A model of the acrosome reaction progression via the acrosomal membrane-anchored protein equatorin

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Abstract

It is important to establish a reliable and progressive model of the acrosome reaction. Here, we present a progression model of the acrosome reaction centering around the acrosomal membrane-anchored protein equatorin (MN9), comparing the staining pattern traced by MN9 antibody immunofluorescence with that traced by Arachis hypogaea agglutinin (PNA)–FITC. Prior to the acrosome reaction, equatorin was present in both the anterior acrosome and the equatorial segment. Since sperm on zona pellucida showed various staining patterns, MN9-immunostaining patterns were classified into four stages: initial, early, advanced, and final. As the acrosome reaction progressed from the initial to the early stage, equatorin spread from the peripheral region of the anterior acrosome toward the center of the equatorial segment, gradually over the entire region of the equatorial segment during the advanced stage, and finally uniformly at the equatorial segment at the final stage. In contrast, the PNA–FITC signals spread more quickly from the peripheral region of the acrosome toward the entire equatorial segment, while decreasing in staining intensity, and finally became weak at the final stage. MN9-immunogold electron microscopy showed equatorin on the hybrid vesicles surrounded by amorphous substances at advanced stage of acrosome reaction. Equatorin decreased in molecular mass from 40–60 to 35 kDa, and the signal intensity of 35 kDa equatorin increased as the acrosome reaction progressed. Thus, the established equatorin-based progression model will be useful for analyzing not only the behavior of equatorin but also of other molecules of interest involved in the acrosome reaction.

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Introduction

The acrosome is a cytoplasmic organelle, a membranous saccule, located at the anterior of a sperm head. The acrosome reaction, which is induced by the zona pellucida, is an essential event for sperm to enter into the perivitelline space. Initiation of the acrosome reaction is characterized by the start of membrane fusion between the plasma membrane and the underlying outer acrosomal membrane (OAM) over the anterior acrosome. During the acrosome reaction, many acrosomal enzymes are released from the acrosome, while some acrosomal molecules are reported to move to the cell surface (Allen & Green 1995, Manandhar & Toshimori 2001, Inoue et al. 2005, Miranda et al. 2009). This evidence implies that the released acrosomal molecules may interact with the pre-existing molecules on the cell surface; this event is thought to be among processes that prime sperm for playing some role(s) during sperm–egg interaction that leads to gamete membrane fusion (reviewed by Yanagimachi (1994) and Toshimori (2009)).

Until now, many studies reported on the acrosome reaction-related acrosomal molecules, e.g. zonadhesin (Hardy et al. 1991, Gao & Garbers 1998, Olson et al. 2004), ACRV1/SP-10 (Foster et al. 1994), AM67/ZP3R/sp56 (Cheng et al. 1994, Foster et al. 1997, Kim et al. 2001a), ZPBP1/sp38/IAM38 (Mori et al. 1995, Yu et al. 2006), AM50 (Westbrook-Case et al. 1994, Kim et al. 2001b), MC41 (Tanii et al. 2002), SAMP32 (Hao et al. 2002), ESP (Wolkowicz et al. 2003), SAMP14 (Shetty et al. 2003), and acrosomal enzymes (reviewed by Moreno & Alvarado (2006)). In previous reports relating acrosomal molecules as described above, the status of the acrosome and the acrosome reaction progression were often measured by acrosome-related lectin stains such as Arachis hypogaea agglutinin (PNA), concanavalin A, and Pisum sativum agglutinin (Kallajoki et al. 1986, Mortimer et al. 1987, Holden et al. 1990, Holden & Trounson 1991, Kinger & Rajalakshmi 1995, Wolkowicz et al. 2003, De Blas et al. 2005, Harper et al. 2008, Mognier et al. 2009), and in transgenic mice such as acrosin-green fluorescent protein (GFP) transgenic mice.
In human sperm, the acrosome reaction progression was identified as having six stages, with the intermediate ones based on transmission electron microscopic observations, in which acrosomal matrix material was lost while outer membranes appeared to retain their integrity (Stock & Fraser 1987, Yudin et al. 1988). However, the structure–molecular relationship during the acrosome reaction is still unclear; this is mainly because acrosomal molecules are nonuniformly (both quickly and slowly) released from the acrosome, showing continuous structural and biochemical changes with various staining patterns (reviewed by Toshimori (2009)). Therefore, when trying to establish a reliable model of the acrosome reaction, it is important to focus on the acrosome domain-specific molecule(s) for which structure and localization are well-characterized. Once such a model is established, we can objectively evaluate the status of the acrosomal molecules of interest, comparing them with the established process model.

