Discovery, Primary, and Crystal Structures and Capacitation-related Properties of a Prostate-derived Heparin-binding Protein WGA16 from Boar Sperm*

Received for publication, December 24, 2014. Published, JBC Papers in Press, January 8, 2015, DOI 10.1074/jbc.M114.635268

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Mammalian sperm acquire fertility through a functional maturation process called capacitation, where sperm membrane molecules are drastically remodeled. In this study, we found that a wheat germ agglutinin (WGA)-reactive protein on lipid rafts, named WGA16, is removed from the sperm surface on capacitation. WGA16 is a prostate-derived seminal plasma protein that has never been reported and is deposited on the sperm surface in the male reproductive tract. Based on protein and cDNA sequences for purified WGA16, it is a homologue of human zymogen granule protein 16 (ZG16) belonging to the Jacalin-related lectin (JRL) family in crystal and primary structures. A glycan array shows that WGA16 binds heparin through a basic patch containing Lys-53/HexNAc, whereas its heparin-binding domain may be involved in binding to sulfated glycosaminoglycans in the female tract, enabling removal of WGA16 from the sperm surface.

**This research was supported in part by Grants-in-Aid for Scientific Research (B) 22380187 and 25292216; Grant-in-Aid for Japan Society for the Promotion of Science (JSPS) fellows relating to JSPS Postdoctoral Fellowship for Foreign Researchers 21-09722 (to K. K. and E. G.); Grant-in-Aid for Scientific Research (C) 23570133 (to C. S.); the JSPS Strategic Young Researcher Overseas Visits Program for Accelerating Brain Circulation (to K. K.) from the Ministry of Education, Science, and Sports; the CNRS and Japan Science and Technology Agency (JST) Strategic Japanese-French Cooperative Program on Marine Genome and Marine Biotechnology (to K. K.); and a grant from the Naito Foundation Subsidy for Promotion of Specific Research Projects in 2012 (to E. G. and K. K.).

The atomic coordinates and structure factors (codes 3WOB and 3WOC) have been deposited in the Protein Data Bank (http://wwpdb.org/).

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB851481.

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‡‡1 3WOB

3 The abbreviations used are: ZP, zona pellucida; GalT, galactosyltransferase; GS-IB4, G. simplicifolia-IB4; JRL, Jacalin-related lectin; NCM, noncapacitating medium; WGA, wheat germ agglutinin; CAPS, 3-(cyclohexylamino)propanesulfonic acid; GAG, glycosaminoglycan; LacNAC, N-acetyl-lactosamine; LacdiNAC, N,N-diacytethylactosamine; HexNAC, N-acetyhexosamine.
fuse with the oocyte plasma membrane. This acquisition process or capacitation only occurs after sperm have spent a period of time in the female reproductive tract (5) and is completed when spermatzoa acquire the ability to respond to ZP proteins, leading to the acrosome reaction (6). Main events include the removal of stabilizing factors that spermatzoa have taken up in the seminal plasma and the remodeling of the sperm plasma membrane, as a prelude to the acrosome reaction and fertilization (4, 6). Capacitation corresponds

**EXPERIMENTAL PROCEDURES**

*Sperm Preparation—* Ejaculated sperm from *Sus scrofa* (Duroc, male, 2 years old) were provided by Ishikawa Pig Farm (Aichi, Japan). Sperm capacitation was performed as described previously (10). Briefly, ejaculated semen samples collected from mature fertile boars were diluted in Beltsville Thawing Solution (0.2 M glucose, 3 mM EDTA, 20 mM sodium citrate-2H2O, 15 mM NaHCO3, 10 mM KCl, pH 7.4). Sperm and seminal plasma were separated by centrifugation (200 × g, 28 °C, 10 min). Seminal plasma was further centrifuged at 9,500 × g for 30 min to remove residual sperm and debris. Sperm were washed twice with the non-capacitation medium (NCM; 100 mM NaCl, 0.36 mM NaH2PO4, 2H2O, 8.6 mM KCl, 23 mM HEPES, 0.5 mM MgCl2-6H2O, 11 mM glucose, pH 7.6) (25). Finally, sperm were resuspended at 1 × 108 cells/ml in NCM. Motile sperm populations were prepared by density gradient on Percoll (GE Healthcare) diluted in NCM. The uncapacitated sperm was applied on the top of the tube containing 35–70% Percoll and centrifuged at 28 °C at 200 × g for 5 min and then at 800 × g for 25 min to remove dead sperm and seminal gel particles (26). The Percoll gradient centrifuged uncapacitated sperm was obtained as a pellet. Percoll gradient centrifuged sperm was preincubated in NCM (30 min, 39 °C, 5% CO2) and then suspended in the capacitation medium (containing all components of NCM plus 10 mM NaHCO3, 2 mM CaCl2-2H2O, 5 mM pyruvate, and 3 mg/ml BSA, pH 7.6) finally at 20 × 107 cells/ml (2 h, 38 °C, 5% CO2) to obtain capacitated sperm. Sperm were then washed three times by NCM (150 × g, 28 °C, 10 min). Capacitation status was monitored visually under microscope to check whether sperm attained hyperactivated motility or monitored biochemically by Western blotting (see below), showing a specific phosphorylation pattern, typical of capacitated sperm (12, 28).

*Preparation of Low Density Detergent-insoluble Membranes—* The low density detergent-insoluble membrane fraction was prepared under 4 °C as described previously (16, 29). In brief, sperm (uncapacitated and capacitated sperm, 200–300 μl as sperm pellet) were suspended in 1.0 ml of 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA (TNE) containing 1% Triton X-100, 1 mM PMSF, and protease inhibitor mixture (1 μg/ml leupeptin, 2 μg/ml antipain, 10 μg/ml benzamidine, 1 μg/ml pepstatin, 0.9 μg/ml aprotonin). The mixture was stood on ice for 20 min and homogenized with 10 strokes by a Dounce homogenizer. After removal of the pellet by centrifugation at 1,300 × g for 5 min, the supernatant was mixed with an equal volume of 85% (w/v) sucrose in TNE. The mixture was layered successively with 6 ml of 30% (w/v) sucrose in TNE and with 3.5 ml of 5% (w/v) sucrose in TNE and centrifuged at 200,000 × g for 18 h (Beckman L-70K centrifuge, SW 41 l rotor). After ultracentrifugation, 1 ml each of 13 fractions was collected from the top to the bottom of the tube. Each fraction was dialyzed against PBS (137 mM NaCl, 8.1 mM Na2HPO4, 2.68 mM KCl, and 1.47 mM KH2PO4, pH 7.4), determined for protein amount by a BCA assay kit (Pierce) using BSA as a standard, and monitored by Western and lectin blotting.

