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

Abhishek Chandra, Kokattam Rama Srinivasan, Farrukh Jamal¹, Puroshottam Kumar Mehrotra, Ram Lakhan Singh¹ and Archana Srivastav

Division of Endocrinology, Central Drug Research Institute, PO Box 173, Lucknow 226001, India and ¹Department of Biochemistry, Dr R M L Avadh University, Faizabad 224001, India

Correspondence should be addressed to A Srivastav; Email: archana1849@gmail.com

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 with specific biotinylated lectins. Polyclonal antiserum raised against affinity-purified MEF3 from caudal epididymal fluid (CEF) cross-reacted 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.

Reproduction (2008) 135 761–770

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,

Figure 1 Densitometric analysis of blots probed with lectins (a) LCA and (b) RCA of epididymal fluids from initial segment (lane 1), proximal caput (lane 2), distal caput (lane 3), corpus (lane 4), and cauda (lane 5) epididymides exhibiting the highest glycosylation for these oligosaccharides in proximal caput and the lowest staining in caudal fluid. Fifty micrograms of total protein were loaded in each lane. Error bars represent s.d. ($N=6$).

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

sera of immunized animals. The Western blots of caudal luminal fluid, caudal spermatozoa of monkey epididymis, and a lane containing human sperm Triton X-100 extracts were probed with this antiserum at 1:100 dilution.

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) epididymides 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).

Figure 3 Immunoblot of caudal epididymal fluid (lane 2), caudal sperm membrane (lane 3), and Triton X-100 human sperm extract (lane 4) probed with 33 kDa antiserum. Lane 1 shows control caudal epididymal fluid probed with preimmune serum and lane 5 shows caudal epididymal fluid probed with lectin LCA. The immune serum cross-reacted specifically with a 33 kDa band in CEF and the caudal sperm membrane and with the Triton X-100 extract of human spermatozoa. Fifty micrograms of total protein were loaded in each lane.

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

Figure 4 Immunofluorescent localization (phase contrast and fluorescence 400X) of 33 kDa protein on (a and b) testicular spermatozoa, (c and d) spermatocytes, and (e and f) spermatids from monkey testis showing that the protein is first expressed in the testis. Scale bar represents 600 μM.

Figure 5 Indirect immunofluorescence localization of 33 kDa polyclonal antiserum (phase contrast and fluorescence 400X) on (a and b) intact, (c and d) capacitated and acrosome-reacted human spermatozoa showing that MEF3 is lost from the sperm flagellum and anterior head when spermatozoa undergo capacitation and acrosome reaction. Scale bar represents 600 μM.

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 (mSA) 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

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.

Groups	No. of animals (n)	Mean litter size*
Control	6	Three males Four females
Immunized	6	No litters

*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 the CEF suggested a loss/alteration of the protein itself and/or deglycosylation/masking of exposed α -D-mannose, and galactose, N-Ac-D-galactosamine (Gal, NAc β -gal) groups found in O-linked oligosaccharides.

Interestingly, the appearance of a faint band of 33 kDa on more mature corpus and caudal spermatozoa as compared with spermatozoa from the initial segment

and caput epididymis on blots probed with lectins WGA (sialylated N-Ac-glucosaminylated residues), PNA (sialylated N-Ac-galactosamine residues), and TGP (fucosylated residues) suggested this protein to be a sperm maturation-dependent glycoprotein. This could indicate either an increase in the expression of the protein itself or an increase in the expression of sialylated units/N-Ac-glucosaminylated/N-Ac-galactosaminylated/fucosylated residues on the protein exterior. Another possibility could be the exposure of the masked components on 33 kDa glycoprotein on membrane exterior of corpus and caudal spermatozoa.

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.

Experiments	Immune-treated			Control (preimmune-treated)		
	Number of eggs	Number penetrated	Percentage	Number of eggs	Number penetrated	Percentage
1	15	2	6	21	17	80.9
2	21	4	19	19	15	78.9
3	24	7	29	27	25	92.5
4	17	2	11.7	15	11	73.3
5	33	9	27	18	16	88.8

Figure 6 Phase contrast photograph of (a) control human spermatozoa, pre-incubated with normal rabbit serum showing no sperm agglutination and (b) showing a tangled agglutination pattern of human spermatozoa with 33 kDa (MEF3) antiserum at 1:1280 (100× magnification).

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 (Diekman *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 antiserum with the approval of the Institutional Animal Ethics Committee for Animals. The rabbits were housed separately under uniform air-conditioned facilities ($24 \pm 1^\circ\text{C}$).

