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The Carbohydrate Structure of DEFB126, the Major Component of the Cynomolgus Macaque Sperm Plasma Membrane Glycocalyx

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Abstract. Based on the amino-acid sequence of the macaque epididymal secretory protein, ESP 13.2 (Q9BEE3/AJ236909), it has now been classified as β-defensin DEFB126. DEFB126 is one of the five β-defensins with genes that are clustered along chromosome 20pl3, and all five proteins have an extended carboxy terminus that continues beyond the 6-cysteine β-defensin core region. This 60-amino acid carboxyl tail extension of the DEFB126 molecule is extraordinarily rich in threonine and serine (40%), many of which appear to be likely candidates for having O-glycosylation. DEFB126 has been shown to coat the entire surface of cynomolgus macaque sperm as they move through the corpus/caudal region of the epididymis. It is a major glycocalyx barrier to the external environment and is retained until the completion of capacitation. Sperm exposed to fluorescein-conjugated poly-L-lysine or Alexa488-histone showed a very uniform fluorescent labeling pattern over the entire sperm surface, almost identical to that observed with anti-DEFB126 Ig label. Sperm surface components that were released following treatment with caffeine/cAMP (in vitro capacitation) were blotted and probed with three different lectins which are known to recognize terminal sialic acid residues, and all three recognized the 35 kDa DEFB126 band. Neuraminidase treatment of sperm shifted the molecular weight of DEFB126 from 34–36 kDa to approximately 38–40 kDa and removed or greatly inhibited sialic acid-specific lectin recognition. O-Glycanase treatment alone was ineffective at removal of the oligosaccharides, but prior treatment with neuraminidase was sufficient to enable the O-glycanase treatment to effectively change the apparent molecular weight to 10 kDa, confirming that a major portion of the molecular mass is associated with the carbohydrate portion. Western blots of neuraminidase-treated DEFB126 showed strong recognition with a number of lectins that identify β-galactose and also lectins that recognize the N-acetylgalactosamine-serine/threonine, the proposed connection site of O-glycosylation. All of the lectins that recognized DEFB126 on Western blots were used to fluorescently probe sperm. The fluorescent patterns that were observed with poly-L-lysine, Alexa488-histone, sialic acid-specific lectins, and galactose-specific lectins showed even distributions over the entire sperm surface and the patterns were identical to sperm labeled with anti-DEFB126 Ig, and all but the antibody did not recognize neuraminidase-treated sperm.

Key words: Sperm — β-Defensin — Glycocalyx — Sialic acid — DEFB126

Introduction

Sperm are the carriers of the male genome. The task of migrating through the female reproductive tract and finding the oocyte is a rigorous challenge for the fertilizing sperm. The fact that the male deposits millions of sperm in the female suggests that many potential hazards await the sperm as they progress to the site of fertilization, in the oviduct (Barratt & Cooke, 1991). Mammalian sperm must reside in the female reproductive tract for an extended, but variable period of time before they attain the capacity to fertilize an oocyte (Austin, 1952). The biological complexity of sperm residence and capacitation in the

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female were first discovered over 50 years ago (Chang & Pincus, 1951) and many of the events involved in capacitation have been identified by experimentation in vitro (Jaiswal & Eisenbach, 2002). The factors that drive capacitation are membrane-related and establish a sperm surface capable of ligand recognition on the zona pellucida and membrane fusion, during and after the acrosome reaction (Yanagimachi, 1994, for review). Loss of membrane cholesterol, an elevation of intracellular pH and Ca^{++} and phosphorylation of a selected population of membrane proteins are all known to contribute to development of a fully capacitated sperm (Evans & Florman, 2002). In addition, researchers have been intrigued for years with the change in the plasma membrane composition and architecture that follows the loss of components of sperm surface coats that take place during capacitation (Yanagimachi, 1994, for review).

Sperm exit the testes incapable of fertilization and must continue their maturation process during epididymal transit (Bedford, 1975; Cooper, 1998). At least two functions of the sperm cell that are required for fertilization (zona recognition and motility) are acquired during passage through the epididymis (Jones, 1998). Because sperm are terminally differentiated in the testes, continued maturation following release from the testis centers on the external surface of the plasma membrane (Toshimori, 2000). How the addition, subtraction or alteration of the epididymal secretions on the sperm surface is accomplished and the relevance of these glycoproteins to sperm maturation continue to be a major research focus (Dacheux, Gatti & Dacheux, 2003; Christova et al., 2004; Gatti et al., 2004). As sperm progress through the male reproductive tract they acquire a substantial glycocalyx by the time of ejaculation. The components of this glycocalyx are likely to have a variety of functions during the period of sperm residence in the female (Schroter et al., 1999; Diekman, 2003). This surface coat is composed of rigorously absorbed and loosely associated glycoproteins (Diekman, 2003). The glycocalyx has been studied with various lectins to demonstrate changes that occur in the carbohydrate composition during in vitro capacitation, but the complexities of a surface coat with numerous elements, positioned somewhere within the 3 dimensions of the 60 nm glycocalyx, presents an insurmountable task of interpretation (Schroter et al., 1999). Macaque sperm possess a glycocalyx in which one of the major components is DEFB126 (formerly known as ESP 13.2), which is a β-defensin that protects sperm from immuno-recognition in the female tract (Yudin et al., 2005), but must be lost during capacitation to enable zona pellucida recognition by sperm (Tollner et al., 2004). The carbohydrate structure of DEFB126 is largely unknown, so we have initially approached our investigation by examining the different glycosylation elements of this unique β-defensin.

Materials and Methods

Reagents

All chemicals and reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), unless otherwise noted.

Semen Collection

Animals were housed at the California National Primate Research Center in compliance with the Federal Health Guidelines for Care and Use of Laboratory Animals. Semen samples were collected by electro ejaculation from seven individually caged cynomolgus macaques (Sarason et al., 1991). Each ejaculate was collected into a 15 ml centrifuge tube containing 5 ml of HEPES-buffered Biggers, Whiten and Whittingham (BWW) medium (Irvine Scientific, Santa Ana, CA). After dispersion of the ejaculate (about one hour) at room temperature (RT), the semen samples were analyzed for motility and samples with >70% actively motile sperm were used in the experiments. The samples were pipetted through a 100 μM mesh filter to remove any of the coagulum or debris. Each sample was diluted to 10 ml with fresh medium and centrifuged at $300 \times g$ for 10 min. The resulting pellet was suspended in 1 ml of fresh medium and used in the following sperm-washing protocols.