*Purification of WGA16 from Sperm and Seminal Plasma—* Unless otherwise stated, all of the experimental procedures were performed under 4 °C. Sperm (10–25 ml of cell pellet) was mixed with lysis buffer (10 mM Tris-HCl, pH 7.5, 1% Triton

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X-100, 150 mM NaCl, 5 mM EDTA, protease inhibitor mixture), stood on ice for 1 h, and centrifuged at 15,000 × g for 30 min. Supernatant was collected and applied onto a CM-Toyopearl 650 M cation exchange column (Tosoh Biosciences, 1.2 × 88 cm) equilibrated with 0.1% Triton X-100, 10 mM Tris-HCl, pH 7.5. The column was extensively washed with 0.1% Triton X-100, 10 mM Tris-HCl, pH 7.5, and eluted with a linear gradient of NaCl (0–0.6 M). At every purification step, the elution profile was monitored by SDS-PAGE visualized by lectin blotting and silver staining. WGA16-positive fractions were pooled and dialyzed two times at 4 °C against WGA-agarose equilibration buffer: 10 mM Tris-HCl, pH 7.5, 150 mM NaCl (membrane size 36, Wako Chemicals). Dialyzed sample was applied on a WGA-agarose column (Seikagaku Biobusiness Corp.) overnight at 4 °C, washed with the equilibration buffer, and successively eluted by 100 mM GlcNac and 1 M GlcNac in the equilibration buffer. Remaining contaminants were removed by passage on Sephacryl S-100 (GE Healthcare; 1.0 × 150 cm) equilibrated in 0.1% Triton X-100, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl. Finally, WGA16-positive fractions were subjected to a 12.5-ml CM-Toyopearl 650 M column equilibrated with 0.1% Triton X-100, 10 mM Tris-HCl, pH 7.5, to remove any remaining contaminants. Elution was performed by a salt gradient (from 0 to 0.6 M NaCl, 20 ml in total). The same procedures were used to purify seminal plasma WGA16.

SDS-PAGE/Lectin Blotting and Western Blotting—Sperm samples were solubilized in a lysis buffer containing protease and phosphatase inhibitors (50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1% deoxycholate, 1 mM EGTA, 150 mM NaCl, 0.4 mM EDTA, 1 mM PMSE, 0.2 mM Na3VO4, 2.5 mg/ml leupeptin, and aprotinin) on ice for 20 min and then sonicated. Pellets were removed by centrifugation at 6,000 × g for 5 min, and supernatant protein concentration was assessed by a BCA assay kit. Samples were incubated with Laemmli buffer containing 10% mercaptoethanol at 100 °C for 3 min. Proteins were separated by SDS-PAGE (15% polyacrylamide gel), followed by either silver staining (Silver Stain Kit II, Wako Chemicals) or electrophoresis onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) using a semidy blotting apparatus for lectin blotting or immunoblotting. One percent of BSA in PBST (0.1% Triton X-100, 10 mM Tris-HCl, pH 7.5, 0.1% Tween 20) was used to dilute antibodies and blocking nonspecific binding to PVDF membrane. Capacitation was monitored by Western blotting using a monoclonal antibody 4G10 (1:10,000 dilution; Santa Cruz Biotechnology, Inc.) that specifically recognizes phosphotyrosine and a specific second antibody (American Qualex, La Mirada, CA; HRP-conjugated goat anti mouse IgG + IgM, 1:5,000 dilution). WGA-lectin epitope-bearing glycoproteins were detected by lectin blotting using WGA lectin (Sigma; 1 mg/ml, 1:2,500 dilution) and anti-WGA lectin serum (Sigma-Aldrich; 1:3,000 dilution), finally detected by the HRP-conjugated anti-rabbit IgG (Seikagaku; 1:4,000 dilution). For immunoblotting for WGA16, the blotted membrane was probed with the primary antibody (anti-WGA16 (see below); 1:1000 dilution) and the HRP-conjugated anti-mouse IgG + IgM (1:5,000 dilution; American Qualex). Visualization in lectin and Western blotting were processed using enhanced chemiluminescence reagent (Amersham Biosciences). In addition to WGA, Maackia amurensis agglutinin (Sigma-Aldrich), Ricinus communis agglutinin (Sigma-Aldrich), peanut agglutinin (J-Oil Mills, Inc.), and Griffonia simplicifolia-IB4 (GS-IB4; Sigma-Aldrich) were also used at 1 µg/ml, followed by incubation with mouse serum to each lectin (1:1,000 dilution) that was prepared in our laboratory.

Preparation of Anti-WGA16—The purified WGA16 mixed with Freund’s complete adjuvant (Wako, Osaka, Japan) was intraperitoneally injected into Ddy mice (female, 8 weeks old; Nippon SLC). The mice were boosted twice with WGA16 (10 µg/mouse) mixed with Freund’s incomplete adjuvant every 2 weeks. Blood was collected 1 week after each boost, and the serum was collected. The anti-WGA16 IgG antibody (anti-WGA16) was purified from the obtained anti-WGA16 serum using Protein G-Sepharose 4 Fast Flow (Amersham Biosciences) according to the manufacturer’s protocol.

Amino Acid Sequencing—Purified WGA16 was digested with either trypsin (Promega) or endopeptidase Lys-C (Sigma), and peptides were further purified by HPLC on an ODS column and submitted to N-terminal sequencing. For amino acid sequence analysis, WGA16 (5 µg) was applied onto a PVDF membrane using a Pro Sorb™ kit (PerkinElmer Life Sciences) and washed with 0.1% trifluoroacetic acid (TFA). The WGA16-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 with methanol. After air drying of methanol, it was subjected to amino acid sequencing by automated Edman degradation on a Procise HT analyzer (Applied Biosystems, Foster City, CA).

cDNA Cloning of WGA16—Preparation of total RNA and oligo(dT)-derived cDNA from pig male reproductive organs (testis, epididymis, seminal vesicles, prostate, and bulbourethral glands) and rapid amplification of cDNA ends (RACE) were performed as described previously (30). Based on a partial amino acid sequence that was identified as homologous to ZG16 homolog B from S. scrofa, the cDNA fragment for WGA16 was amplified by reverse transcription polymerase chain reaction (RT-PCR) from prostate and bulbourethral glands using the following primers: ZG16-F-P (5'-CAGATGTTCGGGAACGGAAAAGGCTCC-3') and ZG16-R-P (5'-GTTGATGGATGGATGTTATATGTGCTCGCCGGGNTGCAGGAT-3'). Amplified fragment was subcloned into a pGEM Easy vector (Promega) for sequencing. An insert was amplified by M13 promoter and terminator and sequenced. Because we observed significant differences between the sequenced amplified fragment and ZG16 mRNA sequence, we assessed the full WGA16 sequence by 5’- and 3’-RACE PCR using primers (3’-GSP1, 5’-CTATCAAGATGATCAGTACCAGGATTTCAGSMART-3’; 3’-GSP2, 5’-GGATCTCCTGCTAGTGGAAATACCGA-3’; 5’-GSP1, 5’-AGTTT ACCCGTCTCCTTTTACTTGAGTGAGTGAT-3’; 5’-GSP2, 5’-GGTGTAGGAAATAGGACCCTTTTCGTGTT-3’). Finally, the full WGA16 cDNA sequence was cloned using sense (5’-GAAGCCATGCTTGCTGTTGGCTA-3’) and antisense (5’-CCCTCCTCAGAGGGTCGTGCTC-3’) primers. The amplified full-length cDNA was cloned into pGEM plasmid (pGEM-WGA16). The sequence of the amplified fragment was investigated by DNA sequencing. The cDNA sequence corresponds to the expressed sequence tag FS704065 sequence (this sequence also corresponds to AK396685.1, AK396699.1, and AK400881.1 from other cDNA.
libraries; all of those expressed sequence tags were obtained from prostate, confirming prostate specificity). For quality evaluation of mRNA prepared from various organs, RT-PCR for actin was done using specific primers: 5’-CTGGAGAAAGGCC-TACGACGTGC-3’ and 5’-CGTGGCACCTTATCATGAGTGA-3’. Real-time PCR was performed as described previously (27), using the above described primers ZG16-F-P and ZG16-R-P and Ssof Fast™ EvaGreen Super mix (Bio-Rad).

