Synthesis, Processing, and Subcellular Localization of Mouse ADAM3 during Spermatogenesis and Epididymal Sperm Transport

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Abstract. To elucidate synthesis, processing, and subcellular localization of mouse ADAM3 (cyritestin) during spermatogenesis and epididymal sperm transport, we carried out immunoblotting and immunohistochemical analysis of testicular germ cells, and epididymal and vas deferens sperm, using affinity-purified anti-ADAM3 antibody. ADAM3 was initially synthesized as a 110-kDa precursor in round spermatids, and the precursor was then processed into a 42-kDa mature protein during the sperm transport into and/or once in the epididymis. The mature ADAM3 was localized on the anterior part of capacitated sperm heads and was rapidly removed from the head region during the calcium ionophore A23187-induced acrosome reaction. These results demonstrate that the mature form of ADAM3 is involved in the binding of sperm to the egg zona pellucida, not in the membrane fusion between sperm and egg.

Key words: ADAM3, Spermatogenesis, Epididymis, Acrosome, Fertilization

family of ADAM transmembranous proteins containing a disintegrin and metalloprotease domain has been implicated in diverse physiological processes such as fertilization, neurogenesis, myogenesis, cancer, and inflammation [1–3]. The members of the ADAM family have multi-functional domains consisting of pro-, metalloprotease, disintegrin, cysteine-rich, epidermal growth factor-like, transmembrane, and cytoplasmic tail domains. The metalloprotease domain exhibits a “sheddase” activity toward the ectodomain of membranous precursor proteins [1, 4], while cell-to-cell adhesion is mediated by the disintegrin domain [5–7]. Although many ADAM members are predominantly or exclusively expressed in the testis, their roles in spermatogenesis and fertilization still remain to be elucidated.

Several ADAM members including ADAM1 (fertilin α), ADAM2 (fertilin β), and ADAM3 (cyritestin) have been shown to play a key role(s) in fertilization. Fertilin is a heterodimeric protein complex of ADAM1 and ADAM2 present on the sperm surface [8–10]. In the mouse, two different isoforms of ADAM1, termed ADAM1a and ADAM1b, are produced in the testis [11, 12]. ADAM1a is localized within the endoplasmic reticulum of testicular germ cells, whereas epididymal sperm contain only ADAM1b on the
cell surface [12]. Male mice lacking ADAM1a [13] and ADAM2 [14] are sterile because sperm are defective in migrating from the uterus into the oviduct, and in binding to the egg zona pellucida. ADAM3-deficient mouse sperm are also incapable of binding to the zona pellucida, although the sperm ascent into the oviduct is normal [15, 16]. Noteworthy is that the disruption of the functional ADAM1a, ADAM2, and ADAM3 genes results in the loss or severe reduction of ADAM3 in epididymal sperm [13, 16]. Thus, ADAM3 may be one of the proteins responsible for the binding of sperm to the zona pellucida.

Because the interaction of sperm with the zona pellucida is a regional process, it is important to know the localization of ADAM3 in sperm. Despite its importance, the ADAM3 localization is still controversial [17, 18]. This uncertainty may result from the nature of the antibodies prepared. Indeed, a commercially available monoclonal antibody against ADAM3 (7C1.2) is restricted to the use only for immunoblot analysis. We have previously prepared an affinity-purified anti-ADAM3 polyclonal antibody applicable to immunoprecipitation analysis [13]. In this study, we thus re-examined synthesis, processing, and subcellular localization of mouse ADAM3 during spermatogenesis and epididymal sperm transport, using the affinity-purified antibody.

Materials and Methods

Materials

Affinity-purified polyclonal antibodies against ADAM1b, ADAM3, sp32, and TPAP were prepared as described previously [12, 13, 19, 20]. Monoclonal anti-ADAM2 (9D2.2) and anti-ADAM3 (7C1.2) antibodies were purchased from Chemicon. Monoclonal antibody MC101 [21] and rabbit anti-mouse AKAP82 antiserum [22] were generous gifts from Drs. K. Toshimori and S. B. Moss, respectively. Horseradish peroxidase-conjugated goat antibodies against mouse IgG (H + L) and rabbit IgG (H + L) were purchased from Jackson Immunoresearch Laboratories. ICR male mice were obtained from Japan SLC Inc., Shizuoka. All animal experiments were carried out according to the Guide for the Care and Use of Laboratory Animals in University of Tsukuba.

