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Characterization, Fate, and Function of Hamster Cortical Granule Components

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ABSTRACT Little is known about the composition and function of mammalian cortical granules. In this study, lectins were used as tools to: (1) estimate the number and molecular weight of glycoconjugates in hamster cortical granules and show what sugars are associated with each glycoconjugate; (2) identify cortical granule components that remain associated with the oolemma, cortical granule envelope, and/or zona pellucida of fertilized oocytes and preimplantation embryos; and (3) examine the role of cortical granule glycoconjugates in preimplantation embryogenesis. Microscopic examination of unfertilized oocytes revealed that the lectins PNA, DBA, WGA, RCA_{120}, Con A, and LCA bound to hamster cortical granules. Moreover, LCA and Con A labeled the zona pellucida, cortical granule envelope, and plasma membrane of fertilized and artificially activated oocytes and two and eight cell embryos. Lectin blots of unfertilized oocytes had at least 12 glycoconjugates that were recognized by one or more lectins. Nine of these glycoconjugates are found in the cortical granule envelope and/or are associated with the zona pellucida and plasma membrane following fertilization. In vivo functional studies showed that the binding of Con A to one or more mannosylated cortical granule components inhibited blastomere cleavage in two-cell embryos. Our data show that hamster cortical granules contain approximately 12 glycoconjugates of which nine remain associated extracellularly with the fertilized oocyte after the cortical reaction and that one or more play a role in regulating cleavage divisions. *Mol. Reprod. Dev. 58:223–235, 2001.*

Key Words: cortical granules; mammals; oocyte; glycoconjugates; embryo; preimplantation development; cleavage

INTRODUCTION

Cortical granules are Golgi-derived organelles that reside in the cortex of mature metaphase II arrested oocytes until fertilization when their contents are secreted into the perivitelline space surrounding the oocyte (reviewed by Cran, 1989; Cran and Esper, 1990). While there is an extensive literature dealing with sea urchin and lower vertebrate cortical granules (Schuel, 1978), relatively little is known about the contents and function of mammalian cortical granules (Hoodbhoy and Talbot, 1994). Mammalian cortical granule components are known to be glycosylated. Specifically, mouse, hamster, and cat cortical granules are labeled by the lectin LCA, which is α-D-mannose-specific and pig cortical granules by PNA, which is specific for β-D-galactosyl (1, 3)-D-*N*-acetylgalactosamine residues (Cherr et al., 1988; Ducibella et al., 1988, 1990; Byers et al., 1992; Yoshida et al., 1993; Ducibella and Buetow, 1994; Wang et al., 1997). Three molecules have been localized directly to mouse cortical granules using cytochemical or biochemical techniques. These include an ovoperoxidase (Gulyas and Schmell, 1980), a 75 Kd glycoprotein known as p75 (Pierce et al., 1990, 1992), and β-*N*-acetylglucosaminidase (Miller et al., 1993). These molecules have not yet been isolated and sequenced; however, p75 and β-*N*-acetylglucosaminidase have been partially characterized biochemically (Pierce et al., 1990; Miller et al., 1993). Mammalian cortical granules may also contain heparin binding placental protein (Sinosich et al., 1990a, 1990b) and proteinases (Gwatkin et al., 1973; Wolf, 1977; Cherr et al., 1988; Moller and Wassarman, 1989; Tawia and Lopata, 1992), including tissue plasminogen activator (Huarte et al., 1985; Zhang et al., 1992), since these molecules are released from oocytes upon artificial activation or fertilization. Direct evidence localizing a trypsin-like proteinase(s) to mammalian cortical granules is limited to the weak labeling of cortical granules in unfertilized hamster oocytes with soybean trypsin inhibitor conjugated to fluorescein isothiocyanate (Cherr et al., 1988).

The fate and function of mammalian cortical granule components have been the subject of several studies. LCA and PNA label the surface of fertilized hamster and pig oocytes, respectively (Cherr et al., 1988; Yoshida et al., 1993), suggesting that some cortical granule components associate with the oocyte surface after fertilization. The proteinases, ovoperoxidase, and β-*N*-acetylglucosaminidase are thought to bring about changes in the zona pellucida that establish the zona block to polyspermy and/or harden the zona by

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unknown mechanisms (Gwatkin et al., 1973; Wolf and Hamada, 1977; Gulyas and Schmell, 1980; Cherr et al., 1988; Moller and Wassarman, 1989; Tawia and Lopata, 1992; Zhang et al., 1992; Miller et al., 1993; Dunbar et al., 1994). Hardening of the zona may also play a role in blocking polyspermy and/or in protection of the preimplantation embryo. Cortical granule proteinases may also help block polyspermy at the plasma membrane, although the evidence for this is inconsistent (Wolf and Hamada, 1977; Wolf et al., 1979; Horvath et al., 1993). Lastly, some cortical granule components remain in the perivitelline space of fertilized hamster, mouse, human, and opossum oocytes and form a new extracellular matrix, called the cortical granule envelope, that persists until blastocyst hatching (Talbot and DiCarlantonio, 1984; Dandekar et al., 1992, 1995; Dandekar and Talbot, 1992). The cortical granule envelope is stabilized by ruthenium red, a polycationic dye, suggesting that it is glycosylated (Talbot and DiCarlantonio, 1984; Dandekar and Talbot, 1992). The persistence of the cortical granule envelope during preimplantation development suggests that it plays a role in early embryogenesis. In echinoderms, the hyaline layer, an extracellular matrix that surrounds fertilized eggs, is derived in part from a cortical granule glycoprotein called hyalin and is necessary for early development (reviewed by Alliegro et al., 1992).

The purpose of this study was to identify the contents of hamster cortical granules using lectins as probes for glycosylated molecules. Cortical granule binding lectins identified using confocal scanning laser microscopy (CSLM) were used to probe blots of oocyte proteins to determine the number, molecular weight, and associated carbohydrates of the glycoconjugates in hamster cortical granules. Lectin blots were also used to determine which cortical granule components are retained with preimplantation embryos and are thus potential components of the cortical granule envelope. Finally, in vivo experiments were done with Con A to determine if the cortical granule components retained by fertilized oocytes function in preimplantation embryogenesis.

MATERIALS AND METHODS

Chemicals and Supplies

Salts used to make all media, human chorionic gonadotropin (hCG), bovine serum albumin (BSA, fraction V), hyaluronidase, paraformaldehyde, paraphenylenediamine, Triton X-100, Tween-20, ammonium persulfate, TEMED, β-2 mercaptoethanol, and polyacrylamide were obtained from Sigma (St. Louis, MO). Pregnant mare's serum gonadotropin (PMSG) was purchased from CalBiochem (La Jolla, CA). Lectins, streptavidin conjugated to Texas Red, and Vectashield mounting medium were obtained from Vector Laboratories (Burlingame, CA). Square capillary tubes were purchased from In Vitro Dynamics (Rockaway, NJ). Nitrocellulose paper and molecular weight standards were obtained from BioRad (Hercules, CA). The enhanced chemiluminescence (ECL) kit was obtained from Amersham Pharmacia Biotech (Piscataway, NJ) and autoradiography film from Du Pont (Boston, MA).

Animals

Mature (8–20 weeks old) female and male golden hamsters (*Mesocricetus auratus*) purchased from Harlan Sprague-Dawley (San Diego, CA) were maintained on a 14:10 light:dark cycle in a room with controlled temperature (22°C) as described previously (Magers et al., 1995). Female hamsters have a 4-day estrous cycle with "Day one" being the day of ovulation indicated externally by the presence of a vaginal discharge.

Solutions

For dissection and oocyte and embryo collection for confocal laser scanning microscopy (CLSM) and SDS-PAGE, a 10× stock solution of Earle's balanced salt solution (EBSS) was made by dissolving the following in deionized water (in g/L): 2.0:$CaCl_2$; 4.0:KCl; 2.0:$MgSO_4 \cdot 7H_2O$; 54.0:NaCl; 1.4:$NaH_2PO_4 \cdot H_2O$ (Morgan et al., 1950). Immediately before use 250 ml of a 1× working solution was made, at which time sodium bicarbonate (2.2 g/L) and HEPES free acid (5.956 g/L) were added, and the pH was adjusted to 7.4. EBSS, pH 7.4 containing 0.5% BSA (EBSS/0.5% BSA) and EBSS containing 0.1% polyvinylpyrrolidone (EBSS/0.1% PVP) were made immediately prior to use.

For the in vivo studies, Con A-rhodamine was diluted using Dulbecco's phosphate-buffered saline (DPBS), pH 7.4, and the preimplantation embryos and uterine horns were evaluated in 0.1 M phosphate-buffered saline (PBS), pH 7.3 containing 0.1% BSA. A 1× working solution of DPBS, pH 7.4 was prepared by dissolving the following salts in deionized water (in g/L): 0.1:$CaCl_2$; 0.2:KCl; 0.2:KH_2PO_4; 0.1:$MgCl_2 \cdot 6H_2O$; 8.0:NaCl; 2.16:$Na_2HPO_4 \cdot 7H_2O$. A 0.2 M PBS solution was prepared by mixing 230 ml of solution A (27.6 g of $NaH_2PO_4 \cdot H_2O$ in 1 L of water) with 770 ml of solution B (28.4 g of Na_2HPO_4 in 1 L of water). A PBS solution (0.1 M) was prepared by dilution and the pH adjusted to 7.3.

