Analysis of Loss of Adhesive Function in Sperm Lacking Cyritestin or Fertilin β

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We produced mice lacking the sperm surface protein cyritestin (ADAM 3) and found mutant males are infertile. Similar to fertilin beta (ADAM 2) null sperm (C. Cho et al., 1998, Science 281, 1857–1859), cyritestin null sperm are drastically deficient in adhesion to the egg zona pellucida (0.3% of wild type) and to the egg plasma membrane (9% of wild type). Thus sperm from male mice with a gene deletion of either ADAM have a loss of adhesive function in at least two steps of fertilization. We found deletion of either ADAM gene resulted in the loss of multiple gene products. This loss of multiple gene products (sperm membrane proteins) appears to result from a novel, developmental mechanism during sperm differentiation. Because the altered sperm protein expression must be responsible for the fertilization defects, our data suggest new models for the molecular basis of the affected steps in fertilization. © 2001 Academic Press

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INTRODUCTION

In mammalian fertilization, sperm adhere first to the egg zona pellucida and later to the egg plasma membrane, followed by sperm–egg fusion. When acrosome-intact mouse sperm adhere to the zona pellucida, they bind to one of the three zona glycoproteins, ZP3, and this binding induces the sperm acrosome reaction (Bleil and Wassarman, 1980; Wassarman, 1999). The best studied sperm protein proposed to interact with ZP3 is β-1,4-galactosyltransferase (GalTase).4 Recent data suggest that GalTase is primarily involved in signal transduction for the acrosome reaction (Gong et al., 1995; Lu and Shur, 1997) and the sperm surface protein(s) that acts in adhesion to the zona remains unknown (Wassarman, 1999).

Several sperm proteins have been proposed to have important roles in sperm–egg plasma membrane adhesion and fusion. Some of these belong to the ADAM family (A Disintegrin And Metalloprotease). The typical domain structure of an ADAM includes a signal sequence followed by pro-, metalloprotease, disintegrin, cysteine-rich, epidermal growth factor-like, transmembrane, and cytoplasmic tail domains (Wolfsberg et al., 1995; Huovila et al., 1996; Bigler et al., 1997; Blobel, 1997; Black and White, 1998; Frayne and Hall, 1999; Primakoff and Myles, 2000). Among the 29 known ADAMs, 12 show testis-specific expression and another three testis-predominant expression; three of these testis-expressed ADAMs have been studied to define their functions in fertilization (Primakoff and Myles, 2000).

Fertilin (a heterodimer with subunits fertilin α, ADAM 1, and fertilin β, ADAM 2) and cyritestin (ADAM 3) are the ADAMs whose roles in fertilization are under investigation. Peptide mimetics of the disintegrin domain active sites of fertilin β and cyritestin inhibit sperm–egg plasma membrane adhesion and fusion (Myles et al., 1994; Almeida et al., 1995; Evans et al., 1995; Yuen et al., 1997; Linder and Heinlein, 1997; Chen and Sampson, 1999; Bronson et al., 1999). This result suggests that fertilin β and cyritestin...
function in sperm–egg plasma membrane adhesion and fusion, although the peptide mimetics could have low specificity and block egg receptors that recognize other native ADAMs. Previously we produced fertilin β-deficient mice and found they have defects in multiple steps in fertilization. Fertilin β−/− male mice are infertile and their sperm fail to adhere to the egg plasma membrane (13% of wild-type level) and, to a lesser degree, to fuse with eggs (45–50% of wild-type level). Surprisingly, deletion of fertilin β also blocked sperm binding to the egg zona pellucida and sperm migration from the uterus into the oviduct. 

In the present study, we generated mice lacking cyritestin and found deletion of cyritestin resulted in similar, but more restricted, effects on fertilization. Cyritestin knockout males are infertile and their sperm show normal migration into the oviduct, fail to bind to the zona pellucida or the egg plasma membrane, but can fuse with eggs.