Preparation of protein extracts

Testicular germ cells and epididymal sperm from ICR mice (3 to 5 months old) were suspended in a lysis buffer consisting of 20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, and 10% protease inhibitor cocktail (Sigma-Aldrich), kept on ice for 20 min, and centrifuged at 10,000 × g for 10 min at 4°C, as described previously [12, 13]. Protein concentration in the supernatant solution was determined using a Coomassie protein assay reagent kit (Pierce).

Immunoblot analysis

Proteins were denatured by boiling for 5 min in the presence of 1% SDS and 1% 2-mercaptoethanol, separated by SDS-polyacrylamide gel electrophoresis (PAGE), and transferred onto Immobilon-P membranes (Millipore). After blocking with 1% skim milk, the blots were incubated with primary antibody for 2 h, and then with horseradish peroxidase-conjugated secondary antibody for 1 h. Immunoreactive proteins were detected by an ECL Western blotting detection kit (Amersham Biosciences), as previously described [23].

Biotinylation of sperm surface proteins

Cauda epididymal sperm (2.5 × 10⁷/ml) were kept at room temperature for 30 min in phosphate-buffered saline (PBS) containing 1 mM sulfo-NHS-LC-biotin (Pierce), as previously described [13]. The biotinylated sperm samples were washed twice with PBS and treated with the above lysis buffer. Proteins were separated by SDS-PAGE under reducing conditions followed by immunoblot analysis.

Immunostaining

Mouse testicular tissues were snap-frozen and embedded in a TissueTek O.C.T compound (Sakura Finetechical Co., Tokyo), as previously described [24]. Sections (8 µm) were prepared by a Leica CM3000 cryostat, mounted on silanized glass slides, air-dried, and fixed in PBS containing 4% paraformaldehyde at 4°C for 30 min. Slides were then washed with PBS, blocked with 2% normal goat serum, and incubated with affinity-purified anti-ADAM3 antibody in PBS containing 0.05% bovine serum albumin at 4°C overnight. After washing with PBS, the slides were treated with 0.3% hydrogen peroxide to remove endogenous
peroxidase activity, washed with PBS, and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG. The slides were stained with 3,3′-diaminobenzidine as a chromogen, counterstained with 2.5% methyl green, and viewed under an Olympus BX50 microscope. For indirect immunofluorescence microscopy, sperm were capacitated in modified Krebs-Ringer bicarbonate solution (TYH medium) containing glucose, sodium pyruvate, bovine serum albumin, and antibiotics under 5% CO₂ in air at 37°C for 2 h, put on slides, and fixed in PBS containing 4% paraformaldehyde at room temperature for 15 min. The slides were then washed with PBS, blocked with PBS containing 3% normal goat serum and 0.05% Tween-20, and incubated with primary antibody at 4°C overnight. The sperm samples were washed, and incubated with goat anti-mouse and anti-rabbit IgGs labeled with Alexa Fluor 488 and 568 (Molecular Probes, Eugene, OR), respectively, as previously described. After washing with PBS containing 0.05% Tween-20, the slides were observed under an Olympus IX-70 fluoromicroscope.

Subcellular fractionation of acrosome-reacted epididymal sperm

Subcellular components of cauda epididymal sperm after the acrosome reaction were prepared as previously described [24, 27]. The acrosome reaction of sperm (5 × 10⁷ sperm/ml) was induced by incubation with 10 µg/ml calcium ionophore A23187 in TYH medium under 5% CO₂ in air at 37°C for 60 min. The sperm samples were centrifuged at 800 × g for 10 min to remove sperm, and the supernatant solution was ultracentrifuged at 100,000 × g for 90 min at 4°C. The resulting supernatant was used as a source of soluble proteins released by the A23187-induced acrosome reaction, including acrosomal components (SPA fraction). The precipitate obtained by ultracentrifugation was washed with PBS, re-suspended in PBS, and used as a source of membranous proteins on the plasma and outer acrosomal membranes fused during the acrosome reaction (MPA fraction). Acrosome-reacted sperm were washed three times with PBS, and sonicated as previously described [28]. To a 2.5-ml suspension, an equal volume of 1.8 M sucrose was added, and the mixture was put onto a discontinuous sucrose gradient solution containing 2.5 ml each of 2.20 and 2.05 M sucrose, and ultracentrifuged at 100,000 × g for 16 h at 4°C. ARH and ART fractions corresponding to the heads and tails of acrosome-reacted sperm were recovered from the bottom of the centrifuge tube and the 2.05 M sucrose layer, respectively, and were precipitated by centrifugation at 100,000 × g. The purities of these two fractions were microscopically more than 95%.