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 Ca^{2+} – Mg^{2+} -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 $\times$ using an Olympus

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 (Srivastav *et al.* 2004). To identify asparagine-linked (N-linked) glycoproteins, lectins LCA, WGA, and TGP were used. LCA binds α -D-mannosyl (α -D-Man) and glucosyl residues present in hybrid and high-mannose N-linked oligosaccharides; WGA exhibits affinity for sialylated terminal N-Ac-D-glucosamine linkage [β -(1-4)-D-Glc. N-Ac] 2 Neu Ac] with particular high affinity for GlcN-Ac trisaccharide-linked β 1-4 linkages; and TGP shows specificity towards α -L-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 [β -1,3, Gal N-Ac] linkages, while RCA binds desialylated galactose, N-Ac-galactosamine (Gal, N-Ac β -gal) groups found in the O-linked oligosaccharides of glycoproteins. Briefly, blots were probed with specific biotinylated lectins (10 μ 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 (Srivastav 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 α -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 (Srivastav 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°C . The animals were bled before immunization for preimmune sera.

The serum was titered using ELISA. In ELISA, dilutions of CEF (2–100 μ 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 μ 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

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 4×10^7 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 20×10^6 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 3 min, and the pooled oocytes were washed by passing the eggs through 20 µl drops of media under mineral oil using pulled and heat-polished Pasteur pipettes. Zona pellucidae were removed by treatment with trypsin (1 mg/ml, Sigma) for 30 s and washed five times. The eggs were then randomly allotted into two groups. Capacitated and acrosome-reacted human sperm (2×10^6 /ml) were incubated with pre- or post-immune sera (de-complemented for 30 min at 56 °C; dilution, 1:10) for 1 h. The zona-free hamster eggs were added to this mix (10 eggs/20 µl droplet) and co-incubated for 3 h at 37 °C with 5% CO₂ under paraffin oil. The eggs were washed five times to remove loosely bound sperm by transferring through 50 µl drops. The washed eggs were stained with 1 mmol

acridine orange in 3% DMSO (Sigma) in capacitation media for 15 s to stain the chromatin and were washed four times in 50 µl droplets. The oocytes were washed once, mounted onto slides, and then examined for evidence of sperm penetration under phase contrast at 400× 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). An average of between 15 and 20 oocytes were examined in each experiment. The results were expressed as the number of eggs penetrated by the sperm. All experiments were repeated five times with five different donors.

mSA antibody assay

Highly motile human sperm were collected by swim-up method as described above. Sperm suspensions (20 µl) were mixed with 20 µl undiluted and diluted antiserum between 1:10 and 1:1280 in flat bottomed 96-well trays. A parallel row of control wells contained NRS with equivalent amount of sperm suspensions. The plates were incubated for 1 h at 37 °C. Aliquots were examined using phase microscopic optics 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). The agglutination pattern was recorded according to the criteria previously defined by Rose *et al.* (1976) to reflect head-to-head, tail-to-tail, tail tip-to-tail tip, mixed, and tangled agglutination.

Statistical analysis

The mean $\pm$ S.D. of the data of densitometer scanning between the experiments was determined by Microsoft Excel.

Acknowledgements

The authors thank Dr C M Gupta, Director, CDRI for taking interest in the study. The authors express their gratitude to the World Health Organization, Geneva, Switzerland (Re-Entry Research Grant No. 92006), Ministry of Health (MOH), Council of Scientific and Industrial Research (CSIR), and the Indian Council of Medical Research (ICMR), Government of India for financial support. They also thank Mr Nabbu Lal, Mr R G Pandey, and Mr Chhatrapal for their expert assistance in handling the monkeys. A portion of this paper was presented in 14th Annual Meeting of the Indian Society for the Study of Reproduction and Fertility (ISSRF) held at Indian Institute of Science, Bangalore, India, 23–25 January 2004. (CDRI Communication No: 7263.) The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

- Benoff S 1997 Carbohydrates and fertilization: an overview. *Molecular Human Reproduction* **3** 599–637.
- Berube B & Sullivan R 1994 Inhibition of *in vivo* fertilization by active immunization of male hamsters against a 26-kDa sperm glycoprotein. *Biology of Reproduction* **51** 1255–1263.
- Boue F, Berube B, De Lamirande E, Gagnon C & Sullivan R 1994 Human sperm-zona pellucida interaction is inhibited by an antiserum against a hamster sperm protein. *Biology of Reproduction* **51** 577–587.

