Isolation and partial characterization of the outer dense fibres and fibrous sheath from the sperm tail of a marsupial: the brushtail possum (*Trichosurus vulpecula*)

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The flagellum of a mammalian spermatozoon consists of a central axoneme surrounded by two cytoskeletal structures, the outer dense fibres and the fibrous sheath, which may aid in sperm motility or stability. In this study the outer dense fibres and fibrous sheath were isolated and partially characterized in a marsupial species, the brushtail possum (*Trichosurus vulpecula*). Spermatozoa from the cauda epididymidis were decapitated by sonication, and the head and tail fractions were separated by centrifugation over a 20, 40 and 60% (w/v) sucrose density gradient. After confirming sperm tail purity by Nomarski microscopy, the tails were incubated in either SDS–dithiothreitol to isolate the outer dense fibres or urea–dithiothreitol to isolate the fibrous sheaths. Purified outer dense fibres and fibrous sheaths were solubilized in SDS and β -mercaptoethanol and proteins were separated by one-dimensional PAGE. Coomassie blue staining showed that the outer dense fibres were composed of seven major proteins (molecular

masses: 73, 58, 55, 54, 52, 41 and 16 kDa), and the fibrous sheath was composed of 12 major proteins (molecular masses: 106, 76, 66, 62, 55, 53, 52, 46, 40, 30, 28 and 16 kDa). A polyclonal antibody to the fibrous sheath proteins showed strong crossreactivity with those of fibrous sheath from spermatozoa of several other marsupial species, as well as those from laboratory rats. Subsequent western blotting identified the immunoreactive 76 and 62 kDa proteins from all species, thus indicating their high conservation between species. No crossreactivity of the fibrous sheath antibody to any other cytoskeletal structures, including the outer dense fibres, mid-piece fibre network or connecting laminae, or to the acrosome or underlying subacrosomal material, was evident, indicating that the fibrous sheath proteins are localized to this structure alone. Further work is in progress to determine the extent of homology of these proteins to those in eutherian mammals.

Introduction

The flagellum of a mammalian spermatozoon contains two cytoskeletal structures: the outer dense fibres and the fibrous sheath. The outer dense fibres connect to the connecting piece anteriorly and extend posteriorly into the principal piece for varying distances (Fawcett, 1975). There are nine outer dense fibres in the mid-piece and each is paired with a corresponding microtubule doublet of the axoneme (Fawcett, 1975). At the annulus, which marks the border between the mid- and principal piece, two of the nine outer dense fibres terminate, and the remaining fibres progressively decrease in size distally (Fawcett, 1970, 1975). The fibrous sheath is a tapering cylinder that surrounds the outer dense fibres in the principal piece and is composed of two structural elements: the longitudinal columns and the circumferential ribs. The longitudinal columns replace the two outer dense fibres that terminate at the annulus, whereas the circumferential ribs bridge the

longitudinal columns. The exact functions of the outer dense fibres and fibrous sheath are not clear, although the outer dense fibres may maintain a passive elastic recoil of the sperm tail (Olson and Sammons, 1980). In addition, the outer dense fibres may interact with the microtubule doublets to increase the bending torque generated by the axoneme (Lindemann, 1996). This increased torque, in conjunction with the additional stiffness provided by the outer dense fibres, may allow the axoneme to generate the forces necessary to produce and propagate bends in long sperm tails (Lindemann, 1996). The fibrous sheath may provide elastic rigidity to the sperm tail or define the shape of its beat by placing a constraint on its plane of bending (Philips, 1972; Fawcett, 1975; Lindemann *et al.*, 1992; Si and Okuno, 1993; Carrera *et al.*, 1994; Jassim, 1995).

The relative insolubility of the fibrous components of the sperm tail has allowed the outer dense fibres and fibrous sheath to be isolated for biochemical analysis. Different numbers of proteins in the outer dense fibres have been reported, with up to seven major proteins being described for the outer dense fibres of rats (Olson and Sammons, 1980; Vera *et al.*, 1984; Oko, 1988; Kim *et al.*, 1999), but only three proteins for the outer dense fibres of bulls

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(Baccetti *et al.*, 1973, 1976; Brito *et al.*, 1986) and humans (Haidl *et al.*, 1991; Henkel *et al.*, 1994). At least 17 major proteins have been identified in the fibrous sheath in rats (Olson *et al.*, 1976; Oko, 1988; Kim *et al.*, 1995a), with 14 and 10 proteins identified for humans (Jassim *et al.*, 1992) and rabbits (Kim *et al.*, 1995a), respectively.

Although the heads of marsupial spermatozoa differ radically from those of eutherian mammals, the structure of the flagellum appears, at least morphologically, to be somewhat similar. However, marsupial sperm tails possess additional cytoskeletal components. In some species these include connecting laminae, which extend from the outer dense fibres to the axoneme, and a mid-piece fibre network, which consists of a helically disposed parallel array of fibres immediately beneath the mid-piece plasma membrane (Cleland and Rothschild, 1958; Harding *et al.*, 1979; Temple-Smith, 1987, 1994). Other than a study demonstrating that there is a 32 kDa protein in the fibrous sheath of spermatozoa from possums and wallabies (Harris and Rodger, 1998), the cytoskeletal components of the flagellum of marsupial spermatozoa have not been isolated or characterized. Therefore, the aim of the present study was to isolate and characterize the outer dense fibres and fibrous sheath proteins of a marsupial species, the brushtail possum (*Trichosurus vulpecula*). Polyclonal antibodies, raised against the possum fibrous sheath proteins, were used to determine the extent of antigen crossreactivity between proteins of fibrous sheath, outer dense fibres, mid-piece fibre network and connecting laminae, as well as between those of fibrous sheath, both within the marsupial lineage and between marsupials and eutherians.

