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Pig sperm membrane microdomains contain a highly glycosylated 15–25-kDa wheat germ agglutinin-binding protein

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ABSTRACT

A highly glycosylated protein, which has unique, novel features in localization, structure, and potential function, is found in pig sperm, and named WGA-gp due to its high binding property with wheat germ agglutinin (WGA). WGA-gp is localized mainly in flagella and enriched in membrane microdomains or lipid rafts. It is not detected by ordinary protein staining methods due to a high content of both *N*- and *O*-glycans consisting of neutral monosaccharides. Interestingly, WGA-gp may be involved in intracellular Ca^{2+} regulation. Treatment of sperm with anti-WGA-gp antibody enhances the amplitude of Ca^{2+} oscillation without changing the basal intracellular Ca^{2+} concentrations. All these features of WGA-gp, except for different carbohydrate structures occupying most part of the molecules, are similar to those of flagellasialin in sea urchin sperm, which regulates the intracellular Ca^{2+} concentration. Presence of carbohydrate-enriched flagellar proteins involved in intracellular Ca^{2+} regulation may be a common feature among animal sperm.

1. Introduction

Membrane microdomains are well recognized as the cell surface sites of cellular interactions and the subsequent signal transductions [1,2]. They are unique areas of biomembranes that are enriched in sphingolipids, cholesterol, and glycosylphosphatidylinositol (GPI)-anchored proteins. Although not so well recognized, an unique feature of microdomains is an enrichment of particular glycan chains derived from glycolipids and glycoproteins [3,4]. However, few studies have focused on functions of those glycan chains on the microdomains. In 1999 the first biochemical characterization of sperm microdomains were reported by us using sea urchin [5]. Many reports have since been made on gamete microdomains in various species [6–8]. For sea urchin sperm microdomains, we have demonstrated their implication in sperm–egg interactions during fertilization [9,10].

Of glycoproteins of sea urchin sperm microdomains, a heavily glycosylated protein localized in sperm flagella, named flagellasialin, is involved in sperm motility by regulating the intracellular Ca^{2+} concentration, $[Ca^{2+}]i$ [11,12]. Flagellasialin contains a high content of glycans (80–90% by weight) on a short polypeptide (7.2 kDa) and it appears as a 40–80 kDa smear on SDS–PAGE [11,13]. Because of the unique structural features and polydispersive nature due to the glycan heterogeneity, flagellasialin cannot be detected by convential methods for protein staining using Coomassie Brilliant Blue (CBB) or silver nitrate. We have recently reported that flagellasialin is involved in Ca^{2+}-dependent mechanosensory behavior of sea urchin sperm when the gametes encounter and try to avoid physical obstacles [12]. In response to mechanical stimulation, the $[Ca^{2+}]i$ of sperm is transiently increased to stop sperm motility and subsequently decreased to the original level to allow sperm to move to a different direction. Flagellasialin in the sperm flagellum is suggested to be involved in the decreasing process by facilitating the Ca^{2+} efflux from the gametes. Regulation of the $[Ca^{2+}]i$ is an important physiological event in animal sperm [14–16]. However,

Abbreviations: APM, anterior plasma membrane; BTS, Beltsville Thawing Solution; CBB, Coomassie Brilliant Blue; CM, capacitated medium; DIC, differential interference contrast; LD-DIM, low density detergent-insoluble membrane; NCM, non-capacitated medium; PGC, Percoll gradient centrifuged; PVDF, polyvinylidene difluoride; WGA, wheat germ agglutinin.

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no report has been made on how heavily glycosylated proteins like flagellasialin are involved in Ca^{2+} regulation through the microdomain. Even whether such glycoproteins are ubiquitously present in sperm microdomains from other animal species remains to be elucidated.

Therefore, an objective of this study is to address the question of whether a similar glycoprotein to flagellasialin exists in mammalian sperm. Here we report the presence of such a glycoprotein in pig sperm.

