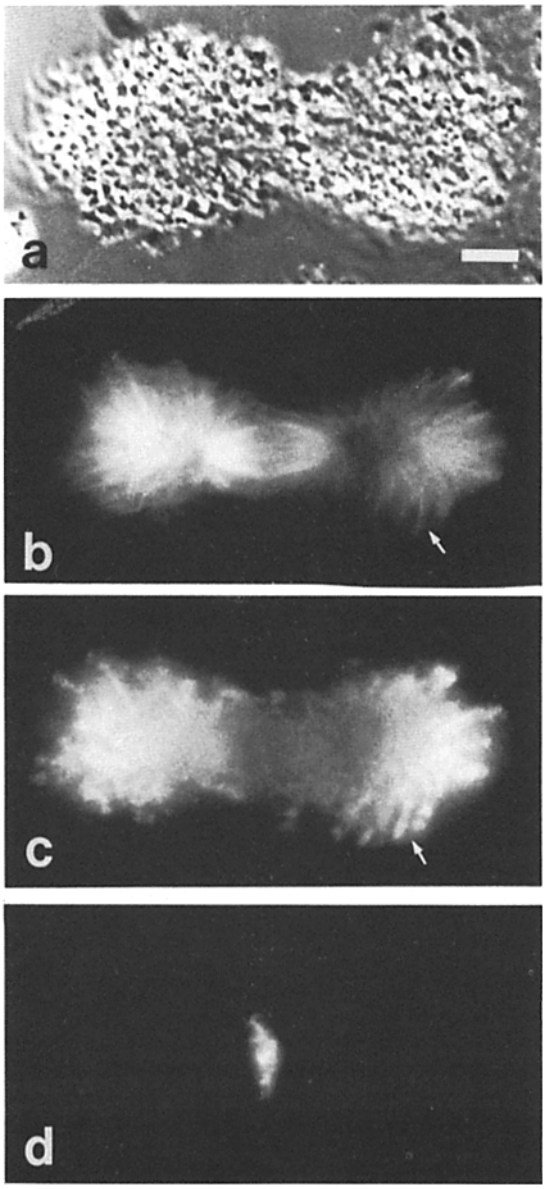


Figure 6. Immunofluorescent localization of the CSL protein in isolated MAs. (*a*) Phase-contrast image of a metaphase MA showing associated membranous material. (*b*) Anti-tubulin staining of this MA shows the microtubules that compose the asters and the spindle. (*c*) Anti-CSL protein staining of this MA shows a concentration of staining in the asters relative to the spindle. Where tubular CSL protein containing elements are evident (*arrow*), they appear to align with microtubules (*arrow* in *b*). (*d*) Hoechst-stained chromosomes. Bar, 5 μm.

characteristic three-way junctions of tubular network elements (Fig. 3 *a*, *inset*). (*b*) The network in isolated cortices demonstrated in this study closely resembles previous ultrastructural images of polygonal ER networks associated with the plasma membrane of isolated egg cortices (7, 57). (*c*) The disruption of the CSL protein containing network in re-