To process oocytes and preimplantation embryos for CLSM, 3.7% paraformaldehyde was made immediately before use in EBSS, pH 7.4. A blocking solution was made fresh by supplementing DPBS, pH 7.4 with 100 mM glycine and 1 mg/ml BSA. Miles' phosphate-buffered saline (Miles' PBS) was prepared by adjusting a 10 mM Na_2HPO_4 solution to pH 8.1. Oocytes and preimplantation embryos were mounted in Vectashield or in a mixture of 10% Miles' PBS, 90% glycerol, and 0.1% phenylenediamine, which was stored at −20°C in foil-wrapped syringes.

Some lectin blots were stripped and reprobed. Stripping buffer was made by adding 2.0g of SDS and 0.78 ml of β-2 mercaptoethanol to 95 ml of a 62.5 mM Tris–HCl solution, pH 6.7 and then adjusting the volume to 100 ml.

Oocyte and Preimplantation Embryo Collection

Female golden hamsters were induced to ovulate by intraperitoneal injection with 25 International Units

(i.u.) of hCG on the evening of Day 3 of their estrous cycle. In some cases, hamsters were superovulated by administering 25 i.u. of PMSG at 10 a.m. on Day 1 of their estrous cycle, followed by 25 i.u. of hCG on Day 3. Unfertilized follicular oocytes were collected for CLSM analysis in EBSS/0.5% BSA by puncturing mature tertiary follicles with an insect pin 12 hr after hCG administration. Unfertilized oviductal oocytes were collected in EBSS/0.5% BSA for CLSM and in EBSS/0.1% PVP for SDS-PAGE by flushing oviducts 14–16 hr following hCG administration. Some oviductal oocytes were artificially activated by incubating them in EBSS/0.5% BSA and 100 i.u. of hyaluronidase for 30 min at 37°C. To collect in vivo fertilized oocytes and preimplantation embryos, female hamsters in Day 4 of their estrous cycle were placed in cages containing 1–2 male hamsters. The following day, fertilized oocytes containing two pronuclei were collected by flushing oviducts with EBSS/0.5% BSA (for CLSM) or with EBSS/0.1% PVP (for SDS-PAGE). Two- and eight-cell preimplantation embryos were collected 2 or 3 days after mating, respectively. Unfertilized and fertilized oocytes were denuded of their cumulus cells by incubating them in EBSS/0.5% BSA (CLSM) or in EBSS/0.1% PVP (SDS-PAGE) containing 100 i.u. of hyaluronidase for 5 min at room temperature, and then washed thoroughly. Zonae were removed if required by incubating oocytes in EBSS/0.5% BSA (CLSM) or 0.1% PVP (SDS-PAGE) containing 900 B.A.E.E. units of bovine pancreatic trypsin.

Confocal Laser Scanning Microscopy (CLSM) of Lectin Labeled Oocytes and Preimplantation Embryos

Oocytes and preimplantation embryos were manipulated under oil to maintain the pH of the solutions and to prevent cytoplasmic crenulation. Oocytes and preimplantation embryos were lectin-labeled according to published protocols (Cherr et al., 1988; Ducibella et al., 1994). Unfertilized and in vivo fertilized hamster oocytes, artificially activated oocytes, and preimplantation embryos were rinsed with EBSS to remove BSA and then fixed with 3.7% paraformaldehyde in EBSS, pH 7.4 for 30 min at room temperature. After washing with the blocking solution, some oocytes and preimplantation embryos were permeabilized for 5 min in blocking solution containing 0.1% Triton X-100 and washed again. The samples were then incubated with 0.2–100 μg/ml of the appropriate biotinylated lectin in a blocking solution for 30 min at room temperature, washed thoroughly, and subsequently incubated with 5 μg/ml Texas Red-streptavidin in a blocking solution for an additional 30 min at room temperature. Some oocytes were incubated with 50 μg/ml Con A conjugated to rhodamine. Control samples were labeled with biotinylated lectins preincubated for 30 min at room temperature with 100 mM of the appropriate control sugar followed by Texas Red-streptavidin or with Texas Red-streptavidin alone. Oocytes and preimplantation embryos were washed with blocking solution overnight at 4°C. For CLSM, the samples were placed in droplets of phenylenediamine or Vectashield and mounted under coverslips with vaseline posts or in square capillary tubes with a wall thickness of 0.1 mm, an inner diameter of 0.2 mm, and a length of 50 mm. Optical sections of the oocytes and preimplantation embryos were examined using a BioRad MRC-600 CLSM or a Zeiss 510 CLSM. All of the CLSM settings including the gain, numerical aperture, and neutral density filter were kept constant for all samples incubated in a given lectin. Images were processed using PhotoImpact (Ulead, Torrance, CA) and printed with a Tektronix Phaser 440 dye sublimation printer (Tektronix, Inc., Wilsonville, OR).

Gel Electrophoresis and Lectin Blotting

Oocytes and preimplantation embryos were solubilized in 2× reducing and denaturing Laemmli sample buffer (Laemmli, 1970). Proteins were separated by one-dimensional SDS-PAGE on a 4% stacking and 7.5% separating Doucet gel (Doucet and Trifaro, 1988). The stacking gel was run at 50 V and the separating gel at 100 V.

Electrophoresed oocyte and preimplantation embryo proteins were blotted onto nitrocellulose at 100 V for 15 min according to previously established procedures (Towbin et al., 1979). The blots were blocked with Tris-buffered saline (TBS) containing 0.5% Tween-20 (blocking solution) for 1 hr at room temperature and incubated with 1–20 μg/ml of the appropriate biotinylated lectin in blocking solution overnight at 4°C with constant agitation. Control blots were incubated overnight at 4°C with biotinylated lectins that were preabsorbed for 2 hr at room temperature with 100 mM of the appropriate control sugar made up in blocking solution, or incubated in blocking solution alone. Experimental and control blots were washed with blocking solution three times at 15-min intervals, and oocyte and embryonic glycoconjugates detected by enhanced chemiluminescence (ECL) according to the manufacturer's instructions, using a 1:20,000 dilution of HRP-streptavidin in blocking solution for 30 min at room temperature. Biotinylated standards (BioRad, Hercules, CA) were used to calibrate molecular weights.

In some cases, lectin blots were reprobed with a different biotinylated lectin after removing the initial lectin–HRP complex from its target carbohydrate with a stripping buffer. ECL was performed on the stripped blots to determine if any HRP-streptavidin was left. If no ECL signal was detected, the blots were rinsed with blocking solution and incubated in 1:20,000 HRP-streptavidin for 30 min at room temperature, and ECL was performed again. This second control determined if there was any biotinylated lectin left on the blot. If the controls worked, the blots were blocked for 1 hr at room temperature before reprobing them with another biotinylated lectin.

In Vivo Functional Studies

Female hamsters were mated and in vivo injections done as described previously (Martin et al., 1981). Two

days after mating, the hamsters were anaesthetized by intraperitoneal injection with 0.3–0.4 ml of Nembutal (50 mg/ml solution of sodium pentobarbital) (Abbott, N. Chicago, IL) delivered gradually. Surgical procedures were performed using a sterile technique. The dorsal surface of the hamster was shaved to reveal the scent glands that mark the position of the ovary. With the aid of a dissecting scope and fiber optic illumination, an incision was made through the skin and muscle medial to the right scent gland, and the right ovary and oviduct were exposed. The position of the ovary and oviduct were secured with the aid of hemostats attached to the ovarian fat pad, and the infundibulum was located through the wall of the bursa. Twenty five or 2.5 μg of rhodamine-Con A in 50 μl of DPBS, 50 μl of DPBS only, 25 μg of unconjugated Con A in 50 μl of DPBS, or 250 μg of normal rabbit serum IgG in 50 μl of DPBS were delivered into the oviduct through the infundibulum using a 1 ml tuberculin syringe fitted with a 30-gauge/1/2 inch needle. The ovary and oviduct were returned to the peritoneal cavity, the incision was sutured and covered with Neosporin, and the animal was allowed to recover under a heat lamp.

The effects of Con A on preimplantation embryonic development were evaluated by examining preimplantation embryos on Day 3 of pregnancy. The stage of the preimplantation embryo was determined by counting the number of blastomeres, and a trypan blue exclusion assay (0.1% trypan blue in PBS/0.1% BSA for 5 min) was used to test for embryo viability. In addition, the binding of Con A to the cortical granule envelope and/or zona was confirmed by examining Vectashield-mounted Day 3 preimplantation embryos with a Zeiss epifluorescence microscope and digital images were captured with a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI). Digital images were processed using Adobe Photoshop (Adobe Systems Inc., San Jose, CA) and printed with a Tektronix Phaser 440 dye sublimation printer (Tektronix, Inc., Wilsonville, OR).

Statistical Analyses

The percentage of cell cleavage occurring in Con A treated vs. saline or normal rabbit serum IgG-treated two cell embryos was analyzed statistically using a 1-way analysis of variance (ANOVA) followed by Dunnet's post hoc test that compared the individual means of the Con A-treated groups to the control groups. P values of less than 0.05 were considered significant.

