Effects of Perturbation of Cell Polarity on Molecular Markers of Sperm-Egg Binding Sites on Mouse Eggs

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ABSTRACT

The mouse germinal vesicle (GV)-intact oocyte is a symmetric cell, with the GV centrally localized and with components of the plasma membrane and cortex symmetrically distributed around the periphery of the oocyte. During oocyte maturation, two distinct regions of the egg plasma membrane and cortex develop: the amicrovillar region overlying the meiotic spindle and the microvillar region. The development of this polarity is significant, since sperm bind to and fuse with the microvillar region. We are interested in the development of egg polarity and have characterized the localizations of several markers for egg polarity in normal metaphase II eggs and GV-intact oocytes. The asymmetric distributions of these markers (including actin, cortical granules, binding sites for the sperm proteins fertilin α and fertilin β, and two different β, integrin epitopes) develop during oocyte maturation in vitro, and this polarity can be perturbed by treatments that disrupt the actin microfilaments or microtubules. In addition, immunoelectron microscopy reveals that binding sites for recombinant fertilin β are specifically localized to the microvillar region, suggesting that the binding sites for this sperm ligand are either specifically localized or activated in this region. These results indicate that structural remodeling of the mouse egg plasma membrane is accompanied by molecular remodeling, resulting in the localization or activation of specific molecules in subdomains of the plasma membrane.

INTRODUCTION

During fertilization, mouse gamete plasma membrane interactions occur in a spatially restricted manner. Specific regions of the sperm head, including the inner acrosomal membrane, the equatorial segment, and the posterior head, are involved in interactions with the egg plasma membrane [1,2]. Likewise, specific regions of the egg plasma membrane mediate these interactions with the sperm. Spatially restricted sperm entry points have been observed in the eggs of several species, including mammals, anurans, and ascidians [2–6]. In several rodent species, sperm are known to bind to and fuse with the microvillar region of the plasma membrane of the metaphase II-arrested mouse egg. The remainder of the egg membrane, overlying the meiotic spindle, is known as the amicrovillar region; this region comprises ~30% of the total surface, and sperm-egg fusion rarely, if ever, occurs in this region of the egg surface [2,4,5,7]. The molecular basis of the spatial restrictions on sperm interactions with the egg plasma membrane (e.g., binding and fusion) is presently unknown, although one possible explanation is that binding sites on eggs for sperm ligands are localized to specific domains on the egg surface.

In the mouse egg, the asymmetrically localized domain for sperm binding develops during meiotic maturation, as the prophase I oocyte becomes a metaphase II egg. The germinal vesicle (GV)-intact oocyte is arrested at prophase I of meiosis prior to maturation; it has a symmetric distribution of actin, cortical granules, and microvilli, and a centrally located nucleus (known as the GV) in the middle of the oocyte (Fig. 1). The distribution of these cellular components changes during maturation such that a number of asymmetries arise in the egg. The chromosomes are condensed and aligned on a metaphase plate, which is localized to one side of the cytoplasm and, in the mouse, positioned perpendicularly to the plasma membrane. Associated with the region over the meiotic spindle are 1) the cortical granule-free domain of the cortex [5], which is generated by the release of a localized subset of cortical granules during oocyte maturation [8]; 2) a cortical actin-rich cap overlying the meiotic spindle [9]; and 3) the amicrovillar region of the plasma membrane [2,10]. The microvillar region of the membrane and the cortical granule-rich and endoplasmic reticulum-rich area of the cortex are localized away from the meiotic spindle [2,11] (Fig. 1). In addition, some molecules appear to be in specific subdomains of the mouse egg, including some integrin subunits [12–14], leptin and STAT3 [15], and myosin heavy chains [16]. Very little is known about the precise mechanism of the development of polarity in the mouse egg. Previous work suggests that two things are important: 1) the actin cytoskeleton [17,18], and 2) an interaction between chromatin and the overlying cortex and/or membrane [19–21].

