Glycodelin-S in Human Seminal Plasma Reduces Cholesterol Efflux and Inhibits Capacitation of Spermatozoa*

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Tight control of sperm capacitation is important for successful fertilization. Glycodelin-S is one of the most abundant glycoproteins in the human seminal plasma. However, its function is unclear. We investigated the role of glycodelin-S on capacitation of human spermatozoa. Binding kinetics experiments demonstrated the presence of two saturable and reversible binding sites of glycodelin-S on human spermatozoa. Differently glycosylated other isoforms of glycodelin, glycodelin-A and -F, did not compete with glycodelin-S for these binding sites, suggesting that the glycodelin-S binding sites are different from those of the other isoforms. Indirect immunofluorescent staining revealed specific binding of glycodelin-S around the sperm head. This immunoreactivity was greatly reduced in spermatozoa that had migrated through the cervical mucus surrogates. Glycodelin-S at physiological concentrations significantly reduced the bovine serum albumin and cyclodextrin-induced cholesterol efflux and down-regulated the adenylyl cyclase/protein kinase A/tyrosine kinase signaling pathway, resulting in suppression of capacitation. Deglycosylation abolished glycodelin-S binding and the effect of glycodelin-S on bovine serum albumin-induced capacitation. This indicates that the carbohydrate moiety of glycodelin-S is critical for the function of the molecule. It is concluded that glycodelin-S in seminal plasma maintains the uncapacitated state of human spermatozoa.

Freshly ejaculated mammalian spermatozoa are not immediately capable of fertilizing an oocyte. They acquire their fertilizing capacity by an ill-defined process termed capacitation that normally occurs during migration of the spermatozoa in the female genital tract (1). After capacitation, the spermatozoa are capable of undergoing acrosome reaction upon stimulation by the zona pellucida (ZP)1 proteins (2) and progesterone (3), thereby releasing acrosomal enzymes for penetration through the ZP. Capacitated human spermatozoa remain responsive to ZP-induced acrosome reaction in vitro for 50–240 min only (4). Thus a tight control in the timing of capacitation is important to ensure the occurrence of appropriate sequence of events in the fertilization process. The male sex accessory glands secrete complex mixture of proteins, glycoproteins, peptides, glycopeptides, and prostaglandins into seminal plasma (1). The seminal plasma is capable of maintaining spermatozoa in an uncapacitated state. This is essential to ensure that capacitation would not start before migration of the spermatozoa to the fertilization site. In fact, seminal plasma can “decapacitate” capacitated spermatozoa (5).

Glycodelin is a glycoprotein belonging to the lipocalin family (6). There are three putative N-glycosylation sites at Asn-28, Asn-63, and Asn-85, of which the first two are glycosylated (7). There are three known isoforms of glycodelin, amniotic fluid glycodelin (glycodelin-A, GdA), follicular fluid glycodelin (glycodelin-F, GdF) and seminal plasma glycodelin (glycodelin-S, GdS) (6, 8). The three isoforms have an identical protein backbone, but their glycosylation profiles are different (6, 8).

GdA is the most extensively studied glycodelin isoform. Its abundance in endometrial glands and its immunosuppressive activity suggest that it may play a role in the fetomaternal defense system (9, 10). GdA is the first endogenous glycoprotein found to inhibit spermatozoa-ZP binding (11). Its absence in the endometrium in the periovulatory period is related to the presence of a fertilization window (6). GdF also inhibits spermatozoa-ZP binding (8). GdF, but not the other glycodelin isoforms, suppresses progesterone-induced acrosome reaction (8, 12), suggesting that it may protect spermatozoa from premature acrosome reaction before binding to the ZP. Glycodelin-S does not adversely affect spermatozoa-zona binding (6), and its action on spermatozoa is not clear.

GdS is one of the most abundant glycoproteins in the seminal plasma with a concentration about 1700 nM (13). Unlike GdA and GdF, GdS does not affect spermatozoa-ZP binding (14). GdS has unusual fucose-rich glycans, and its major complex type glycan structures are bi-antennary with Lewisα and Lewisβ antennae. The glycosylation of GdS is unusual among secreted human glycoproteins, because it has no sialylated glycans, and its Asn-28 contains only high mannose structures, whereas Asn-63 contains only complex type glycans (14). The immunosuppressive properties of the Lewisα and Lewisβ epitopes in GdS may contribute to the low immunogenicity of semen in women despite frequent exposure.

In this report, we studied the role of GdS in regulating capacitation of spermatozoa in view of three objectives. The

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‡ The abbreviations used are: ZP, zona pellucida; GdA, -F, and -S, glycodelina A, F, and S; BSA, bovine serum albumin; EBSS, Earle’s balanced salt solution; PBS, phosphate-buffered saline; CTC, chloroethylthioate triethylammonium salt.
first objective was to investigate the binding of GdS to spermatozoa, a prerequisite for the molecule to exert its effect on spermatozoa. The second objective was to determine the relationship between GdS and capacitation. The third was to study the mechanism of action of GdS on capacitation. In particular, we hypothesize that GdS suppresses capacitation by reducing cholesterol efflux from spermatozoa. We also address the ability of GdS to suppress bovine serum albumin (BSA)-induced or cyclodextrin-induced cholesterol efflux and the effect of GdS on enzymes involved in the intracellular signaling pathways leading to capacitation.