2. Materials and methods

2.1. Sperm preparation

Ejaculated sperm from *Sus scrofa* (Duroc, 2-year old) were provided by Ishikawa Pig Farm (Aichi, Japan). Sperm preparation was performed as described [17]. Sperm were diluted with Beltsville Thawing Solution (BTS: 200 mM glucose, 3 mM EDTA, 20 mM sodium citrate·$2H_2O$, 15 mM $NaHCO_3$, 10 mM KCl, pH 7.4), washed twice with the non-capacitating medium (NCM: 100 mM NaCl, 0.36 mM $NaH_2PO_4 \cdot 2H_2O$, 8.6 mM KCl, 23 mM HEPES, 0.5 mM $MgCl_2 \cdot 6H_2O$, 11 mM glucose, pH 7.6) by centrifugation (200×*g*, 10 min, 28 °C) and were then resuspended in NCM (non-capacitated sperm). The non-capacitated sperm suspension was applied on the top of the 30/70% Percoll (GE Healthcare Bio-Sciences AB, Sweden) gradient and centrifuged (28 °C, 200×*g*, 5 min and then 800×*g*, 25 min) to remove dead sperm and seminal gel particles. The Percoll gradient centrifuged non-capacitated sperm were obtained as a pellet (PGC sperm). PGC sperm were suspended in the capacitating medium (CM: NCM containing 10 mM $NaHCO_3$, 2 mM $CaCl_2 \cdot 2H_2O$, 5 mM sodium pyruvate, 3 mg/ml bovine serum albumin (BSA), pH 7.6) at a final concentration of 2.0×10^7 sperm/ml and incubated (2 h, 38 °C 5% CO_2) (capacitated sperm).

2.2. Preparation of the head and flagellum fractions, and sperm head anterior plasma membranes (APMs)

All the procedures were performed as described previously [17].

2.3. Preparation of the low density detergent-insoluble membrane (LD-DIM)

LD-DIM fraction was prepared from sperm (200–300 μl as sperm pellet) as described previously [5,18].

2.4. Purification of WGA-gp

Non-capacitated sperm was incubated in 0.15 M NaCl, 10 mM Tris–HCl (pH 7.5), 1% Triton X-100, 5 mM EDTA, 1 mM PMSF, and Protease inhibitor cocktail (PIC; 1 μg/ml leupeptin, 2 μg/ml antipain, 10 μg/ml benzamidine, 1 μg/ml pepstain, 0.9 μg/ml aprotinin) at 4 °C for 2 h with gentle rotation. At the end of the incubation, the sperm suspension was centrifuged at 10,000×*g* at 4 °C for 30 min. The collected supernatant was diluted 10-fold with 50 mM sodium acetate buffer (pH 4.0), and applied to a SP-Toyopearl 650 M column (1.2 × 29.5 cm; Tosoh, Tokyo) pre-equilibrated with 50 mM sodium acetate buffer (pH 4.0) containing 0.1% Triton X-100. The flow-through fraction was collected, dialyzed against 10 mM Tris–HCl (pH 8.5) containing 0.1% Triton X-100, and applied to a SP-Toyopearl 650 M (1.2 × 29 cm) pre-equilibrated with 10 mM Tris–HCl (pH 8.5) containing 0.1% Triton X-100. The column was eluted with a linear gradient of NaCl (0–400 mM) in 10 mM Tris–HCl (pH 8.5) containing 0.1% Triton X-100. Each fraction was analyzed by WGA lectin blotting. The obtained WGA lectin-positive fractions were pooled, and dialyzed against 10 mM Tris–HCl (pH 8.0), and quantitated for protein using a BCA assay kit (Pierce, Rockford, IL) using BSA as a standard.

2.5. Chemical analyses and enzyme digestion

For amino acid sequence analysis, WGA-gp (5 μg) was applied onto the polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) using a Pro Sorb™ kit (Perkin–Elmer, Waltham, MA) and washed with 0.1% trifluoroacetic acid (TFA). The WGA-gp containing spot on the PVDF membrane was punched out, and added with a BioBrene solution. It was washed with 0.1% TFA and then added methanol. After air-drying our methanol, it was subjected to amino acid sequencing by automated Edman degradation on a Procise HT analyzer (Applied Biosystems, Foster City, CA). Carbohydrate composition analysis was performed as described [19]. Peptide: *N*-glycanase F (PNGaseF; Takara, Japan) digestion, alkaline treatment, periodate oxidation, and deglycosylation using trifluoromethanesulfonic acid (TFMS; Sigma, St. Louis, MO) were performed as described [20–22].

2.6. Preparation of antisera against WGA-gp

Ddy mice (8-week-old females, Nippon SLC, Japan) were immunized by intraperitoneal injection with the suspension of purified WGA-gp (5 μg) first with Freund's complete adjuvant (Wako, Japan) and subsequently with the incomplete adjuvant (Wako, Japan) at 2-week intervals.

