Post-translational modifications in glycosylation status during epididymal passage and significance in fertility of a 33 kDa glycoprotein (MEF3) of rhesus monkey (Macaca mulatta)

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Abstract

The present study reports data on post-translational modifications in the glycosylation status during epididymal passage and significance in fertility of a 33 kDa glycoprotein of rhesus monkey (Macaca mulatta), designated as MEF3 (monkey epididymal fluid protein 3). MEF3 exhibited strong affinity for N-linked α-D-mannose groups and O-linked N-Ac-galactosamine linkages in epididymal fluids and exhibited moderate affinity for N-Ac-glucosaminylated (wheat germ agglutinin), fucosylated (Tetragonolotus purpurea), and N-Ac-galactosamine (peanut agglutinin) residues on more mature corpus and caudal spermatozoa in a maturation-dependent manner on Western blots probed specifically with CEF and caudal sperm membrane of macaque and with Triton X-100 extract of ejaculated human spermatozoa, suggesting the existence of antigenically related components in both species. The tangled agglutination caused by anti-33 kDa serum of human spermatozoa, along with localization of MEF3 on entire sperm surface of epididymal and testicular sperm of monkey and human spermatozoa, suggest the significance of MEF3 in sperm function. The 100% inhibition of fertility of immunized female rabbits with this protein in vivo and inhibition of human sperm penetration in zona-free hamster eggs in vitro suggests the functional significance of MEF3 in fertility. Together, these results clearly indicate that MEF3 has potential significance as a target for antibodies that inhibit sperm function and fertility.


Introduction

The mammalian epididymis creates a unique microenvironment within the lumen of the duct that helps transform immotile and immature testicular spermatozoa into fully fertile competent cells by post-translational modifications and protects and stores fertile spermatozoa in a viable state within the cauda epididymis. During sperm maturation, specific secretory proteins produced in the epididymis associate with the sperm surface resulting in remodeling of the epididymal sperm plasma membrane correlating with acquisition of fertilizing capacity. There are several ways in which epididymal proteins might be involved in sperm maturation, such as the addition of epididymal secretory components to pre-existing acceptor sites on the sperm surface, the incorporation of antigens in a relatively loose mode of binding as sperm coating or as tightly bound despite their origin outside the cell, the modification of intrinsic sperm membrane molecules or shedding of antigens in the epididymal lumen (reviewed by Cooper 1998, Dacheux et al. 2003, Jones 2004). The proteins present in the epididymal fluid may be the result of (1) rete testis compounds that enter through efferent ducts in the proximal part of the epididymis, (2) epididymal secretion and absorption, (3) proteolysis of pre-existing proteins within the fluid and, to some extent, (4) metabolic activity of the spermatozoa. Many of them are also expressed by testicular germ cells (Gaudreault et al. 2001, Busso et al. 2005).

The role of the carbohydrate portion of glycoproteins is being increasingly recognized for its importance in mediating the adhesion between mammalian sperm and zona pellucida (reviewed by Benoff 1997). During epididymal sperm maturation, glycosylation is one of the important post-translational modifications of sperm surface proteins involving multiple intracellular and extracellular biochemical changes in the spermatozoon, including modification of its surface glycoproteins as evidenced by lectin-binding studies (Srivastava & Olson 1991, Srivastav 2000, Srivastav et al. 2004, Tulsiani 2006). Two sets of glycan-modifying enzymes, namely (1) glycohydrolases, a class of hydrolytic enzymes that cleave sugar residues from existing glycoconjugates and (2) glycosyltransferases, a class of synthetic enzymes that add sugar residues from
a donor sugar (nucleotide sugar) to the acceptor site(s) on existing molecules, are present in high concentrations in the epididymal luminal fluid (EF) that surrounds the spermatozoa. Both classes of glycan-modifying enzymes have a role in modifying sperm surface glycoproteins during epididymal maturation (Tulsiani 2006).