Preparation of Recombinant WGA16—The DNA encoding WGA16 lacking a signal peptide was amplified by PCR with sense primers: 5’-CTCGGATATCAGGCAATGTCGCGG-3’ (EcoRV site underlined) and antisense primer 5’-GGATTCCAAAGCTTTCTCTCAGATG-3’ (HindIII site underlined), using the pGEM-WGA16 as a template. The amplified product was digested with EcoRV and HindIII and inserted into the complementary sites in pET32a (Novagen) to prepare a plasmid, pET-WGA16, that encoded a fusion protein of thioredoxin and His-tagged, signal peptide-deleted WGA16. *Escherichia coli* BL21(DE3) pLysS (Stratagene) was transformed with pET-WGA16 and grown at 37 °C in 2-YT medium to reach A600 = 0.6. After a 4-h induction at 37 °C with 0.5 mM isopropyl β-D-thiogalactoside, cells were harvested and suspended in PBS. After sonication, thioredoxin-His-tagged WGA16 fusion protein was purified using nickel-nitrilotriacetic acid-agarose affinity columns (Qiagen) according to the manufacturer’s protocol. The purified fusion protein was subjected to digestion with recombinant enterokinase (Novagen), followed by application on a second nickel-nitrilotriacetic acid-agarose column. After elution, fractions containing His-tagged WGA16 (WGA16-His) were concentrated on an Amicon Ultrafiltration cell and buffering-exchanged to PBS. WGA16-His was then biotinylated according to the manufacturer’s protocol. Ten μg of WGA16 (final volume 500 μl) in PBS were transferred onto YM-3 membrane. Two μl of 10 mM solution of biotinylation reagent (EZ-Link Sulfo-NHS-LC-Biotin) were added. The mixture was incubated at room temperature for 30 min, extensively washed on YM-3 membrane to remove residual biotinylation reagent, and used as biotinylated recombinant WGA16 (biotin-rWGA16). Biotinification efficiency was tested by dot blot on nitrocellulose membrane using Streptavidin and anti-WGA antibody.

To explore the role of some specific amino acid residues in the carbohydrate binding activity of WGA16, point mutations in pET-WGA16 construct were generated at Trp-138 or Tyr-140 using QuikChange II site-directed mutagenesis kits (Agilent Technologies). Use of the following primers resulted in the construction of pET-WGA16(W138A) (sense, 5’-TACCGGCA-GTTCCGGCTCTATGTCC-3’; antisense, 5’-GATGCCATAGAGCGCGCAACTGCGCCGTA-3’) and pET-WGA16(Y140A) (sense, 5’-CAAGTTCTGCTGCTGCTGCTACGCGCGG-3’; antisense, 5’-GCCGGTGGATGCCAGCGCGCAGACGACTG-3’). Recombinant W138A and Y140A mutants were prepared as described above. To evaluate roles of basic patches, BC1 (Lys-53 and Lys-73) and BC2 (Lys-25, Arg-91, Arg-93, and Lys-117), DNA fragments encoding WGA16 with alanine mutants of BC1 (K53A and K73A = AAG to GCG and AAA to GCA, respectively) and BC2 (K25A, R91A, R93A, and K117A = AAA to GCA, AGG to GCG, AGG to GCG, and AAG to GCG, respectively) flanked by HindIII and XbaI sites were synthesized (Genewiz Inc., Suzhou, China) and cloned into pCold-His6-streptag(st)-PDI vector (31) with HindIII and XbaI sites. Using these plasmids and pCold-WGA16 (see below), recombinant WGA16 and its BC1 and BC2 mutants were prepared as described below.

**Heparin-Agarose Affinity Chromatography—**We performed chromatography according to the manufacturer’s instruction. Briefly, a 1-ml heparin-agarose (Sigma) column was equilibrated in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl. 1 ml of diluted seminal plasma (1 mg/ml) was applied and mixed for 30 min at 4 °C. Flow-through was collected, and the column was sequentially washed with 10 ml of equilibration buffer; washed with 5 ml of 10 mM Tris-HCl, pH 7.5, 1 mM NaCl; and finally eluted with 5 ml of 10 mM Tris-HCl, pH 7.5, 2 mM NaCl. The separation profile was monitored by 15% SDS-PAGE followed by either silver staining or immunoblotting with anti-WGA16. For the heparin binding assay of recombinant BC1 and BC2 mutants of WGA16, supernatants of the lysate of *E. coli* Rosetta2 expressing BC1 and BC2 mutants of WGA16 were mixed with 20 μl of heparin-agarose at 4 °C for 16 h. The pellet was washed with 10 mM sodium phosphate, 150 mM NaCl, and the supernatant and pellet were obtained. The pellet was treated with 1 mM NaCl, sodium phosphate, and the supernatant (1 mM NaCl) fraction and the pellet (gel) were analyzed for the recombinant WGA16 molecules by anti-WGA antibody as described above.

**Glycan Array—**The glycan array was performed using the glycan array Glycan-I (S-Bio Business Division, Sumitomo Bakelite Co., Tokyo, Japan). Biotin-rWGA16 was diluted at a concentration of 10 μg/ml in reaction buffer TBS (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM MnCl2, 1 mM MgCl2) containing 0.5% Tween 20. Seventy μl of the solution were applied onto the glycan array slide surface and incubated at room temperature for 2 h. The slide was washed in TBS for 1 min under gentle agitation and then rinsed with pure water twice. After drying under air blowing, the slide was incubated with 2 μg/ml Cy3-streptavidin (GE Healthcare) in 0.5% Tween 20-TBS at room temperature for 1 h. The slide was washed with TBS and with pure water and dried under air blowing. The fluorescent intensity was immediately quantitated by a Typhoon scanner (λex = 532 nm, λem = 575 nm, PMT 600, medium sensitivity, resolution 50 μm).

**N-Glycosylation Profiling—**Delipidation of seminal plasma was performed by sequential extraction of 500 μl of seminal plasma with 20 volumes of chloroform/methanol mixture (2:1, v/v) and then with the same volume of chloroform/methanol/water (2:1:0.8, v/v/v) solution. The resultant aqueous phase was used as delipidated seminal plasma. Delipidated seminal plasma or purified WGA16 was suspended in a solution of 6 mM guanidinium chloride and 5 mM EDTA in 0.1 M Tris-HCl, pH 8, and agitated at 4 °C for 4 h. Dithiothreitol was added to a final concentration of 20 mM and incubated at 37 °C for 5 h, followed by the addition of iodoacetamide to a final concentration of 50 mM and further incubation in the dark at room temperature for overnight. The reduced and alkylated samples were dialyzed against water at 4 °C and lyophilized. The recovered protein samples were then digested with tosylphenylalanyl chloromethyl ketone-treated trypsin (Pierce) at 37 °C overnight in 50 mM ammonium bicarbonate buffer (pH 8.4). Crude peptides and glycopeptides were loaded onto a C18 Sep-Pak cartridge.
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(Waters). Contaminants were washed off with 5% aqueous acetic acid, and the bound peptides and glycopeptides were eluted with a stepwise gradient of 20, 40, and 60% 1-propanol in water. Eluted fractions were pooled, dried down, and incubated with peptide:N-glycanase F from Flavobacterium meningosepticum (Takara Bio Co.) at 37 °C overnight in 50 mM ammonium bicarbonate buffer, pH 8.4. Released N-glycans were separated from peptides and glycopeptides using the same C18 Sep-Pak procedure and then further purified on a graphitized column. For MALDI-TOF MS glycan profiling, native compounds in water were mixed 1:1 with 2,5-dihydroxybenzoic acid matrix (10 mg/ml in acetonitrile/H2O, 0.1% TFA (50:50)), spotted on the target plate, and dried under vacuum. Data acquisition was performed manually on an Ultraflex III mass spectrometer (Bruker) operated in the reflectron mode. Laser shots were accumulated until a satisfactory signal/noise ratio was achieved when combined and smoothed.