We have been studying gamete plasma membrane interactions with a focus on the sperm surface proteins, fertilin α and fertilin β, both of which are members of the ADAM (a disintegrin and a metallopeptase) family of proteins (also known as MDCs [metalloprotease/disintegrin/cysteine-rich]). These sperm proteins are involved in the cell-cell interactions between the egg and sperm, as evidenced by the abilities of anti-fertilin antibodies [22,23], synthetic peptides [14,23,24], and recombinant fertilin proteins [25–27] to inhibit sperm-egg interactions, as well as by the anal-

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ovulated 6- to 8-wk-old CF1 mice (Harlan, Indianapolis, IN) at 13 h post-hCG in Whitten's medium [29] containing 15 mg/ml BSA (ICN, Costa Mesa, CA) as previously described [25]. Fully grown GV-intact oocytes were collected from ovarian follicles by piercing the ovaries of 6- to 8-wk-old CF1 mice with syringe needles (27-1/2 gauge) in Whitten's medium containing 0.05% polyvinyl alcohol (Sigma, St. Louis, MO). Cumulus cells were removed by pipetting the oocyte-cumulus cell complexes through a thin-bore pipette. Dibutyryl cAMP (dbcAMP) (Sigma) was present at 0.25 mM throughout the oocyte collection procedures to inhibit GV breakdown [30]. Throughout this work, the term “oocyte” refers to GV-intact oocytes at prophase I; the term “egg” refers to a metaphase II egg. The zonae pellucidae of oocytes and eggs were removed by a ≤ 15-sec incubation in acidic medium-compatible buffer (10 mM HEPEs, 1 mM NaH2PO4, 0.8 mM MgSO4, 5.4 mM KCl, 116.4 mM NaCl, final pH 1.5), and then the cells were allowed to recover for 60 min [13].

**Antibodies and Recombinant Fertilin Proteins**

Recombinant fertilin α and recombinant fertilin β have been described previously [25,27]; these recombinant proteins correspond to the complete extracellular domains of these proteins, referred to previously as “DCE” [27]. The anti-human β1 integrin rabbit polyclonal antibody, hereafter referred to as β1-R, was made to a synthetic peptide corresponding to 37 amino acids of the β1 (splice variant A) integrin cytoplasmic tail [32]. It was provided by laboratory of Erkki Ruoslahti (Burnham Institute, La Jolla, CA). Epitopes for this antibody are localized to the amicrovillar region of mouse eggs [13], and the antibody inhibits sperm-egg binding [14,25]. The anti-human β1 integrin rabbit polyclonal antibody, hereafter referred to as β1-R, was made to a synthetic peptide corresponding to 37 amino acids of the β1 (splice variant A) integrin cytoplasmic tail [32]. It was provided by laboratory of Erkki Ruoslahti (Burnham Institute, La Jolla, CA). Epitopes for this antibody are localized to the amicrovillar region of mouse eggs [13].

For studies of the localizations of actin or the epitopes for the β1-R integrin antibody, the eggs were fixed and processed as previously described [13]. For studies of the localizations of binding sites for recombinant fertilin α, recombinant fertilin β, or the β1-R integrin antibody, live ZP-free eggs were incubated for 60 min in Whitten’s medium containing 0.5 mg/ml of the appropriate protein, in the presence of absence or the indicated drugs used to perturb egg polarity (see below). Whitten’s medium was supplemented so that the final concentrations of Ca2+, Mg2+, glucose, and BSA were 2.4 mM, 1.2 mM, 5.5 mM, and 15 mg/ml, respectively. Incubations were performed in a gassed (5% CO2, 5% O2, 90% N2; or 5% CO2 in air) chamber [25]. Following this incubation, the eggs were washed through two 200-μl drops of medium and then fixed in freshly prepared 4.0% paraformaldehyde in PBS for 20–30 min [13,25].

**Cytochalasin D Treatment During In Vitro Maturation**

GV-intact oocytes were matured in vitro at 37°C in CZB medium [30] (10 oocytes per 100-μl drop), containing or lacking 1 μg/ml cytochalasin D (Sigma), in an incubator gassed with 5% CO2 in air. As previously described [17], we also observed that cytochalasin D induced “caving” or indentations in the plasma membrane; this caving disappears upon the removal of the ZP (data not shown). Preliminary experiments showed that identical results were obtained in CZB either devoid of or containing 5–10% fetal...
calf serum (Gibco-BRL, Gaithersburg, MD) (data not shown). Consequently, fetal calf serum was usually omitted. Identical results were also obtained when oocytes were cultured in 5% CO₂ in air or in 5% CO₂, 5% O₂, 90% N₂ (data not shown). Control GV-intact oocytes were cultured in CZB medium containing 0.25 mM dbcAMP. For studies of the localization of recombinant fertilin α, recombinant fertilin β, or the β₁-B antibody, these proteins were included in the culture medium (± cytochalasin D) for the last hour of culture prior to fixation.

