Identification of an ADAM2-ADAM3 Complex on the Surface of Mouse Testicular Germ Cells and Cauda Epididymal Sperm*

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Male mice lacking ADAM2 (fertilin β) or ADAM3 (cyritestin) are infertile; cauda epididymal sperm (mature sperm) from these mutant mice cannot bind to the egg zona pellucida. ADAM3 is barely present in Adam2-null sperm, despite normal levels of this protein in Adam2-null testicular germ cells (TGCs; sperm precursor cells). Here, we have explored the molecular basis for the loss of ADAM3 in Adam2-null TGCs to clarify the biosynthetic and functional linkage of ADAM2 and ADAM3. A small portion of total ADAM3 was found present on the surface of wild-type and Adam2-/- TGCs at similar levels. In the Adam2-null TGCs, however, surface-localized ADAM3 exhibited an increased amount of an endoglycosidase H-resistant form that may be related to instability of ADAM3. Moreover, we found a complex between ADAM2 and ADAM3 on the surface of TGCs and sperm. The intracellular chaperone calnexin was a component of the testicular ADAM2-ADAM3 complex. Our findings suggest that the association with ADAM2 is a key element for stability of ADAM3 in epididymal sperm. The presence of the ADAM2-ADAM3 complex in sperm also suggests a potential role of ADAM2 with ADAM3 in sperm binding to the egg zona pellucida.

A new organism is created through fertilization of an egg by a sperm (1–3). The gametes are equipped with specialized, cell type-specific proteins to complete fertilization. As shown in Fig. 1, testicular germ cells (TGCs)² differentiate into testicular sperm during spermatogenesis in mammals. Subsequently, the testicular sperm moves out of the testis into a long storage tube, the epididymis. Sperm continue differentiation (maturation) as they move along the epididymis. The extent of sperm plasma membrane modification increases as the sperm pass distinct stages of the epididymis, the caput (head), corpus (body), and cauda (tail). Sperm are named according to how far they have progressed along the epididymis and are thus termed caput sperm, corpus sperm, or cauda sperm (see Fig. 1). Many sperm plasma membrane proteins undergo post-translational modification, including proteolytic processing, and have multiple protein-protein interactions during membrane trafficking in the complicated process for differentiation and maturation of the sperm.

The ADAMs (a disintegrin and metalloproteases) compose a gene family distributed in a variety of tissues (4–7). Of ~40 members of this family,³ about half are exclusively or predominantly expressed in the testis. Relevant to our study, ADAM1 (fertilin α), ADAM2 (fertilin β), and ADAM3 (cyritestin) are testis/sperm-specific members. In mice, ADAM1α and ADAM1b were identified as ADAM1 isoforms (8). ADAM1α resides only in the endoplasmic reticulum of TGCs (9) and forms an intracellular heterodimer with ADAM2 (9–13). ADAM1b and ADAM2 are localized together in the endoplasmic reticulum of TGCs (9–13) and on the surface of cauda epididymal sperm (9) as an ADAM1b/ADAM2 heterodimer. ADAM3 is a sperm-surface protein (14, 15).

So far, the mouse genes Adam1a (12), Adam1b (13), Adam2 (16), and Adam3 (17, 18) have been deleted. Cauda epididymal sperm of Adam1a-/- (12) and Adam2-/- (16) mice have defects in migration from the uterus into the oviduct. Thus, mouse sperm enter the oviduct using a mechanism that is ADAM1a/ADAM2-dependent. During direct sperm-egg interactions in the oviduct, one step is sperm binding to the egg’s extracellular matrix, termed the egg zona pellucida (ZP). Adam3-null sperm are incapable of binding to the ZP (17, 18). Adam1a-/- and Adam2-/- sperm also fail to bind to the ZP (12, 16) and contain low levels of ADAM3 (~10% of wild-type levels), despite normal protein levels of ADAM3 in TGCs of the same mutant mice (see Fig. 1 and Refs. 12, 18, and 19). Moreover, the level of ADAM2 is moderately reduced in Adam3-null sperm (see Fig. 1 and Refs. 12, 18, and 19). These findings suggest that ADAM3 plays a critical role(s) in sperm binding to the ZP and is delivered to the cell surface via a functional linkage with ADAM1a/ADAM2 (12, 18, 19). ADAM3 was recently found to bind directly to solubilized ZP (20), supporting its possible function as a biologically significant ZP ligand. However, previous studies have not clarified the relationship of ADAM3 with ADAM1a and/or ADAM2, which could affect the function(s) of ADAM3 (12, 18, 19).