Although the characterization, significance, and role of epididymal glycoproteins has been studied in many primate species including the human (Focarelli et al. 1998, Kirchhoff et al. 1998, Liu et al. 2000, Cohen et al. 2001, Busso et al. 2005), these are poorly understood in the rhesus epididymis (Srivastav 2000, Srivastav et al. 2004), an animal model commonly used for preclinical testing of drugs. Previous work from this laboratory (Srivastav 2000, Srivastav et al. 2004) on the glycosylation status of sperm membrane and EF proteins during sperm maturation in rhesus epididymis led to the identification of three major maturation-dependent, heavily glycosylated proteins viz. 116 (monkey sperm antigen 3, MSA3), 58 (monkey epididymal fluid protein 1, MEF1), and 33 kDa (MEF3) (Srivastav 2000, Srivastav et al. 2004). This prompted us to explore the functional significance of each of these proteins individually. The present study reports data on post-translational modifications in glycosylation status, sperm association, and significance in the fertility of 33 kDa (MEF3) glycoprotein in the epididymal fluid of the rhesus monkey (Macaca mulatta).

Results

Glycosylation status of 33 kDa protein in epididymal fluids

The 33 kDa (MEF3) glycoprotein exhibited staining for N-linked α-D-mannose and for O-linked Gal, N-Ac-galactosamine linkages in fluids from all segments, exhibiting the highest expression and/or the highest glycosylation for these oligosaccharides in proximal caput and the lowest staining in caudal fluid as evidenced by densitometric analysis of blots probed with lectins Lens culinaris agglutinin (LCA) and Ricinus communis agglutinin (RCA; Fig. 1a and b). MEF3 exhibited very faint or no staining for N-Ac-glucosaminylated, fucosylated, and N-Ac-galactosamine residues when probed with lectins peanut agglutinin (PNA), wheat germ agglutinin (WGA), and Tetragonolotus purpurea (TGP) respectively on blots of epididymal fluids (data not shown).

Glycosylation status of 33 kDa protein on maturing sperm membranes

MEF3 exhibited maturation-dependent changes in N-Ac-galactosamine, N-Ac-glucosaminylated, and fucosylated residues on epididymal sperm membranes as evidenced by the appearance of faint band of 33 kDa, identified by lectins PNA, WGA, and TGP respectively on more mature corpus and caudal sperm membranes when compared with sperm membranes from the initial segment and caput epididymis (Fig. 2a–c) with very faint or no staining by lectins LCA and RCA (results not shown).

Immunoblotting

Since MEF3 exhibited affinities for different lectins on epididymal fluids (EFs) and sperm membranes, we were curious to know whether the two forms are different or the same. We raised antiserum against affinity-purified 33 kDa protein from CEF in virgin female albino rabbits. ELISA confirmed the presence of antibodies to this protein in the...
The immune serum cross-reacted specifically with a 33 kDa band in CEF and the membrane of caudal epididymal spermatozoa showing actual association of epididymal fluid and sperm membrane 33 kDa protein. The cross-reactivity of antiserum with the Triton X-100 extract of ejaculated human spermatozoa suggested the existence of antigenically related components in both the species (Fig. 3). The titers of antiserum were confirmed to be 1:12 800 as evidenced by ELISA.

**Immunofluorescent localization of 33 kDa protein on sperm surface**

Fixed and permeabilized spermatozoa from the testis and five segments of epididymis (initial segment, proximal caput, distal caput, corpus, and cauda) of the monkey were probed with polyclonal antiserum against 33 kDa glycoprotein. Indirect immunofluorescence studies demonstrated this protein to be localized on the entire surface of testicular spermatozoa (Fig. 4a and b). A similar localization was observed on spermatozoa from all epididymal regions suggesting its actual association with maturing epididymal spermatozoa. The occurrence of 33 kDa glycoprotein on the surface of spermatocytes and spermatids suggest that the protein is first expressed in the testis (Fig. 4c–f).

The glycoprotein also localized on human spermatozoa as evidenced by the occurrence of fluorescence over the entire surface of acrosome intact, methanol-fixed human spermatozoa (Fig. 5a and b). Together, the results of Western blotting and the cross-reactivity of antiserum with ejaculated human spermatozoa in immunofluorescence studies suggested the existence of antigenically related components in both the species.

When human spermatozoa were capacitated (capacitated for 18 h with BSA) and acrosome-reacted (with calcium ionophore A 23 187), the fluorescence was lost from the anterior head and a major part of the flagellum and was confined to the posterior head and midpiece as shown in Fig. 5c and d. The existence of the 33 kDa glycoprotein on the surface of capacitated and acrosome-reacted human spermatozoa in indirect immunofluorescence assay suggests this protein to be significant for fertilization.