**MATERIALS AND METHODS**

**Semen Samples**—The Ethics Committee of the University of Hong Kong approved the research protocol. Only samples with normal semen parameters according to World Health Organization criteria (15) were used. Spermatozoa were processed by using Percoll (Amersham Biosciences) density gradient centrifugation as described previously (8). The processed spermatozoa were then resuspended in Earl’s balanced salt solution supplemented with 0.3% BSA, 0.3 mM sodium pyruvate, 0.16 mM penicillin-G, 0.05 mM streptomycin sulfate, and antibiotics were added to concentrations (EBSS/0.3% BSA) to a concentration of 2 × 10^6 spermatozoa/ml.

**Purification of Glycodelin Isoforms**—Glycodelin-A (GdA), -S (GdS), and -F (GdF) were purified from amniotic fluid, seminal plasma, and follicular fluid respectively, using a monoclonal anti-glycodelin antibody (clone F43–T79) Sepharose column as described (12). The bound glycodelin was eluted with 0.1% trifluoroacetic acid and dialyzed against 100 mM sodium phosphate buffer (pH 7.2). GdS was further purified by anion exchange chromatography as described (12). GdF was purified from human follicular fluid collected during oocyte retrieval from women undergoing assisted reproduction treatment in Queen Mary Hospital, Hong Kong. The follicular fluid was passed successively through Hi-Trap blue, protein-G, ConA-Sepharose columns (Amersham Biosciences), Amicon-10 concentrator (Amicon Inc., Beverly, CA), Mono-Q, and Superose columns. The mixture of glycodelin isoforms was then added to a Bio-Rad Protein Assay kit.

Deglycosylated GdS was prepared using the N-glycosidase F deglycosylation kit (Bio-Rad) as described (12). The deglycosylated protein was obtained after three successive protein precipitations, resuspended in 20 µl of PBS, and further purified by gel filtration chromatography in a SMART system (Amersham Biosciences). The configuration of the glycodelin protein core is unlikely to be affected by the procedure, because deglycosylated glycodelin prepared as described possesses the same immunosuppressive activity as purified GdA (16) and recombinant glycodelin from *Escherichia coli* without post-translational modifications (17). Moreover, thermodynamic evidence is available showing that the native folding of glycodelin is not influenced by glycosylation (18). The amount of deglycosylated glycodelin was analyzed by SDS-PAGE, and its concentration was determined.

Radioactively labeled GdS was prepared by mixing 50 µg of GdS in 0.2 ml of 0.05 M PBS (pH 7.4) with 2 mM of sodium 2,2'2'-thiodiethanol (22) (20 µl, Amersham Biosciences) and freshly prepared chloramine T (100 µg in 0.2 ml of 0.05 M PBS, pH 7.4) as described (12). The first radioactive peak containing iodinated glycodelin was collected.

**Cervical Mucus Penetration**—Human cervical mucus was not used in this study for three reasons. First, cervical mucus contains glycodelin of unknown isofrom (19). Second, the mucus is not homogeneous, because it may be contaminated with vaginal fluid. Third, its viscosity fluctuates widely depending on the day of menstrual cycle, making interpretation of data difficult. Two commercially available substitutes of cervical mucus were used. They were methylcellulose with a viscosity of 4000 centipoises (MC4000, Aldrich) and hyaluronic acid from rooster comb with molecular mass range of 1–4 × 10^6 Da (Sigma). Previously, MC4000 and hyaluronic acid at concentrations of 10 and 6 mg/ml, respectively, have been used successfully as cervical mucus surrogates (20, 21). The substitutes were dissolved in EBSS/0.3% BSA.

To perform the mucus penetration test, a capillary model similar to that used by Calogero and coworkers (27) was used. Briefly, a sterile glass capillary (Microcaps, Drummond, PA) was successively filled with EBSS/0.3% BSA to a length of 5 cm (medium column), and the mucus was substituted to form a mucus column of 5 cm in length. The end of the capillary with the mucus column was dipped into a 100-µl droplet of liquefied semen overlaid with mineral oil. Another capillary containing only EBSS/0.3% BSA served as a control. Spermatozoa were allowed to migrate through the mucus column into the medium column for 30 min at 37 °C under 5% CO₂ in air before the capillary was cut at position of 1 cm above the interface between the mucus column and the medium column. Spermatozoa in the medium column were collected. Spermatozoa that had swum to the corresponding level in the control capillary were used as controls.

**Determination of Acrosomal Reaction and Capacitation**—fluorescein isothiocyanate-labeled *Pium sativum* agglutinin (Sigma) and Hoechst staining techniques were used to evaluate the acrosomal status of the spermatozoa as described (23). The fluorescence patterns of 300 spermatzoa in randomly selected fields were determined under a fluorescence microscope (Zeiss, Oberkochen, Germany) with 1000× magnification. A digital camera (COOLSNAP, Photometrics, AZ) and its associated software (Photometrics) were used to capture the image at room temperature. The filter set used for Hoechst staining consisted of an excitation filter G365, a chromatic beam splitter FT395, and a barrier filter LP520, whereas that for fluorescein isothiocyanate-labeling of *Pium sativum* agglutinin consisted of an excitation filter BP 450–490, a chromatic beam splitter FT510, and a barrier filter LP520. Spermatozoa without staining or those with fluorescein isothiocyanate-labeled *Pium sativum* agglutinin staining confined to the equatorial segment only were considered as acrosome-reacted spermatozoa.

