Glycodelin-A interacts with fucosyltransferase on human sperm plasma membrane to inhibit spermatozoa-zona pellucida binding

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Summary

Fertilization depends on successful binding of the spermatozoa to the zona pellucida of the oocyte. Glycodelin-A inhibits spermatozoa-zona pellucida binding. Previous data showed that glycodelin-A receptor(s) and zona pellucida protein receptor(s) on human spermatozoa are closely related. Using a chemical cross-linking approach, the glycodelin–A-sperm receptor complex was isolated. The receptor was identified to be fucosyltransferase-5 (FUT5) by mass spectrometry and confirmed with the use of anti-FUT5 antibodies. Sperm FUT5 was an externally oriented integral membrane protein in the acrosomal region of human spermatozoa. Biologically active FUT5 was purified from spermatozoa. Co-immunoprecipitation confirmed the interaction between glycodelin-A and sperm FUT5. Solubilized zona pellucida reduced the binding of glycodelin-A to sperm FUT5. An anti-FUT5 antibody and FUT5 acceptor blocked the binding of glycodelin-A to spermatozoa and the zona binding inhibitory activity of glycodelin-A. Sperm FUT5 bound strongly to intact and solubilized human zona pellucida. The equilibrium dissociation constant of sperm FUT5 binding to solubilized zona pellucida was 42.82 pmol/ml. These observations suggest that human sperm FUT5 is a receptor of glycodelin-A and zona pellucida proteins, and that glycodelin-A inhibits spermatozoa-zona binding by blocking the binding of sperm FUT5 to the zona pellucida.

Key words: Glycodelin, Spermatozoa, Fucosyltransferase, Zona pellucida

Introduction

Glycodelin is a glycoprotein previously known as placental protein-14 or progesterone-associated endometrial protein. The molecule has three reported isoforms that are named according to their source where they are most abundant: glycodelin-A (amniotic fluid isoform), glycodelin-S (seminal plasma isoform) and glycodelin-F (follicular fluid isoform) (Seppala et al., 2002; Chiu et al., 2003a). The glycodelin isoforms have a molecular size of 28-30 kDa. They have the same protein core but differ in their glycosylation.

Glycodelin isoforms modulate sperm functions (Yeung et al., 2006). Glycodelin-A is abundant in the secretory endometrium (Seppala et al., 2002), and is highly upregulated in the endometrium at implantation (e.g. Kao et al., 2002). It is the first endogenous glycoprotein found to inhibit spermatozoa-zona pellucida binding (Oehninger et al., 1995) and its absence in the endometrium in the periovulatory period may be related to the opening of a fertilization window (Seppala et al., 2002). The fallopian tube also expresses glycodelin in a hormone-dependent manner (Seppala et al., 2002). Glycodelin-S maintains the spermatozoa in an uncapacitated state before their migration into the uterine cavity (Chiu et al., 2005), and glycodelin-F prevents premature progesterone-induced acrosome reaction (Chiu et al., 2003b).

The first step in fertilization involves the interaction between molecules on the spermatozoa and the zona pellucida of an oocyte. There is ample evidence that sperm surface carbohydrate-binding proteins mediate gamete recognition and interact with high affinity and specificity to the zona pellucida glycoproteins (Chapman and Barratt, 1996; Wassarman, 1999; Ozgur et al., 1998). Despite the fact that several carbohydrate-binding proteins have been proposed to be responsible for spermatozoa-zona pellucida binding (Nixon et al., 2001; Leyton et al., 1992; Cheng et al., 1994), the exact identity of these sperm surface protein(s), especially those for humans, remains unclear.

The mechanism by which glycodelin-A inhibits spermatozoa-zona pellucida binding is unknown. We hypothesize that glycodelin-A competes with zona pellucida glycoproteins for their sperm membrane receptors. Previous studies showed that glycodelin-A binds specifically to human spermatozoa (Chiu et al., 2003a) and the binding was greatly reduced after acrosome reaction or in the presence of zona pellucida glycoproteins (Chiu et al., 2003b; Chiu et al., 2004). The carbohydrate moieties of glycodelin-A are crucial for its zona-binding inhibitory activity as well as binding on human spermatozoa as deglycosylated glycodelin loses such activities in vitro (Chiu et al., 2003a; Chiu et al., 2004). Coincidentally, the spermatozoa-zona pellucida interaction is also a carbohydrate-mediated event (Wassarman, 1999). These data

suggest that the glycodelin receptor(s) and zona pellucida glycoprotein receptor(s) are closely related. The objectives of this study were to identify the receptor of glycodelin-A in human spermatozoa and to characterize its interaction with human zona pellucida.

Results

Fucosyltransferase 5 (FUT5) is a sperm surface glycodelin-A binding protein

The results of identification of glycodelin-A-bound sperm surface protein(s) are shown in Figs 1, 2. Sulfosuccinimidyl-2-[6-(biotinamido)-2-(*p*-azidobenzamido)-hexanoamido]ethyl-1,3′-dithioproionate (Sulfo-SBED)-conjugated glycodelin-A was covalently bound to spermatozoa after photoactivation.
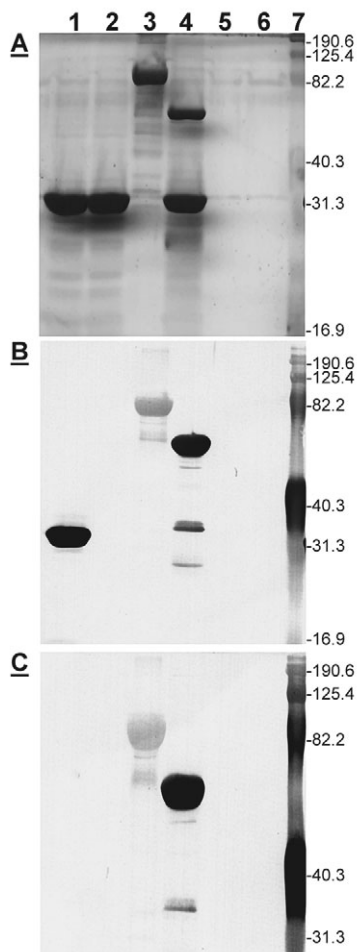

Fig. 1. Isolation of glycodelin-A binding human sperm surface proteins. (A) Silver staining of 12% SDS-PAGE gel. (B) Detection of biotinylated protein using streptavidin-alkaline phosphatase conjugate. (C) Detection of FUT5 using the polyclonal anti-FUT5 antibody, N-18. Lane 1: sulfo–SBED-conjugated glycodelin-A in non-reducing conditions. Lane 2: sulfo–SBED-conjugated glycodelin-A in reducing conditions. Lane 3: immunoaffinity-purified cross-linked glycodelin-sperm protein complex in non-reducing conditions. Lane 4: immunoaffinity-purified cross-linked glycodelin-sperm protein complex in reducing conditions. Lane 5: similar to lane 4 with the use of human fibroblasts instead of spermatozoa. Lane 6: similar to lane 5 with the use of human fibroblasts instead of spermatozoa. Lane 7: molecular mass marker.

Treatment of the glycodelin-A–sperm surface protein complexes with dithiothreitol (DTT; reducing conditions) resulted in reduction of the azo linkage and transfer of the biotin label from glycodelin-A to the sperm proteins. Thus, biotinylated glycodelin-A (Fig. 1A,B, lane 1) showed a single biotin band with molecular size ~30 kDa under non-reducing conditions, which was lost after DTT treatment (Fig. 1A,B, lane 2).

One major biotinylated complex with size ~93 kDa appeared after incubation of glycodelin-A (Fig. 1A,B, lane 3) with the sperm extract. Treatment with DTT revealed a major biotin-labeled complex of size ~65 kDa (Fig. 1A,B, lane 4). Some minor bands of smaller sizes were also seen. By contrast, glycodelin-A did not interact with fibroblasts and non-specifically bound glycodelin-A was removed by washing of the cells after incubation. This is evidenced by the absence of a band in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; Fig. 1A,B, lane 6). Deglycosylated glycodelin-A did not interact with human spermatozoa (data not shown).