To address this issue, we have chosen the acrosomal membrane-anchored molecule equatorin (formerly called MN9 antigen; Toshimori et al. 1992, Yamatoya et al. 2009); MN9/equatorin is a widely distributed acrosomal protein in mammalian sperm, including human sperm. Equatorin is thought to be involved in the process of fertilization (possibly sperm–egg fusion or an early stage of egg activation) because MN9 antibody inhibited fertilization both in vitro (Toshimori et al. 1998) and in vivo (Yoshinaga et al. 2001) without inhibiting zona pellucida penetration or sperm–egg binding. In addition, we have recently analyzed the structure of equatorin (Yamatoya et al. 2009); the molecule is a 40–60 kDa N,O-sialoglycoprotein localized in the acrosome as a type 1 transmembrane protein on the acrosomal membrane. The N-terminus of equatorin faces the acrosomal lumen, while the C-terminus is localized in the cytoplasm. Epitopes of anti-equatorin (MN9) antibody localize around the carbohydrate region on the threonine 138 amino acids (aa); Fig. 1 shows equatorin localization and orientation in the mouse acrosome.

Based on previous work, in this study, we aimed to establish a standard model of the acrosome reaction progression based on imaging (staining pattern) of MN9 immunofluorescence. Our final goal is to apply this model to objectively analyze the structure–molecular relationship of many other molecules of interest involved in the acrosome reaction.

**Results**

**Staining of equatorin of sperm on zona pellucida under IVF**

Next, in order to uncover the physiological behavior of equatorin, sperm on zona pellucida prepared under IVF were analyzed by MN9-IIF. Sperm on zona pellucida

![Figure 1](https://www.reproduction-online.org)

**Figure 1** Illustrations showing the hypothetical molecular structure of mouse equatorin and the localization of the MN9 epitope. This figure was made in reference to the report of Yamatoya et al. (2009). (A) Equatorin is composed of 296 amino acids (aa) (short form) and 337 aa (long form); the conserved domains are 1–20 aa for the signal peptide (SP) and 186–208 aa for the transmembrane domain (TM). The MN9 epitope is present on the region containing sugar chain branching from the threonine (T) at 138 aa. (B) N-terminus of equatorin on the IAM and the OAM faces the acrosomal lumen. IAM, inner acrosomal membrane; OAM, outer acrosomal membrane; PM, plasma membrane.

First, we studied whether or not equatorin staining was the same in the anterior acrosome and equatorial segment; this was done by comparing MN9-indirect immunofluorescence (IIF) pattern changes after permeabilization with methanol (MeOH) and Triton. Intact sperm were found to be negative to MN9 antibody (Fig. 2A and B), as previously reported (Toshimori et al. 1992, Yamatoya et al. 2009). In contrast, MeOH-treated sperm became positive to MN9 antibody; sporadic staining was found at the anterior acrosome, while weak staining was observed at the center of the equatorial segment (Fig. 2C and D). Triton-treated sperm also became positive to MN9 antibody with uniform and strong staining at the equatorial segment, but lost immunogenicity at the anterior acrosome (Fig. 2E and F).
showed various MN9-immunostaining patterns including no MN9 immunostaining (Fig. 3). These staining patterns were classified into four types; type 1–4. The average of number of binding sperm to zona pellucida was $\sim 27.9 \pm 5.2$ sperm/egg ($n=24$ eggs). The percentage of MN9 antibody-positive sperm was $\sim 4.5 \pm 0.7$ sperm/egg. It was difficult to show the statistical analysis of MN9-immunostaining pattern because a small number of MN9-positive sperm was observed. Accordingly, to mimic the progression of spontaneous acrosome reaction under IVF, we aimed to quantitatively analyze large number of sperm under the conditions of spontaneous or artificial acrosome reaction as follows.

**Sequential evaluation of the acrosome reaction progression based on MN9-IIF staining pattern analyses**

To sequentially evaluate the acrosome reaction progression with MN9-immunostaining pattern, MN9-IIF was performed on sperm after spontaneous acrosome reaction at 0, 15, 30, 60 and 120 min, and before artificial induction (termed (Before (−10))) and after artificial acrosome reaction at 0, 15, 30, 60 and 120 min. For this purpose, we used MN9-IIF to first calculate the percentage of sperm classified in each of the four patterns (type 1–4) at each time points (Fig. 4). MN9-immunostaining patterns were classified into four types (as described in the Materials and Methods section) for spontaneous and artificial acrosome reactions.

In brief, the percentages in each type changed as the acrosome reaction proceeded; the type 1 population decreased in number with increasing incubation, while the type 3 and type 4 populations increased in sperm of both spontaneous and artificial acrosome reactions. However, the progression rate or the MN9-positive sperm rate of spontaneous acrosome reaction was low ($\sim 42\%$) when compared with that of artificial acrosome reaction ($\sim 87\%$) at 120 min after incubation.