2.7. Western and WGA lectin blotting

Sperm lysate was prepared as described [5]. The sperm lysate and the LD-DIM were subjected to SDS–PAGE on 15% polyacrylamide gels followed by electroblotting onto the PVDF membrane as described [5]. The membrane was blocked with 1% BSA in PBS containing 0.05% Tween 20 (PBS-T) at room temperature for 1 h. For WGA lectin blotting, the membrane was incubated with WGA (1 μg/ml in PBS; Seikagaku, Japan) at 37 °C for 1 h. After washing, the membrane was incubated with anti-WGA serum (1:3000 dilution; Sigma). As the secondary antibody, the peroxidase-conjugated anti-rabbit IgG (1:4000 dilution; Seikagaku) was used. Washing procedures and the color development were carried out as described [23]. For immunoblotting, the electroblotted membrane was probed with the primary antibody (anti-WGA-gp, 1:1000 dilution), detected with the peroxidase-conjugated anti-mouse IgG + IgM (1:5000 dilution; American Qualex, La Mirada, CA).

2.8. Immunofluorescent microscopy

PGC non-capacitated sperm were fixed with 4% paraformaldehyde in PBS, and deposited onto poly-L-lysine-treated glass coverslip. After 10 min, the coverslip was washed three times with PBS. The blocking was performed with 2% BSA/PBS for 1 h at room temperature. After three washes with PBS, the coverslip was incubated at 37 °C for 30 min with anti-WGA-gp antiserum (1:100 dilution in PBS). After four washes with PBS, the coverslip was incubated with Alexa 488-conjugated goat anti-mouse IgG antibody (1:500) at 37 °C for 30 min. After washing 5 times with PBS and 1 time with water, the coverslip was mounted with PermaFluor™ Aqueous Mounting Medium (Thermo Shandon, Pittsburgh, PA) on a glass slide for observation under an Olympus BX51 microscope equipped with epifluorescence as well as with differential interference contrast (DIC) functions.

2.9. Measurements of intracellular Ca^{2+} concentration

PGC sperm were washed twice with 5 ml of the physiological medium (PM, 145 mM NaCl, 0.5 mM $MgSO_4$, 5 mM KCl, 1 mM

Na_2HPO_4, 1 mM $CaCl_2$, 5 mM glucose, 10 mM HEPES, pH 7.4) by centrifugation at 150×*g* at 24 °C for 10 min. Pelleted sperm were suspended at 1.7×10^7 cell/ml in PM and incubated with 1 μM fura-2 (Molecular Probes, Eugene, OR) at 38 °C for 1 h. The fura-2 loaded sperm were washed and suspended with 5 ml PM, and 600 μl of the sperm suspension was transferred to quarts cuvette, thermostatically maintained at 38 °C under stirring condition. Ca^{2+} measurements were performed on FluroMax-3 florometer (Jobin Yvon-Spex, Park Avenue, NJ) with excitation at 340 and 380 nm, and emission at 500 nm [11]. The fluorescence was monitored for 10 min after addition of appropriately diluted anti-WGA-gp antiserum or preimmune serum to the fura-2 loaded sperm suspension. The ratio of intensities at 340 and 380 nm (F340/F380) reports relative intracellular Ca^{2+}.

3. Results

Based on the nucleotide and amino acid sequences of sea urchin flagellasialin [11], we performed homology search of various gene and protein databases for its structural homolog(s). However, no homologous protein was found. We also performed immunoblotting of pig sperm using monoclonal antibody 4F7, which recognizes the glycan part of flagellasialin [11]. No 4F7 epitope was detected in pig sperm. In light of the involvement of flagellasialin in intracellular Ca^{2+} regulation during fertilization [11,13], we hypothesized that a flagellasialin-like protein, if existing in pig sperm, should contain the following properties: (i) it is a component of membrane microdomains, or the LD-DIM fraction; (ii) it is localized in the sperm flagellum; (iii) it is heavily glycosylated, appearing as a polydispersed smear on SDS–PAGE, which is invisible with CBB and silver staining; and (iv) it is involved in regulation of sperm intracellular Ca^{2+}. To search for a microdomain-localized glycan-rich glycoprotein that gives polydispersed smear on SDS–PAGE, LD-DIM fractions were prepared from fresh non-capacitated and capacitated pig sperm, and analyzed by lectin blotting using various lectins, such as WGA, MAA, SNA, PNA, ConA, GS-I-B4. Only WGA detected an unique smear at 16–30 kDa and 15–25 kDa in non-capacitated and capacitated sperm, respectively (Fig. 1). To further identify these WGA lectin reactive smear components, we sought to purify the components using various chromatographic procedures. We finally established a relatively simple purification method from fresh non-capacitated sperm. Briefly, the sperm lysate obtained in the presence of Triton X-100 was first subjected to SP-Toyoperal chromatography under an acidic condition (pH 4.0). A WGA-epitope-containing smear at 15–25 kDa was obtained in the flow-through fraction (Fig. 2A), and was then subjected to SP-Toyoperal chromatography under an alkaline condition (pH 8.5), followed by elution with a linear salt gradient (Fig. 2B). The WGA-positive glycoprotein was obtained at 50–100 mM NaCl-eluted fraction, and named WGA-gp.