Sperm Preparation

Sperm were washed utilizing three separate methods, all of which preserve their noncapacitated state, but remove seminal plasma components. For the first method, pelleted sperm from the initial semen collection were layered under 3 ml of fresh medium and incubated for 30 min to 1 h at 37°C. The upper 2 ml were collected and centrifuged for 10 min at $300 \times g$. This swim-up washed sperm pellet was suspended in fixative as discussed later. The second and third sperm-washing techniques involved the layering of the pelleted sperm (500 μl) onto 40% or 80% Percoll solution (3 ml) in HEPES-buffered BWW. The 40% Percoll gradient was centrifuged at $300 \times g$ for 20 min and the 80% Percoll was centrifuged for 30 min at $300 \times g$. The resulting pellets were suspended in 10 ml of fresh HEPES-BWW (RT) and centrifuged at $300 \times g$ for 10 min. The pellets were immediately suspended into fixative as described later.

Sperm Fixation

Sperm samples were fixed in 1.6% para-formaldehyde and 0.125% glutaraldehyde in Dulbecco's phosphate-buffered saline (DPBS:Life Technologies, Rockville, MD). Sperm were fixed for 5 min and centrifuged ($100 \times g$) for 5 min and the resulting sperm pellet was suspended into fresh fixative. After fixation for 30 min the sperm were washed through several washes of blocking solution (1%BSA in'DPBS with 0.2% Na azide).

Sperm Activation

Sperm samples resulting from each of the different washing protocols were used to acquire DEFB126 that was released from the sperm surface. Basically, washed sperm (20×10^6/ml) were suspended in DPBS for 1 h at 37°C at which time the chemical

"activators" 1 mM cAMP and 1 mM caffeine were added; the sperm suspension continued to incubate for an additional hour with continuous rocking (Tollner et al., 2004). After 1 h the sperm were pelleted at 1000 × *g* (5 min) and the supernatant was filtered (0.22 μM) before concentration (4–5 ×) with Centricon YM-3 filters (Millipore, Bedford, MA). The concentrated sperm surface components were mixed with sodium dodecyl sulfate (SDS) solubilizing buffer (Pierce, Rockford, ILL) and boiled for 3 min. The sample was chemically reduced with 10 mM dithiothreitol (DTT).

Neuraminidase Treatment of Sperms

Washed sperm from semen were layered over 40% Percoll and centrifuged for 20 min. The Percoll-washed sperm were diluted in 10 ml of HEPES-BWW and centrifuged (30 × *g*) for 10 min. The resulting pellet was suspended in HEPES-BWW with bovine serum albumin (BSA;0.3 mg/ml), typeV neuraminidase was added (1 unit/20×10^6/ml) and sperm were incubated at 37°C for 2 h. Each tube of sperm (20×10^6) was layered over 40% Percoll with BWW-HEPES without BSA. The gradients were centrifuged (300 × *g*) for 20 min, briefly washed in fresh medium and then fixed as previously described, or suspended in DPBS and activated with cAMP and caffeine as described above. The sperm surface components were concentrated and solubilized as previously noted. Sperm were also exposed to typeV neuraminidase (1 unit/20×10^6sperm/ml), or not exposed to neuraminidase and then were washed free of BSA before the whole sperm were solubilized in SDS-buffer (Pierce). The sample was split, with half of the sample being reduced (10 mM DTT), and the other half, not chemically reduced. Percoll-washed sperm were also exposed to another type of neuraminidase (α 2-3, position-specific) at 100 mU/20×10^6/ml (Calbiochem, LaJolla, CA) or the same position-specific neuraminidase from Sigma. In both cases, sperm were SDS-solubilized after washing and subsequently boiled and reduced as previously described (Yudin et al., 2003).

O-Glycosidase (EC.3.2.1.97) Treatment of Sperm and Sperm Surface Components

Neuraminidase-treated sperm were incubated with O-glycosidase (Roche Diagnostics, Mannheim, Germany: Sigma). Sperm were exposed to neuraminidase as previously described and after 1 h O-glycosidase was added (5 mU) and incubation was continued for 2 h at 37°C. Sperm were washed in DPBS to remove the BSA and treated with activators to release the sperm surface components, including DEFB126. The released components were concentrated and solubilized (SDS) as previously described. In other experiments, neuraminidase-treated DEFB126 was released from the sperm for evaluation. Sperm that were previously exposed to neuraminidase (1 unit/20×10^6sperm/ml) for 2 h and then washed into DPBS were activated for 1 h at 37°C. After 2 h the sample was centrifuged (1000 × *g*) for 5 min and the supernatant containing DEFB126 was filtered (0.22 μM). The filtered neuraminidase-treated sperm surface components were incubated overnight with O-glycosidase (2.5 mU/ml:Roche Diagnostics) at room temperature. The sample was concentrated (5 ×) with a Centricon YM-3 filter (Millipore) and was SDS-solubilized and reduced with DTT (10 mM).

Electrophorectic Analysis

Solubilized sperm surface components that had been released by activation (2–5 μg/ml) were separated on an 8–16% gradient Tris-glycine gel (Invitrogen; Carlsbad, CA) and blotted over to nitrocellulose (Yudin et al., 2003). The nitrocellulose blots were blocked for 2 h with Tris-buffered saline (TBS: 50 mM Tris-HCL, pH 7.4, 150 mM NaCl and 0.1% Tween 20), to which 1%BSA was added. Blots were washed (2 ×) in TBS and allowed to dry overnight at room temperature. Each lane represented approximately 2 μg of sperm surfaee components, which were previously shown to be composed primarily of DEFB126 and PSP94 (Tollner et al., 2004). Each lane was cut into individual strips and probed with different lectins. *Limulus polyphemus* (LPA), a lectin that specifically recognizes sialic acid and two other lectins with known specificity to sialic acid *(Macackia amurenesis:* MAL II and *Triticum vulgaris:* WGA), were used. The lectins *Agaricus bisporus* (ABA), *Ricinus communis* (RCA), *Artocarpus integrifolia* (Jacalin) and *Arachis hypogaea* (PNA) were used to show the β-galactose or Galβ(l,3)GalNAc. The different biotinylated lectins (Vector Laboratories; Burlingame, CA: EY Laboratories, San Mateo, CA) were diluted 1 :500 into TBS. When additional ions were required, they were added to the buffer at the appropriate concentration throughout the entire labeling process. The individual strips were placed into 15 ml conical tubes with 10 ml of the biotinylated lectin (20 μg/10 mls) for 2–3 h with constant rolling. Blots were washed with TBS (3 × /10 mls) and then incubated 1 h in alkaline phosphatase –Avidin D (Vector) diluted (1:200) in TBS with 1%BSA. Each strip was individually washed thoroughly with TBS before developing with NBT/BCIP (Pierce). The same blots were also probed with anti-DEFB126 Ig as previously described (Yudin et al., 2003).

