Partial characterization, sperm association and significance of N- and O-linked glycoproteins in epididymal fluid of rhesus monkeys (*Macaca mulatta*)

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Abstract

The present study investigated regional modifications of glycosylation status, sperm association and functional significance of N- and O-linked glycoproteins in epididymal luminal fluid of the rhesus monkey (*Macaca mulatta*). The predominant glycoproteins of the epididymal luminal fluid that increase in the extent of glycosylation or unmasking of exposed epitopes in a region-specific, maturation-dependent manner, included those of 150, 116, 68, 64, 58 (N- and O-linked) and 170 kDa (O-linked). The higher expression of 40 (N-linked), 38 (N- and O-linked) and 60, 56 and 33 kDa (O-linked) glycoproteins in the proximal caput epididymal fluid was followed by alteration or reorganization of 60, 38 and 33 kDa (O-linked) glycoproteins in the distal segments of the epididymis. The association of epididymal fluid glycoproteins with maturing spermatozoa was identified by generating polyclonal antiserum against monkey caudal sperm membrane in female albino rabbits. The antiserum crossreacted strongly with 58 and 33 kDa epididymal fluid glycoproteins of monkeys and also reacted with 116, 68, 58, 56 and 33 kDa glycoproteins from Triton X-100 extracts of human spermatozoa, indicating the presence of antigenically related components in both species. The functional significance of epididymal fluid glycoproteins in sperm functions was investigated by raising antiserum against a heavily glycosylated 58 kDa glycoprotein (MEF1) of caudal epididymal fluid, which crossreacted with the Triton X-100 extracts of epididymal spermatozoa of monkey and ejaculated human spermatozoa on immunoblots. In an *in vitro* micro-sperm agglutination assay, anti-MEF1 serum agglutinated both rat caudal epididymal spermatozoa and human spermatozoa. MEF1 seemed to be involved in fertilization as demonstrated by inhibition of fertility (100%) in female albino rabbits and rats immunized with this protein. A sperm-agglutinating 58 kDa glycoprotein of rhesus monkey epididymis with functional significance in fertility was identified, thus indicating that it is a potential candidate for contraceptive vaccine development.

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Introduction

During sperm maturation, the mammalian epididymis provides a specific intraluminal environment for producing functionally mature spermatozoa, which acquire motility and fertilizing capability (reviewed by Cooper, 1998, Holland & Nixon 1998, Moore 1998). The significance of epididymal function for sperm maturation and male fertility has been highlighted recently in transgenic mice, in which a primary defect in epididymal regionalization and differentiation caused by a dominant negative mutation of retinoic acid receptor- α or disruption of c-Ros tyrosine kinase receptor gene led to male infertility (Sonnenberg-Reithmacher *et al.* 1996, Costa *et al.* 1997, Yeung *et al.* 2000a).

A number of studies have reported modifications of the sperm surface by association of proteins synthesized and secreted by distinct regions of the epididymal epithelium (Xu *et al.* 1997, Chu *et al.* 2000, Gatti *et al.* 2000). A few proteins have been described as playing significant roles in sperm-fertilizing ability, such as enabling capacitation, for example M42 antigen (Lakoski *et al.* 1988); promoting zona pellucida binding and penetration, a 95 kDa protein and P26h (Leyton & Saling 1989, Gaudreault *et al.* 2002); and promoting fusion and fertilizing the ovum, for example FLB1 (Boue *et al.* 1995).

The caput epididymis is a very active region in protein synthesis and secretion and has the highest content of several proteins with functional significance. Many of the

proteins are synthesized by proximal segments of the epididymis, associate with the sperm surface during epididymal transit and later dissociate from spermatozoa to undergo endocytosis by cells of the distal epididymal epithelium and are involved in gamete fusion. These proteins include: clusterin (Mattmueller & Hinton 1991); Crisp 1, AEG, protein D-E, protein B/C and retinoic acid-binding protein (MEP-7) (Lea *et al.* 1978, Brooks 1987, Cohen *et al.* 2000, Turner & Bomgardner 2002); rat glutathione peroxidase (GPX5) (Vernet *et al.* 1997); CRES proteins (Cornwall *et al.* 1995); and CD52 (Kirchhoff & Hale 1996, Kirchhoff *et al.* 1998, Yeung *et al.* 2000b).

Glycosylation is one of the important post-translational modifications of sperm surface proteins that occurs during epididymal sperm maturation. The role of the carbohydrate portion of glycoprotein is becoming increasingly recognized for its importance in mediating the adhesion between the mammalian spermatozoon and the zona pellucida (Loeser & Tulsiani 1999). The post-testicular modification of preformed glycosyl moieties is one of the mechanisms of sperm glycosylation during epididymal transit as demonstrated by CD52, a major maturation-associated sperm membrane antigen (Kirchhoff & Hale 1996, Kirchhoff *et al.* 1998).

Although the characterization, significance and role of epididymal glycoproteins have been studied in many primate species including human (Focarelli *et al.* 1998, Kirchhoff *et al.* 1998, Martin Ruiz *et al.* 1998, Liu *et al.* 2000, Yeung *et al.* 2000b), these are poorly understood in the epididymis of the rhesus monkey (Navneetham *et al.* 1996), an animal model commonly used for preclinical testing of drugs. Previous work from this laboratory has demonstrated maturation-dependent changes in the glycoprotein profile of purified plasma membrane of spermatozoa from rhesus monkey epididymis, and a few of these glycoproteins co-migrated with proteins present in the caudal epididymal fluid. There were marked differences in the extent of glycosylation of some proteins between the immature and mature sperm surface (Srivastav 2000). These differences prompted us to study the identification, characterization, sperm association and significance of *N*- and *O*-linked glycoproteins of epididymal luminal fluid of rhesus monkey to generate base line data in this species as well as to extrapolate these studies towards development of a male contraceptive of epididymal origin in the future.

