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A Monoclonal Antibody That Recognizes Mammalian Cortical Granules and a 32-Kilodalton Protein in Mouse Eggs[1]

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ABSTRACT

The fertilization-induced exocytosis of egg cortical granules (CGs) is responsible for a block to polyspermy, crucial to the viability of many species. The contents of mammalian CGs have been an elusive target for analysis because of picogram quantities of CG proteins. By using media enriched in secreted CG contents from calcium ionophore-induced eggs as an immunogen, a monoclonal antibody was raised that immunolocalized to structures in the mouse egg cortex with all the hallmarks of CGs. These structures were the correct size, absent from the region over the metaphase II spindle, and greatly reduced after fertilization. Double-labeling experiments confirmed that the antibody recognized the same population of CGs as those recognized by *Lens culinaris* agglutinin. On Western blots, the antibody primarily recognized a 32-kDa protein (and secondarily one at ~25 kDa) in mouse eggs. Analysis of biotin-labeled secreted proteins from activated eggs confirmed that CGs release only a small number of major proteins (45, 34, 32, 28, and ~20 kDa by SDS-PAGE). We therefore propose that the 32-kDa protein identified by this antibody is likely to correspond to the 32-kDa protein released from activated eggs and that it may be involved in the block to polyspermy. These methods should make it possible to generate additional antibodies to study the structure of CG components as well as their roles in the polyspermy block and CG biogenesis.

developmental biology, fertilization, gametogenesis, oocyte development, ovum

INTRODUCTION

In order for fertilization to be successful in mammals, only a single sperm must fuse with the egg [1]. The fusion of multiple sperm leads to polyploidy, usually a lethal condition. In mice, humans, and many other mammals, the primary block to polyspermy (BPS) occurs at the level of the interaction between the zona pellucida (ZP) and the sperm, whereas a secondary block may be present at the plasma membrane [2, 3].

Several modifications of ZP proteins make up the ZP BPS, the most important of which is the modification of ZP3. ZP3 is largely responsible for binding the acrosome-intact sperm and inducing the acrosome reaction ([4] and references therein), probably via a carbohydrate-mediated binding event [5]. The acrosome-reacted sperm then penetrates the ZP and fuses with the egg plasma membrane. Alterations of ZP3 after fertilization have been demonstrated functionally by the loss of its ability to bind sperm and induce the acrosome reaction [6]. Although the molecular change in ZP3 has not been determined, there is evidence that a glycosidase activity is involved [7]. Because it has been postulated that ZP2 is required in order to keep acrosome-reacted sperm bound to the ZP [8], the proteolytic cleavage of ZP2 to ZP2f may contribute to the BPS by affecting ZP2-sperm interaction.

The ZP BPS and the modifications of the two ZP proteins are associated with, and most likely caused by, fertilization-induced exocytosis of cortical granules (CGs) from the cortex of mature eggs. Factors released from CGs into the perivitelline space and ZP are thought to modify ZP2 and ZP3, resulting in the ZP block. If CG release is experimentally stimulated within ZP-enclosed eggs, the eggs fail to fertilize when subsequently inseminated. Conversely, polyspermy in porcine eggs fertilized in vitro [9, 10] is associated with failed or delayed CG release. In the mouse egg, CGs are routinely visualized, either by electron microscopy as dense, membrane-bound structures in the cortex or in whole-mount by staining eggs with *Lens culinaris* agglutinin (LCA).

To date, several partially characterized proteins have been reported that are released from CGs or are present in the medium surrounding activated eggs (exudate). A 75-kDa protein immunolocalized to CGs and present in exudate has been described [11], but its function is unknown. A β-hexosaminidase-B (*N*-acetyl-glucosaminidase) activity has also been localized to CGs before exocytosis and is proposed to modify ZP3 [7]. Two bands (at 21 and 34 kDa) identified by SDS-PAGE are proposed to be the CG protein or proteins responsible for cleavage of ZP2 [12], although the localization of these protein(s) before exocytosis has not been established. Other activities released at fertilization have also been described [13, 14], but their localization before fertilization is also unclear. Therefore, although it is known that CG release upon fertilization is associated with modification of ZP2 and ZP3, little is known about the molecules presumably stored in CGs that act directly on the ZP to induce the BPS. Research on putative CG enzymes and the BPS has progressed slowly because mammalian eggs contain only picogram quantities of CG-derived proteins [12], and a highly specific probe for CG protein has not been available.

