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Spermadhesin AQN1 Is a Candidate Receptor Molecule Involved in the Formation of the Oviductal Sperm Reservoir in the Pig[1]

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ABSTRACT

Sperm are stored in the isthmic region of the oviduct under conditions that maintain viability and suppress early capacitation steps until ovulation occurs. The initial contact between sperm and oviductal epithelium is mediated by carbohydrate-protein interactions. In the pig, the carbohydrate recognition system has been shown to involve oligomannosyl structures. The spermadhesins AWN and AQN1 are the dominant porcine carbohydrate-binding sperm proteins. The objective of this study was to demonstrate that AQN1 contributes to sperm binding to the oviductal epithelium. AQN1 showed a broad carbohydrate-binding pattern as it recognizes both α- and β-linked galactose as well as Manα1-3(Manα1-6)Man structures, whereas AWN bound only the galactose species. Binding of ejaculated sperm to oviductal epithelium was inhibited by addition of AQN1 but not by AWN. Mannose-binding sites were localized over the rostral region of the sperm head. Flow cytometry showed that, under capacitating conditions, the population of live sperm was shifted within 30 min toward an increase in the proportion of cells with low mannose- and high galactose-binding. The loss of mannose-binding sites was accompanied by the loss of AQN1 in sperm extracts and the significant reduction in the sperm-oviduct binding. The oviductal epithelium was shown by GNA-lectin histochemistry and by SDS-PAGE and lectin blotting of the apical membrane fraction to express mannose components that could be recognized by AQN1. These results demonstrate that the sperm lectin AQN1 fulfils the criteria for an oviduct receptor in the pig and may play a role in the formation of the oviductal sperm reservoir.

carbohydrate recognition, oligomannose, oviductal sperm reservoir, spermadhesins

INTRODUCTION

Sperm storage in specialized regions of the female genital tract is one of the successful reproductive strategies in the animal kingdom. During vertebrate evolution, a variety of different sperm storage organs have developed to guarantee the availability of a fertile sperm population when a mature oocyte arrives. In lower vertebrates, sperm can be stored in the female reproductive tracts for long periods of time, allowing fertilization at successive ovulations without additional mating [1]. Although in many mammalian species sperm reside in the female genital tract only for hours or at most for a few days, the general principle of the female sperm reservoir also exists in these species. Instead of specialized organs or crypts, higher mammals use a novel tactic involving adhesion to and controlled release from the epithelium of the oviduct [2].

After ejaculation, sperm are deposited in the female reproductive tract. Most of them are lost during their transit through the female tract and only a small number is trapped in the distal portion of the isthmus. This region of the oviduct serves as a storage site in which sperm reside under protective conditions until ovulation occurs. The sperm storage site in the mammalian oviduct is also designated as a functional sperm reservoir [3], which selects the fertilization-competent sperm population, modulates sperm capacitation, and regulates sperm transport to minimize polyspermic fertilization [4–10]. Numerous recent studies using in vitro oviduct-sperm binding assays have shown that viable, uncapacitated sperm are selected by the oviductal epithelium [11–16] and that, when they bind to it, there is suppression of capacitation-related events, such as the influx of calcium ions into the cell and tyrosine phosphorylation of sperm proteins [16–19]. Because the capacitation process is understood as a controlled destabilization process [20], which reduces the life span of sperm, the maintenance of sperm viability within a certain window of time and the control of capacitation are mutually associated events [21, 22]. Conditions within the oviduct generated around ovulation seem to reverse the inhibitory influence of the oviduct on sperm function, initiating capacitation and hyperactivation to allow the sperm to be released from the oviductal storage site and to progress toward the site of fertilization [22–24]. Very little is yet known about the factors and mechanisms that regulate the inhibiting and activating effect of the oviduct on inseminated sperm [22, 25]. Although the ovary has been found to be able to signal the impending ovulation [8, 22, 25], it seems that male and female communication also includes a cross-talk between the oviduct, which recognizes the fertilization-competent sperm, which in turn modulates the oviductal gene expression [26]. This network of interactions within the female reproductive tract may synchronize the fertilizing activity of sperm with the arrival of the ovulated egg(s) to ensure successful fertilization.

The intimate contact between the spermatozoa and the epithelium lining the ducts is a prerequisite for the sperm-oviduct dialogue. Cumulating evidence showed that sperm-oviduct binding is mediated by carbohydrate-recognition mechanisms [27, 28]. The exposed and flexible oligosaccharide chains of the epithelial cells may anchor the spermatozoa entering the oviduct and sequester them in close vicinity to the epithelium, subsequently allowing all down stream events [23]. Inhibition studies of in vitro sperm-

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oviduct binding have identified specific but different sugar signals in various species. Terminal sialic acid residues of glycan side chains, such as those found in fetuin, have been implicated in the sperm-oviduct binding of the hamster [12], and a sialic acid recognition system has recently been shown to be involved in the rat [29]. Bovine sperm are trapped in the isthmus by binding to Le^a-like saccharides containing fucose in α-1,4 linkage [30–32]. In the pig, the establishment of the oviductal sperm reservoir has been found to involve high-affinity binding sites for oligomannose/hybrid-type *N*-glycans [33, 34].

The cell surfaces of both the epithelial cell and of the sperm express a variety of oligosaccharides [35, 36] and lectin-like molecules [21, 37], which may be involved in carbohydrate-mediated sperm oviduct binding. Studies in cattle have shown that the determining carbohydrate-ligands are most likely exposed by the oviductal epithelium and are then recognized by surface-associated lectin-like molecules of the sperm head. Treatment of the oviductal epithelium with fucosidase significantly reduced sperm binding, and a small fucose-binding protein has been found to mediate sperm binding to the oviduct [32, 38]. This protein has been identified as PDC-109 [32, 38], a member of the major heparin-binding proteins of bovine seminal plasma (BSP proteins), which specifically bind via their phospholipid-binding domain to the sperm plasma membrane at ejaculation [39, 40] and has been implicated in bovine sperm capacitation [41]. In the pig, the most abundant secretory proteins of the accessory glands are members of the spermadhesin family, which represents a novel class of carbohydrate-binding proteins and constitutes the major protein portion of porcine seminal plasma [42].

Here, we test the hypothesis that surface-associated spermadhesins may contribute to the formation of the oviductal sperm reservoir in the pig.

