DEVELOPMENTAL BIOLOGY **179,** 320–328 (1996)
ARTICLE NO. 0263

Structural Change of the Endoplasmic Reticulum during Fertilization: Evidence for Loss of Membrane Continuity Using the Green Fluorescent Protein

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Green fluorescent protein (GFP) was targeted to the lumen of the endoplasmic reticulum (ER) of starfish eggs by injecting mRNA coding for a chimeric protein containing a signal sequence and the KDEL ER retention sequence. By confocal microscopy, the GFP chimeric protein was localized in intracellular cisternae (membrane sheets) and the nuclear envelope, showing that it had been successfully targeted to the ER. The labeling pattern closely resembled that produced by the fluorescent dicarbocyanine DiI, which has been used previously to label the ER (Jaffe and Terasaki, *Dev. Biol.* 164, 579–587, 1994). Eggs expressing the GFP chimera were used to examine whether there is a loss of ER continuity at fertilization. The time required for recovery of fluorescence after photobleaching for both the GFP chimera and DiI was much longer in eggs at 1 min postfertilization than in unfertilized eggs or in 20-min-postfertilized eggs. This result provides strong evidence for a transient loss of continuity of the ER associated with Ca release at fertilization.

INTRODUCTION

At fertilization, the sperm initiates a propagated release of Ca in the eggs of many different organisms, including fish (Gilkey *et al.*, 1978), sea urchins (Eisen *et al.*, 1984), frogs (Kubota *et al.*, 1987), ascidians (Speksnijder *et al.*, 1990), mammals (Miyazaki *et al.*, 1992), and starfish (Stricker, 1995). It is very likely that the endoplasmic reticulum (ER) is the internal membrane store from which Ca is released (Eisen and Reynolds, 1985; Han and Nuccitelli, 1990; Terasaki and Sardet, 1991; Miyazaki *et al.*, 1992; Nuccitelli *et al.*, 1993; Mohri *et al.*, 1995). The Ca rise is of central importance to the activation of the egg to begin development (Whitaker and Steinhardt, 1985; Kline, 1988; Kline and Kline, 1992). Among the issues that are still not understood are: how the sperm initiates the Ca wave, how the wave is propagated, and what specific pathways are activated by Ca and by what mechanisms.

One potential clue about the propagation mechanism and about the target of Ca is the change in ER structure that occurs at fertilization in echinoderm eggs. This change was shown in studies in which the ER in living eggs was labeled by injection of DiI, a fluorescent long-chain lipophilic dye (Terasaki and Jaffe, 1991; Jaffe and Terasaki, 1994). At fertilization, an initial change in ER structure proceeded as a wave from the point of fertilization. The membrane sheets in the interior appeared to become more finely divided. The most altered structure was seen at 2–3 min postinsemination in sea urchin eggs. The change was transient, since the ER returned to its original appearance by about 8 min (Terasaki and Jaffe, 1991). The timing and spatial pattern of this transient change correspond well with the rise and fall in cytosolic Ca concentration at fertilization and indicate that there is a large, transient change in ER structure when it releases Ca (Terasaki and Jaffe, 1991; Jaffe and Terasaki, 1994).

We report here studies of the changes in ER at fertilization using a new method for visualizing the ER with improved specificity. The green fluorescent protein (GFP) is a naturally fluorescent protein in the jellyfish *Aequorea victoria* (Prasher, 1995). GFP was cloned and expressed in *Caenorhabditis elegans*, where it was used as a reporter

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0012-1606/96 $18.00

gene for a neuronal promoter (Chalfie *et al.*, 1994). GFP was then used to make a fluorescent chimera of the *exuperantia* gene product in *Drosophila* in order to follow the distribution of this protein during oogenesis (Wang and Hazelrigg, 1994). A mutant GFP has been identified which has a greater fluorescent yield and a fluorescence excitation spectrum that is better suited for microscopy (Heim *et al.*, 1995). With this altered GFP, we have generated a chimeric GFP molecule that is targeted to the ER lumen within starfish eggs and have used this probe in measurements of fluorescence recovery after photobleaching. The results provide strong evidence for a transient loss of ER continuity at fertilization.

MATERIALS AND METHODS

Starfish (*A. miniata*) were obtained from Marinus, Inc. (Long Beach, CA) and were maintained in running seawater tanks at the Marine Biological Laboratory (Woods Hole, MA). Oocytes were collected by use of a 3-mm sample corer (Fine Science Tools, Foster City, CA; see Jaffe and Terasaki, 1994, for further details on obtaining oocytes). In this article, we will use the term ''oocyte'' to refer to the fully grown immature oocyte at meiotic prophase (germinal vesicle stage) and ''egg'' to refer to the mature oocyte at first metaphase after it has been stimulated by 1-methyladenine to resume meiosis. 1-Methyladenine was obtained from Sigma Chemical Co. (St Louis, MO) and used at 1–2 μM. Eggs were fertilized 20–60 min after germinal vesicle breakdown. Microinjection was done as described previously (Terasaki and Jaffe, 1993).

