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Zona-Binding Inhibitory Factor-1 from Human Follicular Fluid Is an Isoform of Glycodelin[1]

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ABSTRACT

Zona-binding inhibitory factor-1 (ZIF-1), a glycoprotein in human follicular fluid, reduces the binding of spermatozoa to the zona pellucida. ZIF-1 has a number of properties similar to those of glycodelin-A from human follicular fluid. The objective of this study was to compare the biochemical characteristics of these two glycoproteins. N-terminal sequencing and protease-digested peptide mapping showed that ZIF-1 and glycodelin-A have the same protein core. However, these glycoproteins differ in their oligosaccharide chains, as demonstrated by fluorophore-assisted carbohydrate electrophoresis, lectin-binding ability, and isoelectric focusing. ZIF-1 inhibited spermatozoa-zona pellucida binding slightly more than did glycodelin-A and significantly suppressed progesterone-induced acrosome reaction of human spermatozoa. Indirect immunofluorescence staining revealed specific binding of glycodelin-A and ZIF-1 to the acrosome region of human spermatozoa, where ZIF-1 produced a stronger signal than did glycodelin-A at the same protein concentration. These data suggest that ZIF-1 is a differentially glycosylated isoform of glycodelin that potently inhibits human sperm-egg interaction. Future study on the function role of ZIF-1 would provide a better understanding of the regulation of fertilization in humans.

female reproductive tract, fertilization, follicle, ovum, sperm

INTRODUCTION

Human follicular fluid enhances various sperm functions, e.g., acrosome reaction [1–3], motility [4], and sperm-oocyte fusion [3]. In contrast, human follicular fluid also inhibits the binding of spermatozoa to zona pellucida (ZP) [5, 6]. This zona-binding inhibitory activity is partly due to the presence of two glycoproteins [7]: zona-binding inhibitory factor (ZIF)-1 and ZIF-2.

The lipocalin superfamily is a group of small extracellular proteins that exhibit structural and functional diversity but share three conserved sequence motifs [8, 9]. Many of its members bind small hydrophobic molecules and bind to specific cell surface receptors [9]. Glycodelin-A, previously known as placental protein 14 or progesterone-associated endometrial protein, belongs to the lipocalin superfamily [10]. Glycodelin-A purified from amniotic fluid inhibits spermatozoa-ZP binding [11] and has immunosuppressive properties [12]. Human seminal plasma contains a glycodelin isoform, glycodelin-S, which is immunologically similar to glycodelin-A with identical primary structure but dissimilar glycosylation [13–16]. Unlike glycodelin-A, glycodelin-S has no spermatozoa-ZP binding inhibitory activity [17, 18]. These observations suggest that glycosylation determines the inhibitory activity of glycodelin.

ZIF-1 has a number of properties similar to those of glycodelin-A. Both have a similar molecular mass; 28 kDa for glycodelin-A [10] and 32 kDa for ZIF-1 [7]. Both bind to concanavalin-A [7, 19] and are found in follicular fluid of women undergoing assisted reproduction treatment with or without hormonal stimulation [7, 20, 21]. Both inhibit binding of human spermatozoa to ZP [7, 11] in a dose-dependent manner. In addition, neither of the glycoproteins affects the motility and spontaneous acrosome reaction of spermatozoa [7, 11, 21]. ZIF-1 is immunologically similar to glycodelin [22]. An understanding of the structure of ZIF-1 and glycodelin-A is important to our understanding of the role these molecules may play in the regulation of fertilization. The objective of this study was to compare the biochemical structure and biological activity ZIF-1 with those of glycodelin-A.

MATERIALS AND METHODS

Semen Samples

The research protocol was approved by the Ethics Committee, University of Hong Kong. Semen samples with normal parameters [23] were obtained from men visiting the subfertility clinics of the Queen Mary Hospital, University of Hong Kong. Spermatozoa were separated by two-step Percoll (Pharmacia, Uppsala, Sweden) density gradient (45% and 90%) centrifugation as described previously [24]. The resulting pellet was washed and then resuspended and incubated for 3 h in Earle balanced salt solution (EBSS; Flow Laboratories, Irvine, U.K.) supplemented with sodium pyruvate, penicillin-G, streptomycin sulfate, and 3% BSA. The spermatozoa were then washed and resuspended in EBSS containing 0.3% BSA (EBSS/BSA) before experimentation.

Human Follicular Fluid

Fifteen batches of human follicular fluid samples (20 samples/batch) were collected during oocyte retrieval from women enrolled in the assisted reproduction program of the Queen Mary Hospital. The follicular fluid samples used were obtained from patients with different infertility indications and ages. Human menopausal gonadotropins after downregulation with buserelin and hCG were used for ovarian stimulation. Only those human follicular fluid samples with no blood contamination and from follicles with a retrieved oocyte were included in the study. The cell debris in human follicular fluid was removed by centrifugation at 300 × *g* for 10 min at room temperature. The samples in each batch were then pooled,

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TABLE 1. Binding of lectins with ZIF-1, glycodelin-A, and glycodelin-S.

Lectin	Specificity	Lectin immunoassay (OD_{490})[a]		
		ZIF-1	Glycodelin-A	Glycodelin-S
PNA	β-gal(1-3)galNAc	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
S-WGA	glcNAc or its oligomer	1.43 ± 0.11[b]	0.20 ± 0.04[c,d]	0.07 ± 0.01[c,e]
ConA	α-Man, α-glc	1.22 ± 0.05	1.24 ± 0.05	1.35 ± 0.06
WFA	galNAc	1.10 ± 0.05[b]	0.92 ± 0.03[d]	0.11 ± 0.01[c,e]
RCA_{120}	β-gal	0.85 ± 0.04[b]	0.97 ± 0.04	1.17 ± 0.08[c]
SNA	α-NeuNAc(2-6)gal/galNAc	1.69 ± 0.04[b]	1.85 ± 0.01[c,d]	0.12 ± 0.01[c,e]
WGA	$(glcNAc)_2$, NeuNAc	1.29 ± 0.07[b]	0.69 ± 0.04[c,d]	0.08 ± 0.01[c,e]
LPA	NeuNAc(GaiNAc, GlcNAc)	0.38 ± 0.03[b]	0.50 ± 0.03[c,d]	0.07 ± 0.01[c,e]
UEA-1	α-L-fuc	0.81 ± 0.02[b]	1.13 ± 0.03[c]	1.30 ± 0.08[c]

[a] Mean ± SEM (n = 3). The control coated with 10 000 ng/ml BSA instead of lectins had OD of 0.06–0.08.
[b,c,d,e] Differences within the same row are significant ($P < 0.05$, ANOVA on rank).

sterilized by filtration with a 0.22-μm filter unit (Millipore, Bedford, MA), and stored at −20°C until used. Before experimentation, human follicular fluid was thawed and diluted with EBSS/BSA to the desired concentration.