**Figure 2** Densitometric analysis of blots probed with lectins (a) PNA, (b) WGA, and (c) TGP of epididymal sperm membranes from initial segment (lane 1), proximal caput (lane 2), distal caput (lane 3), corpus (lane 4), and cauda (lane 5) epididymes exhibiting the appearance of a faint band of 33 kDa, identified by lectins PNA, WGA, and TGP on the more mature corpus and caudal sperm membranes. Fifty micrograms of total protein were loaded in each lane. Error bars represent s.d. (N= 6).
Significance of 33 kDa epididymal fluid glycoprotein in sperm functions and fertility: in vivo inhibition of fertilization by active immunization with MEF3

MEF3 seemed to have an involvement in fertilization as evidenced by the 100% inhibition of in vivo fertility of female albino rabbits (n = 6) immunized with this protein. The immunized female rabbits produced no offspring when they were mated with coeval males of proven fertility as compared with control (n = 6) animals (Table 1).

Inhibition of human sperm penetration of the zona-free hamster eggs by 33 kDa polyclonal antiserum

Since active immunization of female albino rabbits with MEF3 resulted in in vivo inhibition of fertilization and the antiserum cross-reacted with human spermatozoa too, the results of in vivo inhibition of fertilization were confirmed in vitro by studying the inhibition of human sperm penetration of the zona-free hamster eggs by 33 kDa polyclonal antiserum. When zona-free hamster oocytes were co-incubated with capacitated and acrosome-reacted human spermatozoa from five different donors, pretreated with immune serum to MEF3, a significant decrease in the number of oocytes penetrated was observed in five different experiments, when compared with the preimmune control group as shown in Table 2.

Studies on possible mechanism of inhibition of in vivo fertilization by 33 kDa glycoprotein: microsperm agglutination antibody assay

Microsperm agglutination (mA) assay with 33 kDa antiserum was conducted using human spermatozoa to study the mechanism of inhibition of both in vivo and in vitro fertilization by anti-33 kDa sera. The incubation of anti-33 kDa serum caused tangled agglutination of human spermatozoa within 60 min of incubation period at 37 °C up to a dilution of 1:1280. The control lanes with normal rabbit serum (NRS) demonstrated no sperm-agglutinating activity (Fig. 6a and b). Although a majority of spermatozoa


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Table 1 Inhibition of in vivo fertility of female albino rabbits immunized with 33 kDa epididymal fluid glycoprotein on days 10–15 of last immunization.

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of animals (n)</th>
<th>Mean litter size*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>Three males</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Four females</td>
</tr>
<tr>
<td>Immunized</td>
<td>6</td>
<td>No litters</td>
</tr>
</tbody>
</table>

*30 days after mating.

agglutinated, few sperm exhibited flagellar motility. These results of sperm agglutination assay showing a tangled pattern of agglutination including head-to-head, tail-to-tail, and head-to-tail were expected due to the immunolocalization of 33 kDa protein over the entire sperm surface of human spermatozoa.

Discussion

The present study reports data on post-translational modifications in glycosylation status during epididymal passage, sperm association, and significance in fertility of a 33 kDa glycoprotein (MEF3) of the rhesus monkey. The glycoprotein exhibited staining for N-linked α-D-mannose and for O-linked Gal, N-Ac-galactosamine linkages in fluids from all segments. The highest expression and/or the highest glycosylation for these oligosaccharides in proximal caput fluid could probably be due either to addition or to the unmasking of α-D-mannose (LCA) and desialylated terminal Gal, N-Ac-galactosamine (RCA) residues on the protein exterior in this segment of epididymis. This could also indicate the higher expression of the glycoprotein itself as has been reported for cysteine-rich secretory protein 4 (CRISP4) the mouse counterpart of human CRISP-1 (Nolan et al. 2006) showing the highest level of expression in proximal parts of mouse caput epididymis, with a very low signal in the distal parts (Jalkanen et al. 2005). The diminished affinity of the 33 kDa protein for lectins LCA and RCA in CEF along with faint staining for lectins WGA, TGP, and PNA on more mature corpus and caudal spermatozoa suggest that either 33 kDa protein is absorbed by epithelium of the cauda epididymis or is taken up by caudal epididymal spermatozoa with reorganized epitopes exposed with new structures at the oligosaccharide termini on maturing sperm surface. Alternatively, these modifications could result from a progressively greater proportion of these proteins being exposed to and acted upon by epididymal glycosidases, glycosyl transferases, or by the action of luminal proteases (Tulsiani 2006).