Material and Methods

Materials

All chemicals including Tyrode's salts, leupeptin, pepstatin, benzamidine hydrochloride, phenylmethylsulphonyl fluoride, Triton X-100, biotinylated goat anti-rabbit IgG, BSA, 2,2'-azino-bis-(3-ethylbenzthiozoline sulphonic acid), Freund's complete and incomplete adjuvants, haemoglobin, Tween-20, diaminobenzidine (DAB),

endoglycosidase F (EC 3.2.1.96), endo- α -*N*-acetyl galactosaminidase (EC 3.2.1.97), nitrocellulose membranes (0.45 μ m Immobilon-NC, Sigma), biotinylated lectins, including wheat germ agglutinin (WGA), *Lens culinaris* agglutinin (LCA), *Ricinus communis* agglutinin (RCA) and peanut agglutinin (PNA) and their respective inhibitory sugars, including *N*-acetyl-D-glucosamine, α -methyl-D-mannose, β -galactose and L-fucose were purchased from Sigma Chemical Company (St Louis, MO, USA). Chemical Vectastain ABC reagent was obtained from Vector Laboratories Inc. (Burlingame, CA, USA).

Animals

Adult male rhesus monkeys (*Macaca mulatta*), 8–10 kg body weight, from the Institute's primate colony were maintained in air-conditioned rooms ($24 \pm 1^\circ\text{C}$) under uniform husbandry conditions throughout the experimental period. Approval was obtained for the use of rhesus monkeys from the Institutional Animal Ethics Committee for Animal Care and Usage before starting these studies. The monkeys were fed with a fresh fruit, vegetable and pellet diet (Ms. Ashirwad Industries, Chandigarh, India), and water was available freely. The monkeys were anaesthetized by an i.v. injection of Intraval (sodium thiopentone) obtained from May and Baker (Bombay, India) at a dose of 25 mg/ml saline and were subjected to retrograde perfusion with PBS via the testicular artery to clear the epididymides of blood. The testes were exposed and the epididymides were carefully dissected out and cleared free of fat and adhering tissues at room temperature. Each epididymis was divided into five distinct segments for collection of fluids to be included in the study: the initial segment, proximal caput, distal caput, corpus and cauda epididymides (Fig. 1).

Collection of epididymal fluids and spermatozoa

The fluids and spermatozoa from five epididymal segments were obtained as described by Srivastav (2000).

Figure 1 Rhesus monkey epididymis showing five segments: the initial segment, proximal caput, distal caput, corpus and caudal epididymides. The testis and vas deferens have been shown to correlate with the position of the epididymis.

Briefly, each region was minced in Tyrode's solution (pH 7.6) at room temperature and the mixture was gently shaken for 10–15 min to release epididymal spermatozoa and fluid. The cloudy suspension was filtered through cheese-cloth to remove pieces of tissue; the filtrate was centrifuged at 500 *g* for 10 min and the pellets containing spermatozoa were used for isolating sperm membranes. The supernatant fluid containing few spermatozoa was centrifuged at 100 000 *g* for 20 min at 4°C. The clear supernatants representing epididymal fluids were dialysed, lyophilized and preserved at –20°C until further use. In some experiments, pure luminal fluid and spermatozoa from the cauda epididymides were collected by inserting a cannula into the vas deferens and perfusing Tyrode's solution in a retrograde direction. The luminal fluid from the cauda epididymides was centrifuged at 100 000 *g* for 20 min at 4°C and was dialysed, lyophilized and preserved at –20°C until further use.

Isolation of sperm membrane

Purified sperm plasma membranes were isolated as described by Srivastav (2000) with minor modifications. The sperm pellets, resuspended in ice-cold buffer (25.0 mmol/l Tris–HCl, pH 7.5, 150.0 mmol/l sodium chloride, 2.5 mmol/l benzamidinium hydrochloride, 1.0 µg/ml leupeptin, 1.0 µg/ml pepstatin and 0.05% (w/v) sodium azide) were disrupted by nitrogen cavitation at 4°C at 3450 kPa for 10 min. The cavitated sperm suspension was then centrifuged for 15 min at 500 *g* and aliquots of the supernatant fluid containing the released plasma membranes were centrifuged at 100 000 *g* for 60 min on a sucrose cushion consisting of 2 ml 15% (w/v) and 50% (w/v) sucrose in 20 mmol/l Tris–HCl, pH 7.5. The plasma membrane band at the 15:50% interface was centrifuged at 100 000 *g* for 60 min and the resultant pellets were used for estimation of protein (Bradford 1976) and for SDS-PAGE.

SDS-PAGE

SDS-PAGE was performed under reducing conditions using both mini (9 × 8 cm) and extra-wide mini gels (9 × 16 cm) according to the method of Laemmli (1970). The epididymal fluids and sperm plasma membrane preparations were solubilized in Laemmli sample buffer at 100°C for 3 min and proteins were fractionated on 12% (v/v) polyacrylamide gels (1 mm thick) at a constant current of 2 mA per well and were cooled to 4°C. After electrophoresis, the gels were either transferred by electrophoresis on to nitrocellulose sheets (Towbin *et al.* 1979) for lectin blotting or fixed in 50% (v/v) methanol containing 10% (v/v) acetic acid for staining. Proteins were visualized by the silver staining method of Wray *et al.* (1981).