Sperm capacitation was assayed by the chlortetracycline staining (CTC) method as described before (24). The capacitation status and the acrosomal status of 200 spermatozoa were evaluated under a fluorescence microscope (Zeiss) at ×630 magnification with a filter set consisting of an excitation filter BP 450–490, a chromatic beam splitter FT510, and a barrier filter LP520. Five CTC staining patterns of the sperm head were identified (25). CTC4 pattern (uniform head fluorescence) was the main capacitated pattern, and CTC5 pattern (decrease in or loss of uniform head fluorescence) was the acrosome-reacted pattern. The incidence of uncapacitated patterns (CTC1–3) decreased, whereas that of the CTC4 and CTC5 increased with the duration of capacitation of the spermatozoa (24).

**Quantification of Cholesterol**—Sperm cholesterol was extracted by the method of Folch and coworkers (26) adapted for spermatozoa. Briefly, sperm suspension was washed thrice with PBS before vortexing with 8 ml of chloroform:methanol (2:1, v/v). Distilled water (1.5 ml) was then added and the mixture was vortexed and centrifuged at room temperature for 1 h before centrifugation at 500 × g for 10 min at 4 °C. The upper layer was resuspended in 8 ml of chloroform:methanol:water (86:16:1, v/v) and centrifuged at 500 × g for 10 min at 4 °C, after which the chloroform extracts were pooled. The chloroform in the extract was removed in a rotary evaporator (Virtis, Gardiner, NY). The cholesterol content in the dried extract was then determined by a cholesteral assay (Cholesterol Colorimetric Assay Kit; BioVision) according to the manufacturer’s instructions. Sperm cholesterol content was expressed as the amount of cholesterol per million of spermatozoa.

**Determination of cAMP**—Intracellular cAMP was extracted as described by Calogero and coworkers (27). Briefly, spermatozoa that had been incubated for various durations were collected by centrifugation at 3,000 × g for 5 min, and resuspended in 0.5 ml of ice-cold 90% ethanol. After 30 min at −20 °C and 30 min at 4 °C, the samples were centrifuged at 19,000 × g. The supernatants were collected, and the ethanol was evaporated in a rotary evaporator. The dried pellets were stored at −20 °C until used. Intracellular cAMP was then determined using a non-radioactive cAMP enzyme-linked immunosorbent assay Kit (R&D, Minneapolis, MN) according to the manufacturer’s instructions.

**Determination of Protein Kinase Activity**—Spermatozoa were washed thrice with PBS before solubilization by sonication in 100 µl of homogenizing buffer (20 mM PBS, pH 7.4, containing 2 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride, 0.1 mM vanadate, 1 mM MgCl₂, 100 mM NaCl, and 0.05% Triton X-100) for 15 min at 4 °C as described (28). The supernatant was obtained after centrifugation at 15,800 × g for 30 min at 4 °C. The kinase activity in the supernatant was determined at room temperature. Enzyme-linked immunosorbent assay-based tyrosine kinase (Molecular Probes) and protein kinase A (Calbiochem) assay kits were used according to the manufacturer’s instructions. Standard curves were run along with the test samples in each experiment. The protein content in the extracts was determined using commercial kit (Bio-Rad). One unit of protein kinase activity was defined as the amount of enzyme required to catalyze the transfer of 1 pmol of phosphate to the substrates, RFARKSLQKVN and TSETEPQYQPQGENL, respectively, in 1 min at 30 °C.

**Detection of Tyrosine Phosphorylation**—Spermatozoa (2 × 10⁶) were concentrated by centrifugation at 20,000 × g for 2 min at room temperature and washed once in 0.5 ml of PBS at room temperature. The sperm pellet was then resuspended in SDS sample buffer without
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mercaptoethanol and boiled for 5 min. After a further centrifugation at 20,000 × g for 2 min, the supernatant was collected. 2-Mercaptoethanol was added to a final concentration of 5%. The sample was boiled for 3 min before subjected to SDS-PAGE using 10% gels in a Mini-PROTEIN 3 Electrophoresis System (Bio-Rad). The gel was blotted on a polyvinylidene fluoride membrane (Millipore, Bedford, MA) for Western blot analysis using the Chromogenenic Western Blot Kit (Intronig) according to the manufacturer’s instructions. Monoclonal primary antibody, clone 16F4 (Calbiochem) specific for tyrosine phosphorylation was used. In addition, anti-tubulin antibody (Sigma) was used to reveal the equal sample loading of each lane.