³ Refer to www.people.virginia.edu/~jw7g/Table_of_the_ADAMs.html.
⁴ In this work, “epididymal sperm,” “mature sperm,” and “sperm” are used as terms equivalent to “cauda epididymal sperm,” unless particularly stated.
Here, we have extended our previous studies (18, 19) and found elements of the mechanism by which ADAM3 levels may be drastically reduced in cauda epididymal sperm of Adam2-null mice. This study is the first to provide evidence for complex formation between ADAM2 and ADAM3 on the surface of TGCs and cauda epididymal sperm.

**EXPERIMENTAL PROCEDURES**

**Materials**—EZ-Link Sulfo-NHS-LC-Biotin and ImmunoPure immobilized monomeric avidin (monomeric avidin-conjugated beads) were purchased from Pierce. Percoll and protein G-Sepharose 4 Fast Flow were from GE Healthcare. OptiPrep density gradient medium was purchased from Sigma. Novex Tris/glycine gels were from Invitrogen. Peptide-N-glycosidase F (PNGase F) was from New England Biolabs, Inc. (Ipswich, MA), and endoglycosidase H (Endo H) from Roche Applied Science.

**Antibodies**—Rabbit anti-ADAM2 polyclonal antibody and mouse monoclonal antibodies against ADAM2 (9D2) and ADAM3 (7C1) were provided by Chemicon (Temecula, CA). Anti-caveolin-1 polyclonal antibody and anti-α-tubulin monoclonal antibody were obtained from Pharmingen and Sigma, respectively. Horseradish peroxidase-conjugated goat antibodies against rabbit IgG and mouse IgG were products of Santa Cruz Biotechnology, Inc. (Ipswich, MA), and Affinity-purified from the antisera using A3Cys-immobilized agarose beads. This affinity-purified antibody is termed “anti-A3Cys antibody.”

**Animals**—Adam2−/− (16) and Adam3−/− (18) mice were generated previously and have been maintained by our laboratory. Immunoblots—Proteins in samples were separated by 10% SDS-PAGE under reducing conditions and transferred onto Immobilon-P transfer membranes (Millipore Corp., Bedford, MA). The membranes were probed with primary antibodies and horseradish peroxidase-conjugated secondary antibodies, followed by the detection of immunoreactive signals using a SuperSignal West Dura extended duration substrate (Pierce). The signal intensities in Fig. 1 were densitometrically quantified using ImageJ software (available at rsb.info.nih.gov/ij/).

**TGCs**—Testes were taken from 12–16-week-old ICR mice (Charles River Laboratories, Inc., Wilmington, MA), Adam2−/− mice, and Adam3−/− mice. On a 52% Percoll gradient, testicular sperm were separated from testicular cells, which comprise a fraction enriched for TGCs (21).

**Caput, Corpus, and Cauda Epididymal Sperm**—The caput and corpus epididymes were taken from ICR and Adam2−/− mice, minced thoroughly by scissors in phosphate-buffered saline (PBS), and incubated for 15 min at 37 °C in 5% CO2 and 95% air to release sperm into PBS. After filtration of the sperm suspensions using nylon mesh sheets, the sperm cells were washed three times with PBS by centrifugation at 500 × g for 5 min at room temperature. Cauda epididymal sperm of ICR, Adam2−/−, and Adam3−/− mice were highly purified using OptiPrep according to the method of Claassens et al. (22).

**Cell Lysates**—Cells were suspended in PBS and 1% n-octyl β-D-pyranogluconide and incubated on ice for 30 min. After centrifugation of the suspensions at 20,000 × g for 10 min at 4 °C, the supernatants were used for experiments.

**Preparation of Cell-surface Protein Fractions**—To biotinylate cell-surface proteins, TGCs (0.5 × 10⁶ cells/ml) were incubated with 1 μM EZ-Link Sulfo-NHS-LC-Biotin for 30 min at room temperature in 2 ml of 4 mM Hepes-NaOH (pH 7.4), 140 mM NaCl, 4 mM KCl, 10 mM glucose, and 2 mM MgCl₂ (HBSSM). The cells were washed once with HBSSM and then twice with PBS by centrifugation at 500 × g for 5 min at room temperature. The cell pellets were used for preparation of cell lysates or of membrane fractions. Cauda epididymal sperm were also biotinylated by the same method as used for TGCs, except that PBS was consistently used instead of HBSSM.