- Boue F, Duquenne C, Lassalle B, Lefeure A & Finaz C 1995 FLB1, a human protein of epididymal origin that is involved in the sperm-oocyte recognition process. *Biology of Reproduction* **52** 267–278.
- Boue F, Blais J & Sullivan R 1996 Surface localization of P34H, an epididymal protein, during maturation, capacitation, and acrosome reaction of human spermatozoa. *Biology of Reproduction* **54** 1009–1017.
- Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principal of protein dye-binding. *Analytical Biochemistry* **72** 248–254.
- Busso D, Cohen DJ, Hayashi M, Kasahara M & Cuasnicú PS 2005 Human testicular protein TPX1/CRISP-2: localization in spermatozoa, fate after capacitation and relevance for gamete interaction. *Molecular Human Reproduction* **11** 299–305.
- Busso D, Goldweic NM, Hayashi M, Kasahara M & Cuasnicú PS 2007 Evidence for the involvement of testicular protein CRISP2 in mouse sperm-egg fusion. *Biology of Reproduction* **76** 701–708.
- Chandra A, Jamal F, Singh M, Pande A, Das V, Singh RL & Srivastav A 2008 Significance of a fertility inhibiting 33kDa (MEF3) glycoprotein of rhesus monkey (*Macaca mulatta*) in human infertility. *Journal of Ecophysiology and Occupational Health* [in press].
- Cohen DJ, Ellerman DA, Busso D, Morgenfeld M, Piazza A, Hayashi M, Young ET, Kasahara M & Cuasnicu PS 2001 Evidence that human epididymal protein ARP plays a role in gamete fusion through complementary sites on the surface of the human egg. *Biology of Reproduction* **65** 1000–1005.
- Cooper TG 1998 Interaction between epididymal secretions and spermatozoa. *Journal of Reproduction and Fertility* **53** 119–131.
- Dacheux JL, Gatti JL & Dacheux F 2003 Contribution of epididymal secretory proteins for spermatozoa. *Microscopy Research and Technique* **61** 7–17.
- Diekman AB, Westbrook-Case VA, Naaby-Hansen S, Klotz KL, Flickinger CJ & Herr JC 1997 Biochemical characterization of sperm agglutination antigen-1, a human sperm surface antigen implicated in gamete interactions. *Biology of Reproduction* **57** 1136–1144.
- Dube E, Legare C, Gaudreault C & Sullivan R 2005 Contraceptive responses of female hamsters immunized with recombinant sperm protein P26h. *Contraception* **72** 459–467.
- Ellerman DA, Cohen DJ, Da Ros VG, Morgenfeld MM, Busso D & Cuasnicu PS 2006 Potential contraceptive use of epididymal proteins: immunization of male rats with epididymal protein DE inhibits sperm fusion ability. *Biology of Reproduction* **59** 1029–1036.
- Focarelli R, Giuffrida A, Capparelli S, Scibona M, Fabris FM, Francavilla F, Francavilla S, Giovampola CD & Rosati F 1998 Specific localization in the equatorial region of gp20, a 20 kDa sialylglycoprotein of the capacitated human spermatozoon acquired during epididymal transit which is necessary to penetrate zona-free hamster eggs. *Molecular Human Reproduction* **4** 119–125.
- Gaudreault C, Alfí ME, Legare C & Sullivan R 2001 Expression of the hamster sperm protein P26h during spermatogenesis. *Biology of Reproduction* **5** 79–86.
- Jalkanen J, Huhtaniemi I & Poutanen M 2005 Mouse cysteine-rich secretory protein 4 (CRISP 4): a member of the Crisp family exclusively expressed in the epididymis in an androgen dependent manner. *Biology of Reproduction* **72** 1268–1274.
- Jones R 2004 Sperm survival versus degradation in the mammalian epididymis: a hypothesis. *Biology of Reproduction* **71** 1405–1411.
- Kirchhoff C 1999 Gene expression in the epididymis. *International Review of Cytology* **188** 133–202.
- Kirchhoff C, Osterhoff C, Pera I & Schröter S 1998 Function of human epididymal proteins in sperm maturation. *Andrologia* **30** 225–232.
- Knudsen KA 1985 Proteins transferred to nitrocellulose for use in immunogens. *Analytical Biochemistry* **147** 285–288.
- Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **227** 680–685.
- Lamontagne N, Legare C, Gaudreault C & Sullivan R 2001 Identification and characterization of P31m, a novel sperm protein in Cynomolgus monkey (*Macaca fascicularis*). *Molecular Reproduction and Development* **59** 431–441.
- Lasserre A, Gonzalez-Echeverria F, Moules C, Tezon JG, Miranda PV & Vazquez-Levin MH 2003 Identification of human sperm proteins involved in the interaction with homologous zona pellucida. *Fertility and Sterility* **79** 1606–1615.