MATERIALS AND METHODS

Collection of Oocytes, Eggs, and Embryos

Fully grown germinal-vesicle (GV) stage oocytes were collected from CF1 mice (Charles River, Wilmington, MA)

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40–44 h after administration of 5 IU eCG (Sigma, St. Louis, MO). Oocytes were collected in Hepes-buffered Earle's balanced salt solution (EBSS/H) with 0.3% polyvinylpyrrolidone (PVP), and cumulus cells were stripped by passage through a narrow bore pipette. Ovulated mature metaphase II (Meta II) stage eggs were collected from females primed with 5–10 IU eCG followed 44–48 h later with 5–10 IU hCG (Sigma). Fourteen to sixteen hours after hCG application, cumulus masses were released from oviducts. The eggs were stripped of cumulus cells in 0.125 mg/ml hyaluronidase (Sigma). In vivo fertilized preimplantation embryos were collected by flushing the oviduct and uterine horn with EBSS/H 0.3% PVP. Embryos, eggs, and oocytes were treated with 0.25% pronase (Calbiochem, La Jolla, CA) to remove ZP.

Partial Purification of CG Proteins

To isolate CG-derived proteins, Meta II eggs (usually 100–200 per batch) were collected 16–17 h after hCG application in EBSS/H with 0.3% PVP. Eggs without ZP were induced to undergo CG secretion by a 2-min treatment with 2 μM ionomycin (Calbiochem) in Ca^{2+}/Mg^{2+}-free EBSS/H with 0.3% PVP. The CG exudate was collected after a 30-min incubation in 2- to 8-μL drops of EBSS/H with 0.05% PVP under oil at 37°C. Calcium ionophore has been used extensively to stimulate CG exocytosis from mouse eggs [11, 12]. The relatively low ionophore concentration reduced the likelihood of egg lysis in the collection drops, which would contribute non-CG-derived proteins, contaminating the exudate. Lysis was monitored (by changes in egg refractility) in groups of treated eggs, and exudate preparations with lysed eggs were removed. CG release was confirmed by staining eggs for CGs (and DNA [15], as modified in [16]) or ZP2 analysis by SDS-PAGE (when ZP-intact eggs were used). For ZP2 analysis, whole intact ZP were isolated, biotinylated, and separated on 9% SDS-PAGE under reducing conditions [17, 18].

Analysis by SDS-PAGE of CG-Derived Proteins

The exudate and mock exudate (from unactivated eggs) were biotinylated for 1 h at ambient temperature with 0.007 mg/ml E-Z link sulfo-NHS-LC-biotin (Pierce, Rockford, IL) in 50 mM sodium bicarbonate, precipitated with cold 80% acetone to remove unreacted biotin, and separated by SDS-PAGE. Carrier hyaluronic acid (1 μg) was included to prevent loss of the nanogram quantities of exudate protein in the samples during precipitation (kindly donated by Dr. Bryan Toole, Tufts University). The labeled bands were visualized with avidin-horseradish peroxidase (HRP; Vectastain kit from Vector Labs, Burlingame, CA) and enhanced chemiluminescence (ECL kit; Amersham, Arlington Heights, IL).

Generation of Monoclonal Antibodies

Exudates were collected from a total of ~1000 eggs and pooled. Two BALB/c mice were immunized and boosted once with the exudate. Three days after a second boost injection, the spleens were isolated from the immunized mice, and hybridomas were generated according to the protocol of Galfre et al. [19]. Approximately 200 of the resulting hybridomas were screened by immunostaining 5- to 10-μm cryosections of mouse cumulus masses. Those hybridomas that selectively labeled the egg cortex and showed punctate staining were subcloned. The subclones were rescreened by whole-mount immunostaining of intact mouse eggs to confirm that staining was specifically localized to CGs. The positive control was LCA, which labels CGs in whole mount and by electron microscopy [15, 16]. Negative controls for antibody staining were a monoclonal antibody (mAb) specific for sea urchin hyalin [20] which does not stain mouse CGs, as well as fertilized mouse eggs that have undergone CG exocytosis.

Partial Purification of IgM from Ascites

Control, anti-vimentin, and 3E10 IgM (the former two from Sigma) were partially purified from ascites over a Sephacryl S-200 HR (Pharmacia, Piscataway, NJ) column eluted with PBS. The IgM (>900 kDa) eluted in the void volume fractions. The presence of IgM was confirmed by immunoblotting, and the IgM-containing fractions were pooled and concentrated in a Swinnex-13 cell (Millipore, Bedford, MA) with regenerated cellulose membrane of 100 000 molecular weight cutoff (Millipore). The final IgM concentration was 0.2–0.3 mg/mL, in 0.9–1.5 mL of PBS.

Function-Blocking Assay

Dialysis of IgM. 3E10 and control anti-vimentin IgM were dialyzed against M199M (M199 from Gibco BRL with 0.03 mg/mL pyruvic acid [Sigma]) supplemented with 1%, essentially fatty acid-free BSA (Sigma). Dialysis of 20- to 30-μL samples was carried out in Slide-A-Lyzer mini dialysis units with 3500 molecular weight cutoff membrane (Pierce) against two changes of 15 mL of media. Fresh samples were dialyzed for each in vitro fertilization experiment to ensure that the pH of the solution was optimal. Dialyzed 3E10 was used for immunofluorescent staining of eggs to confirm that the antibody was intact. Concentration of IgM in the dialyzed sample was comparable to the starting material and was used without further dilution.