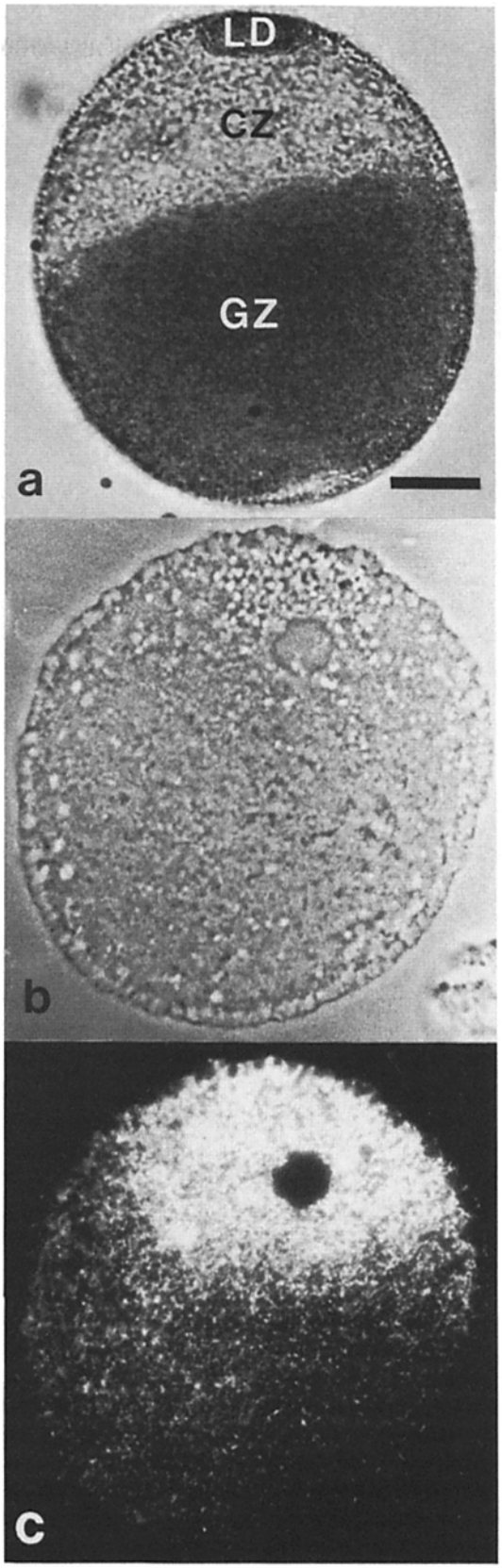


Figure 7. Immunofluorescent localization of the CSL protein in centrifugally stratified unfertilized eggs. (*a*) Stratified unfertilized egg with lipid droplet at the centripetal pole, the granular zone at the centrifugal pole, and the ER-rich and nucleus-containing CZ in between. (*b*) Frozen section of stratified egg showing nucleus embedded in the CZ. (*c*) Anti-CSL protein staining of this section reveals an accumulation of immunofluorescence in the CZ. A faint reticular network still persists in the rest of the egg. Magnifications of *a*, *b*, and *c* are equal. Bar, 10 μm.