Since MEF3 exhibited different affinities for different lectins in EFS and on epididymal sperm membranes, we were curious to know whether the two forms are the same or different. The antiserum raised against affinity-purified protein from CEF in female albino rabbits specifically recognized a 33 kDa band in CEF and also reacted with caudal spermatozoa. These results along with the results of indirect immunofluorescence assay, where the glycoprotein localized on entire sperm surface from all epididymal regions studied using anti-33 kDa serum, suggest its actual association with maturing sperm membranes. This suggestion is further supported by our earlier results where antiserum raised against caudal sperm membrane in female albino rabbits cross-reacted strongly with CEF 33 kDa polypeptide of the rhesus macaque (Srivastav et al. 2004).

In indirect immunofluorescence assay using anti-33 kDa serum, the glycoprotein localized on the entire surface of macaque sperm and showed an intense fluorescent staining over the head and patchy but strong staining over entire flagellum of human spermatozoa.

Table 2 Inhibition of human sperm penetration of zona-free hamster eggs in vitro by 33 kDa polyclonal antiserum.

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Number of eggs</th>
<th>Immune-treated</th>
<th>Control (preimmune-treated)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number penetrated</td>
<td>Percentage</td>
<td>Number of eggs</td>
</tr>
<tr>
<td>1</td>
<td>15</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>21</td>
<td>4</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>7</td>
<td>29</td>
</tr>
<tr>
<td>4</td>
<td>17</td>
<td>2</td>
<td>11.7</td>
</tr>
<tr>
<td>5</td>
<td>33</td>
<td>9</td>
<td>27</td>
</tr>
</tbody>
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The results of the present study are similar to those reported for epididymal secretory protein 13.2 (ESP13.2), a secretory protein of cynomolgus macaque and human epididymis, demonstrated to be involved in modulation of sperm-surface receptor at the time of fertilization (Rodriguez-Jimenez et al. 2003, Tollner et al. 2004).

MEF3 was lost from the anterior head and flagellum and was confined to posterior head and midpiece on capacitated and acrosome-reacted human spermatozoa in immunofluorescence study. This strongly suggests that MEF3 is involved in the sperm–egg interaction. The redistribution of sperm proteins after capacitation and acrosome reaction has been reported in animals (Myles et al. 1987) and humans (Boue et al. 1996, Cohen et al. 2001, Lasserre et al. 2003). Whether changes in relocalization of 33 kDa were the result of protein migration or of removal from the anterior head and major part of flagellum remains to be determined.

MEF3 seemed to have an involvement in fertilization as evidenced by the inhibition of in vivo fertility (100%) of female albino rabbits immunized with this protein. These rabbits produced no offspring when they were mated with coeval males of proven fertility when compared with control animals (Table 1). A similar approach involving the in vivo inhibition of fertilization by active or passive immunization has been used previously (Berube & Sullivan 1994, Srivastav 2000, O’Rand et al. 2004, Srivastav et al. 2004, Dube et al. 2005, Ellerman et al. 2006). These results were confirmed by a subsequent observation that the anti-33 kDa serum significantly decreased the number of zona-free hamster oocytes penetrated with capacitated and acrosome-reacted human spermatozoa. The possible mechanism of inhibition of both in vivo fertilization and in vitro human sperm penetration in the zona-free hamster eggs could be due to sperm agglutination as demonstrated for human spermatozoa pretreated with the 33 kDa antiserum in mSA assay. These observations confirm the involvement of the 33 kDa glycoprotein in sperm function and fertility as reported for other proteins (Boue et al. 1994, Mandal et al. 2003). However, the specific roles of 33 kDa glycoprotein in sperm–egg interaction need to be examined in greater detail.

The results of the present study showed that the 33 kDa glycoprotein is a conserved antigen in monkey and human spermatozoa as evidenced by the cross-reactivity of anti-33 kDa serum with Triton X-100-extracted human spermatozoa on immunoblots and immunofluorescent localization of this protein over the entire surface of human spermatozoa, suggesting the existence of antigenically related components in both species. Similar proteins common to macaque and human spermatozoa have been reported by other investigators. These include ESC42 (Liu et al. 2001), HE1/NPC2 (Kirchhoff 1999), FLB1 (Boue et al. 1995), sperm agglutination antigen-1 (SAGA-1), and CD52 (Diedman et al. 1997, McCauley et al. 2002). However, the epididymal-specific expression of HE1/NPC2, ESC42, ESP13.2, SAGA-1, and CD52 as identified and sequenced by subtractive epididymal cDNA library screening greatly contrasts with the expression of the 33 kDa glycoprotein of rhesus epididymis, which is first expressed at the testicular level on the surface of spermatocytes, spermatids, and testicular spermatozoa.