Materials and Methods

Experimental animals

The following Australian marsupial species were used in this study: brushtail possum (*Trichosurus vulpecula*), tammar wallaby (*Macropus eugenii*), koala (*Phascolarctos cinereus*) and fat-tailed dunnart (*Sminthopsis crassicaudata*). All the animals, with the exception of the dunnarts, were captured from wild populations. The possums were trapped locally and housed at the Central Animal House of the University of Adelaide, whereas the wallabies were obtained on Kangaroo Island, South Australia. The koalas from which tissue was obtained came from the Adelaide Hills. The spermatozoa from dunnarts were obtained from individuals in a colony bred and housed at the Central Animal House. The University of Adelaide Ethics Committee approved all animal experimentation and the South Australian National Parks and Wildlife Service approved the use of protected animals.

The animals were killed by CO₂ asphyxiation. The cauda epididymides were dissected free, minced in 5 ml 0.02 mol PBS I⁻¹, pH 7.4, and the spermatozoa released were centrifuged at 700 *g* for 10 min. The spermatozoa were washed twice with PBS and resuspended in 5 ml buffer.

From this point onwards, all steps were carried out at 4°C unless indicated otherwise and 0.2 mmol phenylmethylsulphonyl fluoride (PMSF) I⁻¹ (Oko, 1988) was added to the buffers.

Isolation of possum sperm tails

The spermatozoa in suspension were decapitated by sonication (Sanophon sonicator, Sydney) for 4–6 15 s bursts at 30 s intervals. The decapitated spermatozoa were washed in PBS–PMSF, resuspended in 20% (w/v) sucrose in PBS–PMSF, layered over a 20, 40 and 60% sucrose gradient, and centrifuged at 3000 *g* for 90 min in a refrigerated swinging bucket rotor (Beckman, model TJ-6). Sperm tail fractions were collected from the 40–60% sucrose interface and purity was assessed by Nomarski microscopy. In most instances there was no more than about 5% contamination with sperm heads. The sperm tail fraction was resuspended in 20% (w/v) sucrose and centrifuged again at 3000 *g* through a 20, 40 and 60% sucrose step gradient for 60 min; in these samples, sperm tails collected from the 40–60% interface contained < 1% sperm head contamination. The sperm were subsequently diluted with PBS–PMSF and pelleted by centrifugation at 15 000 *g* for 10 min.

Isolation of the outer dense fibres

The isolated sperm tails were suspended in 5 ml 1% (w/v) SDS, 2 mmol dithiothreitol I⁻¹ (DTT), in 25 mmol Tris–HCl I⁻¹ buffer, pH 8.0, and were shaken at room temperature for 30, 60, 90 or 120 min. The resultant suspensions were diluted with Tris–HCl, layered over a 20, 40 and 60% sucrose gradient and centrifuged at 3000 *g* for 60 min. The outer dense fibres were collected from the 40–60% sucrose interface, washed in Tris–HCl buffer, and pelleted by centrifugation at 15 000 *g* for 30 min. The pellet was either fixed for routine transmission electron microscopy or frozen at –70°C for later use.

Isolation of the fibrous sheath

The isolated sperm tails were suspended in 2% (v/v) Triton X-100, 5 mmol DTT I⁻¹, in 50 mmol Tris–HCl buffer I⁻¹, pH 9.0, with shaking at 4°C for 15 min. The resultant suspension was centrifuged at 1000 *g* for 10 min; the supernatant was discarded and the procedure was repeated. This procedure was carried out to solubilize all membranous sperm tail components. The pellet was resuspended in 5 ml 4.5 mol urea I⁻¹, 25 mmol DTT I⁻¹, in 25 mmol Tris–HCl buffer I⁻¹, pH 8.0, at 4°C for either 3, 5 or 8 h with gentle shaking. The resultant suspension was diluted with Tris–HCl buffer and layered over a 20, 40 and 60% sucrose gradient and centrifuged at 3000 *g* for 60 min. The fibrous sheaths were collected from the 40–60% sucrose interface, washed in Tris–HCl buffer and pelleted by centrifugation at 15 000 *g* for 30 min. The pellet was either fixed for routine transmission electron microscopy or frozen at –70°C for later use.

SDS-PAGE

Isolated outer dense fibres and fibrous sheaths were solubilized in 2% (w/v) SDS and 5% (v/v) β -mercaptoethanol for 5 min at 100°C, and any insoluble material was removed by centrifugation at 15 000 *g* for 20 min. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA), and the proteins were separated on linear gradient (7.5%–15%) SDS-polyacrylamide gels. Up to 50 μ g protein per lane was used and the gels were stained with Coomassie brilliant blue. Broad range molecular weight standards (Bio-Rad) were used to determine the molecular weights of the proteins.

Polyclonal antibody preparation

The outer dense fibres and fibrous sheath fractions were solubilized as described above, and each denatured component was emulsified in an equal volume of Freund's complete adjuvant (Sigma, St Louis, MO). Approximately 50 μ g protein was injected either i.p. or s.c. into Sprague-Dawley rats. The rats were given a booster injection with 50 μ g solubilized protein in an equal volume of Freund's incomplete adjuvant at 2 week intervals for 6 weeks and test blood collections were carried out 7 days after each booster injection. Serum was collected and stored at –70°C. Pre-immune sera were collected from all rats before immunization.