Fluorescent Labeling

Percoll (40%)-washed sperm, as well as sperm treated with α2,3-neuraminidase, were fixed as previously described and then washed extensively overnight in blocking solution. Fixed sperm were examined for immunorecognition with the anti-DEFB126 Ig as previously described (Tollner et al., 2004). Poly-L-lysine-FITC or Alexa488-histone (10 μg/ml) was incubated in blocking solution for 1 h and centrifuged at 1000 × *g* for 5 min before adding to fixed/blocked sperm (10^4 sperm/ml). After a 15–30 min incubation the sperm were gently pelleted (100 × *g*) and washed (3 ×) before photomicrography as described later. The Alexa488-histone was produced by coupling calf thymus histone (500 μg/ml) with Alexa488 and purified with a gel filtration column (Molecular Probes, Eugene, OR). Each lectin was initially preincubated with the biotinylated lectin in the blocking solution for 1 h prior to adding to the fixed/blocked sperm. The biotin-tagged lectins were the same as those described for the blots and only those that recognized DEFB126 were used in the fluorescent labeling. Each lectin (20 μg/ml) was incubated with fixed sperm (10^5/ml) for 1 h with continual rolling. $Ca^{++}$and Mg^{++} (0.1 mM) were present in the DPBS and Mn^{++} (0.01 mM) was added, if required. Labeled sperm were washed with constant rotation in fresh blocking solution (3 ×, 30 min/each). The secondary label Alexa488-streptavidin (Molecular Probes) was added to the blocking solution (10 μg/ml) for 1 h before it was combined with the sperm. After 1 h of incubation in the fluorescent-labeled streptavidin (rolling continuously) the sperm were centrifuged (300 × *g*), suspended in blocking solution and rolled for 30 min. After three washes sperm were suspended in a fluorescent stabilizing medium (FSM: 50%glycerol, 0.2%NaN_3,1% paraformaldehyde in DPBS).

Photomicrography

Images were taken of representative sperm using a cooled CCD digital camera (Magnafire; Optronics, Santa Barbara, CA) mounted on a Leitz Labolux S microscope (Carl Zeiss Vision, Germany) equipped with 200W mercury fluorescence vertical illuminator and a-1-Lambda Ploemopac incident light fluorescence vertical

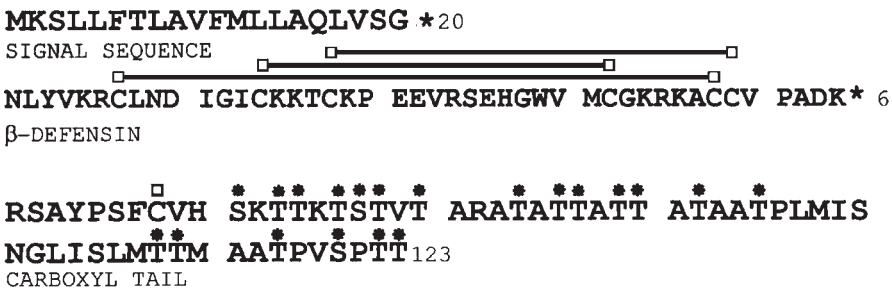


Fig. 1. The amino-acid sequence of ESP13.2/DEFB126 (Q9BEE3:CAC27133) reveals a highly polar initial 20 amino acids characteristic of secretory glycoproteins (Bendtsen et al., 2004). The following 45 amino acids have striking similarities to β-defensins. The β-defensins have a specific six cysteine (*c*) organization and are linked 1-5, 2-4 and 3-6 (*empty squares*). Between c_3 and c_4 there are 13 amino acids, five more than in the other β-defensins and unique to DEFB126 (Schutte et al., 2002). The carboxyl terminus stretching from the β-defensin core is composed of an additional 60 amino acids. The carboxyl terminus is unique in its hydrophobic nature, unpaired cysteine (*empty square*:72aa) and numerous potential sites (serines, threonines, starred) for O-glycosylation (Julenius et al., 2005).

illuminator employing an I3 filter cube with a BP450-490 excitation filter, a RKP 0510 dichromatic mirror and LP515 suppression filter. Optics included a 3.3 × intraocular magnifier (Scientific Instruments, Sunnyvale, Ca) and a Zeiss 63 × oil immersion fluorescence objective (JH Technologies, San Jose, CA). Initial images were captured using Magnafire 2.0 software (Optronics) and processed with Adobe Photoshop (Adobe Systems, San Jose, CA) for production of figures.

Results

The amino acid sequence of DEFB126 reveals the different elements that give this molecule its character. The initial twenty amino acids represent the signal sequence found in all secreted proteins (Fig. 1). Sequence data on DEFB126 from human (Q9BYW3), monkey (Q9BEE3), rat (Q99JD1) and mouse (Q8BVC1) all show a very similar β-defensin domain (45 amino acids), which have an additional five residues between the third and fourth cysteine (Fig. 1). While most β-defensins end shortly after the double cysteine found at or near 60 amino acids, there is a group of β-defensins, like DEFB126, that have an extended (60 amino acids) carboxyl tail (Perry et al., 1999; Zanich, Pascall & Jones, 2003). Another common feature of DEFB 126 from human to mouse is the unused cysteine found in the carboxyl region. The carboxyl terminus has a unique hydrophobic nature with an abundant supply of serines/threonines that are available for O-glycosylation (Fig. 1). There are at least 20 residues (serine and threonine) that are strong candidates for glycosylation (NetOglyc3.1; Julenius et al., 2005). These serine and threonine residues are clustered in groups (3) with each cluster having 6–7 glycosylation sites (Fig. 1). At least 60% of the carboxyl tail is composed of the four amino acids serine, threonine, proline and alanine.