Hemizona Binding Assay

The hemizona binding assay was performed as described previously [25, 26] with slight modifications [27]. Unfertilized oocytes from women in the assisted reproduction program were bisected into two identical hemizonae using a micromanipulator. Each hemizona was incubated with 2×10^5 spermatozoa/ml in a 100-μl droplet of EBSS/BSA under mineral oil for 3 h at 37°C in an atmosphere of 5% CO_2 in air. The number of tightly bound spermatozoa on the outer surface of the hemizonae was counted. The hemizona binding index (HZI) was defined as:

$$HZI = \frac{number\ of\ spermatozoa\ bound\ in\ test\ droplet}{number\ of\ spermatozoa\ bound\ in\ control\ droplet} \times 100$$

Purification of ZIF-1 and Glycodelin

The purification of ZIF-1 was performed as described previously [21]. Human follicular fluid was passed successively through Hi-Trap blue, protein-G column, Con-A sepharose column (Pharmacia), Amicon-10 concentrator (Amicon, Austin, TX), Mono Q, and Superose columns as described previously [7]. The concentration of purified ZIF-1 was measured with a commercially available assay kit (Protein Assay; Bio-Rad, Hercules, CA). The yield of ZIF-1 obtained was 30–100 μg/L follicular fluid used without accounting for the loss during purification.

Glycodelin-A was purified from amniotic fluid, and glycodelin-S was purified from seminal plasma. Amniotic fluid was obtained from women at term pregnancy (n = 10), and seminal plasma was obtained from men in the assisted reproduction program (n = 10). Seminal plasma was diluted 1:4 (v/v) with 50 mM Tris-HCl-buffered saline containing 9 g/L NaCl, pH 7.7 (TBS). Triton X-100 (0.1%, v/v) was added to amniotic fluid or diluted seminal plasma before purification by a monoclonal anti-glycodelin (clone F43-7F9) sepharose column as described previously [28] with a slight modification. After loading of amniotic fluid or seminal plasma onto the affinity column, the column was washed successively with TBS and 1 M NaCl containing 1% isopropanol and 10 mM ammonium acetate. The bound glycodelin was eluted with 0.1% trifluoroacetic acid. The purified protein was dialyzed against 100 mM sodium phosphate, pH 7.2, and the concentration was measured as described above.

SDS-PAGE

2-Mercaptoethanol was used to denature 200 ng of purified glycoprotein (12 μl) by boiling for 5 min. SDS-PAGE was performed on a 12.5% SDS-polyacrylamide gel in a Mini-protein II system (Bio-Rad) at 100 V for 75 min. Protein detection was carried out using a silver-staining kit (Bio-Rad). Silver staining showed that the purified ZIF-1, glycodelin-A, and glycodelin-S all contained a single band with a molecular mass of about 30 kDa (data not shown).

N-Linked Deglycosylation

The *N*-Glycosidase F Deglycosylation Kit (Bio-Rad) was used to deglycosylate ZIF-1 or glycodelin-A. Fifty microliters of ZIF-1 or glycodelin-A (1000 ng/μl in PBS) was denatured according to the manufacturer's instructions and deglycosylated by the addition 2 μl of *N*-glycosidase F and incubated for 24 h at 37°C. Deglycosylated protein was obtained after three successive precipitations steps using three volumes of cold 100% ethanol, each followed by centrifugation for 5 min at 5000 × *g*. The pellet was dried in a vacuum concentrator (Virtis, New York, NY), redissolved in 20 μl PBS, and analyzed by SDS-PAGE. The deglycosylated ZIF-1, glycodelin-A, and glycodelin-S were further purified using the SMART system with a Superdex-75 column (Pharmacia).

N-Terminal Sequence Analysis, Peptide Mapping, and Isoelectric Focusing

After the *N*-linked deglycosylation, 100 μg of deglycosylated ZIF-1 in 50 μl PBS was loaded on a G1000A Protein Sequencing System (Hewlett Packard, Palo Alto, CA) for N-terminal sequencing by Edman degradation. A BLASTp search (http://www.ncbi.nlm.nih.gov:80/) was used to look for similar amino acid sequences in the Swissprot database.

Peptide mapping was performed by digesting deglycosylated glycodelin-A (20 μg) or ZIF-1 (20 μg) in 40 μl of 0.1 M $NaHCO_3$ with 2 units of agarose-bound trypsin (Sigma, St. Louis, MO) and pepsin (Sigma) for 20 h at 37°C. The resulting peptides in the supernatant were analyzed with SDS-PAGE.

Analytical isoelectric focusing was performed on a 5% polyacrylamide gel according to the method of Robertson et al. [29] in the pH range of 3–10. The isoelectric focusing markers used were glucose oxidase (pI 4.15), soybean trypsin inhibitor (pI 4.55), β-lactoglobulin B (pI 5.1), bovine carbonic anhydrase (pI 6.0), human carbonic anhydrase (pI 6.5), and human hemoglobin A (pI 7.1). The gels were stained for proteins with Coomassie blue.