Western blotting

Spermatozoa from the cauda epididymidis of laboratory rats, wallabies, dunnarts and koalas were solubilized in 4.5 mol urea l^{-1} and 2% (w/v) SDS in 25 mmol DTT l^{-1} for 5 h at room temperature. Insoluble material was removed by centrifugation at 15 000 *g* for 20 min and final protein concentration was determined. Proteins were separated on 7–12% (w/v) SDS-polyacrylamide mini gels (Bio-Rad) and up to 10 μ g protein was loaded per lane. Electrophoretic transfer of the proteins from the gels to the nitrocellulose membrane was carried out in a Trans-blot cell (Bio-Rad) in 25 mmol Na_2HPO_4 buffer l^{-1} at pH 6.4, 300 V, for 1 h. After removal from the transfer apparatus, the gels were stained with Coomassie brilliant blue and the nitrocellulose membrane was stained temporarily with 0.2% (w/v) Ponceau in 10% (v/v) acetic acid to determine the effectiveness of the transfers. The blots were destained and blocked by incubating overnight at 4°C with 5% (w/v) non-fat milk powder in TBS-Tween (25 mmol Tris-HCl buffered saline l^{-1} , pH 7.4, containing 0.1% (v/v) Tween-20). The blots were washed in TBS-Tween and incubated with immune serum, diluted 1:100 in TBS-Tween, for 1 h at room temperature. The blots were subsequently rinsed four times in TBS-Tween at 5 min per time, and incubated with biotinylated goat anti-rat immunoglobulin antiserum (Zymed, San Francisco, CA), diluted 1:1000 in TBS-Tween for 1 h at room temperature. The blots were washed in TBS-Tween as described and incubated with streptavidin-

AP (Amersham, Castle Hill, Sydney) diluted 1:3000 in TBS-Tween for a further 1 h. The blots were developed with 10 ml of 5'-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate system (Sigma). Controls consisted of replacing the primary antibody with pre-immune serum or buffer.

Indirect immunofluorescence microscopy

Possum, koala, wallaby, dunnart and rat spermatozoa from cauda epididymidis were washed in PBS, pH 7.4, and fixed in 3% (v/v) paraformaldehyde in PBS at 4°C for 20 min. Reactive aldehyde groups were blocked by resuspending the fixed spermatozoa in PBS containing 50 mmol glycine l^{-1} for 20 min, and 100 μ l aliquots were spotted on glass slides pre-coated with 3-aminopropyl-triethoxysilane (APES), for 2 h at 4°C. The slides were rinsed four times with PBS, pH 7.4, and the spermatozoa were permeabilized with ice-cold acetone for 5 min. The spermatozoa were rinsed four times in PBS and incubated with 10% normal goat serum (NGS) in PBS for 20 min to reduce non-specific binding. The spermatozoa were then incubated overnight at 4°C with antiserum diluted 1:100 in PBS containing 0.1% BSA and 1% NGS. The slides were rinsed four times in PBS, incubated in PBS containing 10% NGS for 15 min and then incubated with FITC-conjugated goat anti-rat IgG (Sigma) diluted 1:50 in PBS containing 0.1% BSA and 1% NGS for 45 min. Non-specific staining was determined by replacing the primary antiserum with pre-immune serum or PBS and autofluorescence was determined by omitting the second antibody.

Immunogold electron microscopy

Adult male possums were anaesthetized with isoflurane, and testes and epididymides were fixed by perfusion for 20 min with 4% (v/v) paraformaldehyde in 0.2 mol PBS l^{-1} , pH 7.4. Tissue was dissected free and immersed in the same fixative for 2 h, washed in two changes of PBS and dehydrated by passing through a graded series of ethanol. The tissue was infiltrated in a 2:1 ratio of LR White:70% (v/v) ethanol for 1 h, followed by two further incubations in pure LR White resin for 1 h and then overnight. The resin was again replaced just before polymerization in gelatin capsules at 60°C for 24 h. Ultrathin sections were cut on a Reichert Jung Ultracut microtome and mounted on nickel grids.

Sections on grids were blocked by incubation in 10% NGS for 30 min and incubated with the polyclonal antibody diluted 1:100 in TBS-Tween containing 1% NGS. The grids were washed six times for 5 min each in TBS-Tween and further blocked in 10% NGS for 15 min before incubation in 10 nm gold-conjugated goat anti-rat immunoglobulin (Sigma) diluted 1:20, for 1 h at room temperature. The grids were washed six times for 5 min each in TBS-Tween and four times for 5 min each in ddH_2O . The grids were finally stained with uranyl acetate

(3 min) and examined on a Phillips CM100 transmission electron microscope at 80 kV.

Transmission electron microscopy

Possum cauda epididymides, and isolated outer dense fibres and fibrous sheaths, were fixed by immersion in 3% (v/v) paraformaldehyde and 3% (v/v) glutaraldehyde in 0.2 mol phosphate buffer l⁻¹ for 4 h, washed in two changes of buffer and post-fixed in 1% (v/v) osmium tetroxide in PBS for 1 h. After washing in buffer, the tissue was dehydrated by passing through a graded series of ethanol, cleared in propylene oxide, and infiltrated in a 1:1 ratio of propylene oxide:resin overnight. Propylene oxide:resin was replaced with pure resin for 24 h before the tissue was polymerized at 60°C for 48 h. Ultrathin sections were cut, mounted on copper grids, stained with uranyl acetate and lead citrate, and examined on a Phillips CM100 transmission electron microscope at 80 kV.