Lectin blotting

Lectin blotting was carried out as described by Srivastava & Olson (1991). Briefly, blots were probed with biotinylated specific lectins (10 µg/ml) after blocking non-specific protein-binding sites with Tris-buffered saline (TBS) containing 25 mmol/l Tris–HCl (pH 7.5), 2 mmol/l magnesium chloride, 2 mmol/l manganese chloride, 2 mmol/l calcium chloride, 150 mmol/l sodium chloride and 1% (w/v) BSA. Lectins WGA and LCA were used to identify asparagine-linked (*N*-linked) glycoproteins. WGA has an affinity for sialylated terminal *N*-acetyl-D-glucosamine linkage [β -(1–4) D-Glc. NAc]₂ Neu Ac] with particular high affinity for GlcNAc trisaccharide-linked β -1–4 linkages; LCA binds α -D-mannosyl and glucosyl residues present in hybrid and high mannose *N*-linked oligosaccharides. PNA and RCA were used to identify serine- and threonine-bound *O*-linked glycoproteins. PNA binds preferentially to a commonly occurring structure containing sialylated galactose *N*-acetyl-galactosamine [β 1,3, Gal NAc] linkages, whereas RCA binds desialylated galactose, *N*-acetyl-galactosamine (Gal, NAc- β -gal) groups found in *O*-linked oligosaccharides of glycoproteins. Lectin blots were then incubated with Vectastain ABC reagent in TBS containing 0.1% Tween-20 (v/v) for 2 h and the lectin-binding bands were identified using 0.5 mg/ml DAB, 0.02% (v/v) hydrogen peroxide and 0.03% (w/v) nickel chloride in 0.05 mol/l Tris–HCl (pH 7.5).

Digestion with endoglycosidase F (*N*-glycosidase F) and endo- α -*N*-acetyl galactosaminidase (*O*-glycosidase)

The presence of *N*- and *O*-linked glycoproteins of epididymal fluid was confirmed by digesting samples (50 µg) separately with 30 mU of *N*-glycosidase F and *O*-glycosidase, as described by Srivastav (2000). After 16 h at 37°C, additional glycosidases (20 mU) were added and incubation was carried out for an additional 8 h at 37°C. At the end of incubation, the reaction mixture was inactivated by heat treatment (60°C for 30 min) and the membrane samples were cooled to room temperature before they were subjected to SDS-PAGE and Western blot analysis. The blot containing *N*-glycosidase F-treated membrane samples was probed with lectin LCA and the blot containing *O*-glycosidase-treated samples was probed with lectin PNA (data not shown).

Antibody production

Polyclonal antiserum against sperm membrane was raised by immunizing virgin female albino rabbits with purified sperm membrane protein (100 µg in 250 µl TBS) from cauda epididymidis emulsified in an equal volume of Freund's complete adjuvant and the mixture was injected into multiple s.c. sites. Another fraction of 100 µg protein solubilized in 250 µl TBS was emulsified with an equal volume of Freund's incomplete adjuvant and was injected

as two boosters at an interval of 15 days. A control batch of rabbits was injected with normal saline in the same way.

Antiserum was raised in virgin female albino rabbits (Knudsen 1985) against a heavily glycosylated 58 kDa glycoprotein (MEF1) of caudal epididymal fluid. SDS-PAGE gels of 500 µg caudal epididymal fluid were transferred on to nitrocellulose and one lane was probed with LCA. The horizontal strip corresponding to the 58 kDa band was excised from the remaining unstained blot and was dissolved in 0.5 ml dimethyl sulphoxide (DMSO). Half of this preparation (0.25 ml) was emulsified with an equal volume of Freund's complete adjuvant and injected into multiple s.c. sites. The other half of the preparation was mixed with 0.25 ml Freund's incomplete adjuvant and was injected as two boosters at an interval of 15 days. A control batch of rabbits was injected with a nitrocellulose strip solubilized in DMSO in the same way. From 4 weeks after the booster, blood and serum samples were collected from the animals at weekly intervals. Blood samples were collected from animals before immunization for preimmune serum.

Mating experiments

Two weeks after the last injection, the control ($n = 3$) and immunized female rabbits ($n = 3$) were mated with normal fertile males. The mated females were allowed to undergo full-term gestation and numbers of live births were recorded 30 days after mating. A fresh batch of female albino rats ($n = 6$) was immunized with 58 kDa protein in a similar way to that described for rabbits to confirm the results in another species. The fertility of these animals was tested on days 10–15 after the last booster injection, and the numbers of implantation sites and corpora lutea in both control and immunized rats were observed after the laparotomy of mated females on day 14.

Antibody analysis

The antiserum generated against the 58 kDa protein from caudal epididymal fluid was analysed for immunoreactivity by both ELISA and Western blot analysis. In the ELISA assay, dilutions of caudal epididymal fluid (2–100 µg/ml in carbonate–bicarbonate buffer, pH 9.6) were coated on to a flat-bottom 96-well microtitre plate overnight at 4 °C. Non-specific 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 µl immune serum at various dilutions between 1:100 and 1:1000 in PBS–1% BSA–0.05% Tween-20 for 2 h at 36 °C. The wells were then washed as described above and incubated with a horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG) at a dilution of 1:2000 for 2 h at 36 °C. Immunoreactivity was visualized using 1 mmol/l 2,2'-azino-bis-(3-ethylbenzthiozoline sulphonic acid) and 0.03% (v/v) H₂O₂ in 100 mmol/l citrate phosphate buffer (pH 4.2). The reaction was quantified by scanning the

plate at 405 nm in a Bio-Rad Benchmark Microplate Reader.

Immunoblot analysis

Western blot analyses of epididymal fluids, caudal sperm membrane and human sperm extract were blocked overnight at 4 °C in PBS–1% BSA. Lanes containing epididymal fluid and membranes were incubated with immune serum at 1:500 dilution in PBS–1% BSA–0.1% Tween-20 for 1 h at room temperature followed by incubations in horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG) at 1:1000 dilution for 2 h at room temperature. After washing three times, reactive bands were visualized with DAB solution containing 0.5 mg DAB, 0.02% (v/v) H₂O₂ and 0.04% (w/v) nickel chloride in 0.05 mol/l Tris–HCl (pH 7.5).