We showed previously that DEFB126 is evenly dispersed over the entire cynomolgus sperm surface (Yudin et al., 2003; Tollner et al., 2004). As with the anti-DEFB126 localization, exposure of cynomolgus sperm to either poly-L-lysine-FITC or Alexa488-histone resulted in an identical, even fluorescent pattern over the entire sperm surface (Fig. 2*A* and *C*). This even distribution of negative charges on the external surface gave reason to examine DEFB126 for sialic acid residues, which are the most common feature of the strong negatively charged carbohydrates. Sperm were exposed to three different lectins, all known to recognize sialic acid in the terminal position of the oligosaccharide (MAL II, LPA and WGA). Each of the lectins gave the same fluorescent pattern as that found for the anti-DEFB126 Ig labeling (Fig. 3). Preincubation of the lectins with released DEFB126 caused an almost complete loss of recognition by the three sialic acid-specific lectins (*data not shown*). The negative charge distribution, sialic acid recognition and anti-DEFB126 Ig labeling, all showed the same uniform labeling over the entire sperm (Figs. 2 and 3). Another lectin, *Sambucus nigra* (SNA), which also recognizes sialic acid although only in the α(2,6) configuration, did not recognize cynomolgus sperm (*data not shown*). Sperm exposed to α2,3-sialidase to remove terminal sialic acids showed no loss of DEFB126 from the sperm surface, based on anti-DEFB126 labelling (Figs. 4*A, B*). Although DEFB126 remained on the sperm surface following sialidase treatment, these sperm showed a major reduction in sialic acid recognition with the MAL II lectin (Figs. 4*C,D*). Furthermore, the sialidase-treated sperm exhibited a major reduction in the negative charge along the sperm surface, as demonstrated by the dramatic reduction in poly-L-lysine-FITC binding (Fig. 4*E* and *F*).

DEFB126 was released from the sperm surface following treatment with activators (''activated-released'') and separated by electrophoresis before blotting onto nitrocellulose. Anti-DEFB126 Ig, MAL II, LPA and WGA all exhibited recognition of a diffuse region between 34–36 kDa (Fig. 5). Attempts to remove the O-glycosylation of the released DEFB126 were initially unsuccessful but eventually,

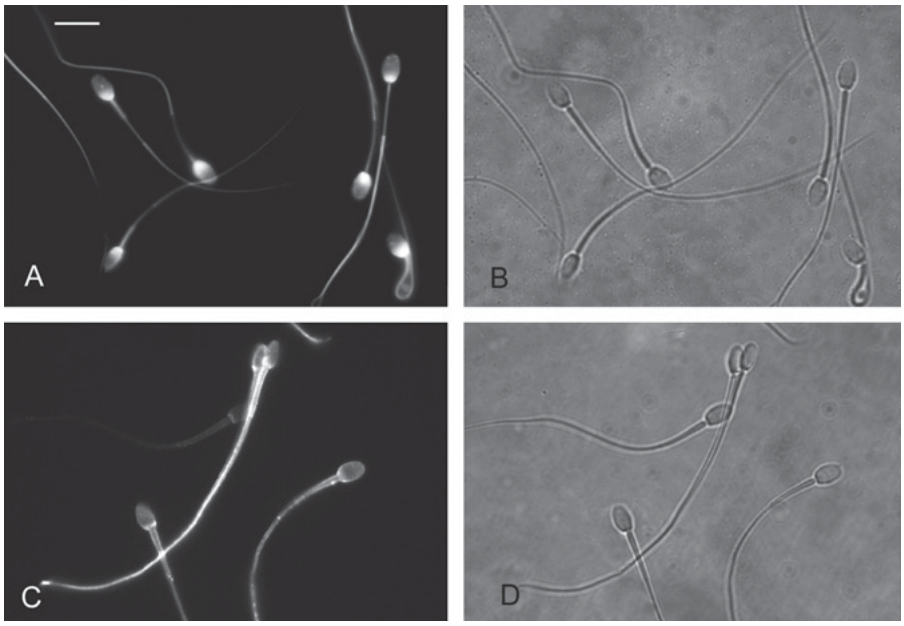


Fig. 2. Noncapacitated washed sperm were exposed to poly-L-lysine-FITC prior to fixation. Poly-L-lysine-FITC binds to negatively charged elements on the sperm surface and gives a uniform fluorescence over the entire sperm surface (*A*, *B*). Another probe that recognizes the negative charges on the sperm surface is Alexa488-histone, which gave the same fluorescent pattern as that seen with poly-L-lysine-FITC (*C*, *B*). Bar = 10 μM.

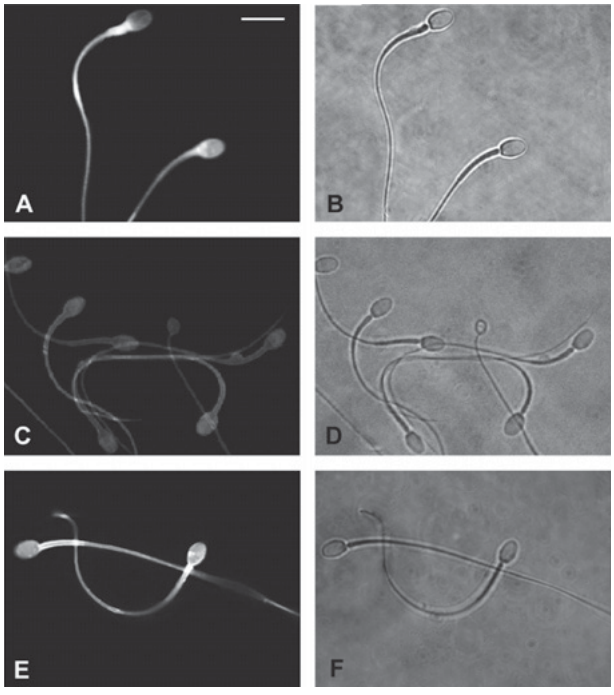


Fig. 3. Non-capacitated washed sperm were exposed to *Limulus polphemus* (LPA), a lectin that specifically recognizes sialic acid (*A*, *B*). Two other lectins with known specificity to sialic acid, *Macackia amurenesis* (MAL II, *C*, *D*) and *Triticum vulgaris* (WGA, *E*,*F*) also appeared to have the same uniform fluorescence pattern over the entire sperm surface as observed with LPA. The sialic acid lectins had the same uniform fluorescent pattern as the poly-L-lysine and histone labeling, which recognized the negatively charged surface molecules (*see* Fig. 2*A–D*). Bar = 10 μM