Lectin-Binding Assay

Flat-bottom, 96-well plates were coated with 10 000 ng/ml lectin overnight at room temperature. The lectins used and their specificities are listed in Table 1. BSA (100 μl/well) in PBS (10 mg/ml) was added to block the unbound sites and incubated for 3 h at room temperature with slow shaking. Twenty-five-microliter aliquots (250 ng) of ZIF-1, glycodelin-A, or glycodelin-S supplemented with 200 μl assay buffer (1 mM $CaCl_2 \cdot 2H_2O$, $MnCl_2 \cdot 4H_2O$, and $MgCl_2 \cdot 6H_2O$) were then added and incubated overnight at 4°C. After washing the wells twice with Tween 20-TBS, anti-glycodelin antibody (2.5 μg) in 200 μl assay buffer (with Ca/Mg/Mn) was added, and the mixture was incubated for 2 h at room temperature. Our unpublished data showed that the antibody bound to both the glycosylated and deglycosylated form of glycodelins, suggesting that the antibody recognized the protein core of the molecules. The wells were washed four times, and 100 μl horseradish peroxidase-conjugated anti-mouse IgG (1:5000; Sigma) was added. After 2 h of slow shaking, the wells were washed four times, and 100 μl/well *o*-phenylenediamine (Sigma) was added to each well. The OD_{490} was measured with a Dynatech MR5000 (Dynatech, Embrach, Switzerland). For the control, the wells were treated in the same way except that BSA was used instead of ZIF-1 or glycodelins.

Fluorophore-Assisted Carbohydrate Electrophoresis

The fluorophore-assisted carbohydrate electrophoresis (FACE) analysis of the samples was performed with an *N*-linked oligosaccharide profiling kit (Bio-Rad) according to the manufacturer's instructions. The glycans obtained from the deglycosylation step described above were labeled with

8-amino-1,3,6-naphthalene trisulfonic acid (ANTS) and separated by gel electrophoresis at 15 mA and 5°C. The separated glycans were visualized in a ultraviolet light box (GDS-8000; UVP, Cambridge, U.K.) with excitation at 365 nm and detection at 515 nm.

The Labwork Analysis Software (UVP) was used to analyze the oligosaccharide patterns. The relative migration of oligosaccharide (RMI_X) was calculated by the equation $RMI_x = [(d_n - d_x)/(d_n - d_{n+1})] + n$, where d is the distance traveled from the top of the resolving gel, x is the oligosaccharide, and n is the number of glucose residues in the oligosaccharide as determined by the glucose ladder provided by the profiling kit (Bio-Rad).

ZP-Binding Inhibitory Activities of ZIF-1 and Glycodelin-A

The hemizona binding assay was used to compare the ZP-binding inhibitory activities of glycodelin-A and ZIF-1. The Percoll-processed spermatozoa (five samples used) were divided into six portions. Because the concentration of ZIF-1 in the follicular fluid is estimated to be about 400 ng/ml, each portion (2×10^6 spermatozoa/ml) was incubated in 10, 100, 1000, 10 000, or 50 000 ng/ml ZIF-1, glycodelin-A, or EBSS/BSA (control) at 37°C in an atmosphere of 5% CO_2 in air for 3 h. After incubation, the spermatozoa were washed with fresh EBSS/BSA. Hemizona binding assays were performed on these treated spermatozoa using the method described above.

Binding of Anti-Glycodelin to ZIF-1

ZIF-1 reacts with anti-glycodelin antibody in Western blot analysis [22]. In the present study, the binding of monoclonal anti-glycodelin antibody (clone F43-7F9) to different concentrations of glycodelins and ZIF-1 was examined using an ELISA. One to 200 ng/ml of ZIF-1, glycodelin-A, or glycodelin-S was coated onto a 96-well plate and incubated for 3 h at room temperature with slow shaking. Excess ZIF-1 and glycodelins were washed away with Tween 20-TBS. Unbound sites in the wells were blocked with 100 μl/well 3% BSA/TBS for 2 h with slow shaking. The wells were then washed with Tween 20-TBS followed by the addition of a limiting concentration of anti-glycodelin in TBS (1000 ng/ml). After overnight incubation at 4°C in a humidified container, the wells were washed three times with Tween 20-TBS, and fluorescein-conjugated goat anti-mouse IgG (Sigma) at a dilution of 1:300 was then added. The bound fluorescence was measured with a fluorometer (FL600; Bio-Tek, Winooski, VT).

Binding of ZIF-1 or Glycodelin-A to Human Spermatozoa

The Percoll-processed spermatozoa (2×10^6 spermatozoa/ml) were incubated in 1000 ng/ml ZIF-1, glycodelin-A, glycodelin-S, or EBSS/BSA at 37°C in an atmosphere of 5% CO_2 in air for 3 h. After incubation, the spermatozoa were washed with fresh EBSS/BSA and then smeared on glass slides, fixed for 30 min in 2% formaldehyde in PBS at room temperature, and washed three times in PBS containing 1% BSA. Anti-glycodelin antibody (clone F43-7F9) in PBS (10 ng/ml IgG) was then added and incubated for 2 h at 4°C in a humidified container. After washing three times in PBS, fluorescein-conjugated goat anti-mouse IgG (Sigma) at a dilution of 1:300 was added and incubated with the slides for 2 h at 4°C. The slides were washed three times with PBS before visualization under a fluorescence microscope.