RESULTS

Lectin Labeling in Oocytes and Preimplantation Embryos

To determine which lectins bind cortical granule components, non-permeabilized and permeabilized unfertilized oocytes were compared using CSLM. The zona pellucida was removed from unfertilized oocytes labeled with PNA, DBA, or WGA since these lectins labeled but did not penetrate the zona. Subsequently, the fate of the cortical granule lectin-binding material was followed in artificially activated and in vivo fertilized zona intact oocytes. The binding characteristics of each lectin in unfertilized and fertilized oocytes are shown in Table 1 and in Fig. 1 and Fig. 2.

RCA labeling. In permeabilized unfertilized oviductal oocytes, 100 μg/ml of RCA_{120}-biotin (specific for D-galactose) labeled the cortical granules, perivitelline space, and zona pellucida (Fig. 1A). Within the oocyte, labeling was confined to the cortical granules, and a cortical granule-free domain adjacent to the first polar body was not labeled (Fig. 1A). In non-permeabilized unfertilized oocytes, RCA_{120}-biotin labeled the zona, perivitelline space, and regions of the plasma membrane, but not the cortical granules, confirming that the granules resided within the oocyte (Fig. 1B). In zona-free fertilized oocytes, RCA_{120} labeled only the oolemma, and this labeling was much greater in non-permeabilized than in permeabilized oocytes (Fig. 1C). Control oocytes treated with RCA_{120}-biotin preincubated with D-galactose followed by Texas Red-streptavidin (Fig. 1D) or treated with Texas Red-streptavidin alone (data not shown) were not labeled.

PNA labeling. At 100 μg/ml, PNA-biotin (specific for β-D-galactosyl(1, 3)-D-*N*-acetylgalactosamine (Gal β(1, 3) GalNAc)) labeled only the cortical granules of permeabilized zona-free unfertilized oviductal oocytes,

TABLE 1. Summary of Lectin Binding to Oviductal Oocytes

	RCA	PNA	DBA	WGA	LCA	CON A
Unfertilized oocytes						
Granules in oocyte cortex	+ +	+ +	+ +	+ +	+ +	+ +
Plasmalemma	+	−	−	−	−	−
Perivitelline space	+	ND	ND	ND	−	−
Zona pellucida	+ +	+ +	+ +	+ +	−	−
Fertilized oocytes						
Granules in oocyte cortex	−	−	−	−	−	−
Plasmalemma	+ +	+ +	−	−	+	+
Perivitelline space	ND	ND	+ +	ND	+ +	+ +
Zona pellucida	ND	ND	+ +	ND	+	+

+ = Some fluorescence.
+ + = Strong fluorescence.
− = No fluorescence.
ND = Not determined.

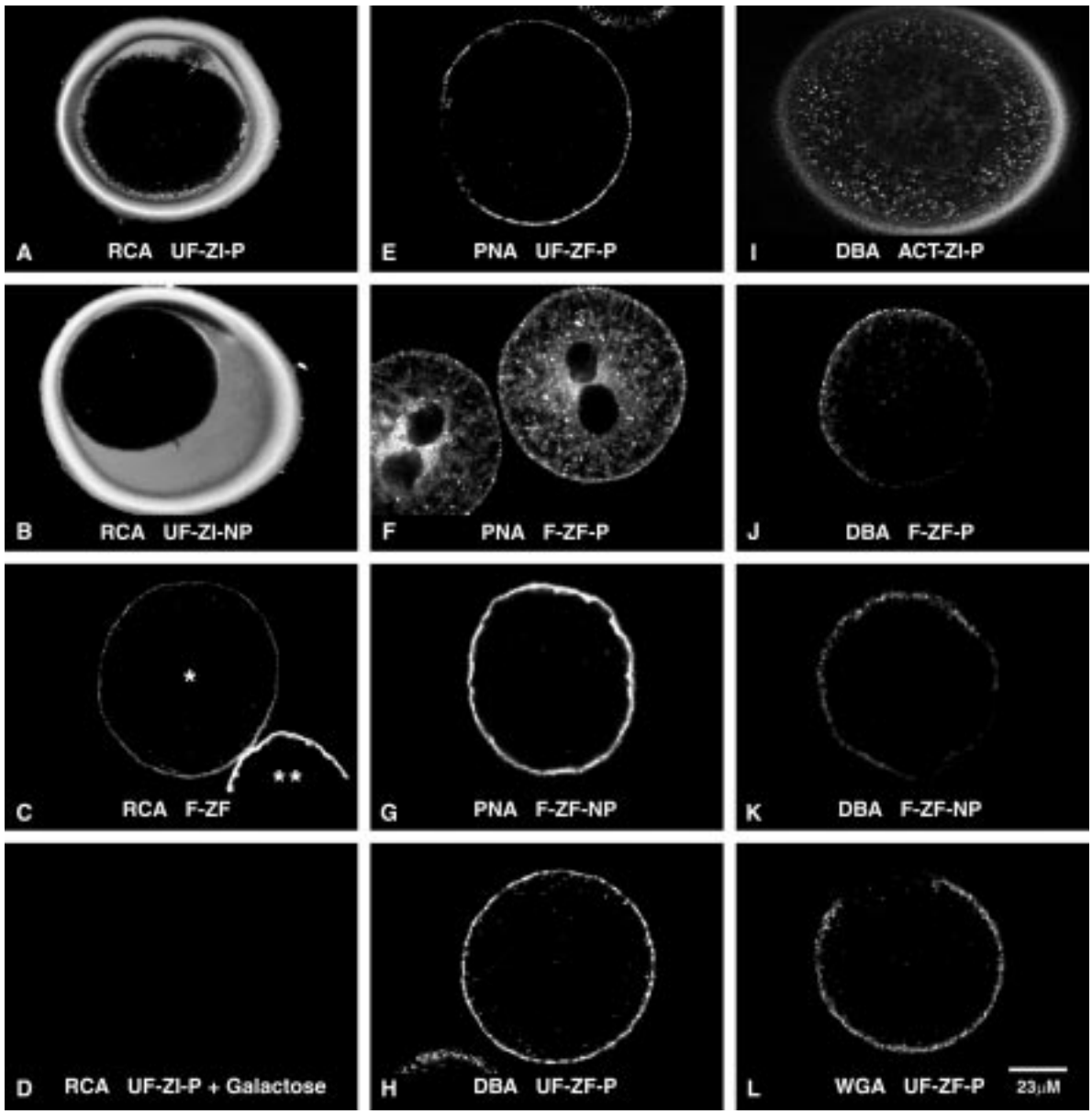

Fig. 1. Confocal laser scanning micrographs of hamster oviductal oocytes labeled with biotinylated RCA (A–D), PNA (E–G), DBA (H–K), and WGA (L). (**A**) A permeabilized zona-intact oocyte showing RCA_{120} labeling in the cortical granules, plasma membrane, perivitelline space, and zona pellucida. The cortical granule-free domain near the first polar body is unlabeled. (**B**) Same as A, except the oocyte is non-permeabilized and cortical granules are not labeled. (**C**) Comparison of RCA_{120}surface labeling of zona-free permeabilized (*) and non-permeabilized (**) fertilized oocytes. (**D**) Control permeabilized unfertilized oocyte labeled with 100 μg/ml of biotinylated RCA_{120} pretreated with 100 mM galactose followed by 5 μg/ml of Texas Red-streptavidin. (**E**) Cortical granules labeled in a permeabilized zona-free unfertilized oocyte treated with PNA. (**F**) Many larger sub-cortical vesicles were labeled with PNA in the cytoplasm of permeabilized fertilized oocytes. (**G**) The plasma membrane of non-permeabilized fertilized oocytes was more strongly labeled than that of permeabilized fertilized oocytes (F). (**H–K**) Zona-free oocytes labeled with 50 μg/ml (H, J, K) or 0.2 μg/ml (**I**) of biotinylated DBA. (H, I) DBA labeled cortical granules in permeabilized zona-free unfertilized oocytes (H) and in the perivitelline space around permeabilized zona-intact artificially activated oocytes (I). DBA slightly labeled the inner region of the zona pellucida of artificially activated oocytes (I). (**J**) Few DBA-labeled cortical granules were found in permeabilized zona-free fertilized oocytes. (**K**) The plasma membrane of non-permeabilized zona-free fertilized oocytes was slightly labeled by DBA. (**L**) A permeabilized zona-free unfertilized oocyte labeled with 50 μg/ml of biotinylated WGA showing label in the cortical granules and a cortical granule-free domain. Unfertilized and fertilized oocytes incubated in a given lectin were co-stained and imaged using identical confocal settings to illustrate true differences in fluorescence labeling. ACT = activated, UF = unfertilized, F = fertilized, P = permeabilized, NP = non-permeabilized, ZF = zona free, ZI = zona intact.