Mass-spectrometry (MS) analysis of the tryptic digests and a database search identified the ~65 kDa glycodelin-A bound protein as FUT5 (Table 1). The six peptide sequences showed >90% match to the MS spectra and confirmed the protein identity at high confidence level. Two of the identified peptides, SFSWALAFCK and TELVAWAVSNWKPDSAR, were unique to FUT5 among fucosyltransferase 3 (FUT3), FUT5 and fucosyltransferase 6 (FUT6). The identity of the protein was confirmed by western blot analysis using the anti-FUT5 antibody, N-18 (Fig. 1C). The immunoreactivity was associated with bands of sizes ~93 and ~65 kDa, corresponding to the glycodelin-A–FUT complex in non-reducing conditions (Fig. 1C, lane 3) and FUT in reducing conditions (Fig. 1C, lane 4), respectively.

Presence of FUT protein in human spermatozoa

Recombinant FUT proteins were expressed in inclusion bodies and constituted for approximately 50% of the total protein of the transformed *Escherichia coli*. The yield of soluble protein was approximately 30-66 mg/l of culture. The expected sizes of recombinant FUT3, FUT5 and FUT6 are 51, 58 and 61 kDa, respectively. These recombinant FUTs were used to determine the specificities of the anti-FUT antibodies against different regions of the enzymes.

Owing to the high sequence similarity (~90%) at the C-terminals, the antibody D-17 raised against this region of FUT5 recognized the three recombinant FUTs (Fig. 2A, lanes 3, 4 and 5). However, antibodies raised against the variable N-terminal of FUT5 (N-18: Fig. 2C, lane 4) and FUT6 (G-16: Fig. 2D, lane 5) reacted only with their respective recombinant isoform only. The antibody N-16 is raised against the N-terminus of FUT6 but cross-reacts with that of FUT3 (manufacturer information). It reacted strongly with FUT3 (Fig. 2B, lane 3) and FUT6 (Fig. 2B, lane 5) and very weakly with FUT5 (Fig. 2B, lane 4).

The antibodies D-17 (Fig. 2A, lane 2) and N-18 (Fig. 2C, lane 2) recognized two major protein bands of sizes ~79 and ~65 kDa in the human sperm extract. Several smaller components of much lower intensity were also found. They might result from proteolysis of sperm FUT during sample preparation. The ~79 kDa band was also recognized by N-16
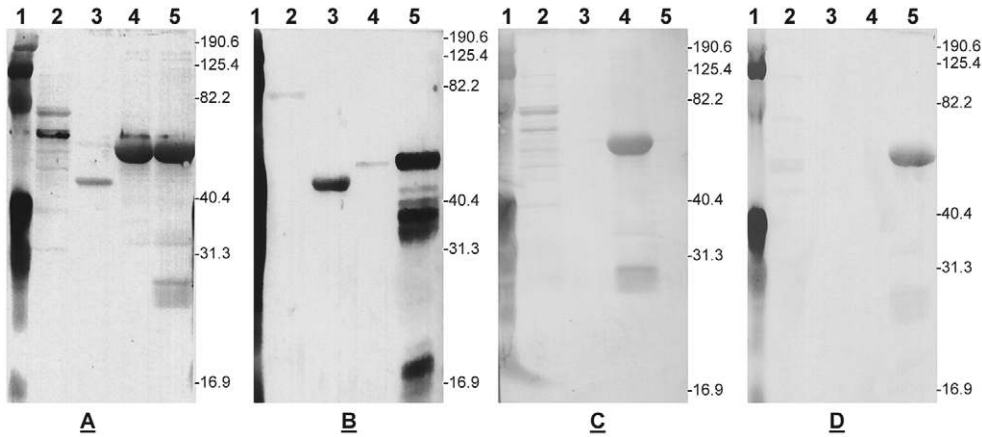
Fig. 2. Western blot analysis of sperm protein extract probed with anti-fucosyltransferase antibodies (1:200). (A) D-17 against the C-terminus of FUT5. N-16(B) N-18 (C) and G16 (D) against the N-terminus of FUT3/FUT6, FUT5 and FUT6, respectively. Lane 1: molecular mass marker. Lane 2: 10 μg sperm protein extracts. Lanes 3-5: 5 μg of recombinant FUT3, FUT5 and FUT6, respectively.

(Fig. 2B, lane 2), although the signal was weaker than that of D-17 and N-18. The antibody G-16 (Fig. 2D, lane 2) did not react with any protein in the sperm protein extract.

The antibody D-17 localized FUT immunoreactivity to the acrosome region and the midpiece (Fig. 3A) in 90-95% of spermatozoa with or without permeabilization. Immunoreactivity was found in the midpiece in 88-96% of non-permeabilized spermatozoa when N-18 antibody was used (Fig. 3C). The acrosome region in 83-90% of the spermatozoa was also strongly stained after permeabilization, suggesting that the N-terminal epitope of FUT5 in the acrosome region was intracellular. The omission of FUT antibodies and the use of antibodies preabsorbed with the appropriate blocking peptide greatly reduced the signal (data not shown). The antibodies N-16 and G-16 did not produce any signal (Fig. 3B,D).

Acceptors of FUT reduced the binding of glycodelin-A to spermatozoa

Unlabeled glycodelin-A was the best competitor ($LogEC_{50}$: 2.31±0.08 pmol/ml) in the binding of ^{125}I-glycodelin-A to spermatozoa (Fig. 4A), consistent with previous observations (Chiu et al., 2003b; Chiu et al., 2004). Among the FUT-acceptors, 2′-fucosyllactose, lacto-N-fucopentaose-I and asialofetuin competed with ^{125}I-glycodelin-A for binding sites with $LogEC_{50}$ of 2.81±0.14, 3.48±0.20 and 2.50±0.12 pmol/ml, respectively (Fig. 4B). Phenyl-β-D-galactoside melibiose, RNAse and glycodelin-S did not affect the binding of ^{125}I-glycodelin-A to spermatozoa. The competitors at the concentrations used did not affect sperm viability, acrosomal status and motility (data not shown).

Purification of the sperm FUT

Ion-exchange chromatography separated the sperm protein extracts into ten fractions (fractions 1-10; Fig. 5A). The FUT in these fractions were further purified by guanosine 5′-diphosphate (GDP)-agarose affinity chromatography. The purified sperm FUT appeared as a single major band of size ~65 kDa in fractions 5-8 in silver-stained SDS-PAGE (Fig. 5B) and in western blot analysis using antibody N-18 (Fig. 5C). The identity of the isolated band was confirmed to be FUT5 by the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; data not shown). The FUT activity in these fractions positively correlated with the appearance of the protein band (Table 2). There were 36.3-88.7-fold increases in FUT activity in fractions 5-8 relative to that in the total detergent-extractable protein (data not shown). Fractions 5-7 with a higher percentage of enzyme activity recovery (10.2-38.7%) were pooled for further experimentation.

Co-immunoprecipitation of glycodelin-A with FUT

The interaction between FUT and glycodelin-A was confirmed by co-immunoprecipitation using the anti-glycodelin antibody and the anti-FUT antibody, N-18. Glycodelin-A co-precipitated with recombinant FUT3 and FUT5, but not with FUT6 (Fig. 6A). It also interacted strongly with sperm FUT (Fig. 7). Deglycosylated glycodelin-A did not co-precipitate with recombinant and purified sperm FUTs (Fig. 6B), consistent with the previous observation that the interactions between glycodelin-A and spermatozoa were glycosylation dependent (Chiu et al., 2003b; Chiu et al., 2004). The addition of solubilized zona pellucida reduced the binding of

Table 1. Identification of glycodelin-A interacting protein by MALDI-TOF MS

Peptides	Mass-to-charge ratio submitted (*m/z*)	Mass-to-charge ratio matched (*m/z*)	Start	End	Peptide sequence
1	847.6733	847.4228	329	334	YLSYFR
2	**1158.9422**	**1158.5532**	**343**	**352**	**SFSWALAFCK**
3	1678.3245	1677.9202	276	291	NALEAWAVPVVLGPSR
4	**1929.9542**	**1928.9744**	**203**	**219**	**TELVAWAVSNWKPDSAR**
5	2087.8442	2086.0047	256	272	FYLAFENSLHPDYITEK
6	3210.3452	3209.5458	174	202	SDSDIFTPYGWLEPWSGQPAHPPLNLSAK