MATERIAL AND METHODS

Chemicals and Media

Unless otherwise stated, chemicals were obtained from Merck (Darmstadt, Germany) and Sigma Chemical Company (Steinheim, Germany) and were of suitably high purity. Second antibodies (anti-rabbit IgG and anti-chicken IgY conjugated to alkaline phosphatase) and streptavidin conjugates were purchased from Dianova (Hamburg, Germany). Biotinylated lectins were purchased from Alexis/Vector (Grünberg, Germany) and sugar-polymeric probes (sugar-PAA-biotin and sugar-PAA-FITC) from Lectinity (Lappenranta, Finland). TBS is composed of 50 mM Tris-HCl, pH 7.4, and 150 mM NaCl; TBST is supplemented with 0.5% Tween 20. Hepes-balanced salts (HBS) solution was prepared by adding 20 mM Hepes, 10 mM glucose, and 2.5 mM calcium hydroxide (pH 7.5). A complete bicarbonate Tyrode balanced salt solution (pH 7.4, 300 mOsm/kg), consisting of 96 mM NaCl, 3.1 mM KCl, 5 mM glucose, 0.4 mM $MgSO_4$, 15 mM $NaHCO_3$, 2 mM $CaCl_2$, 0.3 mM NaH_2PO_4, 1 mM sodium pyruvate, 21.6 mM sodium lactate, 3 mg/ml bovine serum albumin (BSA), and 20 mM Hepes (Tyrode medium) [43] was used for sperm capacitation after equilibration at 39°C in 5% CO_2 for 1 h. Standard medium for oviductal explant assay (OEA) was modified Tyrode balanced solution (TALP-medium) [44] containing 6 mg/ml BSA and 2.2 mg/ml Na-pyruvate. Media were passed before use through a 0.2-μm single-use filter unit (Minisart Sartorius, Göttingen, Germany).

The investigations reported in this article were conducted after approval of the Ethical Committee for Animal Experiments of our institution and were in accordance with the International Guiding Principles for Biochemical Research Involving Animals as promulgated by the Society for the Study of Reproduction.

Isolation and Purification of Spermadhesins AQN1 and AWN

The spermadhesins AQN1 and AWN were isolated from porcine seminal plasma of normospermic ejaculates as described by Sanz et al. [45]. In brief, the heparin-binding proteins of porcine seminal plasma were isolated by affinity chromatography on Heparin-Sepharose CL-6b (Amersham Biosciences, Freiburg, Germany) by elution with 3 M sodium chloride in 20 mM phosphate buffer, pH 7.3. This fraction was collected, dialyzed against distilled water, and further separated by reverse-phase HPLC on a Nucleosil 300-5 C18 column (Macherey & Nagel, Düren, Germany) with a gradient of 0.1% trifluoric acid in (A) water and (B) acetonitrile, between 10 min from 0% to 30% B and between 75 min from 30% to 45% B. The HPLC-purified proteins were characterized after SDS-PAGE by Western blotting with anti-AQN1 and anti-AWN antibodies (see below) and by MALDI-ToF-MS (Kratos Analytical V5.2, Manchester, UK). Proteins were cocrystallized with α-cyano-4-hydroxycinnamic acid on the target as previously described [46].

Sperm Preparation

The sperm-rich phase of ejaculates was collected from healthy fertile boars from the Institute's colony and processed within 30 min after collection. Each ejaculate was routinely assessed for sperm concentration, progressive motility, and sperm morphology. The quality of the ejaculates was in the normal range as judged by conventional spermatological parameters (motility of ejaculates, >70%; percentage of morphologically abnormal sperm, < 25%; total number of sperm in the collected fraction, $40–90 \times 10^9$ sperm cells). Semen samples were washed by Percoll (Amersham Biosciences, Freiburg, Germany) density gradient centrifugation as described in detail elsewhere [43]. The sperm fraction was resuspended in HBS buffer or Tyrode medium and adjusted to the desired sperm concentration.

Sperm Capacitation

Sperm concentration was adjusted to 10^7 cells/ml in Tyrode medium, and sperm samples were incubated at 39°C in a humid atmosphere with 5% CO_2. After 3, 15, and 30 min, the aliquots were immediately used in the oviduct explant assay or extracted as described below.

Sperm Extraction

Sperm samples (10^7 cells/ml) were centrifuged at $10\,000 \times g$ for 5 min. The sperm pellets were resuspended in extraction buffer (10 mM Tris-HCl, pH 7.4, 10% saccharose, 1 mM phenylmethylsulfonylfluoride [PMSF], 1 mM ethylenediaminetetraacetic acid [EDTA] containing 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfontate [CHAPS] hydrate) and allowed to extract for 2 h on ice. Sperm were removed by centrifugation at $10\,000 \times g$ for 20 min, and the sperm-free extracts were used for SDS-PAGE and dot blot analysis or stored at −20°C until further use. Protein contents of the extracts were determined using the Bradford assay [47].

Isolation of Oviductal Apical Membranes

Oviducts were collected from a local abattoir and divided into the isthmic and ampulla regions, as recently described [9]. The apical plasma membrane fractions of oviductal epithelial cells (OAPM) were isolated using a modified version of the method of Smith and Nothnik [18], as described in detail by Fazeli et al. [48]. In brief, sections were longitudinally opened and the epithelia scraped onto a Petri dish in ice-cold PBS. Epithelial cells were washed and resuspended in Tris-HCl, pH 7.4, containing 60 mM mannitol, 5 mM EGTA, and 1 μM PMSF. The cells were snap frozen in liquid nitrogen until OAPM preparation. The thawed cells were homogenized and supplemented with 1 M $MgCl_2$ solution to a final concentration of 10 mM and allowed to stand on ice for 30 min. After centrifugation for 15 min at $3000 \times g$, the supernatant was centrifuged for 30 min at $90\,000 \times g$. The resulting pellet was homogenized in 20 ml buffer (Tris-HCl, pH 7.4, containing 60 mM mannitol, 7 mM EGTA) with 10 strokes in a Potter homogenizer, after addition of 1 M $MgCl_2$ solution to a final concentration of 10 mM and incubation for 30 min on ice, the homogenate was again sequentially centrifuged, first at $3000 \times g$ for 15 min and then the supernatant at $90\,000 \times g$ for 30 min. The resulting pellet was homogenized in TALP medium, centrifuged for 30 min at $90\,000 \times g$, and the pellet then resuspended in the desired medium and snap frozen until further use. Protein contents were determined according to Bradford et al. [47].

Carbohydrate Dot Blot Analysis

Serial dilution of the spermadhesins, AQN1 and AWN (0.5–0.12 μg/dot) in water were spotted onto polyvinylidene fluoride (PVDF) mem-

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branes (Millipore, Eschborn, Germany). After blocking in 1% (w/v) blocking Reagent (Roche, Mannheim, Germany) in TBS overnight, the strips were incubated with a solution of sugar-conjugated biotinylated polymeric probes (sugar-PAA-biotin, 20 μg/ml in TBS for 60 min). After three washing steps in TBS containing 1% blocking solution, the strips were incubated with alkaline phosphatase-conjugated streptavidin (1:5000 for 60 min). After additional washing steps, the reaction was developed with NBT/BCIP (AppliChem, Darmstadt, Germany) for 5 min following the manufacturer's instructions. All incubations were carried out at room temperature.