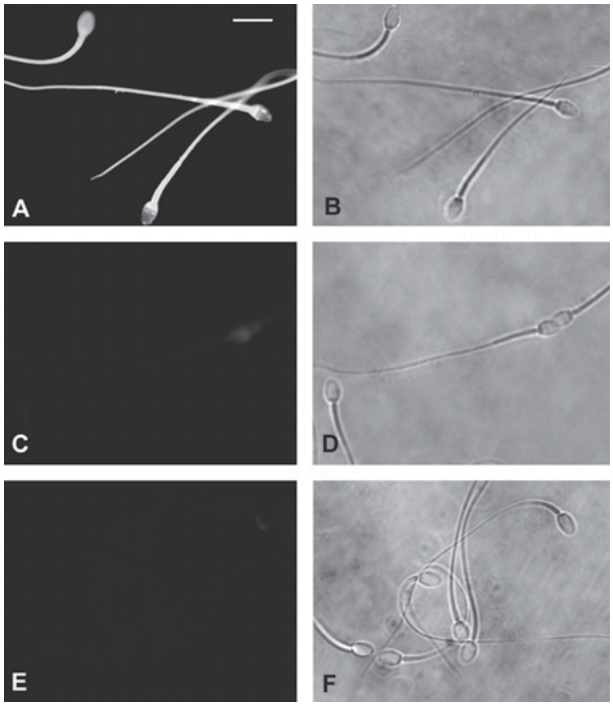


Fig. 4. Macaque sperm were incubated with α2,3-sialidase before exposure to anti-DEFB126 (*A*,*B*), MAL II (*C*, *D*), which recognizes sialic acid, or to poly-L-lysine-FITC (*E*, *F*), which binds to negatively charged components. Anti-DEFB126 recognized the entire sperm surface, even following enzymatic removal of the terminal sialic acid (*A*, *B*), but the removal of the sialic acid residues resulted in a major reduction of the MAL II (*C*, *D*) recognition of sialic acid as well as the poly-L-lysine-FITC recognition of the surface negative charge (*E*, *F*). Bar = 10 μm

complete removal of all of the carbohydrates was accomplished when sequential sialidase and glycanase were used (Fig. 6). Incubation with O-glycanase for 2 h before SDS-solubilization resulted in the broadening of the DEFB126 band from 34–36 kDa to 28–36 kDa, but there was neither a radical shift in molecular

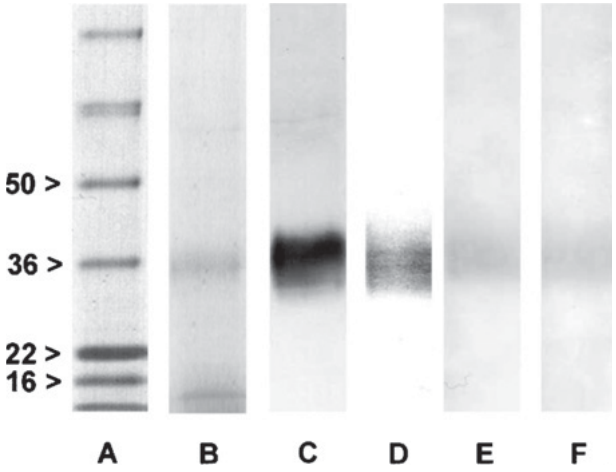


Fig. 5. SDS gel electrophoresis of activated released (cAMP and caffeine) components stained for protein with Gel Code Blue. Western blots of surface components released from cynomolgus macaque sperm after treatment with activators (caffeine and cAMP) and probed with anti-DEFB126 Ig (*A*) or with the sialic acid-specific lectins LPA (*B*), MAL II (*C*) and WGA (*D*). The three sialic acid-specific lectins recognized the whole cynomolgus sperm (3*A–F*) and also the DEFB126 glycoprotein on a Western blot (4*A–4D*).

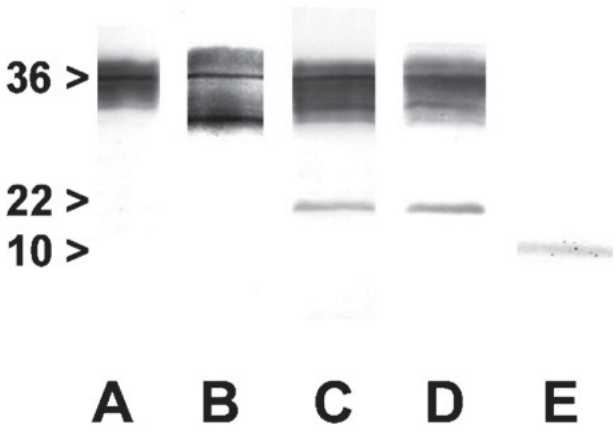


Fig. 6. Whole cynomolgus sperm solubilized in SDS and chemically reduced with DTT, appears as a single broad band (31–35 kDa) when probed with anti-DEFB126 Ig (*A*). Noncapacitated sperm were incubated with O-glycanase before solubilization and reduction. O-glycanase-exposed sperm appeared as a much broader and diffuse band (28–36 kDa) when probed with anti-DEFB126 Ig (*B*). Activation-released sperm surface components were incubated with O-glycanase overnight at 37°C (*C*) and 24°C (*D*) before solubilization. The Western blots were probed with anti-DEFB126 Ig and there was a second band (20 kDa) in addition to the prominent 30–35 kDa band for both treatments (*C*, *D*). On the other hand, if sperm were treated with neuraminidase prior to activation, the released components were readily degraded with O-glycanase (*E*). Neuraminidase and O-glycanase treatment of DEFB126 resulted in a 10 kDa peptide, the deduced molecular weight of DEFB126 without glycosylation (*E*).

weight nor the appearance of obvious low molecular weight degradation products (Figs. 6*A* and 6*B*). When the DEFB126 released by sperm activation was incubated overnight with either O-glycanase at 24°C or 37°C, a thin band appeared at 20 kDa, but a major band remained at 30–35 kDa (Fig. 6*C* and *D*). When the sperm were first treated with neuraminidase to remove sialic acid, washed, and then activated in order to release the DEFB126 prior to incubation with O-glycanase, a ''new'' band appeared at 10 kDa and the 34–36 kDa band disappeared (Fig. 6*E*). Curiously, as presented elsewhere (Yudin, et al., 2005) and as shown in Fig. 7, when sperm were treated with neuraminidase, there was a marked increase in the molecular weight of the 34–36 kDa band to 38–40 kDa.