Results and Discussion

To examine synthesis and processing profiles of ADAM3 during spermatogenesis and epididymal sperm transport, we carried out immunoblot analysis of proteins from purified populations of testicular germ cells, and from epididymal and vas deferens sperm, using affinity-purified anti-ADAM3 antibody (Fig. 1A). ADAM3 was initially synthesized as a 110-kDa precursor in postmeiotic round spermatids. No processing of the precursor occurred in elongating spermatids. Sperm from caput, corpus, and cauda epididymides, and vas deferens contained a 42-kDa protein corresponding to the mature form of ADAM3. Consistent with previous results reported by Linder et al. [17], epididymal sperm lacked the 110-kDa ADAM3 precursor (Fig. 1A). However, when monoclonal anti-ADAM3 (7C1.2) antibody was used as a probe, a 50-kDa protein as well as the 42-kDa mature ADAM3 was found only in caput and corpus epididymal sperm (data not shown). The 50-kDa protein may correspond to an intermediate form of ADAM3 that is then converted into the mature protein. Thus, the ADAM3 precursor is processed into the 42-kDa mature protein possibly via the 50-kDa intermediate during the sperm transport into and/or once in the epididymis.

We next examined whether ADAM3 is localized on the surface of acrosome-intact sperm (Fig. 1B). Biotinylation of surface proteins on cauda epididymal sperm resulted in slow migration of ADAM1b, ADAM2, and ADAM3 on SDS-PAGE. Two acrosomal proteins, a proacrosin-binding protein, sp32 [19], and a 155-kDa protein (termed MC101) recognized by monoclonal antibody MC101 [21], were not biotinylated. Therefore, these three ADAMs including ADAM3 are localized on the cell surface of cauda epididymal sperm.

Immunohistochemical analysis of testicular
sections indicated that ADAM3 is localized in round and elongating spermatids (Fig. 2). No significant signal for ADAM3 was detected in spermatogonia, spermatocytes, and Sertoli cells. These data are consistent with the results obtained by immunoblot analysis (Fig. 1A). Monoclonal antibody 7C1.2 commercially available gave no immunoreactive signal.

The information on the localization of ADAM3 in sperm is essential for understanding the functional role of ADAM3 in sperm/egg interaction. Linder et al. [17] first reported that an antibody against the cytoplasmic tail domain of ADAM3 recognizes this protein in the acrosomal region of acrosome-intact sperm, and on the inner acrosomal membrane of acrosome-reacted sperm. However, indirect immunofluorescence analysis using another antibody against the active site (residues 453–475) of ADAM3 prepared by Yuan et al. [18] indicated that the immunoreactive signal is restricted to the equatorial region of sperm, and the staining pattern is unchanged after the induction of the acrosome reaction. In the present study, our affinity-purified anti-ADAM3 antibody gave immunoreactive signals on the anterior part of the head of capacitated, acrosome-intact cauda epididymal sperm, including the apical tip of the sperm head (panel a in Fig. 3). The localization of ADAM3 on cauda epididymal sperm was essentially similar to those on sperm from caput and corpus epididymides, and vas deferens (data not shown). The staining patterns with monoclonal antibody MC101 and 4′-6-diamidino-2-phenylindole (DAPI) revealed the absence of ADAM3 in the acrosome and nucleus of acrosome-intact sperm (panels b-d). Unexpectedly, the immunoreactive signals for ADAM3 rapidly disappeared from the sperm head during the calcium ionophore A23187-induced

![Fig. 1. Synthesis and processing of ADAM3 precursor during spermatogenesis and sperm maturation.](image-url)
Fig. 2. Immunohistochemical analysis of testicular sections. Sections of mouse testis were probed by monoclonal antibody 7C1.2 (anti-ADAM3 antibody, see a and c) or affinity-purified anti-ADAM3 antibody (b and d), and observed under a microscope (magnification, ×200 for a and b, and ×400 for c and d). No significant signal was detected when pre-immune serum was used as the probe.