SDS-Polyacrylamide Electrophoresis and Protein Blot Analysis

A polyclonal monospecific antibody directed against purified AWN was raised in chicken and tested for specificity as described [49]. The anti-AQN1 antibody raised in rabbit was a gift of Dr. V. Jonakova, Praha [50].

Aliquots of sperm extracts (equivalent to 7.5×10^6 or 7.5×10^5 cells/lane) or OAPM extracts (~20 μg protein/lane) were subjected to SDS-PAGE on 15% or 8% polyacrylamide gels, using a discontinuous buffer system according to Laemmli [51]. Proteins were transferred to PVDF membranes with a semidry system [52] and probed with antibodies (anti-AWN in chicken and anti-AQN in rabbit) as recently described [53]. In brief, after saturation with 1% (w/v) blocking reagent (Roche, Manheim, Germany) in TBS overnight at 4°C, the membranes were sequentially incubated with anti-AQN1 in rabbit (1:1000 [v/v] in TBS for 60 min) and with alkaline phosphatase-conjugated anti-rabbit-IgG (1:5000 [v/v] in TBS for 60 min), or the membranes were probed with anti-AWN in chicken (1:2000 in TBS) and anti-chicken IgY-alkaline phosphatase (1:2500 in TBS for 60 min). Alternatively, the membranes were probed with biotin-conjugated Galanthus nivalis agglutinin (GNA) (2 μg lectin/ml) in TBS containing 0.5% Tween 20 (TBS-Tween) for 60 min. After three washing steps in TBS-Tween, the lectin blots were incubated with alkaline-conjugated streptavidin (1:50 000 [v/v] in TBS-Tween). After additional washing steps, the different pretreatments of the protein blots (Western blot and lectin blot) were visualized by the addition of NBT/BCIP for 5 min following the manufacturer's instructions.

Oviductal Explant Assay

Explants were prepared from oviducts collected from the local abattoir from sows as previously described [9]. In brief, at each time, pairs of pieces between 0.5 and 1 mm long were cut from the longitudinal folds of the isthmic region of the oviductal epithelium and placed into TALP medium. The viability of the oviductal explants was assessed by evaluating their ciliary activity. Two pairs of explants were equilibrated in 60 μl TALP medium at 39°C in a humidified atmosphere containing 5% CO_2 in air. Percoll-washed sperm were added to the explants, resulting in a sperm concentration of 1×10^5 cells/80 μl final volume. In case of competitive inhibition, the spermadhesins (AQN1, AWN) were added to the explants in TALP medium in the desired concentration (serial dilution of between 20 μM and 1.25 μM for IC_{50} determination) before coincubation with the sperm for 15 min at 39°C in 5% CO_2 in air.

Alternatively, Percoll-washed sperm were precapacitated in Tyrode medium for 3, 15, and 30 min as described above. Two pairs of explants for each sampling time were equilibrated at 39°C in 5% CO_2. Sperm were added to the explants, resulting in a sperm concentration of 1×10^5 cells/80 μl final volume. As a control, spermatozoa of the Percoll-washed non-capacitated samples were tested for their capacity to bind to explants.

The explants were freed of loosely attached sperm by vigorous washing twice in TALP medium, transferred to prewarmed slides, covered with cover slips supported by silicon grease, and analyzed by videomicrography as described elsewhere [9, 35]. A video monitor WV-BM 1400 equipped with a video camera CF 8/1 (Kappa, Gleichen, Germany) were used to observe and videotape three separate fields in each explant of the pairs with constant focusing on the tissue. The numbers of bound sperm were counted by direct observation at the edges and surface of the explants. The surface area of each observed particular explant region was calculated from the videotape using image analysis software (Mika Medical GmbH, Version 2.0, Bad Feilnbach, Germany), a monitor (Trinitron, Sony, Japan), and a computer system (Dell 450/M). Furthermore, the number of bound sperm/0.01 mm^2 was defined for each explant within the pair as the sum of the sperm number bound to each region divided by the sum of the areas of the three regions as $BI_E = (N_{R1} + N_{R2} + N_{R3})/(S_{R1} + S_{R2} + S_{R3})$ and the arithmetic mean for two explants was calculated (for more details, see [9]).

Fluorescent Labeling of Sperm

The sperm suspension (10^7 cells/ml) were incubated with α-D-mannose-PAA-biotin (20 μg/ml) in PBS containing 10% goat serum (PBS-GS) for 15 min at 39°C. After washing and centrifugation (3×2 min at $150 \times g$) in PBS-GS, the loose sperm pellet was incubated with streptavidin-FITC (1:1000 [v/v] in PBS-GS) for an additional 15 min at 39°C. Following additional washing steps (3×2 min at $150 \times g$) in PBS-GS, 5 μl of the sperm suspension were dropped on a slide, covered with cover slips, and examined under an Axioskop microscope equipped with epifluorescence optics (Zeiss) using a 565-nm filter (magnification: 1000×) with integrated digital camera (Olympus DP 50 CU, Olympus Optical Co, Ltd. Melville, NY). The documentation was made with the Olympus AnalySIS soft imaging system. Specificity of the binding was tested by incubation with α-D-mannose-PAA biotin in the presence of 0.5 M α-methyl-mannopyranoside.

Quantification of Sugar-Binding Sites by Flow Cytometry

Sperm (10^6 cells/ml) were preincubated in Tyrode capacitation medium containing 5 μl/ml propidium iodide for 3, 15, and 30 min at 39°C in a humid 5% CO_2 atmosphere. Three minutes before sampling time, FITC-conjugated sugar probes (α-D-mannose, Galα1-3GalNAcα, and Galβ1-3GalNAcβ) were added to a final concentration of 3 μg/ml. Fluorescence intensity (FI) of sugar-labeled sperm was measured using a DAKO Galaxy flow cytometer (DakoCytomation GmbH, Hamburg, Germany) with a 100-mW argon ion laser. The cells were excited at 488 nm, and FITC fluorescence was detected using a 520-nm band-pass filter (FL 1), while propidium iodide was detected using a 610-nm band-pass filter (FL 3). The settings for recording FITC/PI fluorescence were as recently described [54]. Fluorescence data were collected in the logarithmic mode, while forward- and side-scattered light (FSC and SSC) data were collected in the linear mode. Ten thousand cells were analyzed per sample. Specificity of the binding was controlled by coincubation of the sperm samples with FITC-conjugated sugar-PAA in the presence of 0.5 M α-methyl-mannopyranoside (for FITC-conjugated α-D-mannose-PAA) and 0.5 M lactose (for FITC-conjugated Galα1-3GalNAcα- and Galβ1-3GalNAcβ-PAA).