The free cysteine of DEFB126 is thought to contribute to the aggregation of DEFB126 glycoprotein, as reported in the rat (defb22/2D6: Zanich et al., 2003). DEFB126 isolated from whole cynomolgus sperm samples that were chemically reduced, always exhibited a molecular weight of 34–36 kDa, however, non-reduced, whole sperm showed a clear laddering or aggregation of the DEFB 126 glycoprotein (Fig. 7*A* and *B*). Sialidase treatment of sperm prior to solubilization and reduction resulted in a marked increase in the molecular weight of DEFB126 to 38–40 kDa (Fig. 7*C*). Thus, the charge contributed by sialic acid did not appear to affect the aggregation of DEFB126, suggesting that the laddering is related to the free cysteine. When the reducing agent (10 mM DTT) was continuously present as whole sperm were activated to release DEFB126, a single band was apparent, but when DTT was removed after DEFB126 release and sperm removal, DEFB126 appeared to aggregate and form a laddering of molecular weights (Fig. 7*E* and *F*). Sperm exposed to α2,3-sialidase did not show any change in the apparent molecular weight of DEFB126 (Fig. 7*G* and *H*), even following overnight treatment.

O-linked oligosaccharides typically possess sialic acid in the terminal and/or penultimate positions and it is common to have β-galactose residues between the sialic acid and the connection to the peptide. Neuraminidase treatment of DEFB126 released from sperm after treatment with activators resulted in a slight increase in apparent molecular weight to 38–40 kDa and, with concomitant recognition by both PNA and RCA, which are specific for β-galactose (Fig. 8*A*, *B* and *C*). Neuraminidase treatment of the DEFB126 released by capacitation activators also revealed the N-acetyl-galactosamine that is linked either to the serine or threonine, the linkage common to all O-linked oligosaccharides. The use of the lectins Jacalin and ABA clearly showed the recognition to the N-acetyl-galactosamine (Fig. 8*D* and *E*). Fluorescent labeling showed that both lectins recognized either β-galactose or N-acetylgalactosamine in an even distribution (fluorescence) over the entire sperm (Fig. 9*A–G*).

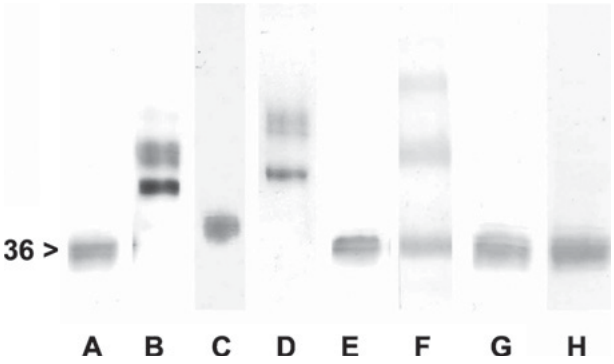


Fig. 7. As previously shown, solubilized/reduced whole sperm when probed with anti-DEFB126 Ig appeared as a single broad band (31–35 kDa); on the other hand, non-reduced whole sperm DEFB126 appeared as multiple bands with the 53–55 kDa and the 64–72 kDa bands being the most prominent (compare *A* and *B*). Neuraminidase treatment of sperm resulted in a marked increase in molecular weight of DEFB126 (38–40 kDa) (*C*), but if the whole sperm were not chemically reduced, the neuraminidase treatment resulted in a shift in DEFB126 molecular weight (56 kDa and 76–84 kDa) (*D*). The loss of terminal sialic acid resulted in DEFB126 having a much slower molecular mobility regardless of the state of sulfhydryl linkages. Activation-released DEFB126 had a molecular weight of 34–36 kDa (*E*), but if reducing agents were not continually present, DEFB126 aggregated, appearing as a series of 35 kDa, 72 kDa and 125 kDa bands (*F*). Activation-released DEFB126 was also incubated with two different α(2,3)-specific neuraminidases and there was no shift in molecular weight, even after prolonged incubation (*G* and *H*).

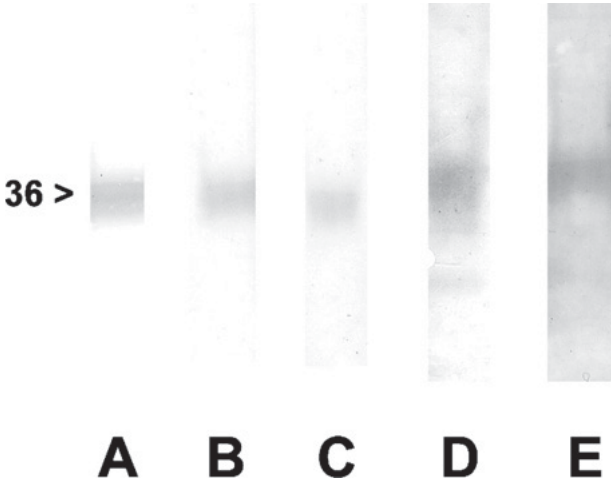


Fig. 8. Neuraminidase-treated sperm were incubated with activators to release DEFB126. The neuraminidase-treated DEFB126 was probed with anti-DEFB126 (*A*), as well as β-galactose-specific lectins (PNA, *B* and RCA, *C*). In both cases, the neuraminidase-treated DEFB126 was positive for β-galactose. Two lectins, Jacalin and ABA, are known to recognize the site specific for O-linked glycosylation (galactose-N-acetylgalactosamine-serine/threonine). In each case, the lectins recognized DEFB126, but with much greater intensity after neuraminidase treatment (*D* and *E*).

Discussion

Sperm are terminally differentiated cells incapable of generating any new biosynthetic products after exiting the testes (Yanagimachi, 1994, for review). The ability of sperm to acquire a surface coat or glycocalyx results from the absorption of multiple products that were secreted by the various reproductive tract organs (Martins et al., 2003; Gatti et al., 2004). Sperm exit the testes morphologically complete, yet epididymal passage is necessary for sperm to acquire motility and develop fertilization competence, demonstrating the requirement for fully functional plasma membranes (Toshimori, 2003). Under hormonal regulation, these significant (glyco)proteins are synthesized, secreted and incorporated into the sperm plasma membrane in a well-orchestrated manner. Three dimensional matrices as much as 30–60 nm thick are established over the entire sperm surface, a coating that is dramatically thicker than the 7 nm coat described for erythrocytes and the 30 nm coat found along endothelial cells (Levine et al., 1983; Adamson & Clough, 1992; Bearer & Friend, 1990; Schroter et al., 1999). Some of the unique sperm-specific molecules have been identified and evidence has been put forward to explain the potential functions of the sperm extracellular matrix up to the final stages of sperm maturation (Eddy & O'Brien, 1994; Jones, 1998; Cooper, 1998).