Protein sequence of human FUT5 (SwissProt accession no. Q11128) as retrieved from a ProteinProspector MS-Fit search shows a sequence coverage of 25% and MOWSE score of 69 (prospector.ucsf.edu). Peptides 2 and 4 (in bold) were unique to FUT5 among FUT3, FUT5 and FUT6.
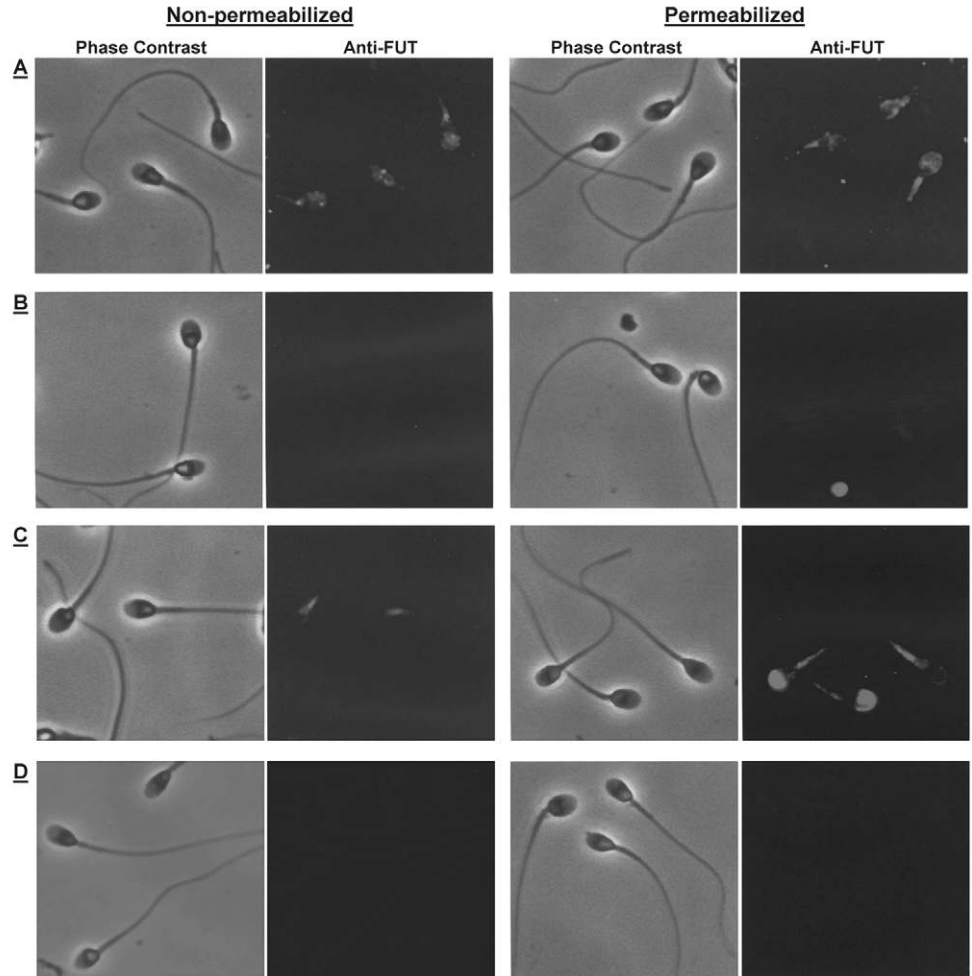
Fig. 3. Immunolocalization of fucosyltransferase in human spermatozoa with or without Triton X-100 permeabilization. Spermatozoa were incubated with different polyclonal anti-FUT antibodies: D-17 (A), N-16 (B), N-18 (C) and G-16 (D). They were visualized using FITC-conjugated mouse anti-goat IgG.

glycodelin-A to sperm FUT in a dose-dependent manner (Fig. 7, Lane 1-3).

Anti-FUT antibody and FUT acceptors inhibited spermatozoa-zona pellucida binding

An anti-FUT antibody D-17 at concentrations of 1, 5, 10 and 20 μg/ml significantly decreased the number of spermatozoa bound to hemizona ($P<0.05$) when compared with the control.
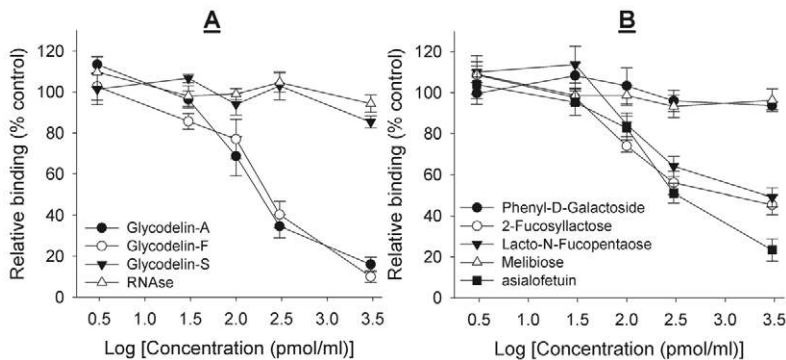

Fig. 4. Competition binding of spermatozoa to saturated concentration (300 pmol/ml) of ^{125}I-glycodelin-A in the presence of glycodelin ($n=3$) (A) and fucosyltransferase acceptors ($n=3$) (B).

The hemizona binding index (HZI) decreased in a dose-dependent manner (Fig. 8A). Preabsorption with blocking peptide abolished the inhibitory effect of the antibody on spermatozoa-zona pellucida binding (Fig. 8A).

The results of different concentrations of FUT acceptors on spermatozoa-zona pellucida binding are shown in Fig. 8B. The zona-binding capacity of treated spermatozoa was inhibited by 2′-fucosyllactose, lacto-N-fucopentaose-I and asialofetuin in a dose-dependent manner ($P<0.05$). At 3000 pmol/ml, the HZI was 60.6±4.1%, 72.6±2.8% and 52.8±4.3%, respectively. No significant inhibition was observed with phenyl-β-D-galactoside, melibiose and RNAse. Anti-FUT antibodies with or without blocking peptide preabsorption and FUT acceptors at the concentrations used did not affect sperm viability, acrosomal status and motility (data not shown).

Sperm FUT bound to human zona pellucida

Fluorescent-labeled sperm FUT specifically bound to the zona pellucida of human oocytes (Fig. 9A). The bound signal was removed in the presence of an excess of unlabeled sperm FUT (Fig. 9B). Fluorescent-labeled ovalbumin did not bind to intact zona pellucida (Fig. 9C). The binding of labeled sperm FUT to solubilized zona pellucida
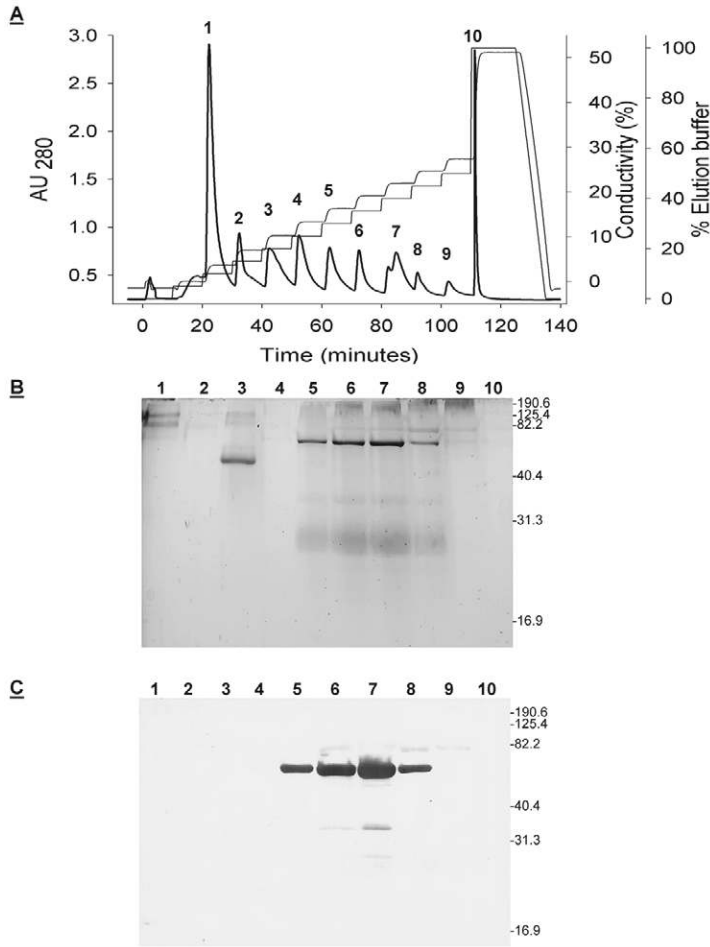
Fig. 5. Purification of sperm FUT. (A) Representative Mono-Q chromatogram of sperm extracts. The protein fractions (1-10) were desalted and further purified using a GDP-agarose column. They were then subjected to 12% SDS-PAGE analysis with silver staining (B) and western blot analysis using the anti-FUT5 antibody N-18 (B).