A green fluorescent protein mutant with serine for threonine at position 65 (Heim *et al.*, 1995) was obtained from Roger Heim and Roger Tsien (UCSD). A partial clone encoding the first 44 amino acids of the cDNA for sea urchin ER calcistorin–protein disulfide isomerase (ECast/PDI) (Lucero *et al.*, 1994) was obtained from Hector Lucero and Benjamin Kaminer (Boston University). To construct the chimeric protein (Fig. 1), we first obtained a fragment from the sea urchin ECast/PDI plasmid by PCR, using as primers TCGAGAATTCCTTAAAAATGAAGTATTTGGCTCTTTG (forward) and CATGCTCGAGAGCGACATCTTCTTCGATTTC (reverse). The fragment generated contained an *Eco*RI restriction site at the 5′ end, the 7-nucleotide sequence immediately 5′ of the ATG start codon of ECast/PDI, the first 28 amino acids of ECast/PDI, and an *Xho*I restriction site at the 3′ end. To minimize the error rate in the PCR reaction, PFU polymerase (which has proofreading ability), high template concentration, and low cycle number were used. The PCR amplified product was cut with *Eco*RI and *Xho*I, gel purified, and cloned into the *in vitro* transcription vector pSP64R1 (Kreig and Melton, 1984; Tang *et al.*, 1995). A second fragment was made from the mutant GFP clone from Heim and Tsien by PCR using as primers GCTACTCGAGATGAGTAAAGGAGAAGAACTTTTC (forward) and GCTAGATCTTTAAAGTTCATCCTTTTTGTATAGTTCATCCATGC (reverse). This generated a fragment containing an *Xho*I restriction site at the 5′ end, the mutant GFP, the KDEL ER retention sequence, stop codon, and a *Bgl*II restriction site at the 3′ end. The fragment was amplified by PCR as above, cut with *Xho*I and *Bgl*II, gel purified, and cloned into the plasmid resulting from the previous steps. The final construct contains a 2-amino-acid linker (glutamate–leucine) resulting from the added restriction site between the ECast/PDI and GFP sequences.

For *in vitro* transcription, the plasmid was linearized with *Bam*HI and transcribed with SP6 RNA polymerase using an RNA Transcription kit (Stratagene, La Jolla, CA). The mRNA was resuspended in water at a concentration of 1–3.6 mg/ml. It was injected at 1–4% of the oocyte volume, resulting in a final concentration of 10–144 μg/ml (31–450 pg per oocyte). A concentration of 144 μg/ml caused 4/4 oocytes to lyse within a few hours, while 36 μg/ml caused lysis in 2/4 oocytes overnight. Lower concentrations seemed to have no toxic effect. We therefore typically injected to a final concentration of 10–20 μg/ml.

$DiIC_{16}(3)$ 3,3′ dihexadecyloxacarbocyanine iodide (DiI) was obtained from Molecular Probes (Eugene, OR) and was made as a saturated solution in Wesson oil (100% soybean; Super Stop and Shop, Falmouth, MA). A droplet about 70 μm in diameter was injected (the oocyte diameter is about 180 μm). DiI appears to leave the droplet completely within 5–10 min; since this large droplet can sometimes interfere with observations, most of it was removed before the experiment using a microinjection pipette (''oocyte liposuction'').

Fluorescein dextran (70 kDa) with six fluorescein molecules per dextran molecule was obtained from Molecular Probes. A 1% injection of a 100 μg/ml solution in injection buffer resulted in a 1 μg/ml final concentration in the egg; this corresponds to a fluorescein concentration of 84 nM. Total oocyte protein was determined by BCA assay (Pierce Chemical Co., Rockford, IL).

A Bio-Rad MRC 600 confocal microscope (Oxnard, CA) with upgraded photomultiplier tubes was coupled with a Zeiss Axioskop (Thornwood, NY). A Zeiss 63X 1.4 N.A. lens was used for imaging the ER structure. A Zeiss 10X 0.3 N.A. lens was used for quantitating the increase in fluorescence. Photobleaching experiments were performed in the following manner. An egg was imaged using a Zeiss 40X plan-neofluar 1.3 N.A. objective. The confocal microscope was set to scan continuously at the 1-sec rate. At the start of the experiment, an area approximately 35 $\times$ 50 μm (the area scanned at zoom 6 setting) was irradiated for 8 $\times$ 1 sec scans at unattenuated laser intensity. At the end of the 8th scan, the magnification was changed to the lowest magnification (zoom 1) and the laser light was attenuated 33-fold; with the Bio-Rad confocal microscope, these changes can be made manually without interrupting the continuous scan. With this procedure, all but the 1st scan after photobleaching provided useful data. The light intensity (energy/area) used for bleaching was ~1200 times as high as the light intensity used for imaging (i.e., 33 $\times$ 6 $\times$ 6). Throughout this procedure, each scanned image was recorded manually on an optical memory disk recorder (Model TQ-3038F; Panasonic, Secaucus, NJ). The video signal was passed through a ForA VTG-33 time date generator (Cypress, CA) in order to mark the time the images were recorded. For quantitation, images were transferred to a Macintosh computer with a video capture card. Measurements of average fluorescence intensity were done using NIH Image 1.57 (Wayne Rasband, Research Services Branch, NIH, Bethesda, MD). For making figures, images were cropped and arranged in Photoshop (Adobe Systems Inc., Mountain View, CA) and printed on a Tektronix Phaser 440 dye sublimation printer (Wilsonville, OR).

