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Putative sperm fusion protein IZUMO and the role of *N*-glycosylation

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ABSTRACT

IZUMO is the mouse sperm protein proven to be essential for fusion with eggs. It contains one immunoglobulin-like domain with a conserved glycosylation site within. In the present paper, we produced transgenic mouse lines expressing unglycosylated IZUMO (N204Q-IZUMO) in *Izumo1* −/− background. The expression of N204Q-IZUMO rescued the infertile phenotype of IZUMO disrupted mice, indicating glycosylation is not essential for fusion-facilitating activity of IZUMO. The N204Q-IZUMO was produced in testis in comparable amounts to wild-type IZUMO, but the amount of N204Q-IZUMO on sperm was significantly decreased by the time sperm reached the cauda epididymis. These data suggest that glycosylation is not essential for the function of IZUMO, but has a role in protecting it from fragmentation in cauda epididymis.

Glycosylation is the most common post-translational modification of proteins, with glycans involved in many key biological processes such as cell adhesion, molecular trafficking and clearance, receptor activation, signal transduction, and endocytosis [1].

The importance of glycans has been demonstrated in reproductive biology. For example, when the enzyme α-mannosidase IIx (MX) is disrupted, spermatogenic cells fail to adhere to Sertoli cells and are prematurely released from the testis, resulting in male infertility [2]. Disruption of the testis-specific, lectin-type molecular chaperone CALMEGIN leads to the disappearance of various glycoproteins from sperm surface and results in male infertility [3–5].

On the other hand, the importance of *O*-linked glycan of the zona component ZP3, which forms a sperm receptor [6], has been reported. This was reinforced by the experiment in which mouse ZP3 gene was replaced by human gene. The result indicated that the peptide sequence of ZP3 is not the crucial factor for species-specific binding of sperm [7]. Moreover, it has been suggested for many years that D(+)-glucosamine, bovine–albumin–glucosamide and D(+)-galactosamide, fucoidan and dextran sulphate have inhibitory effects on sperm–egg fusion [8], but the mechanism of their actions is not clarified.

Recently, we reported IZUMO null sperm cannot fuse with eggs [9]. Since IZUMO is a member of an immunoglobulin superfamily protein and possesses a well-conserved putative *N*-glycosylation site, we examined the role of the glycosylation in IZUMO. For this purpose, we produced transgenic mouse lines that have IZUMO with no *N*-glycosylation site and crossed to the IZUMO disrupted mouse line. The fertilizing ability of these mice with unglycosylated IZUMO was analyzed both *in vitro* and *in vivo* to elucidate the role of glycosylation in IZUMO.

Materials and methods

Animals, cells, and antibodies. IZUMO-null mice were prepared as indicated in our previous paper [9]. BDF1 male and female mice were purchased from SLC. Chinese hamster ovary (CHO) cells were obtained from RIKEN Cell Bank (RIKEN, Saitama, Japan). Anti-IZUMO monoclonal antibody (#125) was previously generated in our laboratory according to the standard method [3]. All of the experiments were performed with the approval of the Animal Care and Use Committee of Osaka University.

Western blotting. Proteins from testis, corpus epididymis and epididymal sperm were solubilized with 1% Triton X-100, 150 mM NaCl, 50 mM Tris–HCl (pH 8.0) and 1% protease inhibitor cocktail (nakalai tesque, Kyoto, Japan), and was centrifuged at 15,000 rpm for 30 min at 4 °C. The supernatants were denatured by boiling for 5 min in the presence of 1% SDS with or without 6% 2-mercaptoethanol, separated by SDS–PAGE, and transferred onto Immobilon-P membranes (Millipore, MA, USA). After blocking with 10% skimmed milk, the blots were incubated with primary antibodies for 2 h and then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. Immunoreactive proteins were detected by an ECL Western blotting detection kit (GE Healthcare, Little Chalfont, England).