Micro-sperm agglutination (mSA) antibody assay

The mSA assay with anti-58 kDa antiserum was conducted using both human and rat spermatozoa to study the functional significance of epididymal fluid glycoproteins in sperm fertility. Semen samples obtained from healthy, fertile men were evaluated for sperm count, motility and viability according to WHO (2000). Samples free of agglutination and with 40% or more live spermatozoa were used for the experiment. Motile human spermatozoa were diluted with Ham's F-10 media to 4×10^7 cells/ml. Sperm suspensions (20 µl) were mixed with 20 µl undiluted and diluted antiserum between 1:10 and 1:1280 in flat-bottom 96-well trays. A parallel row of control wells contained normal rabbit serum with equivalent amount of sperm suspensions. The plates were incubated for 1 h at 37 °C and in a parallel row, the sperm suspension was pre-incubated with primary antiserum. Aliquots were examined using phase microscopic optics on a BX-60 Olympus microscope and agglutination pattern was recorded according to the criteria defined by Rose *et al.* (1976) to reflect head-to-head, tail-to-tail, tail tip-to-tail tip, mixed and tangled agglutination. Similarly, rat caudal epididymal spermatozoa, diluted in Tyrode's solution supplemented with 0.5% BSA, were incubated with undiluted antiserum and antiserum serially diluted up to 1:1280 to confirm the results of agglutination of human spermatozoa in another species.

Results

Analysis by SDS-PAGE and silver staining of epididymal fluid from five segments of rhesus monkey epididymis revealed many polypeptides (Fig. 2). Biotinylated specific lectins WGA, LCA, PNA and RCA bound specific glycoproteins on blots of SDS-PAGE-fractionated epididymal fluid samples and several of these glycoproteins displayed an affinity for more than one lectin, indicating diversity in their exposed carbohydrate residues (Table 1). The specificity of lectin interaction was evaluated by including

appropriate saccharide inhibitors in the lectin solutions: 0.2 mol/l α -methyl mannose for LCA, 0.2 mol/l *N*-acetyl-D-glucosamine for WGA and 0.2 mol/l D-galactose for PNA and RCA. Each inhibitor resulted in a marked reduction in lectin binding (data not shown).

The specific lectins LCA and WGA were used to identify asparagine-linked (*N*-linked) glycoproteins. Both LCA (Fig. 3a) and WGA (Fig. 3b) recognized a prominent heavily glycosylated protein of 58 kDa in fluids from all segments of the epididymis. Another prominent heavily glycosylated protein of 33 kDa that also stained with LCA showed qualitatively less staining in the fluid from the cauda epididymidis compared with preceding segments. The glycoproteins that showed very faint or negligible staining in fluids from the initial segment and caput epididymides, but revealed dark prominent staining in the corpus and cauda epididymidis, included components of 150 (LCA), 116 (LCA and WGA), 68 (LCA and WGA) and 64 kDa (WGA) reflecting region-specific changes in glycosylation status of proteins. In contrast, two glycoproteins of 40 kDa (LCA) and 38 kDa (WGA) showed much higher staining in the proximal caput epididymidis compared with other parts of the epididymis, indicating either unmasking or addition of sugar moieties on exposed epitopes of these glycoproteins in the proximal caput epididymides and masking or absence of these sugars on protein in the initial segment, the distal caput and corpus epididymides followed by complete loss or masking in the cauda epididymis, or that the proteins simply become more concentrated due to fluid resorption. The absence of LCA staining for a glycoprotein of 40 kDa and the presence of a new protein band of 38 kDa adjacent to the 40 kDa in caudal epididymal fluid indicates that the

40 kDa glycoprotein possibly undergoes a shift in molecular mass from 40 to 38 kDa in the cauda epididymidis. Alternatively, the presence of the 38 kDa protein may also be attributed to region-associated qualitative or quantitative variations in the oligosaccharide side chains of the carbohydrate moieties as a result of changes in the molecular weights of the proteins.

Lectins RCA (Fig. 4a) and PNA (Fig. 4b) were used to identify serine- and threonine-bound *O*-linked glycoproteins. A very prominent glycoprotein of 58 kDa showed intense RCA staining in fluids from all segments of the epididymis. The protein showed a prominent increase in staining intensity in fluid from the cauda epididymidis in response to lectin PNA. The glycoproteins that revealed dark prominent staining in the fluid from the cauda epididymidis, but showed very faint or negligible staining in fluids from the initial segment and caput epididymides, included components of 170 (RCA and PNA), 150 (RCA), 116 (RCA and PNA), 68 (RCA), 64 (RCA) and 58 kDa (PNA), indicating an increase in the extent of glycosylation or unmasking of exposed epitopes of glycoproteins containing *N*-acetyl-galactosamine groups in a region-specific, maturation-dependent manner. In contrast, three RCA-staining glycoproteins of 56, 38 and 33 kDa that appeared faintly in the fluid from the initial segment revealed much darker staining in the proximal caput epididymal fluid. Although the 33 kDa protein showed diminished activity in cauda epididymal fluid only, the 38 kDa protein showed decreased staining in fluids from the distal caput, corpus and cauda epididymides, indicating loss or alteration of the protein itself and deglycosylation or masking of exposed oligosaccharides on the protein exterior. A very faint PNA-staining band of 60 kDa was seen only in fluid from the proximal caput epididymidis.