The biotinylated proteins in samples (equivalent to 1 × 10⁶ cells) were precipitated with 50 μl of monomeric avidin-conju-
gated beads in 4 ml of PBS, 500 mM NaCl, 5 mM EDTA, and 1% n-octyl β-D-pyranogluco-side during incubation for 30 min at room temperature. After washing three times with the same buffer, the beads were mixed with 200 μl of PBS, 500 mM NaCl, 5 mM EDTA, 1% n-octyl β-D-pyranogluco-side, and 5 mM d-biotin (total of 250 μl) and kept for 30 min at room temperature. The biotinylated proteins were recovered in the supernatants (equivalent to 4 × 10⁶ cells/ml) after centrifugation of the suspensions at 500 × g for 5 min at room temperature.

**Separation of Membrane Fractions from TGCs**—Biotinylated TGCs (2 × 10⁶ cells) were suspended in 500 μl of 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 5 mM EDTA (TNE) containing 1% Triton X-100; kept on ice for 1 h; and centrifuged at 20,000 × g for 10 min at 4°C. To prepare fractions of detergent-insoluble membranes (DIMs) and detergent-soluble membranes (DSMs), the supernatants were mixed with 1.5 ml of TNE, 0.33% Triton X-100, and 52% OptiPrep and overlaid with 6 ml of TNE and 30% OptiPrep and subsequently with 3.5 ml of TNE and 5% OptiPrep. After centrifugation at 200,000 × g for 18 h at 4°C in an SW 41 Ti rotor (Beckman Coulter, Fullerton, CA), 1-ml fractions were taken from the top to the bottom of the gradient (total of 12 fractions). Based on the immunoblotting of each fraction for the DIM marker protein caveolin-1 (data not shown), fractions 4 and 5 were pooled as DIMs. Fractions 9–12 were judged to correspond to DSMs from the fractionation results for ADAM2, ADAM3, calmegin, and calnexin (data not shown), pooled together, and termed as DSMs. To prepare biotinylated proteins in the DIM and DSM fractions for affinity isolation with monomeric avidin, n-octyl β-D-pyranogluco-side was added to these membrane fractions (1% final). The fractions were incubated for 30 min at room temperature and then centrifuged at 20,000 × g for 10 min at 4°C. Biotinylated proteins in the supernatants were precipitated with monomeric avidin-conjugated beads to isolate cell-surface proteins as described above.

**Glycosidase Digestion**—Glycosidase digestion was carried out as described previously (9). Briefly, glycoproteins in samples were denatured by boiling for 5 min in the presence of 0.4% SDS and 1% 2-mercaptoethanol and digested for 12 h at 37°C with 15,000 units/ml PNGase F in PBS, 5 mM EDTA, 0.2% SDS, and 0.5% 2-mercaptoethanol or with 0.2 units/ml Endo H in 200 mM sodium phosphate (pH 6.0), 0.2% SDS, and 0.5% 2-mercaptoethanol.

**Immunoprecipitation**—Lysates (equivalent to 1 × 10⁷ cells) and cell-surface protein fractions (equivalent to 1 × 10⁸ cells) were kept on ice for 2 h with 20 μg of anti-A3Cys antibody, 20 μg of rabbit anti-ADAM2 monoclonal antibody, 10 μl of anti-calmegin antisera, or 10 μl of anti-calnexin antisera in 1 ml of PBS, 500 mM NaCl, 5 mM EDTA, and 1% n-octyl β-D-pyranogluco-side. Immune complexes that formed in the reaction mixtures were captured with protein G-Sepharose 4 Fast Flow beads (5 μg of IgG or 1 μl of antisera/μl of beads) during 30-min incubation at room temperature, followed by washing three times with the same buffer. ADAM3 and coprecipitated proteins were eluted from the beads by incubation for 30 min at room temperature in 50 μl of the wash buffer containing 500 μg/ml A3Cys (the peptide used for immunization). The beads carrying the immune complexes of calmegin or calnexin were boiled for 5 min in 50 μl of reducing SDS sample buffer. The eluted proteins were then analyzed by immunoblotting. When proteins contained in the immune complexes of ADAM2 or calnexin were required for digestion with PNGase F and Endo H, they were eluted from the beads by boiling for 5 min in 50 μl of PBS, 0.3% SDS, and 0.75% 2-mercaptoethanol.