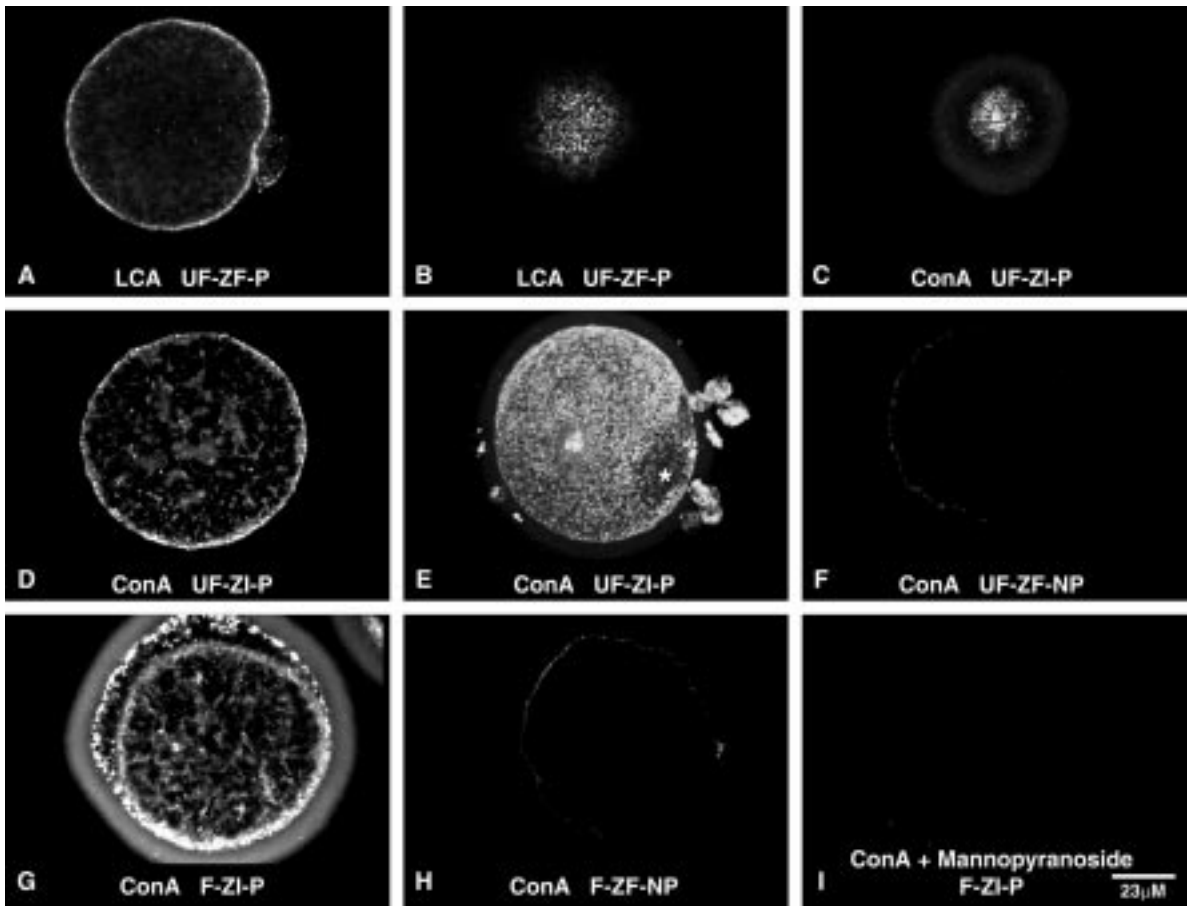

Fig. 2. Confocal laser scanning micrographs of unfertilized and fertilized hamster oviductal oocytes labeled with LCA and Con A. (**A**) Equatorial and (**B**) polar sections through a permeabilized zona-free unfertilized oocyte labeled with 50 μg/ml of biotinylated LCA demonstrating labeled cortical granules. (C–H) Unfertilized and fertilized oocytes labeled with 50 μg/ml of Con A-rhodamine. (**C**) Polar and (**D**) equatorial sections through a zona-intact unfertilized oocyte demonstrating label in the cortical granules but not the zona pellucida or the perivitelline space. (**E**) Projection image of 95 optical sections through a permeabilized zona-intact unfertilized oocyte showing the cortical granule-free domain (*). (**F**) A zona-free non-permeabilized unfertilized oocyte with virtually no surface label. (**G**) A zona-intact permeabilized in vivo fertilized oocyte demonstrating that Con A binds to exocytosed cortical granule material in the perivitelline space and slightly to the zona. (**H**) A non-permeabilized zona-free in vivo fertilized oocyte showing little surface label. (**I**) Control permeabilized in vivo fertilized oocyte labeled with 50 μg/ml biotinylated Con A preincubated with 100 mM α-D-methyl-mannopyranoside followed by 5 μg/ml Texas Red-streptavidin. The unfertilized and fertilized oocytes were co-stained with Con A and imaged using identical confocal settings to illustrate the true differences in fluorescence labeling. UF = unfertilized, F = fertilized, P = permeabilized, NP = non-permeabilized, ZF = zona free, ZI = zona intact.

and labeling was absent from a cortical granule-free domain (Fig. 1E). No labeling was observed in non-permeabilized zona-free unfertilized oocytes (data not shown). In permeabilized zona-free fertilized oocytes, PNA-biotin positive granules were found throughout the oocyte, especially near the nucleus (Fig. 1F). The oolemma of non-permeabilized zona-free fertilized oocytes was more heavily labeled (Fig. 1G) than that of permeabilized fertilized oocytes (Fig. 1F). Zona-free oocytes were not labeled by PNA pretreated with lactose followed by Texas Red-streptavidin (data not shown).

DBA and WGA labeling. At 50 μg/ml, DBA-biotin (specific for *N*-acetylgalactosamine (GalNAc)) and WGA-biotin (specific for *N*-acetylglucosamine (GlcNAc)) labeled only the cortical granules of permeabilized zona-free unfertilized oviductal oocytes (Figs. 1H, 1L respectively). At 100 μg/ml, both lectins labeled a few sub-cortical vesicles (data not shown). No label was seen in non-permeabilized zona-free unfertilized oocytes labeled with DBA-biotin or WGA-biotin (data not shown). Following artificial activation, DBA-biotin labeled the inner aspect of the zona pellucida and freshly exocytosed cortical granules in the perivitelline space (Fig. 1I) at concentrations (0.2 μg/ml) that did not label cortical granules in unactivated oocytes. Fertilized zona-free oocytes whether permeabilized (Fig. 1J) or non-permeabilized (Fig. 1K) had little DBA-positive material associated with the oolemma. Likewise, WGA did not label the surface of unfertilized or fertilized oocytes irrespective of permeabilization (data not shown). Oocytes were not labeled by DBA-biotin pre-

treated with GalNAc or by WGA-biotin preincubated with GlcNAc, followed by Texas Red-streptavidin (data not shown).

LCA and Con A labeling. LCA-biotin (specific for α-D-mannose) bound to cortical granules of the permeabilized zona-free unfertilized oviductal oocytes (Figs. 2A, B). However, LCA-biotin did not label the zona and perivitelline space of zona-intact unfertilized oocytes or the cortical granules in non-permeabilized oocytes (data not shown). Con A (specific for α-D-methyl-mannopyranoside), whether biotinylated or conjugated directly to rhodamine, also labeled the cortical granules, but not the zona or perivitelline space of permeabilized zona-intact unfertilized oviductal oocytes (Figs. 2C, D). The cortical granule-free domain (*) is shown in a three-dimensional reconstruction of a serially sectioned Con A-labeled oocyte (Fig. 2E). In contrast to other lectins, little Con A bound to the surface of non-permeabilized zona-free fertilized oocytes (Fig. 2F).

Fate of Con A-binding cortical granule components after fertilization. To determine what cortical granule material remains associated extracellularly with fertilized oocytes, zona-intact artificially activated and in vivo fertilized oviductal oocytes were examined after labeling with LCA-biotin followed by Texas Red-streptavidin or with Con A-rhodamine (Fig. 2G, Table 1). Con A and LCA were used because the previous lectin screen showed that these lectins penetrate the zona pellucida of unfertilized oocytes without labeling the zona or perivitelline space. Con A strongly labeled the perivitelline space and weakly labeled the zona of permeabilized fertilized (Fig. 2G) and artificially activated (data not shown) oocytes. A little Con A binding was also observed in the cytoplasm of fertilized oocytes (Fig. 2G). Little Con A was observed on the plasma membrane of non-permeabilized zona-free fertilized oocytes (Fig. 2H); however, Con A labeled the plasma membrane of non-permeabilized zona-intact fertilized oocytes (data not shown). LCA-biotin also bound to the zona, oolemma, and perivitelline space of fertilized oocytes; however, the perivitelline space labeling was more diffuse than in the Con A-labeled oocytes (data not shown). Control unfertilized and fertilized oocytes incubated with LCA-biotin pretreated with α-D-mannose (data not shown) or with Con A-biotin pretreated with α-D-methyl-mannopyranoside (Fig. 2I) followed by Texas Red-streptavidin, or with Texas Red-streptavidin alone (data not shown), were not labeled.

Retention of mannosylated cortical granules components by preimplantation embryos. To determine if mannosylated cortical granule components are retained extracellularly during development, preimplantation embryos were labeled with Con A-biotin (Fig. 3). The zona, perivitelline space, and plasma membrane of two (Fig. 3A) and eight (Fig. 3B) cell embryos were labeled by Con A-biotin. Control two (Fig. 3C) and eight (data not shown) cell embryos were not labeled by Con A-biotin that was preincubated with α-D-methyl-mannopyranoside followed by Texas Red-streptavidin (Fig. 3C) or by Texas Red-streptavidin alone (data not shown).