Localization of Mannose-Containing Oligosaccharides at the Oviductal Epithelium by Lectin Histochemical Analysis

The tissue preparation of the female genital tracts was performed substantially as previously described [53]. In brief, uterotubal junction, isthmus, and ampulla of adult estrous sows were dissected, fixed in 4% PBS-buffered formalin, and embedded in paraffin. Tissue preparations were cut into 7-μm sections and mounted on poly-lysine-coated slides. After rehydration, endogenous peroxidase activity was blocked by incubation with 0.6% hydrogen peroxide. To avoid unspecific binding, the sections were treated with PBS-GS for 30 min at 37°C before the preparations were sequentially incubated for 30 min at 37°C with the biotinylated lectin (15 μg/ml) and with streptavidin-peroxidase (1:10 000 v/v) in PBS containing 0.1% BSA. Between and after each incubation steps, the sections were washed and the lectin reactions were visualized with 3.3′diaminobenzidine chromogen solution (Bio Genex, Hamburg, Germany) for 10 min. The sections were washed in distilled water and mounted with Kaiser glycerin gelatine (Merck, Darmstadt, Germany) and observed under an Axioskop microscope with integrated digital camera. Control sections were tested in the presence of the inhibiting sugar at a concentration of 0.5 M and by omitting the lectin-binding step.

Statistical Evaluation

Analysis of variance and nonparametrical analysis (SAS Software: General Linear Model; Means; Npar1way) were used to verify the results of the experiments: the effect of incubation under capacitating conditions and coincubation with spermadhesins on the binding index, and that of coincubation with FITC-conjugated sugars on fluorescence intensity and fluorescence signal distribution within the sperm population.

Data were calculated and analyzed with the SAS-program package (V. 8, SAS Institute Inc., Cary, NC), Microsoft Excel software (Microsoft Windows 98), FlowMax software (V. 1999, Partec GmbH, Münster, Germany).

Unless otherwise stated, values presented here are means ± SEM. Differences were considered to be significant if the calculated probability of their occurring by chance was less than 5% ($P < 0.05$).

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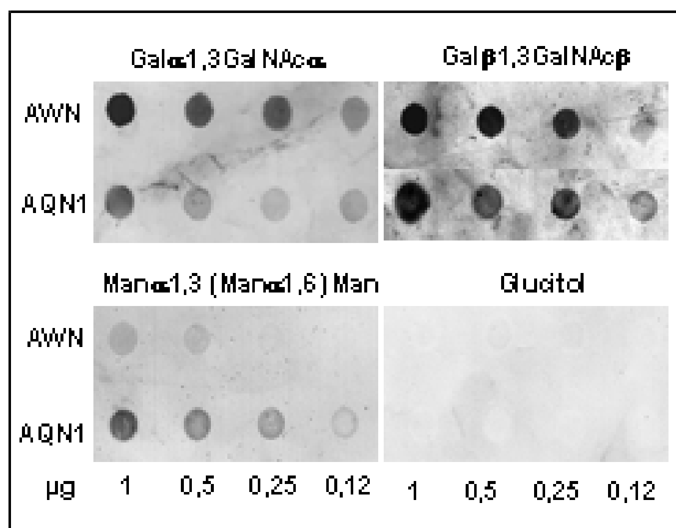

FIG. 1. Carbohydrate binding of boar spermadhesins by dot blot analysis. Decreasing amounts of HPLC-purified AWN and AQN1 were spotted onto nitrocellulose membrane and incubated with biotinylated PAA-galactose moieties [Galα1-3GalNAcα], [Galβ1-3GalNAcβ], mannose moieties [Manα1-3 (Manα1-6) Man] and glucitol. Both spermadhesins bound galactose in α and β linkage. AQN1 bound to mannosyl oligosaccharides in a concentration-dependent manner, whereas AWN did not. As expected, glucitol was bound neither by AQN1 nor AWN.

RESULTS

Carbohydrate-Binding Activity of Spermadhesins and of Sperm Extracts

Spermadhesins were isolated from the heparin-binding fraction of porcine seminal plasma by reverse-phase HPLC. AQN1 (*m/z* 11 833) and AWN (*m/z* 14 775) were identified by MALDI-ToF-MS and by Western blotting (not shown). AWN and AQN1 have been shown to bind preferentially with slightly different specificities to the oligosaccharides sequences Galβ1,3GalNAc and Galβ1,4GlcNAc [42, 55, 56]. By using PAA-immobilized Manα1-3(Manα1-6)Man in a dot blot analysis, it was also shown that AQN1 bound mannosyl-oligosaccharides in a concentration-dependent manner (Fig. 1), whereas AWN did not. Both spermadhesins bound immobilized Galα1-3GalNAc and Galβ1-3GalNAc sequences (Fig. 1). To exclude interference of the PAA-backbone with the protein, AQN1 and AWN were also tested against glucitol-PAA biotin, which was completely negative for both proteins (Fig. 1).

Percoll-washed sperm samples were used for the oviductal explant assay and other binding assays to partially remove seminal plasma and sperm-coating substances. In order to verify that spermadhesins are not completely lost during the washing procedure, the sperm extracts were analyzed by Western blotting. The anti-AWN and anti-AQN1 did not cross-react with AQN1 and AWN, respectively [50, 57], as also shown in Fig. 2. AQN-type proteins were identified in the CHAPS extracts, where they appeared as a triple band at 12 kDa (Fig. 3A), while AWN was detected only in the SDS extracts as a small but distinct band at 15 kDa (Fig. 3, B and C). This signal intensity did not change substantially during 30 min of incubation under capacitating conditions.

Inhibitory Activity of Spermadhesins in the Oviductal Explant Assay

In the pig, oligomannosyl residues have been implicated in the formation of the female sperm reservoir [34]. The

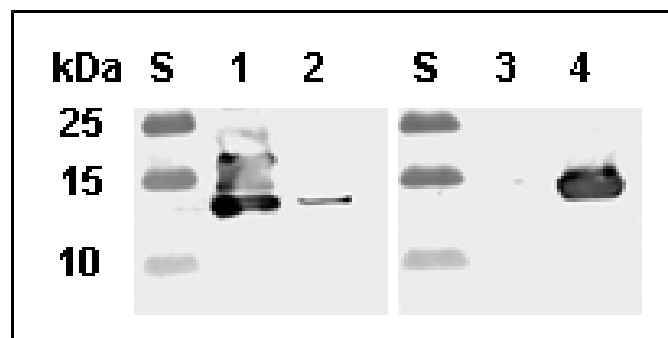

FIG. 2. Cross-reactivity of the anti-AQN1 and anti-AWN antibodies. AQN1 and AWN (1 μg purified protein/lane) were subjected to 15% SDS-PAGE, blotted, and probed with the anti-AQN1 antibody (AQN1, lane 1; AWN, lane 2) and with the anti-AWN antibody (AQN1, lane 3; AWN, lane 4). Protein standards, lane S. Both proteins showed no cross-reactivity. The small band in lane 2 is due to a minor contamination of the isolated AWN.

mannose-binding and non-mannose-binding spermadhesins AQN1 and AWN were tested for their inhibitory activity in the oviductal explant assay. AQN1 showed a typical inhibition curve with $IC_{50} \sim 4.5$ μM, whereas AWN did not specifically inhibit the sperm-oviduct binding (Fig. 4).