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Deglycosylation assay of IZUMO. The methods for analyses using deglycosidase were described previously [10]. Briefly, sperm were solubilized with solubilization buffer containing 1% Triton X-100, 20 mM Tris-maleate (pH 7.4), 1 mM PMSF for *N*-glycosidase F, or 1% Triton X-100, 20 mM sodium citrate (pH 5.9), 1 mM PMSF for Endglycosidase H, or 1% Triton X-100, 20 mM Tris–maleate (pH 6.0), 1 mM PMSF for *O*-glycosidase. Solubilized proteins were centrifuged at 15,000 rpm for 30 min at 4 °C and the supernatants were treated with 250 mU of *N*-glycosidase, or 250 mU of Endglycosidase, or 1 mU of *O*-glycosidase for 16 h at 37 °C. The samples were subjected to SDS–PAGE followed by Western blotting. IZUMO protein was detected with IZUMO polyclonal antibody.

Production of transgenic mice. A construct was prepared in the pBluescript SK II+ plasmid. We designed testis-specific expression construct inserting *Izumo1* cDNA between *Calmegin* promoter and a rabbit β-globin polyadenylation signal. Point mutation (N204Q) was inserted using the QuikChange site-directed mutagenesis system (Stratagene, CA, USA). All constructs were verified by DNA sequencing using an ABI 310 sequencer (Applied Biosystems, CA, USA).

Transgenic mouse lines were produced by injecting 2.3 kb AseI–XhoI DNA fragment into pronuclei of BDF1 × BDF1 fertilized eggs. Offspring carrying the transgene were identified by PCR using Primer A (5′-CCTTCCTGCGGCTTGTTCTCT-3′) and Primer B (5′-GG TCTCAGAACTTTGCTCCCAAACCCTGTA-3′) for wild-type *Izumo1* and *N204Q-Izumo1* cDNA. The endogenous *Izumo1* and their mutated alleles were detected by PCR using Primer C (5′-GGGTT CACTCTCCAGCTACCCCAAACTCAC-3′) and Primer D (5′-CAGAAC CCCGAACCCAGCCTATGCC-3′), and Primer E (5′-GCTTGCCGAATATC ATGGTGGAAAATGGCC-3′) and Primer D, respectively.

Sperm–egg fusion assay. Mouse sperm were collected from cauda epididymides and capacitated *in vitro* for 2 h in 200 μl drop of TYH medium covered with paraffin oil. Female mice (>8-weeks-old) were superovulated with injection of 5 IU of hCG (human chorionic gonadotropin) 48 h after a 5 IU injection of equine chorionic gonadotropin (eCG). The eggs were collected from the oviduct 14 h after the hCG injection. Eggs were placed in a 200 μl drop of TYH medium. After being freed from cumulus cells with 0.01% (w/v) hyaluronidase, the zona pellucida was removed from mouse eggs using a piezo-manipulator as previously reported [11]. The zona-free mouse oocytes were pre-loaded with Hoechst 33342 by incubating them with 1 μg/ml of the dye in TYH for 10 min. After washing, the eggs were incubated with 2×10^5 sperm/ml incubated for 30 min at 37 °C in 5% CO_2, and unbound sperm were washed away. The eggs were observed under a fluorescence microscope (UV excitation light) after fixing with 0.25% glutaraldehyde. This procedure enabled staining of only fused sperm nucleus by transferring the dye into sperm after membrane fusion as in Fig. 2C.

Observation of zona pellucida-penetrated sperm. B6D2F1 females were superovulated by intraperitoneal injection of 5 IU of eCG, followed 48 h later by 5 IU of hCG. Superovulated females were caged together with test males after hCG injection, and the formation of vaginal plug was observed 20 h later. Eggs were collected from the oviduct and placed in a 200 μl drop of TYH medium. After being freed from cumulus cells with 0.01% (w/v) hyaluronidase, these eggs were washed several times by transferring them into fresh medium. Immunostaining was performed by incubating the fertilized eggs with 1 μg/ml #125 Mab for 1 h at 37 °C in 5% CO_2 in 100 μl TYH medium and subsequently secondary antibody staining was done with 10 μg/ml Alexa fluor 546-conjugated anti-rat IgG (Invitrogen, CA, USA) in 100 μl drop of TYH medium for 1 h at 37 °C in 5% CO_2. After repeated washing to observe zona pellucida-penetrated sperm, four small dabs of Vaserine mix (Vaseline: solid paraffin = 9:1) were applied on a slide glass. The eggs were gently pressed with cover glass to flatten them under the stereoscopic microscope, and were then viewed using a fluorescence microscope.