In addition, we discovered that deletion of the gene for either fertilin β or cyritestin leads to the loss of multiple sperm membrane proteins (at least three sperm membrane proteins are reduced or absent in each of the knockouts). The loss of multiple sperm membrane proteins in the ADAM knockouts may explain why the knockout sperm have multiple fertilization defects. By comparing the fertilization defects and the absent proteins in the cyritestin and fertilin β knockouts, we are able to propose new models for the affected steps in fertilization.

MATERIALS AND METHODS

Antibodies

Mouse anti-mouse fertilin β (9D2.2) and cyritestin (7C1.2) monoclonal antibodies (mAbs) were produced by immunizing female mice in collaboration with Chemicon (Tecaluma, CA) and shared with us by Chemicon. Antiserum-to-mouse PH-20 was obtained by immunizing female mice with the purified, recombinant mouse PH-20, expressed in a baculovirus system, as described (Lin et al., 1998). Anti-cyritestin rabbit antiserum (mCyri-DC1, mouse cyritestin disintegrin, cysteine-rich domain) was obtained by immunizing a rabbit with a purified, recombinant construct containing the disintegrin domain plus the cysteine-rich domain of mouse cyritestin, expressed in a baculovirus system. Affinity-purified rabbit polyclonal antibodies to the entire extracellular domain of mouse fertilin α and to the cytoplasmic tail of mouse fertilin β, named mα-EC1 and mβ-CT1, respectively, were described elsewhere (Cho et al., 2000). Rabbit antiserum to mouse testase 1 was also raised and affinity-purified by our laboratory (Zhu, G.-Z., Myles, D. G., and Primakoff, P., unpublished data). Rabbit preimmune and immune antiserum-to-mouse GalTase were kind gifts from Dr. Barry D. Shur (Emory University).

Production of Mice Lacking Cyritestin

A genomic clone of mouse cyritestin and a targeting vector were isolated by Genome Systems (St. Louis, MO) in collaboration with our laboratory. In the targeting vector, exon 14 of the endogenous cyritestin gene (corresponding to the disintegrin active site) was replaced with a neomycin-resistance gene. To obtain the targeting vector, a 4.4-kb (5′ arm) containing exon 13 and a 2.0-kb (3′ arm) containing exons 15 and 16 were cloned into a pGt-N28 vector carrying the neomycin-resistance gene cassette (New England Biolabs) (Fig. 1A). Using this targeting vector, the Cyn gene was disrupted in mouse embryonic stem cells (ESC) by homologous recombination. Genotypes of the ESC or the mice were determined by PCR (Fig. 1B). All positive ESC clones, screened by PCR, were confirmed by Southern blotting (Fig. 1C). Homozygous mutant mice were obtained by standard methods (Fig. 1 legend). We also checked that cyritestin was deleted in the homozygous mutant mice at the protein level. Sperm from the wild-type (+/+) or mutant (−/−) mice were subjected to Western blotting using anti-mouse cyritestin mAb (7C1.2). As expected, there was an ~45 kD band in wild-type sperm, corresponding to the known size of mature (processed) cyritestin, while the mutant sperm yielded no band in the Western blotting (Fig. 1D). With these tests, production of mice lacking cyritestin was confirmed.

PCR Screening

Genotypes of ESC clones or mice were determined by PCR and/ or Southern blotting. The wild-type and targeted cyritestin gene sequences were amplified in separate tubes. The forward PCR primers were 5′-ATAGCCGGAAGAGGACGCTATG-3′ (wild type) and 5′-GCCTGCTCTTTACTGAAAGGCTCTT-3′ (targeted). An oligonucleotide primer 5′-TTGTTCCAAATTCTGTCC-TGAGCC-3′ was used as a reverse primer to amplify products from both the wild-type and the targeted gene. The reaction was carried out in a Stratagene RoboCycler Gradient 40 (1 cycle at 94°C for 3 min; 34 cycles at 94°C for 0.5 min, 60°C for 1 min, and 72°C for 4 min; and 1 cycle at 72°C for 10 min).