Effect of antibody on the BPS. Zona pellucida-intact Meta II eggs were preincubated under oil in 18-μL drops of M199M with 3E10, with control IgM, or with medium alone for 90 min. This length of incubation has previously been demonstrated to be sufficient to allow IgM to pass through the ZP [21]. Bovine serum albumin was added to bring the final concentration to ~0.3%, and eggs were inseminated with $1–1.5 \times 10^6$ capacitated mouse sperm per mL. Sperm from CF1 mice were collected and capacitated essentially as described [22] in M199M with 3% essentially fatty acid-free BSA (Sigma). Eggs were incubated with sperm for 5.5–6 h at 37°C under oil in a humidified atmosphere of 5% CO_2. After incubation, eggs in all groups had many sperm attached to the surface of the ZP that were removed by pipetting before eggs were fixed. DNA was stained as described above, and the number of sperm that had penetrated into the egg cytoplasm was recorded. The assay was repeated three times, with 30–50 eggs per group, giving a total of ~100 eggs per treatment. For analysis, all the data were pooled and averaged.

Western Blotting

Mouse ovaries were collected from 6- to 8-wk-old female mice and homogenized in RIPA lysis buffer (10 mM Tris, pH 8.0; 1% NP-40; 0.1% SDS; 8.7 mg/ml NaCl; 1% deoxycholic acid; and 0.02% sodium azide) with benzamidine, aprotinin, leupeptin, and PMSF and stored frozen. ZP-free Meta II eggs were collected and stored frozen in 2× SDS-PAGE sample buffer with 2-mercaptoethanol [23].

Pooled samples containing >1000 eggs were separated along with prestained standards (Bio-Rad, Hercules, CA) on 10% SDS-PAGE minigels. The proteins were transferred to Immobilon-P (Millipore) and blocked in 6% non-fat dry milk (Carnation) in PBS with 0.2% Tween-20 (Bio-Rad). Overnight incubation at 4°C with partially purified IgM diluted 1:100 in blocking solution was followed by incubation with secondary antibody for 2 h. A mixture of secondary antibodies coupled to HRP: goat anti-mouse IgM (Sigma) and horse anti-mouse IgG (Bio-Rad) was used to maximize the signal. Blots were developed with an ECL kit (Amersham). Blots were stripped and reprobed with either the negative control anti-vimentin IgM or the positive control anti-p34^{cdc2} protein kinase IgG (Pharmingen, San Diego, CA). Alternatively, two blots were run simultaneously and probed with either 3E10, or secondary antibody alone. Resulting film exposures were quantified by densitometry, and integrated optical density (IOD) was calculated for each of the 15 bands. The ratio of the 3E10 IOD to the control IOD was calculated for each band in order to determine which of the bands were specific to the 3E10-probed blot and which were nonspecific (or due to the secondary antibodies reacting with endogenous antibodies in the lysate).

Immunostaining and LCA Staining of Eggs and Embryos

Zona pellucida-free eggs and embryos were fixed in 3% paraformaldehyde as previously described [24]. Eggs and embryos were blocked for 1–1.5 h (PBS with 0.2% nonfat dry milk, 2% normal goat serum, 1% BSA, 0.1 M glycine, 0.01% Triton-X 100, and 0.02% azide), permeabilized for 20 min in Dulbecco PBS with 0.1% Triton-X 100 and 0.3% BSA, and incubated in primary antibody for up to 2 h. The samples were then washed and incubated with secondary antibody (either goat anti-mouse IgG coupled to fluorescine isothiocyanate [FITC; Sigma] or donkey anti-mouse IgG coupled to Texas-Red [Jackson Immunoresearch, West Grove, PA]). The anti-IgG (whole molecule) antibodies cross-react strongly with IgM and yielded results superior to those with IgM-specific (anti-μ chain) secondary antibody. DNA was stained as previously described [25], and the samples were washed for 1–2 h in PBS. Eggs and embryos were mounted with 50% glycerol in Dulbecco PBS supplemented with 25 mg/ml sodium azide (to reduce photobleaching). For double-staining of CGs, biotinylated LCA was visualized with streptavidin Texas-Red (Gibco BRL, Gaithersburg, MD), and the primary antibody was visualized with secondary antibody coupled to FITC. Cortical granules were visualized on a Nikon inverted microscope with a Nikon 100× PlanApo oil objective (NA = 1.4). Confocal microscopy was performed by NORAN-2D intervision software.