Results

Glycosylation status in IZUMO

IZUMO has four putative *O*-glycosylation sites at the 268th, 274th, 279th and 282nd threonine according to computer analysis using NetOGlyc 3.1 Server (http://www.cbs.dtu.dk/services/NetOGlyc/). However, when we treated IZUMO with *O*-glycosidase, we could not observe any decrease in molecular weight, suggesting IZUMO does not have any *O*-glycans (Fig. 1B). Since IZUMO was also predicted to possess a well-conserved *N*-glycosylation site in the middle of an immunoglobulin-like loop among species (Fig. 1A), we treated mouse sperm IZUMO with two kinds of *N*-glycosidases and examined the change of molecular weight. The molecular weight of Endo

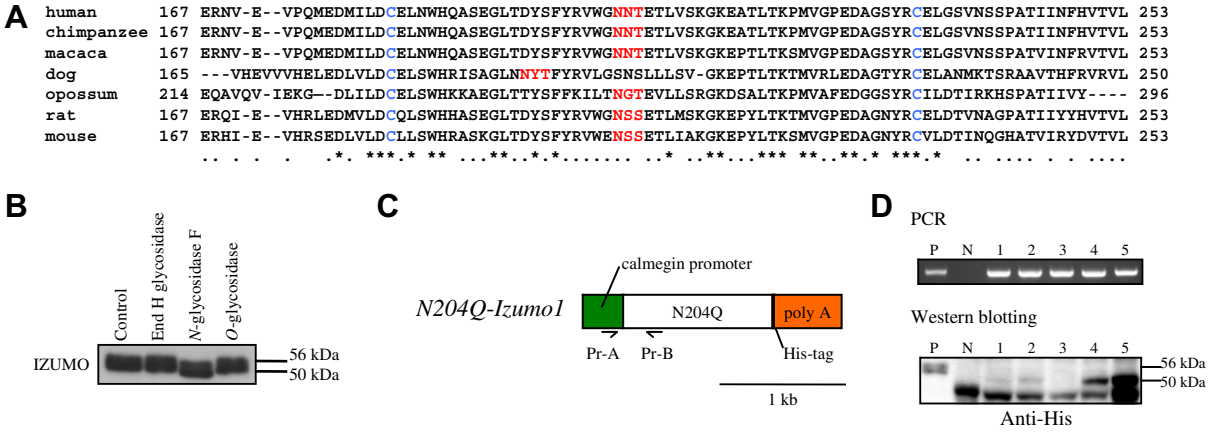

Fig. 1. Deglycosylation study and establishment of N204Q-IZUMO mice. (A) Amino acid sequences of Immunoglobulin-like domain of IZUMO from human, chimpanzee, macaca, dog, opossum, rat, and mouse. A *N*-glycoside link motif and putative cysteine residues that form a disulphide bridge are shown in red and blue, respectively. The similar and identical amino acid residues are shown by a dot and asterisk, respectively. (B) Deglycosylation study of IZUMO. Solubilized sperm proteins (10 μg) were treated with End H or *N*- or *O*-glycosidase. The samples were subjected to SDS–PAGE followed by Western blotting and detected with anti-IZUMO polyclonal antibody under non-reduced conditions. (C) The constructs of transgene to express mouse N204Q-IZUMO were under the control of a *Calmegin* promoter. The locations of a pair of primers (A and B) to clarify the transgene were indicated by arrows. (D) Identification of N204Q-IZUMO-expressing male mice. The upper panel shows 671-bp PCR band using Primer set A and B. The lower panel shows Western blot analysis of testes extracts (50 μg) from transgenic mice. Western blot analysis under reduced conditions was performed by detecting with anti-His tag antibody (Qiagen). The testes extracts from wild-type IZUMO rescue and wild-type were used as a positive and negative control, respectively.