Southern Blotting

To prepare a probe for Southern blotting, PCR was carried out to amplify the 3′ flanking region of the 3′ arm. PCR primers were 5′-GTGTTTTTACCCCCATATATTTT-3′ (forward) and 5′-CGGATCCTAAACCATCATGTTCTTACA-3′ (reverse). The resulting 0.8-kb PCR product was then subcloned into a pCR II vector (Invitrogen), followed by EcoRI digestion to excise a 500-bp DNA probe.

Fertility of Homozygous Cyritestin−/− Male and Female Mice

Each male (8–16 weeks old) was placed with two C57BL/6N females for 5–7 days. Two females (6–12 weeks old) were placed with one C57BL/6N male for three weeks. The number of pups born was counted to calculate fertility rate.

Sperm Transit into the Oviduct

The number of sperm present in the oviduct of mated females was determined as previously described (Cho et al., 1998).

In Vitro Sperm-Zona Pellucida Binding and Sperm-Egg Fusion Assay

The in vitro assays for sperm binding to the zona pellucida or sperm binding to or fusing with the egg plasma membrane were performed as previously described (Cho et al., 1998).
Western Blotting

Western blotting was carried out as previously described (Cho et al., 2000). To determine expression levels of sperm proteins, band intensity was quantified by Scion Image software for a PC computer (Scion Corp., http://www.scioncorp.com/).

Expression Levels of Proteins in Testicular Cells

Testes from cyritestin\(^{+/+}\) or cyritestin\(^{-/-}\) mice were chopped thoroughly with a razor blade in ice-cold 4 mM Hepes-NaOH, pH 7.4, containing 140 mM NaCl, 4 mM KCl, 10 mM glucose, and 2 mM MgCl\(_2\); (1× Hepes). Debris was removed through a small mesh cloth and cells were washed once with 1× Hepes. The cell pellet was suspended in 2 ml of 1× Hepes and overlaid with a gradient of Percoll (Amersham-Pharmacia) in 1× Hepes. (The gradient was formed in advance by centrifuging a 52% Percoll suspension at 15,000 rpm for 1 h at 4°C.) After centrifugation at 15,000 rpm for 10 min at 4°C, fractions containing testicular cells were collected; 5 × 10\(^6\) testicular cells/lane were subjected to 7.5% SDS-PAGE under nonreducing conditions, followed by Western blotting using m\(^a\)-EC1, m\(^b\)-CT1, or 7C1.2.

Immunoaffinity-Purification of Cyritestin from Biotinylated Sperm

Washed cauda and vas sperm (2.0 × 10\(^8\)) were biotinylated with Sulfo-NHS-LC-Biotin (Pierce) according to the manufacturer’s instructions. The biotinylated sperm were lysed by incubating on ice in 1.3 ml of PBS, containing protease inhibitors and 30 mM octyl glucoside (OG). The extract was centrifuged at 20,000g for 10 min at 4°C and the supernatant was collected. Cyritestin was immunoaffinity-purified from the extract with 1 ml of Sepharose 4B (Amersham-Pharmacia) coupled with mCyri-DC1 IgG (2 mg IgG/ml Sepharose). As a negative control, the same extract was also applied to 1 ml of the Sepharose 4B beads coupled with control mCyri-DC1 preimmune IgG (2 mg IgG/ml Sepharose). After extensive washing with PBS containing 30 mM OG, proteins were eluted in 50 mM diethylamine, pH 11.5, containing 30 mM OG, and concentrated 10-fold. An aliquot from the eluate (equivalent to 1 × 10\(^7\) sperm) from mCyri-DC1 IgG-Sepharose or mCyri-DC1 preimmune IgG-Sepharose was separated by 10% SDS-PAGE under reducing conditions followed by Western blotting. Blots were probed with 7C1.2 mAb or alkaline phosphatase-conjugated streptavidin.
Detection of Proteins Associated with the Cyritestin Precursor in Testicular Cells