- Liu HW, Lin YC, Chao CF, Chang SY & Sun GH 2000 GP-83 and GP-39, two glycoproteins secreted by human epididymis are conjugated to spermatozoa during maturation. *Molecular Human Reproduction* **6** 422–428.
- Liu Q, Hamil KG, Sivashanmugam P, Grossman G, Soundararajan R, Rao AJ, Richardson RT, Zhang YL, O'Rand MG, Petrusz P *et al.* 2001 Primate epididymis specific proteins: characterization of ESC42, a novel protein containing a trefoil-like motif in monkey and human. *Endocrinology* **142** 4529–4539.
- Mandal A, Klotz KL, Shetty J, Jayes FL, Wolkowicz MJ, Bolling LC, Coonrod SA, Black MB, Diekman AB, Haystead TAJ *et al.* 2003 SLLP1, a unique, intra-acrosomal, non-bacteriolytic, c lysozyme-like protein of human spermatozoa. *Biology of Reproduction* **68** 1525–1537.
- McCauley TC, Kurth BE, Norton EJ, Klotz KL, Westbrook A, Rao AJ, Herr JC & Diekman HB 2002 Analysis of a human sperm CD 52 glycoform in primates: identification of an animal model for immunocontraceptive vaccine development. *Biology of Reproduction* **66** 1681–1688.
- Myles DG, Hyatt H & Primakoff P 1987 Binding of both acrosome-intact and acrosome-reacted guinea pig sperm to the zona pellucida during *in vitro* fertilization. *Developmental Biology* **121** 559–567.
- Nolan MA, Wu L, Bang HJ, Jelinsky SA, Roberts KP, Turner TT, Kopf GS & Johnston DS 2006 Identification of rat cysteine-rich secretory protein 4 (Crisp 4) as the ortholog to human CRISP1 and mouse Crisp 4. *Biology of Reproduction* **74** 984–991.
- O'Bryan MK, Sebire K, Meinhardt A, Edgar K, Keah H, Hearn MT & De Kretser DM 2001 Tpx-1 is a component of the outer dense fibers and acosome of rat spermatozoa. *Molecular Reproduction and Development* **58** 116–125.
- O'Rand MG, Widgren EE, Sivashanmugam P, Richardson RT, Hall SH, French FS, VandeVoort CA, Ramachandra SG, Ramesh V & Jagannadha Rao A 2004 Reversible immunocontraception in male monkeys immunized with eppin. *Science* **306** 1189.
- Richardson RT, Sivashanmugam P, Hall SH, Hamil KG, Moore PA, Ruben SM, French FS & O'Rand M 2001 Cloning and sequencing of human Eppin: a novel family of protease inhibitors expressed in the epididymis and testis. *Gene* **270** 93–102.
- Rodriguez-Jimenez F, Krause A, Schilz S, Forssmann WG, Conejo-Garcia JR, Schreeb R & Motzkus D 2003 Distribution of new human beta-defensin genes clustered on chromosome 20 in functionally different segments of epididymis. *Genomics* **81** 175–183.
- Rose NR, Hjort T, Rumke P, Harper MJK & Vyazov OP 1976 Techniques for detection of iso- and autoantibodies to human spermatozoa. *Clinical and Experimental Immunology* **23** 175–199.
- Srivastav A 2000 Maturation-dependent glycoproteins containing both N- and O-linked oligosaccharides in epididymal sperm plasma membrane of rhesus monkey (*Macaca mulatta*). *Journal of Reproduction and Fertility* **119** 241–252.
- Srivastav A, Singh B, Chandra A, Jamal F, Khan MY & Chowdhary SR 2004 Partial characterization, sperm association and significance of N- and O-linked glycoproteins in epididymal fluid of rhesus monkey (*Macaca mulatta*). *Reproduction* **127** 343–357.
- Srivastava A & Olson GE 1991 Glycoprotein changes in the rat sperm plasma membrane during maturation in the epididymis. *Molecular Reproduction and Development* **29** 357–364.
- Tollner TL, Yudin AI, Treece CA, Overstreet JW & Cherr GN 2004 Macaque sperm release ESP13.2 and PSP94 during capacitation: the absence of ESP13.2 is linked to sperm-zona recognition and binding. *Molecular Reproduction and Development* **69** 325–327.
- Towbin H, Staehlin T & Gordon J 1979 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *PNAS* **76** 4350–4354.
- Tulsiani DRP 2006 Glycan-modifying enzymes in luminal fluid of the mammalian epididymis: an overview of their potential role in sperm maturation. *Molecular and Cellular Endocrinology* **250** 58–65.
- WHO 1999 *WHO Laboratory Manual for the Examination of Human Semen-Cervical Mucus Interaction*. Cambridge: Cambridge University Press.

Received 21 November 2007

First decision 21 January 2008

Revised manuscript received 26 February 2008

Accepted 7 March 2008