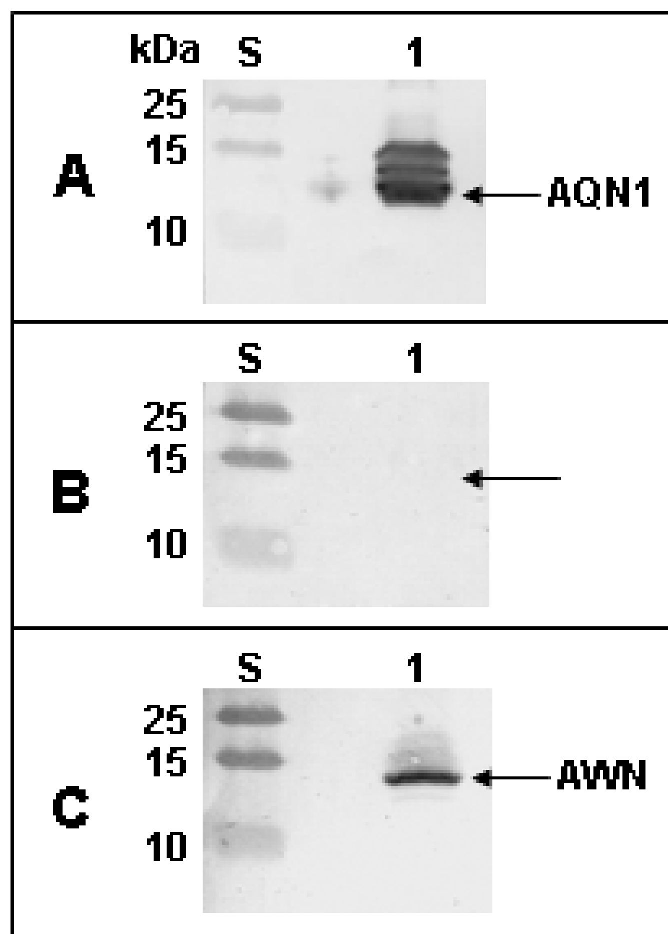

FIG. 3. Identification of AQN1 and AWN in extracts of washed ejaculated sperm. Aliquots of the extracts, equivalent to 7.5×10^6 sperm/ml were subjected to 15% SDS-PAGE and Western blotting. **A**) CHAPS extract probed with anti-AQN1 antibody (lane 1). At high protein concentration, AQN proteins appear as a triple band. **B**) CHAPS extract probed with anti-AWN antibody (lane 1). No protein was detectable. **C**) SDS extract probed with anti-AWN antibody (lane 1). Protein standards, lane S.

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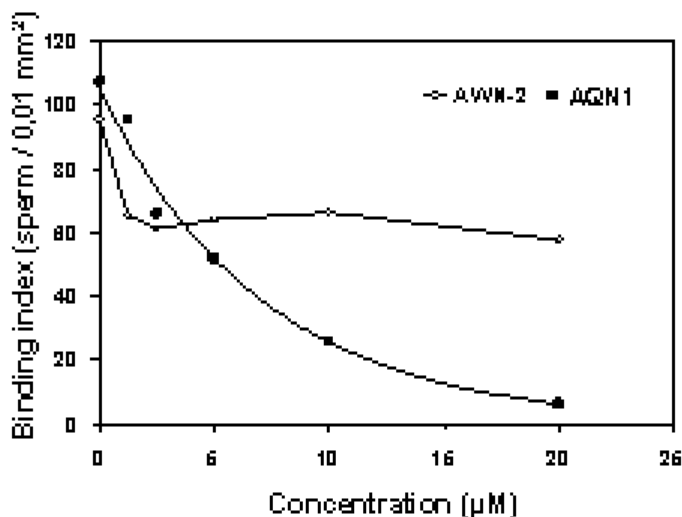

FIG. 4. Dose-dependent inhibition of sperm binding to explants of oviductal epithelium by boar spermadhesins. HPLC-purified AQN1 and AWN were added to the oviductal explants in concentrations varying from 1.25 to 20 μM. AQN1 inhibited sperm-oviduct binding in a concentration-dependent manner with $IC_{50} \sim 4.5$ μM. No specific inhibition of sperm-oviduct binding by AWN was observed. Circles and squares represent the mean ± SEM for at least three replicates.

AQN1 may therefore be the likely candidate protein for mediation of the sperm interaction with exposed oligomannosyl residues of the oviductal epithelium.

High-Mannosyl Glycoproteins of the Porcine Oviductal Epithelium

Lectin histochemical analysis of the female reproductive tract was undertaken only for the mannose-specific Galanthus nivalis agglutinin (GNA) to document the presence of mannosyl oligosaccharide ligands in the oviduct. A detailed lectin histochemical screening of the porcine female genital tract has already been described [35]. The apical glycocalyx of the epithelium of the uterine tubal junction (UTJ) and the isthmic and ampulla regions of the oviduct (Fig. 5, A–C) were shown to interact with the lectin, indicating the presence of mannose-rich glycoconjugates. In the presence of hapten (α-methyl-mannopyranoside), the interaction was completely inhibited, indicating the specificity of the binding (Fig. 5D). Isolation of the apical plasma membrane fraction of the isthmic epithelial cells allowed the electrophoretic identification of the GNA-binding glycoproteins. Lectin blotting of the electrophoretically separated plasma membrane proteins made it possible to identify a subset of mannose-containing glycoproteins with a molecular mass ranging from 180 to 35 kDa (Fig. 6, lane 3). Dominant signals were apparent in the high molecular-mass range at 180, 150, 145, 120, 110, and 105 kDa. Weak but distinct signals also appeared at 98, 84, 76, 64, 45, and 43 kDa (Fig. 6, lane 3). A signal at about 94 kDa could not be inhibited in the presence of the hapten sugar (Fig. 6, lane 4) and also appeared when the lectin binding step was omitted (Fig. 6, lane 5); this indicates the unspecificity of the interaction of the 94-kDa band with the streptavidin conjugate.

Mannose-Binding Sites of Boar Sperm

Mannose-binding sites were localized by fluorescence microscopy chiefly at the apical ridge of the acrosomal region of the sperm head in about 70% of the sperm cells (Fig. 7, A and B). The binding was completely inhibited in the presence of α-methyl-mannopyranoside, which indicates the specificity of this interaction (Fig. 7, C and D).