Ruthenium red staining of spermatozoa

Possum spermatozoa from the cauda epididymidis were fixed in 3% (v/v) glutaraldehyde containing 0.5% (w/v) ruthenium red in 0.2 mol cacodylate buffer l⁻¹, pH 7.4, for 4 h, washed in two changes of buffer containing 0.5% (w/v) ruthenium red, and post-fixed in 1% (v/v) osmium tetroxide in buffer containing 0.5% (w/v) ruthenium red. After washing in buffer, the spermatozoa were processed and stained for routine transmission electron microscopy.

Results

Morphology of possum sperm tails

The flagellum of possum spermatozoa consists of: (i) a mid-piece, in which a mitochondrial sheath surrounds nine outer dense fibres, together with a fibre network that is composed of parallel rows of fibres beneath the plasma membrane (Fig. 1a,b,c); and (ii) a principal piece, consisting of outer dense fibres, which progressively decreases in size distally, surrounded by a fibrous sheath (Fig. 1a,b). Ruthenium red fixation of possum spermatozoa from the cauda epididymidis showed that the fibrous sheath appeared homogeneous (not shown), whereas the outer dense fibres contained two distinct regions: a narrow, electron dense, outer C-shaped cortex and a central medulla (Fig. 1c,d).

Isolation of possum sperm tails

Sonication of possum spermatozoa from the cauda epididymidis for six 15 s bursts at 100% output resulted in the decapitation of about 95% of the spermatozoa. Subsequent sucrose gradient centrifugation (Fig. 2a) resulted in the deposition of relatively pure fractions of red blood cells and what appeared to be cytoplasmic droplets at the 20–40% sucrose interface (Fig. 2b), sperm tails at the

40–60% sucrose interface (Fig. 2c) and sperm heads at the bottom of the 60% sucrose layer (Fig. 2d).

The red blood cells originated from epididymal blood vessels that were severed at the time of obtaining spermatozoa from the cauda epididymidis when the duct was cut into several pieces, whereas the cytoplasmic droplets were shed from spermatozoa during epididymal transit.

Sonication and subsequent centrifugation removed most of the plasma membrane and mid-piece fibre network from the spermatozoa, but the mitochondrial sheath, outer dense fibres and fibrous sheath remained intact (Fig. 2c). The plasma membrane and most of the acrosomal material were similarly removed from the isolated sperm heads, although in some sperm heads acrosomes with marked vacuolation of acrosomal material or remnants of acrosomal matrix were observed (Fig. 2d).

Isolation of possum outer dense fibres

Incubation of the isolated sperm tails in 1% (w/v) SDS and 2 mmol DTT l⁻¹ for 30 min resulted in complete solubilization of the axoneme and fibrous sheath, although remnants of the mitochondria remained (Fig. 3a). After 60 min of incubation the outer dense fibres and the connecting piece were still present (not shown) but by 90 min of incubation only the outer dense fibres remained (Fig. 3b), with some thin fibres occurring that were presumed to contain outer dense fibre material (Fig. 3c). Incubation of this material in SDS–DTT for an additional 30 min resulted in complete solubilization of the thin fibres and swelling of the remaining outer dense fibres, although they retained their overall shape (Fig. 3d).

Isolation of possum fibrous sheath

Incubation of the sperm tails in 2% (v/v) Triton X-100 and 5 mmol DTT l⁻¹ for two 15 min periods resulted in complete solubilization of the remaining mid-piece fibre networks, as well as the mitochondrial sheath, which exposed the outer dense fibres of the mid-piece (Fig. 4a). Subsequent treatment of sperm tails with 4.5 mol urea l⁻¹ and 25 mmol DTT l⁻¹ for 3 h resulted in complete solubilization of the axoneme and disruption and partial solubilization of the outer dense fibres (Fig. 4b). After 5 h the fibrous sheath remained with occasional remnants of outer dense fibres persisting (Fig. 4c). Prolonged exposure of sperm tails to urea–DTT (up to 8 h) to remove remnants of outer dense fibres resulted in the disruption of the fibrous sheath with some of the outer dense fibre remnants still remaining (Fig. 4d).

Polypeptide composition of possum outer dense fibres and fibrous sheath

One-dimensional SDS polyacrylamide gels of isolated outer dense fibres after 90 min of incubation revealed the presence of seven major Coomassie brilliant blue-stained protein bands (Fig. 5, lane 1) (molecular masses: 73, 58, 55,

Fig. 1. Transmission electron micrographs of (a) transverse and (b) longitudinal sections of possum spermatozoa from cauda epididymidis. The outer dense fibres (odf) in the mid-piece are surrounded by a mitochondrial sheath (ms) and mid-piece fibre network (fn), whereas the outer dense fibres in the principal piece are surrounded by the fibrous sheath (fs). The annulus (an) marks the border between the mid- and principal piece. (c,d) Transmission electron micrographs of transverse sections through the mid-piece of possum spermatozoa from cauda epididymidis fixed in the presence of ruthenium red. The outer dense fibres are composed of a narrow electron-dense peripheral, C-shaped cortex and a central medulla that is less electron-dense. Scale bars represent (a,b,c) 0.5 and (d) 1.5 μm .