increased with the amount of FUT used and reached a plateau at concentrations >200 pmol/ml (data not shown). Scatchard plotting revealed an equilibrium dissociation constant (K_d) of 42.82 pmol/ml.
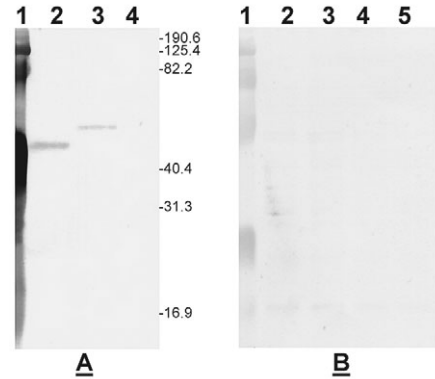

Fig. 6. Co-immunoprecipitation of FUT with glycodelin-A or deglycosylated glycodelin-A. (A) Glycodelin-A. Lane 1: marker. Lane 2: 5 μg FUT3 + 30 pmol/ml glycodelin-A. Lane 3: 5 μg FUT5 + 30 pmol/ml glycodelin-A. Lane 4: 5 μg FUT6 + 30 pmol/ml glycodelin-A. (B) Deglycosylated glycodelin-A. Lane 1: marker. Lane 2: 5 μg FUT3 + 30 pmol/ml deglycosylated glycodelin-A. Lane 3: 5 μg FUT5 + 30 pmol/ml deglycosylated glycodelin-A. Lane 4: 5 μg FUT6 + 30 pmol/ml deglycosylated glycodelin-A. Lane 5: 5 μg sperm FUT + 30 pmol/ml deglycosylated glycodelin-A.

Discussion

Experimental evidence suggests that lipocalins, e.g. glycodelin-A (Rachmilewitz et al., 2003), β-lactoglobulin (Mansouri et al., 1998) and retinol-binding protein (Sundaram et al., 1998) bind to cell surface receptors. Glycodelin-A inhibits T-cell activity partly by binding to CD45, a major surface tyrosine phosphatase in T-cells (Rachmilewitz et al., 2003). Chemical cross-linking followed by mass spectrometric analysis of the cross-linked molecules is useful to elucidate ligand-receptor interactions (Williams et al., 2000; Bleil and Wassarman, 1990). Using such an approach, we identified FUT5 as a glycodelin-A binding protein on human spermatozoa. Several observations support this conclusion: (1) sulfo-SBED linked glycodelin-A to a sperm surface protein of size ~65 kDa, which was identified as FUT5 by MS; (2) an anti-FUT5 antibody recognized glycodelin-A–sperm protein complexes and a ~65 kDa protein band in sperm extract; (3) indirect immunofluorescence staining localized FUT5 to the

Table 2. Purification of sperm FUT

	Fractions	Protein (μg)	FUT activities (pmol product/h)	Specific activities (activity/μg protein)	Recovery (% activity)
Sperm protein extract		822.8	248.2	0.302	
Mono-Q + GDP-agarose	1	12.8	0.5	0.039	0.2
	2	3.6	0.4	0.111	0.2
	3	3.8	0.5	0.132	0.2
	4	3.4	0.6	0.174	0.2
	5	2.3	25.2	10.957	10.2
	6	3.3	46.8	14.182	18.9
	7	3.6	96.0	26.778	38.7
	8	1.0	18.4	18.4	7.4
	9	0.5	0.1	0.2	0.0
	10	2.1	0.4	0.190	0.2

The table shows the typical activity of FUT from 100×10^6 spermatozoa in different fractions of the purification protocol. A similar pattern was seen in two other experiments.
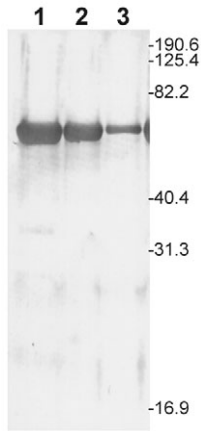
Fig. 7. Co-immunoprecipitation of sperm FUT with glycodelin-A in the presence of zona pellucida glycoproteins. The amount of zona pellucida glycoprotein used is expressed as the number of zona pellucida solubilized per microlitre of the final incubation mixture (ZP/μl). Lane 1: 5 μg sperm FUT + 30 pmol/ml glycodelin-A. Lane 2: 5 μg sperm FUT + 30 pmol/ml glycodelin-A + 0.02 ZP/μl. Lane 3: 5 μg sperm FUT + 30 pmol/ml glycodelin-A + 0.1 ZP/μl.
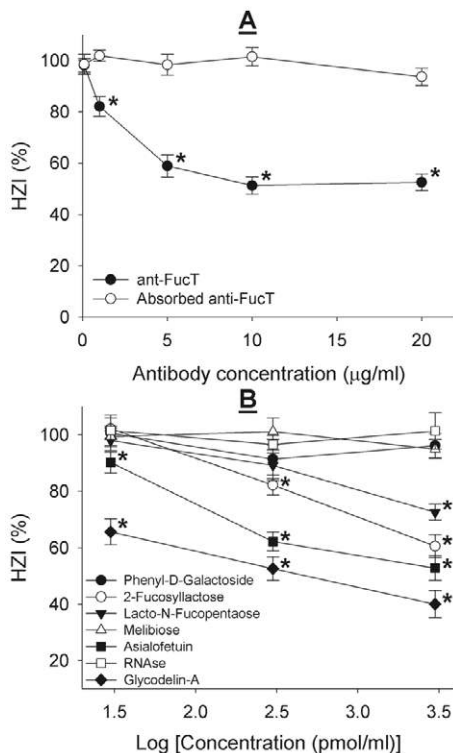
Fig. 8. Effect of anti-FUT5 antibody and FUT acceptors on zona pellucida binding of spermatozoa. Spermatozoa was incubated in EBSS/BSA (control) and in (A) EBSS/BSA containing 0.1, 1, 5, 10 and 20 μg/ml anti-FUT antibody D-17 with or without blocking peptide preabsorption, and (B) EBSS/BSA containing FUT acceptors at concentrations of 30, 300 and 3000 pmol/ml. Each point represents the mean of the results of five hemizona binding assays using five zona pellucida and five different sperm samples. *$P<0.05$, when compared with the control without any treatment.

acrosome region of human spermatozoa, a region that binds glycodelin-A (Chiu et al., 2003a); (4) $\alpha(1,3)$ and $\alpha(1,4)$, but not $\alpha(1,2)$ FUT acceptors, non-FUT-related sugars (melibiose) and glycodelin-S, inhibited the binding of glycodelin-A to human spermatozoa; and (5) co-immunoprecipitation of glycodelin-A with recombinant FUT5 and purified sperm FUT. Moreover, in a cross-linking experiment, the glycodelin-A–FUT5 complex could only be obtained from spermatozoa, but not from fibroblasts, consistent with our previous finding that the binding of glycodelin is cell type-specific (Tse et al., 2002). The inability of deglycosylated glycodelin-A to co-immunoprecipitate with sperm FUT and recombinant FUT5 is also in line with the documented importance of carbohydrate moieties on spermatozoa-glycodelin interaction (Chiu et al., 2003b; Chiu et al., 2004).