Initial Response of Spermadhesins and Carbohydrate-Binding Activity to Capacitating Conditions

Uncapacitated sperm have been shown preferentially to interact with the oviduct, whereas after capacitation sperm are released from the oviductal epithelium [15, 16, 19]. The following experiments were designated to correlate the

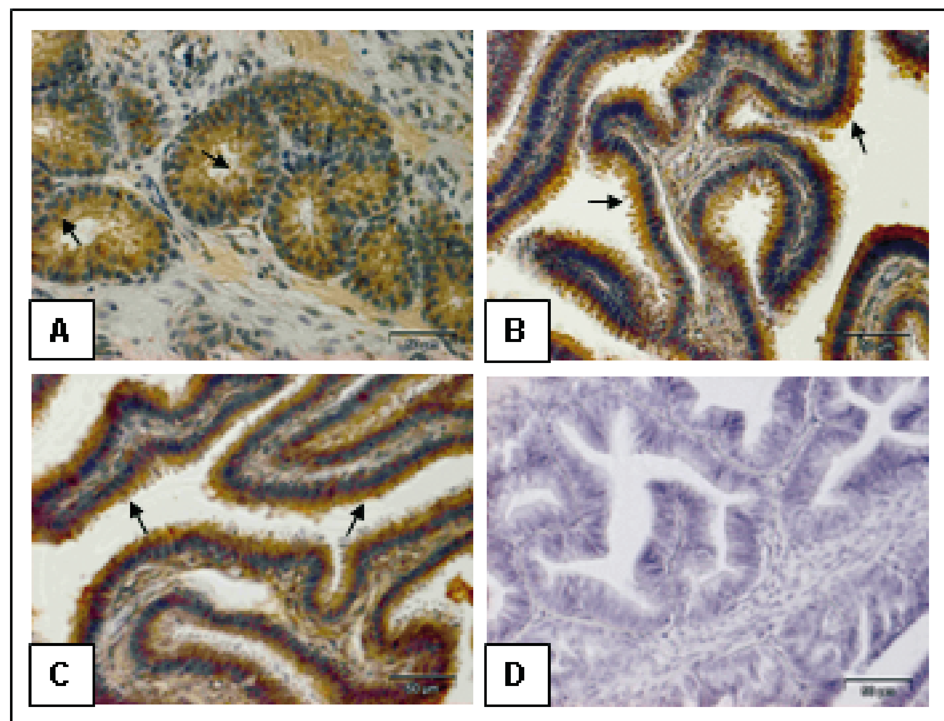

FIG. 5. Lectin histochemical analysis of the porcine oviductal tissue. Histological localization of high-mannosyl glycoproteins of porcine oviductal epithelium was performed using biotinylated GNA and streptavidin-peroxidase. Arrows indicate specific regions within the apical glycocalyx of the epithelium of (**A**) the uterine tubal junction, showing slight staining of the epithelium, (**B**) the isthmic, and (**C**) the ampulla region of the oviduct, showing prominent staining of the epithelium and cilia (**D**). As documented here, specific binding was completely inhibited in the presence of α-methyl-mannopyranoside. Bar = 50 μm.

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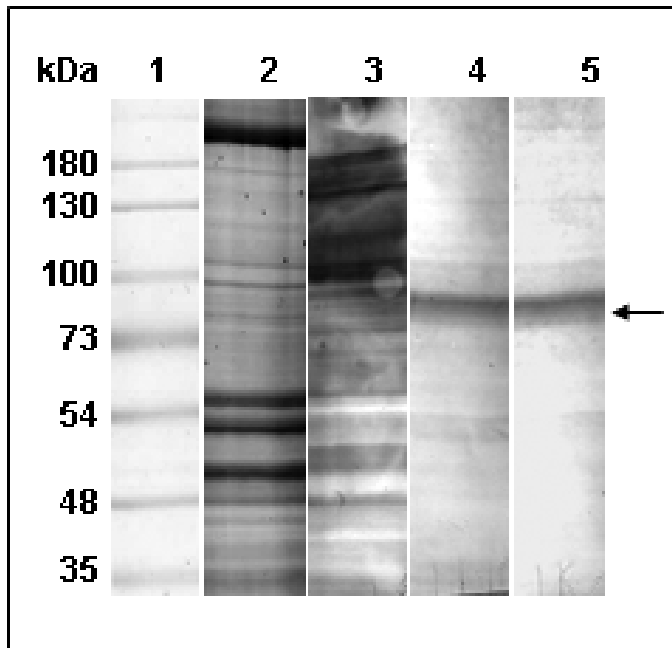

FIG. 6. SDS-PAGE and lectin blotting of the apical membrane fraction of the oviductal epithelium. The solubilized apical plasma membrane fraction of the oviductal epithelium were size fractionated by 8% SDS-PAGE. Identical protein amounts (20 μg/lane) were stained with Coomassie (lane 2) or probed with biotinylated GNA (lane 3). Specific binding of GNA was inhibited by preincubation of the membrane section with α-methyl-mannopyranoside (lane 4). As a control, an additional membrane section was probed only with the streptavidin conjugate (lane 5), and this demonstrates the unspecific staining of the 94-kDa band in lanes 4 and 5 (arrow). Protein standards, lane 1.

binding activity of capacitating sperm with the fate of spermadhesins as measured by Western blot techniques and with the carbohydrate-binding capacity of sperm as determined by flow cytometry. During the initial 30 min of sperm incubation under capacitating conditions, the amount

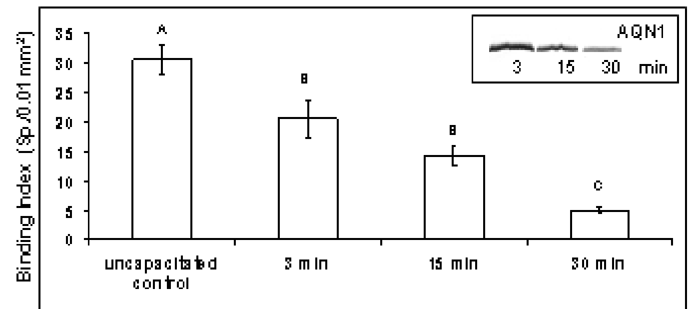

FIG. 8. Sperm binding to explants of oviductal epithelium and loss of AQN1 under capacitating conditions. After incubation under capacitating conditions for 3, 15, and 30 min, identical numbers of sperm were subjected to the oviductal explant assay. Within 30 min, sperm showed a rapid decline in the binding index (BI). Different letters denote significant differences among sampling points ($P < 0.05$). Means ± SEM are shown at least for three replicates. In parallel, detergent extracts of the precapacitated sperm samples (equivalent to 7.5×10^5 cells/lane) were resolved by SDS-PAGE, blotted, and probed with anti-AQN1 antibody showing a considerable loss of AQN1 within 30 min (inset).

of AQN1 in the sperm extracts decreased significantly (Fig. 8, inset). Concomitantly, the binding index decreased from 20.6 sperm/0.01 mm^2 (3 min) to 5.0 sperm/0.01 mm^2 (30 min) (Fig. 8). Parallel to this decrease in sperm numbers (BI), there was a decrease in mannose binding in the viable sperm population and an increase in galactose binding. The relative intensity of the fluorescence signal for Galα1-3GalNAcα and for Galβ1-3GalNAcβ was about two to three times higher after 30 min incubation, while that of the mannose binding sites decreased by half (not shown). In terms of population distribution, the population of live spermatozoa was shifted toward an increased proportion of cells with high galactose and with low mannose binding after 30 min of incubation (Fig. 9).