H-treated IZUMO was not altered, but *N*-glycosidase F-treated IZUMO showed higher electrophoretic mobility and the estimated molecular weight changed from approximately 56–50 kDa (Fig. 1B). Since Endo H *N*-glycosidase is reported to hydrolyze only high mannose and some hybrid *N*-glycans, whereas *N*-glycosidase F is reported to cleave most *N*-glycans, including high mannose, hybrid, and complex structures, the digestion results indicate that IZUMO had a complex *N*-glycan structure. Since the stoichiometric molecular mass of IZUMO deduced from its amino acid sequence is 43.5 k, there remains an additional possibility that IZUMO has been modified by some means other than glycosylation, such as phosphorylation or palmitoylation etc.

Establishment of mutated IZUMO expressing mouse line under IZUMO-null background (N204Q-IZUMO)

Since glycan composition is known to be involved in many molecular interaction mechanisms [1], we tried to examine the role of *N*-glycan on IZUMO. Accordingly we produced mouse lines expressing mutated IZUMO by replacing 204th putative *N*-glycosylation site (Asn-X-[Thr/Ser]) aspargine to glutamine by site-directed mutagenesis, and inserted between a testis-specific *Calmegin* promoter and rabbit β-globin polyadenylation signal [4] (Fig. 1C). Since our previous studies showed the addition of His-tag to the carboxy terminal of IZUMO did not affect its function, we performed the above task to make the transgenic protein distinguishable from endogenous IZUMO. After microinjection of the transgene into fertilized eggs and transplantation into pseudopregnant female, we obtained 68 pups and among those, 12 mice were shown to have the transgene by PCR (Fig. 1D; upper panel). Transgenic mouse lines obtained were tested for their expression of mutated IZUMO by Western blot analysis. As illustrated in Fig. 1D, in 4 out of tested 5 transgenic lines, we could detect the production of mutated ~50 kDa IZUMO by anti-His tag antibody. The apparent expression levels of IZUMO in testes of lines #4 and #5 were greater than those of the His-tagged wild-type *Izumo1* transgene that was already shown to rescue the sterility of IZUMO-null male mice [9] (Fig. 1D; lower panel). In the present experiment, lines #4 and #5 were backcrossed to IZUMO-null mice to produce mutant mouse lines that completely lack *N*-glycan of IZUMO (referred to hereafter as N204Q-IZUMO).

The localization of transgenic N204Q-IZUMO and the fertility in male mice

We established N204Q-IZUMO males of #4 and #5 transgenic mouse lines with *Izumo1* −/− background to evaluate the necessity of *N*-glycosylation in IZUMO. Three males from each of the transgenic mouse lines were each caged with three wild-type females and kept for 3 months. Although the litter sizes were smaller compared to the rescued *Izumo1* −/− mice with wild-type IZUMO, the N204Q-IZUMO of both #4 and #5 lines could rescue the infertile sperm back to a fertile state (Fig. 2A). We then performed sperm–