Effects of ZIF-1/Glycodelin-A on Progesterone-Induced Acrosome Reaction

Spermatozoa (five samples of 2×10^6 spermatozoa/ml) were incubated with 0.1, 1, and 10 ng/ml ZIF-1, glycodelin-A, or EBSS/BSA (control) at 37°C under 5% CO_2 in air for 3 h. After washing with fresh EBSS/BSA, these spermatozoa were further incubated with 1000 ng/ml progesterone or EBSS/BSA (control) for 30 min. The acrosomal status of the spermatozoa was evaluated by fluorescein isothiocyanate (FITC)-PSA (Sigma) and Hoechst staining as described previously [21]. Spermatozoa without Hoechst staining and without FITC staining or with FITC staining confined to the equatorial segment only were considered acrosome-reacted spermatozoa. Progesterone-induced acrosome reaction (PIAR) was defined as the difference in the percentage of acrosome-reacted spermatozoa with and without progesterone treatment. The percentage inhibition of PIAR was calculated as

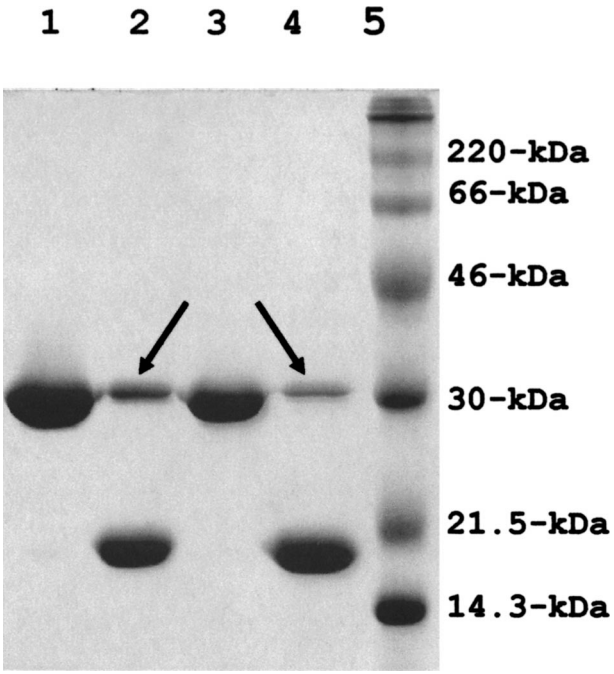

FIG. 1. Detection of purified and deglycosylated ZIF-1 and glycodelin-A in 12% SDS-polyacrylamide gel. Lane 1: 5 μg ZIF-1; lane 2: 5 μg degycosylated ZIF-1; lane 3: 5 μg glycodelin-A; lane 4: 5 μg deglycosylated glycodelin-A; lane 5: molecular mass marker. The thinner bands (arrow) in lane 2 and lane 4 are PNGaseF (34 kDa).

$$\frac{(\%\ \text{PIAR in EBSS/BSA} - \%\ \text{PIAR in ZIF-1/glycodelin-A treated group})}{\%\ \text{PIAR in EBSS/BSA}} \times 100$$

Data Analyses

All the data were expressed as mean ± SEM. The data were analyzed by Sigmastat statistical software (Jandel Scientific, San Rafael, CA). Nonparametric ANOVA on rank analyses were used for all the multiple comparisons, e.g., binding of ZIF-1 or glycodelins to lectins in the lectin-binding assay and the HZI for different glycoproteins and at different concentrations. The parametric Student *t*-test was subsequently used as a post test and is reported here because the software detected that the data were normally distributed. The nonparametric Mann-Whitney *U*-test was also used as a post test. The conclusions are identical to those using the parametric method (data not shown). Paired Student *t*-tests were used to compare the number of spermatozoa bound to ZP between matching hemizona.

RESULTS

Protein Cores of Glycodelin-A and ZIF-1

The size of ZIF-1 and glycodelin-A was 30 kDa, as determined by SDS-PAGE. After glycosidase digestion, both glycoproteins decreased to 19 kDa (Fig. 1). Using Edman degradation, the sequence for the first 25 amino acids from the N-terminal of deglycosylated ZIF-1 was MDI-PQTKQDLELPKLAGTWHSMAMA, i.e., identical to that reported for glycodelin-A [10]. After proteolytic digestion with trypsin and pepsin, deglycosylated glycodelin-A and ZIF-1 gave identical banding patterns (Fig. 2).

After isoelectric focusing, glycodelin-S was separated into several bands with pIs of 4.8–5.5, whereas glycodelin-A yielded bands with pIs of 4.4–5.2 (Fig. 3). These results are similar to that reported previously by Koistinen et al. [14]. ZIF-1 had bands with more acidic pIs; several poorly resolved bands were seen in the pH range of 4.2–4.8. Thus,

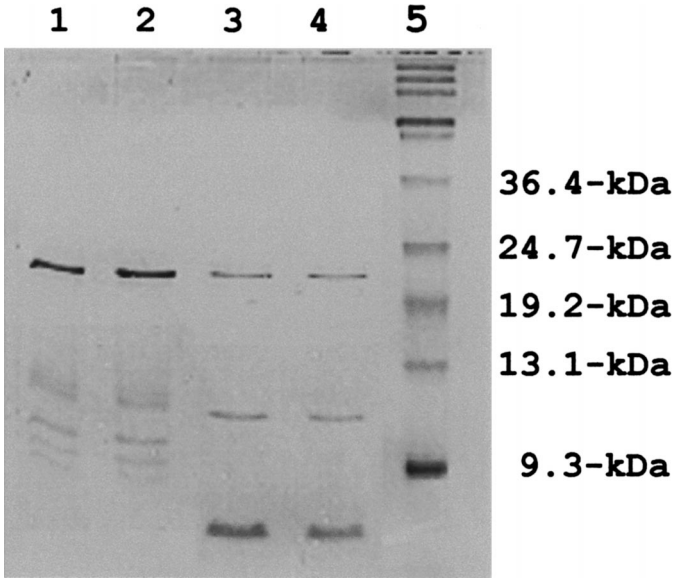

FIG. 2. Peptide mapping of ZIF-1 and glycodelin-A after trypsin or pepsin digestion. Lane 1: trypsin digestion of 1 μg glycodelin-A; lane 2: trypsin digestion of 1 μg ZIF-1; Lane 3: pepsin digestion of 1 μg glycodelin-A; Lane 4: pepsin digestion of 1 μg ZIF-1; Lane 5: molecular mass marker.

the net charges in ZIF-1, glycodelin-A, and glycodelin-S were different.