Several epididymal proteins of testicular origin showing significant similarity to MEF3 are reported in primates. P31m, a 31 kDa protein of cynomolgus macaque sperm, is acquired by maturing spermatozoa from corpus epididymis. Northern blot analysis of P31m mRNA distribution revealed that the transcript was mainly expressed in the epididymis and at a lower level in the testis and was undetectable on testicular spermatozoa (Lamontagne et al. 2001). This greatly contrasts with the 33 kDa glycoprotein, which is strongly expressed at the testicular level over the entire surface of spermatozoa. TPX1/CRISP2, a human testicular protein localized to specific regions in the spermatozoa, is reported to be involved in mouse sperm–egg fusion (O’Bryan et al. 2001, Busso et al. 2007). The intra-acrosomal localization of protein in fresh sperm and lack of glycosylation discriminates TPX1/CRISP2 from MEF3. Another human protein – designated ARP (AEG-related protein)/CRISP1, an epididymal secretory glycoprotein
of 30 kDa – binds to the post-acrosomal region of the sperm head and participates in sperm–egg fusion. However, the epididymis-specific secretion of ARP/CRISP1 and lack of cross-reaction of anti-ARP/CRISP1 with testicular cytosol (Cohen et al. 2001) precludes the possibility of both 33 kDa and ARP/CRISP1 as homologous proteins. Epididymal protease inhibitor (EPPIN), an androgen-regulated sperm-binding protein, is expressed specifically in the testis and epididymis and is bound to the ejaculated spermatozoa. Western blots of extracts of human caput and corpus epididymal tissue and washed ejaculate spermatozoa demonstrate that EPPIN occurs predominantly as a dimer (36–46 kDa); however, both monomer (18–23 kDa) and multimer forms are present (Richardson et al. 2001, O’Rand et al. 2004). The finding that MEF3 is a monomer as compared with EPPIN, which is a dimer, and that the molecular masses of MEF3 on Western blots recognized by lectins and on immunoblot (33 kDa) recognized by anti-33 kDa serum is slightly different from the molecular mass reported for EPPIN (36–46 kDa) differentiates MEF3 from EPPIN.

In conclusion, the present study has provided data on glycosylation status during epididymal passage, sperm association, and significance in fertility of a 33 kDa glycoprotein (MEF3), a protein common to macaque and human spermatozoa. The 100% inhibition of fertility of immunized female rabbits with this protein in vivo and inhibition of human sperm penetration in the zona-free hamster eggs in vitro suggests the functional significance of MEF3 in fertility. This is further supported by our observation that MEF3 is poorly expressed in patients suffering from idiopathic infertility (Chandra et al. 2008). Together, these results clearly indicate that MEF3 has potential significance as a target for antibodies that inhibit sperm function and fertility.

**Materials and Methods**

**Animal ethics**

The studies were conducted using adult male rhesus monkeys (8–10 kg) of the Institute’s primate colony with the approval of the ‘Institutional Animal Ethics Committee for Animal Care and Usage’. The experiment was conducted in two consecutive breeding seasons of monkeys (September to March) and three monkeys per breeding season were used. Adult female albino rabbits weighing 2.5–3.0 kg from the Institute’s Animal Resource Central facility were used for raising antisera with the approval of the Institutional Animal Ethics Committee for Animals. The rabbits were housed separately under uniform air-conditioned facilities (24 ± 1 °C).

Human semen samples were obtained from healthy, fertile men with normal sperm count, motility, and viability according to the ‘WHO laboratory manual for the examination of human semen–cervical mucus interaction’ (WHO 1999) with the prior consent of the donors from King George Medical University, Lucknow.