Carbohydrate Analyses—The monosaccharide compositions were determined by gas-liquid chromatography (GLC) as described previously (32). To determine the sialic acid content of WGA16, sialic acids were liberated directly by strong hydrolysis in 0.1 N TFA at 80 °C for 2 h and then reacted with a volume of 1,2-diamino-4,5-methylenedioxybenzene reagent at 50 °C for 2 h (33). The monomeric 1,2-diamino-4,5-methylenedioxybenzene-sialic acid derivatives were analyzed by a JASCO LC-900 HPLC system equipped with a JASCO FP-920 fluorescence detector (λex = 373 nm, λem = 448 nm) operating iso- crahically at 1.0 ml/min on a TSK-gel ODS-120T (250 × 4.6 mm) with a solvent mixture of acetonitrile/methanol/water (7:9:84, v/v/v) and identified by referring to the elution positions of standard Neu5Ac and Neu5Gc derivatives. Digestion with peptide:N-glycanase F, sialidase from Arthrobacter ureafaciens (Nacalai), or N-acetylhexosaminidase from jack bean (Seikagaku Co.) was performed manually on an Ultraflex III mass spectrometer (Bruker) operated in the reflectron mode. Laser shots were accumulated until a satisfactory signal/noise ratio was achieved when combined and smoothed.

Expression and Purification of Recombinant WGA16 for Crystallization—DNA fragments encoding pig WGA16 (amino acid residues 18–166) were subcloned into pCold-His 6- streptag(st)-PDI vector (31) for production of recombinant protein (pCold-WGA16). E. coli Rosetta2 (DE3) (Novagen) was transformed with the plasmid, and the cells were grown in LB medium at 37 °C. After induction with 0.1 mM isopropyl β-D-thiogalactoside (Wako), the cells were cultured at 15 °C for 12 h. The harvested cells were resuspended in a buffer containing 50 mM Tris-HCl (pH 8.0) containing 50 mM NaCl and 12 h. The harvested cells were resuspended in a buffer containing 50 mM Tris-HCl (pH 8.0) containing 50 mM NaCl and 1 mg/ml doxycycline, and the bound peptides and glycopeptides were eluted with 8 mM Na2HPO4, 1 mM KH2PO4, 177 mM NaCl, and 3 mM KCI, 30 mM arginine (pH 7.4) for crystal form A or 10 mM CAPS buffer (pH 9.0) for crystal form B and used for the crystallization.
Glycan/Heparin-mediated Removal of WGA16 from Sperm Surface—Uncapacitated sperm were incubated with NCM or with 10 mM monosaccharide (Gal, GlcNAc, or Neu5Ac), 10 mM activated sugar (UDP-Gal, UDP-GlcNAc, or CMP-Neu5Ac), or with increasing concentrations (0.5, 1, and 2 mg/ml) of heparin in NCM at 39 °C in 5% CO₂ for 30 min. The sperm suspensions were centrifuged (150 × g, 10 min) to pellet down intact sperm and washed twice with NCM. Sperm lysates were quantified by a BCA assay and applied onto a 15% SDS-polyacrylamide gel at 15 μg of protein/lane. WGA16 was quantified by the intensity of the immunostaining band on Western blotting with anti-WGA16.

RESULTS

Discovery and Purification of WGA16—To examine whether glycosylation changes occur on the sperm surface during capacitation, sperm lysate and the lipid raft that was prepared as low density detergent-insoluble membranes from uncapacitated and capacitated pig sperm were analyzed by lectin blotting. Sperm capacitation was monitored by antiphosphotyrosine assay and applied onto a 15% SDS-polyacrylamide gel at 15 μg of protein/lane. WGA16 was quantified by the intensity of the immunostaining band on Western blotting with anti-WGA16.

This 16–25-kDa protein was initially purified from sperm lysate. Then a protein with similar properties was also detected in seminal plasma (Fig. 2, A–D). Sperm lysate was applied to a CM-Toyopearl 650 M column (Fig. 2A). The 16–25-kDa protein was eluted around 0.4 M NaCl, as revealed by WGA lectin blotting. The WGA lectin-positive fractions were then applied onto a WGA-agarose column. The 16–25-kDa protein was eluted with 100 mM or 1 M GlcNAc. These elution fractions were separated on a Sephacryl S-100 column (Fig. 2C). Based on the silver staining and WGA lectin blotting, the WGA lectin-positive components were eluted at positions corresponding to their molecular weights, indicating that they are homogeneous in that other contaminants are absent and that they are present as a monomer with a different glycosylation state. We named the 16–25-kDa protein WGA16. Notably, other low molecular weight proteins, such as 16-kDa PSP-I/II, which were identified by proteomic analysis, were eluted as oligomers prior to WGA16 (fractions 38–48; Fig. 2C). When contaminants were present, WGA16 was further purified on a CM-Toyopearl 650 M column to finally obtain pure WGA16. Similar procedures were used in purification of the seminal plasma-derived WGA16 (Fig. 2B for the anion exchange chromatography; Fig. 2D for Sephacryl S-100 gel filtration). From 200 ml of semen, 5 ml of sperm cells and 195 ml of seminal plasma were obtained. Purification yields were, respectively, 0.4 mg from 5 ml of sperm and 22 mg from 195 ml of seminal plasma. Thus, we can conclude that about 2% of WGA16 in semen were present on the sperm cells.