WGA-gp was detected as a 15–25 kDa smear only by WGA lectin blotting, but not by CBB (data not shown) or silver staining (Fig. 2C). The purified WGA-gp is of high homogeneity in that it did not contain any contaminating proteins detected by a sensitive silver staining. Edman degradation of the intact WGA-gp showed that the first 4 amino acid residues from the N-terminus were of an unique sequence, i.e., X-Arg-Thr-Ser-, where X was an unidentified residue. When TFMS-treated WGA-gp was analyzed, the N-terminal sequence was Asp/Asn-Arg-Thr-Ser-, with Asp and Asn detected in the first cycle. These results suggest that the N-terminal amino acid is a modified Asn residue, which may be changed to Asn and Asp residues by the strong acid treatment. The N-glycosylation might be linked to the Asn residue, because the first 3-amino-acid sequence, Asn-Arg-Thr, corresponds to the consensus triplet of N-glycosylation sites. The N-terminal sequence of WGA-gp is thus uniquely determined to be Asn-Arg-Thr-Ser-, suggesting that WGA-gp is not a mixture of heterogeneous peptides, but a homogeneous peptide (Fig. 2C).

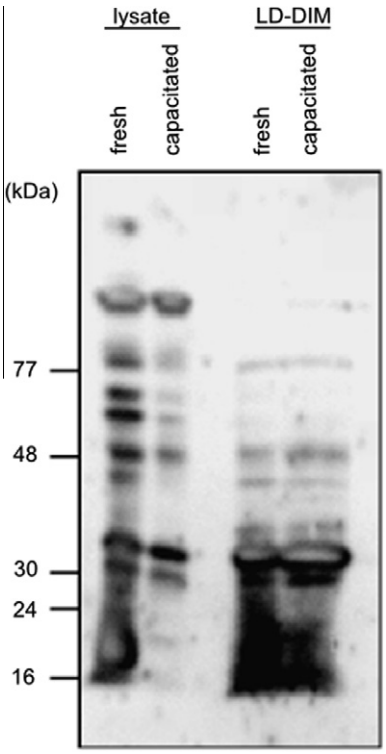

Fig. 1. WGA lectin blotting of fresh and capacitated sperm and their LD-DIM fractions.

GLC analysis showed that carbohydrate composition of WGA-gp was Man/Gal/GalNAc/GlcNAc = 3.0:5.3:3.4:7.6 in molar ratios where Man was set at 3.0 mol. This composition suggests the presence of both *N*- and *O*-glycans. The presence of *N*-glycan is consistent with the presence of a consensus N-glycosylation site as described above. However, PNGase F digestion had no effect on the behavior on SDS–PAGE/WGA lectin blotting (Fig. 2D), suggesting that the *N*-glycan(s) appears to be resistant to PNGase F digestion, and this might be due to specific resistance of the *N*-glycan at the N-terminal Asn residue to the enzyme. On the other hand, alkaline treatment, which facilitates β-elimination of *O*-glycans, resulted in a large reduction of intensity of the lectin blotting (Fig. 2E). These results suggest that WGA-gp contains at least *O*-glycans with the WGA-epitope in its major part. Provided that all the GalNAc residues are attached on Ser or Thr residues of WGA-gp, the molar proportion of *O*- to *N*-glycan would be about 3:1. These glycosylation features of WGA-gp suggest that a polydisperse nature of WGA-gp on SDS–PAGE is due to the heterogeneity of the glycan structures. WGA lectin recognizes non-reducing terminal GlcNAc and Neu5Ac residues [24]. Since only a little Neu5Ac was detected in WGA-gp, the WGA-epitope might be due to terminal GlcNAc residues.