54, 52, 41 and 16 kDa). Subsequent silver staining of these outer dense fibre gels revealed an additional 11 minor bands (Fig. 5, lane 2) (molecular masses: 106, 64, 43, 34, 32, 30, 29, 28, 26, 25 and 21 kDa). The 39–42 kDa and 15–17 kDa protein smears observed after silver staining

presumably originated from the 41 and 16 kDa outer dense fibre protein bands, respectively, as identified by Coomassie staining. The most prominent outer dense fibre protein bands had molecular masses of 73, 55, 41 and 16 kDa. SDS–polyacrylamide gels were also run of fractions of outer

Table 1. Molecular masses of fibrous sheath proteins of several species identified by western blotting with anti-possum fibrous sheath serum

Possum fibrous sheath	Possum outer dense fibres	Rat spermatozoa	Wallaby spermatozoa	Dunnart spermatozoa	Koala spermatozoa
106	–	106	–	106	106
76	–	76	76	76	76
62	–	62	62	62	62
–	–	–	48	–	48
–	–	45	–	45	–
40	–	–	–	–	–
–	–	–	39	–	–
–	–	–	37	–	–
–	–	–	–	35	–
–	–	–	34	–	–
–	–	–	32	–	–
–	–	27	27	–	–
–	–	20	20	–	20
16	–	–	16	–	16
–	–	14	–	14	–

All molecular masses are in kDa.

dense fibres incubated in SDS–DTT for 120 min, at which stage the cortex-like fragments of the outer dense fibres had solubilized and only the medulla appeared to remain. Silver staining of these gels revealed that only two of seven major possum outer dense fibre protein bands, the 41 and 16 kDa proteins, remained (Fig. 6).

One-dimensional SDS–polyacrylamide gels of isolated fibrous sheath after 5 h of incubation revealed the presence of 12 major protein bands stained by Coomassie blue (molecular masses: 106, 76, 66, 62, 55, 53, 52, 46, 40, 30, 28 and 16 kDa) (Fig. 5, lane 3). Subsequent silver staining revealed three additional minor bands (molecular masses: 35, 29 and 22 kDa) (Fig. 5, lane 4). The most prominent fibrous sheath protein bands have molecular masses of 76, 66, 62, 52 and 40 kDa.

All gels were repeated at least twice with samples from five different incubations of outer dense fibre and fibrous sheath, and the protein bands described consistently appeared in all gels.

Immunocytochemistry

Only a weak antibody response was observed in laboratory rats immunized with solubilized outer dense fibres and, thus, further work with these antibodies was not performed. In contrast, a very strong antibody response was observed in each of the rats immunized with solubilized fibrous sheath proteins.

The possum fibrous sheath antiserum reacted strongly and specifically with proteins of the principal piece of permeabilized possum spermatozoa from cauda epididymidis (Fig. 7a,b), with no staining observed for any of the proteins of the mid-piece or sperm head. This observation indicates that the possum fibrous sheath proteins to which the antibody bound are different from those of the

outer dense fibres and mid-piece fibre network, and from those of the acrosome and underlying subacrosomal material. Intense fluorescence was also observed over the principal piece in permeabilized koala (Fig. 8a,b), dunnart (Fig. 8c,d) and wallaby (Fig. 8e,f) spermatozoa, with less intense and patchy, but still strong, fluorescence over the principal piece of laboratory rat spermatozoa (Fig. 8g,h). No staining of the sperm head or mid-piece components was evident in any of these species.

Ultrastructural localization of the fibrous sheath anti-serum to possum spermatozoa from the cauda epididymidis showed distinct immunogold labelling over the entire fibrous sheath (Fig. 7c,d), but no antibody reactivity was observed over the outer dense fibres, mitochondrial sheath, mid-piece fibre network, acrosome or subacrosomal material. The same pattern of immunogold labelling was also observed over spermatozoa from the dunnart cauda epididymidis (not shown). These dunnart spermatozoa also have connecting laminae that occur between the outer dense fibres and axoneme, and the antibody did not bind to material in these structures.

Western blotting

Immune serum raised against the fibrous sheath proteins reacted strongly with the major fibrous sheath polypeptides of molecular masses 106, 76, 62, 40 and 16 kDa (Fig. 9, lane 1). No crossreactivity was observed with the possum outer dense fibre proteins (Fig. 9, lane 2). In addition, the antibody crossreacted with proteins from whole laboratory rat, wallaby, dunnart and koala sperm extracts (Fig. 9, lanes 3–6; Table 1). The antibody crossreacted with the 76 and 62 kDa proteins from all four species, as well as the 106 kDa protein from rats, dunnarts and koalas, the 45 kDa proteins from rats and dunnarts, and the 48 kDa proteins from wallabies and koalas

Fig. 2. (a) Sonication and sucrose density gradient centrifugation of possum spermatozoa from cauda epididymidis resulted in the deposition of (b) red blood cells (rbc) and cytoplasmic droplets (cd) at the 20–40% (w/v) sucrose interface, (c) sperm tails at the 40–60% interface and (d) sperm heads at the bottom of the 60% sucrose layer. Acrosomes on the isolated sperm heads are vacuolated or their matrix is dispersed (arrows). pp: principal piece; mp: mid-piece. Scale bars represent (b,c) 0.5 and (d) 1.0 μm .

(Fig. 9, lanes 3–6). Additional proteins were immunostained in each species, especially in extracts of wallaby spermatozoa (see Table 1). No staining was observed when immune serum was replaced with pre-immune serum (Fig. 9, lanes 7–12).