Identification of Lectin Binding Cortical Granule Components

The number, molecular weight, and associated carbohydrate residues of the lectin-binding cortical granule components were determined by comparing lectin-blotted zona-free unfertilized oocytes to zona-free fertilized oocytes (Fig. 4). Since lectin binding is largely confined to the cortical granules at the CLSM level, bands that are present in zona-free unfertilized oocytes but lost or reduced in zona-free fertilized oocytes should include cortical granule components. Zona-intact fertilized oocytes and eight cell embryos were also analyzed to determine which of the lectin-binding bands remained associated extracellularly with the fertilized oocyte and preimplantation embryo. Con A was used as the main probe in this study since CSLM had shown that it bound only to cortical granules in unfertilized oocytes and preliminary trials showed that it recognized more bands on blots than the other lectins.

In unfertilized zona-free oocytes, Con A recognized 11 major mannosylated bands (142, 110, 100, 62, 56, 52, 48, 45, 42, 39, and 33 Kd) and at least three minor bands (86, 75, and 66 Kd) (Fig. 4, lane 1). All of the major Con A binding bands were either absent or partially reduced in zona-free fertilized oocytes (Fig. 4, lane 2) and in oocytes that were artificially activated with the calcium ionophore A23187 (data not shown). Nine of the Con A binding bands in zona-free unfer-

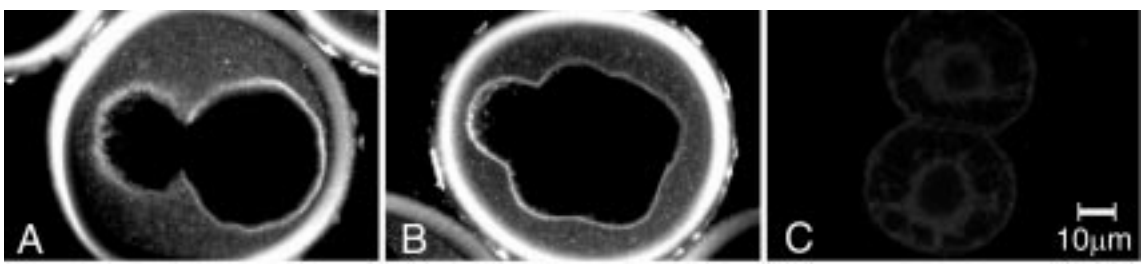

Fig. 3. Confocal laser scanning micrographs of preimplantation embryos labeled with 50 μg/ml of biotinylated Con A which bound to the zona, the plasma membrane, and the cortical granule envelope of two (**A**) and eight (**B**) cell embryos. (**C**) Control two-cell embryos were not labeled by 50 μg/ml of biotinylated Con A preincubated with 100 mM α-D-methyl-mannopyranoside followed by 5 μg/ml of Texas Red-streptavidin. The two and eight-cell embryos were co-stained with Con A and imaged using identical confocal settings. The zona appears brighter in the embryos compared to fertilized oocytes (Fig. 2G) since a higher gain and numerical aperture were used to image the embryos.

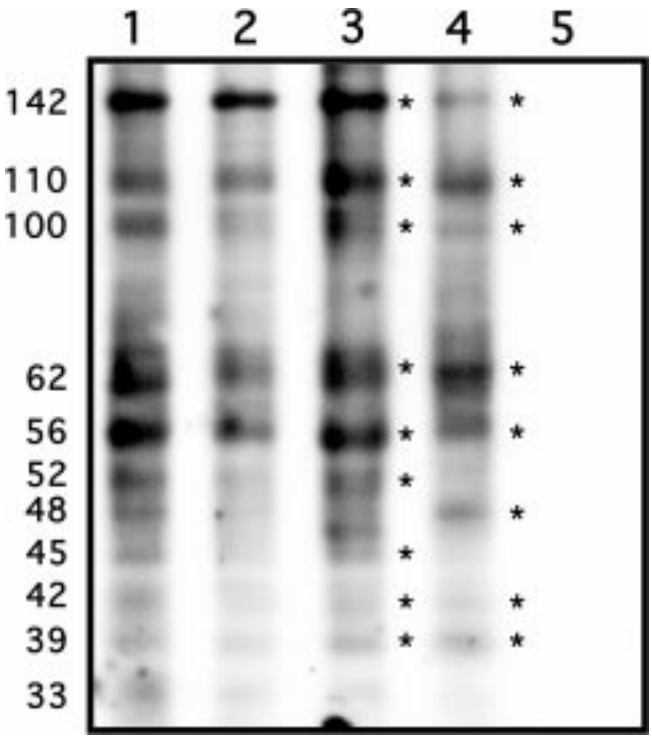

Fig. 4. Lectin blot analysis of Con A-biotin binding bands in hamster oocytes and eight-cell preimplantation embryos. Fifty zona-free unfertilized (lanes 1 and 5), fifty zona-free (lane 2) and fifty zona-intact (lane 3) fertilized oocytes, and fifty zona-intact eight-cell embryos (lane 4) were solubilized in reducing and denaturing Laemmli sample buffer and polypeptides were electrophoresed, lectin blotted with 0.5 μg/ml biotinylated Con A, and visualized by enhanced chemiluminescence (ECL). ECL film was exposed for 15 sec. Biotinylated Con A bound to 11 bands (142, 110, 100, 62, 56, 52, 48, 45, 42, 39, and 33 Kd) in zona-free unfertilized oocytes (lane 1). All except for the 142 Kd band were either absent or markedly reduced in zona-free fertilized oocytes (lane 2). Nine bands (142, 110, 100, 62, 56, 52, 45, 42, 39 Kd) were still associated with zona-intact fertilized oocytes in addition to a 47 Kd band (lane 3). Seven of the Con A-biotin-binding bands (142, 110, 100, 62, 56, 42, 39 Kd) were detected on blots of zona-intact eight-cell embryos; however, three of these bands (142, 100, 42 Kd) were reduced in appearance (lane 4). A 48 Kd band appeared in the eight-cell stage but not in zona-intact fertilized oocytes. Control blots of unfertilized oocytes were not labeled by Con A-biotin pretreated with α-D-methyl-mannopyranoside followed by HRP-streptavidin (lane 5).

tilized oocytes (142, 110, 100, 62, 56, 52, 45, 42, 39 Kd) as well as a 47 Kd band were detected on blots of zona-intact fertilized oocytes (Fig. 4, lane 3). Seven of the Con A binding bands (142, 110, 100, 62, 56, 42, 39 Kd) were still associated with zona-intact eight cell embryos, although three of them (142, 100, 42 Kd) were reduced in appearance (Fig. 4, lane 4). In addition, a 48 Kd band, not seen in zona-intact fertilized oocytes, appeared in eight cell stages (Fig. 4 lane 4). Control blots of fertilized oocytes (data not shown) and zona-free unfertilized oocytes incubated with Con A-biotin pretreated with α-D-methyl-mannopyranoside followed by HRP-streptavidin (Fig. 4, lane 5) or with HRP streptavidin alone (data not shown) were not labeled.

Similar comparisons were made for blots of zona-free unfertilized and fertilized oocytes probed with LCA, PNA, and DBA (Fig. 5). LCA, PNA, and DBA recognized subsets of the Con A binding bands, with PNA binding additionally to an 87 Kd band (Fig. 5). A very broad ~200 Kd band of oviductal origin was detected on blots of zona-intact fertilized oocytes probed with PNA or DBA, but not with Con A or LCA (data not shown). Because RCA_{120}-biotin produced a high level of background and WGA-biotin bound inconsistently and weakly to only two oocyte polypeptides (62 and 56 Kd), they were not used further.