The percentage of sperm classified in each of the four stages at each time point is shown at the bottom of the line graph (Fig. 4); in spontaneous acrosome reaction (Fig. 4A), the percentage of each pattern of spontaneous acrosome reaction was indicated as follows; type 1 sperm started at $\sim 15\%$ and gradually decreased to $10\%$ at 120 min. The percentage of type 2 started at $\sim 5\%$ and gradually increased to $14\%$ with incubation time. The percentage of type 3 gradually increased up to $13\%$, but the rate of increase was slower than that of type 2. The percentage of type 4 could not be detected until about 30 min and rose to $\sim 3\%$ at 120 min.

In artificial acrosome reaction (Fig. 4B), the percentage of type 1 sperm started at $\sim 25\%$ (highest at 0 min) and gradually decreased to $3\%$ at 120 min. The percentage of type 2 started at $\sim 5\%$ and gradually increased to $14\%$ with incubation time. The percentage of type 3 gradually increased up to $13\%$, but the rate of increase was slower than that of type 2. The percentage of type 4 could not be detected until about 30 min and rose to more than $35\%$ at 120 min.

The percentage of MN9 antibody-positive sperm at ‘0 min’ time point in spontaneous acrosome reaction and the ‘before induction (Before (−10))’ time point in artificial acrosome reaction indicated the amount of sperm that had already started the acrosome reaction not only due to spontaneous acrosome reaction but also due to unavoidable acrosome damage through experimental
procedure(s) such as centrifugation. Basically, the MN9 antibody could not recognize intact sperm acrosomes, as shown in Fig. 2A and B, which show sperm before the acrosome reaction.

In acrosome reaction inhibition experiments with EGTA, which could remove Ca$^{2+}$ from extracellular medium and inhibit the influx of Ca$^{2+}$ into sperm, the immunostaining pattern proportion of four types at 120 min after incubation in 10 μM EGTA in TYH-treated sperm was different from that of artificial acrosome reaction. In the EGTA-treated sperm, the proportion of types 1 and 2 was higher, while that of types 3 and 4 was lower, than those of artificial acrosome reaction (data not shown). Therefore, we thought that the staining pattern change was induced with the acrosome reaction progression, not induced with cell damage or death caused by ionophore's toxicity.

Based on the change of each staining pattern proportion with the acrosome reaction progression, each staining pattern was renamed as follows; type 1 is for 'initial stage', type 2 for 'early stage', type 3 for 'advanced stage', and type 4 for 'final stage'.

**Comparison of staining pattern changes during the acrosome reaction progression by IIF with MN9 antibody and PNA**

We next analyzed the change in fluorescent staining pattern of MN9 antibody during the spontaneous and artificial acrosome reactions, compared with that of PNA. The result was as follows: when the acrosome reaction progressed from the initial stage to the early stage, equatorin became noticeable at the center of the equatorial segment, and the staining then spread over the entire region of the equatorial segment. In contrast, PNA signals were gradually reduced as the acrosome reaction advanced. Typical features of fluorescence images at each stage are described below and shown in Fig. 5.

At the initial stage (Fig. 5A–C), both MN9 antibody and PNA showed diffuse staining patterns on the anterior acrosome (Fig. 5A and B). At the early stage (Fig. 5D and F), MN9 antibody showed almost the same staining pattern as in the initial stage, while PNA signals were reduced at the anterior acrosome compared to the initial stage (Fig. 5B). The equatorial segment was slightly stained with MN9 antibody and PNA (Fig. 5D and E). At the advanced stage (Fig. 5G and I), MN9 antibody showed a rather patchy staining pattern extending toward the center of the equatorial segment (arrow in Fig. 5G), while PNA staining signals were wider and more uniform at the equatorial segment than MN9 antibody signals. Both MN9 antibody and PNA signals were reduced in intensity at the anterior acrosome compared to the initial and early stages (Fig. 5A–E). In the final stage (after acrosome reaction; Fig. 5J–L), MN9 antibody showed a diffuse staining pattern over the entire equatorial segment (Fig. 5I), while the PNA signal was weak and narrow, restricted to only the equatorial segment; the anterior acrosome became barely detectable (Fig. 5K). Since PNA signals of the advanced (Fig. 5H) and the final stages (Fig. 5K) were low in the fluorescence intensity, it took longer exposure time to take the fluorescence images of the advanced and final stages than to take those of the initial and early stages.