WGA16 Is Related to the Jacalin-related Lectin Family—For purified WGA16 from sperm lysate and seminal plasma, N-terminal amino acid sequence on the native protein as well as internal sequences on tryptic or Lys-C-digested peptides were determined. The results for seminal plasma WGA16 are shown in Table 1, A–D. Further analysis established that sperm surface and seminal plasma WGA16 correspond to the same protein because they share the same proteolytic profiles and amino acid sequence. A computer-assisted search indicated that some peptide fragments match the deduced amino acid sequences from mRNA sequence XM_003124749.1, corresponding to S. scrofa zymogen granule protein 16 (ZG16) homolog-B like. In PCR analysis using primers after this nucleotide sequence, a 204-bp product was amplified for prostate, although no product was obtained for testis, epididymis, seminal vesicles, or bulbourethral gland. This partial cDNA sequence coded for a 68-amino acid peptide (underlined in Table 1D). The sequence of WGA16 full mRNA was determined by a 3′- and 5′-RACE approach. Information obtained by RACE PCR was registered as GenBank™ AB851481, which matched the sequence at accession number FS704065: full-length enriched swine cDNA library, adult prostate S. scrofa cDNA clone PST010091G03 5′ mRNA sequence. The corresponding deduced amino acid sequence is shown in Table 1D. This sequence matches peptides obtained after tryptic or proteinase-Lys-C treatment (Table 1, A–C). WGA16 open reading frame consists of 498 bp (nucleotides 69–566 in the FS704065 sequence), encoding a protein of 166 amino acids with a predicted 17-amino acid signal peptide (Table 1D). The deduced mature WGA16 corresponds to a 149-amino acid polypeptide chain with a molecular weight of 16,200 with a calculated pI of 8.94, consistent with the biochemical characteristics of WGA16. N-terminal sequencing on native protein confirmed that first 17 amino acids correspond to signal peptide because native WGA16 starts at Gly-18. The nucleotide sequence of WGA16 shows 70 and 43% identi-
ties with the predicted ZG16 homolog B-like from *S. scrofa* and with human protein ZG16 homolog b (hZG16b), respectively. Those proteins belong to a large family of plant and animal lectins, grouped as Jacalin-related lectins (JRLs). In animals, the function of JRLs such as ZG16 remains unclear. So far, those proteins have been exclusively found in tissues with high secretory activity (pancreas, submaxillary glands, liver, and intestine) (41, 42). This is the first case of the existence of a JRL member in sperm and reproductive organs.

**WGA16 Shows a Unique Glycosylation Profile**—Although major seminal plasma glycoproteins can be revealed using either WGA or GS-IB4 lectin blotting, WGA16 was exclusively observed using WGA (Fig. 3, A and B), suggesting a specific glycosylation pattern. The glycosylation profile of PSP-I/PSP-II heterodimer, the major seminal plasma glycoproteins, shows diantennary complex-type *N*-glycans bearing α1,3-fucosylated or α2,6-sialylated *N*-acetyllactosamine (LacNac) as well as di-*N*-acetyllactosamine (LacdiNac) structure. The β-GlcNac residues of non-sialylated antennae are usually substituted by α1,3-Fuc residues, and a majority of the non-sialylated LacNac antennae are terminated by α1,3-Gal residues (43). In the case of WGA16, the lack of reactivity toward GS-IB4 lectin (Fig. 3B) indicates that WGA16 glycans are devoid of terminal α-Gal residues.

*N*-glycan profiling of WGA16 and seminal plasma proteins by mass spectrometry confirmed observations made by lectin blotting. Glycan structures were assigned by combining data from MALDI-MS with monosaccharide composition obtained by gas chromatography as well as information from the literature (43). Seminal plasma glycoproteins were confirmed to contain *N*-glycans bearing terminal α-Gal residues but also terminal LacNac or LacdiNac units that can be substituted.
by Fuc (Fig. 3C and Table 2). On the other hand, MS profiling of purified WGA16 exhibited a complex N-glycosylation pattern, with more than 10 different N-glycan structures (Fig. 3D). Assignment of WGA16 N-glycans confirmed the absence of terminal α-Gal residues but a rather high content of LacdiNAc antennae, with and without fucosylation. The prominent peak at m/z 1891.9 corresponds to core-fucosylated diantennary LacdiNAc N-glycan (Fig. 3D and Table 2). Finally, both lectin blotting and MS analysis indicate that WGA16 presents a very unique glycosylation pattern among seminal plasma proteins, remarkably characterized by the absence of terminal α1,3-Gal.

Carbohydrate compositions of WGA16 and seminal plasma glycoproteins are shown in Table 3. The content of HexNAc (GalNAc and GlcNAc) is higher for WGA16 than for seminal plasma glycoproteins. The WGA epitope was lost after N-acetylhexosaminidase treatment but not after sialidase treatment (Fig. 3E), suggesting that terminal HexNAc but not Neu5Ac residues are responsible for the WGA epitope of WGA16. Peptide:N-glycanase F treatment gave a sharp band at 16 kDa on SDS-PAGE/anti-WGA blotting, whereas WGA epitope was lost on the 16-kDa product (Fig. 3E). These results indicate that the WGA epitope exclusively resides on N-glycans, which mainly contributes to the heterogeneity in molecular mass of WGA16. Based on the deduced amino acid sequence (Table 1D), there are two potential N-glycosylation sites at Asn-35 and Asn-124. At least Asn-35 is glycosylated, because this residue could not be assigned on the N-terminal and internal sequence analyses (Table 1, A–C). It should be noted that WGA epitope remained at 20–23 kDa, in addition to the 16-kDa product, after peptide:N-glycanase treatment, suggesting the presence of O-glycans with the WGA epitope. Because the mannose amount decreased but it was still detected in WGA16 after peptide:N-glycanase treatment (data not shown), some N-glycans may be peptide:N-glycanase-resistant. Therefore, it remained unclear whether O-glycans also contain the WGA epitope. Structural analysis of the O-glycans should be performed in detail in the future.
Crystal Structure of WGA16—We determined the crystal structures of WGA16 (residues 18–166) in two different crystal forms, A (Protein Data Bank code 3WOB) and B (Protein Data Bank code 3WOC) at 2.6 and 2.4 Å resolution, respectively (Table 4, Fig. 4 (A–D), and Fig. 5 (A–C)). In crystal form A, one WGA16 molecule is included in the asymmetric unit, whereas in the crystal form B, six WGA16 molecules (chains A, B, C, D, E, and F) were included in the asymmetric unit (Fig. 5C). WGA16 was found as a monomer in crystal form A, whereas dimer formation was observed in crystal form B. Both structures are similar except for the N-terminal region, which is involved in dimerization in crystal form B (Fig. 5B). The dimer formation in the crystal form B is likely to be an artifactual, because WGA16 eluted as a monomer on size exclusion chromatography (data not shown). Hereafter, we describe crystal form A unless otherwise stated.

The overall structure is similar to JRL family proteins, such as Banlec (44), human ZG16p (37), and human ZG16b (37). WGA16 assumes a β-prism fold consisting of three β-sheets (Fig. 4A). Each β-sheet is made up of 3–4 β-strands (sheet I: β2, β11, and β12; sheet II: β3, β4, and β6; sheet III: β7–β10), forming three Greek key motifs. The N-glycosylation site, Asn-35, is located at the loop between β2 and β3 strands and solvent-exposed. In Jacalin-related mannos-binding lectins, the sugar-binding site is composed of three loops (GG loop, binding loop, and recognition loop) and a key aspartic acid residue (Asp-151 in human ZG16p) (Fig. 4B, bottom). Asp-151 in human ZG16p is replaced with Thr-143 in WGA16 (Fig. 4B, top). Characteristically, hydrophobic residues (Trp-138, Leu-139, and Tyr-140) are clustered in the region corresponding to the canonical sugar-binding site of Jacalin-related mannos-binding lectins. It is therefore suggested that WGA16 does not bind to mannos-type sugars.