**Biological materials**

**Collection of epididymal fluids (EFs) and spermatozoa from monkey epididymis**

The monkeys were anesthetized by sodium thiopentone, i.v. injection (Intraval; May and Baker, Bombay, India) and were subjected to retrograde perfusion with PBS via the testicular artery to clear the epididymides of blood. The epididymis was divided in five segments viz., initial segment (Is), proximal caput (Pr Ca), distal caput (Ds Ca), corpus (Cs), and cauda (Cd) epididymides to collect epididymal fluids (EFs) and epididymal spermatozoa as described previously (Srivastav et al. 2004). Each epididymal region was dissected in Tyrode’s solution (pH 7.6) and the fluid was collected by retrograde perfusion with Tyrode’s solution. The cloudy suspension was filtered through a cell dissociation sieve (Sigma) to remove tissue debris. The filtrate was centrifuged (500 g, 10 min) and the pellets containing spermatozoa were utilized for sperm membrane isolation. The supernatant fluid containing few spermatozoa was centrifuged (100 000 g, 20 min) at 4 °C. The clear supernatants representing epididymal fluids (EFs) were dialyzed, lyophilized, and preserved at −20 °C till further use.

**Sperm membrane isolation**

Purified sperm plasma membranes were isolated as described previously (Srivastav 2000) with minor modifications. The sperm pellets, resuspended in ice-cold TNI buffer (25 mmol Tris–HCl/l (pH 7.6), 150 mmol sodium chloride per l, 2.5 mmol benzamidine hydrochloride per l, 1 µg leupeptine per ml, 1 µg pepstatin per ml, and 0.05% (w/v) sodium azide), were disrupted by nitrogen cavitation at 4 °C, at a pressure of 3450 kPa and an equilibration period of 10 min. The cavitated sperm suspension was centrifuged at 500 g for 15 min and aliquots of the supernatant fluid containing the released plasma membranes were centrifuged onto a sucrose cushion consisting of 2 ml of 15% (w/v) and 50% (w/v) sucrose in 20 mmol Tris–HCl per l (pH 7.6) at 100 000 g for 60 min. The plasma membrane band at the 15:50% interface was centrifuged at 100 000 g for 60 min and the resultant pellets were utilized for estimation of protein (Bradford 1976) and for SDS-PAGE.

**Isolation of spermatids and spermatocytes**

Testicular tissue was washed twice with Dulbecco’s PBS (D-PBS) and minced in Ca2+–Mg2+-free PBS, followed by repeated pipetting. The cells were treated for 3 min with 0.2 mg/ml Pronase in Dulbecco’s modified Eagle’s medium (DMEM) and centrifuged at 400 g for 5 min after being filtered through a 20 μm nylon mesh to remove cell aggregates and tissue debris. The cell pellet was washed twice with D-PBS by centrifugation at 200 g for 5 min and then on a discontinuous Percoll gradient (45, 40, 35, and 20%) in DMEM. After centrifugation (650 g, 25 min), the cell populations recovered in the Percoll fractions were washed twice with DMEM supplemented with 10% fetal bovine serum and observed at 400× using an Olympus microscope.
microscope (BX 60) equipped with a digital imaging camera (DP-12) with the aid of the Micro Image Lite software 4.0 (Olympus, Tokyo, Japan). Spermatid-enriched cells were observed from each of the Percoll fractions by morphological characteristics and counted for cell populations using hemocytometer. Microscopic observation showed that 40% of cells collected from 35% Percoll gradient were round spermatids, whereas in 40% Percoll gradient, mostly primary spermatocytes were observed.