As epididymal sperm maturation involves interaction of proteins and glycoproteins secreted or synthesized by the epididymal epithelium with maturing sperm membranes during their passage through the epididymis, the co-migration of common glycoproteins of epididymal fluids and epididymal sperm membranes from the same segment was observed on lectin blots. The co-migration of *N*-linked LCA-binding proteins of epididymal fluids and of sperm membranes was compared (Fig. 5a). The common LCA-staining glycoproteins co-migrating in epididymal fluids and sperm membranes included a 116 kDa polypeptide in corpus and cauda epididymides, a prominent 56 kDa in the initial segment, the caput and corpus epididymidis, a heavily stained luminal fluid component of 33 kDa glycoprotein appearing very faintly on the sperm membrane from the distal caput epididymidis, which became darker on the sperm membranes from corpus and cauda epididymidis and lastly, a band of 22 kDa co-migrated in fluid and membrane of caudal epididymidis only. The common co-migrating WGA-staining glycoproteins of epididymal fluids and sperm membranes were compared (Fig. 5b). These included a prominent band of 58 kDa in all segments of the epididymis

Figure 2 Silver-stained gel showing proteins from the epididymal luminal fluid of rhesus monkeys. The gel shows epididymal fluid proteins from the initial segment (lane 1), proximal caput (lane 2), distal caput (lane 3), corpus (lane 4) and cauda (lane 5) epididymides. Ten micrograms of total protein were loaded into each lane. Molecular mass is indicated on the left side of the figure.

Table 1 Lectin staining intensity profile of glycoconjugates of epididymal fluids of the resus monkey based on visual assessment and densitometric scanning of bands by the Gel Doc 2000 System (Bio-Rad).

Glycoconjugates (kDa)	Lectin specificity	Intensity profile				
		Initial segment (lane 1)	Proximal caput epididymides (lane 2)	Distal caput epididymides (lane 3)	Corpus epididymides (lane 4)	Caudal epididymides (lane 5)
170	LCA	—	—	—	—	—
	WGA	—	—	—	—	—
	RCA	—	v.f.	—	++	++
	PNA	—	—	v.f.	++	+++
150	LCA	—	v.f.	v.f.	+++	+++
	WGA	—	—	—	—	—
	RCA	—	—	—	++	++
	PNA	—	—	—	—	—
116	LCA	—	v.f.	v.f.	+++	+++
	WGA	—	—	—	—	++
	RCA	—	v.f.	—	+++	+++
	PNA	—	—	—	—	+++
68	LCA	v.f.	v.f.	+	++	++
	WGA	v.f.	—	+	+	++
	RCA	+	+	+	++	+++
	PNA	+	++	+	—	—
64	LCA	—	—	—	—	—
	WGA	++	+++	++	+++	+++
	RCA	—	—	—	+	+
	PNA	—	—	—	—	—
60	LCA	—	—	—	—	—
	WGA	—	—	—	—	—
	RCA	—	—	—	—	—
	PNA	—	+++	—	—	—
58	LCA	++++	++++ +	++++ +	+++++	+++++
	WGA	+	++	+	++	+++
	RCA	++++	++++ +	++++ +	+++++	+++++
	PNA	v.f.	++	+	++	+++
56	LCA	+++	++++ +	++++	+++	++
	WGA	+	++	—	++	—
	RCA	+++	++++	+++	+++	++
	PNA	—	—	—	—	—
40	LCA	++++	++++ +	++++	++++	+
	WGA	+	—	—	—	—
	RCA	—	—	—	—	—
	PNA	—	—	—	—	—
38	LCA	—	—	—	—	++++
	WGA	—	++++ +	+	++	—
	RCA	+++	++++	v.f.	+	+
	PNA	—	—	—	—	—
33	LCA	++++ +	+++++	++++ +	++++ +	+++
	WGA	v.f.	+	—	—	+
	RCA	++++	++++ +	++++	++++	++
	PNA	—	—	—	—	—
29	LCA	—	++	—	v.f.	++
	WGA	—	+	++	++	++
	RCA	v.f.	++	v.f.	v.f.	v.f.
	PNA	—	—	—	—	—
22	LCA	—	—	—	+	++
	WGA	—	—	—	—	—
	RCA	—	—	—	—	—
	PNA	—	—	—	—	—

v.f.: very faint staining; +: faint staining; ++: moderate staining; +++: moderate-strong staining; ++++: very strong staining; +++++: moderate-intense staining; ++++++: very intense staining.

Figure 3 Blot probed with LCA (a) and WGA (b) showing *N*-linked glycoproteins of rhesus monkey epididymal luminal fluids from the initial segment (lane 1), proximal caput (lane 2), distal caput (lane 3), corpus (lane 4) and cauda (lane 5) epididymides. Fifty micrograms of total protein were loaded into each lane. Molecular mass is indicated on the left and molecular mass of specific proteins is indicated on the right side of the figure.

and a 38 kDa protein, which first appeared in sperm membranes of distal caput epididymides and later persisted in both fluids and membranes of corpus and caudal epididymides. Common glycoproteins of epididymal fluids and sperm membranes with affinity for lectin PNA, staining *O*-linked oligosaccharide residues are shown (Fig. 6a). A maturation-dependent polypeptide of 116 kDa, showing gradual increase on sperm membranes as spermatozoa

Figure 4 (a) Blot probed with RCA showing *O*-linked glycoprotein of rhesus monkey epididymal luminal fluids from the initial segment (lane 1), proximal caput (lane 2), distal caput (lane 3), corpus (lane 4) and cauda (lane 5) epididymides. Fifty micrograms of total protein were loaded into each lane. (b) PNA-binding *O*-linked glycoproteins on a blot of rhesus monkey epididymal luminal fluids from the initial segment (lane 1), proximal caput (lane 2), distal caput (lane 3), corpus (lane 4) and cauda (lane 5) epididymides. Fifty micrograms of total protein were loaded into each lane. Molecular mass is indicated on the left and molecular mass of specific proteins is indicated on the right side of the figure.

migrate from the initial segment to the caudal epididymides, was present in fluid from the caudal epididymides only. Another polypeptide showing co-migration was 58 kDa staining with PNA appearing in proximal caput

Figure 5 Blot probed with LCA (a) and WGA (b) showing co-migration of N-linked glycoproteins of rhesus monkey epididymal luminal fluids with purified sperm membrane from the initial segment (lanes 1 and 2), proximal caput (lanes 3 and 4), distal caput (lanes 5 and 6), corpus (lanes 7 and 8) and cauda (lanes 9 and 10) epididymides. Fifty micrograms of total protein were loaded into each lane. Molecular mass is indicated on the left and the molecular mass of specific proteins is indicated on the right side of the figure.