In Vivo Functional Studies

The function of mannosylated cortical granule components associated with the pre-embryonic surface was examined in vivo by treating early two-cell stage embryos with Con A-rhodamine on Day 2 of pregnancy, then examining development 1 day later. Con A was used since it recognized the greatest number of cortical granule components and did not bind to oviductal glycoproteins. In live Con A-treated two-cell embryos recovered on Day 3 of pregnancy, fluorescence was detected between blastomeres that were in physical

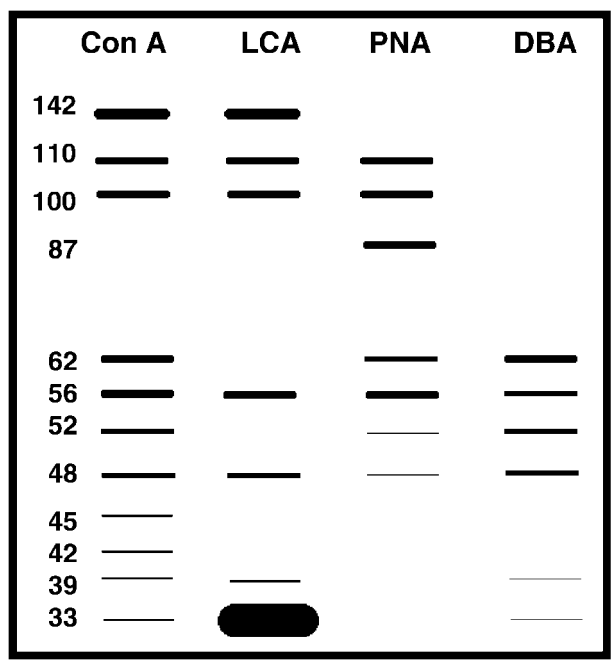

Fig. 5. Schematic diagram showing probable cortical granule components and their glycosylation patterns. Bands in the diagram were present in unfertilized zona-free oocytes but reduced or absent in fertilized zona-free oocytes. LCA-biotin and Con A-biotin bound to seven mannosylated bands (142, 110, 100, 56, 48, 39, and 33 Kd) and Con A-biotin bound to an additional four bands (62, 52, 45 Kd, and 42 Kd). Six of the Con A-biotin binding bands (110, 100, 62, 56, 52, 48 Kd) also bound PNA-biotin and were thus glycosylated by Gal β(1-3) GalNAc. PNA-biotin also recognized an 87 Kd band that was not detected on Con A blots. Six of the Con A-biotin binding bands (62, 56, 52, 48, 39, 33 Kd) bound DBA-biotin and, therefore, were glycosylated by GalNAC. Band thickness indicates relative abundance of each band for each lectin.

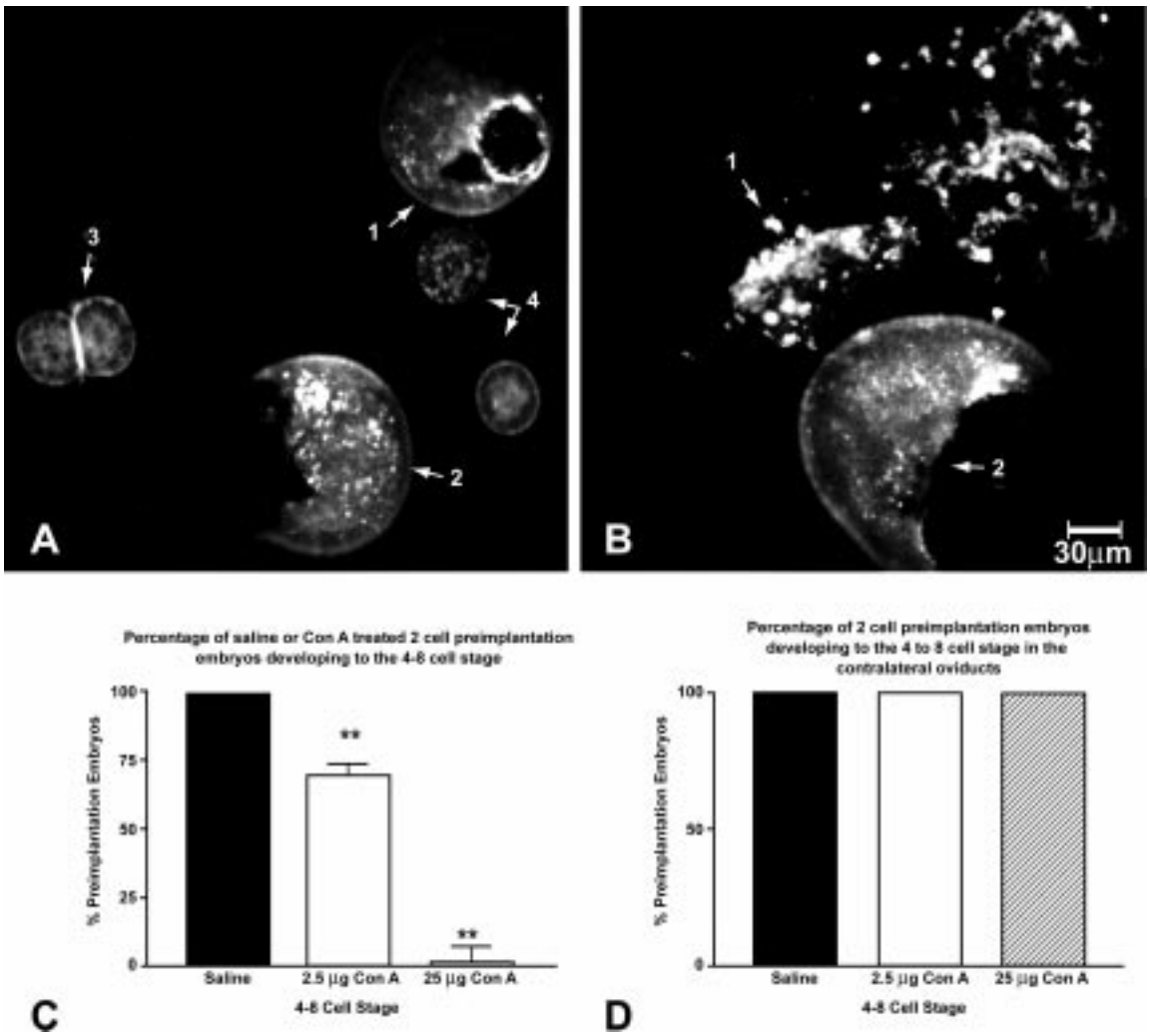

Fig. 6. In vivo development of early two-cell preimplantation embryos treated with saline or Con A-rhodamine on Day 2 of pregnancy. The percentage of embryos reaching the four-to-eight cell stage was determined on Day 3 of pregnancy. (**A**, **B**) Epifluorescent micrographs of live embryos from an oviduct injected with 25 μg of Con A-rhodamine. Each embryo was arrested at the two-cell stage of development. Con A-rhodamine bound to: the zona pellucida (A: 1, 2 and B: 2), the embryonic surface if the blastomeres were in contact (A: 3) but not if they were separated from each other (A: 4), and the cortical granule envelope which can be clearly seen in embryos whose zonae were ruptured during mounting (B: 1). (**C**) All of the saline-treated two-cell embryos developed to the four-to-eight cell stage; however, treatment with 2.5 or 25 μg of Con A-rhodamine caused significant arrest of the embryos at the two-cell stage. (**D**) All of the embryos reached the four-to-eight cell stage in the contralateral oviducts in all treatment groups. n = 5 for each group. ** = $P < 0.01$.

contact, as well as in the zona pellucida and perivitelline space (Fig. 6A, B). Labeled perivitelline space components were especially well observed after their liberation following rupture of the zona (Fig. 6B). Treatment of two cell embryos with Con A-rhodamine prevented their development to the four-to-eight-cell stage in a dose-dependent manner (Fig. 6C). All of the two-cell preimplantation embryos that were treated with saline reached the four-to-eight-cell stage by Day 3 of pregnancy. However, in oviducts injected with 2.5 and 25 μg of Con A-rhodamine, 31 and 100% of the two-cell embryos were arrested in their development (Fig. 6C). To control for negative effects of rhodamine on cell division, two-cell embryos (n = 18) were exposed to unconjugated Con A, and 83.8% arrested at the two-to-three cell stage. Inhibition of cell division was at the level of the cell cycle not cytokinesis since a single nucleus could be detected in each blastomere by CLSM. None of the Con A-treated embryos were stained by trypan blue. When two-cell embryos were treated with 250 μg of normal rabbit serum IgG to control for "protein load," there was no significant effect on embryonic development (data not shown). Finally, all of the two-cell embryos reached the four-to-eight-cell stage in the contralateral oviducts irrespective of the treatment of the other oviduct (Fig. 6D).

DISCUSSION

We have used lectins to: (1) estimate the number and molecular weight of the glycoconjugates present in hamster cortical granules and show which sugars are associated with each glycoconjugate; (2) identify cor-

tical granule components that remain associated with the oolemma, perivitelline space, and/or zona pellucida of fertilized oocytes and preimplantation embryos; and (3) examine the role of cortical granule components in preimplantation development.

All lectins tested in this study labeled organelles in the cortex of unfertilized hamster oocytes. These organelles were interpreted to be cortical granules for the following reasons. LCA was previously shown at the ultrastructural level to label hamster cortical granules (Cherr et al., 1988), and the cortical labeling of unfertilized oocytes for all lectins in our study was similar to that for LCA. In addition, lectin-labeled granules were the expected size and shape of cortical granules, were observed in unfertilized oocytes only after permeabilization and hence were inside the oocyte, were located close to the oolemma, were absent from a domain interpreted to be the cortical granule-free domain, and were absent in permeabilized oocytes after fertilization. In all oocytes optically sectioned by CSLM, lectins bound only to the cortical granules in unfertilized oocytes, with the exception of a few interior vesicles labeled by DBA and WGA only at 100 μg/ml. No lectin labeling was observed inside fertilized oocytes except for PNA, which labeled many larger vesicles found mainly near the pronuclei. These vesicles may contain endocytosed oviductal components such as the ~200 Kd PNA binding oviductal glycoprotein observed on lectin blots of fertilized oocytes (data not shown). Since PNA bound uniquely to the 87 Kd band and since only PNA labeled fertilized oocytes internally, the 87 Kd protein could also be internalized after fertilization. Con A and LCA were the most useful lectins for tracing the fate of the cortical granule after exocytosis since they did not label the zona pellucida, perivitelline space, or oviductins prior to fertilization. Con A was used to follow cortical granule glycoconjugates after fertilization since it bound to the greatest number of glycoconjugates on lectin blots.