**Structure–molecular relationship of equatorin; translocation and interaction with hybrid vesicles**

To understand the details of how equatorin is translocated over the sperm surface of the equatorial segment plasma membrane during the acrosome reaction, as we previously suggested (Manandhar & Toshimori 2001), we used MN9-immunogold electron microscopy (IEM) to analyze the behavior (localization and interaction with surroundings) of equatorin; sperm analyzed were recovered from early (Fig. 6A) and advanced stages.
At the early stage, immunogold particles (equatorin) were dense on the inner acrosome membrane and sparse on the outer acrosome membrane (Fig. 6A). Interestingly, some particles were found in association with the surrounding amorphous substances (Fig. 6A). At the advanced stage, immunogold particles were still found on the inner acrosome membrane (Fig. 6B). Some particles were found in association with hybrid vesicles formed by fusion of plasma membrane and outer acrosome membrane; interestingly, we also found these hybrid vesicles accompanying amorphous substances (Fig. 6B). In order to quantitatively analyze, we tried to determine the quantity of whole equatorin around the acrosome reaction-related components by counting the gold particles during the acrosome reaction, but it was difficult to accurately quantify about all of these components. This was because the acrosome (sperm head) structure and the surrounding environments were so dramatically altered during the acrosome reaction progression that it was difficult to accumulate enough data for statistical analyses.

Reduction of equatorin molecular weight

Next, we were interested in how the molecular size of equatorin changes during the acrosome reaction. This analysis was done by MN9 western blot for sperm that had been incubated for 0, 60 and 120 min after induction of the acrosome reaction. At 0 min, immediately after induction of the acrosome reaction, the antibody detected several bands of equatorin complexes between 40 and 60 kDa (Fig. 7). Interestingly, however, a new 35 kDa band outside of the 40–60 kDa range appeared and increased in signal intensity as the acrosome reaction advanced (Fig. 7).

Discussion

This study provides a progression model of the acrosome reaction based on the immunostaining pattern of equatorin, an acrosomal membrane-anchored protein.

To understand the staining properties of the acrosomal domains (anterior acrosome and equatorial segment) or the outer and inner acrosomal membranes (IAMs), we probed the nature of each acrosomal domain using MeOH and Triton X-100 in the first part of this study. Interestingly, the immunoreactivity between MeOH and Triton X-100 treatments was different in staining pattern, as shown in Fig. 2. Importantly, mild treatment by MeOH allowed MN9 antibody to enter the anterior acrosome, which enabled recognition of equatorin. This is because MeOH-soluble materials in the membrane such as glycolipids were extracted, which eventually increased the access of MN9 antibody in the anterior acrosomal region. Interestingly, however, MeOH did not increase the access of the antibody in the equatorial segment (Fig. 6B).
region; presumably, this is due to not only the different biochemistries but also the region-specific structures of the two domains, since there are periodic cross bridges between the inner and OAMs that span the lumen at the equatorial segment, where the lumen becomes quite narrow (\(\sim 7\)–\(9\) nm in width; Toshimori & Ito 2004). The reason why the MN9 epitope was not easily detected by MN9 antibody might be partially attributed to the nature or high glycosylation around the epitope region (Yamatoya et al. 2009). On the other hand, since Triton X-100 is harsher than MeOH in its effect on plasma membrane, MN9 antibody could easily enter into the equatorial segment lumen. However, it is important to point out the result that Triton treatment resulted in loss of MN9 antigenicity specifically located at the anterior acrosome. This result indicates that Triton treatment is not beneficial for correctly evaluating the localization and behavior of equatorin.

In addition to the different status of different domains between the anterior acrosome and equatorial segment, we have shown differently changed staining pattern at the anterior acrosome and equatorial segment during the acrosome reaction. As shown in Figs 4 and 5, the MN9-IIF changing pattern was more stable and clearer than that of the PNA–FITC pattern (Fig. 4). In fact, when the MN9-IIF pattern was compared with PNA, the MN9 antibody was found to be more appropriate for PNA lectin in terms of stainability and immunostaining stability. In addition, it is apparent that the sugar moiety recognized by PNA (Pereira & Kabat 1976) disappeared more quickly than that of equatorin. In other mammalian sperm, the equatorial segment of acrosome-reacted sperm was stained by PNA, but the staining was reduced or disappeared as the acrosome reaction progressed (Mortimer et al. 1987, Cross & Watson 1994, Cheng et al. 1996, Valcárcel et al. 1997). Since it is known that PNA is more specific to the OAM (Mortimer et al. 1987), such reduction and disappearance of PNA signals could be caused by the loss of OAM. Therefore, the assessment of the acrosome reaction progression by PNA mainly reflects the status of the OAM. This information is important when we consider that the MN9 antibody recognizes the region including carbohydrate moieties on the threonine 138 aa as at least a part of the MN9 epitope (Yamatoya et al. 2009), where the functional domain leading to egg activation is supposed to be localized (Toshimori et al. 1992).