Colchicine Treatment of Metaphase II Eggs

ZP-free metaphase II eggs were cultured in Whitten’s medium, containing or lacking 10 µM colchicine (Sigma) for 4–5 h, as has been previously described for studies with nocodazole [20]. For studies of the localization of recombinant fertilin α, recombinant fertilin β, or the β₁-B antibody, these proteins were included in Whitten’s medium (± colchicine) for the last hour of culture prior to fixation.

Cytochalasin B-Induced Pseudocleavage of GV-Intact Oocytes

In this study, GV-intact oocytes were induced to undergo “pseudocleavage” essentially as described previously by Wassarman et al. [33], with the following modifications. ZP-intact GV-intact oocytes were cultured in Whitten’s medium containing 0.05% polyvinylalcohol (Sigma), 0.25 mM dbcAMP, and either 5 µg/ml cytochalasin B (Sigma; 2 mg/ml stock in DMSO) to induce pseudocleavage, or 0.05% DMSO for control oocytes. The oocytes were then cultured 13–14 h at 37°C in 5% CO₂, in air. At the end of this culture period, a subset of the cytochalasin B-treated GV-intact oocytes (20–50%) showed varying degrees of pseudocleavage, from complete pseudocleavage to a partially cleaved, “snowman-like” appearance. When the ZP were removed from these oocytes, the majority of the oocytes completed pseudocleavage. (A portion of these oocytes blebbled, as was observed previously [33]; blebbing oocytes were not used for further analysis.) For studies of the localization of recombinant fertilin α, recombinant fertilin β, or the β₁-B antibody, these proteins were included in the culture medium (± cytochalasin B) at 0.5 mg/ml for the last hour of culture prior to fixation.

Immunoelectron Microscopy

ZP-free eggs or oocytes were incubated in the presence of 0.5 mg/ml maltose binding protein (MBP) or recombinant fertilin β-EC for 60 min and then washed twice. Paraformaldehyde fixation and antibody labeling steps were performed at room temperature. The eggs were fixed in freshly prepared 4.0% paraformaldehyde in PBS for 30–45 min. Following fixation, the eggs were washed twice in PBS and then incubated in PBS containing 1% casein (Tro pix, Bedford, MA) (blocking solution) for 45 min, and then washed three times in PBS containing 0.05% casein (washing solution). The eggs were incubated in a 18-nm gold particle-conjugated goat anti-rabbit IgG antibody (Jackson Immunoresearch, West Grove, PA; diluted 1:50 in blocking solution) for 45–90 min, and then washed three times in washing solution and once in PBS. The eggs were then fixed in PBS containing 1% osmium tetroxide (Electron Microscopy Sciences, Ft. Washington, PA) for 60 min at 4°C, washed three times in PBS, dehydrated through an ethanol series (50%, 75%, 85%, 95%, and 100%) at −20°C for 60 min each incubation, and embedded in LR Gold resin (Electron Microscopy Sciences). Grids were counterstained with 7% aqueous uranyl acetate and Reynold’s lead citrate. Specimens were observed and photographed using a Philips 201 transmission electron microscope (Eindhoven, The Netherlands). These experiments were performed three times, with at least 5–10 eggs viewed in each experiment. Representative micrographs were chosen for presentation.

Immunocytochemistry and Confocal Microscopy

Eggs were fixed in freshly prepared 4.0% paraformaldehyde in PBS for 20–60 min. All steps were performed at room temperature in a humidified chamber as described previously [13]. Recombinant fertilin α and β were detected using an anti-MBP polyclonal antibody (New England Biolabs; diluted 1:300) followed by a goat anti-rabbit IgG Texas Red-conjugated secondary antibody (Jackson Immunoresearch) diluted 1:500 as previously described [25–27]. The anti-β₁ integrin antibody β₁-B was detected with a goat anti-rabbit IgG Texas Red-conjugated secondary antibody (Jackson Immunoresearch) diluted 1:500 as previously described [25]. Staining with the anti-β₁ integrin cytoplasmic tail antibody, β₁-R, and FITC-conjugated phalloidin (final concentration 0.025–5 µg/ml, depending on the microscope used; Sigma) was performed as previously described [13]. BODIPY 650/665-conjugated phalloidin (Molecular Probes; Eugene, OR: 0.1–1 µg/ml final concentration) was also in some experiments to label F-actin. DNA in the GV or associated with the meiotic spindle was labeled with either DAPI (4',6-diamidino-2-phenylindole; Sigma) or YOYO-1 (Molecular Probes), depending on the microscope used. DAPI was either included with the secondary antibody (2 µg/ml) or in the mounting medium (1.5 µg/ml). When YOYO-1 labeling was used, eggs and oocytes were treated with RNase A (50–100 µg/ml in 40 mM Tris-HCl, pH 8.0, 10 mM NaCl, 6 mM MgCl₂, 0.1% PVA, for 30–60 min) prior to staining with 10 nM YOYO-1.