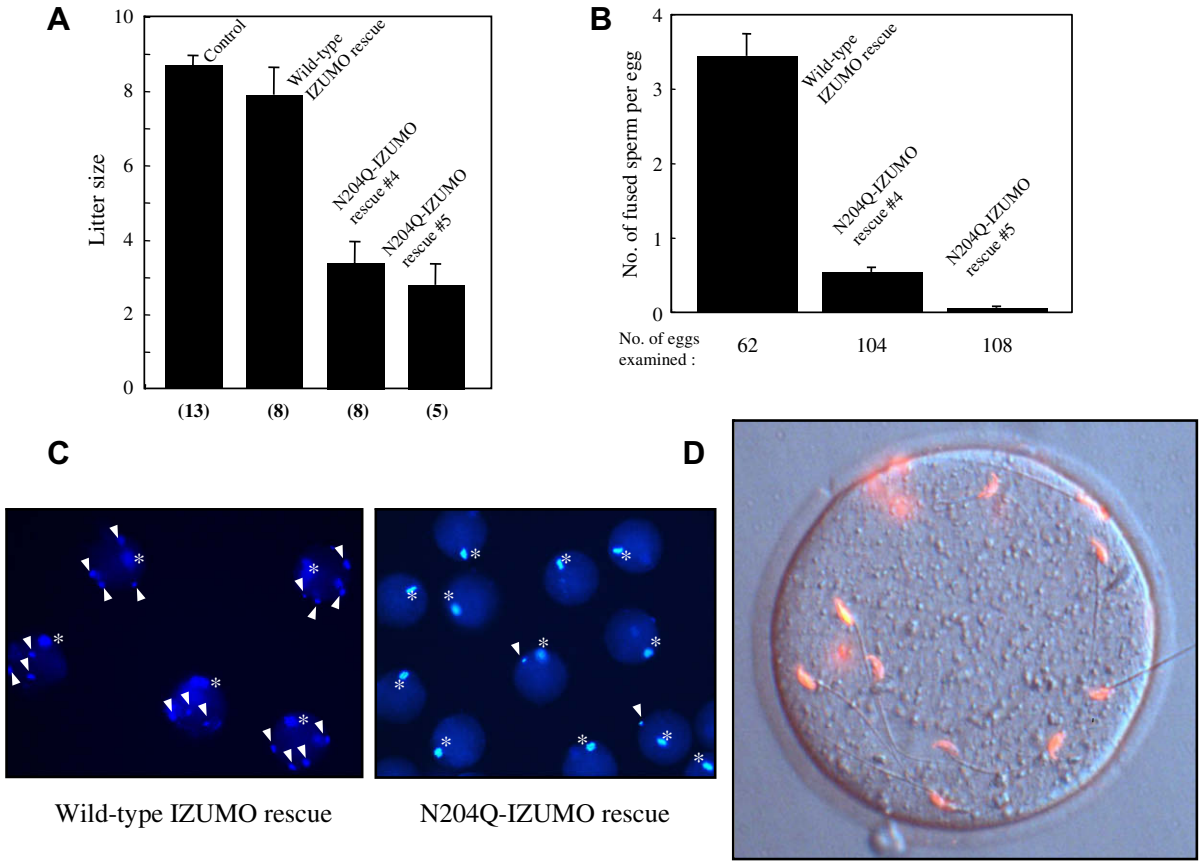

Fig. 2. Fusing ability and subcellular localization of N204Q-IZUMO. (A) Litter size of male mice of control wild-type, and IZUMO-null rescued by wild-type and N204Q-IZUMO (#4, #5 lines) as a transgene. The numbers in parentheses indicate the numbers of mating pairs. Values are presented as means ± standard error of mean (SEM). (B and C) Comparison of the fusing ability of wild-type and N204Q-IZUMO-rescued sperm. Average numbers of fused sperm observed 30 min after insemination ($n = 4$). Values are presented as means ± SEM. Representative photos were shown in C. The arrowheads and asterisks indicate fused sperm and metaphase II-arrested chromosomes, respectively. (D) The localization of N204Q-IZUMO after zona pellucida penetration in naturally acrosome reacted sperm. The migration of N204Q-IZUMO on zona penetrated sperm (acrosome reacted sperm) was normal and seen in the entire head region as observed in wild-type sperm (red signal).

egg fusion assay using zona-free eggs prepared by the piezo-manipulator method [11]. The sperm from both #4 and #5 lines were able to bind to the plasma membrane of eggs as well as to wild-type IZUMO-rescued sperm when observed 30 min after the sperm insemination. The efficiency was low, but sperm from N204Q-IZUMO could fuse with eggs (Fig. 2B and C).

IZUMO is located inside acrosome and exposed on sperm surface only after acrosome reaction. After IZUMO is exposed on the surface, it migrates and spreads across the entire head area including the equatorial segment where sperm–egg fusion takes place [9]. We then examined whether the loss of glycosylation in IZUMO affects the nature of translocation of IZUMO after acrosome reaction. When we stained sperm inside the perivitelline space, N204Q-IZUMO was found to be localized on entire sperm head in the same manner with wild-type IZUMO (Fig. 2D). These data suggest that the absence of *N*-glycan on IZUMO had little or no influence on the transposition of the antigen on sperm surface which accompanied acrosome reaction. However, the staining intensities were dimmer than those of wild-type IZUMO.