Oligosaccharide Chains of Glycodelin-A and ZIF-1

The lectin-binding properties of the carbohydrate moieties of ZIF-1, glycodelin-A, and glycodelin-S are shown in Table 1. ZIF-1 reacted with succinylated wheat germ agglutinin (S-WGA), concanavalin A (ConA), *Wisteria floribunda* agglutinin (WFA), *Ricinus communis* agglutinin (RCA_{120}), *Sambucus nigra* bark agglutinin (SNA), wheat germ agglutinin (WGA), and *Ulex europaeus* agglutinin (UEA-1) but did not react or reacted only weakly with peanut agglutinin (PNA) and *Limulus polyphemus* agglutinin (LPA), respectively. These results indicated the presence of antennae with carbohydrate residues of N-acetyl-D-glucosamine or N-acetyl-D-glucosamine oligomers, α-D-mannose, N-acetyl-D-galactosamine, β-galactose, NeuNAc(α2–6)-D-Gal/D-GalNAc, and L-fucose in ZIF-1-derived oligosaccharides. Glycodelin-A had a similar lectin-binding spectrum except that it showed much weaker binding to S-WGA and WGA compared with ZIF-1, which suggests a difference in glycosylation between glycodelin-A and ZIF-1. The possible interference among different layers in the sandwich assay had been checked by appropriate removal of individual layers. The nonspecific binding in this experiment was negligible (data not shown). Glycodelin-S, with a known glycan structures that differs from that of glycodelin-A, was used to check the specificity of the assay. Glycodelin-S bound strongly to ConA, RCA_{120}, and UEA-1 only.

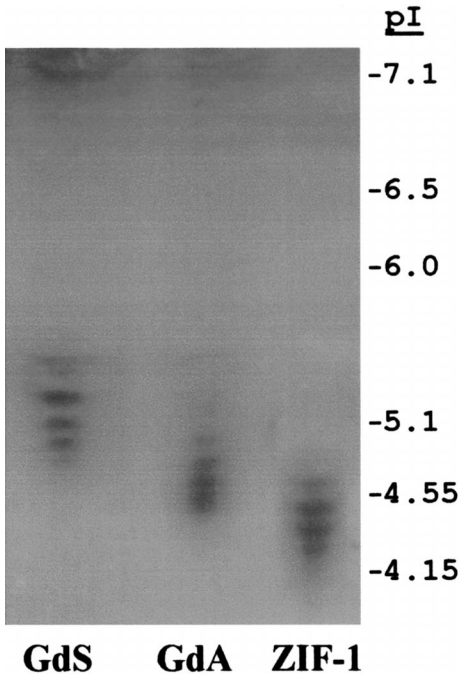

FIG. 3. Isoelectric focusing analysis of glycodelins and ZIF-1 on 5% polyacrylamide gel in the pH range of 3–10. Lane 1: 10 μg glycodelin-S; lane 2: 10 μg glycodelin-A; lane 3: 10 μg ZIF-1. GdA, Glycodelin-A; GdS, glycodelin-S.

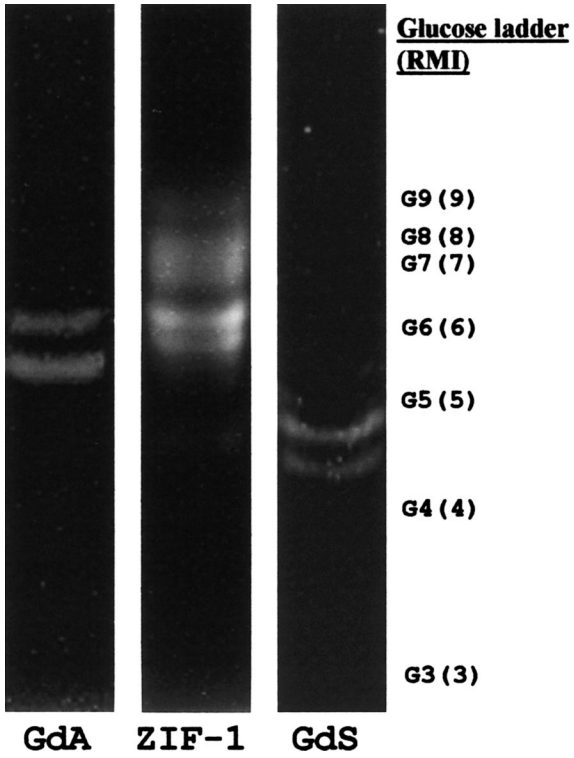

FIG. 4. FACE analysis of oligosaccharides derived from 50 μg ZIF-1 and 50 μg glycodelins. The glycans derived from glycodelin-A (GdA) and glycodelin-S (GdS) were labeled with ANTS. The relative migration of oligosaccharide (RMI_x) was calculated by the equation $RMI_x = [(d_n - d_x)/(d_n - d_{n+1})] + n$, where d is the distance traveled from the top of the resolving gel, x is the oligosaccharide, and n is the number of glucose residues in the oligosaccharide as determined by the glucose ladder. GdA possesses two oligosaccharide bands, with relative RMI_x of 6.48 and 5.84. There are three oligosaccharide bands for ZIF-1 with RMI_x at 7.90, 6.50, and 6.00. GdS possesses two bands with RMI_x of 4.80 and 4.50.

The oligosaccharide chains released after PNGase F digestion of ZIF-1, glycodelin-S, and glycodelin-A were assessed with FACE. With this method, the reducing end of the oligosaccharides was covalently linked to ANTS by reductive amination, and the resulting fluorescent ANTS-oligosaccharide conjugates were resolved electrophoretically on high-density polyacrylamide gels. Figure 4 shows the fluorescent banding patterns of ANTS-labeled oligosaccharides derived from ZIF-1, glycodelin-A, and glycodelin-S.

Glycodelin-A and ZIF-1 had different banding patterns

with FACE. Glycodelin-A possessed two oligosaccharide bands, with relative RMI_x of 6.48 and 5.84. There were three oligosaccharide bands for ZIF-1 with RMI_x at 7.90, 6.50, and 6.00. Glycodelin-S possessed two bands, with RMI_x of 4.80 and 4.50, that were different from those found in ZIF-1 and glycodelin-A. Compare with the glycodelins, the resolution of the ZIF-1 bands was poorer.