Equilibrium Binding of 125I-Glycodelin-S to Spermatozoa—Four semen samples were used (n = 4). Spermatozoa (200,000 in 100 µl of EBSS/0.3% BSA) were incubated with different concentrations (0.16–4700 nM) of 125I-GdS at 37 °C for 3 h. The binding was terminated by the addition of 1.5 ml of ice-cold PBS followed by centrifugation at 300 g for 3 min. The supernatant was then further washed with fresh EBSS/0.3% BSA. The radioactivity associated with the spermatozoa was counted with a gamma counter (Model 5500B, Beckman). Specific binding of GdS was determined by subtracting the radioactivity bound on the spermatozoa in the presence of a 100-fold concentration of unlabelled GdS from that in the absence of unlabelled protein (12). The determinations of total binding and non-specific binding were done in triplicate.

Effect of Glycodelin-S on Spermatozoa—Glycodelin-s binding kinetics was studied by incubation of 2000 nM 125I-GdS with 200,000 spermatozoa in 100 µl of EBSS/0.3% BSA (n = 4) at 37 °C for 0, 2, 5, 10, 15, 20, 30, 60, 90, 120, 180, or 210 min (12). The concentration of glycodelin used was the concentration for saturation binding (see below). Ice-cold buffer (1.5 ml) was added to terminate the binding. Non-specific binding was determined by the inclusion of 100-fold excess of unlabelled GdS. Bound and free glycodelin was separated by centrifugation at 300 × g for 3 min. The sperm-bound radioactivity was measured. For the dissociation kinetics, 200,000 spermatozoa (n = 4) in 100 µl of EBSS/0.3% BSA were incubated with 125I-GdS (2,000 nM) at 37 °C for 30 min, washed with fresh EBSS/0.3% BSA, and smeared on glass slides before fixation in 2% formaldehyde in PBS for 30 min at room temperature. The slides were washed in PBS, stained with 25 µg/ml filipin and glycodelin, and observed under a fluorescence microscope (Zeiss) with 100× magnification. The filters used for filipin staining were excitation filter G365, chromatic beam splitter FT395, and barrier filter LP420.

Specificity of Glycodelin-S Binding to Spermatozoa—Competition binding analysis was used to compare the affinity of GdS binding sites for glycodelin and other lipocalins (12). The binding of 2000 nM of 125I-GdS to 200,000 spermatozoa (n = 4) in 100 µl of EBSS/0.3% BSA was determined in the presence of an increasing concentration (200, 2000, 20,000, and 1 × 105 nM) of unlabelled GdS, GdA, GdF, bovine β-lactoglobulin A (Sigma), and human retinol-binding protein (Sigma) or buffer alone at 37 °C for 360 min. The amount of zona pellucida used is expressed as the number of zona pellucida solubilized per microliter of the final incubation medium (ZP/ml). Percoll processed spermatozoa (n = 5) were first capacitated in EBSS/3% BSA for 3 h, washed in EBSS/3% BSA, and incubated for 30 min in EBSS/3% BSA containing solubilized ZP (5 ZP/ml). The percentage of acrosome-reacted spermatozoa was then determined by P. sativum agglutinin-fluorescein isotheiocyanate staining.

Investigation of Cholesterol Distribution by Filipin—To confirm the effect of GdS on sperm cholesterol content, in situ double staining of GdS and cholesterol with monoclonal anti-glycodelin antibody and filipin, respectively, was performed. Filipin is an antibiotic that forms complexes with non-esterified cholesterol (31). Percoll-processed spermatozoa were incubated with 2000 nM GdS, deglycosylated GdS, GdA, or EBSS/0.3% BSA (control) at 37 °C under 5% CO2 in air for 60 min. They were then capacitated in EBSS/3% BSA for 3 h or MßCD for 30 min, washed with EBSS/0.3% BSA, and incubated for 3 min in EBSS/3% BSA containing solubilized ZP (5 ZP/ml). The percentage of acrosome-reacted spermatozoa was then determined by P. sativum agglutinin-fluorescein isothiocyanate staining.

EFFECTS OF Sperm Capacitation on the Intracellular Distribution of Cholesterol—To evaluate the potential site of action of GdS in these signaling pathways, Percoll-processed spermatozoa (n = 5) were incubated with 2000 nM GdS, deglycosylated GdS, GdA, or EBSS/0.3% BSA (control) at 37 °C under 5% CO2 in air for 60 min. They were capacitated in EBSS/3% BSA for 3 h or MßCD for 30 min, with or without 1 mM Sp-cAMPS, a membrane-permeable cAMP analogue resistant to cyclic nucleotide phosphodiesterase that activates protein kinase A. The intracellular cAMP level, protein kinases activities, and protein tyrosine phosphorylation pattern of the treated spermatozoa were evaluated as described above.

Data Analysis—All the data were expressed as mean ± S.E. The data were analyzed by using statistical programs (SigmaPlot 8.02, Ligand Binding Analysis Module & SigmaStat 2.03, Jandel Scientific, San Rafael, CA). For all experiments, the non-parametric analysis of variance on rank test for multiple comparisons was used. The parametric
Student's t test or the non-parametric Mann Whitney U test were used where appropriate as the post-test. A probability value of \( P < 0.05 \) was considered to be statistically significant.