In the literature, there are some reports relating to the present work, the acrosome reaction progression, especially concerning at the initial to early stage. According to a report by transmission electron microscopy that showed the acrosome structural change of human sperm up to hybrid vesicle formation stage (Zanetti & Mayorga 2009), it would be possible that the changes of the acrosomal molecules per se including equatorin and the surrounding substances had occurred before starting the initial stage. However, we found it difficult to accurately address this issue at present mainly because of technical difficulties.

Another report by Buffone et al. (2009b) using acrosin-GFP transgenic mice showed the differences of the rate and region of intra-acrosomal GFP signals loss during the acrosome reaction after comparison with those induced by solubilized zona pellucida proteins.
and A23187 calcium ionophore. The rate of GFP signals loss induced by solubilized zona pellucida proteins was slower than that by A23187. In addition, GFP signals loss induced by solubilized zona pellucida proteins started uniformly at the posterior acrosomal region, while that by A23187 randomly started. Although the details are unknown, these results are thought to be essentially similar to our results, since in this study, we found only small number of MN9-immunopositive sperm on zona pellucida (i.e. under spontaneous acrosome reaction) and since the progression speed of the acrosome reaction was slower than that of artificial acrosome reaction. In addition, Buffone et al. (2009b) proposed that the acrosome reaction orderly occurs with membrane fusion between plasma membrane and OAM under the controlled receptor-mediated manner, forming hybrid vesicles and releasing acrosomal components. In this context, the present model of the acrosome reaction progression based on the equatorin behavior will be useful to further analyze the molecular mechanism during the acrosome reaction.

As for the acrosomal membrane, the IAM has a unique extracellular coat (IAMC; Yu et al. 2006). In this study, we have shown detailed images of equatorin translocation to the sperm surface, i.e. over the equatorial segment plasma membrane at an ultrastructural level (Fig. 6). This evidence suggests that equatorin interacts with the surrounding matrix; in fact, equatorin was surrounded by amorphous substances, which are also found around the hybrid vesicles derived from the IAM (Fig. 6). The other molecule such as G11 antigen (Allen & Green 1995) was relocated to the cell surface accompanying with amorphous substances. This amorphous substance could be regarded to be IAMC. In this context, it will be noteworthy to point out some acrosomal matrix substances (proteins) that could be related to IAMC, as described in the introduction and reviewed by Buffone et al. (2008) and Toshimori (2009). Unfortunately, however, most of these molecules besides equatorin cannot be retained on the sperm surface that can reach the perivitelline space; equatorin can be retained on sperm that have reached the perivitelline space where sperm-egg fusion initiates (C Ito, K Yamatoya, K Yoshida & K Toshimori, 2007–2009, unpublished data, which will be published separately). This line of evidence indicates that re-locatable molecules such as G11 antigen (Allen & Green 1995), equatorin (this study and Manandhar & Toshimori 2001), and Izumo (Inoue et al. 2005, Buffone et al. 2009a) are possibly involved in sperm-egg fusion or the oolemma-binding process, which leads to egg activation.

Another interesting point is the appearance of low molecular mass 35 kDa equatorin with the acrosome reaction progression; the amount or intensity of the low molecular weight equatorin appeared to increase as the acrosome reaction proceeded, as shown in Fig. 7. This evidence strongly suggests that some unknown molecular mechanism modifies equatorin during the acrosome reaction; one possibility is a deglycosylation mechanism, since equatorin is highly glycosylated and sialylated protein and molecular weight of equatorin decrease during maturation in the epididymis possibly by deglycosylation (Yamatoya et al. 2009). Another
possibility is a proteolytic cleavage by acrosomal proteases. Various enzymes including proteases and glycosidases exist in the acrosomal matrix and content (Yanagimachi 1994, Tulsiani et al. 1998, Moreno & Alvarado 2006, Toshimori 2009). Some acrosomal matrix and content proteins such as acrosin (Honda et al. 2002), AM50 (Westbrook-Case et al. 1994), and ZP3R (Buffone et al. 2009a) were processed and/or cleaved to be functionally activated during the acrosome reaction. Although there were several reports analyzing the role of sperm glycosidase(s) at sperm–zona pellucida interaction (Miller et al. 1993, Skudlarek et al. 1993, Tulsiani et al. 1998), we found no reports in which acrosomal glycosidases enzymatically affected on the acrosomal matrices and contents. Therefore, to our knowledge, this study is the first report showing the possibility of the effect of acrosomal glycosidase(s) on the acrosomal matrices and contents. However, at this time, it is unclear how such a deglycosylation and/or proteolytic cleavage might be induced; thus, we are interested in addressing this issue in the next project.