RESULTS

Analysis of Cortical Granule Exudate

Cortical granule exudates and mock exudates (from eggs that had not been exposed to ionophore) from 50–100 eggs were biotinylated and separated by SDS-PAGE. Five prominent bands, as well as several minor bands, were identified in the exudate samples (Fig. 1A, lane 2). The mock exudates were either negative or had very faint bands, which may be due to a low level of spontaneous CG release in culture (Fig. 1A, lane 1). The apparent molecular weights of the bands were interpolated from prestained standards

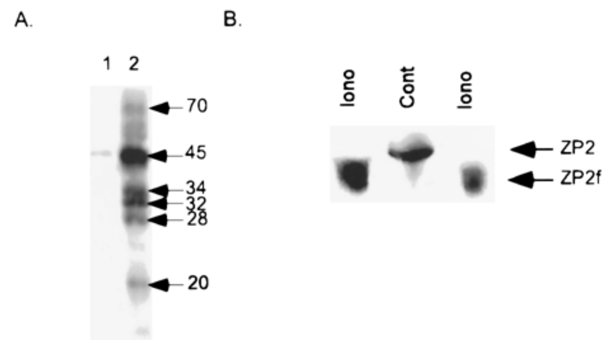

FIG. 1. Analysis by SDS-PAGE of biotinylated exudate and ZP from ionophore-treated eggs. **A**) Biotinylated exudate (lane 2) and mock exudate (lane 1) from 60 eggs each were separated on a 12.5% acrylamide minigel. The exudate exhibits bands at 20, 28, 32, 34, 45, and ~70 kDa, as well as fainter bands in the 50- to 60-kDa range. The mock exudate collected from eggs that were cultured without exposure to ionomycin contains trace amounts of CG materials, possibly due to a low level of spontaneous CG release. **B**) Examples of ZP from eggs treated with or without 2 μM ionomycin (iono and cont). Ionomycin treatment induced ZP2 proteolysis, whereas control egg ZP exhibit little, if any, ZP2f.

separated on the same gel. Stronger bands were present at ~45, 34, 32, 28, and 20 kDa and fainter ones, at ~50 and ~70 kDa. Release of CGs from ionophore-treated eggs was confirmed by two approaches. The eggs were either stained with LCA to confirm the presence or absence of CGs, or else ZP were isolated (from ZP-intact eggs) and analyzed by SDS-PAGE for ZP2 conversion [8], which is an indicator of CG release [26]. Ionophore-treated eggs underwent CG exocytosis (87.5% of ionophore-treated eggs [n = 32], compared with 18% of control eggs [n = 33] exhibited CG loss) and consistent conversion of ZP2 to ZP2f (Fig. 1B).

Screen for Anti-CG Monoclonal Antibodies

The primary antibody screen of ~200 hybridomas, using sections of ovulated cumulus-intact eggs (Fig. 2), yielded three positive hybridomas, which were subcloned. The clone 3E10 is an IgM-secreting hybridoma that has been grown in cell culture and ascites. This antibody labeled Meta II-stage eggs (Fig. 3a) in a pattern very similar to that seen with LCA (Fig. 3b), with CGs present throughout the cortex, except for in the region overlying the meiotic spindle [16]. Fertilized eggs that had undergone CG release exhibited almost no 3E10 staining (Fig. 5, G, I, and K).

Specificity of 3E10 Epitope to CGs

Eggs that were stained with both 3E10 IgM and LCA exhibited nearly identical patterns of fluorescence when the two reagents were visualized with separate fluorochromes using confocal microscopy (Fig. 4). Analysis of the overlap of the CGs stained by the two reagents indicated that >90% of LCA-positive CGs were also 3E10-positive, although a few LCA-positive, 3E10-negative spots were observed in all double-stained eggs. Only rarely were LCA-negative, 3E10-positive structures observed. In whole-mount immunostaining (Fig. 5), the 3E10 IgM localized to CGs in GV-stage oocytes and Meta II-stage eggs. In fertilized embryos at the two-pronuclear and two-cell stages, staining was restricted to a few granules that are likely to be remnants of unreleased CGs or to be CG material adhering to the egg surface (similar staining was observed with LCA, not shown). In eight-cell and morula and blastula stage embryos, no specific staining was observed (some clumps of