**Electrophoresis (SDS-PAGE) and lectin blotting**

The epididymal fluids and sperm plasma membrane proteins were fractionated under reducing condition on 12% polyacrylamide gels (Laemmli 1970) and the gels were electrophoretically transferred to nitrocellulose sheets (Towbin et al. 1979) for lectin blotting (Srivastava & Olson 1991). The glycosylation status of the 33 kDa protein was evidenced on western blots of epididymal fluids and sperm membranes from five segments of epididymis using biotinylated lectins as specific surface probes as described earlier (Srivastava et al. 2004). To identify asparagine-linked (N-linked) glycoproteins, lectins LCA, WGA, and TGP were used. LCA binds \( \alpha-D\)-mannosyl \( (\alpha-D-Man) \) and glucosyl residues present in hybrid and high-mannose N-linked oligosaccharides; WGA exhibits affinity for sialylated terminal N-acetylglucosamine linkage \([\beta-(1-4)-\text{Glc. N-Ac}] 2 \text{ Neu Ac}\) with particular high affinity for GlcN-ac trisaccharide-linked \( \beta-1-4 \) linkages; and TGP shows specificity towards \( \alpha-D\)-fucose residues present on N-linked oligosaccharides. To identify serine- and threonine-bound O-linked glycoproteins, lectins PNA and RCA were used. PNA binds preferentially to a commonly occurring structure containing sialylated galactose \( N-ac\)-galactosamine linkage \([\beta-(1-3,4)-\text{Glc. N-Ac} 2 \text{ Neu Ac}] \) with particular high affinity for GlcN-ac triosaccharide-linked \( \beta 1-4 \) linkages; and TGP shows specificity towards \( \alpha-D\)-fucose residues present on N-linked oligosaccharides. Briefly, blots were probed with specific biotinylated lectins (10 \( \mu g/ml \)) after blocking nonspecific protein-binding sites with Tris-buffered saline and 1% (w/v) BSA. Blots were then incubated with Vectastain ABC reagent (Vector Laboratories Inc., Burlingame, CA, USA) for 2 h containing 0.1% Tween-20 (v/v) and the lectin-staining bands were identified using diaminobenzidine (DAB) substrate. The specificity of lectin staining was confirmed as described earlier (Srivastava 2000).

**Purification of 33 kDa by affinity chromatography and antibody production**

The 33 kDa protein was purified from CEF by single-step purification using LCA-affinity chromatography. Lyophilized CEF was reconstituted in 2 ml TNI buffer containing 0.1% Triton X-100 (pH 7.6) and was applied to a column (0.6 \( \times \) 1.5 cm) of LCA-agarose. The column was washed with 10 ml TNI buffer containing 0.1% (v/v) Triton X-100. The LCA-binding glycoproteins were eluted with 10 ml of the same buffer containing 100 mM methyl \( \alpha-D\)-mannopyranoside. The eluent containing LCA-binding glycoproteins was desalted on PD-10 column (Pharmacia), lyophilized, and was subjected to SDS-PAGE. Ten SDS-PAGE gels were run, transferred onto nitrocellulose membranes, and one lane from each blot was probed with lectin LCA. The horizontal strips corresponding to the 33 kDa band were excised from the remaining unstained blots, dissolved in 0.5 ml DMSO, and used for raising antiserum in female albino rabbits as described by Knudsen (1985). The animals were immunized initially with half of this preparation (0.25 ml) emulsified with Complete Freund’s adjuvant injected into multiple s.c. sites around the neck and shoulders, followed by injections with another half (0.25 ml) emulsified with Incomplete Freund’s adjuvant as two boosters at 15-day intervals as described earlier (Srivastava 2000). A control batch of rabbits was injected with plain nitrocellulose strip in DMSO in the same way. Commencing 4 weeks after the booster, the animals were bled from the ear vein at weekly intervals and the serum was separated by centrifugation and stored at \(-20^\circ C\). The animals were bled before immunization for preimmune sera.

The serum was titered using ELISA. In ELISA, dilutions of CEF (2–100 \( \mu g/ml \)) were coated onto a flat-bottomed 96-well microtiter plate overnight at 4°C. Nonspecific binding sites were blocked by incubating the wells in blocker solution (PBS–1% BSA) for 1 h at 36°C. The wells were incubated with 100 \( \mu l \) immune serum at various dilutions between 1:100 and 1:12 800 in PBS/BSA/Tween-20 for 2 h at 36°C. The wells were incubated with HRP-conjugated secondary antibody (goat anti-rabbit IgG) at 1:2000 for 2 h at 36°C. The immunoreactivity was visualized using 1 mM of 2,2'-azino-bis-(3-ethylbenzthiazoline sulfonic acid) substrate. Scanning the plate at 405 nm in a Bio-Rad Benchmark Microplate Reader quantitated the reaction.

**Mating experiments**

Two weeks after the last injection, the control (n=6) and the test group of immunized female rabbits (n=6) were mated with normal fertile males. The mated females were allowed to undergo full-term gestation period and the number of live births were recorded 30 days post mating.

**Immunoblotting**

The specificity and cross-reactivity of antiserum generated against CEF 33 kDa protein were analyzed by immunoblotting. Blots containing lanes of CEF, caudal epididymal sperm membrane, and human sperm Triton X-100 extract (2% for 30 min at 4°C) were blocked overnight at 4°C in PBS/3% BSA and the membranes were incubated with immune sera at 1:100 dilution in PBS/BSA/Tween-20 for 1 h at room temperature (RT) followed by incubations in HRP-conjugated secondary antibody (goat anti-rabbit IgG) at 1:1000 dilution for 2 h at RT. After washing thrice, reactive bands were visualized using DAB substrate.