**RESULTS**

**Equilibrium Binding of \(^{125}\text{I}-\text{Glycodelin-S} to Spermatozoa**—Specific binding of \(^{125}\text{I}-\text{GdS}\) to spermatozoa increased with the concentration of GdS used up to 2000 nM, after which no further increase was observed (Fig. 1). This result indicated that the binding was saturable. Analysis of the saturation data revealed a curvilinear plot. Scatchard plots best fitted by non-linear regression analysis \((R^2 = 0.98)\) suggested the presence of two specific binding sites (Fig. 1A). The low affinity binding sites \((K_D = 1413 \pm 316 \text{ nM}; B_{\text{max}} = 17 \pm 3 \text{ pmol/}2 \times 10^6 \text{ spermatozoa})\) were more abundant than the high affinity binding sites \((K_D = 104 \pm 20 \text{ nM}; B_{\text{max}} = 8 \pm 1 \text{ pmol/}2 \times 10^6 \text{ spermatozoa})\). Hill equation analysis of the binding data yielded a Hill coefficient of less than unity \((0.75 \pm 0.07)\) (Fig. 1B), further suggesting binding heterogeneity.

**Binding Kinetics**—Fig. 2 shows the binding kinetics of \(^{125}\text{I}-\text{GdS}\) to spermatozoa at 37 °C. Specific binding increased rapidly for the first 10 min, reaching equilibrium after about 30 min. These association data (Fig. 2A) were best fitted by a double-exponential equation \((R^2 = 0.97)\), indicating the presence of two populations of binding sites with different observed association rate constants \((K_{\text{obs1}} \text{ and } K_{\text{obs2}})\). The major population (69.4% of the total) had a slow observed association rate constant of \(0.11 \pm 0.05\) per minute \((K_{\text{obs1}})\) compared with the minor population (30.6% of the total) with an observed association rate constant of \(0.06 \pm 0.08\) per minute \((K_{\text{obs2}})\).

The dissociation kinetics at 37 °C was also best described by two-exponential functions \((R^2 > 0.98)\) (Fig. 2B), supporting the presence of two binding site populations. These binding sites had different dissociation constants \((K_{\text{off1}} \text{ and } K_{\text{off2}})\). The larger population (65.7% of the total) had a dissociation rate constant of \(0.028 \pm 0.002\) min\(^{-1}\) \((K_{\text{off2}})\) corresponding to a dissociation half-life of 25 min (obtained from natural log 2/\(K_{\text{off2}}\)). The smaller population (34.3% of the total) had a dissociation rate constant of \(0.007 \pm 0.0006\) min\(^{-1}\) \((K_{\text{off2}})\) and a half-life of 99 min.

The kinetic data indicated that the binding of GdS was reversible. The true association rate constant \((K_{\text{on}})\) was calculated from the equation, \((K_{\text{obs}} = K_{\text{off}}/L)\), where \(L\) is the concentration of \(^{125}\text{I}-\text{GdS}\) used in the binding kinetic experiment. The \(K_D\) values of the two binding sites derived from the rate constants according to the relationship: \(K_D = K_{\text{off}}/K_{\text{on}}\) were 136 and 1700 nM. These values closely agreed with that obtained in the equilibrium binding study.

**Specificity of Glycodelin-S Binding to Human Spermatozoa**—Fig. 3 shows the results of competitive binding to human spermatozoa between \(^{125}\text{I}-\text{GdS}\) and different lipocalin family proteins.
members. As expected, unlabeled GdS inhibited the binding of $^{125}$I-GdS in a dose-dependent manner with a half-maximal inhibition (IC$_{50}$) of 2300 nM. Glycodelin-F, glycodelin-A, and deglycosylated GdS inhibited the binding at high concentrations (IC$_{50}$ = 105 nM), whereas the other lipocalin proteins tested did not affect the binding of $^{125}$I-GdS to spermatozoa at all (IC$_{50}$ > 20 × 10$^5$ nM).

**Localization of Glycodelin Immunoreactivity on Human Spermatozoa**—Glycodelin immunoreactivity was localized to the whole head of swim-up-processed spermatozoa (Fig. 4A). A vast majority (85–90%) of these spermatozoa possessed glycodelin immunoreactivity. The immunoreactivity of the sperm head disappeared after Percoll processing (Fig. 4G). It was greatly reduced after passing through the cervical mucus surrogates (Fig. 4, B and C), but regained after incubation with GdS (Fig. 4E). Percoll-processed spermatozoa incubated with deglycosylated GdS (Fig. 4F) did not possess glycodelin immunoreactivity.

**Effect of Glycodelin-S on Acrosomal Status of Human Spermatozoa**—Glycodelin-S, GdA, or deglycosylated GdS at concentrations of 0.3–3000 nM did not affect spontaneous acrosome reaction as determined by *P. sativum* agglutinin-fluorescein isothiocyanate and CTC staining (data not shown). GdS in all the concentrations tested did not affect sperm capacitation, whereas it inhibited 3% BSA- and MβCD-induced capacitation of Percoll-processed spermatozoa at concentrations >1500 nM and >2000 nM, respectively (Fig. 5A). Using the CTC staining, the percentages of 3% BSA- and MβCD-induced capacitated spermatozoa were 37 ± 2% and 49 ± 4%, respectively. These values decreased with the addition of GdS. The corresponding values at GdS concentration of 3000 nM were 14 ± 3% and 29 ± 3%.
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Effects of different concentrations of GdS on the 3% BSA- or 3 mM MβCD induced capacitation of human spermatozoa as determined by CTC staining (A) and zona pellucida-induced acrosome reaction assay (B). *p < 0.05 when compared with the corresponding control without glycodelin treatment (dashed region).