**RESULTS**

**Level of Surface ADAM3 in Adam2-null TGCs**—To examine why the level of ADAM3 is so low in Adam2-null sperm, we investigated when ADAM3 first appears on the cell surface. A previous study was unable to detect ADAM2 and ADAM3 on the plasma membrane of TGCs (19). To improve the sensitivity of cell-surface protein detection, we developed a technique using vectorial labeling of the cell-surface proteins with biotin, followed by isolation of the biotinylated proteins with monomeric avidin-conjugated beads.

Whole cell lysates and cell-surface protein fractions prepared from wild-type, ADAM2−/−, and ADAM3−/− TGCs were analyzed by immunoblotting for several testicular proteins such as ADAM2, ADAM3, ADAM24, calmegin, calnexin, and PH-20 (Fig. 2). Densitometry of immunoreactive signals was used to quantify the amounts of specific protein in each lane. To set up the experimental conditions in Fig. 2, we used PH-20 as a positive control. Tryptic digestion of cell-surface proteins on intact TGCs revealed that PH-20 was almost completely localized on the plasma membrane of TGCs (19). To improve the sensitivity of cell-surface protein detection, we developed a technique using vectorial labeling of the cell-surface proteins with biotin, followed by isolation of the biotinylated proteins with monomeric avidin-conjugated beads. When band intensities of PH-20 were compared between “total” and

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5 H. Nishimura, D. G. Myles, and P. Primakoff, unpublished data.
levels in the mutant sperm. Previously, we had found that a substantial population of ADAM3 partitioned into lipid rafts (detergent-insoluble membranes/microdomains) on sperm (18). To investigate whether ADAM3 is targeted into the incorrect membrane microdomains on Adam2-null TGCs, we examined the amount of surface ADAM3 in DIMs and DSMs of wild-type and Adam2−/− TGCs. TGCs were first biotinylated, and after separation of DIMs from DSMs, the cell-surface proteins were isolated from the membrane fractions using monomeric avidin-conjugated beads.

Total fractions of DIMs and DSMs contained ADAM2 and ADAM3 in wild-type TGCs (Fig. 3).

The profiles for distribution of ADAM2 and ADAM3 into DIM and DSM fractions from wild-type cells were similar to those for distribution in Adam3−/− and Adam2−/− TGCs, respectively. Even when cell-surface proteins were isolated from the total DIMs and DSMs, little difference was observed for ADAM2 between wild-type and Adam3−/− TGCs and for ADAM3 between wild-type and Adam2−/− TGCs. Other tested proteins in the Adam2−/− and Adam3−/− cells were also normally present in the total and surface fractions of DIMs and DSMs. Thus, Adam2-null TGCs are apparently indistinguishable from wild-type TGCs in localization of ADAM3 in cell-surface membrane microdomain(s).

Another possibility is that ADAM3 is altered in its modifications during trafficking of ADAM3 to the cell surface in Adam2-null TGCs. We investigated by Endo H digestion of ADAM3 whether glycosylation of cell-surface ADAM3 is altered in Adam2-null TGCs. Endo H resistance of glycans on surface glycoproteins is often acquired as the glycoproteins complete their passage through the Golgi apparatus and achieve normally modified carbohydrate moieties. Endo H cleaves off high-mannose N-linked oligosaccharides and can be compared with another glycosidase, PNGase F, which splits all N-linked glycans.

Cell-surface proteins in DIMs and DSMs were digested with PNGase F and Endo H. We used DIMs and DSMs to increase the resolution of this analysis, i.e. to enhance detection of non-abundant forms of tested proteins. Immunoblot analysis of the digests (Fig. 4) revealed that ADAM2 was mostly sensitive to Endo H in DIMs and DSMs of wild-type TGCs. Adam3-null TGCs exhibited essentially the same digestion profiles of ADAM2 as the wild-type cells. Cell-surface ADAM3 was also investigated after Endo H digestion. In wild-type TGCs, approximately half of ADAM3 was Endo H-resistant in DIMs, whereas this enzyme almost completely cleaved ADAM3 in DSMs. Interestingly, most of ADAM3 became Endo H-resistant in DIMs of Adam2-null TGCs. Also in DSMs of Adam2-null cells, about half of the ADAM3 protein was Endo H-resistant. Thus,
ADAM3 is different in wild-type and Adam2<sup>−/−</sup> TGCs with respect to its glycosylation pattern. In both DIMs and DSMs of Adam2-null TGCs, there is more Endo H-resistant ADAM3 compared with wild-type cells. The control protein PH-20, for which trafficking is presumably independent of ADAM2 and ADAM3, was completely Endo H-resistant in any sample tested (Fig. 4).