Intriguingly, the crystal structure of WGA16 in crystal form B contains a sulfate ion at the β3–β4 loop region (Fig. 4C, top). The bound sulfate was observed at the same site in chains A, B, C, D, and F, and the sulfate-binding site is close to the second sugar binding site in Banlec (45). The sulfate ion interacts with Nε of Lys-53 and main chain nitrogens of Pro-48 and Val-49. Similarly, the crystal structure of ZG16b contains phosphate at the same site consisting of Leu-83, Leu-84, Leu-85, and Lys-87 (Fig. 4C, bottom). These negatively charged ions may mimic the sulfate group of glycosaminoglycan (GAG). In fact, WGA16 possesses a positively charged surface, as seen in human ZG16p and ZG16b (Fig. 4D), suggesting that the region is involved in binding to GAGs, such as heparin, and is apart from the N-glycosylation site, Asn-35 (see “Discussion”).

WGA16 Has the Heparin-binding but Not Jacalin-related Lectin Activity—During the course of purification of WGA16, we first noticed that WGA16 came to the heparin-binding fraction of seminal plasma proteins. On the heparinagarose chromatography of seminal plasma (Fig. 6A), the elution profiles of the 75- and 90-kDa proteins confirmed that the separation procedure worked efficiently and indicated that heparin-binding proteins started being eluted at 1 M NaCl. Anti-WGA16 immunoblotting showed that highly glycosylated forms of WGA16 were rapidly eluted, whereas less glycosylated forms were slowly eluted in 1 M and 2 M NaCl-eluted fractions, establishing WGA16 affinity to heparin. To further explore WGA16 lectinic activity, recombinant WGA16 with an N-terminal His6 tag (rWGA16-His) was expressed in E. coli and purified as a single band at 17 kDa on SDS-PAGE. Glycan binding specificity of WGA16 was analyzed using a glycoconjugate microarray comprising 29 immobilized compounds, including several GAGs, as well as N- and O-glycans. WGA16 unambiguously exhibits a selective binding to heparin (Fig. 6B). Additionally, when the exposure time was extended, we could observe the weak binding to partially desulfated heparin D2S and D6S but not to non-sulfated GAGs.

In the region corresponding to the canonical sugar-binding site of the JRL family, WGA16 is devoid of the typical GXXXD motif. Instead, WGA16 contains hydrophobic amino acid residues Trp-138 and Tyr-140 in this region (Table 1D). As predicted by the crystal structure of this region (Fig. 4B), WGA16 had no binding activity to high mannnose type glycans (Fig. 6B). To investigate whether those residues were involved in the sugar-binding domain of WGA16, we performed point mutations and explored heparin-binding properties of W138A and Y140A mutants. Because no changes in heparin affinity were observed for either W138A or Y140A, we concluded that those residues were not involved in heparin binding in WGA16.

The crystal structure of WGA16 exhibits two basic amino acid clusters BC1 and BC2 that are presumably responsible for heparin-binding: BC1 contains Lys-53 and Lys-73 that are close to the site corresponding to the second sugar binding site in Banlec (45), and BC2 contains Lys-25, Arg-91, Arg-93, and Lys-117 (Fig. 6C). The two alanine mutants for BC1 and BC2 were bacterially expressed and tested for binding ability to heparinagarose resins (Fig. 6D). BC2 mutant bound to heparin-agarose like WGA16, whereas BC1 mutant did not. These results indicate that BC1 is involved in the heparin-binding activity, consistent with the observation that Lys-53 interacts with sulfate ion in the WGA16 crystal structure (Fig. 4C).
Distribution of WGA16 in Male Reproductive Organs—Specific polyclonal anti-WGA16 antibody showed the occurrence of WGA16 protein in tissues of the prostate and bulbourethral glands. Tissue homogenates from boar reproductive organs were tested for the presence of WGA16 using both anti-WGA16 and WGA lectin (Fig. 7, A and B). The strongest immunostaining was detected in prostate. The high molecular weight and intense WGA lectin signal indicate that it corresponds to heavily glycosylated forms of WGA16. A weaker signal was detected in the bulbourethral gland, corresponding to underglycosylated WGA16. Real-time PCR results showed that the WGA16 transcript was exclusively detected in prostate (Fig. 7C).

FIGURE 3. Comparative analysis of glycosylation profile from seminal plasma proteins and WGA16. Comparative lectin staining of seminal plasma proteins separated on cation exchange column. Shown is lectin blotting with GS-IB4 (A) and WGA (B). Arrowheads, fractions containing WGA16. Shown are MALDI-MS profiles of N-glycans from seminal plasma proteins (C) and from seminal plasma-derived WGA16 (D). E, SDS-PAGE of WGA16 after treatment with β-hexosaminidase, sialidase, endo-β-galactosidase, and peptide-N-glycanase F (PNGaseF). Left, WGA lectin blotting; right, silver staining.
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In situ hybridization and immunohistochemistry using anti-WGA16 were performed on prostate, epididymis, and testis (Fig. 8). Whereas expression of WGA16 mRNA was detected in testis, cell layer of epididymis, and epithelial cells of prostate, the protein-specific signal was exclusively detected in epithelial cells of prostate. Based on the results of Western blotting and immunohistochemistry, it appears that WGA16 is a protein characteristic of the prostate gland. Taken together, the results indicate that WGA16 secretion mainly takes place in the prostate to be deposited on the sperm surface.

WGA16 is associated with sperm through sperm surface galactosyltransferase—As described above, WGA16 is secreted from prostate and deposited on the sperm surface, specifically on the lipid rafts, during passage through genital tracts until capacitation (Fig. 1). One striking structural feature of WGA16 lies in its very particular glycosylation profile, characterized by the absence of terminal α-Gal residues, and the presence of terminal GalNAc/GlcNAc residues, contrasting with other seminal plasma glycoproteins (Fig. 3). To explore whether the surface association of WGA16 is mediated through a specific interaction with a sperm membrane lectin, uncapacitated sperm were incubated with NCM containing 10 μg/mL Gal, GlcNAc, or Neu5Ac or their activated forms (UDP-Gal, UDP-GlcNAc, and CMP-Neu5Ac). After incubation and extensive washing by NCM, sperm lysate was analyzed by SDS-PAGE and Western blotting with anti-WGA16 (Fig. 9A). Surprisingly, sperm surface WGA16 was efficiently removed with UDP-Gal, a donor substrate for galactosyltransferase (GalT). This is of particular interest because the sperm surface β-galactosyltransferase (β-GalT) has been reported in various species, including pigs (46, 47). To confirm the presence of an active GalT at the sperm surface and to validate that this GalT interacts with WGA16, sperm and the supernatant at the washing step were analyzed by the SDS-PAGE/lectin blotting using either R. communis agglutinin (Galβ1,4GlcNAc-specific) or GS-IB4 lectin (α-Gal/GalNAc-specific) as well as by Western blotting with anti-WGA16 (Fig. 9B). After incubation with UDP-Gal, sperm surface WGA16 decreased, whereas WGA16 in the supernatant increased, suggesting that the presence of UDP-Gal induces WGA16 transfer from the sperm surface to the supernatant. In addition, the lectin blotting revealed that upon UDP-Gal incubation, supernatant WGA16 became R. communis agglutinin-, but not GS-IB4-reactive (i.e. the β-Gal, but not α-Gal, residues were newly formed). These