Fucosyltransferases catalyze fucose transfer from GDP-fucose to different oligosaccharide acceptors in $\alpha1,2$-, $\alpha1,3$-, $\alpha1,4$- and $\alpha1,6$-linkages. They are type II transmembrane proteins consisting of a small N-terminal cytoplasmic tail, a transmembrane region and a C-terminal catalytic domain usually oriented towards the lumen of the Golgi complex. Six human FUTs have been cloned (de Vries et al., 2001). Three FUTs, namely FUT3, FUT5 and FUT6, cluster on human chromosome 19p13.3 (Reguigne-Arnould et al., 1995; McCurley et al., 1995). They share ~90% sequence homology; the main differences between them are located in the N-
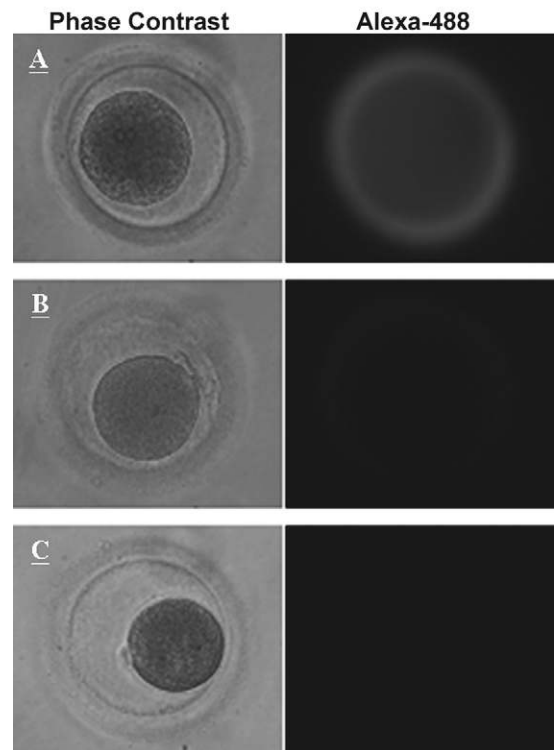

Fig. 9. Binding of sperm FUT5 to intact human zona pellcuida. Human oocytes were incubated with (A) 150 pmol/ml Alexa-Fluor 488 sperm FUT5, (B) 150 pmol/ml Alexa-Fluor 488 sperm FUT5 + 50-fold excess of unlabeled sperm FUT5, or (C) 150 pmol/ml Alexa-Fluor 488 ovalbumin. The results shown are representative of two replicate experiments.

terminal region, whereas their C-terminal regions are almost identical (de Vries et al., 2001).

Fucosyltransferase 3, FUT5 and FUT6 are found only in humans and chimpanzees (Costache et al., 1997). The expression of FUT3 and FUT6 are abundant in gastrointestinal tissues and minimal in spleen, lung and cervix uteri. Compared with FUT6, FUT3 is not expressed in liver and is less abundant in kidney. The expression of FUT5 is weak and restricted to liver, colon, spleen, testis and hematopoietic cells (Cameron et al., 1995). Human FUTs differ in their acceptor substrate specificity (de Vries et al., 2001); FUT3 and FUT5 are α1,3/1,4-FUTs and interact with both type 1 (Galβ1-3GlcNAc) and type 2 (Galβ1-4GlcNAc) acceptors, whereas FUT6 is a α1,3-FUT and uses only type 2 acceptors. Among this group of enzymes, FUT5 has the broadest acceptor specificity. In addition to type 1 and 2 acceptors, FUT5 also works well with 3-sulfo-, 6-sulfo-, α2,3-sialylated and α1,2-fucosylated forms of these acceptors as well as glycans having the GalNAcβ1-4GlcNAc structure (Pykari et al., 2000; de Vries et al., 2001).

Among the human FUTs, only FUT3, FUT5 and FUT6 are inhibited by N-ethylmaleimide, a method for discriminating FUTs (de Vries et al., 1995). In a separate experiment, we found that treatment with 10 mM N-ethylmaleimide for 1 hour at 4°C abolished the enzymatic activity of sperm FUT. This sensitivity towards N-ethylmaleimide treatment together with their high similarity in protein sequence and exclusive presence in higher primates meant only FUT3 and FUT6 were considered as possible alternatives to FUT5 in this study.

Glycodelin-A contains approximately 17.5% of carbohydrate moieties (Bohn et al., 1982) with two glycosylation sites, Asn-28 and Asn-63, containing mainly complex-type glycans (Dell et al., 1995). The major non-reducing epitopes in the complex-type glycans include Galβ1-4GlcNAc and antennae containing GalNAcβ1-4GlcNAc (Dell et al., 1995). The latter is rare in glycoproteins of higher animals. Both are substrates of FUT5, which may serve as the site of interaction between glycodelin-A and sperm FUT5. By contrast, glycodelin-S has little Galβ1-4GlcNAc and no GalNAcβ1-4GlcNAc structure (Morris et al., 1996). Its glycans are unusually rich in fucose. The differences in carbohydrate moieties may explain the inability of glycodelin-S to bind to sperm FUT, and is consistent with the observation that the binding sites of glycodelin-S are different from other glycodelin isoforms (Chiu et al., 2005). However, glycodelin-F competes with glycodelin-A for binding to spermatozoa. This is in line with our previous binding kinetic experiments demonstrating that human spermatozoa possess two receptors for glycodelin-F, one of which is common with glycodelin-A (Chiu et al., 2003; Chiu et al., 2004). Our unpublished observation also shows that sperm FUT binds glycodelin-F.

This report provides the first demonstration of the isolation of FUT5 from human spermatozoa. Soluble FUTs are present in semen (Tulsiani et al., 1990) and adsorbed onto the sperm surface (Tulsiani et al., 1993). These exogenous FUTs are unlikely to be involved in spermatozoa-zona pellucida binding as our preliminary data showed that they are easily removed by density gradient centrifugation, consistent with the previous observation that Percoll processing shed loosely attached seminal plasma components including glycosyltransferase from spermatozoa (Flesch et al., 2001; Tulsiani et al., 1990). In this study, these exogenous FUTs were further removed from percoll-processed and capacitated spermatozoa with high-salt solutions known to release peripheral but not integral membrane proteins (Khunsook et al., 2003). Triton X-100 was then used to solubilize the integral membrane proteins (Shur and Neely, 1988). Our results showed that human sperm FUT activity was detergent but not high-salt extractable. The purified protein with FUT activity had a size (~65 kDa) similar to that of the FUT5 band identified in the cross-linking experiment. Its identity was confirmed to be FUT5 based on western blot analysis using an anti-FUT5 antibody and MS analysis. These observations indicated that the purified sperm FUT5 was an integral membrane component on the sperm surface. Immunostaining using antibodies against different regions of FUT5 with and without permeabilization further suggested that FUT5 had its C-terminal oriented externally whereas its N-terminal was intracellular.

Although the antibodies D-17 and N-18 recognized two major protein bands with masses of ~79 and ~65 kDa in human sperm extracts, we were successful in purifying the ~65 kDa protein only. The reason for this is unclear. One possibility is that the ~79 kDa protein may be a new isoform of FUT highly similar to FUT5 but with different surface-charge properties and/or GDP-binding affinities making it unable to be purified by the present purification protocol. It has been shown that the specificity and affinity of FUTs to substrate and donor can be greatly altered by mutating a few or even one amino acid residues (Jost et al., 2005; Vo et al., 1998). This hypothesis is being investigated in our laboratory.