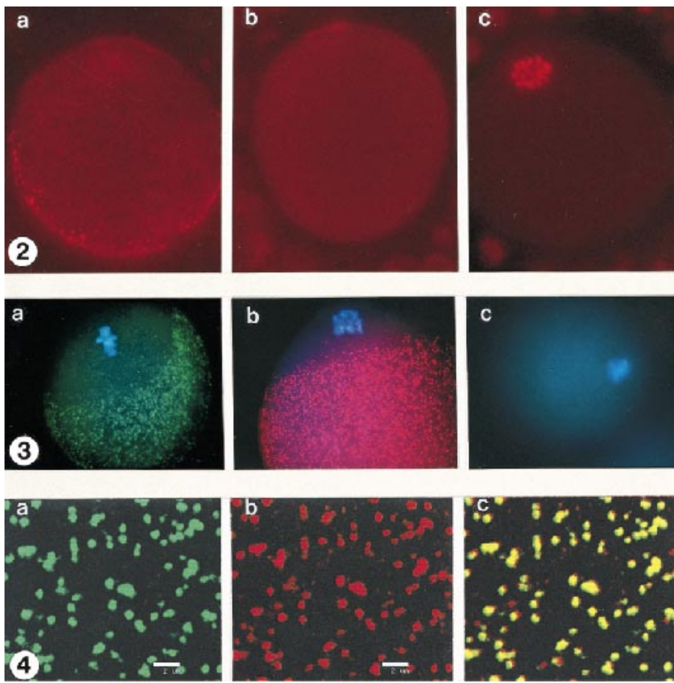

FIG. 2. Primary screening of hybridomas (×860). Paraformaldehyde-fixed cryosections of mouse cumulus masses. **a**) 3E10 hybridoma-stained egg. Note the crescent-shaped punctate cortical staining and the CG-free domain. **b**) Negative-control section probed with anti-sea urchin hyalin antibody. **c**) Positive-control section stained with an anti-histone H1 IgG (Leinco Technologies, Ballwin, MO). Photographed with a 60× oil objective.

FIG. 3. 3E10 localizes to CGs in whole-mount staining of Meta II eggs. Metaphase DNA shown in blue (DAPI/Hoechst). **a**) Egg labeled with 3E10 IgM and visualized with a FITC-coupled secondary antibody. **b**) Egg labeled with LCA-biotin and visualized with streptavidin-Texas-Red. **c**) Egg labeled with control IgM (which does not localize to CGs) and visualized as in **a**. Note the CG-free domain over the spindle in **a** and **b**. Eggs were flattened between slide and coverslip to obtain a large cortical field of CGs in a single optical plane, whereas DNA was photographed in a different plane (100× PlanApo oil objective). The variations in the apparent size of the egg and the CG-free domain are due to differences in the extent of egg compression and orientation, respectively.

FIG. 4. 3E10 immunostaining co-localizes with LCA to CGs in double-labeled eggs. **a**) 3E10 visualized with FITC-coupled secondary antibody. **b**) Same region of cortex as in **a** but visualized with LCA-biotin and streptavidin-Texas-Red. **c**) Computer-generated overlap (yellow) of **a** and **b**; note the three red spots in **c** that are LCA-positive but 3E10-negative structures. Note the size of the CGs, 0.1–0.5 μm, as predicted from electron microscopy.

staining were present in the blastocele in both 3E10- and control IgM-stained embryos, indicating that these clumps were not specific).

Test of Function-Blocking Activity of 3E10

In order to determine whether 3E10 exhibited function-blocking activity, its effect on polyspermy in vitro was assayed at the highest antibody concentration available (see *Materials and Methods*). No increase in the percentage of fertilized eggs that were polyspermic (polyspermy rate) was observed in 3E10-treated eggs when compared with the case of controls fertilized in the absence of antibody (Table 1). The overall percentages of fertilized and polyspermic eggs were similar in both control and 3E10-treated groups. The mean polyspermy rate was 11.9% in controls and was 8.5% in 3E10-treated groups. Almost all polyspermic eggs

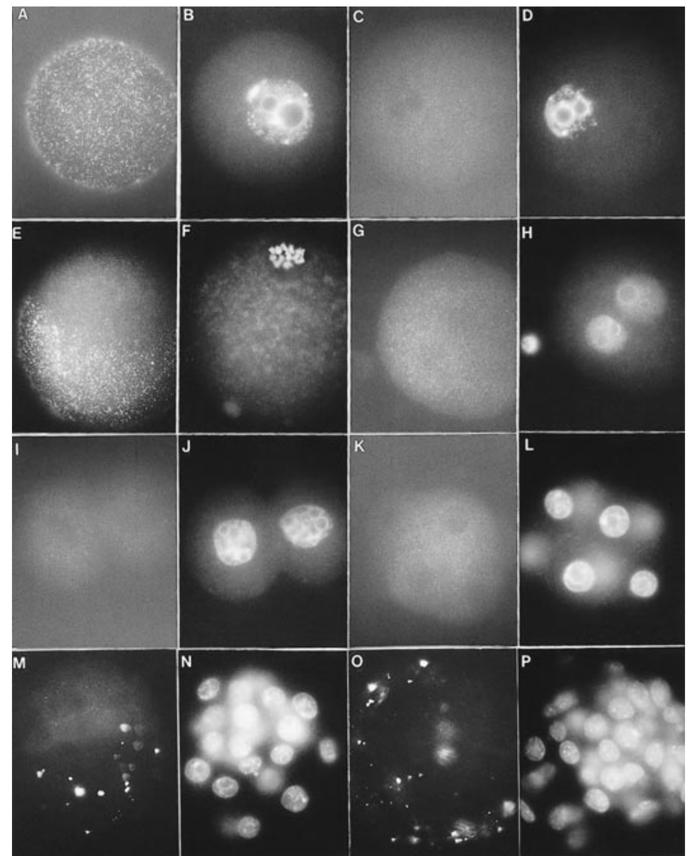

FIG. 5. 3E10 staining is significantly reduced after fertilization. Indirect-immunofluorescent staining with 3E10 (**A, E, G, I, K, M**) and control IgM (**C, O**) detected with FITC-coupled secondary antibody. DNA was stained in the same eggs and embryos with DAPI and Hoechst (**B, D, F, H, J, L, N, P**). **A, B**) Germinal vesicle-stage oocyte. **C, D**) Germinal vesicle-stage oocyte; note the absence of CG staining in **C**. **E, F**) Metaphase II egg. **G, H**) Fertilized 2-pronuclear stage embryo; note the few remaining CGs in **G**. **I, J**) Two-cell embryo. **K, L**) Eight-cell embryo; four nuclei are out of the plane of focus in **L**. **M, N**) Early blastula. **O, P**) Early blastula; note the patches of bright fluorescence in the blastocoele, as seen in **M**.