Testicular cells (9.3 x 10⁶) were prepared from CD1 male mice as described above and lysed as described above for biotinylated sperm. After cyritestin was immunoadfinity-purified as described above (in this case proteins were eluted in 100 mM glycine-HCl, pH 2.5, containing 30 mM OG), an aliquot of the eluate (equivalent to 4.7 x 10⁵ cells) from the mCyri-DC1 IgG-Sepharose or from control mCyri-DC1 preimmune IgG-Sepharose was electrophoresed on 10% SDS–PAGE under reducing or nonreducing conditions followed by Western blotting. Each fraction were electrophoresed on 10% SDS–PAGE under reducing conditions. The eluted proteins were then detected by Western blotting using 7C1.2, mα-EC1, or 9D2.2 to detect copurified proteins.

Isolation of Microdomains from the Sperm Membrane

Sperm used for isolation of membrane microdomains were obtained from the cauda epididymis and vas deferens and released into PBS. After washing twice with PBS at 5,000 rpm for 2 min at 4°C, the sperm pellet (1.7 x 10⁷) was suspended in 500 μl of 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA (TNE) containing 1% Triton X-100, and 10% protease inhibitor cocktail (Sigma). The suspension was allowed to stand on ice for 1 h followed by centrifugation at 10,000 rpm for 2 min at 4°C. The supernatant was mixed well with an equal volume of 80% sucrose and overlaid by 1 ml each of 30, 20, 10, and 5% sucrose in TNE. After ultracentrifugation at 200,000 g for 18 h at 4°C in an SW 55 Ti rotor (Beckman), a total of 14 fractions (350 μl each) were collected from the top to the bottom of the gradient. Twenty microliter aliquots of each fraction were electrophoresed on 10% SDS–PAGE under reducing or nonreducing conditions followed by Western blotting.

RESULTS

Using standard methods, we isolated mice carrying a knockout of the cyritestin gene (see Materials and Methods, Fig. 1). Though they are viable and healthy, we found that male mice lacking cyritestin were 100% infertile in vivo. Five cyritestin null males mated with 30 wild-type females gave no offspring. Cyritestin−/− sperm were equivalent to wild-type sperm in number, morphology, motility, and rate of spontaneous acrosome reaction. Unlike fertilin β-null sperm which fail to migrate from uterus to oviduct (Cho et al., 1998), cyritestin−/− sperm, examined in three matings, had normal transit into the oviduct. We also found that cyritestin−/− sperm cannot bind to the zona pellucida. A mean of ~20 wild-type sperm bound per zona in three in vitro sperm-zona binding assays. The mean level of cyritestin-null sperm binding to the zona, compared to wild type in the three assays, was ~0.06 sperm bound per zona, i.e., only 0.3% of the level of wild-type sperm. Similar findings on another cyritestin knockout have been reported (Shamsadin et al., 1999).

In their report, Shamsadin and co-workers did not test the ability of cyritestin−/− sperm to bind to the egg plasma membrane. We found that sperm–egg plasma membrane binding and fusion were differentially affected by deletion of the cyritestin gene. The number of cyritestin−/− sperm bound to the egg plasma membrane was 9% of the number of wild-type sperm bound (Fig. 2A). Nonetheless, mutant and wild-type sperm fused with the egg plasma membrane with essentially the same fertilization rate (Fig. 2B) and index (Fig. 2C). In fertilin β-null sperm, adhesion to the egg plasma membrane was also strongly reduced (13% of wild type) and this was coupled with a modest reduction of fusion with the egg plasma membrane (45-50% of wild type; Cho et al., 1998).
TABLE 1
Expression Levels of Sperm Surface Proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Genotype of sperm</th>
<th>+/+</th>
<th>C&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>β&lt;sup&gt;-/-&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fertilin α</td>
<td></td>
<td>100</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Fertilin β</td>
<td></td>
<td>100</td>
<td>60</td>
<td>n.d.</td>
</tr>
<tr>
<td>Cyritestin</td>
<td></td>
<td>100</td>
<td>n.d.</td>
<td>11</td>
</tr>
<tr>
<td>Testase 1</td>
<td></td>
<td>100</td>
<td>96</td>
<td>90</td>
</tr>
<tr>
<td>GalTase</td>
<td></td>
<td>100</td>
<td>89</td>
<td>130</td>
</tr>
<tr>
<td>PH-20</td>
<td></td>
<td>100</td>
<td>99</td>
<td>100</td>
</tr>
</tbody>
</table>