The purified WGA was detected at 15–25 kDa by Western Blotting using the polyclonal antibodies anti-WGA-gp (Fig. 2F). The immunostaining with anti-WGA-gp largely disappeared after periodate oxidation of WGA-gp (Fig. 2F), thus suggesting that the carbohydrate part of WGA-gp is at least involved in anti-WGA-gp epitope. Western Blotting of the LD-DIM fractions prepared from PGC sperm and PGC capacitated sperm showed that WGA-gp was present as a component of LD-DIM before and after capacitation (Fig. 2G), consistent with the results obtained for the 15–25 kDa smear by WGA lectin blotting (Fig. 1).

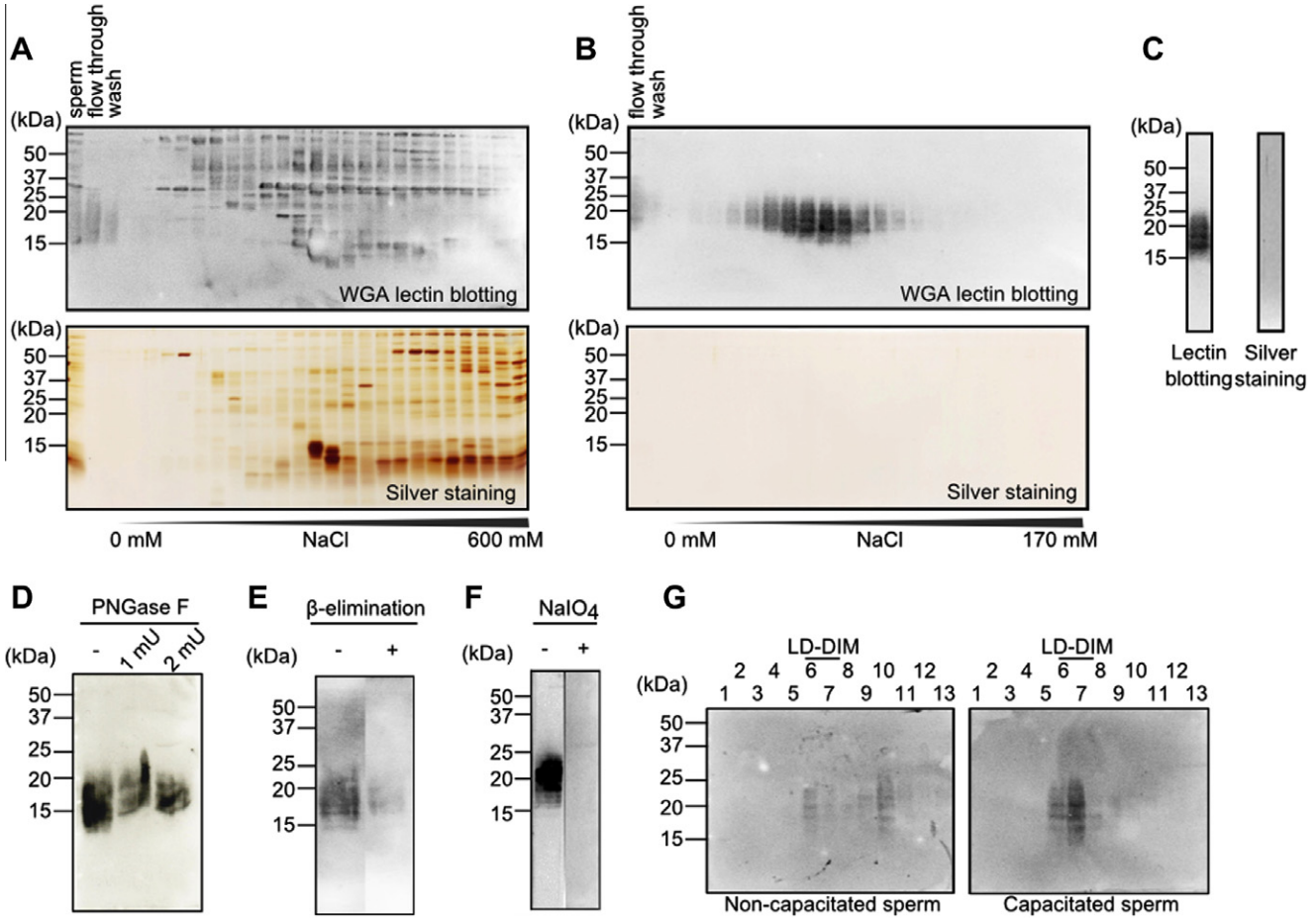

Fig. 2. Purification of WGA-gp. (A) SP-Toyopearl chromatography at pH 4.0. The lysate of non-capacitated sperm was applied to the column, and proteins were washed, and eluted with a linear gradient of NaCl (0–400 mM) as indicated at the bottom of lower panels. On the top, the fraction names and tube numbers collected on the chromatography are shown. Molecular weight markers in kDa are shown on the left. Upper, WGA lectin staining; Lower, silver staining. (B) SP-Toyopearl chromatography at pH 8.5. The flow-through fraction in (A) was applied to the column, and proteins were washed and eluted with a linear gradient of NaCl (0–400 mM). (C) WGA lectin blotting and silver staining of purified WGA-gp. (D) WGA lectin blotting of purified WGA-gp untreated (−) and treated with 1 and 2 milliunits (mU) of PNGase F. (E) WGA lectin blotting of purified WGA-gp treated with (+) and without (−) β-elimination. (F) Western Blotting using anti-WGA-gp antibody for purified WGA-gp untreated (−) and treated (+) with sodium periodate ($NaIO_4$). (G) Western Blotting of each fraction obtained during preparation of the LD-DIM by sucrose-density gradient centrifugation of the Triton X-100 lysates of non-capacitated (*left panel*) and capacitated sperm (*right panel*). Underlined fractions 6 and 7 correspond to the LD-DIM fraction.