The eggs were mounted in VectaShield mounting medium (Vector Labs, Burlingame, CA) and viewed by epifluorescence and confocal laser scanning microscopy, using a Leica (Deerfield, IL) or a Bio-Rad (Hercules, CA) confocal microscope. All images shown (Figs. 2–5, and 7) are optical sections of ~0.6 µm, with the exception of Figure 6, which is a three-dimensional reconstruction from multiple optical sections. The immunocytochemistry experiments were repeated 3–6 times, with 8–24 oocytes viewed in each experiment. In all instances of GV-intact oocyte labeling, identical results were obtained with oocytes that were freshly retrieved from follicles and oocytes that were cultured in parallel with maturing oocytes for up to 16 h in the presence of dbcAMP to inhibit progression to metaphase (data not shown).

RESULTS

Localization of Binding Sites of Recombinant Fertilin β Observed by Immunoelectron Microscopy

Using immunofluorescent staining, we observed recombinant fertilin β bound to the microvillar region of the mouse egg plasma membrane [25], the region of the egg to which sperm bind and fuse [2,4,5]. There are two possible
explanations for this apparent localization. The increased immunofluorescence on the microvillar region as compared to the amicrovillar region could be due to the greater surface area in the microvillar of the egg membrane compared to the amicrovillar region. Alternatively, the increased fluorescence observed in the microvillar region could be due to a higher density of recombinant fertilin β binding sites on that region as compared to the amicrovillar region. To distinguish between these two possibilities, immunoelectron microscopy was used to observe the localization of recombinant fertilin β binding sites on metaphase II eggs. This demonstrated that recombinant fertilin β was bound almost exclusively to the microvillar region of the mouse egg membrane, indicated by the decoration of the microvillar region by numerous gold particles (Fig. 2A). In contrast, the amicrovillar region had virtually no gold particles (Fig. 2B); of all the sections that were examined, only two gold particles were observed on the amicrovillar region of the eggs with bound recombinant fertilin β. In contrast to eggs incubated in recombinant fertilin β, eggs incubated in the control protein MBP showed very low levels of labeling with gold particles. On MBP-labeled eggs, occasional small patches of clusters of 2–6 gold particles were observed on both the microvillar and amicrovillar regions, with the vast majority of the plasma membrane being very sparsely labeled (data not shown), in agreement with our previous findings of very low binding of MBP to ZP-free eggs as detected by immunofluorescence or a quantitative lumino- metric immunoassay [25].

**Binding of Recombinant Fertilin α and β to GV-Intact Oocytes**

GV-intact oocytes have not yet developed the asymmetric membrane and cortical domains that metaphase II eggs have, i.e., the amicrovillar region, the cortical granule-free domain, and the actin-rich domain over the meiotic spindle of metaphase II-arrested eggs (Fig. 1) [34]. Therefore, since recombinant fertilin α and recombinant fertilin β bind to the microvillar region of metaphase II eggs [25–27], we wanted to determine if GV-intact oocytes were capable of binding recombinant fertilin α and β and, if so, where the binding sites were localized. In agreement with the finding that sperm bind to the entire surface of the GV-intact oocyte [35], the binding sites for both recombinant fertilin α (Fig. 3A) and recombinant fertilin β (Fig. 3D) to GV-intact oocytes were distributed evenly over the entire surface of GV-intact oocytes. The asymmetric pattern of recombinant fertilin α and β binding sites developed during meiotic progression to metaphase II, since GV-intact oocytes matured in vivo [25–27] and in vitro (Fig. 3, B and E) to metaphase II showed binding of recombinant fertilin α and β on the amicrovillar region.