**ADAM2 and ADAM3 Form a Complex on the Surface of TGCs and Cauda Epididymal Sperm**—An association between ADAM2 and ADAM3 could be a key factor in understanding the severe loss of ADAM3 in Adam2<sup>−/−</sup> and Adam3<sup>−/−</sup> mice. If ADAM2 and ADAM3 are physically associated at some points in their biosynthesis or trafficking, it would be possible that the absence of ADAM2 might directly affect the survival of ADAM3. Furthermore, the existence of such a complex on mature sperm could be important in the sperm function(s) required for sperm-ZP interaction.

Previously, Ikawa et al. (11) reported that testicular ADAM2 is associated with, at least, calmegin, and Yamaguchi et al. (26) reported that this chaperone also forms a protein complex with ADAM3 in TGCs. In Figs. 2 and 3, both calmegin and calnexin were found on the surface of TGCs as well as in the endoplasmic reticulum. Thus, we carried out immunoprecipitation of calmegin and calnexin in whole cell lysates (equivalent to 3 × 10<sup>6</sup> TGCs/lane) and surface protein fractions (equivalent to 30 × 10<sup>6</sup> TGCs/lane) from wild-type, Adam2<sup>−/−</sup>, and Adam3<sup>−/−</sup> TGCs. In wild-type and Adam3<sup>−/−</sup> cells, immune complexes of calmegin contained ADAM2 in total lysates and, at lower levels, in surface fractions (Fig. 5A). ADAM3 was also coprecipitated with calmegin in wild-type and Adam2<sup>−/−</sup> lysates; surface fractions of the same cell types contained calmegin-bound ADAM3 at barely detectable levels (Fig. 5B). Thus, most of the ADAM2-calmegin and ADAM3-calmegin complexes are intracellularly localized in TGCs.

In contrast to calmegin, the wild-type surface fractions contained a calnexin-bound form of ADAM3 equally or more abundantly compared with the corresponding whole cell lysates. We also found that ADAM2 was associated with calnexin in the surface fractions of wild-type TGCs. The cell-surface association between ADAM3 and calnexin was found in the Adam2-null cells, but to a lesser degree than in the wild-type cells. ADAM2 was not detected in the immunoprecipitates of calnexin from Adam3-null surface fractions. Thus, association of calnexin and ADAM2 is ADAM3-dependent. Hence, these data suggest that a protein complex containing ADAM2, ADAM3, and calnexin is present on the TGC plasma membrane.

To confirm the presence of the tertiary protein complex on the cell surface, ADAM3 was immunoprecipitated with anti-A3Cys antibody in surface fractions of wild-type TGCs (Fig. 5C). As expected, ADAM2 and calnexin, but not calmegin, were included in the immune complexes of ADAM3. When surface fractions of Adam3-null TGCs were used instead of the wild-type samples, coprecipitation of ADAM2 and calnexin was barely found.

We also explored the protein complex containing ADAM2 and ADAM3 in cauda epididymal sperm (Fig. 5D). When immunoprecipitation of ADAM3 was performed in sperm lysates using anti-A3Cys antibody, both ADAM2 and ADAM3 were immunoprecipitated with the antibody from wild-type sperm, but not from Adam2<sup>−/−</sup> and Adam3<sup>−/−</sup> sperm. Thus, the ADAM2-ADAM3 complex is present in cauda epididymal sperm. When cell-surface protein fractions of wild-type and Adam3<sup>−/−</sup> sperm were used for the immunoprecipitation, ADAM2 was found in the immune complexes of ADAM3 from solely the wild-type surface fractions. These data indicate that the ADAM2-ADAM3 complex is localized on the surface of cauda epididymal sperm. In contrast to TGCs, no calnexin was coprecipitated with the immune complexes of ADAM3 in any sperm-derived sample, despite the presence of this chaperone in mouse sperm. Immunoprecipitation of calnexin in lysates and surface fractions of wild-type sperm also failed to coprecipitate ADAM2 or ADAM3 (data not shown). However, the possibility remains that the calnexin that was associated with the sperm ADAM2-ADAM3 complex, if any, was of too small an amount to be detected by our immunoblot analysis.