To localize WGA-gp on sperm, the head and flagellum fractions were prepared and subjected to Western Blotting with anti-WGA-gp. The head and flagellum fractions were successfully prepared with high purity based on microscopic observations (Fig. 3A). WGA-gp was detected in flagellum fraction, but not in the head fraction (Fig. 3B). These results suggested that WGA-gp is mainly localized in the flagellum. To examine if WGA-gp is also present in sperm head, the APM, a tip of sperm head, fractions derived from PGC non-capacitated and capacitated sperm [9] were analyzed by Western Blotting with anti-WGA (Fig. 3C). Although mostly present in the sperm particulate that contained flagellar fragments, WGA-gp was detected in the APM fraction. Immunostaining of sperm was also performed using anti-WGA-gp (Fig. 3D). The flagellum was immunostained, while immunostaining of the head part was sometimes weak or missing. Taken all together, these results indicate that WGA-gp is present mainly in flagellum, but also in APM of sperm head.

Finally, to ask if WGA-gp is involved in the intracellular Ca^{2+} regulation, the effect of anti-WGA-gp serum on the intracellular Ca^{2+} concentration of sperm was examined. As shown in Fig. 4C, the anti-WGA-gp serum caused enhanced amplitudes of oscillation of intracellular Ca^{2+}, although the overall drift of Ca^{2+} concentration remained unchanged. Preimmune serum (Fig. 4B) and antibodies against WGA16 (data not shown), a glycoprotein unrelated with WGA-gp in pig sperm, had no effect on the amplitude change of Ca^{2+} oscillation, compared with no addition control (Fig. 4A). Therefore, it is suggested that the change of Ca^{2+} oscillation is WGA-gp-dependent. Considering that the anti-WGA-gp antibody recognizes the glycan parts (Fig. 2F), these results suggest that WGA-gp may be involved in intracellular Ca^{2+} regulation through the glycan moieties.

4. Discussion

In this study, we found a microdomain-enriched unique glycoprotein from pig sperm, which shows similarities to flagellasialin from sea urchin sperm in localization, overall structural features, and potential physiological functions [11–13]. First, WGA-gp is mainly localized in sperm flagellum, as is the case with flagellasialin. Second, WGA-gp is heavily glycosylated and shows polydispersive nature in SDS–PAGE probably due to a high heterogeneity differences of glycan sizes. WGA-gp contains both *N*- and *O*-glycan chains consisting of neutral monosaccharides. In contrast, flagellasialin contains several acidic *O*-glycan chains with α2,9-linked poly-*N*-acetylneuraminic acid (α2,9-polySia) moieties