**Effects of Cytochalasin D on the Development of Cell Polarity During Oocyte Maturation**

Cytochalasin D disrupts the actin cytoskeleton by binding to the barbed ends of actin filaments by depolymerizing existing filaments and inhibiting the polymerization of new filaments [36]. Treatment of mouse oocytes with cytochalasin D during oocyte maturation results in “hypo-polarized” eggs, arrested in M-phase with a centrally located meiotic spindle (arrest is at metaphase I since cytokinesis is inhibited) [17,37]. These hypo-polarized eggs lack an amicrovillar region and have a low density of microvilli distributed symmetrically over the egg surface [17,37]. We hypothesized that oocytes matured in the presence of cytochalasin D would have mislocalized markers of egg polarity; markers that would normally be asymmetrically distributed would be symmetrically localized in the cytochalasin D-treated eggs.

Four markers of egg polarity were examined in these experiments. Two were binding sites for recombinant fertilin α and recombinant fertilin β, described above. The other two were the epitopes of two anti-β1 integrin antibodies: β1-R, which becomes localized to the amicrovillar

![Figure 2](image2.png)

**FIG. 2.** Immunoelectron microscopy of recombinant fertilin β binding to eggs. ZP-free eggs were incubated in medium containing 0.5 mg/ml recombinant fertilin β and then processed and viewed by immunoelectron microscopy as described under Materials and Methods. A) Microvillar region of a metaphase II egg that had been incubated in recombinant fertilin β, showing gold particles decorating the plasma membrane. B) Amicrovillar region of a metaphase II egg incubated in recombinant fertilin β, showing no gold particle labeling.

![Figure 3](image3.png)

**FIG. 3.** Binding of recombinant fertilin α and recombinant fertilin β to GV-intact oocytes, in vitro-matured oocytes, and oocytes matured in the presence of cytochalasin D. GV-intact oocytes were cultured in the presence of dbcAMP (to inhibit meiotic maturation; A and D), in the absence of dbcAMP (to allow meiotic maturation; B and E), or in the absence of dbcAMP and in the presence of cytochalasin D (C and F). Following ~13 h of culture and removal of the ZP, the oocytes were incubated with 0.5 mg/ml recombinant fertilin α or β, and then processed for immunocytochemistry as described under Materials and Methods. The asterisks in B and E indicate the location of the amicrovillar region, as localized by observing the meiotic spindles with DAPI. A) GV-intact oocyte showing surface binding of recombinant fertilin α. B) In vitro-matured oocyte showing binding of recombinant fertilin α to its microvillar region. C) In vitro-matured oocyte cultured in the presence of cytochalasin D showing symmetric binding of recombinant fertilin α. D) GV-intact oocyte showing surface binding of recombinant fertilin β. E) In vitro-matured oocyte showing binding of recombinant fertilin β to its microvillar region. F) In vitro-matured oocyte cultured in the presence of cytochalasin D showing symmetric binding of recombinant fertilin β.
region during in vitro maturation [13], and β₁-B, which is localized to the microvillar region [13,14] and has been implicated as a binding partner for recombinant fertilin β [25]. As anticipated, cytochalasin D treatment during maturation in the symmetric localizations of these four markers, mimicking the symmetric localizations observed in GV-intact oocytes and contrasting the polarized distributions in control in vitro-matured metaphase II eggs (Fig. 3, C and F, and Fig. 4, C and F). It should be noted that oocytes treated with cytochalasin D and control oocytes both showed normal increases in cdc2/cyclin B kinase (as assessed by an H1 kinase reaction) and MAP kinase (as assessed by an electrophoretic shift to the active phosphorylated form) activities at 15 h post-dbcAMP removal (data not shown).

Effects of Colchicine Treatment of Metaphase II Eggs on Markers of Egg Polarity

Another method to perturb polarity is to treat metaphase II eggs or maturing oocytes with microtubule-disrupting drugs such as colchicine or nocodazole. These drug treatments cause the meiotic spindle to break down, resulting in the dispersal of clusters of condensed chromosomes throughout the cortex of the egg. This results in the “hyperpolarization” of the eggs, with small amicrovillar regions forming over each chromosome cluster [19,20]. We tested the hypothesis that the four molecular markers for egg polarity described above would segregate with respect to the localization of these spindle-less chromosome clusters. In these studies, the molecular markers for egg polarity segregated as anticipated. Binding sites for recombinant fertilin α, recombinant fertilin β, and epitopes for the β₁-B anti-integrin antibody were localized away from chromosome clusters (Figs. 5 and 6), and F-actin and epitopes for the β₁-R antibody were localized over each chromosome cluster (Fig. 5).