were dispermic; only one egg penetrated by more than two sperm (four pronuclei) was observed. Therefore, in this assay, 3E10 did not interfere with the ability of fertilized eggs to establish the BPS. At comparable IgM concentrations, control anti-vimentin IgM inhibited fertilization to levels below those seen in controls (25.5%; n = 106 eggs).

Western Blot Analysis with 3E10

Western blots of whole egg lysates probed with partially purified 3E10 IgM exhibited a specific band at 32 kDa (Figs. 6 and 7). No 32-kDa band was observed when blots were probed with secondary antibody alone (Fig. 7A) or

TABLE 1. Effects of 3E10 IgM on the rate of polyspermy in in vitro-fertilized eggs.

Treatment group	Number of eggs	Fertilization rate[a,c]	Polyspermy rate[b,c]
Control	94	45 (42)	12 (5)
3E10 IgM	125	38 (47)	9 (4)

[a] Fertilization rate: percentage of inseminated eggs exhibiting ≥1 sperm head in cytoplasm.
[b] Polyspermy rate: percentage of fertilized eggs containing ≥2 sperm heads.
[c] Numbers in parentheses indicate numbers of eggs.

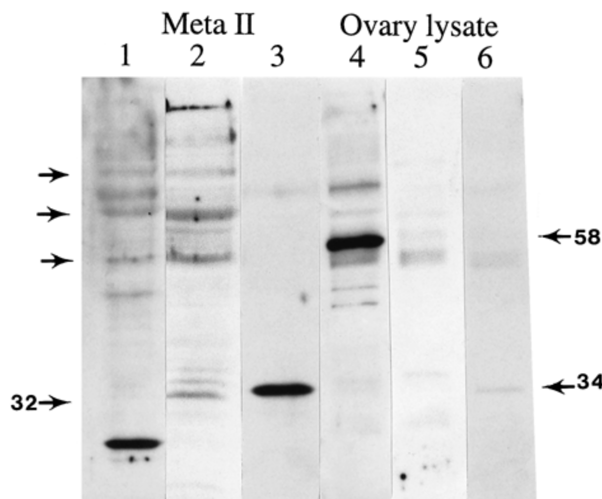

FIG. 6. Western blot of Meta II egg lysate (lanes 1–3) and whole-ovary lysate (lanes 4–6) probed with 3E10 and control antibodies. Lane 1: >1000 Meta II eggs probed with anti-vimentin monoclonal IgM (negative control). Lane 2: >1000 Meta II eggs probed with 3E10; note the stronger band at 32 kDa and a fainter band at ~34 kDa. The 58-, 65-, and 74-kDa bands (unlabeled arrows) in lanes 1 and 2 are not 3E10-specific. Lane 3: >1000 Meta II eggs probed with an anti-p34^{cdc2} antibody (positive control for p34 in eggs). Lane 4: In the ovary lysate, anti-vimentin IgM detects the 58-kDa vimentin protein. Lane 5: 3E10 mAb; the 32-kDa band is not detected in ovary lysate, which is composed predominantly of stromal cells. Lane 6: Anti-p34^{cdc2}; only a faint p34 band is detected in ovary.

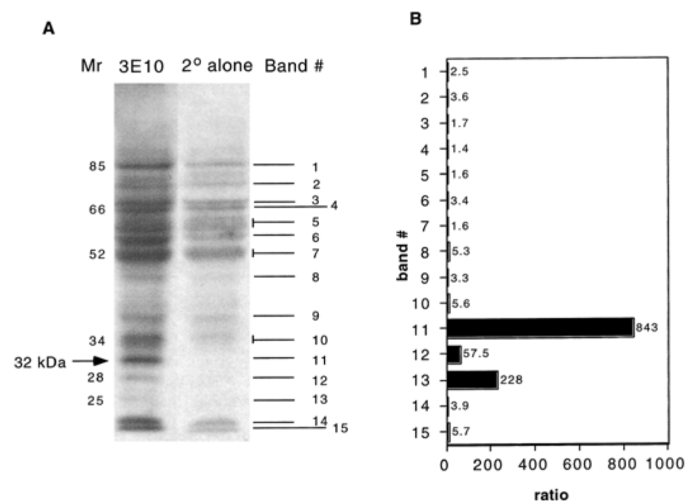

FIG. 7. Comparison of Western blots of Meta II egg lysate probed with 3E10 and secondary antibody alone. **A**) Lane 1: ~900 Meta II eggs probed with 3E10. Lane 2: ~900 Meta II eggs probed with secondary antibody alone. Apparent molecular weights in kilodaltons are indicated on the left. **B**) Ratio of the IOD for each of the 15 quantifiable bands. Band 11 corresponds to the 32-kDa protein; band 7 migrates to the location predicted for IgG heavy chain.