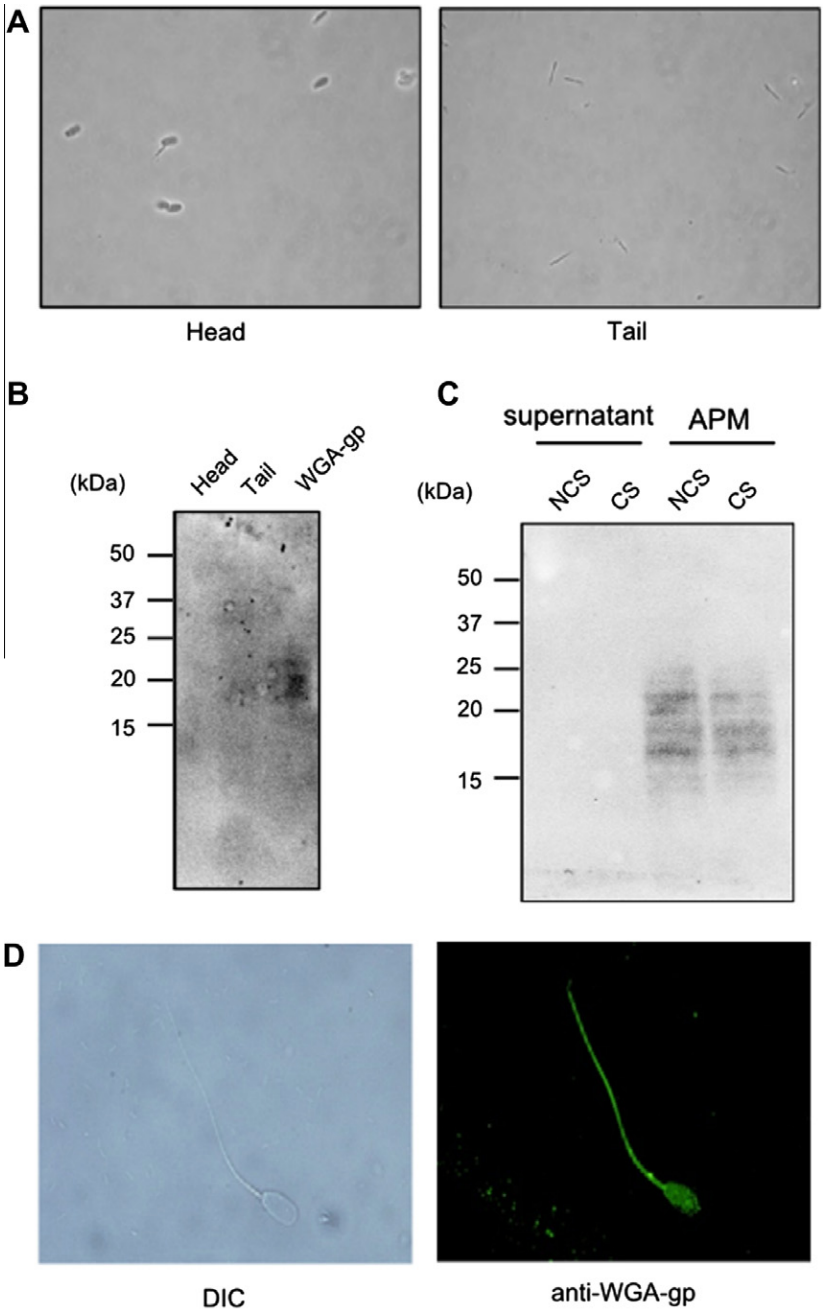

Fig. 3. Immunolocalization of WGA-gp in sperm. (A) DIC microscopy of head and tail preparations from non-capacitated sperm. (B) Western Blotting of the head and tail fractions with anti-WGA-gp. (C) Western Blotting of cytosolic fraction (*supernatant*) and APM (*APM*) from non-capacitated sperm (*NCS*) and capacitated sperm (*CS*) with anti-WGA-gp. (D) Immonofluorescent and DIC microscopy of non-capacitated pig sperm using anti-WGA-gp and Alexa488-labeled anti-mouse IgG antibody as the first and secondary antibodies. Preimmune serum instead of anti-WGA-gp had no staining (data not shown).