Discussion

In the present study, the outer dense fibres and fibrous sheath of the sperm tail of a marsupial species, the brushtail

Fig. 3. Incubation of possum sperm tails in SDS–dithiothreitol for (a) 30 min resulted in solubilization of all sperm tail components with the exception of the outer dense fibres (odf), connecting piece (cp) and fragments of the mitochondrial sheath (ms). (b) After 90 min of incubation, only the outer dense fibres remained and (c) thin fibres of the outer dense fibres (arrows) were detached from the main portion. (d) Incubation for 120 min resulted in solubilization of the thin fibres and swelling of the larger outer dense fibres. Scale bars represent (a) 0.50, (b) 0.10 and (c,d) 0.05 μm .

possum, were isolated and the protein composition determined by one-dimensional PAGE. The techniques used to isolate these cytoskeletal structures were adapted from those used for similar studies on laboratory rats (see Oke, 1988). In addition, a polyclonal antibody raised

against the possum fibrous sheath proteins showed strong crossreactivity by immunofluorescence, immunogold electron microscopy and western blotting with proteins of the fibrous sheath of spermatozoa from dunnarts, koalas, wallabies and laboratory rats. This finding indicates that

Fig. 4. (a) Incubation of possum sperm tails in Triton X-100–dithiothreitol resulted in solubilization of the mitochondrial sheath. Subsequent incubation in urea–dithiothreitol for (b) 3 h resulted in solubilization of axoneme and disruption of the outer dense fibres (arrows), whereas (c) incubation for 5 h resulted in solubilization of most of the outer dense fibres (arrows). (d) After 8 h of incubation, the fibrous sheath (fs) began to degenerate but small fragments of outer dense fibres still persisted (arrow). Scale bars represent 0.5 μm .

there is high conservation of at least some of these proteins across marsupial, and even a eutherian, species. However, there was no crossreactivity of this antibody to any outer dense fibre, mid-piece fibre network or connecting laminae

proteins from the sperm tail, or to the acrosome or underlying subacrosomal material, indicating that these structures have very different proteins from those that make up the fibrous sheath.

Fig. 5. One-dimensional SDS-PAGE of (a) total possum outer dense fibres stained with Coomassie brilliant blue (lane 1) and silver (lane 2), and (b) total possum fibrous sheath stained with Coomassie brilliant blue (lane 3) and silver (lane 4). Seven major and 11 minor outer dense fibre protein bands are visible, and 12 major and three minor fibrous sheath protein bands are visible. S: molecular mass standards.

The results of the present study demonstrate that techniques developed by Olson *et al.* (1976) and Olson and Sammons (1980) and later modified by Oko (1988) to isolate outer dense fibres and fibrous sheath from eutherian spermatozoa can be satisfactorily applied, with minor modifications, to extract a relatively pure protein fraction of these cytoskeletal components from possum spermatozoa. In the initial separation of possum sperm heads from tails, shorter and fewer bursts of sonication were needed than has been required in studies carried out on spermatozoa of laboratory rats (Oko, 1988; Kim *et al.*, 1995a, 1999). The relative ease with which the heads separate from the tails of marsupial spermatozoa may be due to the mode of attachment of the tail to the fossa of the sperm head that

occurs at the capitulum on the ventral surface of the head. This attachment may relate to less disulphide bonding of the material in this region in marsupials compared with in eutherian mammals (Bedford and Hoskins, 1990). Similarly, the force of centrifugation required to separate possum sperm heads from tails in a sucrose density gradient was only 3000 *g* (for 90 min) compared with 100 000 *g* (for 60 min) required for rat spermatozoa (Oko, 1988; Kim *et al.*, 1995a), indicating great differences in density of the possum sperm head and tail components compared with those of rats. Similar results have been reported in fractionation studies of tammar wallaby spermatozoa (Lin *et al.*, 1998).

Incubation of isolated possum sperm tails in 1% (w/v) SDS and 2 mmol DTT l^{-1} resulted in a relatively pure

Fig. 6. One-dimensional SDS-PAGE of possum outer dense fibres after 120 min incubation of spermatozoa in SDS-dithiothreitol and staining with silver (lane 1). S: molecular mass standard.

fraction of outer dense fibres as determined by transmission electron microscopy. Seven major polypeptides were observed by Coomassie staining after solubilization and one-dimensional PAGE, with molecular masses of 73, 58, 55, 54, 52, 41 and 16 kDa. This number is similar to the number of major outer dense fibre proteins identified in laboratory rats (molecular masses: 84, 79, 66.2, 57.2, 29, 21 and 15.5 kDa) (Kim *et al.*, 1999), although their molecular masses are somewhat different. Silver staining of the possum outer dense fibre gels revealed an additional 11 minor protein bands (molecular masses: 106, 64, 43, 34, 32, 30, 29, 28, 26, 25 and 21 kDa), compared with only five additional protein bands in silver-stained gels of rat outer dense fibres (molecular masses: 190, 150, 54, 48.5 and 44.6 kDa) (Kim *et al.*, 1999). This finding indicates that either there are significantly more minor proteins in the outer dense fibres of possum spermatozoa than in rat spermatozoa or that there was minor contamination of the outer dense fibre fraction in the present study with, for example, proteins from remnants of the mitochondrial sheath. Unfortunately, our attempts to raise polyclonal antibodies to the possum outer dense fibres did not succeed and, therefore, we could not determine by immunogold labelling whether some of these proteins were from the mitochondrial sheath. Only three major outer dense fibre proteins have been reported in bulls (molecular masses: 85,

33 and 11 kDa) (Baccetti *et al.*, 1973, 1976; Brito *et al.*, 1986) and humans (molecular masses: 150, 67 and 55 kDa) (Haidl *et al.*, 1991; Henkel *et al.*, 1994). However, the detergent cetyltrimethylammonium (CTAB) rather than SDS was used in these studies, so it is possible that not all proteins were extracted and that additional proteins are present in the outer dense fibres of these species.