The same conclusion was obtained from the filipin staining. In Percoll-processed but non-capacitated spermatozoa, the intensity of filipin staining was uniformly high in the acrosome and equatorial regions and weak in the postacrosomal region and the tail (Fig. 7A). These data are consistent with a previous report (36). Capacitation in 3% BSA reduced the intensity of staining on the sperm head. In addition, the fluorescent signal became aggregated and gave a heterogeneous appearance (Fig. 7B). Compared with 3% BSA, the cholesterol removal effect of MβCD was higher resulting in almost complete disappearance of the signal (Fig. 7D). The binding of GdS on spermatozoa suppressed the effect of 3% BSA and MβCD as demonstrated by the strong filipin staining after capacitation treatment (Fig. 7, C and E). Deglycosylated GdS and GdA had no effect on sperm cholesterol content (data not shown).

Effects of Glycodelin-S on Intracellular cAMP Concentration—Fig. 8 shows the effect of GdS on intracellular levels of cAMP in capacitated and non-capacitated spermatozoa. GdS alone had no effect on the cAMP concentration as demonstrated by similar cAMP concentrations in spermatozoa with or without prior GdS treatment. On the other hand, both 3% BSA and MβCD induced a significant increase in cAMP concentration, the effect of MβCD being more rapid. The cAMP level increased from 35 ± 4 fmol/10⁶ spermatozoa to 52 ± 2 fmol/10⁶ spermatozoa after 180 min of 3% BSA treatment, and from 39 ± 3 fmol/10⁶ spermatozoa to 76 ± 2 fmol/10⁶ spermatozoa after 30 min of MβCD treatment. Incubation with 2000 nM GdS significantly reduced the 3% BSA (36 ± 1.8 fmol/10⁶ spermatozoa after 180 min)- and MβCD (61 ± 2.4 fmol/10⁶ spermatozoa after 30 min)-induced up-regulation of cAMP level. Neither deglycosylated GdS nor GdA had any effect on BSA- or MβCD-mediated increase in protein kinase activities and tyrosine phosphorylation (data not shown).

Effects of Glycodelin-S on Protein Kinase Activities—Spermatozoa incubated with 3% BSA or MβCD had significantly higher protein kinase A and tyrosine kinase activities than those in EBSS/0.3% BSA (Fig. 9, A and B). GdS at a concentration of 2000 nM significantly inhibited BSA- and MβCD-induced up-regulation of protein kinase A and tyrosine kinase activities (p < 0.05). The protein kinase A activity decreased from 61 ± 5 to 44 ± 5 units/2 × 10⁶ spermatozoa and from 88 ± 7 to 68 ± 5 units/2 × 10⁶ spermatozoa for 3% BSA and MβCD, respectively (Fig. 9A). The corresponding values for tyrosine kinase were from 8.9 ± 1.4 to 5.7 ± 0.6 units/2 × 10⁶ spermatozoa for 3% BSA and from 11.0 ± 0.9 to 7.7 ± 1.0 units/2 × 10⁶ spermatozoa for MβCD (Fig. 9B).

The results of tyrosine phosphorylation also demonstrated that 2000 nM GdS inhibited the capacitation-associated increase in protein tyrosine phosphorylation mediated by 3% BSA (Fig. 10, lanes 5 and 6) and MβCD (Fig. 10, lanes 9 and 10). On the other hand, the protein kinase activities (Fig. 9, A and B) and tyrosine phosphorylation (Fig. 10, lanes 1 and 2) of
Fig. 7. **Double staining for glycodelin and cholesterol using monoclonal anti-glycodelin and Filipin, respectively, on human spermatozoa.** A, incubation with EBSS/0.3% BSA for 240 min (control); B, preincubation with EBSS/0.3% BSA for 60 min plus capacitation in 3% BSA for 180 min; C, preincubation with 2000 nM GdS for 60 min plus capacitation in 3% BSA for 180 min; D, preincubation with EBSS/0.3% BSA for 60 min plus capacitation in 3 mM MβCD for 30 min; E, preincubation with 2000 nM GdS for 60 min plus capacitation in 3 mM MβCD for 30 min.
spermatozoa in EBSS/0.3%BSA were not affected by GdS.

The effects of GdS on BSA and MβCD-induced kinase activities were not due to abrogation of BSA and MβCD function, because Sp-cAMPS could overcome the effect of GdS. The addition of Sp-cAMPS supported protein kinase activity (Fig. 9, A) and protein tyrosine phosphorylation (Fig. 10) to levels similar to those seen with 3% BSA (Fig. 10, lane 7) or MβCD (Fig. 10, lane 11) incubation with or without GdS. This demonstrates that the effect of GdS is directed upstream of protein kinase A and tyrosine kinase activation in the signal transduction cascade. Again, neither deglycosylated GdS nor GdA had any effect on the BSA- or MβCD-mediated increase in protein kinase activity and tyrosine phosphorylation (data not shown).