Note. +/+, wild-type sperm; C<sup>-/-</sup>, cyritestin<sup>-/-</sup> sperm; β<sup>-/-</sup>, fertilin β<sup>-/-</sup> sperm; n.d., not detected.

* Expression levels were densitometrically determined from the data in Fig. 3, assuming that the level of each protein on +/+ sperm is 100%.

One possible explanation for the relatively high rates of sperm-egg fusion that remain after targeting of cyritestin or fertilin β is that both genes must be deleted to seriously impair fusion. To test this we did appropriate crosses to generate double knockout males. We found double knockout sperm gave fertilization rates with zona-free eggs in the range 50–70% of wild-type sperm (data not shown) and thus were not more impaired in fusion than the single knockout of fertilin β.

Protein Expression Phenotype of Male Mice Lacking Fertilin β or Cyritestin

Since the fertilin β and cyritestin knockouts affect sperm function in more than one step in fertilization, we asked if multiple sperm membrane proteins might be affected by the deletion of a single gene. We used densitometric analysis (Table 1) following Western blotting of wild-type (+/+), cyritestin<sup>-/-</sup>, and fertilin β<sup>-/-</sup> sperm (Fig. 3). Among the sperm membrane proteins tested, three showed no change in mutant sperm: PH-20 [a glycosylphosphatidylinositol (GPI)-anchored hyaluronidase], testase 1 (ADAM 24) (Zhu et al., 1999), and β-1,4-galactosyl transferase (a zona-binding/signaling protein) were expressed normally on both fertilin β<sup>-/-</sup> and cyritestin<sup>-/-</sup> sperm. Fertilin α and fertilin β are present on the sperm surface, at least in part, as an α/β heterodimer (Primakoff et al., 1987; Blobel et al., 1990; Waters and White, 1997; Cho et al., 2000). We had previously found that mature fertilin α was absent from fertilin β<sup>-/-</sup> sperm (Cho et al., 2000) and that result was confirmed here (Fig. 3). In addition to the absence of fertilin β and fertilin α from fertilin β-null sperm, we found that mature cyritestin was very reduced (11% of wild-type sperm, Fig. 3, Table 1). Furthermore on cyritestin-null sperm, in addition to the absence of cyritestin, fertilin α was absent and fertilin β was reduced to 60% of the level on wild-type sperm (Fig. 3, Table 1).

These results show that sperm from mice with a deletion of the gene for fertilin β or for cyritestin lack more than one gene product. The data suggest two possibilities. First,
Cyritestin does not associate with fertilin α or fertilin β. We also asked if cyritestin forms a multi-subunit protein with fertilin α and fertilin β. The cyritestin precursor was isolated from a testicular cell lysate using an immunoaffinity column and electrophoresed on SDS–PAGE under reducing conditions. The eluted protein was tested in Western blots with antibodies to cyritestin or fertilin α or fertilin β to determine if fertilin α and/or fertilin β coeluted with cyritestin from the immunoaffinity column. A positive result, showing the immunoaffinity procedure was working, was that a large amount of cyritestin (relative to the load) was detected in the immune column eluate (Fig. 5). However, fertilin α was not detected (Fig. 5). A small amount of fertilin β (relative to the load) was detected, but a similar low level of fertilin β was present in the preimmune column eluate, indicating fertilin β may have low, nonspecific binding to Sepharose or preimmune IgG (Fig. 5). These results suggest that cyritestin is not associated with either fertilin α or fertilin β in a multi-subunit protein.