RESULTS

GFP Construct Targeted to the ER

A chimeric GFP protein was designed so that it would be inserted into the ER upon synthesis and retained there as a

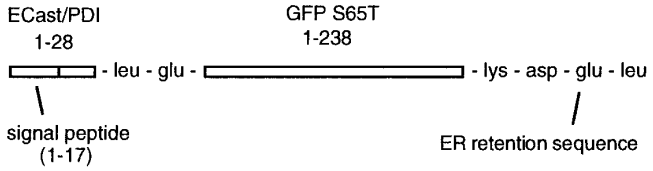

FIG. 1. Map of GFP–KDEL construct. The N terminal end consists of the first 28 amino acids of sea urchin egg ECast/PDI (Lucero *et al.*, 1994). The first 17 amino acids of this fragment serve as a signal sequence, which targets the protein for the ER and is afterward cleaved by signal peptidase. There is a 2-amino-acid linker to the GFP sequence (serine 65 to threonine mutant; Heim *et al.*, 1995), followed by the KDEL ER retention sequence at the C terminal end.

lumenal protein (Fig. 1). The chimeric protein had a signal sequence at its N-terminus and an ER retention signal at its C-terminus. In the construction of this chimeric GFP, we made use of a recently sequenced ER protein from sea urchin, ECast/PDI (Lucero *et al.*, 1994). This protein contains a signal sequence at its N-terminus and the ER retention signal KDEL (Munro and Pelham, 1987) at its C-terminus. The chimeric GFP protein contains the first 28 amino acids of ECast/PDI (comprising the N-terminal 17-amino-acid signal sequence, which is cleaved by signal peptidase, and the first 11 amino acids of the mature form of ECast/PDI), all 238 residues of the mutant form of GFP (65 SΔT) with higher fluorescence yield (Heim *et al.*, 1995), and KDEL at the C terminus. For convenience, we will refer to this protein as GFP–KDEL.

mRNA coding for GFP–KDEL was injected into immature starfish oocytes and resulted in expression of the protein as demonstrated by an increase in fluorescence. Under our culture conditions (18–20°C), the time course of the fluorescence increase consisted of a lag of approximately 1 hr, followed by a linear increase for about 8 hr, after which the rate of increase slowed (Figs. 2 and 3).

When the oocytes were imaged at high magnification

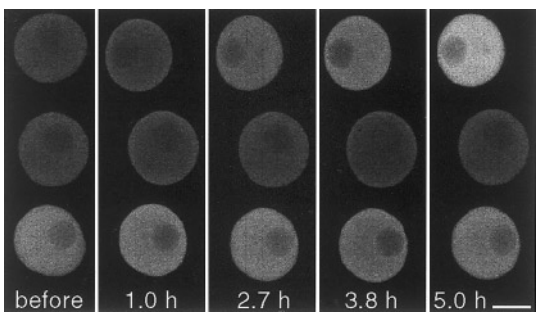

FIG. 2. Time course of the fluorescence increase in an oocyte injected with mRNA for GFP–KDEL. The top oocyte was injected with a final concentration of 10 μg/ml mRNA for GFP–KDEL. The middle oocyte was uninjected and served as the autofluorescence control. Prior to the start of the experiment, the bottom oocyte was injected with a final concentration of 1 μg/ml fluorescein dextran (70 kDa). The left-most panel shows an image obtained before injection of GFP–KDEL mRNA. The other panels show images obtained at the indicated times after injection of mRNA. The large dark circle is the germinal vesicle (nucleus). Bar, 100 μm.

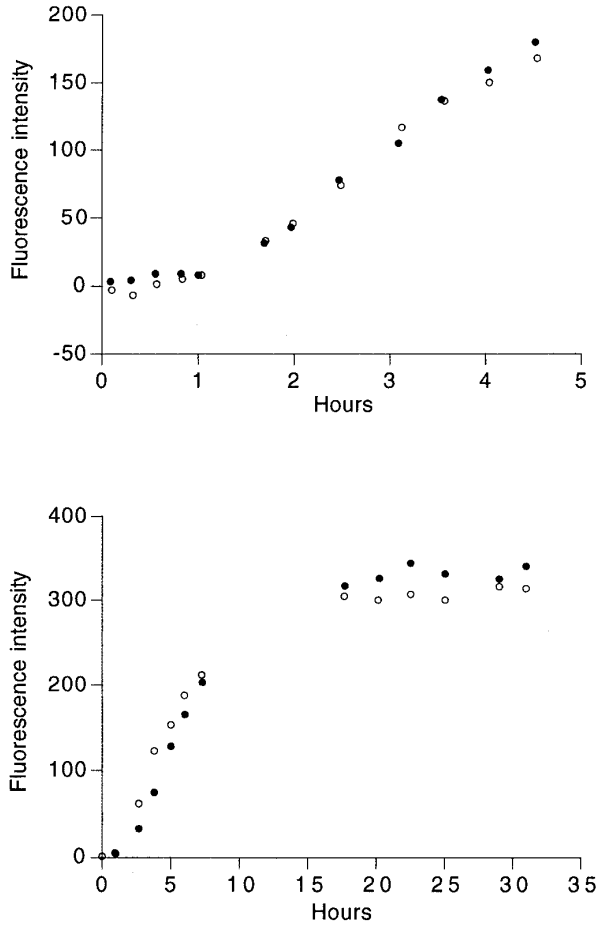

FIG. 3. Graph of fluorescence increase in oocytes injected with mRNA for GFP–KDEL. (Top) An experiment showing the early time points for the increase. There is a lag period of about 1 hr before a linear increase in fluorescence. Two mRNA-injected oocytes were observed and are denoted by the filled and open circles. (Bottom) An experiment showing a longer time course, taken from the experiment shown in Fig. 2. The amount of GFP–KDEL levels off at later time points. Two mRNA-injected oocytes were observed and are denoted by the filled and open circles.