with a control IgM to vimentin, although the 58-kDa vimentin band was strongly positive in ovary lysates (Fig. 6). As predicted, blots of Meta II-stage eggs that were stripped and reprobed with the positive control anti-p34^{cdc2} protein kinase antibody exhibited a strong band at 34 kDa (Fig. 6). In contrast, there was only a faint p34 band detected in ovary lysate.

Densitometry analysis of duplicate blots probed with 3E10 or secondary antibody alone (Fig. 7A) indicated that the p32 band is specifically recognized by the 3E10 antibody. The 32-kDa band was the only one seen consistently and specifically on blots probed with 3E10, whereas a weaker band at ~34 kDa was detected on longer exposures of some blots (compare Figs. 6 and 7). Densitometry analysis indicated that the most significant difference between the 3E10 and control blot was the 32-kDa band, which was increased >800-fold in the 3E10 blot over control (Fig. 7B, band 11). Interestingly, densitometry analysis also indicated that band 13 may be increased in the 3E10 blot, >200-fold over control.

DISCUSSION

In mammals, our understanding of CG biogenesis and the biochemical analysis of the BPS has been hampered by the paucity of information available about CG components, which have been difficult to study because of the lack of specific probes. Here, we confirm that there are likely to be only a small number of released exudate proteins and demonstrate a method for generating mAbs that will provide sensitive and specific probes for the examination of CG biogenesis and function, including the BPS.

This is the first report of a mAb made specifically against mammalian CG-derived proteins. The 3E10 antibody described herein was raised against CG exudate and specifically stains CGs in mouse eggs. It recognizes a 32-kDa band by Western blot analysis that likely corresponds to a protein of similar molecular weight seen in exudate blots. Because the CGs contain relatively few proteins and because there are only a small number of known CG-induced ZP modifications, it is possible that the protein recognized by the mAb described is responsible for at least one of the ZP modifications involved in the BPS.

Our strategy for exudate collection was to induce the eggs to perform a 200-fold purification of CG materials by stimulating them to undergo CG exocytosis. In this way, each egg containing ~20 ng of total protein released an estimated 50–100 pg of CG proteins [12] into a small volume of medium that was analyzed or used for immunization. We demonstrate that exudate from ionophore-activated eggs contains bands that range from ~20 to ~70 kDa on SDS-PAGE under reducing conditions. There are two advantages of this biotinylation method for studying the picogram quantities of CG material in eggs. The first is its sensitivity; when coupled with ECL, exudate proteins from only 50 eggs can be routinely visualized. The second advantage is that like iodination, but unlike metabolic labeling, proteins whose synthesis has ceased or is very low can be detected. This last point is relevant because CG protein synthesis and CG formation occur primarily during oocyte growth, with little detected during meiotic maturation [24, 27].

The exudate bands visualized with this method are consistent with other, previously published methods for labeling and visualizing exudate proteins. Following exudate iodination with ^{125}I-Bolton-Hunter reagent, four bands were described with apparent M_r of 76, 45, 34, and 21 kDa [12]. Exudate gels visualized by biotinylation and described herein also exhibit bands at ~45, ~34, and ~20 kDa, as well as an upper band at ~70 kDa, which may correspond with the 76-kDa band. Proteins at ~30, 40–45, 50–55, and 75 kDa, as well as several other bands, were observed in exudate when oocytes were metabolically labeled with ^{35}S-methionine [11]. Exudate labeled with [^{125}I]-Na using chloramine T exhibited bands at 75, ~50, and ~40 and a lower band at ~30 kDa (Fig. 5 in [11]). The above results and those in this study are in general concordance and indicate

that activated egg exudate contains a small number of proteins. The observation that all four labeling methods visualize a protein in the 30- to 35-kDa range in exudate supports our conclusion that such a protein is released from CGs and is likely to correspond to the 3E10 antigen. At present, it is not known whether each of the observed bands represents a distinct gene product or is the result of processing or proteolysis.

3E10 recognizes CGs in mouse eggs by immunofluorescence, specifically by staining structures that are localized to the egg cortex, absent from the region overlying the meiotic spindle, and the correct size and shape for CGs. The 3E10 staining colocalizes with LCA and is lost after CG release at fertilization. It is not clear whether the $<10\%$ of LCA-stained structures that are 3E10-negative are a genuine population of non-CG, LCA-positive structures or a subpopulation of 3E10-negative CGs. Alternatively, perhaps there are differences in the relative permeability of CGs (during the staining protocol) to LCA and IgM that account for the observed results.