At the completion of sperm maturation or plasma membrane modification, the sperm are exposed to the female reproductive tract, which begins a process to remove many of the sperm surface additions acquired in the male (Yanagimachi, 1994, for review). Capacitation is a set of coordinated events including elevation of intracellular Ca^{++}, phosphorylation of specific proteins, stimulation of cAMP, loss of membrane cholesterol and loss of a surface coat that exposes the zona pellucida receptors (Jaiswal & Eisenback, 2002; Baldi et al., 2002). Primate sperm are ejaculated into the vagina and gain entrance into the cervical canal and mucus, where it would appear that loosely adhering sperm surface coats are "scrubbed off", leaving a formidable coat capable of protecting the sperm on its journey to the oocyte (Yudin et al., 1989; Overstreet, Katz & Yudin, 1991; Chretien, 2003; Feki et al., 2004). DEFB126 is a glycoprotein that is adsorbed to the monkey sperm surface in the caudal epididymis (Yudin et al., 2003) and is retained on the entire sperm surface even after sperm have reached the uterus (*unpublished data*). DEFB126 has been shown to be the macaque sperm surface coat that must be lost for sperm recognition of the zona pellucida (Tollner et al., 2004).

The presence of a "cell coat" is known to be a common feature of many cell types, but determination of the carbohydrate structure of such coats and their contribution to the negative cell surface charge has been challenging (Rambourg & Leblond, 1967; Yanagimachi et al., 1972; Schauer, 2004). The carbohydrate typically associated with the negative charge is sialic acid, which is known to exist in nature

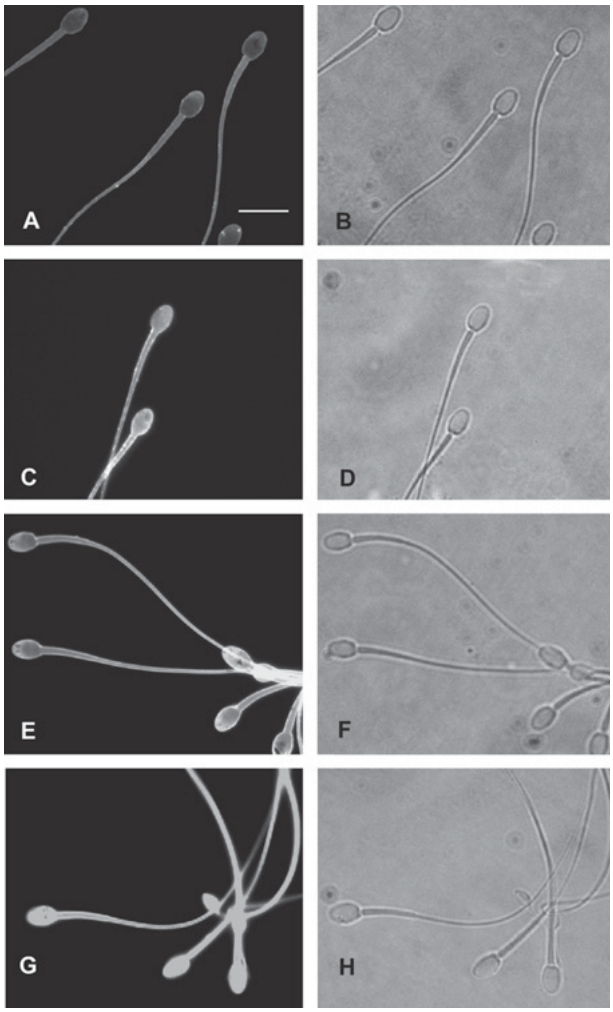


Fig. 9. Neuraminidase-treated sperm were fixed and exposed to the lectins specific for β-galactose (RCA, *A*, *B* and PNA, *C*, *D*). In each case the fluorescence was uniformly distributed over the entire sperm surface, as was found with the sialic acid-specific lectins (Fig. 3*A*, *C* and *E*). Jacalin and ABA, the lectins specific to the O-linked glycosylation site, also gave a uniform fluorescent pattern over the entire sperm surface (*E*, *F* and *G*, *H*). Bar = 10μm

in one of 40 different configurations (Schauer, 2001). Sialic acid is most often found as the terminal sugar in the oligosaccharide, thereby creating the negative shell around the molecule and extending its life (Traving & Schauer, 1998). In the present study, three different lectins that are specific for sialic acid recognized cynomolgus sperm DEFB126 on a Western blot and they each showed a uniform fluorescence pattern over the entire sperm surface. The lectin, SNA, is specific for the α(2,6)-linked sialic acid and does not recognize DEFB126. The lectins that were specific to the α(2,3) linkage recognized the sialic acid, suggesting that the sialic acid is linked to β-galactose in the α(2,3) configuration. When sperm or released DEFB126 was incubated with α(2,3),-specific neuraminidase, there was no shift in molecular weight, which is most likely due to the fact that this enzyme will only recognize the terminal sialic acids in unbranched oligosaccharides. Since this enzyme was ineffective, it is probable that the DEFB126 oligosaccharide has a branched configuration. The sialic acid of DEFB126 is externally exposed and could be associated with cell recognition events, providing protection from enzymatic degradation or imparting protection from immune attack (Yudin et al., 2005), as has been proposed for other cell types (Ashwell & Morell, 1974; Schauer et al., 1985; Kelm & Schauer, 1997; Schauer, 2004). Sperm possess a net negative charge, as is the case with most cells. The fluorescent pattern of sperm exposed to poly-L-lysine or Alexa488-histone revealed a uniform distribution of negative charges similar to that observed following lectin labeling of sialic acid and DEFB126 localization with the anti-DEFB126 Ig. Removal of the sialic acid, but not DEFB126 itself from the sperm surface clearly demonstrates that DEFB126 and its sialic acid(s) are responsible for most, if not all of the negative charge on sperm. Release of DEFB126 from sperm during capacitation also results in a dramatic decrease in poly-L-lysine-FITC labeling (*data not shown*).