To obtain the fluorescence intensity values shown in the graph, the autofluorescence of the uninjected oocyte was subtracted from the readings for the Fl dextran and GFP–KDEL oocytes. The Fl dextran value fluctuated up to 15% relative to the initial reading, probably due to laser power fluctuations and microscope focal plane differences; the value at each time point was used to normalize the GFP–KDEL value relative to the initial value for the Fl dextran fluorescence.

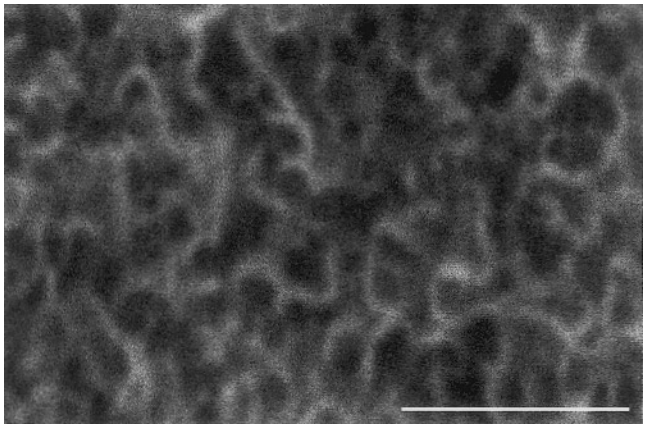

FIG. 4. High-resolution confocal microscope image of an immature oocyte expressing GFP–KDEL (63X 1.4 N.A. oil immersion lens; confocal aperture setting 3). Staining within the cytoplasm shows the presence of membranes sheets (cisternae), corresponding very well with images obtained previously in DiI-labeled oocytes (Jaffe and Terasaki, 1994). The image was taken 14 hr after the oocyte was injected with a final mRNA concentration of 20 μg/ml. Bar, 10 μm.

with confocal microscopy, a complex pattern was seen throughout the interior (Fig. 4). This pattern is very similar to that seen previously using DiI to label the ER (Jaffe and Terasaki, 1994) and in sea urchin eggs (Terasaki and Jaffe, 1991), where it was shown by serial optical sections to correspond to cisternae (equivalently, membrane sheets). The germinal vesicle (nucleus) of GFP-labeled oocytes had a sharp outline around it, corresponding to labeling of the nuclear envelope (not shown), also corresponding to the previous DiI results.

An important advantage of the GFP method over the DiI method is that the labeling remains specific to the ER. DiI-injected oocytes begin to develop bright spots about 2 μm in diameter as early as 1 hr after injection (Terasaki and Jaffe, 1991). These spots probably correspond to labeling of the Golgi apparatus by dye transfer due to membrane traffic from the ER (Terasaki and Jaffe, 1991). This difference in labeling can be seen by comparing the GFP images in Figs. 4 and 5 with the DiI images in Figs. 1A, 3, and 6 of Jaffe and Terasaki (1994). Apparently, the C terminal KDEL sequence is sufficient to retain the GFP chimera in the ER.

Estimation of the Amount of GFP–KDEL Protein

Fluorescence is proportional to the product of the extinction coefficient and quantum yield (e.g., Haugland, 1992). Values for the extinction coefficient and quantum yield of the mutant GFP are 39,200 M^{-1} cm^{-1} and 0.68 (Heim *et al.*, 1995) and for fluorescein are 75,000 M^{-1} cm^{-1} and 0.71 (Tsien and Waggoner, 1995). Therefore, a GFP–KDEL solution should be 0.5 times as fluorescent as a solution of the same concentration of fluorescein. To determine the quantities of GFP–KDEL protein in the oocyte cytoplasm, three oocytes were imaged in the same field: an uninjected oocyte to determine autofluorescence, an oocyte injected with a known amount of fluorescein dextran, and an oocyte injected with 36 pg mRNA for GFP–KDEL (see Fig. 2). Based on the relative fluorescence values for GFP and fluorescein, and the known amount of fluorescein in the oocyte, the amount of GFP–KDEL protein at plateau (~17 hr) was about 60 pg (two experiments, three oocytes). The total protein for a starfish oocyte was determined to be ~400 ng, so this represents ~0.02% of the oocyte protein. Based on the oocyte volume of 3.1 nl, the plateau value of GFP was 0.7 μM; the true concentration in the ER is at least several times higher than this since the 0.7 μM figure assumes equal distribution in the cytoplasm. The plateau fluorescence corresponds to about 40 molecules of GFP–KDEL per mRNA molecule injected. During the time the fluorescence was increasing linearly, the rate of increase corresponded to a rate of accumulation of fluorescent GFP–KDEL of ~6 pg/hr (Fig. 3). It would be interesting to determine the rate of GFP–KDEL synthesis, but this would require data on the rate of its degradation. Another factor that should be considered is that development of fluorescence involves an autooxidation reaction (Heim *et al.*, 1994); for the serine 65 to threonine mutant GFP used here, the development of fluorescence has a half time of 25 min in a bacterial expression system (Heim *et al.*, 1995).