No other biotin-labeled sperm surface proteins are detected in association with mature cyritestin. A second approach was used to see if cyritestin is a subunit in a multi-subunit protein. Sperm surface proteins were biotinylated; sperm were lysed and cyritestin was isolated using an immuno-affinity column. Immunoblots of the isolated cyritestin with the anti-cyritestin mAb 7C1.2 revealed a strong band ~45 kD and a very faint band (possibly a cyritestin degradation product, ~30 kD; Fig. 6). This posi-
tive result showed that the immuno-affinity column was working. When the eluted material from the immune column was blotted with streptavidin, the strongest band corresponded to cyritestin; 45 kD. Although other major bands were detected by streptavidin, these major bands were also present in the eluate from the preimmune beads (Fig. 6). Since cyritestin itself is the only major band uniquely present in the streptavidin blot of the immune eluate, the experiment indicates that no other biotin-labeled surface polypeptide copurifies with cyritestin. This result suggests that cyritestin may be a monomer (or homomultimer).

Cyritestin and fertilin α are localized in both detergent-insoluble microdomains and detergent-soluble fractions of the sperm membrane. The findings described above show that both fertilin α and cyritestin are either very low or absent in fertilin β and cyritestin knockout sperm (Table 1) and in these sperm, adhesion to the egg zona pellucida and the egg plasma membrane is impaired. A possible explanation is that there is an organized structural unit or assembly in the sperm plasma membrane that is essential for sperm adhesive functions. This structural unit may contain fertilin α and cyritestin and be incapacitated in their absence. Microdomains, also called lipid rafts or low-density detergent-insoluble membrane (LDD-IM) fraction, have been reported to be this type of structural and functional unit for signal transduction and cell–cell adhesion. In various cell types, cholesterol and sphingolipids are major lipid components of microdomains and signaling and adhesion molecules such as GPI-anchored proteins, integrins, other adhesion proteins and G proteins are often included in these structures (Simons and Ikonen, 1997; Hooper, 1999; Horejsi et al., 1999; Jacobson and Dietrich, 1999).

We tested for the presence of microdomains in the sperm plasma membrane by the standard technique of isolating a membrane fraction insoluble in 1% Triton that migrates at a low density on sucrose gradients. Sperm were lysed in TNE including 1% Triton X-100 and the lysate was mixed with sucrose and ultracentrifuged to separate the membrane fractions by density. When each fraction obtained was analyzed by Western blotting, a pattern typical of many other cell types was obtained. Two peaks of plasma membrane proteins were evident, one of low density, the insoluble microdomain or raft fraction (fractions 8–10), and one of high density, the soluble membrane proteins (fractions 11 or 12–14, Fig. 7). Most of the PH-20 was found in microdomains (LDD-IM fractions, fractions 8, 9, and 10, Fig. 7). This was expected since PH-20 is a GPI-anchored protein. Fertilin β, testase 1, and GalTase were predominantly localized in high-density detergent-soluble fractions (fractions 11 or 12 through 14, Fig. 7). In contrast fertilin α and cyritestin were localized in both detergent-soluble and detergent-insoluble fractions. These findings indicate that a fraction of the fertilin α is not complexed with fertilin β and fertilin α may form a complex with some other subunit that

![FIG. 6. Immunoprecipitation of cyritestin from biotinylated sperm. After the sperm surface was biotinylated, immunoprecipitation (IP) was carried out by using mCyri-DC1 immune (I) and preimmune (PI) Sepharose beads. Aliquots of the eluates from these beads (equivalent to 1 × 10^7 sperm) were analyzed by Western blotting using 7C1.2 or alkaline phosphatase-conjugated streptavidin, after 10% SDS-PAGE under reducing conditions.](image)