Localization of Molecular Markers for Egg Polarity in Pseudocleaved Oocytes

Wassarman et al. [33,38,39] observed that GV-intact eggs treated with cytochalasin B will “pseudocleave” into two compartments, an anucleate, microvillar half and a nucleated, amicrovillar half. Thus, induction of pseudocleavage is another experimental treatment that perturbs normal oocyte/egg polarity by inducing inappropriate polarity, making a cell that would normally be symmetric into a polarized cell. It should be noted that several differences from the original methods used to induce pseudocleavage [33] were observed. First, in our hands, 5 μg/ml was the most effective concentration of cytochalasin B for inducing pseudocleavage (data not shown). Second, the time course of pseudocleavage was much slower than previously described. Pseudocleavage was not observed for periods up to 5 h, but apparent asymmetries were present in up to 50% of the oocytes after culture periods of >12 h. Third, pseudocleavage did not always go to completion in ZP-intact oocytes. However, pseudocleavage often completed after ZP removal, although a portion of oocytes maintained a partially cleaved, snowman-like appearance even after ZP removal. Fourth, some oocytes never responded to cytochalasin B treatment. A portion of each batch of oocytes (generally 50% and up to 80%) never showed signs of pseudocleavage, even after culture in medium containing cytochalasin B for >15 h. Finally, the pseudocleaved oocytes observed were not always cleaved into two equal parts. Frequently one compartment was approximately one-third the size of the other compartment, with the nucleate compartment (containing the GV) being the larger or the smaller compartment with equal frequency. Examples of the variations in shapes of pseudocleaved oocytes are shown in Figure 7.

Markers of egg polarity segregated to different compartments pseudocleaved oocytes. Interestingly, markers of the amicrovillar region responded differently from microvillar markers. As anticipated, markers of the microvillar region of the egg membrane, including cortical granules, β₁-B epitopes, and binding sites for recombinant fertilin α and β₁-R segregated to the anucleate, microvillar half of the pseudocleaved oocytes (Fig. 7, A–E). Recombinant fertilin α and β₁-R binding were often quite patchy; the reason for this is unclear, although it may be associated with perturbation of the actin cytoskeleton by the cytochalasin B. In contrast, markers for the amicrovillar region did not segregate to the nucleated compartment as would have been predicted. In the metaphase II egg, actin and β₁-R epitopes are localized over the meiotic spindle in the amicrovillar region. Thus, it might be hypothesized that these two markers would segregate to the nucleated, amicrovillar compartment of the pseudocleaved oocyte. However, actin (Fig. 7F), as detected by two different phalloidin preparations (see Materials and Methods), and the epitopes for the β₁-R antibody (Fig. 7, G–I) showed varying localizations. In the majority of pseudocleaved oocytes (~65%), actin and β₁-R epitopes were observed in the anucleate compartment (Fig. 7, F and H), although localizations of these markers in the nucleated, amicrovillar compartment (Fig. 7G) (~13% of the oocytes) and diffusely distributed through...
the cortices of both compartments (Fig. 7I) (~22% of the oocytes) were also observed.

**DISCUSSION**

This study examines the development of cell polarity in the mouse egg. One important result of this process is the establishment of a specialized microvillar region for sperm-egg membrane interactions [2,4,5]. Similar asymmetrically localized binding sites for sperm have been observed in the eggs of other species [3,6], although the molecular basis of these polarized sperm entry sites is not known. In addition, cell polarity in eggs and early embryos can have important consequences during embryonic development. There are several examples of this. The development of cell polarity following fertilization can affect embryonic axis formation, such as the cortical rotation with respect to the sperm entry point that occurs in early *Xenopus* embryos [40]. In mouse oocytes, the endoplasmic reticulum redistributes from cytoplasmic stores to a cortical location; this cortical localization of intracellular calcium reserves could be important for early events of egg activation [11]. Finally, polarity in mouse eggs could have important effects on embryonic patterning as development progresses, as axis determination in the blastocyst has been found to correlate with the animal-vegetal axis in the one-cell embryo [41].