Putative function of N-glycan in IZUMO

We previously produced monoclonal antibody (Mab) against mouse IZUMO (#125) [3]. We extracted proteins from testis and sperm from male mice in lines #4 and #5 and analyzed them by Western blot analysis using Mab#125. The N204Q-IZUMO was migrated as a 50 kDa band probably due to the lack of *N*-linked glycan at N204 site. However, in sperm, a severe fragmentation of N204Q-IZUMO was observed while it was not apparent in wild-type IZUMO or testicular N204Q-IZUMO (Fig. 3A). The major fragmented bands were observed at ~30 and 35 kDa area. This fragmentation was not observed in sperm until sperm reached the cauda epididymis (Fig. 3B). It should be noted that the 30 kDa band disappeared when anti-His tag antibody was used, indicating that 35 kDa fragment contains carboxy-terminal His tag, but that the 30 kDa band did not (Fig. 3C).

Although N204Q-IZUMO could rescue the infertile phenotype, the amount of intact N204Q-IZUMO presented on sperm was significantly small compared to wild-type IZUMO in spite of an abundance of N204Q-IZUMO in testis (Fig. 3A). This decrease was

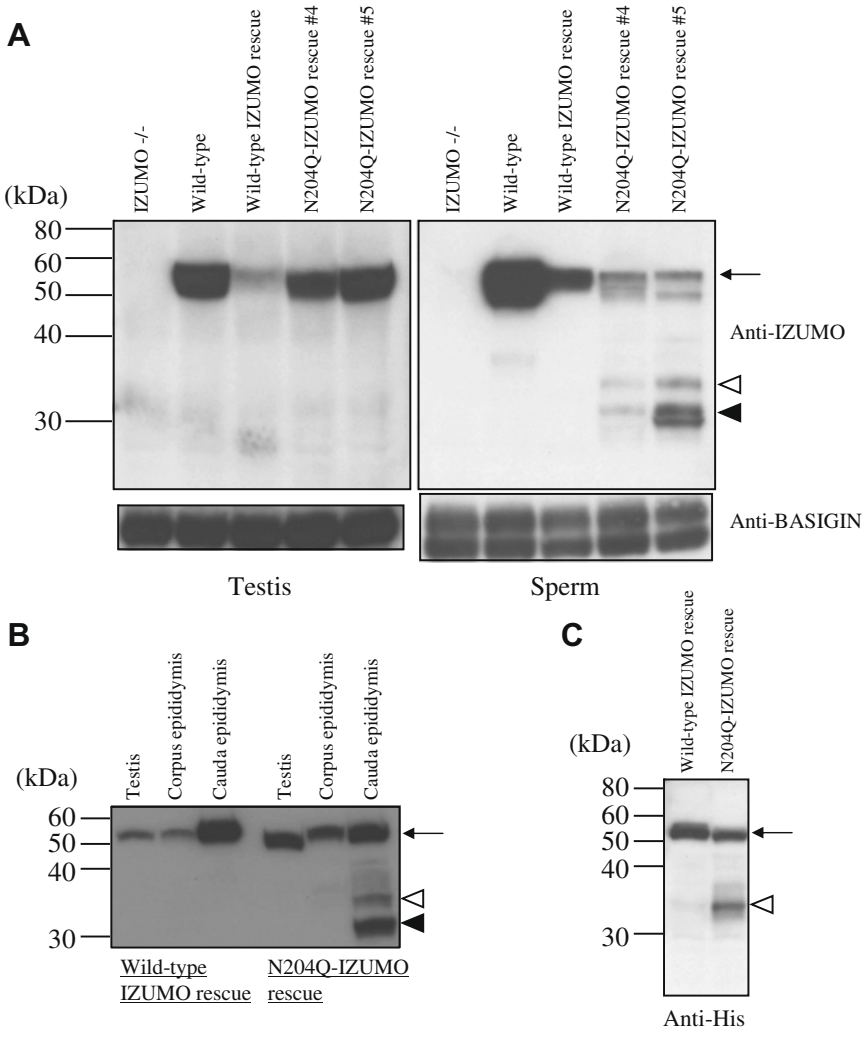

Fig. 3. Fragmentation of N204Q-IZUMO protein in cauda epididymal sperm. (A) N204Q-IZUMO is fragmented by protease in cauda epididymal sperm. Solubilized proteins (30 μg) from testes and sperm were separated by SDS–PAGE in a 10% polyacrylamide gel, and detected with #125 Mab. Immunoglobulin superfamily protein BASIGIN (CD147) was shown in the lower panel as a control. The arrow and filled and unfilled arrowheads are indicated by 50 kDa unglycosylated IZUMO and ~30 and 35 kDa fragmented products, respectively. (B) IZUMO reactivity in testis and epididymis. The 30 μg of proteins from extracts of testis (testicular cell), corpus epididymis (tissue) and sperm from cauda epididymis were separated on a 10% SDS–PAGE and subjected to Western blot analysis. The bolts were incubated with #125 Mab. (C) Western blotting with carboxy terminal antibody. The 30 μg of proteins from cauda epididymal sperm were immunoreacted with anti-His tag antibody. All Western blot analyses were performed under reduced conditions in this figure.

characteristic to N204Q-IZUMO and was not observed in the wild-type IZUMO-rescued mice (Fig. 3A and C).

Discussion

Fragmentation of unglycosylated IZUMO in cauda epididymis

The membranous proteins of sperm are exposed to proteases, and are modified during epididymal transit. For example, testicular angiotensin I-converting enzyme (tACE) is released from the testicular sperm membrane to epididymal fluid when sperm enter the epididymis [12] and this is indispensable for sperm to bind to zona pellucida properly [13].