**DISCUSSION**

This is the first report on characterization of the binding of GdS to human spermatozoa. The binding is time- and concentration-dependent. There are two binding sites for GdS; a low and a high affinity binding site. Three observations indicate that, on human spermatozoa, these binding sites are different from those of GdA and GdF (8, 12). First, the affinity of GdS to spermatozoa is much lower than that of GdF. Even the KD of the high affinity binding site of GdS (about 100 nM) is four times higher than that of the low affinity binding site of GdF (about 25 nM). The low binding affinity of GdS explains why it is readily removed from the sperm surface and has a short half-life of dissociation. Second, the binding sites of GdA and GdF are confined to the acrosomal region (8), whereas the binding sites of GdS are spread all over the sperm head. Third, in the competition binding assay, GdA and GdF cannot significantly compete with GdS for its binding sites. Likewise, the other lipocalin family members are ineffective in competing with GdS. Thus, the binding of GdS to spermatozoa is specific. This is in line with the suggestion that lipocalins do not promiscuously bind to human spermatozoa (12).

The ejaculated spermatozoa have to traverse through the cervical mucus to enter the upper female genital tract for fertilization. The cervical mucus has several proposed functions, including regulation of sperm transport, selection of spermatozoa according to their motility and morphology, protection of the endocervical epithelium against bacterial invasion and fluid loss (37), and initiation of capacitation (38). However, the molecular mechanism of action of these functions is poorly understood.

The present study provides a molecular basis for the initiation of capacitation by cervical mucus, i.e. removal of seminal plasma factors that maintain spermatozoa in an uncapacitated state (uncapacitation factors). The glycodelin immunoreactivity of spermatozoa became greatly reduced after cervical mucus penetration. This result is consistent with the observation that interaction between spermatozoa and cervical mucus results in loss of decapacitation factors (39). Data derived from the present study suggest that GdS is an uncapacitation factor.

Although the swim-up process retains glycodelin immunoreactivity on ejaculated human spermatozoa, Percoll gradient centrifugation removes the immunoreactivity. The main difference between the two sperm-processing procedures is that spermatozoa are forced through a colloid solution by centrifugal force in the latter, but not in the former. The passage of spermatozoa through the Percoll gradient could be similar to the penetration through cervical mucus. A previous study also showed that Percoll processing reinforced shedding of loosely attached seminal plasma components from spermatozoa (40). Transmission electron microscopy study suggested that Percoll processing removed a coating envelope from the head and tail regions of spermatozoa, which lead to higher zona-free hamster egg-penetration ability of Percoll processed spermatozoa, com-
Capacitation is generally assessed by the ability of the spermatozoa to undergo acrosome reaction in response to physiological inducers, such as ZP or progesterone (1–3). Capacitation starts in the female reproductive tract, and acrosome reaction marks completion of the process. Some components of the seminal plasma are adsorbed on the surface of ejaculated spermatozoa (43). The fact that most of these seminal plasma components are shed from spermatozoa during their ascent to the oviduct is consistent with the proposal that the removal of the seminal plasma components is necessary for capacitation (44).

Spermatozoa are exposed to uncapacitation factors in seminal plasma, but only after ejaculation. A rapid binding of these factors to spermatozoa is important to ensure that ejaculated spermatozoa will remain uncapacitated in the vagina. On the other hand, it is also essential that these factors can be removed from spermatozoa during their passage through the cervical mucus to allow initiation of capacitation. The rapid association to and dissociation of GdS from spermatozoa is compatible with its role as an uncapacitation factor. Of note, cervical mucus contains measurable level of glycodelin (19), but its glycosylation pattern and physiological role are unknown.

The present study demonstrates for the first time that GdS neither affects the acrosome reaction nor decapacitates capacitated spermatozoa, but it inhibits BSA-induced capacitation, shown by both CTC- and ZP-induced capacitation assay. Because GdA has no similar activity, this biological activity of GdS is isoform-specific. The concentration of GdS (>1500 nM) required to elicit the biological activity is compatible with the concentration of GdS in seminal plasma (1700 nM) (13). This suggests that such activity may have physiological relevance. These observations strongly indicate that one of the functions of GdS in seminal plasma is to maintain the uncapacitated state in human spermatozoa.

Coincidentally, recombinant glycodelin from yeast, Pichia pastoris, at a concentration of 1500 nM inhibits capacitation of human and hamster spermatozoa (45). However, the degree of similarity of the glycans of this recombinant glycodelin with GdS is unknown. Glycosylation is important for the biological activity of other glycodelin isoforms (6, 8, 12, 45), and like in the case of the other glycodelin isoforms, the biological activity of GdS is also glycosylation-dependent. Interestingly, carbohydrate residues are also involved in the activity of decapacitation factors in the human spermatozoa (46, 47).

It is interesting to note that albumin is present in high concentration in the uterine fluid and follicular fluid, but is virtually absent from the seminal plasma (48). In the follicular fluid, albumin acts as a sterol acceptor to induce cholesterol efflux from the sperm plasma membrane leading to capacitation (33, 49). The disappearance of glycodelin immunoreactivity after cervical mucus penetration and the ability of GdS to suppress BSA-induced capacitation are consistent with a regulatory role of GdS on capacitation in vivo, such that the removal of GdS during cervical mucus penetration allows albumin in the uterine fluid to initiate capacitation.