The 3E10 epitope-containing protein appears to be distinct from previously reported CG components identified with other antibodies. The lack of 3E10 immunoreactivity in fertilized eggs and embryos suggests that the antigen is not synthesized early in embryonic development. This is in contrast to the rabbit polyclonal ABL2 [11, 27] that recognizes a 75-kDa protein in CGs and is still present as late as the early blastocyst stage [11]. ABL2 also recognizes embryo-specific proteins at 65 and 70 kDa [11]. Western blot data, indicating a 32-kDa antigen rather than one at 75 kDa, support the conclusion that the 3E10 recognizes a CG component distinct from the one recognized by ABL2. These results also imply that 3E10 may be more specific for CGs than the ABL2 polyclonal antibody. Another antibody that has been used to study mouse CGs [7] is a goat antiserum made to β-hexosaminidase purified from human placenta [28]. This polyclonal antibody localizes to CGs by immunoelectron microscopy, but there is no direct evidence of a protein of the predicted 63-kDa molecular weight [28] in CGs or exudate, even though the hexosaminidase activity was detected in exudate [7].

At the present time, the BPS is known to include the following events: the modifications of ZP3 that reduce sperm binding and acrosome reaction induction, the proteolytic cleavage of ZP2, and possibly oolemma changes that mediate the plasma membrane BPS [3, 6]. Some or all of these BPS-associated activities are predicted to correlate with the protein bands in exudate, implying that there are likely to be few CG-localized proteins that are not involved in the BPS. For this reason, we chose to examine whether the 3E10 antibody was capable of interfering with the establishment of the BPS, which would indicate that the epitope-containing protein contributes to the BPS. Interference by 3E10 was predicted to result in an increased rate of polyspermy in an in vitro fertilization assay. However, the 3E10 mAb IgM did not affect the BPS, as assessed by polyspermy of ZP-enclosed eggs. These results indicate that the 3E10 epitope is not crucial for establishing a functional BPS. Because 3E10 is a mAb, it may bind its target protein in a region distinct from the active site, thereby not affecting its activity. Another plausible explanation is that the 3E10 target is involved in some other aspect of the BPS, such as modification of the plasma membrane. Alternatively, the 32-kDa protein may be involved in packaging of proteins in the CGs and may not be directly involved in the BPS after exocytosis. Future experiments will address these questions in more detail. Whatever the case, the 3E10 antibody is the first reagent generated specifically to mouse CG-derived materials and is likely to yield new insights into CGs and possibly the BPS.

3E10 has several advantages over previously available reagents for the study of CGs. The lectin, LCA, has been useful for examining mouse and other mammalian CGs and presumably recognizes a high concentration of LCA binding sites in CGs relative to other parts of the egg. However, because LCA recognizes alpha-mannose and, to a lesser extent, alpha-glucose and alpha-*N*-acetylglucosamine, it may recognize multiple proteins in eggs and other cell types [29]. The observation in eggs that LCA and 3E10 recognize virtually the same population of granules confirms that LCA labels primarily CGs, originally shown by LCA localization to CGs by electron microscopy [15, 16]. Therefore, LCA continues to be an excellent reagent for localization and quantification of CGs, but an antibody is likely to be more useful in elucidating the identity of specific CG proteins, for example by screening expression libraries and studying CG biogenesis.

Little is known about CG biogenesis in mammals, although much research has focused on invertebrates such as sea urchins [30–32]. The ABL2 antibody was used to quantitatively examine the synthesis of the 75-kDa CG protein in growing oocytes [27], but its site of synthesis before packaging into CGs was not reported. Localization of components such as the 3E10 antigen during CG biogenesis could reveal spatial and temporal regulation of both protein synthesis as well as CG translocation to the cortex. LCA has also been used to examine CG localization and production during oogenesis [24]. However, because LCA reactivity relies on protein glycosylation, the synthesis of CG proteins that have not been glycosylated cannot be studied with LCA. In addition, because the proteins of the ZP are heavily glycosylated and made during a similar timeframe in oogenesis, some lectins may recognize both CG and ZP proteins in developing oocytes. In this regard, 3E10 does not appear to recognize the proteins of the mature ZP (Fig. 2a).

It is noteworthy that 3E10 is an IgM. Previous attempts to generate mAbs to mammalian egg proteins have often resulted in IgMs rather than IgGs [33, 34]. In fact, in one study, 75% of mAbs (6 of 8) generated against ZP-free mouse oocytes were of the IgM isotype [34]. The relative frequency of IgMs may be due in part to the relatively small amounts and/or low antigenicity of protein available when working with egg materials.

In future experiments, this antibody will be used to characterize the 3E10 antigen and elucidate its function. This can be done by immunodepletion or immunoprecipitation of the antigen from exudate. Another question to be addressed is whether the 3E10 antigen is an egg-specific protein or is expressed in other tissues. If another source of the protein is available, then other biochemical methods, requiring more protein, can be employed to address the protein's structure and function.

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