Oocyte Maturation, Fertilization, and Early Development

Immature oocytes expressing GFP–KDEL were stimulated to undergo maturation by addition of the hormone 1-methyladenine. Images taken at 20–60 min after germinal vesicle breakdown (GVBD) showed circular profiles (Fig. 5A; n = 6 eggs). Similar profiles in mature eggs were seen previously with DiI and seem to correspond to incomplete spherical shells of ER associated with yolk platelets (Jaffe and Terasaki, 1994). When mature eggs were inseminated, the circular profiles appeared to fragment within 1 min (Fig. 5B), remained in this form for several minutes, and then gradually returned to a form like that of the unfertilized egg (Fig. 5C). These changes in ER structure during fertilization were seen in 5/5 fertilized eggs from two animals and are similar to results obtained using DiI labeling (Jaffe and Terasaki, 1994).

Fertilized eggs expressing GFP–KDEL went on to divide and developed normally. The embryos formed blastulae that were indistinguishable from those of uninjected embryos (Fig. 6A) and were fluorescent (Fig. 6B). The labeling appeared to be localized to the ER (Fig. 6C).

Evidence for Loss of ER Membrane Continuity at Fertilization

Previous evidence obtained from the spreading rate of DiI from an injected DiI-saturated oil drop suggested that there

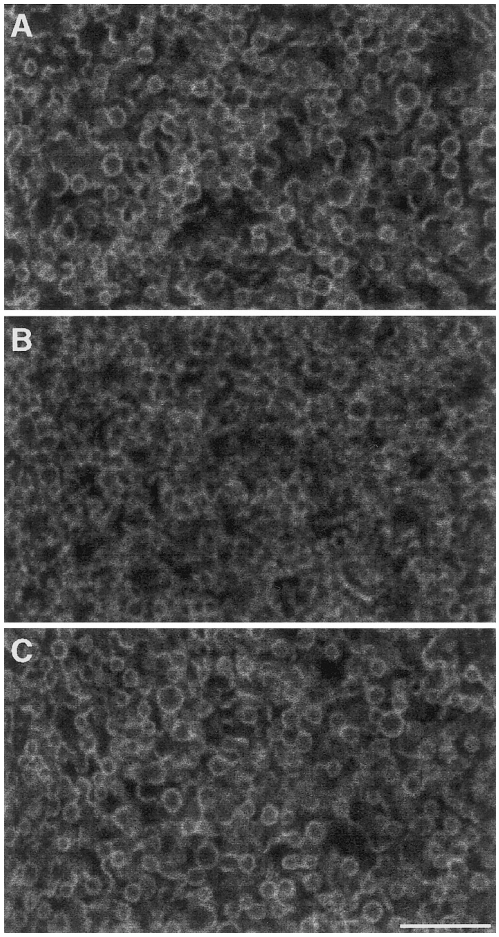

FIG. 5. Imaging of GFP–KDEL-labeled ER during fertilization. Images were obtained approximately 16 μm below the cell surface, 24 hr after injection of GFP–KDEL mRNA (20 μg/ml final concentration). (A) Mature egg, before fertilization. The circular profiles appear during maturation, as previously seen with DiI; the profiles seem to be spherical shells of ER associated with yolk platelets (Jaffe and Terasaki, 1994). (B) Fertilized egg, 1 min 40 sec after the ER change that occurs at fertilization was first detected; the circular profiles are less distinct. (C) 30 min after fertilization; the form of the ER is similar to that before fertilization. The image brightness in (B) is noticeably dimmer than that in (A) and (C), probably due to the photobleaching effect demonstrated in Fig. 7; image B was obtained as part of a series taken at 15-sec intervals. Bar, 10 μm.

is a large loss of continuity of the ER at fertilization (Jaffe and Terasaki, 1993, 1994). We used fluorescence recovery after photobleaching (FRAP) of eggs expressing GFP–KDEL to test this hypothesis. An area of cytoplasm ($\sim$35 $\times$ 55 μm) was bleached in eggs before fertilization, 1–2 min after fertilization, and 20–30 min after fertilization. In unfertilized eggs, the fluorescence recovered rapidly to approximately the original level (Figs. 7 and 8A). This indicates that most, if not all, of the GFP–KDEL diffuses freely in the ER lumen and that there are numerous ER pathways for diffusion between the bleached and unbleached areas. Fluorescence recovered with a similar time course when GFP–KDEL was bleached 20–30 min after eggs were fertilized (Figs. 7 and 8C). However, fluorescence recovered much more slowly when bleached 1–2 min after fertilization (Figs. 7 and 8B), indicating that there is a loss of connections among ER membranes. The time required for recovery of half of the original fluorescence within an $\sim$8 $\times$ 30 μm bleached rectangle was $\sim$ 1 min for both unfertilized eggs and eggs at 20–30 min after fertilization. In contrast, for eggs at 1–2 min after fertilization, the half time for recovery was $\sim$7 min (Table 1). This effect was seen in 6/6 fertilized eggs from two animals.

We also examined ER membrane continuity by photobleaching of DiI, which is incorporated in the ER membrane bilayer. Similar FRAP behavior was seen in DiI-labeled eggs that were photobleached at these times (Fig. 7). The times taken for the fluorescence to recover halfway to its original value were $\sim$2 min in unfertilized eggs and in eggs bleached 20 min after fertilization compared to $\sim$8.5 min when eggs were bleached at 1–2 min after fertilization (Table 1). This was seen in 5/5 fertilized eggs from two animals.