The molecular mass of the purified sperm FUT5 (~65 kDa) differs from that of recombinant FUT5 (~58 kDa). The difference could be because of glycosylation of the native enzyme, whereas the recombinant FUT5 derived from E. coli did not possess glycosylation. The human FUT5 contains four potential N-glycosylation sites with two of them in the N-terminal region and the other two closer to the C-terminal region (Christensen et al., 2000a; Christensen et al., 2000b). The N-linked glycans of glycosyltransferases are involved in protein folding, prevention of proteolytic degradation and intracellular transport and localization. In addition, glycosylation in FUTs affects the biochemical activity of the enzyme (Christensen et al., 2000a; Christensen et al., 2000b).

Glycodelin-A inhibits spermatozoa-zona pellucida binding (Chiu et al., 2003a; Oehninger et al., 1995). Evidence suggests that there is a close relationship between glycodelin-A receptors and zona pellucida glycoprotein receptors (Chiu et al., 2003b; Chiu et al., 2004). The present study showed that FUT5 was involved in spermatozoa-zona pellucida binding and that glycodelin-A inhibited such binding by interacting with FUT5. This conclusion comes from three observations: (1) sperm FUT5 bound to intact or solubilized human zona pellucida; (2) co-incubation with solubilized zona pellucida dose-dependently reduced the binding of glycodelin-A to sperm FUT5; (3) an anti-FUT5 antibody and FUT acceptors dose-dependently decreased the number of bound spermatozoa in a hemizona binding assay. It was noted that an anti-FUT5 antibody could not completely block spermatozoa-zona pellucida binding. This may be because of the presence of multiple receptors for zona pellucida proteins (see below).

It is generally accepted that the specific interaction between spermatozoa and zona pellucida is a carbohydrate-mediated event (Wassarman, 1999; Ozgur et al., 1998; Chapman and

Barratt, 1996). In the past two decades, several carbohydrate-binding proteins on the sperm plasma membrane such as β1,4-galactosyltransferase (Nixon et al., 2001), α-mannosidase (Tulsiani et al., 1989), β-N-acetylglucosaminidase (Godknecht and Honegger, 1991) and selectin-like receptors (Oehninger, 2001) have been proposed to be involved in such interaction. However, none has received wide acceptance (Wassarman, 1999) and no carbohydrate-binding protein has so far been purified from human spermatozoa and confirmed to bind to human zona pellucida. The exact identity of the sperm protein(s) responsible for spermatozoa-zona pellucida binding in human remains unknown.

The evidence presented in this study shows that human sperm FUT5 has all the characteristics required to play a role in spermatozoa-zona pellucida binding. It is an intrinsic component of the sperm plasma membrane located in the acrosomal region of human spermatozoa and has its catalytic domain (C-terminal) oriented towards the sperm surface. Its ability to bind to intact zona pellucida raises the possibility that it may be a zona pellucida glycoprotein receptor. This is in line with our previous conclusion that the glycodelin-A receptor is closely related to a zona pellucida glycoprotein receptor (Chiu et al., 2003b; Chiu et al., 2004).

Apart from their usual intracellular location, FUT activities have been demonstrated on the cell surface of diverse cell types, such as prostatic epithelial cells (Marker et al., 2001) and rat Sertoli cells (Raychoudhury and Millette, 1997). It is generally believed that glycosyltransferases on the cell surface do not perform the glycosyltransferase function because of a lack of sugar nucleotide donors. Consequently, their function is confined to their carbohydrate-binding ability (Colley, 1997). These cell surface FUTs mediate adhesion of spermatogenic cells to Sertoli cells (Raychoudhury and Millette, 1997) and among neurons during synaptogenesis in culture (Matsui et al., 1986).

The spermatozoa of rat (Raychoudhury and Millette, 1995), mouse (Ram et al., 1989; Cardullo et al., 1989) and human (Tulsiani et al., 1990) express FUT activities. In human and mouse, FUT activity resides in the plasma membrane and is retained after capacitation (Ram et al., 1989; Cardullo et al., 1989; Tulsiani et al., 1990). Carbohydrate moieties, sialyl and non-sialyl N-acetyllactosamines recognized by FUT are present on the zona pellucida of boar (Mori et al., 2000), mouse (Dell et al., 2003; Chalabi et al., 2006) and pig (Yonezawa et al., 1999). Oligosaccharides or artificial carbohydrate chains with N-acetyllactosamine terminals inhibit the binding of spermatozoa to the mouse oocyte (Litscher et al., 1995). Our unpublished observation also shows that glycodelin-A suppresses spermatozoa-zona pellucida binding in mouse. Although the glycan structure of human zona pellucida is not known, a recent study using lectins and antibodies against different carbohydrate moieties has demonstrated the presence of FUT-binding glycans on human zona pellucida (Jimenez-Movilla et al., 2004).

Spermatozoa-zona pellucida interaction is largely species-specific (Wassarman, 1999; Oehninger et al., 1993; Yanagimachi, 1994). How this can be accomplished in view of the presence of FUT and its ligands in spermatozoa and zona pellucida respectively in different species is not known. It has been suggested that multiple receptors are necessary for the many complex events associated with spermatozoa-zona

pellucida binding (Wassarman, 1999; Thaler and Cardullo, 1996; Focarelli et al., 2001). This is exemplified in studies of β1,4-galactosyltransferase as a candidate zona pellucida protein-3 receptor in mouse (Nixon et al., 2001). The antibody of the enzyme induces acrosome reaction (Lu and Shur, 1997). Spermatozoa from β1,4-galactosyltransferase-deficient mice are able to bind to the oocyte, but do not respond to zona pellucida protein-3-induced acrosome reaction (Lu and Shur, 1997). These results indicate that spermatozoa-zona pellucida interaction involves at least two receptors, one for binding and one for induction of acrosome reaction. We observed that an anti-FUT antibody and a FUT5 substrate did not induce acrosome reaction in human spermatozoa, suggesting that sperm FUT was related to binding. There is recent evidence for the occurrence of transitional stages of acrosomal reaction with acrosomal contents partially exposed or released during capacitation (Kim and Gerton, 2003). Whether an anti-FUT antibody and FUT5 substrate would affect these transitional stages is unknown. The possible presence of other receptor(s) that determine species-specificity remains to be investigated.

Spermatozoa are not exposed to uterine glycodelin-A as it is absent in the periovulatory period. However, spermatozoa are exposed to oviductal glycodelin, the production of which increases in the luteal phase (Seppala et al., 2002). Our unpublished observation also shows that human oviductal cells secrete glycodelin-A in vitro. The exact physiological significance of the zona pellucida-binding inhibitory activities of glycodelin-A around the time of ovulation is not exactly known. In mammalian species, the isthmus of the oviduct serves as a temporary sperm reservoir before ovulation with spermatozoa bound to the ciliated oviductal epithelial cells through a carbohydrate-mediated event (Suarez and Pacey, 2006; Topfer-Petersen, 1999). Although definitive evidence for a distinct sperm reservoir in human Fallopian tubes is lacking, human spermatozoa attached to the endosalpingeal epithelium intermittently in vitro (Pacey et al., 1995). Our unpublished data also show that purified sperm FUT binds to the ciliated oviductal epithelial cells. Taken together, we hypothesize that luteal-phase progesterone increases oviductal production of glycodelin-A, which binds to sperm FUT to regulate spermatozoa-endosalpingeal epithelium interaction in humans. Experiments are being performed to confirm this hypothesis.

In summary, the present investigation provides evidence for the presence of FUT5 on the plasma membrane of human spermatozoa. Its unusual plasma membrane location and its specific interaction with glycodelin-A and zona pellucida protein suggest that it is a candidate receptor molecule for the zona pellucida proteins and glycodelin-A on human spermatozoa. Further characterization of this protein is necessary to reveal the details of interaction of spermatozoa with the zona pellucida. Data showed that glycodelin-A interacts with sperm FUT to inhibit spermatozoa-zona pellucida binding.