IZUMO is a membrane protein and is not exposed to the outside of sperm, but is hidden under the plasma membrane before acrosome reaction. Therefore, the fragmentation of unglycosylated IZUMO during epididymal maturation must be caused by acrosomal proteases such as ACROSIN, TESP1, TESP2, PRSS2 and PRSS21, etc. [14–17]. We reported the rise of pH level inside acrosome during the incubation of epididymal sperm in capacitation medium [18]. This pH change could facilitate some enzymes to be activated, but the enzyme that cleaves IZUMO must be activated before capacitation. As far as we know, there are no precedent papers reporting activation of proteases inside acrosome during epididymal maturation. Although the processing of unglycosylated IZUMO is not a physiological phenomenon, the result indicates that some acrosomal enzymes are activated during maturation in epididymis and process membrane proteins. As of today, disruptions of a few acrosomal enzymes are reported, but defects in sperm fertilizing ability is not reported *in vivo* [15,19]. However, as indicated in the present paper, active protease(s) exist inside acrosome. This suggests that a processing of some membrane proteins during epididymal maturation is essential for fertilization.

Subcellular localization and function of unglycosylated IZUMO

In virus, if the fusion (F) proteins are unglycosylated by mutation, it is reported to be transported to the cell surface and consequently not able to mediate cell–cell fusion [20,21]. However, unglycosylated IZUMO was transported to its proper location and spread to the entire head surface after acrosome reaction as in the normal IZUMO, indicating the glycosylation of IZUMO is not essential for proper protein trafficking in sperm.

It was clear that N204Q-IZUMO could rescue the sterile male to fertile without the aid of glycosylation of IZUMO (Fig. 1A). This indicates that the glycosylation is not "essential" for fusion. However, the N204Q-IZUMO rescue was not as effective as wild-type IZUMO rescue. We think there are three possible explanations for the inefficient rescue: (i) the lack of glycosylation decreased the fusion-facilitating ability of IZUMO, (ii) the disappearance of glycan in IZUMO evoked the fragmentation system and only a small amount of IZUMO was retained on sperm, (iii) a mixture of (i) and (ii).

Numerous glycoproteins have been proven to be involved in sperm–egg interaction. [6,10]. However, the role of their glycan moiety remains unclear. Using the gene disruption and its rescue by mutant proteins, we could demonstrate that at least one of the roles of *N*-glycan in IZUMO is to protect IZUMO from fragmentation during sperm maturation in epididymis. As far as we searched, this is the first *in vivo* indication of glycans proteolytic fragmentation protecting activity.

Acknowledgments

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