In lectin blot analysis of zona-free unfertilized oocytes, 12 major bands and several minor bands were recognized by the lectins Con A, LCA, PNA, and/or DBA. Each of these bands could contain multiple polypeptides that were not resolved by one-dimensional SDS-PAGE. Con A and LCA both bind mannosylated groups; however, LCA did not interact with several Con A-binding bands, and LCA produced a much stronger signal than Con A with the 33 Kd band. All of the major lectin-binding bands were either no longer detectable or reduced in amount on blots of zona-free fertilized oocytes, and nine of the lectin-binding bands were retained extracellularly in zona-intact fertilized oocytes. These bands most likely represent exocytosed cortical granule components that form the cortical granule envelope and/or associate with the oolemma and/or zona pellucida. Seven of the lectin-binding bands were still observed on blots of zona-intact eight-cell embryos. The retention of cortical granule components extracellularly during preimplantation development is consistent with electron microscopic observations showing that the cortical granule envelope remains until blastocyst hatching (Dandekar and Talbot, 1992). The 48 Kd band associated with zona-intact eight-cell embryos could be a modified cortical granule envelope component or a newly synthesized embryonic glycoprotein. The bands that were reduced after fertilization were not deglycosylated glycoconjugates or degraded non-cortical granule cytoplasmic components since these would in all likelihood be missing from both zona-free and zona-intact fertilized oocytes.

There are several possible reasons why some lectin-binding bands (142, 110, 100, 62, and 56 Kd) were reduced but not completely absent from blots of zona-free fertilized oocytes. First, each of the 12 major lectin-binding bands could contain multiple glycoconjugates, some of which may be membrane-associated or cytoplasmic glycoproteins that are not lost at fertilization and are not detected by CSLM. Secondly, our data show that some exocytosed cortical granule glycoconjugates adhere tightly to the oolemma after fertilization and would thus be expected to appear on blots. For example, the PNA and RCA_{120}-binding cortical granule glycoconjugates were present on the plasma membranes of fertilized oocytes after zona removal but not after the zona-free oocytes had been permeabilized by Triton X-100, indicating that PNA and RCA_{120}-binding cortical granule glycoconjugates associate tightly with the oocyte surface. Some of the residual bands seen in lanes of zona-free oocytes would include these oolemma-associated glycoconjugates since the lectin-blotted oocyte samples were not permeabilized.

Both lectin blots and CSLM revealed that significant amounts of Con A positive cortical granule material remained associated extracellularly with fertilized oocytes and preimplantation embryos. In confocal images, Con A and LCA labeled the plasma membrane, perivitelline space, and zona pellucida of fertilized hamster oocytes and preimplantation embryos, with most of the label localizing to the perivitelline space. Previous studies focused on the plasma membrane have shown that a variety of lectins labels the oolemma of fertilized or artificially activated oocytes from the rabbit (Con A) (Gordon et al., 1975), mouse (UEA I, FBP, LPA) (Lee et al., 1988), hamster (LCA, Con A) (Cherr et al., 1988; Hoodbhoy and Talbot, 1993), pig (PNA) (Yoshida et al., 1993; Wang et al., 1997), and human (UEA I) (Tam et al., 1990). These lectins are thought to bind to exocytosed cortical granule components that adhere to the oolemma, although it is possible that they also label cortical granule integral membrane proteins that get incorporated into the oolemma. Some of the glycoconjugates on the oolemma of fertilized oocytes could be part of the cortical granule envelope which interacts with plasma membrane of zygotes and blastomeres (Dandekar and Talbot, 1992; Dandekar et al., 1992). Hyalin, a major component of the echinoderm hyaline layer, which is equivalent to the mammalian cortical granule envelope, also interacts directly with the embryonic surface, and this

interaction is essential to early echinoderm development (Adelson and Humphreys, 1988; Adelson et al., 1992; Wessel et al., 1998).

Prior lectin studies on fertilized mammalian oocytes were generally done using zona-free oocytes and have not addressed possible retention of Con A-binding cortical granule glycoconjugates in the cortical granule envelope or the zona pellucida. Our data are the first to show that mannosylated cortical granule glycoconjugates are retained in the perivitelline space following fertilization and are consistent with these components being part of the cortical granule envelope. Although the specific glycoconjugates that make up the cortical granule envelope have not yet been identified, they would probably be included in the nine bands that are retained by fertilized oocytes. Our study demonstrated enhanced binding of Con A to the hamster zona pellucida following fertilization. Post-fertilization labeling of the zona could be the result of Con A and LCA-binding mannosylated cortical granule components that had diffused into the zona and/or binding to mannose that had been exposed in the zona by cortical granule enzymes. In either case, it is a clear demonstration that mannosylation of the zona is altered after fertilization.

Con A bound to the zona, cortical granule envelope, and blastomere surfaces of two-to-eight-cell preimplantation embryos indicating that mannosylated cortical granule glycoconjugates are retained by cleavage stage embryos. These data are consistent with electron microscopy observations demonstrating that the cortical granule envelope exists throughout preimplantation development (Dandekar and Talbot, 1992; Dandekar et al., 1992) and with our lectin blot data showing that cortical granule components are still associated extracellularly with the eight-cell stage. However, the 52 and 45 Kd lectin-binding bands disappear or are deglycosylated by the eight-cell stage, and four of the seven–eight remaining mannosylated components (142, 100, 56, and 42 Kd), are reduced in amount. Moreover, one or more of the seven to eight bands found on lectin blots of eight-cell embryos could be newly synthesized glycoproteins. In echinoderms, components of the hyaline layer are replenished during embryogenesis by de novo synthesis (reviewed by Alliegro et al., 1992). The cortical granule envelope, like other extracellular matrices, may be a dynamic structure whose components are modified and/or replenished.

In vivo treatment of two-cell stage embryos with Con A-rhodamine inhibited their development to the four-to-eight cell stage in a dose-dependent fashion without showing evidence of cytotoxicity. These data suggest that one or more of the cortical granule components plays a role in the development of the mammalian preimplantation embryo. Inhibition of cleavage was not due to the presence of rhodamine in the oviduct since unconjugated Con A was as inhibitory as Con A-rhodamine. Our in vivo study supports and extends earlier work showing that the development of mouse embryos is inhibited in vitro by Con A (Pienkowski and Koprowski, 1974; Pratt et al., 1982; Reeve, 1982), but not by Con A preincubated with α-D-methylmannopyranoside (Pienkowski and Koprowski, 1974). In vitro Con A treatment retards the rate of blastomere cleavage in zona-free two and four-cell mouse embryos and inhibits compaction in eight-cell embryos (Pratt et al., 1982; Reeve, 1982). The earlier in vitro Con A inhibition studies were carried out using the late two stages (46–48 hr old, just prior to division) while we used earlier (30-hr-old) stages. In the late two-cell stage, the perivitelline space may contain non-cortical granule-derived components secreted by embryo after the activation of its genome. Our study was therefore more specific for cortical granule-derived components and is the first to demonstrate that inhibition of cleavage by Con A is likely mediated through its binding to an exocytosed cortical granule component. Neither the in vitro nor in vivo studies eliminate the possibility that Con A, which is a tetravalent molecule, inhibits embryonic development by cross-linking neighboring mannosylated targets. Since our functional studies were carried out in vivo, Con A may have inhibited blastomere cleavage by binding to oviductal glycoproteins (oviductins) that facilitate preimplantation embryonic development (St-Jacques et al., 1992; Malette and Bleau, 1993; El-Mestrah and Kan, 1999). However, this possibility is unlikely since the hamster oviductal epithelium does not secrete glycoconjugates carrying terminal mannose (El-Mestrah and Kan, 1999), and Con A did not bind to secreted oviductal glycoproteins on our lectin blots. Further studies will be needed to identify the cortical granule glycoconjugates that appear to play a role in regulating the preimplantation cleavage.

CONCLUSIONS

All six lectins tested in this study bound to the cortical granules of unfertilized hamster oocytes indicating diverse glycosylation of the granule components. Our CSLM and lectin blot data are consistent with the idea that hamster cortical granules contain at least 12 heterogeneously glycosylated components. Nine of these glycoconjugates associate with the blastomere surface, cortical granule envelope, and/or zona pellucida after fertilization, and seven of them were still detected at eight-cell stage of embryogenesis. Our data further show that Con A inhibits cleavage of hamster two-cell stage embryos in vivo and suggests that preimplantation mammalian embryos regulate their own cleavage divisions in an autocrine manner, either directly or indirectly, through glycoconjugates released from the cortical granules at fertilization.

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REFERENCES

Adelson DL, Humphreys T. 1988. Sea urchin morphogenesis and cell–hyalin adhesion are perturbed by a monoclonal antibody specific for hyalin. Development 104(3):391–402.

Adelson DL, Alliegro MC, McClay DR. 1992. On the ultrastructure of hyalin, a cell adhesion protein of the sea urchin embryo extracellular matrix. J Cell Biol 116(5):1283–1289.