The ability of GdS in inhibiting capacitation is related to suppression of BSA- and MβCD-induced capacitation. Because BSA might be contaminated by traces of other serum components, MβCD was used to confirm that the effect of GdS on capacitation was via modulating cholesterol efflux. MβCD is a non-physiological cholesterol acceptor that promotes cholesterol efflux, tyrosine phosphorylation, and capacitation of spermatozoa (29–33). MβCD is more effective than 3% BSA in inducing capacitation. This may be due to the smaller size of MβCD, which allows a more rapid kinetics of cholesterol removal from cells.

Cholesterol is a plasma membrane lipid component that is important in regulating membrane fluidity and permeability, and the mobility of integral proteins and functional receptors in the membranes. Loss of cholesterol from the plasma membrane initiates capacitation (1, 33, 49). It begins when the spermatozoa leave the seminal plasma and is associated with a decrease in the cholesterol/phospholipid ratio and an increase in fluidity and permeability of the sperm membrane (1, 49). Incubation with exogenous cholesterol reduces cholesterol loss and suppresses progesterone-, calcium ionophore-, and ZP-induced acrosome reaction (32, 33, 49) and inhibits the fertilizing ability of spermatozoa (50). It has been suggested that cholesterol in the seminal plasma inhibits capacitation (49). This is supported by the observations that cholesterol acceptors such as serum albumin and cyclohextrin stimulate sperm capacitation (30–33, 49, 51, 52). Pretreatment of cholesterol acceptors with excessive cholesterol blocks the capacitation promoting activity of the acceptors (30, 31). Given this background, results of the present study demonstrate that GdS in seminal plasma inhibits capacitation by reducing cholesterol loss.

The present study expands upon previous reports (30–32, 35) suggesting that capacitation of human spermatozoa may involve the loss of cholesterol and the subsequent increase in cAMP concentration, leading to the activation of protein kinase A and tyrosine kinase and to protein tyrosine phosphorylation. Here, we provide direct evidences on the existence of such signaling pathway by determining the change in intracellular protein kinase activities and cAMP concentration of human spermatozoa after induction of cholesterol efflux. This regulation of protein tyrosine phosphorylation by cAMP and protein kinase A pathway is unique to spermatozoa. It is in contrast to somatic cells, in which the activation of protein tyrosine phosphorylation is mediated through plasma membrane receptors with intrinsic tyrosine kinase activity or associated with tyrosine kinases.

This is the first report demonstrating the ability of GdS to suppress 3% BSA- and MβCD-induced capacitation-associated increases in cholesterol efflux, cAMP concentration, protein kinase activities, and protein tyrosine phosphorylation. This effect was isoform-specific, as GdA was unable to produce such effect even at high concentrations. GdS alone had no effect on these intracellular signaling events, suggesting that the activity of GdS on 3% BSA- and MβCD-induced changes in protein kinase activities and protein tyrosine phosphorylation is not due to a direct action of GdS on these molecules. The observation that exogenous cAMP agonists, Sp-CAMPS, overcome such activity of GdS further indicates that the effect of GdS is directed upstream of protein kinase activation in the signal transduction cascade.

How GdS prevents cholesterol loss is unknown. The plasma membrane of sperm head has a glycocalyx of 100–150 Å in thickness with glycoproteins anchoring to the lipid bilayer of the membrane. The glycocalyx retains a layer of immobilized water around the sperm head. It has been proposed that cholesterol efflux from membranes involves the entrance of the cholesterol acceptor into the immobile water layer and subsequent binding of the acceptor with cholesterol (53). Therefore, one possibility of GdS action is that it covers the glycocalyx and prevents entrance of cholesterol acceptors, thus preventing removal of cholesterol. As glycodelin has significant homology with another lipocalin member, β-lactoglobulin, which binds cholesterol (54), it is also possible that sperm-bound glycodelin-S may bind to cholesterol directly, thereby reducing cholesterol efflux from the sperm plasma membrane. Another possibility is that the binding of GdS on sperm cells causes cholesterol redistribution on the sperm membrane, thereby
reducing accessibility of cholesterol to its acceptors. A similar condition exists in bovine seminal plasma. It contains a glycoprotein, PDC-109, that immobilizes membrane lipid, including cholesterol and retards capacitation upon binding to spermatozoa (55). Interestingly, albumin-mediated cholesterol depletion only occurs after bicarbonate induced membrane lipid scrambling (40, 56). The effect of GdS on bicarbonate-induced capacitation is under investigation in our laboratory.

Taken together, the present results and previous observations show that tissue-specific glycosylation of glycololin modulates sperm function for fertilization. GdS in the seminal plasma acts as a natural uncapacitation factor by modulating sperm cholesterol efflux. Based on current data from this and previous reports, the sequence of events appears as follows: GdS is removed from spermatozoa as they migrate across the cervical mucus, initiating capacitation. Subsequently, follicular fluid-derived GdS in oviductal fluid attaches onto the acrosome region of the sperm head and prevents premature acrosome reaction (8, 12). GdS is then removed by the cumulus/corona cells (42), whereby progesterone-induced acrosome reaction is restored, and sperm-egg binding capacity is induced initiating the fertilization process.

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