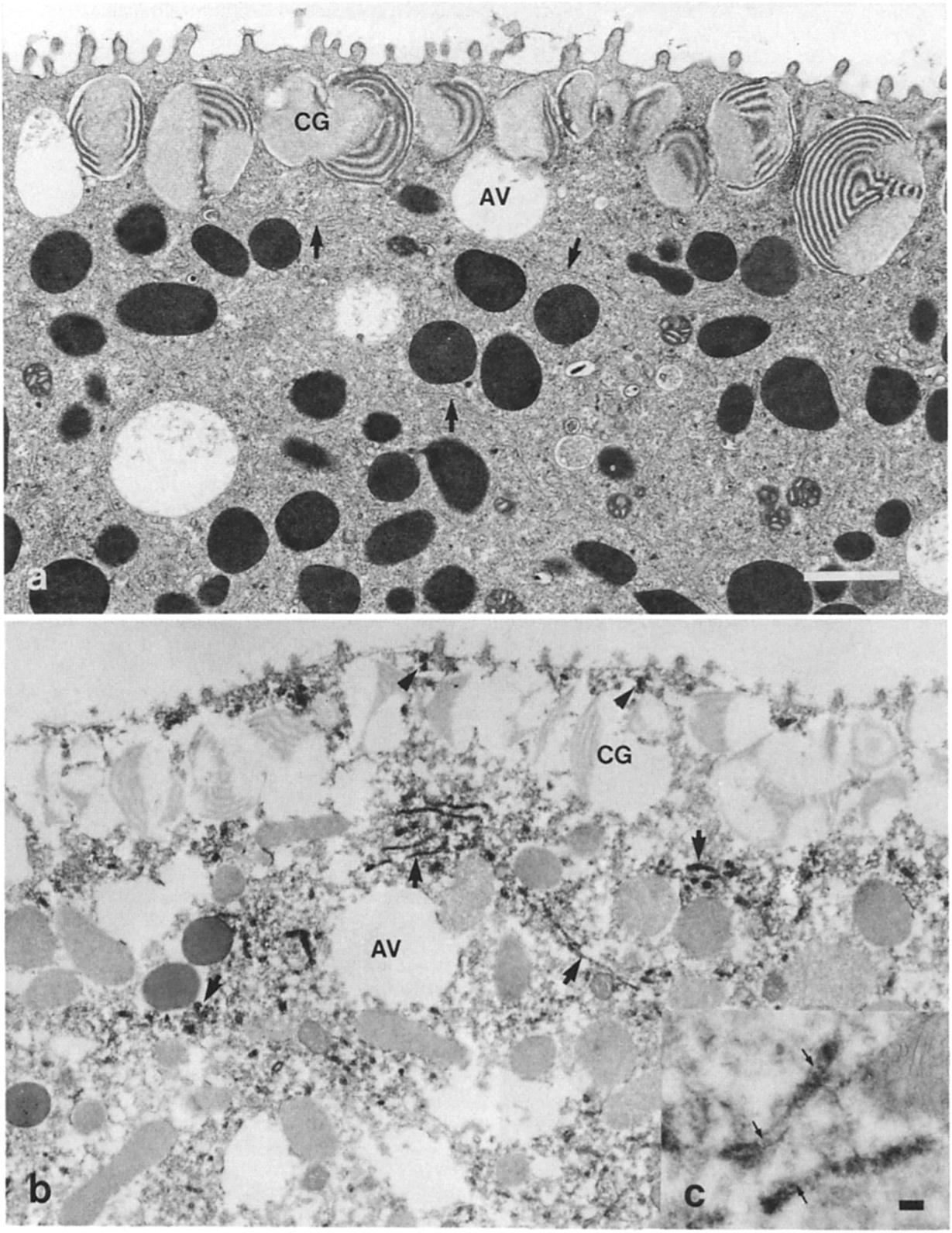


Figure 8. Electron microscopic localization of the CSL protein in unfertilized eggs. (a) Control, nondetergent extracted unfertilized egg showing the surface microvilli, CG, ER (*arrows*), AV, and mitochondria typical of the egg cortex. (*b*) Immunoperoxidase localization of the CSL protein in a detergent extracted unfertilized egg shows the electron dense labeling associated with ER-like tubules, cut in longitudinal and cross section (*arrows*). Cross sections of labeled tubules appear just below the plasma membrane (*arrowheads*). CSL protein label is not associated with the CGs, AVs or mitochondria. (*c*) At higher magnification, the CSL protein label can be seen to reside in the lumen between opposing membranes of ER-like tubules (*arrows*). Magnifications of *a* and *b* are equal. Bar, 0.1 μm.

sponse to CG discharge after fertilization (Fig. 3 *b*) resembles published electron microscopic observations of cortical ER network changes after fertilization (57). (*d*) Immunoelectron microscopic localization shows the presence of the CSL protein in the luminal compartment of ER-like tubules in sections of whole eggs (Fig. 8, *b* and *c*). The cytoplasmic, immunofluorescent network present in frozen sections of eggs (Fig. 2 *b*) is similar in organization to ER networks seen electron microscopically in thick sections of osmium-zinc-iodide stained eggs (39). (*e*) Elements of the reticulum containing the CSL protein concentrate in the asters of the MA of dividing first cell cycle embryos (Fig. 4), resembling the known aggregation of ER in the MA of echinoderm embryos and other cells (19, 24). The punctate nature of the CSL protein staining in the asters and the linear tubular elements in the peripheral cytoplasm (Fig. 4 *e*) agrees with previous studies showing aster-associated vesicular ER and alignment of ER along astral ray microtubules (19). (*f*) In centrifugally stratified eggs, the immunofluorescent staining is concentrated in the CZ (Fig. 7), a layer known to be highly enriched in ER (1). (*g*) Finally, the sea urchin CSL protein was originally purified from a microsomal fraction of unfertilized eggs containing the ER marker enzymes glucose-6-phosphatase and nicotinamide adenine dinucleotide phosphate-cytochrome C reductase (44).

Unlike the sarcoplasmic reticulum in muscle cells, where calsequestrin is restricted to the terminal cisternae (30), our results show a uniform distribution of the CSL protein in the ER of the egg without any observable concentration sites. The protein appears to be present in both rough and smooth ER in the egg since ultrastructural studies have shown the cortex associated ER network to be ribosome studded (7, 57) while MA-associated ER is predominantly smooth in nature (19, 24). A recent study provides immunological and biochemical evidence for the presence of a CSL protein in the ER of mammalian cells (10). In addition, Koch and coworkers have documented the presence of a number of Ca^{++} storage proteins in the ER of cultured mammalian cells (33, 40). The results of these studies and of our experiments contrast with the recent findings by Volpe et al. (76) who report that in mammalian cells, calsequestrin is localized in distinct cytoplasmic organelles (termed "calciosomes") rather than in the ER.

The CSL protein containing ER appears to be a dynamic mobile network responding to the various functional events of the cell. This network is disrupted and reforms during fertilization (Fig. 3) and concentrates in the asters of the MA during division (Fig. 4). Staining is diminished in the interzone during anaphase, suggesting that the ER network is relocalized during chromosome movement. Recent studies using video microscopy have revealed the dynamic nature of ER networks in living cells (36) as well as in a reconstituted system (9). ER organization appears to be highly dependent upon microtubule distribution in cells (66), and the movement of ER may be mediated by microtubule-associated mechanochemical enzymes (9). Our results indicate that the organization of the CSL protein containing ER follows that of the microtubules of the MA (Figs. 4, 5, and 6), and suggests that the dynamic reorganization of this network during mitosis may be microtubule dependent.