**Endo H-sensitive ADAM3 Is Associated with ADAM2 and Calnexin on the TGC Surface and Is Also Localized in Cauda Epididymal Sperm**—In Adam2-null TGCs, cell-surface ADAM3 was normal in amount (Fig. 2) and distribution into DIMs and DSMs (Fig. 3), but became partially Endo H-resistant.
FIGURE 5. ADAM2 and ADAM3 form a complex on the surface of TGCs and epididymal sperm. Whole cell lysates (Total) were prepared in PBS and 1% n-octyl ß-D-pyranoglucoside from biotinylated TGCs of wild-type (WT), Adam2−/− (A2−/−), and Adam3−/− (A3−/−) mice. Cell-surface proteins (Surface) were subsequently isolated from the lysates. A and B, immunoprecipitation (IP) of calnexin (CN) and calnexin (CN) was carried out in the lysates and cell-surface protein fractions using antisera against these two chaperones. Negative control experiments (Mock) were also performed using normal rabbit serum instead of the specific antisera. Coprecipitation of ADAM2 (A) and ADAM3 (B) was analyzed by immunoblotting. Total, 0.3 &times; 10^7 TGCs/lane (Input) and 3 &times; 10^6 TGCs/lane (IP); Surface, 3 &times; 10^6 TGCs/lane (Input) and 30 &times; 10^6 TGCs/lane (IP). C, ADAM3 (A3) was immunoprecipitated with rabbit anti-A3Cys antibody in cell-surface protein fractions of wild-type and Adam3−/− TGCs. The immune complexes were examined to test whether they contain ADAM2, calnexin, or ADAM3. D, mouse sera were prepared from wild-type, Adam2−/−, and Adam3−/− cauda epididymal sperm in PBS and 1% n-octyl ß-D-pyranoglucoside. Surface protein fractions (Surface) were also prepared from wild-type and Adam3−/− sperm after biotinylation of these sperm cells. ADAM3 was then immunoprecipitated in the total and surface samples, and coprecipitated proteins were examined by immunoblotting as described for C. In cauda epididymal sperm, ADAM2 and ADAM3 are proteolytically processed into 44- and 42-kDa proteins, respectively, as mature forms (14, 16–19). Total, 0.5 &times; 10^6 sperm/lane (Input) and 5 &times; 10^6 sperm/lane (IP); Surface, 5 &times; 10^5 sperm/lane (Input) and 50 &times; 10^5 sperm/lane (IP). All results are representative of two or three independent experiments.

As summarized in Fig. 7, these data demonstrate that Endo H-resistant ADAM3 corresponds to an ADAM3-calnexin complex, localized on the surface of wild-type and Adam2−/− TGCs. ADAM3 that is in a tertiary Adam2-Adam3-calnexin complex is likely to be Endo H-sensitive and to be found on the wild-type cell surface. Endo H-sensitive ADAM3 is also present on the surface of Adam2-null TGCs, but the ADAM3 is probably free from Adam2 and calnexin.

We next investigated the Endo H sensitivity of ADAM3 in caput, corpus, and cauda epididymal sperm (Fig. 6) to examine ADAM2-associated ADAM3 at late developmental stages. As expected, most of ADAM3 was digested with Endo H in all caput, corpus, and cauda epididymal sperm. In Adam2-null mice, ADAM3 was almost absent at any sperm stage tested. Thus, these results emphasize again the importance of association with ADAM2 for survival of ADAM3 in mature sperm. The ADAM3-calnexin complex may be lost during normal development into wild-type sperm (Fig. 7) because Endo H-resistant ADAM3 was barely present in any tested epididymal sperm of wild-type mice as well as of Adam2-null mice (Fig. 6).