DISCUSSION

Use of GFP to Label the Endoplasmic Reticulum

Chimeric GFP proteins have been targeted to mitochondria (Rizzuto *et al.*, 1995), Golgi apparatus (Cole *et al.*,

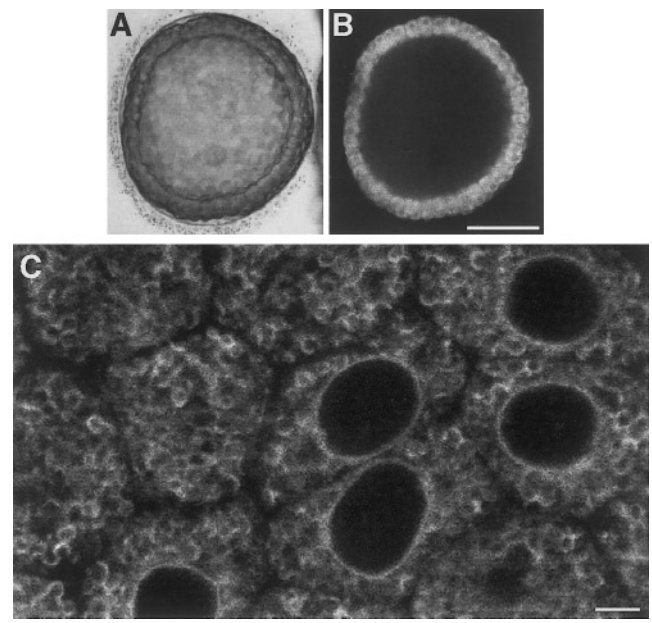

FIG. 6. GFP–KDEL distribution in the blastula stage of an embryo (22 hr after fertilization). (A) Scanning transmission image. (B) Fluorescence image showing labeling of the cells of the blastula. Bar, 100 μm. (C) High-magnification image. The nuclear envelope, which is part of the ER, is stained. Bar, 10 μm.

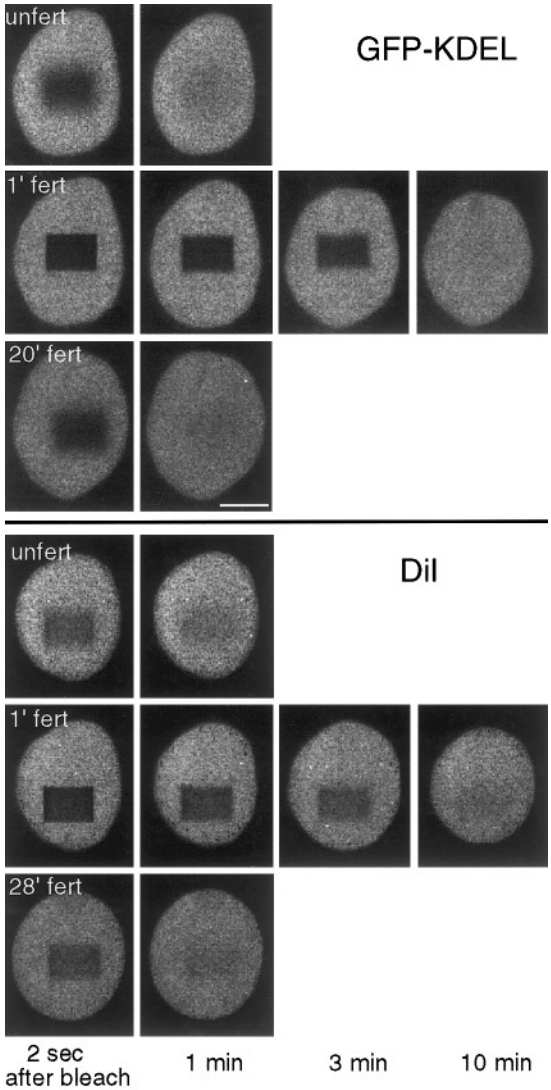

FIG. 7. FRAP of GFP–KDEL and DiI to investigate ER continuity during fertilization. The top set of images are from eggs expressing GFP–KDEL, and the bottom set are from eggs whose ER was labeled by injection with a DiI-saturated oil drop. A rectangular area was bleached, and then the recovery was observed by collecting images at 1-sec intervals. The bleach was done at the times shown on the far left panels. For the ''1-min'' time point, bleaching was begun at 1 min after the structural change in the ER was first detected. The images were collected at the times shown along the bottom of the figure. Bar, 50 μm. In both GFP–KDEL and DiI-labeled eggs, the recovery takes much longer when the bleaching is at 1 min after fertilization. Note also the contrast in the boundary of the bleached areas at 2 sec after bleach; the diffuse boundary in the unfertilized or 20- to 30-min-fertilized eggs indicates that diffusion is rapid during the bleaching period, whereas the sharp boundary in the 1-min-fertilized egg indicates that there is little diffusion of GFP–KDEL or DiI. These results provide evidence that the continuity of ER membranes is disrupted at fertilization.