The demonstration of a CSL protein lying within the ER of the egg adds to our understanding of the role of this organelle in Ca^{++} regulation. Cortical preparations, shown here to contain a network of CSL protein containing ER (Fig. 3), are known to take up Ca^{++} in an ATP-dependent manner and to release it in response to IP_3 and the calcium ionophore A23187 (44, 46). IP_3 is known to release Ca^{++} from the ER in a number of cell types (3) including the egg (72, 73, 77). Isolated ER in microsomal fractions is also known to take up Ca^{++} in an ATP-dependent manner (26, 44) and to release it in response to IP_3 (8). Ultrastructural studies have localized Ca^{++} within ER-like compartments in the unfertilized egg (50). Furthermore, the ability of the MA in embryos (32) and in vitro (61) to take up Ca^{++} correlates with the demonstrated concentration of CSL protein containing ER in the MA (Figs. 4 and 6). Previous studies have localized intracellular Ca^{++} in the ER in the MA of cells at both the light (24, 79) and electron microscopic level (24, 78).

In addition to a CSL protein, a Ca^{++} ATPase transport enzyme may also be present in the ER of the egg. An egg protein that cross reacts with an antibody against the Ca^{++} ATPase transport enzyme of the sarcoplasmic reticulum of muscle (60) and a 46-kD Ca^{++} ATPase transport protein of eggs (47, 48) have been immunocytochemically localized in the asters of the egg MA. This suggests that additional elements of a SR-like Ca^{++} regulatory system may be associated with the ER in the egg.

The organization and dynamics of the CSL protein containing ER has fundamental implications for the regulation of fertilization and mitosis by intracellular Ca^{++}. Our demonstration of an extensive, three-dimensional ER network containing the CSL protein and distributed throughout the unfertilized egg cytoplasm fits the requirements of a widespread calcium uptake and release system needed for the generation of the Ca^{++} wave at fertilization. This wave travels across the cytoplasm of the sea urchin egg, originating at the site of sperm fusion (15, 18). Furthermore, the fertilization-dependent Ca^{++} release has been shown to emanate from the CZ of stratified eggs (14). The concentration of immunofluorescence in the CZ (Fig. 7) implicates the CSL protein containing ER as being the source of this Ca^{++} release. However, since additional cytoplasmic organelles have also been implicated in Ca^{++} storage in the egg (14, 58), the ER cannot be considered the exclusive Ca^{++} regulating organelle.

Fluxes in intracellular Ca^{++} levels have also been shown to occur at the stage of chromatin condensation and nuclear envelope breakdown during prophase of the first cell cycle in sea urchin embryos (63, 74). Elimination of a transient intracellular Ca^{++} elevation with microinjected Ca^{++} buffers block chromatin condensation and nuclear envelope breakdown, while artificial increases in intracellular Ca^{++} via microinjection of Ca^{++} or IP_3 induce premature chromatin condensation and nuclear envelope breakdown (63, 74). In our study, CSL protein containing ER has been shown to concentrate around the nuclear envelope during the time of chromatin condensation and nuclear envelope breakdown in prophase (Fig. 4 *b*). This accumulation of ER in the perinuclear region may be significant in the precise control of Ca^{++} concentrations in the vicinity of the occurrence of those events.

The concentration of the CSL protein containing ER in the MA, as we have observed, is consistent with the need for the precise control of Ca^{++} in the MA during mitosis. The regulation of mitosis by intracellular Ca^{++} has been implied

from the sensitivity of the progression of mitosis to Ca^{++} levels (22, 27, 31), the location of Ca^{++} sequestering activity in the MA (61) and the sensitivity of MA microtubules to Ca^{++} (32, 37, 56). The actual nature of the intracellular Ca^{++} fluctuations during mitosis is not clear; however, there appears to be agreement on a general increase in intracellular Ca^{++} levels. A recent report describes brief, transient elevations in Ca^{++} at the metaphase to anaphase transition (52), another study describes the occurrence of multiple transient elevations that do not coincide with this transition point (55), and still other evidence points to a gradual increase in calcium starting at metaphase and continuing into telophase (23, 55). Increases in Ca^{++} during mitosis could be directly responsible for spindle microtubule depolymerization (23, 37, 56). In vitro studies have shown that microtubule depolymerization alone is sufficient to allow for the poleward movement of chromosomes (34) supporting a long standing hypothesis advanced by Inoué (25). Alternatively, the elevation of Ca^{++} concentrations may induce microtubule depolymerization indirectly by Ca^{++}-dependent phosphorylation of a 62-kD MA-associated sea urchin egg protein (11). Furthermore, the ER concentrated in the asters of the MA may be involved in the apparent regulation of cytokinesis by the MA that provides the stimulus for and determines the orientation of the cleavage furrow (53).

In summary, the results reported here demonstrate an extensive network of ER containing a CSL protein in sea urchin eggs and first cell cycle embryos. The distribution of this network and its organizational dynamics during the first cell cycle suggest that it may function in the generation of Ca^{++} fluxes responsible for the regulation of fertilization and cell division.

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