![FIG. 7. Distribution of sperm surface proteins in sperm membrane fractions. After sperm (1.7 × 10^8) were lysed in TNE containing 1% Triton X-100 and 10% protease inhibitor cocktail, sperm membrane fractions were separated by ultracentrifugation. Each fraction obtained was resolved by 10% SDS-PAGE under reducing conditions or nonreducing conditions (for PH-20), followed by Western blotting. Fractions corresponding to microdomains (8, 9, and 10) are indicated by an asterisk. A band observed in fraction number 8 of the GalTase blot was also observed in Western blots using the corresponding preimmune serum (data not shown), so this molecule is probably unrelated to GalTase. Antibodies used here are the same as those used in Fig. 5.](image)
The fact that there is no fertilin b sperm in microdomains but fertilin b binds to both the zona pellucida and the egg plasma membrane. This altered protein expression phenotype results in fertilin b a gene product. In the fertilin b deletion of a single gene results in loss of more than one protein. In the fertilin b heterodimer and in part due to other novel reasons. In our analysis of knockouts of two testis-specific ADAMs, cyritestin and fertilin b, we found sperm from each of the knockouts has an altered expression of multiple sperm proteins. This altered protein expression phenotype results in a fertilization phenotype that includes male infertility and loss of sperm adhesive function: knockout sperm fail to bind to both the zona pellucida and the egg plasma membrane.

The Protein Expression Phenotype

A remarkable finding for both ADAM knockouts is that deletion of a single gene results in loss of more than one gene product. In the fertilin b knockout, fertilin b is deleted, fertilin a is absent, and cyritestin is reduced to 11% of wild type. In the cyritestin knockout, cyritestin is deleted, fertilin a is absent, and fertilin b is reduced to 60% of wild type. Precedents for this type of result exist when the deleted gene encodes, for example, a transcription factor whose activity is needed to express various other gene products. However we are not aware of a previous finding of loss of multiple gene products when a single deleted gene encodes a plasma membrane protein, unless the encoded polypeptide is a subunit of a multi-subunit protein.

We tested whether the two deleted ADAMs are subunits of a larger protein. We found that when cyritestin is immunopurified, it does not appear to be associated with any other polypeptide and, in particular, does not copurify with fertilin a or fertilin b. These data support the idea that cyritestin may be a monomer or homomultimer. Although fertilin a and fertilin b are present on mouse sperm as an a/b heterodimer (Cho et al., 2000), they also seem to be present outside the heterodimer. This is supported by the finding that fertilin b is present in testicular cells in excess of fertilin a (Cho et al., 2000) and fertilin b is on cyritestin knockout sperm (at 60% wild-type level) where fertilin a is absent. Furthermore, fertilin a is present on wild-type sperm in microdomains but fertilin b is not. Thus in fertilin b−/− sperm, the absence of fertilin a may in part be due to the fact that there is no fertilin b with which to form a heterodimer and in part due to other novel reasons. In cyritestin−/− sperm, the absence of fertilin a and reduction in fertilin b also appear to be due to a novel, unknown mechanism.

The mechanism may be triggered because the deleted plasma membrane protein is synthesized in a developing system and its absence may block particular further developmental steps. During sperm cell differentiation, some plasma membrane proteins may be tagged for retention and others tagged for loss from the cell surface and this process could be perturbed by the ADAM knockouts. The phenomenon, loss of multiple gene products resulting from deletion of one gene for a membrane protein, may prove important in many areas of development to investigators who are making knockouts of membrane receptors.

The Fertilization Phenotype

Because of the loss of not just one but several gene products, the mutant (fertilin b−/− and cyritestin−/−) mice can be thought of as “single,” “double,” or “triple knockouts” in interpreting their fertilization phenotypes. Perhaps still other unidentified plasma membrane proteins may be lost from the sperm of these mutant mice and these absent proteins could be responsible for aspects of the fertilization phenotype.