Such a negative coat is reported to repel other cells or the negative components of the external environment (Mehrishi & Bauer, 2002). It is easy to envision how the universal negative coat established by the sialic acids of DEFB126 enable sperm to enter and colonize the highly negatively charged cervical mucus found in primates (Nasir-ud-Din et al., 2003). The coulombic forces created by these repulsive shells allow for the continued residence of sperm in the viscoelastic cervical mucus.

DEFB126 has been shown to remain on the surface of cynomolgus sperm recovered from the uterus (*unpublished data*), and during capacitation in vitro it is rigorously retained, even after overnight incubation in high BSA (30 mg/ml); however, physiological stimulation with cAMP and caffeine induces the final stages of capacitation and the release of DEFB126 from the sperm (Tollner et al., 2004). The loss of a glycoprotein (decapacitation factor) has been the hallmark of sperm capacitation and its presence is thought to mask the zona pellucida receptors (Yanagimachi, 1994, for review; Diekman, 2003). A sialoglycoconjugate has been reported to be shed during human sperm capacitation in vitro, but only in the presence of high concentrations of BSA (Focarelli, Rosati & Terrana, 1990; Lassalle & Testart, 1994; Focarelli Giuffrida & Rosati, 1995).

The human sperm surface is thought to contain as many as 228 (glyco)proteins (Naaby-Hansen, Flickinger & Herr, 1997). Most, if not all, of the sperm surface glycoproteins are uniquely organized into five different functional domains (Friend, 1982; Cowan et al., 1997). DEFB126 is not restricted to one of the five domains, but is evenly dispersed along the entire surface and retained during passage through

cervical mucus and/or incubation in vitro with high concentrations of BSA. This may be accomplished by association of DEFB126 with the lipid bilayer, which was proposed by Zanich et al., (2003) for defb22 (2D6), the rat homologue of DEFB126. Zanich et al., (2003) believed that the highly hydrophobic region (amino acids 58–72) may associate with the lipid bilayer and, in fact, a good portion of the defb22 (2D6) was found in the lipid fraction.

DEFB126 possesses a polypeptide backbone of about 10 kDa, but exhibits an electrophoretic mobility of 34–36 kDa with SDS-PAGE, indicating that a major portion of the molecular mass resides in the O-linked oligosaccharides. Our attempts to remove the O-glycosylation with O-glycanase were unsuccessful until neuraminidase treatment was first used to remove the sialic acid, which eventually resulted in the carbohydrate-free 10 kDa DEFB126. O-Glycosylation is a post-translational modification found in less than 10% of proteins and is most often associated with glycoprotein components of extracellular matrices or mucins (Apweiler, Hermjakob & Shaton, 1999). Shogren, Gerken & Jentoft (1989) pointed out that O-linkages are often clustered and highly branched. Molecules with a high number of O-linked carbohydrates consume a great deal of space (volume) and the sugars maintain an extended, rigid polypeptide backbone. DEFB126 is one of the unique β-defensins that possess the extended apolar carboxyl tail and represents the major external coat in rat sperm, as well as macaque sperm (Zanich et al., 2003; Rao et al., 2003; Yudin et al., 2003). The rat defb22 and monkey DEFB126 are homologues and share the common feature of having a free cysteine in the carboxyl region, which causes the molecule to aggregate upon release from the sperm (Zanich et al., 2003; Rao et al., 2003; Yudin et al., 2003). In both species DEFB126/defb22 appear to be the dominant molecules that are externally accessible to either immunological probes or carbohydrate labeling with galactose oxidase/NaB_3H_4 (Jones et al., 1981; Zeheb and Orr, 1984; Rao et al., 2003; Tollner et al., 2004). It is common for antibodies to be directed specifically to the carbohydrate portion of a molecule, in fact, 2D6 is the monoclonal antibody to the carbohydrate element of the rat defb22 (Zanich et al., 2003). Rat defb22 is found to be incorporated into the oolemma at the time of fertilization and spreads out from the fused sperm (Brown, von Glos & Jones, 1983). The incorporation of defb22 into the oolemma would indicate that not all of the defb22 is lost during capacitation and that it is most likely inserted in the lipid bilayer in some way, thereby enabling transfer to the oolemma.

Defensins, and more specifically β-defensins, appear to be the primary means of protection against microbial attack in the male reproductive tract (Zaballos et al., 2004). β-Defensins disrupt the membranes of pathogens, but appear not to associate with normal healthy cells, except for a few examples of β-defensins that associate with sperm (Com et al., 2003; Semple,Rolfe & Dorin, 2003). β-Defensin genes are clustered at four separate chromosomal locations and those found at 20q11.1 (DEFB115-124) and 20pl3 (DEFB25-29) have an extended carboxyl tail and with the exception of DEFB118, they are not known to have antimicrobial activity or to be associated with sperm (Rodriques-Jimenez et al., 2003; Yenugu et al., 2004; Shen et al., 2005; Radhakrishnan et al., 2005). It is known that DEFB126 and defb22 are major secretory products of the caudal epididymis and that they readily associate with sperm, but whether or not they have an antimicrobial function like their ancestral family has yet to be determined (Xiao et al., 2004).

Clearly, DEFB126 is a major component of the cynomolgus sperm glycocalyx. The numerous O-linked oligosaccharides add significantly to its molecular mass and the sialic acids α(2,3)-linked to the β-galactose give DEFB126 a pI of <3.5, which is similar to that observed for defb22 (Rao et al., 2003; Yudin et al., 2005). Lectins have been used in the past to observe changes in the sperm surface during capacitation, but this approach only detects general shifts in the surface carbohydrates and does not define an individual sperm surface glycoprotein (Cross & Overstreet, 1987; Navaneetham, Sivashanmugan & Rajalakshmi, 1996; Srivastav, 2000; Srivastav, et al., 2004). It would be assumed that as capacitation removes DEFB126 there is an appearance of CD-52, as well as other well-characterized GPI-anchored sperm surface glycoprotein (Yeung et al., 2000; Yudin et al., 2005). Unlike DEFB126, CD-52 is rich in N-linked carbohydrates and has numerous sialic acids, but like PH20 and other GPI-anchored glycoproteins, they are localized in specific domains on the sperm surface (Della Giovampeola et al., 2001; McCauley et al., 2002). DEFB126 (and defb22 in the rat) is the monkey and the rat sperm surface coat that provides the sperm its uniform negatively charged coat and is the shield that protects the sperm during residence in the female (Yudin et al., 2005).

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