**TABLE 2**
Comparison of PNGase F-released N-glycans from pig seminal plasma and WGA16

| m/z  | Oligosaccharide composition | Seminal plasma glycoproteins | WGA16 |
|------|---------------------------|-----------------------------|-------|
| 1282.1 | Hex,HexNAc,Fuc,       | +                           |       |
| 1444.2 | Hex,HexNAc,Fuc,       | + + + + +                   |       |
| 1485.2 | Hex,HexNAc,Fuc,       | +                           |       |
| 1606.3 | Hex,HexNAc,Fuc,       | + + + +                     |       |
| 1631.3 | Hex,HexNAc,Fuc,       | +                           |       |
| 1647.3 | Hex,HexNAc,Fuc,       | +                           |       |
| 1683.3 | Hex,HexNAc,Fuc,       | + + + +                     |       |
| 1809.6 | Hex,HexNAc,Fuc,       | +                           |       |
| 1891.3 | Hex,HexNAc,Fuc,       | + + + + + +                 |       |
| 1955.7 | NeuAc,Hex,HexNAc,     | (+)                         |       |
| 1997.7 | Hex,HexNAc,Fuc,       | +                           |       |
| 2038.7 | Hex,HexNAc,Fuc,       | + + +                       |       |
| 2054.7 | Hex,HexNAc,Fuc,       | +                           |       |
| 2133.3 | Hex,HexNAc,Fuc,       | +                           |       |
| 2184.4 | NeuAc,Hex,HexNAc,Fuc, | + +                         |       |
| 2237.7 | Hex,HexNAc,Fuc,       | +                           |       |
| 2259.8 | Hex,HexNAc,Fuc,       | +                           |       |
| 2261.4 | Hex,HexNAc,Fuc,       | +                           |       |
| 2701.4 | Hex,HexNAc,Fuc,       | (+)                         |       |
| 2743.5 | Hex,HexNAc,Fuc,       | (+)                         |       |
| 2946.7 | Hex,Hex,HexNAc,Fuc    | (+)                         |       |

**TABLE 3**
Carbohydrate composition of WGA16 and seminal plasma glycoproteins

| Monosaccharide | Seminal plasma glycoproteins | WGA16 |
|---------------|-----------------------------|-------|
| Fuc           | 10.2 % μmol                  | 3.4   |
| Man           | 39.9 % μmol                  | 24.9  |
| Gal           | 16.1 % μmol                  | 17.8  |
| GalNAc        | 4.0 % μmol                   | 5.0   |
| GlcNAc        | 27.4 % μmol                  | 36.3  |
| Neu5Ac        | 2.3 % μmol                   | 12.5  |

**TABLE 4**
Data collection and refinement statistics of WGA16 structures

| Crystal form A | Crystal form B |
|---------------|---------------|
| Data collections |                |
| Protein Data Bank code | 3WOB | 3WOC |
| Space group | P21 | C2 |
| Unit cell parameters a, b, c (Å), β (degrees) | 20.88, 52.33, 45.30, 103.18 | 134.48, 77.85, 87.19, 91.30 |
| Resolution (last shell data in parentheses) (Å) | 50.0-2.60 (2.64-2.60) | 50.0-2.40 (2.44-2.40) |
| Rmerge (last shell) | 16.2 (51.0) | 6.9 (61.2) |
| Mean (I/σ(I)) (last shell data in parentheses) | 3.1 (2.1) | 22.4 (2.2) |
| Completeness (last shell data in parentheses) (%) | 99.8 (100.0) | 99.7 (99.3) |
| No. of unique reflections | 3,847 | 35,861 |
| Average multiplicity (last shell data in parentheses) | 3.7 (3.6) | 5.7 (5.7) |

| Crystallographic refinement statistics |                |
| No. of reflections used for refinement | 3,654 | 33,943 |
| Reflections marked for Rfree | 28.3 | 32.0 |
| Rfree | 21.4 | 25.2 |
| Average B-factors (Å²) | 24.7 | 39.5 |
| Root mean square deviation of bond length (Å) | 0.006 | 0.006 |
| Root mean square deviation of bond angle (degrees) | 0.935 | 1.015 |
| Ramachandran plot: most favored/additional allowed/generously allowed/disallowed (%) | 88.5/9.6/1.9/0.0 | 89.2/9.0/1.9/0.0 |
results suggest that sperm surface GalT is present and specifically interacts with WGA16 through its terminal HexNAc residues. It is unknown whether UDP-Gal is extracellularly present in genital tracts. The UDP-Gal-dependent WGA16 release that we observed requires high concentrations of UDP-Gal (e.g. 1–10 mM) (Fig. 9A). Because UDP-Gal is not present extracellularly in vivo, we point out that UDP-Gal-dependent WGA16 release does not occur under physiological conditions and therefore is of no physiological relevance. This experiment only allows confirmation that WGA16 retention occurs through the H9252-GalT under physiological conditions. Another mechanism, probably involving female tract components, may explain WGA16 release from the sperm surface during in vivo capacitation.

Removal of WGA16 from Sperm Surface Is Mediated by Heparin—In vitro observations established that WGA16 was removed from the lipid rafts during capacitation (Fig. 1). However, the molecular basis of in vivo capacitation remains unclear. In the case of bovines, it has been clearly demonstrated that heparin induces capacitation (48), and heparin-binding proteins have been shown to modulate capacitation (49). The presence of heparin, heparan sulfate, and other sulfated glycosaminoglycans has been demonstrated in follicular fluid of sows (50). Unlike in the case of bovines, heparin does not induce capacitation in sows but rather enhances in vitro capacitation of porcine sperm only under capacitating conditions (51). Our results demonstrated the strong affinity of WGA16 toward heparin (Fig. 6, A and B). We thus hypothesized that in vivo heparin or sulfated glycosaminoglycans could be involved in the removal of WGA16 from the sperm surface. To test this hypothesis, uncapacitated pig sperm was incubated with NCM containing increasing concentrations of heparin, and both sperm and supernatant obtained from the washing step were analyzed for the amount of WGA16 by Western blotting with anti-WGA16 (Fig. 10). Whereas it decreased in sperm lysate, the WGA16 signal increased in supernatant in a heparin-dependent manner, indicating that heparin can be responsible for WGA16 removal, probably through a direct WGA16-heparin interaction.