Fig. 3. Indirect immunofluorescent analysis of capacitated epididymal sperm. The acrosome reaction of capacitated cauda epididymal sperm was induced by calcium ionophore A23187 for 60 min. Acrosome-intact (AI) and reacted (AR) sperm were probed by affinity-purified anti-ADAM3 antibody (a and e) and monoclonal antibody MC101 (b and f). Sperm were also stained with 4′,6-diamidino-2-phenylindole, DAPI (c and g). Arrows indicate acrosome-reacting sperm. Note that when affinity-purified anti-testis-specific poly(A) polymerase, TPAP, antibody and Alexa Fluor 568-conjugated secondary antibody were used as controls, no significant staining was observed (not shown). Moreover, only the staining patterns of ADAM3 on cauda epididymal sperm are shown here, because the localization of ADAM3 was similar among capacitated, acrosome-intact sperm from caput, corpus, and cauda epididymides, and vas deferens.
acrosome reaction (panels e-h). We occasionally observed acrosome-reacting sperm where acrosomal protein MC101 was being dispersed from the acrosome (arrows in panels f and h). Even on the head of acrosome-reacting sperm, most ADAM3 had already disappeared. Thus, ADAM3 is localized on the anterior part of the sperm head, and is removed during the acrosome reaction.

To obtain more definitive data concerning the localization of ADAM3, proteins in four subcellular fractions (SPA, MPA, ARH, and ART fractions) of cauda epididymal sperm after the acrosome reaction were analyzed by immunoblotting using antibodies against ADAM3, ADAM1b, sp32, and AKAP82, and monoclonal antibodies 9D2.2 (anti-ADAM2 antibody) and MC101. SPA, soluble proteins released by A23187-induced acrosome reaction, including acrosomal components; MPA, membranous proteins on plasma and outer acrosomal membranes fused during the acrosome reaction; ARH, proteins in acrosome-reacted sperm heads; ART, proteins in acrosome-reacted sperm tails.

Fig. 4. Subcellular localization of ADAM3 in cauda epididymal sperm after the acrosome reaction. Subcellular fractions from acrosome-reacted epididymal sperm were analyzed by immunoblotting using antibodies against ADAM3, ADAM1b, sp32, and AKAP82, and monoclonal antibodies 9D2.2 (anti-ADAM2 antibody) and MC101. SPA, soluble proteins released by A23187-induced acrosome reaction, including acrosomal components; MPA, membranous proteins on plasma and outer acrosomal membranes fused during the acrosome reaction; ARH, proteins in acrosome-reacted sperm heads; ART, proteins in acrosome-reacted sperm tails.

[22], in the flagellum fibrous sheath, were used as controls (Fig. 4). ADAM3 as well as two acrosomal proteins, sp32 and MC101, was found in the SPA (soluble proteins released by the acrosome reaction including acrosomal components) fraction. The localization of ADAM1b and ADAM2 on acrosome-reacted sperm was totally different from that of ADAM3; ADAM1b and ADAM2 were still present in the ARH (acrosome-reacted sperm heads) fraction. These data are consistent with the fact that ADAM3 is removed from the sperm head during the acrosome reaction (Fig. 3). However, it is uncertain at present that the molecular size of the mature form of ADAM3 (42 kDa) remains unchanged (Fig. 4) after the removal from the sperm heads.

As noted above, ADAM3-deficient mouse sperm show a severe defect in binding to the egg zona pellucida, despite normal membrane fusion between sperm and egg [15, 16]. Our data presented here strongly support the functional role of ADAM3 in the sperm/egg interaction. Notably, the loss of ADAM3 from the surface of sperm head during the acrosome reaction (Figs. 3 and 4) demonstrates the involvement of ADAM3 not in the membrane fusion between sperm and egg, but in the binding of sperm to the egg zona pellucida.

Acknowledgements

The contributions of E. K. and H. N. to this study are equal. This work was partly supported by Grant-in-Aids for Scientific Research on Priority Areas, for Scientific Researches (A) and (B), for Exploratory Research, for Young Scientists (B) from the Japan Society for the Promotion of Science (JSPS) and the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT), and by the 21st Century COE Program from MEXT. S. I. was supported by a Research Fellowship for Young Scientists from JSPS. We thank Drs. K. Toshimori (Chiba University) and S. B. Moss (University of Pennsylvania) for kind gifts of monoclonal antibody MC101 and anti-AKAP82 antibody, respectively, and Drs. M. Okabe (Osaka University), G. L. Gerton (University of Pennsylvania), and R. Yanagimachi (University of Hawaii) for helpful discussion.
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