Materials and Methods
Semen samples

Determination of acrosomal status and motility of sperm

Fluorescein isothiocyanate-labeled peanut (*Pisum sativum*) agglutinin (FITC-PSA; Sigma, St Louis, MO, USA) and Hoechst staining techniques were used to evaluate the acrosomal status of spermatozoa (Chiu et al., 2002). The fluorescence patterns of 300 spermatozoa in randomly selected fields were determined under a fluorescence microscope (Zeiss, Oberkochen, Germany) with 600× magnification. Spermatozoa without Hoechst and FITC-PSA staining or with FITC-PSA staining confined to the equatorial segment only were considered as acrosome reacted. A Hobson Sperm Tracker System (Hobson Tracking Systems, Sheffield, UK) was used to study sperm motility. The set-up parameters of the system and the procedures were described elsewhere (Chiu et al., 2003b).

Hemizona binding assay

The hemizona binding assay was performed as described (Yao et al., 1996). Unfertilized oocytes from our assisted reproduction program were micro-bisected into two identical hemizonae. Each hemizona was incubated with 2×10^5 spermatozoa/ml in a 100 µl droplet of EBSS-BSA under mineral oil for 3 hours at 37°C in an atmosphere of 5% CO_2 in air. The numbers of tightly bound spermatozoa on the outer surface of the hemizonae were counted. The HZI was defined as the ratio of the number of bound spermatozoa in the test droplet to that in the control droplet multiplied by 100.

Purification of glycodelin isoforms

A monoclonal anti-glycodelin antibody (clone F43-7F9) sepharose column was used to purify glycodelin-A and glycodelin-S from amniotic fluid and seminal plasma, respectively (Riittinen et al., 1991; Chiu et al., 2003b). The bound glycodelin was eluted with 0.1% trifluoroacetic acid and dialysed against 100 mM sodium phosphate buffer (pH 7.2). Glycodelin-S was further purified by anion-exchange chromatography (Chiu et al., 2005). The concentrations of the purified glycodelin isoforms were determined with a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA). The N-Glycosidase F Deglycosylation Kit (Bio-Rad) was used to deglycosylate glycodelin-A (Chiu et al., 2003b). The deglycosylated protein obtained after three successive protein precipitations was redissolved in 20 µl phosphate buffered saline (PBS) and purified by gel filtration chromatography in an AKTA system (Pharmacia). The purity of the deglycosylated protein was analyzed by SDS-PAGE.

Radioactively labeled glycodelin was prepared by mixing 50 µg of glycodelin in 0.02 ml of 0.05 M PBS (pH 7.4) with 2 mCi of sodium ^{125}I (20 µl; Amersham Biosciences, Little Chalfont, UK) and freshly prepared chloramine-T (100 µg in 0.02 ml of 0.05 M PBS, pH 7.4) as described (Chiu et al., 2003b). The first radioactive peak containing iodinated glycodelin was collected.

Expression and purification of FUT3, FUT5 and FUT6

The full-length cDNAs coding for FUT3, FUT5 and FUT6 were PCR amplified using gene-specific primers (Table 3) and human testis Marathon cDNA (Clontech, Palo Alto CA, USA). The amplified fragments were column purified and cloned into pET100-D/TOPO vector (Invitrogen, Carlsbad, CA, USA) for protein expression. Expression of the FUT fusion proteins were performed in *E. coli* BL21 (DE3) Star by 0.5 mM IPTG induction for 3 hours. The FUT proteins were expressed as inclusion bodies in the bacteria and purified with the Protein Refolding kit (Novagen; EMD Biosciences, Madison, WI, USA) according to the manufacturer protocol. In brief, bacterial cell pellet was resuspended in 0.1 culture volume of 1× IB wash buffer (20 mM Tris-HCl; 10 mM EDTA, pH 7.5, 1% Triton X-100) and lysozyme at a final concentration of 100 µg/ml was added. The mixture was incubated at 30°C for 15 minutes and sonicated for 5×20 seconds on ice. Inclusion bodies were collected by centrifugation at 10,000 *g* for 10 minutes, washed twice with 1× IB wash buffer and dissolved in 1× IB solubilization buffer containing 1 mM DTT and 0.3% N-lauroylsarcosine in 50 mM CAPS (pH 11.0). The soluble enzyme fraction in the supernatant was collected after centrifugation and dialyzed with 500 ml of 20 mM Tris-HCl (pH 8.5) containing 0.1 mM DTT, and then with 500 ml of the same buffer but without

DTT. Quantification of the recombinant proteins was performed as described above.

Solubilization of human spermatozoa

Capacitated spermatozoa (100×10^6) were washed three times in PBS. Non-integral, peripheral membrane-associated proteins on spermatozoa were removed by incubating the washed spermatozoa in 1 M NaCl in PBS with gentle stirring for 30 minutes at 25°C as described (Khunsook et al., 2003). The spermatozoa were then collected by centrifugation at 600 *g* for 10 minutes before the sperm membrane proteins were extracted by 1% Triton X-100 (Sigma) in the presence of a cocktail of protease inhibitors (Calbiochem, San Diego, CA, USA) (Chiu et al., 2003b). The insoluble fraction was discarded after centrifugation at 15,000 *g* for 40 minutes. The concentration of the soluble proteins was determined by Coomassie Plus-200 protein assay reagent (Pierce, Rockford, IL, USA). The supernatant was diluted in a solution of MOPS-NaOH buffer (pH 7.3) containing 0.2% Triton X-100 and 6 mM $MnCl_2$ (buffer A).

Identification of glycodelin-A binding protein

Glycodelin-A and glycodelin-S were conjugated to Sulfo-SBED by a ProFound™ Sulfo-SBED Biotin Label Transfer Kit (Pierce) according to the manufacturer's instructions. Sulfo-SBED contains a sulfonated *N*-hydroxysuccinimide active ester, a photoactivatable aryl azide and a transferable biotin handle. It was conjugated to glycodelin at a molar ratio of 4:1 for 2 hours at 4°C in the dark. Non-reacted cross-linker was removed by dialyzing the reaction mixture in PBS overnight at 4°C.

Capacitated spermatozoa (100×10^6/ml) that had their non-integral, membrane-associated proteins removed by incubation in 1 M NaCl in PBS as described above were incubated with 300 pmol/ml Sulfo–SBED-linked glycodelin-A. After 90 minutes, the spermatozoa were washed three times in PBS and cross-linked glycodelin-A with its binding proteins by UV irradiation at 302 nm (High Performance Transilluminator TFM-26; UVP, Upland, CA, USA) for 15 minutes at room temperature. The integral membrane proteins of spermatozoa were isolated by 1% Triton X-100 with a cocktail of protease inhibitors (Calbiochem). The soluble proteins were diluted in buffer A, and the protein concentration was determined by Coomassie Plus-200 protein assay reagent (Pierce). The glycodelin covalently linked sperm surface proteins were then purified by immunoaffinity chromatography using a monoclonal anti-glycodelin antibody sepharose column as described above. One-half of the purified protein complex was treated with reducing sample buffer provided by the ProFound Sulfo-SBED Biotin Label Transfer Kit containing 100 mM DTT to cleave the crosslinks between glycodelin and the sperm proteins at the azo linkage and to transfer the biotin label from the glycodelin to the sperm proteins. The remainder was treated with non-reducing sample buffer. Both samples were heated at 90°C for 5 minutes and analyzed by SDS-PAGE. Biotinylated protein was detected with streptavidin-AP conjugate (Flura, Newport, TN, USA). Chromogenic reaction was developed by incubation with 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium solution (Calbiochem).

MS and database search

The reduced and silver-stained glycodelin-interacting protein bands in SDS-PAGE were excised and digested in situ with trypsin (0.1 mg/ml in 25 mM NH_4HCO_3, pH 8.0). The peptides formed were recovered with Millipore C18 ZipTips and 60% acetonitrile-0.1% trifluoroacetic acid (TFA) containing the α-cyano-4-hydroxycinnamic acid matrix. The peptide-matrix samples were then analyzed with MALDI-TOF MS to obtain the peptide mass spectra (MDS Sciex, South San Francisco, CA, USA) and compared with the protein sequences in the public protein databases at the Swiss-Prot Knowledgebase (http://www.ebi.ac.uk/swissprot/) as described (Lee et al., 2004).