epididymidis, stained very faintly with PNA in epididymal fluids compared with sperm membranes. RCA stained heavily more common components present in fluid from the caudal epididymides as well as on sperm membranes (Fig. 6b). The prominent RCA-staining polypeptides of 170 and 150 kDa co-migrated in both epididymal fluid and sperm membrane of caudal epididymides. Another prominent 116 kDa glycoprotein co-migrated in corpus and cauda epididymides. Similarly, a faint RCA-staining 68 kDa protein, first seen in proximal caput epididymal fluid, was seen co-migrating with sperm membranes from corpus and cauda epididymides. The protein showed comparative darker staining in cauda epididymidis compared with the preceding segment. The most heavily stained polypeptide of 58 kDa was seen co-migrating in caput, corpus and cauda epididymides.

Polyclonal antiserum was raised against purified sperm membranes from cauda epididymides in female albino rabbits to determine the actual association of epididymal fluid proteins synthesized and secreted by epididymis with maturing sperm surface. Western blot analysis of epididymal luminal fluids from five segments of monkey epididymis and a lane containing human sperm extracts with Triton X-100 were probed with this antiserum (Fig. 7). Polyclonal antiserum (1:500 dilution) reacted strongly with the 58 kDa polypeptide in fluids from all segments of the epididymis, a 38 kDa glycoprotein from fluid from the initial segment and a 33 kDa protein in fluid from the initial segment, corpus and caudal luminal epididymides with no reactivity in proximal caput epididymides and

very faint reactivity in distal caput epididymal fluid. The antiserum also reacted faintly with 116, 68, 56 and 40 kDa polypeptides in fluids from all segments of the epididymis.

The polypeptides strongly recognized by both antiserum and lectins on blots included a 58 kDa polypeptide (LCA, WGA, RCA and PNA staining) in the fluids from all segments of the epididymis; a 38 kDa protein (RCA staining) from the fluid from the initial segment; a 33 kDa protein in fluids from the initial segment, proximal caput, distal caput, corpus (LCA and RCA staining) and cauda (LCA, WGA, RCA staining) epididymides. The polypeptides, 116, 68, 56 and 40 kDa, moderately recognized by antiserum in fluids from all segments showed diversity in lectin staining in different segments of epididymis. The 116 kDa polypeptide from the corpus epididymal fluid was recognized moderately by lectins LCA and RCA and by LCA, WGA, RCA and PNA in caudal epididymal fluid. The 68 kDa polypeptide showed faint staining by all the lectins in the distal caput epididymal fluid, but moderate staining by LCA, WGA and RCA in corpus and caudal epididymal fluids. The 56 kDa polypeptide showed moderate to strong staining by LCA and RCA in fluids from all segments, but showed faint WGA staining in the fluids from the initial segment, proximal caput and corpus epididymides. The 40 kDa polypeptide showed intense staining by LCA in the initial segment, proximal and distal caput and corpus epididymides with faint staining in cauda epididymidis. The antiserum raised against caudal sperm membrane of rhesus monkey epididymis also crossreacted

Figure 6 Blot probed with PNA (a) and RCA (b) showing co-migration of *N*-linked glycoproteins of rhesus monkey epididymal luminal fluids with purified sperm membrane from the initial segment (lanes 1 and 2), proximal caput (lanes 3 and 4), distal caput (lanes 5 and 6), corpus (lanes 7 and 8) and cauda (lanes 9 and 10) epididymides. Fifty micrograms of total protein were loaded into each lane. Molecular mass is indicated on the left and the molecular mass of specific proteins is indicated on the right side of the figure.

strongly with polypeptides of 116, 68, 58, 56 and 33 kDa in Triton X-100 extracts of human spermatozoa, indicating the presence of common glycoproteins in both species.

The functional significance of epididymal fluid glycoproteins in sperm functions and fertility, was studied by selecting a major 58 kDa protein from caudal epididymal luminal fluid as a candidate for raising polyclonal antibodies in female albino rabbits as this protein was heavily glycosylated with exposed *N*- and *O*-linked oligosaccharides on protein exteriors namely β -Gal, *N*-acetyl-galactosamine, *N*-acetyl-glucosamine and α -D-mannose, and exhibited major maturation-dependent increases in exposed *N*- and *O*-linked oligosaccharides namely *N*-acetyl-glucosamine and β -Gal, *N*-acetyl-galactosamine residues on sperm membranes from the initial segment to cauda epididymis. ELISA results confirmed the presence of antibodies to this protein in the serum of immunized animals. The immune serum specifically recognized a major band of 58 kDa on epididymal luminal fluid and caudal sperm membrane on immunoblots (Fig. 8) indicating a possible interaction between epididymal fluid and sperm membrane glycoproteins. In addition, antiserum cross-reacted with a similar band in human sperm Triton X-100 extracts showing that this protein is a common antigen of monkey and human spermatozoa. No pregnancies

occurred when female rabbits immunized with the 58 kDa protein were mated with coeval males of proven fertility compared with control animals (Table 2). The 100% inhibition of fertility in female albino rabbits indicates that these common antigens of epididymal origin can be developed as sperm antigens for immunocontraceptive purpose. The results were confirmed in another species using a fresh batch of female albino rats ($n = 6$) that were immunized with 58 kDa protein in a similar way to that described for rabbits. The fertility of these animals was tested on days 10–15 after the last booster injection. No implantations were observed in immunized female rats at day 13 after mating despite normal ovulation in these females, as demonstrated by normal number of corpora lutea (Table 3).