The results presented here provide evidence that polarized sperm entry points in the mouse egg could be regulated by the asymmetrical localization of receptors for sperm ligands. In this work, we have focused on fertilin β, one of several sperm ligands that mediates sperm-egg binding [42]. By immunoelectron microscopy, we find that the binding sites for recombinant fertilin β on the mouse egg plasma membrane are found almost exclusively on the microvillar region (Fig. 2A); virtually no binding is observed on the amicrovillar region (Fig. 2B). This result is significant for two reasons. First, this is, to our knowledge, the first insight into the possible molecular basis of polarized sperm entry sites. The localization of binding sites for a sperm ligand to specific egg plasma membrane domains could clearly contribute to the spatial restriction of sperm entry points. Second, this observation of recombinant fertilin β binding sites on the microvillar region contrasts the results from similar ultrastructural studies of other markers of egg asymmetry. While other markers, including Con A [4], an anti-β1 integrin antibody epitope [12], and an anti-α6 integrin antibody (GoH3) epitope...
pseudocleavage in the presence of 5 μg/ml cytochalasin B. The localizations of cortical granules, the binding sites for recombinant fertilin α and β, β1-B, and β1-R epitopes were determined by immunocytochemistry and confocal laser-scanning microscopy as described under Materials and Methods. The label “GV” indicates the presence of the GV and thus which compartment is the nucleate half of the pseudocleaved oocyte. Markers that are normally microvillar or amicrovillar are shown in the upper or lower row, respectively. A) Control GV-intact oocyte stained with LCA to label cortical granules. B-I Pseudocleaved oocytes showing the localization of B) cortical granules to the anucleate compartment, C) β1-B integrin epitopes to the anucleate compartment, D) recombinant fertilin α in the anucleate compartment, E) recombinant fertilin β in the anucleate compartment, F) F-actin in the anucleate compartment, G) β1-R epitopes in the nucleate compartment, H) β1-R epitopes in the anucleate compartment, I) β1-R epitopes in the both the anucleate and nucleate compartments.

[12], show some preferential localization to the microvillar region, immunoelectron microscopy reveals that these markers of egg asymmetry are present on both the microvillar and amicrovillar surfaces of the mouse egg plasma membrane [12,43] (although the amicrovillar localized α6 integrin epitope is at a lower density as compared to the microvillar region [12]). Both α6 [14,44] and β1 [25,44] integrin subunits on eggs have been implicated as a receptor/acceptor for fertilin β. As a recent study implicates that the ligand specificity of egg α6β1 can be regulated between laminin-binding and fertilin-binding states [45], it is interesting to speculate that there could be different forms or differently regulated forms of these receptors in microvillar and amicrovillar regions of the egg plasma membrane.

The microvillar and amicrovillar regions of the egg plasma membrane normally develop during meiotic maturation. In this study, binding sites for recombinant fertilin α and recombinant fertilin β were found to be symmetrically localized on GV-intact oocytes (Fig. 2, A and D). This is not surprising, since sperm [35] and the epididymal, sperm-associated protein DE/CRISP-1 [46] bind to the entire surface of the GV-intact oocyte. It should also be noted that the localizations of fertilin binding sites are the same on oocytes that are freshly retrieved from follicles (data not shown) and oocytes that are cultured for 16 h in the presence of dbcAMP to inhibit progression to metaphase (in parallel with maturing oocytes, Fig. 2), indicating that binding sites for recombinant fertilin α and β are not synthesized and/or transported to the surface during culture.