Ruthenium red fixation of possum spermatozoa from the cauda epididymidis showed that while the fibrous sheath was homogeneous, the possum outer dense fibre was composed of a narrow, electron dense, outer C-shaped cortex together with a central medulla. Similar morphological findings have been observed in the outer dense fibres of spermatozoa from several eutherian species, including the laboratory rat (Telkka *et al.*, 1961; Bawa, 1963; Fawcett, 1970), indicating that there are ultrastructural similarities between the outer dense fibres of eutherians and the brushtail possum. Incubation of possum sperm tails in SDS and DTT for 90 min resulted not only in the isolation of the outer dense fibres, but also in the detachment of a thin fibre from the rest of the outer dense fibres. It is possible that this may, in fact, be the outer dense fibre cortex, which has become detached from the medulla during incubation. Prolonged incubation of the possum outer dense fibres in SDS and DTT resulted in solubilization of the thin segments, but the main part of the outer dense fibres, while swollen, remained largely intact and SDS-PAGE of this material revealed that only two of seven major possum outer dense fibre proteins (41 and 16 kDa) remained, indicating that these two proteins may be present in the medulla of the possum outer dense fibres.

In the present study, incubation of possum sperm tails in 2% (v/v) Triton X-100 and 5 mmol DTT l^{-1} followed by 4.5 mol urea l^{-1} and 25 mmol DTT l^{-1} solubilized the membranous flagella components and isolated the fibrous sheath as determined by TEM, although some remnants of the outer dense fibres remained. Residual outer dense fibre material has also been observed after identical incubations using spermatozoa from laboratory rats (Kim *et al.*, 1995a) and rabbits (Kim *et al.*, 1997). The possum fibrous sheath fraction had 12 major proteins, as shown by Coomassie staining, with molecular masses of 106, 76, 66, 62, 55, 53, 52, 46, 40, 30, 28 and 16 kDa, and an additional three protein bands (molecular masses: 35, 29 and 22 kDa) were identified by silver staining. In comparison, 17, 14 and 10 major fibrous sheath proteins have been identified in spermatozoa from rats (Kim *et al.*, 1995a), humans and rabbits (Kim *et al.*, 1997), respectively. Although the molecular masses of the fibrous sheath proteins in each species are somewhat different, the most prominent fibrous sheath proteins in rats and rabbits have molecular masses between 72 and 85 kDa. Likewise, in the present study on the brushtail possum, the most prominent protein had a molecular mass of 76 kDa. Whether this possum protein is homologous to the proteins of similar molecular masses in eutherian species has yet to be ascertained.

It is possible that the 106, 55, 52 and 16 kDa possum

Fig. 7. Immunocytochemistry of possum spermatozoa incubated with anti-possum fibrous sheath serum. (a) Phase-contrast micrograph and (b) corresponding fluorescent micrograph of a possum spermatozoon probed with possum fibrous sheath anti-serum. There is positive staining of the principal piece (pp), but no crossreactivity with the sperm head (h) or mid-piece (mp) was observed. an: annulus. Scale bar represents 10 μ m. (c) Longitudinal and (d) transverse sections showing immunogold localization of the antibody to the fibrous sheath (fs) with no specific gold labelling observed over the outer dense fibres (odf) and mitochondrial sheath (ms). Scale bars represent 0.5 μ m.

Fig. 8. Phase-contrast (a,c,e,g) and corresponding immunofluorescent (b,d,f,h) localization of the possum fibrous sheath antiserum to the principal piece of fixed, permeabilized spermatozoa from the cauda epididymidis of a (a,b) koala (c,d) dunnart, (e,f) wallaby and (g,h) laboratory rat. Strong fluorescence was observed on the principal piece (pp) of all species. mp: mid-piece; arrow: annulus. Scale bars represent 10 μ m.

Fig. 9. Western blot of possum fibrous sheath (lane 1), possum outer dense fibres (lane 2) and laboratory rat (lane 3), wallaby (lane 4), dunnart (lane 5) and koala (lane 6) sperm polypeptides probed with a polyclonal antibody against the possum fibrous sheath. (a) and (b) are corresponding blots stained with immune and pre-immune sera, respectively. The antibody recognized most of the major possum fibrous sheath proteins (lane 1), but did not cross-react with the possum outer dense fibres (lane 2). Numerous proteins from rat, wallaby, dunnart and koala spermatozoa were immunostained in the blots, including the 76 and 62 kDa polypeptides in each species (lanes 3–6). No immunoreactive bands were detected in blots probed with pre-immune sera controls (lanes 7–12).