The mSA assay with 58 kDa antiserum was conducted in a rodent (rat) and a primate (human) species to study the significance of epididymal fluid glycoproteins in sperm function and fertility. Incubation with anti-58 kDa serum caused head-to-head agglutination of both human (Fig. 9a–c) and rat (Fig. 10a–c) caudal epididymal spermatozoa within a 60 min incubation period up to dilution of 1:1280. Control lanes with normal rabbit serum demonstrated no sperm-agglutinating activity in both these species.

Figure 7 Immunoblot of rhesus monkey epididymal fluid proteins probed with polyclonal antiserum against purified caudal sperm membrane proteins showing sperm association. The blot shows epididymal luminal fluids from the initial segment (lane 1), proximal caput (lane 2), distal caput (lane 3), corpus (lane 4), cauda (lane 5) epididymides and Triton X-100 human sperm extract (lane 6). Fifty micrograms of total protein were loaded into each lane. Molecular mass is indicated on the left and the molecular mass of specific proteins is indicated on the right side of the figure.

Figure 8 Immunoblot of rhesus monkey caudal epididymal fluid (lane 2) and caudal epididymal sperm membrane (lane 3), and Triton X-100 human sperm extract (lane 4) probed with antiserum to 58 kDa glycoprotein. Lane 1 shows control caudal epididymal fluid probed with LCA and lane 5 shows caudal epididymal fluid probed with preimmune serum. Fifty micrograms of total protein were loaded into each lane.

Table 2 Fertility testing of rabbits immunized with 58 kDa epididymal fluid glycoprotein on days 10–15 after last immunization.

Group	Number of animals	Mean litter size*
Control	3	Three males and three females
Immunized	3	No litters

* Observed at 30 days after mating.

Table 3 Fertility testing of rats immunized with 58 kDa epididymal fluid glycoprotein on days 10–15 after last immunization.

Group	Number of animals	Implantation sites* (mean)		Corpora lutea (mean)	
		Right	Left	Right	Left
Control	6	6	7	7	5
Immunized	6	0	0	6	5

* Observed at 13 days after mating.

Discussion

The present study in rhesus monkeys has focused on identification, partial characterization, sperm association and significance of epididymal fluid glycoproteins containing both *N*- and *O*-linked oligosaccharides using a panel of biotinylated lectins. Lectins have been widely used as probes in the examination of glycocomponents of the cell surface and secretions in a variety of species (Young *et al.* 1986, Arenas *et al.* 1996, Navneetham *et al.* 1996, Calvo *et al.* 2000). The use of lectins to probe Western blots of SDS-PAGE-fractionated plasma membranes of spermatozoa from various segments of the epididymis in mouse, rat, rhesus monkey and human spermatozoa (Rankin *et al.* 1989, Srivastava & Olson 1991, Liu *et al.* 2000, Srivastav 2000) examines a wide diversity of carbohydrate moieties and permits direct analysis of glycocomponents on Western blots of fractionated proteins, within specific epididymal segments and, furthermore, provides evidence for different mechanisms that may generate these changes.

A prominent heavily glycosylated protein of 58 kDa with strong staining for *N*-linked α -D-mannose and *N*-acetyl-glucosamine linkages was identified in fluid from all segments of the epididymis. The protein showed a gradual increase in exposed *O*-linked desialylated *N*-acetyl-galactosamine groups on sperm membranes from the initial to caudal segments of rhesus monkey epididymis. An increase in lectin staining for 170, 150, 116, 68, 64 and 58 kDa proteins in cauda epididymis indicates an increase in the extent of glycosylation of these proteins or unmasking of exposed epitopes containing *N*- and *O*-linked sugar residues in a region-specific, maturation-dependent manner. A decrease in α -D-mannose and desialylated galactose, *N*-acetyl-galactosamine binding by another prominent glycosylated component of 33 kDa indicates either a decreased amount of protein or masking of residues on exposed epitopes of this glycoprotein in the cauda epididymidis indicating reorganization of exposed oligosaccharide residues on this protein. The regional modification in the extent of glycosylation of both 58 and 33 kDa proteins seems to be significant for sperm maturation.

The higher expression of exposed oligosaccharides on glycosylated components of 40, 38, 60, 56 and 33 kDa protein in the proximal caput epididymal fluid indicates

Figure 9 Phase-contrast photograph showing agglutination of human spermatozoa with 58 kDa (MEF1) antiserum. (a) Control incubated with normal rabbit serum showing no sperm agglutination at $\times 100$ magnification; (b) 58 kDa antiserum diluted 1:1280 showing a tangled agglutination pattern at $\times 100$ magnification; (c) 58 kDa antiserum diluted 1:1280 showing tangled agglutination pattern at $\times 400$ magnification.

Figure 10 Phase contrast photograph showing agglutination of rat spermatozoa with 58 kDa (MEF1) antiserum. (a) Control incubated with normal rabbit serum showing no sperm agglutination at $\times 40$ magnification; (b) 58 kDa antiserum diluted 1:1280 showing tangled agglutination pattern at $\times 40$ magnification; (c) 58 kDa antiserum diluted 1:1280 showing agglutination pattern at $\times 100$ magnification.

either higher expression of the glycoproteins themselves or addition or unmasking of α -D-mannose and sialylated terminal *N*-acetyl-D-glucosamine linkages on 40 and 38 kDa proteins and sialylated galactosyl, *N*-acetyl-galactosamine residues on 60, 56 and 33 kDa proteins in proximal caput epididymidis. A further decrease in or absence of lectin staining of these proteins in fluid from distal caput, corpus and cauda epididymides indicates loss or alteration of the proteins themselves or deglycosylation or masking of exposed α -D-mannose, sialylated terminal *N*-acetyl-D-glucosamine and sialylated galactosyl, *N*-acetyl-galactosamine oligosaccharides on these glycoproteins. On the basis of these results, it is also possible that either these glycoproteins are absorbed by the epithelium of the corpus and cauda epididymides or are taken up by spermatozoa. Alternatively, reorganization resulting in changes in molecular mass or a shift in electrophoretic mobility could be another mechanism as reflected by very faint LCA staining by *N*-linked 40 kDa protein with simultaneous appearance of an adjoining protein band of 38 kDa in caudal epididymal fluid indicating that the glycoprotein may undergo a shift in molecular mass from 40 to 38 kDa in the cauda epididymidis.