DISCUSSION

The intimate contact between sperm and the oviductal epithelium seems to be essential for the cross-talk between

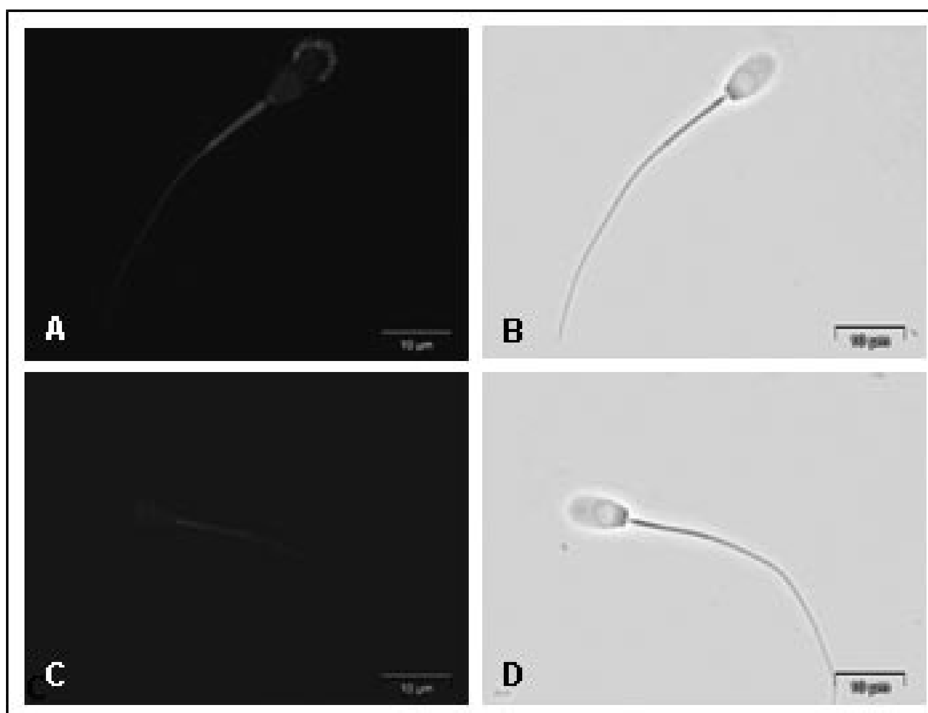

FIG. 7. Localization of mannose binding sites on sperm. **A**) Mannose-binding sites were detected by fluorescence microscopy at the apical ridge of the acrosomal region of the sperm head. **B**) Brightfield image of **A**. **C**) Specific binding of mannose sites on sperm were inhibited in the presence of α-methyl-mannopyranoside. **D**) Brightfield image of **C**. Bar = 10 μm.

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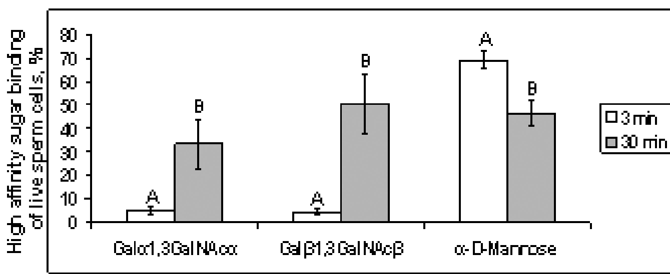

FIG. 9. Changes in the sugar-binding ability during incubation under capacitating conditions. During 30 min of incubation in Tyrode medium, the proportion of spermatozoa with high α-galactose binding increased from 5% to 33% of the live sperm population. Similarly, there was a redistribution within the live sperm population toward an almost 10-fold increase in the proportion of cells with high β-galactose binding. In contrast, the proportion of cells able to bind mannose decreased by ~30% during 30 min of incubation under capacitating conditions. Different letters denote significant differences among sampling points ($P < 0.05$). Means ± SEM are shown at least for three replicates.

the male gamete and the oviduct that aids successful fertilization [26]. Carbohydrate-mediated adhesion has been found to keep sperm in the vicinity of the oviductal epithelial cells. Intensive studies in cattle support the existence of sperm surface-associated receptor molecules that function in the carbohydrate-mediated binding event. A small fucose-binding protein isolated from ejaculated bull sperm has been shown to recognize Le^a-like saccharides of epithelial glycoconjugates [30, 32, 38]. This protein has been identified as the major BSP protein, PDC-109 (also named BSPA1/2). BSPs are characterized by two tandemly arranged conserved fibronectin-II (Fn-2) modules [58] and short highly acidic (BSP-A1/2, BSP-A3) or long repeatedly glycosylated (BSP-30 kDa) N-terminal extensions [59–61]. Homologous proteins have been also described in other ungulates, e.g., pB1 in the pig [62] and SP proteins in stallion [63]. PDC-109 is secreted by the seminal vesicles and associates with the sperm surface at ejaculation via its specific phosphorylcholine-binding sites [39, 40], a property common to seminal Fn-2 type proteins [62, 63]. Recently, the crystal structure of the dimeric PDC-109-phosphorylcholine complex has been successfully identified, indicating that the phosphorylcholine-binding pockets and the heparin-binding domains are located at opposite sides of the PDC-109 dimer [64]. Upon binding to the sperm surface via its phosphorylcholine-binding domain, the heparin-binding sites of PDC-109—and probably its fucose-binding sites as well—are exposed and may therefore be available for the interaction with the oligosaccharides from and within the oviduct.

The efficiency of in vitro binding to oviductal explants has been reported to be significantly reduced in capacitated sperm of various species [13, 15, 16, 65, and this work]. Furthermore, ejaculated porcine sperm also have shown an about 2-fold higher binding capacity than did epididymal sperm from the same animal, indicating that, upon ejaculation, components of the accessory glands tightly bind to the sperm surface, thereby promoting sperm binding to the oviduct [38]. The same characteristic has been reported for cattle. In this species, the oviduct-binding capacity of epididymal and capacitated sperm can be restored by pretreatment with PDC-109 [38]. These observations points to the role of secretory seminal plasma proteins in the formation of the female sperm reservoir. Posttesticular secretion and multifunctionality seem to be mandatory inasmuch as the putative receptor molecule should have both sperm-binding and carbohydrate-binding abilities.