Finally, based on the overall results we have obtained so far, we present a schematic drawing (Fig. 8) describing the proposed localization and molecular size change of equatorin during the acrosome reaction. Our current explanations are as follows: before the acrosome reaction, equatorin is rich on the IAM and rather sparse on the outer acrosome membrane in its highly glycosylated 40–60 kDa form (Yamatoya et al. 2009). As the acrosome reaction proceeds, equatorin on the outer acrosome membrane at the anterior acrosome extracellularly released with hybrid vesicle and amorphous substance (IAMC; Fig. 6), and a part of released equatorin may reassociate to sperm surface (reassociation model; Yanagimachi 1994, Toshimori 2009). Alternatively, equatorin remaining on the IAM (Fig. 6) may diffuse from the acrosomal lumen along the outer acrosomal and plasma membranes to the surface (lateral diffusion model). In the literature, reorganization (redistribution) of lipids and putative associated proteins of sperm head, which occurred during capacitation and acrosome reaction, had been mainly explained by the lateral diffusion model (Gadella et al. 1995, Flesch & Gadella 2000, Boerke et al. 2008). However, we have no data to prove or disprove the translocation mechanism of equatorin at this time. When the acrosome reaction approaches the final stage, some amounts of equatorin appear on the sperm surface of the plasma membrane over the equatorial segment (Manandhar & Toshimori 2001). However, as described above, the details of the translocation mechanism–how equatorin remaining on the acrosomal membranes moves to the sperm surface

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**Figure 8** A schematic drawing showing the change in localization (translocation) of equatorin during the acrosome reaction. Before the acrosome reaction started (intact), equatorin (red) was rich on the IAM and rather sparse on the OAM. There are several possible routes of the appearance of equatorin on the sperm surface. Firstly as indicated by A (reassociation model), as the acrosome reaction proceeds, equatorin on the IAM at the anterior acrosome (AA) detaches and reassociates with the sperm surface, presumably undergoing molecular processing as shown in Fig. 6; some amounts of equatorin were associated with the hybrid vesicles (asterisk) and surrounded by amorphous substance (acrosomal contents). Secondly as indicated by B (lateral diffusion model), equatorin in the acrosomal lumen diffuses to the sperm surface along the acrosomal and plasma membranes. When the acrosome reaction was completed, equatorin was found localized on the surface of the PM over the equatorial segment (ES; also shown in Manandhar & Toshimori 2001); this mechanism is unclear. Some amounts of equatorin remained on the IAM on the AA. During the acrosome reaction progression, low molecular mass equatorin (35 kDa) appeared in addition to the 40–60 kDa range equatorin and increased in the signal intensity (Fig. 7). This process may be attributed to the biochemical modifications such as deglycosylation and/or cleavage. Many of these issues are unclear at present and should be clarified in the future. IAM, inner acrosomal membrane; N, nucleus; OAM, outer acrosomal membrane; PM, plasma membrane.
or how equatorin localized on the hybrid vesicles surrounding amorphous substances reassociates with plasma membrane of the equatorial segment—are unclear. Furthermore, during the acrosome reaction progression, lower molecular mass equatorin (35 kDa) appeared in addition to the 40–60 kDa equatorin and increased in the signal intensity (Fig. 7). Although this molecular weight reduction may be attributed to the biochemical modifications such as deglycosylation and/or cleavage, this issue is totally unclear and remains to be cleared in the future as well as an issue how the reduction of molecular weight relates to the acquisition of function(s) for gamete membrane fusion. In any case, the translocated equatorin is suggested to be involved in the next step leading to egg activation (Toshimori et al. 1992), possibly modifying not only the equatorin per se but also the pre-existing molecules (Toshimori 2009). After the acrosome reaction, some amounts of equatorin remain on the IAM. Thus, the role(s) of equatorin, which could be carried out on the sperm surface and/or on the exposed IAM, should be further clarified in the future.

Previously, lectin probe such as PNA has been used in comparative studies performed under cross-species fertilization conditions to evaluate the status of the acrosome (e.g. Mugnier et al. 2009). When performing such comparative studies, equatorin will be a good index of the acrosome reaction progression, because equatorin is conserved in all mammalian species examined so far including human (Yamatoya et al. 2009; K Toshimori & C Ito, unpublished data).

In summary, the equatorin-based progression model will be useful for analyzing when and where acrosomal molecules change during the acrosome reaction. The information about equatorin translocation will be useful for analyzing how acrosomal molecules interact with the surrounding substance.

Materials and Methods

Animals and chemicals

Animals

Male ICR mice (16 weeks old) and female ICR mice (8–10 weeks old) were purchased from Charles River Japan (Yokohama, Japan) and Takasugi Experimental Animal Supply Company (Saitama, Japan). The animals were killed by cervical dislocation just before beginning experiments. This study was conducted according to the guidelines of Chiba University for the care and use of laboratory animals.