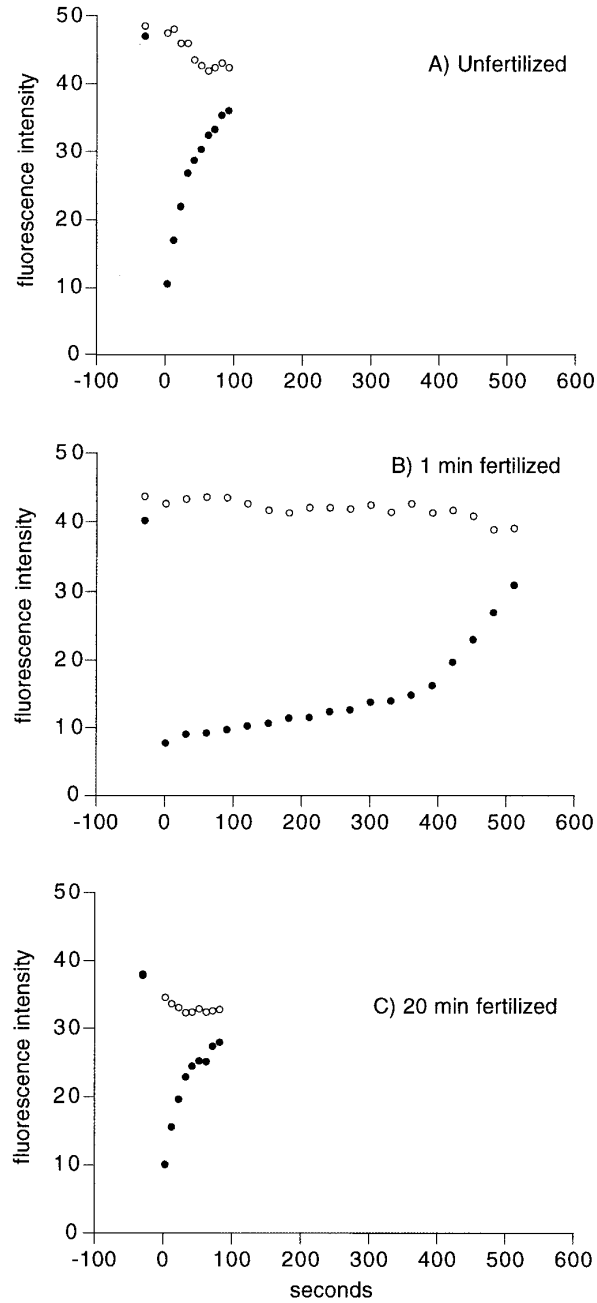

FIG. 8. Recovery of fluorescence after photobleaching in a GFP–KDEL expressing egg. The data are taken from the egg shown in Fig. 7. The average fluorescence intensities in an area 8×30 μm within the bleached region (filled circles) and in an area outside of the bleached region (open circles) were determined. Unfertilized eggs (A) and 20-min-fertilized eggs (C) recover from bleaching rapidly compared to 1-min-fertilized eggs (B). The bleach consisted of six consecutive 1-sec scans and ended at time 0. The *y* axis shows fluorescence intensity (scale, 0–255).

TABLE 1
FRAP of Fluorescent Molecules in the ER: Time Taken for Half Recovery

	Before fertilization	1–2 min postfertilization	20 min postfertilization
GFP–KDEL	55 ± 7* sec	404 ± 101	44 ± 7
DiI	117 ± 29	509 ± 134	107 ± 34

* Standard deviation; $n = 6$ for GFP–KDEL, $n = 5$ for DiI.

1996), and the ER membrane, in which GFP was attached to the cytosolic C terminal end of the membrane spanning portion of hydroxymethylglutaryl–CoA reductase (Hampton *et al.*, 1996). In order to target GFP to the ER lumen, we made a chimeric protein construct with the signal sequence of a sea urchin egg ER resident protein (ECast/PDI; Lucero *et al.*, 1994) and the ER retention sequence KDEL (lysine–aspartate–glutamate–leucine; Munro and Pelham, 1987). A similar strategy was previously used to target horseradish peroxidase to the ER lumen (Connolly *et al.*, 1994). mRNA for the GFP chimeric protein was injected into immature starfish oocytes, which are an efficient system for expression of foreign mRNA (Shilling *et al.*, 1994). There was some doubt whether GFP would be able to fold correctly within the ER lumen, an environment that is more oxidizing than the cytosol (Hwang *et al.*, 1992). The fluorescence patterns that developed included large membrane sheets in the interior and the nuclear envelope, which is part of the ER. This showed that GFP–KDEL was successfully targeted to the ER and that it had folded correctly there.

The fluorescence pattern of GFP–KDEL had a very similar appearance to that produced by the DiI labeling technique. The DiI technique has been used to label the ER in echinoderm eggs (Terasaki and Jaffe, 1991; Jaffe and Terasaki, 1993, 1994; Terasaki, 1994) as well as in ascidian and mouse eggs (Speksnijder *et al.*, 1993; Mehlmann *et al.*, 1995; Shiraishi *et al.*, 1995) and in neurons (Feng *et al.*, 1994; Terasaki *et al.*, 1994). The argument that DiI labels the ER depends on the reasoning that DiI only diffuses in continuous membrane bilayers and that the ER is the only organelle in eggs that has extensive continuity in cells (Terasaki and Jaffe, 1991). This is open to possible objections, for instance, that other organelles such as endosomes may have extensive continuity or that some parts of the ER may not be continuous with the rest of the ER. In addition, DiI can be transferred from one compartment to another by membrane traffic; after 1–2 hr, there is significant labeling of organelles other than ER, probably by dye transfer from the ER to the Golgi apparatus (Terasaki and Jaffe, 1991). The use of GFP–KDEL to label the ER does not depend on membrane continuity for its specificity; rather, its specificity is based on the well-established ER retention sequence. The similar staining patterns of GFP–KDEL and DiI gives greater confidence in both of these methods for labeling the ER.