[11,13]. Irrespective of these differences, they share a common feature of having a small protein part but large glycan moieties. Third, most interestingly, WGA-gp may be involved in $[Ca^{2+}]i$ regulation. Anti-WGA-gp antibody enhanced the amplitude of Ca^{2+} oscillation without changing the basal $[Ca^{2+}]i$. On the other hand, anti-flagellasialin antibody recognizing α2,9-polySia structure increases the $[Ca^{2+}]i$ [12,13]. Thus, the mode of action of the specific antibody to each protein on the $[Ca^{2+}]i$ is different; however, both of these molecules affect the $[Ca^{2+}]i$ regulation. These common features lead us to consider somewhat general roles of a glycan chain-enriched protein in sperm flagellar microdomains. Experiments are underway in our laboratory to elucidate its roles in the regulation of intracellular Ca^{2+} oscillation.

Capacitation occurs in the female reproductive tract *in vivo* to prepare sperm for the full fertilizing ability. Capacitation can also be induced *in vitro*, thus facilitating the gaining of information related to the capacitation process. All evidence indicates that the sperm surface membrane remodeling is a major event taking place during capacitation. This remodeling includes removal and acquisition of specific proteins, glycoproteins and lipids [16,17]. As shown in Fig. 1, WGA lectin detected at least two polydispersive components at 16–30 kDa and 15–25 kDa in non-capacitated and capacitated sperm, respectively. As we showed in this study, the 15–25 kDa component was identified as WGA-gp. WGA-gp is consistently localized in the microdomain of pig sperm during capacitation. On the other hand, the 16–30 kDa component is

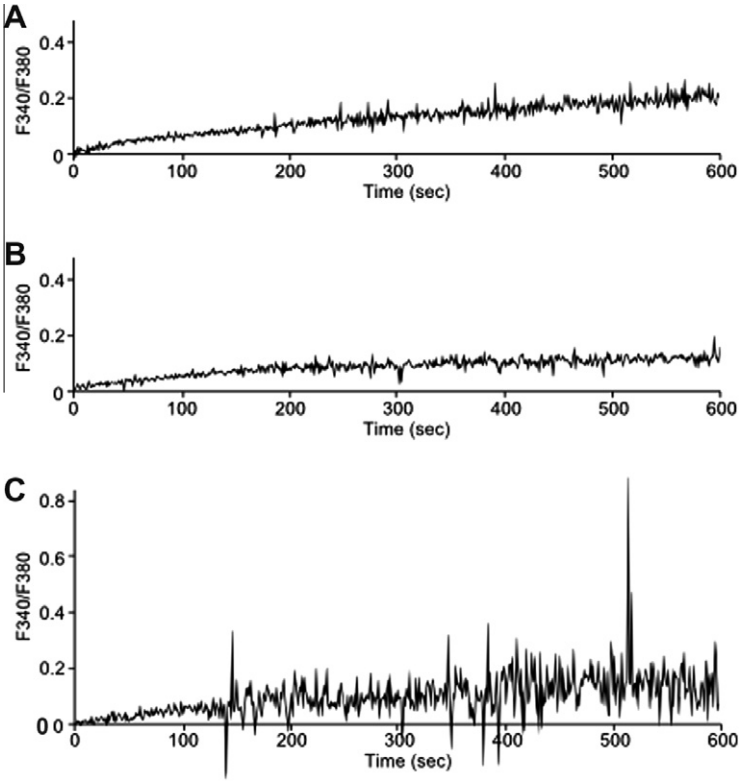

Fig. 4. Effects of anti-WGA-gp antibody on the $[Ca^{2+}]i$. To Fura-2-loaded sperm in a cuvette added with (A) PM (negative control), (B) preimmune serum, 1:100 (0.5 μg/μL), or (C) anti-WGA-gp antiserum, 1:100 (0.5 μg/mL) at time 0, and monitored for 10 min. Fura-2 loading was confirmed by an increase of $[Ca^{2+}]i$ with 50 μM A23187 calcium ionophore (data not shown).

easily removed during capacitation, and appears to be loosely associated with microdomains. Therefore, WGA-gp and the 16–30 kDa component are totally different in physicochemical properties and capacitation-dependency, although they shared similar size and WGA-positive staining.

Several reports have described physiological significance of WGA-epitope in mammalian sperm. Decreased expression of WGA-epitope on the sperm surface is associated with infertility in pig and human [25,26]. Co-incubation of human sperm with WGA activates the tyrosine phosphorylation cascade associated with capacitation, and this response can be abolished by the removal of either Neu5Ac or GlcNAc residues from the sperm surface [27]. In most cases, no protein identification of WGA-bearing components has yet been done. It would be interesting to link such phenomena to functions of WGA-gp and the 16–30 kDa component, although it has to be taken into account that glycan structures of gametic cells are often species-specific.

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