Chemicals

Calcium ionophore A23187 (Nakalai Tesque, Kyoto, Japan) was used to induce the acrosome reaction. Hoechst 33258 (Molecular Probes, Prague, Czech Republic) were used for nuclear staining. FITC-conjugated PNA (PNA–FITC; Seikagaku Biobusiness Co., Tokyo, Japan) was used for acrosome staining. Pregnant mare serum gonadotropin (PMSG; ASKA Pharmaceutical Co., Ltd, Tokyo, Japan) and human chorionic gonadotropin (hCG; ASKA Pharmaceutical Co., Ltd) were used for superovulation of mice.

Antibodies

Primary antibody

Anti-equatorin antibody, MAB MN9 (IgG2a), which specifically recognizes the antigenic molecule equatorin, was produced in female BALB/c mice by immunizing them with cauda epididymal spermatozoa of CD1 mice; production, purification, and characterization of MN9 antibody have been previously reported (Toshimori et al. 1992, 1998, Manandhar & Toshimori 2001, Yoshinaga et al. 2001). Epitopes of MN9 antibody (MN9 epitope) have recently been found on the carbohydrate region around 138 aa (threonine: Yamatoya et al. 2009) as shown in Fig. 1A. MN9 antibody was used at a dilution of 1/20 000 (v/v) (~0.02 μg/ml) for IIF microscopy, at 1/100 (v/v) (~4 μg/ml) for immunogold staining (IGS) for IEM, and at 1/4000 (v/v) (~0.1 μg/ml) for western blot analysis.

Secondary antibodies

Alexa 546-conjugated goat anti-mouse IgG (H+L; Molecular Probes) was used at a 1/4000 (v/v) dilution for IIF. Five nanometer colloidal gold-conjugated goat anti-mouse IgG (BB International, Cardiff, UK) was used at a 1/20 (v/v) dilution for IEM. HRP-conjugated sheep anti-mouse IgG antibody (GE Healthcare Ltd, Buckinghamshire, UK) was used at a 1/10 000 (v/v) dilution for western blot.

Collection of mature sperm

Mature sperm were collected from cauda epididymides by pricking with a fine needle (23 gauge), rinsed twice with PBS or TYH medium (Toyoda et al. 1971), and used for this study. To induce capacitation, some sperm were incubated in TYH medium for 2–3 h in a humidified atmosphere of 5% (v/v) CO2 in air at 37 °C.

Indirect immunofluorescence of mature sperm with MN9 antibody (MN9-IIF)

Some of the collected sperm were treated with 0.1% (v/v) Triton X-100 in PBS at RT for 10 min. Other sperm were put on slides, air-dried, and then treated with MeOH at RT for 10 min for IIF. Triton X-100 or MeOH treatment was done to permeabilize the membrane to increase antibody access. These samples were incubated in PBS containing MN9 antibody overnight at 4 °C, washed in PBS twice, and incubated with secondary antibody alone at RT for 90 min. Nuclei were stained with Hoechst 33258 (1/400 (v/v) dilution). Observation was performed with an Olympus B×50 microscope with a UPlanApo 40× NA 0.85 dry objective lens (Olympus Co., Tokyo, Japan) equipped with an imaging system composed of appropriate filters for fluorescence and a RETIGA Exi FAST 1394 CCD camera.
(Qimaging, Surrey, BC, Canada). Acquisition and storage of the data were controlled by SlideBook 4 software (Intelligent Imaging Innovations, Denver, CO, USA).

**Preparation of sperm on zona pellucida under IVF**

Female mice were stimulated to superovulate by injecting 5 IU PMSG and 48 h after 5 IU hCG injection. Cumulus–egg complexes were collected from the oviductal ampulla 12–14 h after hCG injection. In brief, egg–cumulus complexes were treated with 0.05% hyaluronidase in TYH medium for 10 min to remove cumulus cells, and then the cumulus-free eggs were washed twice in cold PBS, and fixed with 1% (w/v) PFA in PBS for 15 min at RT. These samples were subjected to IIF. These sperm were then incubated in TYH containing 5 nm MN9 antibody overnight at 4 °C. After washing in TYH, the sperm were incubated in TYH containing 0.05% hyaluronidase in TYH medium until the use for IIF.

**Spontaneously induced acrosome reaction (spontaneous acrosome reaction)**

Sperm prepared as above were incubated in a humidified atmosphere of 5% (v/v) CO2 in air at 37 °C in TYH. The sperm were collected after incubation for 0, 15, 30, 60, and 120 min, washed twice in cold PBS, and fixed with 1% (w/v) PFA in PBS for 15 min at RT. These samples were subjected to IIF.