The GFP–KDEL approach has the significant advantage over the DiI method that fluorescence appears to remain specific to the ER for a much longer time. We observed no obvious signs that GFP–KDEL labels the Golgi apparatus or any post-Golgi membranes even after many hours. There are two possible limitations to the use of GFP–KDEL as a marker for the ER. KDEL containing proteins are thought to cycle through intermediate compartments between the Golgi and ER (Pelham, 1988), so that some of the staining we observed may be due to GFP–KDEL in those compartments. Second, some parts of the ER might exclude GFP–KDEL and be unlabeled. These possible limitations notwithstanding, it appears that GFP–KDEL will be widely applicable for observing the dynamics of ER organization in other systems. GFP–KDEL has been successfully expressed in the ER of *Xenopus* cells (Mark Terasaki, unpublished results).

Our success in expressing GFP–KDEL makes it seem likely that functional GFP chimeric proteins, such as calcium binding proteins or chaperone proteins, can be expressed within the ER lumen. Our experiments show that it would be feasible to use such GFP chimeric proteins to investigate their compartmentalization or mobility. In addition, we have shown that the amount of a GFP chimeric protein can be estimated by comparing GFP fluorescence to that of a fluorescein standard. Since the protein levels can be monitored with time, it may also be possible to investigate protein synthesis and degradation rates in single cells.

Evidence for Loss of Continuity of ER Membranes at Fertilization

When DiI-saturated oil drops were injected into eggs at 1 min after fertilization, the rate of spreading from the oil drop was much slower than in eggs before fertilization or 10 min after fertilization (Jaffe and Terasaki, 1993, 1994). The slower rate of spreading can be explained by a reduction in the number of diffusion pathways for DiI. However, it can also be explained by an inhibition of DiI transfer from the oil drop to the ER. This transfer process, which probably involves a form of contact between the oil drop and ER membranes, is not well understood. In unfertilized eggs, DiI begins as a uniformly dissolved solute in the unsaturated triglycerides and somehow transfers completely out of the oil drop within 5 min (Terasaki and Jaffe, 1991). In the first few minutes after fertilization, little dye moves out of the oil drop. This reduction in the transfer process could depend on a physical property of ER membranes that becomes altered at fertilization or could be due to a change in cytosolic proteins that interferes with the transfer.

Fluorescence recovery after photobleaching is a better technique for testing whether ER continuity is transiently lost at fertilization, because the fluorescent label is already present in the ER at the time of the experiments. When a region of an unfertilized egg expressing GFP–KDEL was bleached, the fluorescence recovered rapidly (Fig. 7). This is consistent with GFP–KDEL being a soluble protein that can

diffuse rapidly in the lumen of interconnected ER membranes. An alternative interpretation of the observed fluorescence recovery could be cytoplasmic mixing, but this possibility has been discounted by time lapse observation of the pattern of individual spherical elements of the ER (Jaffe and Terasaki, 1994). Comparison of a composite of 10 images accumulated over a period of 30 sec with the image obtained at the beginning of the time lapse sequence showed insufficient translocation of the ER elements to account for the observed FRAP results (see Figs. 1B and 1E of Jaffe and Terasaki, 1994). An additional reason for discounting cytoplasmic mixing as a factor in the fluorescence recovery is the $\sim 2\times$ difference in 50% recovery time when two diffferent fluorescent markers were used (Table 1).

At 1–2 min after fertilization, when the appearance of the ER has undergone a large change, the time for recovery from photobleaching was much longer than that in the unfertilized egg. The longer recovery time is consistent with a large decrease in pathways for diffusion of the GFP–KDEL molecules in the lumen of the ER. At 20 min after fertilization, when the appearance of the ER has returned to that of unfertilized eggs, the recovery time for GFP–KDEL fluorescence was similar to that in unfertilized eggs. Similar changes in recovery times were obtained in FRAP experiments using DiI. Since DiI behaves like a membrane lipid (Haugland, 1992), this indicates that pathways for diffusion of membrane lipids are transiently reduced at fertilization. The accumulated data using a lumenal protein marker (GFP–KDEL) and a molecule in the membrane bilayer (DiI) now provide strong evidence for a transient loss of continuity of ER membranes at fertilization.

The functional significance of the disruption of the interconnected ER membranes of echinoderm eggs at fertilization is not known. It is possible that the disruption is a part of the mechanism of Ca wave propagation. For example, a change in ER membrane structure associated with Ca release could open a mechanosensitive Ca channel in the ER, or increase phospholipase C activity (Boguslavsky *et al.*, 1994), thereby contributing to propagation of a Ca wave. It is also possible that the disruption of ER structure is somehow involved in activating metabolic pathways in the ER, since many processes such as protein synthesis are activated at fertilization (e.g., Rees *et al.*, 1995). The other instance in which a disruption in ER continuity has been reported is during mitosis of some mammalian cells (Zeligs and Wollman, 1979; reviewed in Warren, 1993). The function of this change in continuity is also not known, but the change at mitosis supports the view that changes in ER structure in general, and in ER continuity in particular, have an important role in regulation of cellular processes.

ACKNOWLEDGMENTS

We thank Hector Lucero and Ben Kaminer for the partial clone of sea urchin ECast/PDI and Roger Heim and Roger Tsien for the clone of the mutant GFP. We also thank Tom Sargent for technical advice, Jennifer Lippincott-Schwartz, Nelson Cole, and Noah Sciaky for discussions, and Edward D. Korn for his comments on the manuscript. The confocal microscope was obtained with a grant from the Patrick and Catherine Weldon Donaghue Foundation. Partial support was provided by NIH Grant HD-14939 and NSF Grant DCB-9023987 to L.A.J. We thank Tom Reese for partial support of this work.

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Received for publication May 3, 1996
Accepted August 5, 1996