Some of the surface differences demonstrated by spermatozoa as they migrate through the epididymis have been shown to result from the binding of epididymal secretory proteins to the sperm surface, by using approaches such as: coincident migration of epididymal

and sperm proteins on polyacrylamide gels (Rankin *et al.* 1989, Srivastava & Olson 1991, Srivastav 2000); radio-labelling techniques using both *in vitro* and *in vivo* binding of labelled epididymal proteins to spermatozoa (Moore *et al.* 1994); direct binding of protein to spermatozoa (Fournier-Delpech *et al.* 1997); and immunochemical techniques (Xu *et al.* 1997, Gatti *et al.* 2000) in several species, including both non-human primates (Fröhlich & Young 1996) and human (Tezon *et al.* 1985, Focarelli *et al.* 1998, Martin Ruiz *et al.* 1998, Liu *et al.* 2000).

Polyclonal antiserum was raised in female albino rabbits against whole monkey caudal sperm membranes to determine the association of epididymal fluid glycoproteins with the maturing sperm surface. The antiserum crossreacted strongly with 58 and 33 kDa polypeptides. The 58 kDa glycoprotein was detected in luminal fluids from all epididymal segments, whereas the 33 kDa glycoprotein was present in fluids from the initial segment, corpus and caudal epididymal luminal fluid and there was no reactivity in caput epididymal fluid. The antiserum reacted faintly with 116, 68, 40 and 38 kDa proteins exhibiting stronger crossreactivity on the initial segment and corpus epididymal fluid. This finding indicates that these proteins are absorbed on the maturing sperm surface from epididymal secretions, and are released from spermatozoa during epididymal transit, or alternatively are processed by endoproteolysis in the lumen.

Several mechanisms have been shown in which epitopes synthesized by principal cells are initially secreted into the epididymal fluid and are then expressed on the surface of spermatozoa either through direct covalent binding via enzymes, such as transferases (Tulsiani *et al.* 1998), or by direct membrane interchange mediated by glycoposphatidyl inositol (GPI) lipid anchors as demonstrated by CD52, a major maturation-associated highly glycosylated sperm membrane antigen. CD52 is produced by the distal epididymis, released into the lumen where exogenous luminal glycoprotein is inserted into the outer lipid layer of sperm membrane either as part of a GPI oligomer of a membrane vesicle or bound to a lipid carrier coinciding with the sperm acquiring and maintaining fertilizing capacity (Kirchhoff & Hale 1996, Kirchhoff *et al.* 1998).

The common proteins of monkey and human spermatozoa identified by polyclonal antiserum raised against monkey caudal sperm membrane in female albino rabbits included proteins of 116, 68, 58, 40 and 33 kDa, indicating that these proteins were present in two species. Similarly, interaction of the human epididymal protein CD52 (HE5) with epididymal spermatozoa from men and cynomolgous monkeys has been reported by Yeung *et al.* (1997). Another human protein, FLB1, of epididymal origin, involved in the sperm–oocyte recognition process, has been shown by Boue *et al.* (1995) to bind specifically to human, macaque and rodent spermatozoa.

In the present study the significance of glycosylation in sperm function and fertility was studied by selecting a heavily glycosylated 58 kDa glycoprotein (MEF1) of caudal epididymal fluid for raising antiserum in virgin female albino rabbits since the protein was heavily glycosylated with exposed *N*- and *O*-linked oligosaccharides on the protein exterior namely, β -Gal, *N*Ac-galactosamine, *N*Ac-glucosamine and α -D-mannose, exhibited major maturation-dependent increases in exposed *N*Ac-glucosamine and β -Gal, *N*Ac-galactosamine residues on initial to caudal sperm membranes of rhesus monkey epididymis and crossreacted with human spermatozoa. The 58 kDa glycoprotein seems to have functional significance in sperm function and fertility as anti-MEF1 serum resulted in head-to-head agglutination of both rodent (rat) and primate (human) spermatozoa within 60 min of incubation up to a dilution of 1:1280; MEF1 localized on the entire head region of rat caudal epididymal and human ejaculated spermatozoa in immunofluorescence studies (B Singh, A Chandra & A Srivastav, unpublished observations) and the antibody to MEF1 inhibited fertility (100%) of female albino rabbits and rats immunized with this protein. The results of the present study are supported by other findings in which immunization of male rats with protein DE inhibits sperm fusion ability (Ellerman *et al.* (1998). In another study, a 26 kDa protein, acquired during epididymal transit and localized on the sperm acrosome, has been shown to have immunocontraceptive properties in active immunization of male hamsters

(Berube & Sullivan 1994). The human homologue of hamster P26h, P34h, associated with male infertility, has been proposed as a marker of male fertility (Boue & Sullivan 1996). A maturation-related 27 kDa protein in caudal epididymal fluid and on the surface of epididymal spermatozoa in chimpanzees has been shown to be a good immunocontraceptive candidate (Fröhlich & Young 1996).

The current study provides information on regional modification of glycosylation status, sperm association and significance of *N*- and *O*-linked glycoproteins of epididymal luminal fluid of rhesus monkey. The study further identifies a heavily glycosylated 58 kDa caudal epididymal fluid glycoprotein, the antiserum to which crossreacts with human spermatozoa and causes head-to-head agglutination of both human and rat spermatozoa. The protein seems to be significant in sperm fertility and, hence, may be a potential candidate for immunocontraception. Further studies on the role of this protein in sperm-fertilizing ability are in progress in this laboratory.

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