**Artificially induced acrosome reaction by A23187 calcium ionophore (artificial acrosome reaction)**

Sperm prepared as above in cold TYH were treated with 0.01 mol/l A23187 in TYH in a humidified atmosphere of 5% (v/v) CO2 in air at 37 °C for 10 min to induce the acrosome reaction, and washed in TYH. This study was done in IVF dishes. The treated sperm were incubated for 0, 15, 30, 60, and 120 min in a humidified atmosphere of 5% (v/v) CO2 in air at 37 °C, washed twice in cold PBS, and fixed with 1% (w/v) PFA in PBS for 15 min at RT. These samples were subjected to IIF and western blot. Samples without 1% PFA fixation were subjected to western blot.

**MN9-IIF of sperm on zona pellucida**

This study was done in IVF dishes. The samples were incubated in TYH containing MN9 antibody 30 min at RT, washed in TYH twice, and incubated with secondary antibody at RT for 30 min. Nuclei were stained with Hoechst 33258 (1/400 (v/v) dilution). Observation was done as described above. Number of sperm bound to zona pellucida was counted in differential interference contrast and Hoechst images. The number of binding sperm on zona pellucida was expressed as mean±S.E.M.

**Evaluation of the acrosome reaction progression**

The acrosome reaction progression was analyzed by comparing the fluorescence pattern changes of MN9-IIF and PNA–FITC staining of more than 600 sperm at each point described below.

**Classification of the staining pattern of the acrosome reaction progression based on MN9-IIF**

The acrosome reaction was classified into four patterns based on the IIF images obtained from sperm on zona pellucida and at each point. ‘Type 1’ was for sperm that had diffuse staining on the anterior acrosome region, ‘type 2’ was for sperm that had slight staining on the equatorial region in addition to staining on the anterior acrosome region similar to the initial stage, ‘type 3’ was for sperm that had immunofluorescence signals extending from the anterior acrosome and sometimes attenuated toward the equatorial segment, and ‘type 4’ was for sperm that had clear immunostaining on both the anterior acrosome and the equatorial segment, but had much stronger staining on the latter.

**Statistical analysis**

The percentage of sperm in each of the above four patterns was calculated, and the results were expressed as mean±S.E.M. The results were shown in a line graph.

**Analysis of the translocation mode of equatorin during the acrosome reaction**

This analysis was done using IGS for IEM with MN9 antibody (MN9-IEM). First, sperm were incubated in TYH medium for 4–6 h in a humidified atmosphere of 5% (v/v) CO2 in air at 37 °C; this longer time incubation (4–6 h) was aimed to increase the number of spontaneously acrosome-reacted sperm. These sperm were then incubated in TYH containing MN9 antibody overnight at 4 °C. After washing in TYH, the treated sperm were incubated in TYH containing 5 nm colloidal gold-conjugated goat anti-mouse IgG (H+L; 1.4 µg/ml) at 4 °C for 1 h. Then, these sperm were rinsed in TYH, fixed with 1% (v/v) glutaraldehyde, and postfixed with 2.5% (w/v) osmium tetroxide. The fixed sperm were embedded in 2% (w/v) agar, routinely dehydrated through a graded ethanol series, and embedded in Epon 812 (TAAB Laboratories Equipment, Berks, UK). Ultrathin sections were routinely made via free access.
using an ultramicrotome (Ultracut E; Reichert-Jung) and stained with uranyl acetate and lead citrate. The sections were observed with a transmission electron microscope (JEM-1200 EX TEM; JEOL, Tokyo, Japan).

**Western blot with MN9 antibody (MN9 western blot)**

This assay was performed to observe the change in molecular size of equatorin in sperm subjected to the artificial acrosome reaction; the analyses were done at 0, 60, and 120 min after induction of the acrosome reaction. These samples were lysed in SDS sample buffer (0.05 mol/l Tris–HCl (pH 6.8), 2% SDS (w/v), 0.1 mol/l dithiothreitol, 10% (v/v) glycerol, and 0.002% (w/v) bromophenol blue). The lysates were heated for 10 min at 98°C and centrifuged at 16 000 g for 10 min to remove insoluble materials. The lysates (20 μg/lane) were separated in a 12.5% gel by SDS-PAGE and blotted onto a PVDF membrane (Millipore, Bedford, MA, USA). The membrane was soaked in 5% (w/v) skim milk in TBS–T (0.02 mol/l Tris–HCl, (pH 7.6), 0.137 mol/l NaCl, and 0.1% Tween 20 (w/v)) for 30 min or overnight to suppress non-specific background and incubated with MN9 antibody at RT for 60 min. After washing in TBS-T, the membrane was incubated with secondary antibody (HRP-conjugated sheep anti-mouse IgG antibody) at RT for 60 min. The bound antibody was detected by an ECL Western Blotting Detection System (GE Healthcare Ltd) and exposed on X-ray films (GE Healthcare Ltd).

**Declaration of interest**

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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