Previously it was found that peptide mimetics of the fertilin b and cyritestin disintegrin domains inhibit sperm adhesion to the plasma membrane of zona-free eggs (Myles et al., 1994; Almeida et al., 1995; Evans et al., 1995; Yuan et al., 1997; Linder and Heinlein, 1997; Chen and Sampson, 1999; Bronson et al., 1999). Coupled with these data, the very poor ability of mutant sperm to bind to the egg plasma membrane suggests that the ADAMs have a direct role in this process. A single ADAM, e.g., cyritestin, which is missing or very low in both mutants, may be the key adhesion protein. The ADAM would use its disintegrin-active site to bind to an egg integrin (or other receptor). We are currently testing this idea by making “knock in” mice that express mutant fertilin b or cyritestin with an altered disintegrin active site.

In the cyritestin knockout it seems paradoxical that mutant sperm bind to the egg plasma membrane at 9% of the wild-type level but fuse with the eggs at 100% of the wild-type level. A possible explanation for the differential effects on these two processes is that sperm adhesion to the plasma membrane of a zona-free egg is mostly an adhesion/signaling event for activating the egg. The mutant sperm, though unable to perform this putative signaling/adhesion step, can accomplish another type of adhesion that leads directly to fusion.

Both cyritestin−/− and fertilin b−/− sperm are unable to bind to the zona pellucida. The protein expression data show that the two knockouts have in common the absence of fertilin a and the absence or low level (11%) of cyritestin. The connection between the loss of zona binding ability and loss of these proteins is currently unclear.

In the case of the cyritestin knockout, wild-type sperm binding is ~300-fold higher than mutant sperm binding. Shamsadin and co-workers also reported that cyritestin knockout sperm cannot bind to the zona. They found that a peptide mimetic (Cyripep457) of the cyritestin disintegrin-active site blocked wild-type sperm binding to the zona pellucida and proposed that cyritestin has a direct role in zona binding through its disintegrin active site (Shamsadin et al., 1999). Although cyritestin could have a direct role, certain problems remain to be clarified: (1) there is no
evidence that an integrin is present in the egg zona pellucida; (2) it is a carbohydrate structure on ZP3 to which sperm bind (Wassarman, 1999), and it is unclear how a disintegrin active site loop would bind to carboxyhydrate; (3) we were unable to observe inhibition of sperm binding to the zona using a peptide with the same sequence as that of Cyripep457. The reasons for these differing experimental results with the peptide Cyripep457 remain to be resolved. Because we found that multiple proteins are absent in the ADAM knockouts, there is no reason to focus on cyretinin. It is possible that another key membrane protein(s), the actual zona binding molecule, is also missing in cyretinin-null and fertilin β-null sperm.

An alternative model is that sperm adhesion to both the zona pellucida and the egg plasma membrane depends upon unique protein/lipid assemblies in the sperm cell membrane. In the absence of sperm membrane proteins that are lost in the knockouts, these membrane assemblies might become nonfunctional and sperm adhesion would fail. In an initial attempt to explore this idea, we found that cyretinin and fertilin α are present in wild-type sperm microdomains. Since microdomains have been found in other cell types to participate in adhesion and signaling, it is possible that these have activities in sperm. If the absence of cyretinin and fertilin α from microdomains results for unknown reasons) in loss of their adhesive function, the sperm adhesive failures we observe could be the result.

The most striking difference between the two ADAM knockouts in fertilization performance is that cyretinin β-sperm migrate into the oviduct while fertilin β-sperm do not. The protein expression data show that the cyretinin β-sperm express 60% of the wild-type levels of fertilin β (whereas fertilin β-null sperm express no fertilin β). These results suggest that fertilin β, or possibly another protein present in cyretinin knockout but not fertilin β knockout sperm, is directly involved in the process that allows sperm to migrate from the uterus to the oviduct. The nature of this process is unknown though it is clear that sperm, in addition to motility, must have some property or receive some signal that makes them competent to move into the oviduct (Cho et al., 1998). The direct involvement of fertilin β in the mechanism suggests that a fertilin β adhesion activity to epithelium (in the uterus or uterotubal junction) may be required for sperm to achieve competence to progress into the oviduct.

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