In addition to examining normal GV-intact oocytes and metaphase II eggs, we investigated the effects of three different experimental treatments that perturb mouse egg/oocyte polarity on the localizations of four molecular markers for egg polarity. In vitro maturation of oocytes in the presence of cytochalasin D results in hypopolared eggs (i.e., a cell that would normally become polarized remains symmetric). These eggs have symmetrically localized binding sites for recombinant fertilin α, recombinant fertilin β, and epitopes for the β1-B and β1-R integrin antibodies (Figs. 2 and 3). Hypopolared eggs were generated by treatment of metaphase II eggs with colchicine, resulting in the breakdown of the meiotic spindle and the formation of a mini-amicrovillar domain over each cluster of condensed chromosomes that scatter throughout the cortex [19,20,37]. In these eggs, all molecular markers of egg polarity that were examined in this study were localized with respect to the location of the clusters of chromosomes: binding sites for recombinant fertilin α, recombinant fertilin β, and epitopes for the β1-B antibody segregated in membrane regions away from the chromosomes, and β1-R epitopes were localized over the chromosomes (Figs. 5 and 6). Myosin IIA, which is localized to the amicrovillar region of metaphase II eggs, also is localized adjacent to chromosomes in eggs treated with microtubule-disrupting drugs [16]. Finally, inappropriate polarity was induced in GV-intact oocytes by treating them with cytochalasin B to induce the cells pseudocleave [33,38,39]. In these cells, the molecular markers of the microvillar region of the egg are localized as anticipated with respect to the location of GV. Binding sites for recombinant fertilin α, recombinant fertilin β, and epitopes for the β1-B antibody segregated to the anucleate compartment of the pseudocleaved oocyte. However, the two molecular markers of the amicrovillar region, F-actin and β1-R epitopes, are not localized as would be anticipated. F-actin and β1-R epitopes are observed unexpectedly in the anucleate compartments of the majority of pseudocleaved oocytes (Fig. 7, F and H), away from nuclear material, in contrast to the localization of F-actin in metaphase II eggs over the nuclear material (Fig. 1). Moreover, in a subset of pseudocleaved oocytes, F-actin and β1-R epitopes are localized diffusely between both compartments or in the anucleate compartment (Fig. 7, G and I). The reasons for this are unknown, but they perhaps are due to the disruption of the actin cytoskeleton with cytochalasin B. However, with the exceptions of these two amicrovillar markers in pseudocleaved oocytes, we find that molecular markers of egg
polarity sort out as predicted in response to the three treatments that perturb egg polarity and with respect to the location of nuclear material (condensed chromatin in the case of cytochalasin D- and colchicine-treated eggs, the GV in the case of pseudocleaved GV-intact oocytes).

From these and other studies, it is clear that polarity in the membrane and cortex of the mouse female gamete is determined by the localization of nuclear material [19–21,33,37]. Spindle microtubules are not required, as clusters of chromosomes in nocodazole-treated eggs lack microtubules and foci of pericentriolar material associated with microtubule-organizing centers [19], and yet these chromosomes still have the ability to induce cortical and membrane polarity (e.g., cortical granule-free domain, actin-rich cap, etc.; see Fig. 1). It is also interesting that polarity in female gametes orients with respect to both condensed M-phase chromat and to chromatin enclosed in a nuclear envelope in pseudocleaved GV-intact oocytes, with the exception of the amicrovillar markers in the pseudocleaved oocytes. This raises two intriguing possibilities: 1) that polarizing signals can transverse the nuclear envelope or that nuclear envelope components (e.g., lamins, lamina-associated polypeptides, etc.) are associated with condensed chromosomes in eggs and are perhaps involved in the establishment of egg polarity; and 2) that microvillar markers can sense and respond to GV-associated polarizing signals, but amicrovillar markers cannot. At this time, it is unclear what chromatin-associated factor(s) are involved in this process. It should also be noted that an intact actin cytoskeleton is important in polarity establishment, as actin microfilaments appear to be required for cortical translocation of both the meiotic spindle and of chromosomes of nocodazole-treated eggs [19,37,47]. In addition, recent work shows that myosin II heavy chains cooperate with actin microfilaments in these processes. Myosin IIA appears to participate in the translocation of the meiotic spindle during oocyte maturation, and myosin IIB mediates the movement of chromosomes to the cortex of colcemid-treated metaphase II eggs [16].

Actin microfilaments could be also involved in the establishment of egg polarity via interactions with the cytoplasmic domains of transmembrane proteins, including integrins [48]. Two different β1 integrin epitopes have different subcellular localizations in mouse eggs (Fig. 4); such differences have also been observed in mouse eggs with other anti-β1 integrin antibodies [12,13]. The reasons for these differences are unclear, although alternative splicing of the mouse egg β1 integrin is a possible explanation. Recent studies of the mouse genome have revealed the presence of two of the four β1 integrin cytoplasmic tail splice variants that have been identified in humans [49]. The differences in the localizations of the two β1 integrin epitopes examined in this study suggest that the mouse egg possesses the ability to regulate the subcellular redistribution that occurs during oocyte maturation, possibly through cytoskeletal interactions with these potentially different β1 integrin forms. The observation that cytochalasin D perturbs the re-localization of the two β1 integrin epitopes during maturation (Fig. 4) suggests that actin microfilaments participate in the regulation of the subcellular distributions of these antigens.

The results of this work indicate that structural remodeling of the mouse egg plasma membrane is accompanied by molecular remodeling, resulting in the segregation and/or activation of specific molecules in specific subdomains of the plasma membrane or cortex of the egg. These data provide a foundation for future investigations into the interactions between the nuclear material and the membrane and cortex of the mouse egg that direct the polarization of this cell.

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