DISCUSSION

Targeted deletion of the gene coding for ADAM2 or ADAM3 results in infertile male mice, the epididymal sperm of which are incapable of binding to the egg ZP. Additionally, Adam2-null sperm fail to migrate from the uterus into the oviduct and contain very low levels of ADAM3 (~10% of wild-type levels) (Fig. 1), revealing a pleiotropic phenotype. Adam3-null sperm enter the oviduct normally and show moderately reduced levels of ADAM2 (60–75% of wild-type levels) (Fig. 1). We investigated the relationship between ADAM2 and ADAM3 to gain further insight into the null phenotypes and the biosynthetic and functional linkage between these two ADAM proteins.

Here, we discovered a key element to explain the Adam2-null pleiotropic phenotype: ADAM2 and ADAM3 form an essential complex early in sperm development. By disruption of the association between these two ADAM proteins, ADAM3 could be both structurally unstable and impaired in its function in fertilization. Thus far, there had been no evidence for the association of ADAM2 and ADAM3 (12, 18, 19). To test for the presence of the protein complex in TGCs, we used enriched cell-surface protein fractions in addition to or instead of whole cell lysates,
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which had been investigated in previous studies. In the surface fractions of TGCs, a complex of ADAM2, ADAM3, and calnexin was identified. We also found an ADAM2–ADAM3 complex on the surface of cauda epididymal sperm.

The identification of the complex of testicular and sperm proteins implies their functional roles in sperm development and/or fertilization. During spermatogenesis and sperm maturation, the association with ADAM2 may affect the stability of ADAM3 in epididymal sperm. Fig. 7 shows a schematic model for loss of ADAM3 in Adam2-null mice according to our present findings. Surface ADAM3 is present at normal levels on Adam2-null TGCs (Fig. 2), but the compositions of protein complexes containing ADAM3 are different between wild-type and Adam2−/− TGCs (Fig. 6): ADAM3-calnexin and ADAM2–ADAM3-calnexin are on the wild-type cells, and ADAM3 and ADAM3-calnexin are on the Adam2-null cells. The surface of cauda epididymal sperm might lose any form of ADAM3, except for the ADAM2–ADAM3 complex, in wild-type and Adam2−/− mice. The ADAM2-free forms of ADAM3 are possibly degraded on the cell surface by spermatogenic or epididymal protease(s). In the absence of ADAM2 as a partner in the complex, ADAM3 might be more susceptible to such aberrant degradation.

We suggested previously that loss of ADAM3 from Adam2-null TGCs might occur in a post-Golgi compartment or by proteolytic release or degradation immediately upon arrival at the plasma membrane (19). In that work, we could not find ADAM3 on the TGC surface and therefore could not study the idea that surface-resident ADAM3 might be degraded. Development of a more sensitive technique, which is to use enriched cell-surface protein fractions, allowed us to determine that ADAM3 is measurable on the surface of wild-type and Adam2−/− TGCs at equivalent levels.

Relevant to a role(s) of the ADAM2–ADAM3 complex in fertilization, ADAM3 has been so far thought to act in sperm binding to the egg ZP from investigation using gene-targeted mice (12, 16–18). Indeed, recent work showed that ADAM3 binds directly to solubilized ZP (20), implying the function of ADAM3 as a biological ZP ligand. Our finding of the sperm ADAM2–ADAM3 complex is a significant result because it suggests for the first time that ADAM2 might function in sperm-ZP binding as a part of the ZP ligand with ADAM3.

Complex formation between ADAM2 and ADAM1a (9–13), ADAM1b (9–13), and now ADAM3 (this study) has been demonstrated. Moreover, Adam2-null mouse sperm have been found recently to contain a severely reduced amount of ADAM5 (15), suggesting an interaction between ADAM2 and ADAM5. Hence, as the ADAM2–ADAM3 complex is implied to act in sperm binding to the ZP, we can suggest that ADAM2 regulates the localization and functions of multiple ADAM proteins through associ-
ation with them. Based on the phenotypes of Adam2-null sperm, other ADAM protein(s) associated with ADAM2 could be responsible for sperm transit into the oviduct. ADAM2 is thus probably a key molecule to elucidate the functional roles of the testis/sperm-specific ADAM family members in spermatogenesis and fertilization.

Overall, our findings suggest that studies of the ADAM family represent a major resource for continued advances in spermatogenesis and fertilization. The propensity of these proteins to associate (particularly ADAM2 with other ADAM proteins and with chaperones) will continue to provide clues about cell-surface activities on developing and mature sperm cells.

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