Our results demonstrate that AQN1, a member of the spermadhesin family, meets these criteria and may be a candidate receptor molecule that contributes to the binding of sperm to the oviductal epithelium in the pig. Spermadhesins are related to a superfamily of developmentally regulated proteins, which share the CUB domain within a modular structure [66]. The spermadhesins spanning about 110–133 amino acids form a subgroup comprising a single CUB domain, in which different ligand-binding abilities are imprinted [42]. They are predominantly found in the seminal plasma and/or peripherally associated with sperm of pig, bull, and horse [42]. The spermadhesins designated AQN1, AQN3, AWN, PSP-I, PSP-II, and their glycoforms are the major secretory products of the seminal vesicle and form the bulk of the seminal plasma proteins in the pig. AWN is the only member of its family, which is synthesized in the epididymis and is present on epididymal sperm [53, 67]. AWN and the AQN-type spermadhesins AQN3 and AQN1 have been found tightly attached to the sperm surface, presumably via a direct or indirect phospholipid-binding mechanism [62, 68, 69]. In their monomeric forms, AWN and AQN3 seem to interact directly with membrane phospholipids [68, 69], whereas AQN1 forms a heteromer with pB1, the porcine member of the seminal Fn-2 type protein family [62]. At ejaculation, the binding of the $(pB1)_2/(AQN1)_2$ complex via the phosphorylcholine-binding sites of pB1 may link AQN1 to the sperm surface. This hypothesis is supported by the finding that pB1 and AQN1 attach tightly to the acrosomal region of epididymal sperm [50].

The most striking features of porcine spermadhesins are their carbohydrate-binding abilities to glycoproteins containing Gal-GalNAc and Gal-GlcNAc sequences in *O*-glycans and in *N*-glycans, respectively [55, 56]. Interestingly, *N*-glycosylation of AWN and AQN3 has been found to interfere with their carbohydrate-recognition ability and could be established by cleavage of the glycan chains [70]. AWN- and AQN-type spermadhesins seem to meet the demands for a sperm-associated oviduct receptor, i.e., the ability to bind sperm and carbohydrates.

In this study, nonglycosylated AWN and AQN1 were tested for their properties as putative oviduct receptors. Recently, the high-affinity sugar signal for sperm-oviduct binding in the pig was shown to be mannose in high-mannose or hybrid-type *N*-glycans [34]. The re-evaluation of the sugar specificity with a defined neoglycoconjugate indicated that AQN1 recognizes a broad spectrum of carbohydrates, including mannose and galactose structures, while the galactose specificity of AWN was confirmed. Our findings that AQN1 but not AWN inhibited the in vitro binding of sperm to oviductal explants in a concentration-dependent manner ($IC_{50} \sim 4.5$ μM) seem to be in line with the idea that AQN1 may attach sperm to the oviductal epithelial cells via its mannose-binding site.

By Western blot analysis of the CHAPS sperm extracts, AQN-type proteins were demonstrated as strong signals, while AWN was detected as a distinct signal only in the SDS extracts, and the signal intensity did not change substantially during the first 30 min of sperm in vitro capacitation. This corroborates earlier observations that only small amounts of AWN molecules are present on viable sperm [57]. This AWN has been shown to remain bound in vivo until the sperm reaches the ovulated oocyte and has been detected on sperm bound to the zona pellucida of

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surgically collected embryos after insemination [71]. In contrast, AQN-type proteins were lost during the beginning of in vitro sperm capacitation. This loss was accompanied by a significant decrease in the ability of the sperm to bind to oviductal explants and by a reduction in the number of mannose-binding sites on viable sperm as determined by flow cytometry. Concomitantly, the number of binding sites for terminal α-D-galactose and β-D-galactose residues increased, most probably by unblocking of these sugar-binding sites. Flow cytometric studies carried out in parallel showed that, during in vitro capacitation, the percentage of viable acrosome-reacted sperm remained stable and low ($<2\%$) (Edda Töpfer-Petersen, unpublished observations), indicating that the loss of mannose binding was not due to an ongoing acrosome reaction. These results demonstrate that ejaculated boar sperm expose mannose-binding sites and that the mannose-binding proteins, e.g., AQN1 begin to detach from the surface at an early stage of capacitation.

Lectin histochemical studies have demonstrated the occurrence of several lectin receptors within different substructure of the oviductal epithelium, including glycoconjugates with terminal *N*-acetylgalactosamine, *N*-acetylglucosamine, galactose, and fucose residues [35]. To illustrate the availability of potential ligands for the peripheral sperm lectins, we conducted histochemical analysis with Galanthus nivalis agglutinin (GNA), which recognizes α-1,3 mannose-linked residues in high-mannose and hybrid-type *N*-glycans, to show the distinct localization of GNA receptors in the apical glycocalyx of the epithelial cells of the isthmus and ampulla. Electrophoresis of the oviductal apical membrane fraction confirmed the existence of glycoproteins, particularly in the high molecular mass range of between 180 and 80 kDa. Heparin-like glycosaminoglycans are known to be potent inducers of capacitation in bull sperm [72, 73] and have recently been shown to detach bovine sperm from oviductal explants and oviductal cell cultures [10, 74]. Bovine Fn-2 type proteins (BSPs) mediate the effects of capacitating factors, such as heparin, and the high-density lipoproteins (HDLs) and have been shown to stimulate a significant efflux of cholesterol and phospholipids in artificial and natural membranes [75, 76]. It has, for example, been shown that PDC-109 intercalates into the sperm membrane after binding, first causing an effective rigidification and then inducing membrane perturbation and stimulating the efflux of cholesterol and phosphatidylcholine from the cell membranes, resulting in the formation of protein (PDC-109)-lipid particles [40, 41, 76, 77, 78]. The release of cholesterol/phospholipid complexes from sperm membranes can be enhanced by the capacitating factors and is a crucial early step in the capacitation process [41]. Bovine Fn-2 type proteins are thus multifunctional in that they link sperm to the oviductal epithelium [38], remodel membrane architecture [77, 78], mediate the effects of capacitating factors on sperm, most probably by extracting cholesterol/phospholipid complexes from the membrane [41], and thus prime the capacitation machinery. Although it is known that porcine pB1, like its bovine counterparts, can bind phosphorylcholine and heparin [62] and that AQN1 can bind heparin [42], the mechanisms initiating capacitation in the pig are not yet clarified. As heparin has been shown to be unable to release porcine sperm from oviductal explants, it may not be the major capacitating factor in the porcine oviduct [79]. It remains to be investigated how the pB1/AQN1 complex influences sperm membranes in regard to the capacitation process in the pig.

In conclusion, different peripherally associated sperm proteins may be involved in the formation of the female sperm reservoir in various species. In cattle PDC-109, a member of the short seminal Fn-2 type proteins [32] and in the pig the spermadhesin AQN1 (this work) were shown to initiate sperm binding to the oviductal epithelium. PDC-109 and AQN1 belong to different protein classes, which both are predominantly characterized by a β-domain structure [64, 80] and which share carbohydrate-binding ability.

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