4 E. Garénaux, C. Sato, and K. Kitajima, unpublished results.
DISCUSSION

If their implication in fertilization is clearly established, the function of seminal plasma proteins during capacitation, acrosome reaction, and sperm-egg interaction remains largely unclear. Especially, no functional significance of their glycan chains has ever been elucidated. Because glycan chains on glycolipids and glycosylphosphatidylinositol-anchored glycoproteins are enriched in lipid rafts (52), particular glycan structures may need to be concentrated in the lipid rafts to be functional. Thus, our hypothesis is that lipid rafts must give a platform for glycan-involved interactions (18). Sperm capacitation is characterized by a drastic remodeling of sperm plasma membrane (9–14). In our approach, we focused on lipid raft-localized glycoproteins whose localization changed upon capacitation in order to link the sperm lipid rafts to their involvement in sperm capacitation and in the function of seminal plasma glycoproteins. In this study, we discovered a particular glycoprotein that disappeared from the sperm surface during capacitation. We named this protein WGA16 because of its enrichment in the WGA lectin epitope. WGA16 is shown to be a prostate-derived seminal plasma glycoprotein that is deposited on the sperm surface in the male genital tract at the moment of ejaculation. We show that this deposition process is glycan-dependent and involves the binding of sperm surface β-GalT to the N-glycan of WGA16. WGA16 is the only WGA-positive seminal plasma glycoprotein that undergoes capacitation-dependent redistribution on the sperm surface, whereas other WGA-positive glycoproteins, such as spermadhesins PSP-I/II and AQN-I, remain at the sperm surface (Fig. 1). Isolation and functional characterization of WGA16 evidenced common features with the spermadhesin family (PSP-I/II, AQN-3, AWN). The main seminal plasma proteins, 18/20-kDa spermadhesins PSP-I and PSP-II, form heterodimers and are described as decapacitation factors. They may be involved in several sequential steps of fertilization through a multifunctional ability to bind to carbohydrates, sulfated glycosaminoglycans, or phospholipids (53, 54). We established, based on comparison of both primary and crystal structure, that WGA16 was not a spermadhesin, although it shares common features, particularly affinity toward heparin or sulfated GAGs. WGA16 belongs to the JRL family; however, WGA16 does not form a dimer, confirming that, unlike JRLs from plants that form oligomers by non-covalent interaction (55), animal JRLs, such as hZG16 or WGA16, consist of monomers (33).
One of the striking features of WGA16 is the exclusive absence of α-Gal residues and abundance of terminal GlcNAc and GalNAc residues, which enables an interaction of WGA16 with the sperm surface 1,4-GalT (Fig. 11). For the past 30 years, several studies have been dedicated to defining the function of 1,4-GalT in sperm-egg interaction (54). Results obtained on knock-out mice suggest that the most important role of 1,4-GalT in fertilization is to induce the acrosome reaction (56). Before the acrosome reaction occurs, sperm has to bind to ZP3 through the surface receptors, which induce exocytosis of acrosomal contents. Several studies to understand the nature of sperm receptors for the murine zona pellucida have been conducted (57–59). The most compelling supporting evidence is in favor of the binding of β1,4-GalT to terminal GlcNAc residues but that β1,4-GalT does not have a required function in porcine zona adhesion (47, 62). Our results suggest that porcine β1,4-GalT would instead be responsible for modulation of capacitation by retaining seminal plasma-derived stabilizing (decapacitating) factors like WGA16 on the sperm surface. By incubating sperm
with UDP-Gal, we could observe the modification of the WGA16 glycosylation profile, with a great increase of R. communis agglutinin lectin signal, as well as the specific release of WGA16 from the sperm surface. We thus hereby identify and describe for the first time a binding partner for boar sperm 1,4-GalT and address the molecular basis of one particular aspect of boar sperm capacitation.

Apart from its unique glycosylation profile, another striking aspect of WGA16 is its high homology with ZG16 family proteins, which belong to the JRL family. In humans, ZG16p has been found in the liver, small intestine, and pancreas (42). ZG16b, also called PAUF, has been described in the pancreas and in saliva (63, 64). For the first time, we report the presence of a ZG16-like protein in reproductive tissues. WGA16 is highly glycosylated, whereas human, mouse, and rat ZG16p sequences do not contain any potential N-glycosylation sites. Although numerous serine and threonine residues are present in the ZG16p sequence, rZG16p was purified from the pancreas as a clear sharp band, suggesting the absence of glycosylation (65).

Sequence homology indicates that WGA16 is closer to human and pig ZG16b than human ZG16p. A recent study (66) reported that ZG16b facilitates tumor growth and metastasis through enhancing cell adhesiveness by regulating interactions between tumor cells and extracellular matrix molecules.

Rat ZG16p has been demonstrated to bind to heparin, whereas human ZG16p binds to mannose. Classically, the sugar-binding site of mannose-binding type JRL consists of three exposed regions (the GG loop, recognition loop, and binding loop) at the top of the β-prism fold (31). In human ZG16p, mannose binding is mediated by the binding loop with a conserved motif, GXXXD. Rat ZG16p interaction with heparin has been associated with a secondary sugar binding site between β-sheets 3 and 4 (N-terminal) but not with the classical binding loop. In WGA16, as in human ZG16b, most of the conserved motifs responsible for lectinic activity are absent or replaced. Particularly, in human ZG16b, the mannos-binding loop between β-sheets 11 and 12 containing the GXXXD motif is modified (36). In WGA16, the binding loop between β-sheets 11 and 12 is absent (i.e. there are no basic residues susceptible to bind to heparin). However, the site-directed mutagenesis studies could clearly define the heparin-binding site, showing that basic cluster of Lys-53 and Lys-73 is involved. This binding site is different from conventional mannos-binding sites but close to the secondary binding site of Banlec or the corresponding site of human ZG16b (Fig. 4D). It is striking that as for sperma-

![Image](image-url)
dhesins, the modality of their interaction with sulfated GAGs has not yet been completely deciphered. Data suggest that, as it was already observed for PSP-I (67), glycosylation of WGA16 might influence its affinity toward heparin. Complex type N-glycan-bearing PSP-I was recovered in the non-heparin-binding fraction, whereas highly mannose-substituted PSP-I was eluted in the heparin-binding fraction. However, mechanisms explaining the influence of glycosylation on heparin binding are still unclear, and a future comparative study to establish the molecular basis of heparin binding of seminal plasma proteins will be of interest.

Finally, from a functional point of view, it would be interesting to point out that N-glycosylation sites and a potential lectin site (sulfate binding site) in WGA16 were present at two opposite sides of the protein. As summarized in Fig. 11, we hypothesize that this functional asymmetry illustrates the functional duality of WGA16. Indeed, WGA16 is deposited at the sperm surface upon ejaculation via recognition of its N-glycan by H9252,1,4-GalT. Seminal plasma contains numerous factors that modulate the capacitation process in vivo during sperm residence in the female's genital tract (4). We hereby provide for the first time a model explaining the molecular mechanism of deposit and release of a particular seminal plasma protein. We hypothesize that WGA16 blocks and protects the substrate-binding site of H9252,1,4-GalT until capacitation occurs and that these phenomena are regulated by GAG content (i.e. heparin, chondroitin sulfate, and so on) of the female tract environments. Once in the high GAG concentration region of the oviduct, WGA16 is removed from the sperm surface through interaction with sulfated GAGs; only then can the substrate-binding site of H9252,1,4-GalT be exposed to interact with the acro-
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some reaction induced by ZP glycoproteins. It has been determined that in mice, β1,4-GaIT seems to directly interact with ZP components, implying a direct role in fertilization. So far, based on sequence homology, we have not been able to identify the WGA16 equivalent in mice. Deeper studies on murine seminal plasma content, particularly WGA lectin-positive, heparin-binding proteins, might allow us to further study the role of β1,4-GaIT in fertilization. Deciphering and comparing the molecular mechanisms underlying capacitation in several species may provide answers about the cascade of events that must take place before fertilization occurs.

Acknowledgments—We thank Masahiko Tanizawa, Kozoh Ishikawa, and Yasutoshi Ishikawa (Ishikawa Pig Farm Co., Ltd., Aichi, Japan) for kind and constant provision of fresh ejaculated sperm. We are grateful to Drs. Satoshi Okhura and Tsukasa Matsuda (Nagoya University) and Dr. Nongnuj Tanphaichitr (Ottawa Hospital Research Institute) for valuable discussions. We also thank Drs. Kay-Hooi Khoo and Chia-Wei Lin (Adademia Sinica, Taiwan) for technical advice and critical reading of the manuscript.

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