Localization of sperm FUT

Triton X-100-extracted sperm proteins (see above) or recombinant FUTs were separated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane for western blot analysis as described (Lee et al., 2004). Polyclonal

Table 3. Primer sequences used for PCR amplification of the full-length FUT cDNA for protein expression in *E. coli*

Target	Primer[a]	Sequence	Product size
FUT3	FUT3-5′-TOPO FUT3-3′-TOPO	5′-CACC<u>ATG</u>GATCCCCTGGGTGCAGC-3′ 5′-<u>TCA</u>GGTGAACCAAGCCGCTA-3′	1086 bp
FUT5	FUT5-5′-TOPO FUT5-3′-TOPO	5′-CACC<u>ATG</u>GATCCCCTGGGCCCAGC-3′ 5′-<u>TCA</u>GGTGAACCAAGCCGCTA-3′	1124 bp
FUT6	FUT6-5′-TOPO FUT6-3′-TOPO	5′-CACC<u>ATG</u>GATCCCCTGGGCCCGGC-3′ 5′-<u>TCA</u>GGTGAACCAAGCCGCTA-3′	1080 bp

[a]The primer sequences were complementary to FUT3 (accession no. NM_0000149), FUT5 (accession no. NM_002034) and FUT6 (accession no. NM_000150). A TOPO-directional cloning sequence (CACC) was added at the 5′-end of the forward primers, the start codon (ATG) is underlined and the stop codons (TGA, in reverse direction) are double underlined.

antibodies against FUTs (D-17 against the C-terminus of FUT5; N-16, N-18 and G16 against the N-terminus of FUT6, FUT5 and FUT6, respectively; Santa Cruz) were used. The dilutions of the primary antibodies used were 1:200, whereas that of alkaline phosphatase conjugated anti-goat immunoglobulin G (IgG) secondary antibody was 1:5000. Chromogenic reaction was developed by incubation with 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium solution (Calbiochem).

For immunostaining, capacitated spermatozoa that had been treated with 1 M NaCl as described above to remove the non-integral surface protein were fixed with 2% paraformaldehyde and either directly incubated with 1:100 of different anti-FUT antibodies (D-17, N-16, N-18 and G16; Santa Cruz) or permeabilized by 0.2% Triton X-100 for 20 minutes before incubation with the anti-FUT antibodies. Bound antibodies were detected by FITC-conjugated mouse anti-goat IgG (Sigma).

Purification of sperm FUT

The purification of FUT was performed as described (Muramatsu et al., 1986; Martin et al., 1988) with modifications. In brief, sperm proteins were fractionated on a Mono Q column in a SMART System (Pharmacia) pre-equilibrated in buffer A as described (Yao et al., 1998). Sperm proteins were stepwise eluted with buffer A containing 0.5 M NaCl and desalted through a Sephadex G-25 column (Pharmacia). They were further purified by a GDP-agarose column (Sigma) and eluted with buffer A containing 10 mM guanosine-monophosphate and 0.5 M NaCl. The eluates from five runs were combined and desalted. The entire procedure of purification was performed at 4°C and within 24 hours to minimize enzyme denaturation. The purity of the purified proteins was checked by SDS-PAGE, MS and western blotting using the N-18 antibody (1:200).

The FUT activity in the fractions was measured as described (Rabina et al., 1997). Briefly, sialyl-α2,3-N-acetyllactosamine-BSA (sLN-BSA; Calbiochem)-coated wells were blocked with SuperBlock (Pierce) in Tris-buffered saline (TBS) and washed with TBST and water immediately before use. Purified FUTs were added and incubated at 37°C in assay buffer containing 0.1 M MOPS-NaOH (pH 7.3), 50 μM GDP-fucose, 6 mM $MnCl_2$, 5 mM ATP, 10 mM fucose and 0.2% Triton X-100. The product, Sialyl Lewis x (sLex) was detected at OD_{490} after successive incubation with mouse monoclonal anti-sLex immunoglobulin M (IgM) (Calbiochem), horseradish peroxidase (HRP)-conjugated goat anti-mouse IgM (Sigma) and o-phenylenediamine.

Co-immunoprecipitation of glycodelin with FUT in the presence or absence of solubilized zona pellucida

To confirm the binding relationship between glycodelin, FUT and zona pellucida, co-immunoprecipitation was performed. Human zona pellucida was separated from the oocytes using glass micropipettes under a microscope (Franken et al., 1994) and heat-solubilized at 70°C for 90 minutes in distilled water with the pH adjusted to 9 using Na_2CO_3 (Dunbar et al., 1980). A Seize X Mammalian Immunoprecipitation Kit (Pierce) was used to detect the interaction between glycodelin and purified sperm FUT or recombinant FUTs according to the manufacturer's instructions. Briefly, recombinant or purified FUT was incubated with glycodelin-A in the presence of different concentrations of solubilized zona pellucida glycoprotein at 37°C for 3 hours in buffer A. After 2 hours of incubation with gentle shaking, monoclonal anti-glycodelin antibody immobilized on Protein G beads was used to precipitate the glycodelin-A–FUT complex, which was analyzed by western blot using the N-18 antibody. The procedure for the control was similar to that described for glycodelin-A except that deglycosylated glycodelin-A was used instead.

Effects of FUT acceptors and anti-FUT antibody

The binding of saturation concentration of ^{125}I-labeled glycodelin-A to human spermatozoa was determined (Chiu et al., 2003b) in the presence of different concentrations of low molecular mass FUT acceptors, including phenyl-β-D-galactoside, 2′-fucosyllactose and lacto-N-fucopentaose-I (Sigma), which had been used to demonstrate the presence of α(1-2)- (Scudder and Chantler, 1981), α(1-3)- (Prieels et al., 1981) and α(1-4)-FUT activity (Prieels et al., 1981), respectively, or the high molecular mass acceptor, asialofetuin (Sigma) (de Vries et al., 1995). Non-FUT substrates, melibiose, RNAse, unlabeled glycodelin-A and glycodelin-S were used as controls. The cell-bound radioactivity was measured after incubation for 180 minutes at 37°C in an atmosphere of 5% CO_2 in air.

Capacitated spermatozoa were incubated either in EBSS/BSA or EBSS/BSA supplemented with various concentrations (0.1, 1, 5, 10 and 20 μg/ml) of D-17 antibody at 37°C for 3 hours. The spermatozoa were washed with fresh EBSS/BSA. A Hemizona binding assay was performed on these treated spermatozoa as described above. The antibody preabsorbed with 1:100 blocking peptide (Santa Cruz) was used as control. The ability of different concentrations of FUT acceptors to inhibit spermatozoa-zona pellucida binding was also studied. The effect of an anti-FUT antibody and FUT acceptors on sperm motility, viability and acrosomal status was determined as described above.

Binding of sperm FUT to zona pellucida

Intact human oocytes were incubated with Alexa Fluor 488 (Alexa Fluor® 488 Protein Labeling Kit; Molecular Probes, Carlsbad, CA, USA)-conjugated sperm

FUT in buffer A for 30 minutes at 37°C, and observed under a fluorescence microscope. Oocytes incubated with labeled sperm FUT in the presence of 50-fold excess of unlabeled sperm FUT or labeled ovalbumin were used as controls. The binding kinetics of sperm FUT to zona pellucida were studied by incubation of Alexa Fluor 488-labeled sperm FUT (0-500 pmol/ml) in solubilized zona pellucida (10 zona pellucida/well)-coated well microtiter plates for 2 hours at 37°C. The fluorescence intensity of bound FUT was determined using a fluorometer with excitation and emission wavelengths of 425 and 520 nm, respectively. Specific binding of sperm FUT was determined by subtracting the optical density (OD) in the presence of a 50-fold excess of unlabeled FUT from the OD in the absence of unlabeled FUT. The experiments were performed in triplicate.

Data analysis

All the data were expressed as the mean and standard error of the mean (s.e.m.). The data were analyzed by statistical software (SigmaPlot 8.02 Ligand Binding Analysis Module and SigmaStat 2.03; Jandel Scientific, San Rafael, CA, USA). For all experiments, the non-parametric analysis of variance on rank test for multiple comparisons followed by the Mann-Whitney U-test were used. A probability value <0.05 was considered to be statistically significant.

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