fibrous sheath proteins are contaminants from the outer dense fibres, as proteins of identical molecular masses are present in gels of the outer dense fibres. However, the outer dense fibre proteins did not immunostain when western blotting was carried out using the possum fibrous sheath antiserum. This finding indicates strongly that these proteins are not outer dense fibre contaminants and that the outer dense fibres and fibrous sheath are composed of different proteins. In rats, both the outer dense fibres and fibrous sheath were shown to contain 14.4 kDa proteins (Okó, 1988), and antibodies affinity-purified to each of these proteins were used to demonstrate immunocytochemically that these proteins, while antigenically similar, are not cross-contaminants (Okó, 1988). The possum fibrous sheath antiserum also did not bind to the mid-piece fibre network or connecting laminae of the marsupial sperm tails, indicating that the fibrous sheath proteins are unique and that a different suite of proteins is present in the other cytoskeletal structures. Harris and Rodger (1998) demonstrated that a 32 kDa protein, identified by a monoclonal antibody PSA-10, occurs in the fibrous sheaths of possums and wallabies. This antibody was raised against possum acrosomal proteins, indicating that a fibrous sheath and acrosomal protein may be related. However, in the present study no binding of possum fibrous sheath antiserum to the acrosome or subacrosomal material was observed. In addition, no 32 kDa protein was found in the gels run of the possum fibrous sheath, although minor proteins with molecular masses of 28, 29 and 30 kDa were detected, and it is possible that one of these proteins corresponds to the protein recognized by the PSA-10 antibody.

In the present study, western blotting of possum outer dense fibres with anti-possum fibrous sheath serum demonstrated that, although there were no common proteins in the outer dense fibres, mid-piece fibre network or connecting laminae to which the possum fibrous sheath antiserum bound, there was marked crossreactivity with the fibrous sheath from spermatozoa of the dunnart, koala, wallaby and laboratory rat. The possum fibrous sheath antiserum recognized seven proteins from rats with molecular masses of 106, 76, 62, 45, 27, 20 and 14 kDa, which correspond roughly to the molecular masses of most of the major rat fibrous sheath proteins reported previously (Okó, 1988; Kim *et al.*, 1995a). This finding indicates that the possum fibrous sheath antiserum may have recognized many of the fibrous sheath proteins from the dunnart, koala and wallaby, including the 76 and 62 kDa proteins in all four species. The fact that the antibody recognized proteins of identical or similar molecular masses in spermatozoa of laboratory rats, dunnarts, koalas and wallabies indicates that these proteins may be highly conserved in the fibrous sheath both between marsupial species of three different families and laboratory rats. As eutherians and marsupials diverged over 100 million years ago, this finding indicates a remarkably high degree of conservation of these proteins across both eutherian and marsupial mammals, although these proteins do not appear to be present in the other cytoskeletal structures of the sperm head and tail.

For further determination of the degree of similarity between marsupial cytoskeletal proteins and those in eutherians, it is necessary to sequence the genes that code

for the major marsupial proteins. To date, a small number of eutherian sperm outer dense fibres and fibrous sheath proteins have been sequenced although the insolubility of these proteins and blocking at the N-terminus have hampered progress in this field. The most prominent rat outer dense fibre protein, termed Odf1 with a molecular mass of 27 kDa, has been cloned (van der Hoorn, 1990; Burfeind and Hoyer-Fender, 1991; Morales *et al.*, 1994), and the sequence of this protein is highly conserved in mice (Hoyer-Fender *et al.*, 1995), humans (Gastmann *et al.*, 1993), bulls and boars (Kim *et al.*, 1995b). The C-terminus of Odf1 contains a repetitive cysteine–glycine–proline motif that is also present in the *Drosophila* Mst87F gene product (Schäfer *et al.*, 1993), a member of a gene family that encodes satellite fibres in the *Drosophila* sperm tail, which may be structural homologues of the mammalian outer dense fibres (Kuhn *et al.*, 1988; Schäfer *et al.*, 1993). These resemblances may reflect evolutionary conservation of these proteins from insects to mammals (Schäfer *et al.*, 1993; Morales *et al.*, 1994), although no 27 kDa possum outer dense fibre protein was detected in the present study. Other outer dense fibre proteins the genes of which have been sequenced include an 84 kDa rat outer dense fibre protein, termed Odf2, which has been proposed to interact with Odf1 via leucine zippers (Shao *et al.*, 1997; Schalles *et al.*, 1998), and a third rat outer dense fibre gene that encodes highly variable transcripts that translate into proteins of 65–75 kDa (Brohmann *et al.*, 1997). Of the fibrous sheath proteins sequenced, most interest surrounds the major 82 kDa mouse fibrous sheath protein, as it is an A-kinase anchoring protein (AKAP) (Carrera *et al.*, 1994; Fulcher *et al.*, 1995), which anchors protein kinase A, the major downstream target of cyclic AMP, to the sperm tail cytoskeleton; therefore, it may be involved in sperm motility (Miki and Eddy, 1988; Mei *et al.*, 1997; Vijayaraghaven *et al.*, 1997). Homologues of this protein have also been found in human (Turner *et al.*, 1998), bull (Moss *et al.*, 1999) and rat (El-Alfy *et al.*, 1999) spermatozoa, and it would be interesting to determine whether the major possum fibrous sheath protein, which has a similar molecular mass, is also an AKAP.

In conclusion, the outer dense fibres and fibrous sheath have been isolated from the sperm tail of a marsupial species, the brushtail possum, and the molecular masses of the major proteins have been determined. Antiserum raised against the major fibrous sheath proteins did not crossreact with proteins in the outer dense fibres or other sperm cytoskeletal structures, thus indicating that these fibrous sheath proteins are unique. However, these fibrous sheath proteins appear to be conserved across marsupials and even a eutherian species.

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