Spermatozoa-ZP Binding Inhibitory Activity of ZIF-1 and Glycodelin-A

Five sperm samples were used. Glycodelin-A and ZIF-1 at concentrations of 100, 1000, 10 000, and 50 000 ng/ml significantly decreased the number of spermatozoa bound to the hemizona ($P < 0.05$) as compared with the control. The HZI decreased in a dose-dependent manner for both glycoproteins (Fig. 5). Although both glycodelin-A and ZIF-1 reduced the number of spermatozoa bound to hemizonae at concentrations ≥100 ng/ml, comparison of HZI between ZIF-1 and glycodelin-A at the same concentration showed that ZIF-1 inhibited significantly more binding than did glycodelin-A at concentrations ≥1000 ng/ml.

Binding of ZIF-1 or Glycodelin-A to Human Spermatozoa and the Effects on PIAR

The dilution curves of the immobilized glycodelin-A, glycodelin-S, and ZIF-1 from the ELISA were identical, indicating similar immunoreactivity of the three glycoproteins with the antibody used (Fig. 6). Indirect immunofluorescence staining with the same antibody revealed binding of glycodelin-A and ZIF-1 to the acrosome region of human spermatozoa (Fig. 7). The percentages of spermatozoa with positive immunoreactivity were about 94–98% for both ZIF-1 and glycodelin-A treatments. ZIF-1 produced a stronger signal than that of glycodelin-A at the same protein concentration. The control spermatozoa incubated with glycodelin-S and EBSS/BSA gave no signal.

ZIF-1 or glycodelin-A at concentrations of 0.1, 1, and 10 ng/ml did not affect the spontaneous acrosome reaction of human spermatozoa (data not shown), consistent with previous observations [18, 21]. Although preincubation with glycodelin-A at the concentrations tested did not affect PIAR, ZIF-1 produced a dose-dependent suppression of PIAR (Fig. 8).

DISCUSSION

The spermatozoa-ZP binding inhibitory activity of ZIF-1 is contrary to the general belief that follicular fluid promotes fertilization. An understanding of the structure of the molecule would give hints on its physiological role in fertilization. In this study, we compared the structural and functional properties of ZIF-1 and its related molecules, the glycodelins. ZIF-1 was compared with both glycodelin-A and glycodelin-S because these glycodelins have different carbohydrate moieties, which are known to be important for spermatozoa-ZP binding inhibitory activity.

Results of several of the experiments indicated that ZIF-1 and glycodelin-A are isoforms with the same protein core. The two glycoproteins have identical N-terminal amino acid sequence. Proteolytic digestion of ZIF-1 and glycodelin-A produced identical profiles of peptide fragments. Deglycosylation of ZIF-1 and glycodelin-A with PNGase F each yielded a single band with identical molecular mass, corresponding to the size reported for glycodelin-A [10].

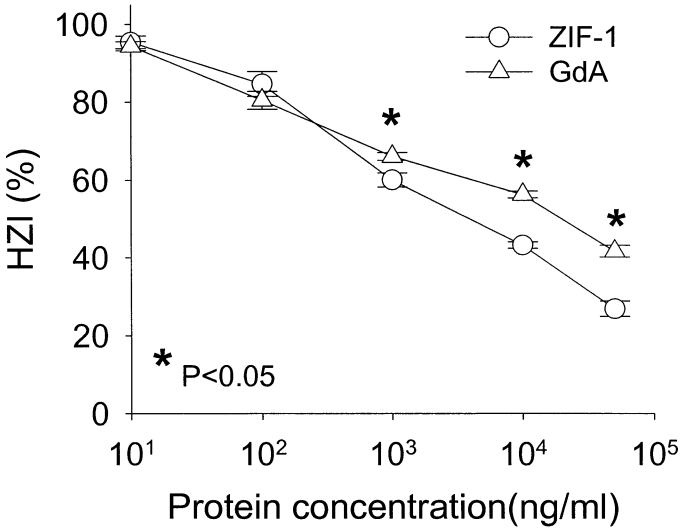

FIG. 5. Effect of different concentrations of purified ZIF-1 or glycodelin-A on the ZP-binding capacity of human spermatozoa. Each point represents the mean of the results of five hemizona binding assays using five ZP and five different sperm samples. One sperm sample and one ZP were used in each hemizona binding assay. *$P < 0.05$ for HZI when comparing ZIF-1 and glycodelin-A at the same concentration.

Both ZIF-1 and glycodelin-A bind to the acrosome region of the human spermatozoa.

Contrary to their immunological and peptide sequence similarities, ZIF-1 and glycodelin-A are dissimilar with respect to their carbohydrate moieties, as shown by the differential binding of glycodelin-A and ZIF-1 to certain lectins and the differences in FACE banding patterns and pI values. In the FACE analysis, the resolution of the ZIF-1 bands was poorer than that of bands derived from glycodelins possibly because of the more complex oligosaccharide chains and probably more side branches in the chains from ZIF-1. These possibilities are consistent with the observation that ZIF-1 has more bands in the FACE gel. The

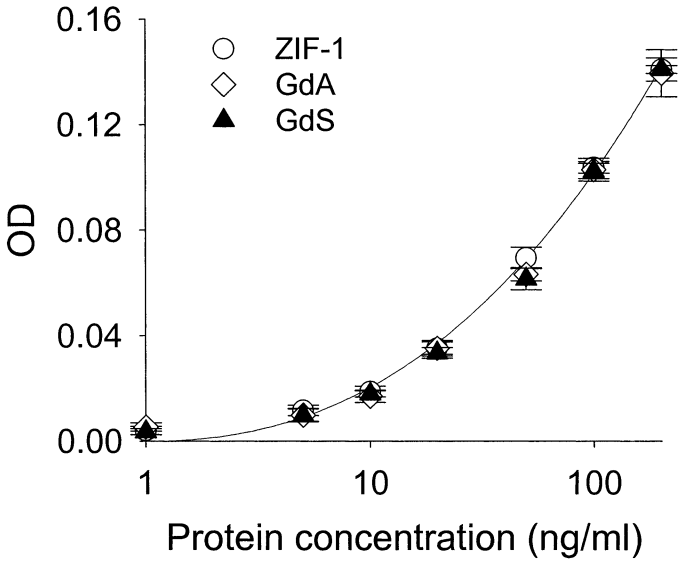

FIG. 6. Dilution curves of ZIF-1, glycodelin-A, and glycodelin-S binding to anti-glycodelin from an ELISA. ZIF-1 and the glycodelins (1–200 ng/ml) were coated onto a 96-well plate and incubated for 3 h at room temperature followed by the addition of anti-glycodelin. The bound fluorescence was measured with a fluorometer. Values are mean ± SEM from three replicates.