Alliegro MC, Black SD, McClay DR. 1992. Deployment of extracellular matrix proteins in sea urchin embryogenesis. Microsc Res Tech 22(1):2–10.

Byers AP, Barone MA, Donoghue AM, Wildt DE. 1992. Mature domestic cat oocyte does not express a cortical granule-free domain. Biol Reprod 47(5):709–715.

Cherr GN, Drobnis EZ, Katz DF. 1988. Localization of cortical granule constituents before and after exocytosis in the hamster egg. J Exp Zool 246(1):81–93.

Cran DG. 1989. Cortical granules during oocyte maturation and fertilization. J Reprod Fertil Suppl 38:49–62.

Cran DG, Esper CR. 1990. Cortical granules and the cortical reaction in mammals. J Reprod Fertil Suppl 42:177–188.

Dandekar P, Talbot P. 1992. Perivitelline space of mammalian oocytes: extracellular matrix of unfertilized oocytes and formation of a cortical granule envelope following fertilization. Mol Reprod Dev 31(2):135–143.

Dandekar P, Aggeler J, Talbot P. 1992. Structure, distribution, and composition of the extracellular matrix of human oocytes and cumulus masses. Hum Reprod 7(3):391–398.

Dandekar P, Mate KE, Talbot P. 1995. Perivitelline space of marsupial oocytes: extracellular matrix of the unfertilized oocyte and formation of a cortical granule envelope following the cortical reaction. Mol Reprod Dev 41(3):368–373.

Doucet JP, Trifaro, JM. 1988. A discontinuous and highly porous sodium dodecyl sulfate-polyacrylamide slab gel system of high resolution. Anal Biochem 168(2):265–271.

Ducibella T, Buetow J. 1994. Competence to undergo normal, fertilization-induced cortical activation develops after metaphase I of meiosis in mouse oocytes. Dev Biol 165(1):95–104.

Ducibella T, Anderson E, Albertini DF, Aalberg J, Rangarajan, S. 1988. Quantitative studies of changes in cortical granule number and distribution in the mouse oocyte during meitoic maturation. Devel Biol 130:184–197.

Ducibella T, Duffy P, Reindollar R, Su B. 1990. Changes in the distribution of mouse oocyte cortical granules and ability to undergo the cortical reaction during gonadotropin-stimulated meiotic maturation and aging in vivo. Biol Reprod 43(5): 870–876.

Ducibella T, Duffy P, Buetow J. 1994. Quantification and localization of cortical granules during oogenesis in the mouse. Biol Reprod 50(3):467–473.

Dunbar BS, Avery S, Lee V, Prasad S, Schwahn D, Schwoebel E, Skinner S, Wilkins B. 1994. The mammalian zona pellucida: its biochemistry, immunochemistry, molecular biology, and developmental expression. Reprod Fertil Dev 6(3):331–347.

El-Mestrah M, Kan FW. 1999. Differential distribution of lectin-binding glycoconjugates in the secretory granules of hamster oviductal ampulla during the estrous cycle: a quantitative cytochemical analysis. Histochem Cell Biol 111(1):23–32.

Gordon M, Fraser LR, Dandekar P. 1975. The effect of ruthenium red and concanavalin A on the vitelline surface of fertilized and unfertilized rabbit ova. Anat Rec 181:95–112.

Gulyas BJ, Schmell ED. 1980. Ovoperoxidase activity in ionophore treated mouse eggs. I. Electron microscopic localization. Gam Res 3:267–277.

Gwatkin RBL, Williams DT, Hartmann JF, Kniazuk M. 1973. The zona reaction of hamster and mouse eggs: production in vitro by a trypsin-like protease from cortical granules. J Reprod Fertil 32:259–265.

Hoodbhoy T, Talbot P. 1993. The use of confocal scanning laser microscopy (CSLM) to study the fate of the cortical granule (CG) components following fertilization in the hamster. Biol Reprod Suppl 48:166.

Hoodbhoy T, Talbot P. 1994. Mammalian cortical granules: contents, fate, and function. Mol Reprod Dev 39(4):439–448.

Horvath PM, Kellom T, Caulfield J, Boldt J. 1993. Mechanistic studies of the plasma membrane block to polyspermy in mouse eggs. Mol Reprod Dev 34(1):65–72.

Huarte J, Belin D, Vassali JD. 1985. Plasminogen activator in mouse and rat oocytes: induction during meiotic maturation. Cell 43:551–558.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(259):680–685.

Lee SH, Ahuja KK, Gilburt DJ, Whittingham DG. 1988. The appearance of glycoconjugates associated with cortical granule release during mouse fertilization. Development 102(3):595–604.

Magers T, Talbot P, Carlantonio GD., Knoll M, Demers D, Tsai I, Hoodbhoy T. 1995. Cigarette smoke inhalation affects the reproductive tract of female hamsters. Reproductive Toxicology 9:513–525.

Malette B, Bleau G. 1993. Biochemical characterization of hamster oviductin as a sulphated zona pellucida-binding glycoprotein. Biochem J 295(Pt 2):437–445.

Martin GG, Talbot P, Pendergrass P. 1981. An intrabursal injection procedure for the in vivo study of ovulation in hamsters. J Exp Zool 216(3):461–468.

Miller DJ, Gong X, Decker G, Shur BD. 1993. Egg cortical granule *N*-acetylglucosaminidase is required for the mouse zona block to polyspermy. J Cell Biol 123(6):1431–1440.

Moller CC, Wassarman PM. 1989. Characterization of a proteinase that cleaves zona pellucida glycoprotein ZP2 following activation of mouse eggs. Dev Biol 132:103–112.

Pienkowski M, Koprowski H. 1974. Study of the growth regulation of preimplantation mouse embryos using concanavalin A. Proc Soc Exp Biol Med 145(2):464–469.

Pierce KE, Siebert MC, Kopf GS, Schultz RM, Calarco PG. 1990. Characterization and localization of a mouse egg cortical granule antigen prior to and following fertilization or egg activation. Dev Biol 141(2):381–392.

Pierce KE, Grunvald EL, Schultz RM, Kopf GS. 1992. Temporal pattern of synthesis of the mouse cortical granule protein, p75, during oocyte growth and maturation. Dev Biol 152(1):145–151.

Pratt HP, Ziomek CA, Reeve WJ, Johnson MH. 1982. Compaction of the mouse embryo: an analysis of its components. J Embryol Exp Morphol 70:113–132.

Reeve WJ. 1982. Effect of concanavalin A on the formation of the mouse blastocyst. J Reprod Immunol 4(1):53–64.

Schuel H. 1978. Secretory functions of egg cortical granules in fertilization and development. Gamete Res 1:299–382.

Sinosich MJ, Lanzendorf SE, Hodgen HG. 1990a. Immunocytochemical studies of hamster oocyte activation. Mol Reprod Dev 26:331–336.

Sinosich MJ, Lazendorf SE, Saunders DM. 1990b. A protein marker of hamster oocyte fertilization. Hum Reprod 5(6):729–736.

St-Jacques S, Malette B, Chevalier S, Roberts KD, Bleau G. 1992. The zona pellucida binds the mature form of an oviductal glycoprotein (oviductin). J Exp Zool 262(1):97–104.

Talbot P, DiCarlantonio G. 1984. The oocyte–cumulus complex: ultrastructure of the extracellular components in hamsters and mice. Gam Res 10:127–142.

Tam PPL, Loong EPL, Chiu TTY. 1990. Localization of fucosyl glycoconjugates in human oocytes following cortical granule discharge in the presence of a protease inhibitor. Human Reprod 7:1004–1009.

Tawia SA, Lopata A. 1992. The fertilization and development of mouse oocytes following cortical granule discharge in the presence of a protease inhibitor. Hum Reprod 7(7):1004–1009.

Towbin H, Staehelin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76(9):4350–4354.

Wang WH, Sun QY, Hosoe M, Shioya Y, Day BN. 1997. Quantified analysis of cortical granule distribution and exocytosis of porcine oocytes during meiotic maturation and activation. Biol Reprod 56(6):1376–1382.

Wessel GM, Berg L, Adelson DL, Cannon G, McClay DR. 1998. A molecular analysis of hyalin—a substrate for cell adhesion in the hyaline layer of the sea urchin embryo. Dev Biol 193(2):115–126.

Wolf DP. 1977. Involvement of a trypsin-like activity in sperm penetration of zona- free mouse ova. J Exp Zool 199(1):149–156.

Wolf DP, Hamada M. 1977. Induction of zonal and egg plasma membrane blocks to sperm penetration in mouse eggs with cortical granule exudate. Biol Reprod 17(3):350–354.

Wolf DP, Nicosia SV, Hamada M. 1979. Premature cortical granule loss does not prevent sperm penetration of mouse eggs. Dev Biol 71(1):22–32.

Yoshida M, Cran DG, Pursel VG. 1993. Confocal and fluorescence microscopic study using lectins of the distribution of cortical granules during the maturation and fertilization of pig oocytes. Mol Reprod Dev 36(4):462–468.

Zhang X, Rutledge J, Khamsi F, Armstrong DT. 1992. Release of tissue-type plasminogen activator by activated rat eggs and its possible role in the zona reaction. Mol Reprod Dev 32(1): 28–32.