**Immunofluorescent localization**

Indirect immunofluorescence studies were performed using permeabilized spermatozoa from testis and epididymis (initial segment, proximal caput, distal caput, corpus, and cauda) of the rhesus monkey. The spermatozoa were fixed in 2% (w/v) paraformaldehyde in PBS for 30 min, washed in PBS, air-dried, and fixed onto slides precoated with poly-l-lysine. Nonspecific protein-binding sites were blocked by incubating in PBS with 3% BSA containing 10% normal goat serum (NGS) for 1 h. The slides were incubated with polyclonal antiserum against 33 kDa glycoprotein at a dilution of 1:40 in PBS/BSA/NGS for 2 h followed by incubation in secondary fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG for 1 h at RT. The slides

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were washed in PBS and covered with an antifade solution containing DABCO (Sigma). They were visualized and fluorescence images were recorded using a BX 60 Olympus microscope equipped with a digital imaging camera (DP-12) with the aid of the Micro Image Lite software 4.0 (Olympus).

**Induction of capacitation and acrosome reaction**

The swim-up method was used to isolate highly motile human spermatozoa. Briefly, semen was diluted with the human tubal fluid medium containing 0.5% BSA in a 1:2 ratio (Irvine Scientific, Santa Ana, CA, USA) and centrifuged at 400 g for 10 min. The supernatant was then carefully discarded, until about 1 ml fluid was left (for sperm counting), and the tube was placed in a 36 °C incubator for 30 min. During incubation, the motile sperm migrated into the supernatant, while the immotile and sluggish sperm and the particulate matter of semen remained at the bottom. After the incubation period, the top 0.5 ml of the supernatant, which is enriched in the motile sperm, was withdrawn carefully. Highly motile human sperm were diluted with Ham’s F-10 media to 4 × 10⁷ cells/ml and after washing were allowed to capacitate overnight in 50 μl drops of Biggers–Whitten-Whittingham medium containing 30 mg/ml human serum albumin at a concentration of 20 × 10⁶ sperm/ml.

Calcium ionophore A23187 (Sigma Chemical Co.) stock solution was prepared as 5 mmol/l stock in DMSO. Aliquots of 50 μl were frozen at −20 °C. Before use this was thawed, diluted 1:10 with PBS, and added to the sperm giving a final ionophore concentration of 10 μmol/l. For each ionophore challenge, 20 μl sperm solution was diluted with PBS to give a volume of 500 μl. A total of 10 μl A23187-diluted stock was added to it (10 μmol/l final concentration) and the tube was incubated at 37 °C. After 30 min, it was centrifuged at 600 g for 12 min. The supernatant with PBS was removed and 20 μl sperm pellet was obtained. The acrosome reaction was confirmed by FITC-PSA staining.

**Sperm penetration assay of zona-free hamster eggs**

The sperm penetration assay of the zona-free hamster eggs was carried as described by Mandal et al. (2003) with minor modifications. Golden Syrian hamsters were superovulated with i.p. injections of 30 IU equine chorionic gonadotropin (eCG) followed by 30 IU human chorionic gonadotropin (hCG) after 72 h. Oviducts were flushed with swim-up medium following 14–16 h of hCG injection and cumulus–oocyte complexes were isolated. Cumulus cells were removed by treating the complex with hyaluronidase (1 mg/ml, Sigma) for 12 min. The supernatant was then carefully discarded, until about 1 ml fluid was left (for sperm counting), and the tube was placed in a 36 °C incubator for 30 min. During incubation, the motile sperm migrated into the supernatant, while the immotile and sluggish sperm and the particulate matter of semen remained at the bottom. After the incubation period, the top 0.5 ml of the supernatant, which is enriched in the motile sperm, was withdrawn carefully. Highly motile human sperm were diluted with Ham’s F-10 media to 4 × 10⁷ cells/ml and after washing were allowed to capacitate overnight in 50 μl drops of Biggers–Whitten-Whittingham medium containing 30 mg/ml human serum albumin at a concentration of 20 × 10⁶ sperm/ml.

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**Statistical analysis**

The mean ± s.d. of the data of densitometer scanning between the experiments was determined by Microsoft Excel.

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