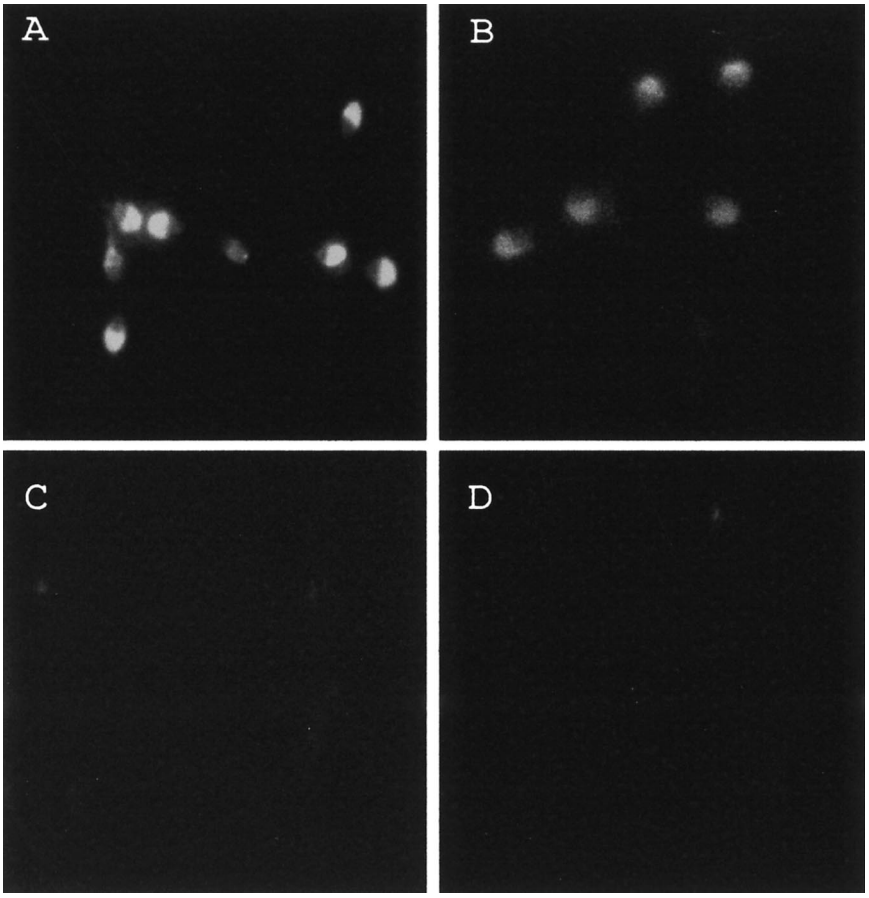

FIG. 7. Immunofluorescence staining of human spermatozoa. Spermatozoa were incubated with 1000 ng/ml ZIF-1 (**A**), 1000 ng/ml glycodelin-A (**B**), 1000 ng/ml glycodelin-S (**C**), and EBSS/BSA (**D**) at 37°C in an atmosphere of 5% CO_2 in air for 3 h. The treated spermatozoa were then incubated successively with anti-glycodelin antibody and fluorescein-conjugated goat anti-mouse IgG. The slides were washed three times with PBS between each step, and reaction was visualized under a fluorescence microscope.

composition of complex mixtures of oligosaccharide chains cannot be achieved in a single FACE analysis [30], and manipulation of electrophoretic parameters is required to optimize the separation of the oligosaccharide chains for a particular sample [31, 32]. ZIF-1 bound strongly to S-WGA, whereas glycodelin-A bound only weakly to this lectin. S-WGA binds specifically to N-acetylglucosamine or its oligomers, suggesting that the N-acetylglucosame content in the two glycoproteins is different. These data indicate that ZIF-1 is an isoform of glycodelin. Because ZIF-1 is found in the follicular fluid, it would be appropriate to rename it glycodelin-F to be consistent with the nomenclature of the other glycodelins [13].

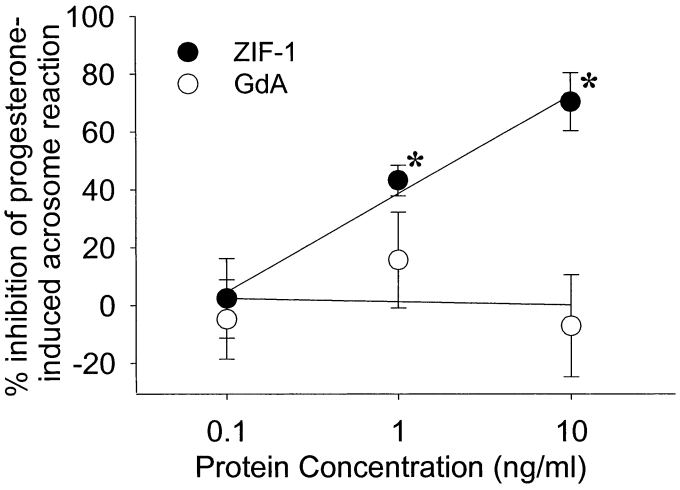

FIG. 8. Effect of different concentrations of ZIF-1 or glycodelin-A on the acrosomal status of human spermatozoa. Spermatozoa were incubated with either ZIF-1/glycodelin-A (0.1, 1, or 10 ng/ml) or EBSS/BSA for 3 h followed by 30 min of treatment with progesterone (1000 ng/ml) or EBSS/BSA. The results are expressed as the percent inhibition of PIAR. Each point represents the mean of results from five different sperm samples. *$P < 0.05$ when compared with the control without ZIF-1/glycodelin-A treatment.

We previously demonstrated specific binding of radioactively labeled ZIF-1 to human spermatozoa [21]. The present immunofluorescence staining experiment further indicates that both ZIF-1 and glycodelin-A bind to the acrosomal region of human spermatozoa. About 94–98% of spermatozoa bound to ZIF-1 and glycodelin-A, and only 6–8% of spermatozoa had spontaneous acrosome reaction under the same experimental condition (data not shown). Therefore, at most only a small percentage of the spermatozoa with positive immunoreactivity are acrosome reacted. Both ZIF-1 and glycodelin-A probably bind mainly to acrosome-intact spermatozoa; the percentages of positively stained spermatozoa are greatly reduced after calcium ionophore treatment to induce acrosome reaction (unpublished results). The acrosome is involved in spermatozoa-ZP binding [33], and the presence of acrosomal binding sites on these glycoproteins is consistent with their inhibitory activity on ZP binding. Two possibilities may account for the difference in intensity of the immunofluresecent staining between ZIF-1- and glycodelin-A-treated spermatozoa at the same concentration. First, our binding kinetics study using ^{125}I-labeled ZIF-1 demonstrated two binding sites for ZIF-1 on spermatozoa, a low-affinity site and a high-affinity site [34]. Glycodelin-A has one binding site on human spermatozoa with affinity close to that of the low-affinity bind-

ing site of ZIF-1. The presence of an additional receptor on spermatozoa may account for the more intense immunoreactivity and stronger spermatozoa-ZP binding inhibitory activity of ZIF-1 when compared with glycodelin-A. Second, the access of the antibody to spermatozoa-bound glycodelin-A may be impaired, e.g., by steric hinderence, although the antibody used in this experiment has the same affinity for ZIF-1 and glycodelin-A. The present data do not allow us to distinguish between these possibilities.

There is compelling evidence that carbohydrate-binding proteins on the sperm surface mediate gamete recognition by binding with high affinity and specificity to complex glycoconjugates on the ZP [35–38]. The results of this study show that ZIF-1 contains antennae with terminal N-acetyl-D-glucosamine or N-acetyl-D-glucosamine oligomers, N-acetyl-D-galactosamine, NeuNAc(α2–6)-D-gal/D-GalNAc, α-D-mannose, β-galactose, and L-fucose. The latter three types of glycans are also present in glycodelin-S and are therefore unlikely to be related to spermatozoa-ZP recognition.

N-acetylglucosamine reduces the binding of human capacitated spermatozoa to ZP [39]. The number of spermatozoa bound on the ZP is also significantly reduced in the presence of hexosaminidase, which hydrolyzes terminal N-acetylglucosamine [40]. A Ca^{2+}-dependent lectin galactosyl receptor is present on the sperm membrane in some animals [41, 42] and in humans [43]. This receptor binds preferentially to N-acetylgalactosamine rather than to galactose and has been suggested to play a role in spermatozoa-ZP binding [44]. This suggestion is consistent with the observation that N-acetylgalactosamine treatment inhibits the binding of hamster spermatozoa to ZP [45]. The precise role of these carbohydrates within the oligosaccharide chains of ZIF-1 and glycodelin-A in inhibiting spermatozoa-ZP binding remains to be elucidated.

The hypothesis that human spermatozoa-ZP binding involves a selectin-like interaction has been proposed [46–48]. Fucoidan inhibits spermatozoa-ZP binding and reduces selectin-mediated adhesions [49, 50]. Glycodelin-A carries fucosylated lacdiNAc antennae, which block selectin-mediated adhesions and inhibit spermatozoa-ZP binding in the same range of concentrations as found in the present study [11, 51]. Glycodelin-S does not have similar oligosaccharide chains and does not interfere with the spermatozoa-ZP binding. Its oligosaccharide chains carry mainly $Lewis^x$ and $Lewis^y$ epitopes that are not involved in selectin-mediated processes [13, 15, 18, 52].

Our finding of a substance in follicular fluid that inhibits spermatozoa-ZP binding was unexpected. Although the presence of binding sites on the acrosome region of spermatozoa is highly suggestive for a functional role of ZIF-1 in fertilization, the physiological significance of this observation can only be speculated upon. Using a lectin immunoassay, our preliminary data indicate that the concentration of ZIF-1 in the follicular fluid is about 400 ng/ml, and the concentration of glycodelin-like substances in the cumulus matrix is at least 10–12 times higher. The spermatozoa-ZP binding inhibitory effect of ZIF-1 may serve to reduce the incidence of polyspermic fertilization or to select spermatozoa with stronger ZP-binding capacity for fertilization. ZIF-1 also may have other effects on sperm functions in the cumulus mass, where local high concentrations of the molecule may exist.

The results of the present study show that ZIF-1, but not glycodelin-A, suppresses PIAR in a dose-dependent manner. At a concentration of 10 ng/ml, ZIF-1 inhibits about 70% of the progesterone action on the acrosome reaction. Progesterone, which is found in the cumulus matrix and follicular fluid, stimulates the acrosome reaction [53], but this action may not be beneficial to fertilization, because the ZP-binding capacity of spermatozoa is reduced after the acrosome reaction [54]. The other possible function of ZIF-1 may be to protect the spermatozoa from premature acrosome reaction before they bind to the ZP. In addition, cumulus cells take up and modify glycodelin obtained from the surrounding fluid [22]. The function of the modified glycodelin remains to be determined. The exact physiological function of different glycosylated forms of the glycodelin is unknown. However their differential expression in various fluids, e.g., ZIF-1 in follicular fluid, glycodelin-A in endometrium and amniotic fluid, and glycodelin-S in seminal plasma, is highly suggestive that they have different functions in different tissues.

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