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Actin polymerisation during morphogenesis of the acrosome as spermatozoa undergo epididymal maturation in the tammar wallaby (*Macropus eugenii*)

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ABSTRACT

In the tammar wallaby (*Macropus eugenii*), post-testicular acrosomal shaping involves a complex infolding and fusion of the anterior and lateral projections of the scoop-shaped acrosome into a compact button-like structure occupying the depression on the anterior end of the sperm nucleus. The present study has generated cytochemical and histological evidence to demonstrate that the occurrence of actin filaments (F-actin, labelled by Phalloidin-FITC) in the acrosome of tammar wallaby spermatozoa is temporally and spatially associated with the process of acrosomal shaping in the epididymis, through a pool of monomeric actin (G-actin, labelled by Rh-DNase I) present in the acrosome throughout all stages of epididymal maturation. F-actin was not detected in the acrosome of testicular spermatozoa, but was found in the infolding and condensing acrosome of caput and corpus epididymal spermatozoa. When the spermatozoa completed acrosome shaping in the cauda epididymidis, F-actin disappeared from the acrosomal area. The strong correlation between the occurrence of F-actin and the events of acrosomal shaping suggested that the post-testicular shaping of the acrosome might depend on a precise succession of assembly and disassembly of F-actin within the acrosome as the spermatozoa transit the epididymis. Thus, actin filaments might play a significant role in the acrosomal transformation, as they are commonly involved in morphological changes in somatic cells.

Key words: Sperm maturation; actin; testis; epididymis; marsupials.

INTRODUCTION

The mammalian acrosome is a membrane-bound organelle covering the anterior portion of the sperm nucleus. It contains hydrolytic enzymes necessary for spermatozoa to penetrate the glycoprotein coat (zona pellucida, ZP) of the egg, and plays an important role in sperm-egg binding (Bleil et al. 1988; Jones et al. 1988; Gilbert, 1997). It is known that gross defects to the acrosome can seriously impair sperm penetration through the ZP (Krzanowska & Lorenc, 1983), as exemplified by globozoospermia, a human condition in which sterility is associated with a failure of acrosomal morphogenesis. Even minor defects, such as abnormalities in the presentation of ZP binding proteins on the surface of plasma membrane overlying the acrosomal domain, are sufficient to impair fertility (Yanagimachi, 1994; Ikawa et al. 1997). Thus the normality of acrosomal formation in the testis and its post-testicular maturation in the epididymis is vital for fertility in mammals.

The development of the acrosome occurs during spermiogenesis, a radical reshaping of the sperm cell involving the repositioning of organelles when the round spermatid is transformed into the characteristic elongated shape of the spermatozoon in the testis. However, mammalian spermatozoa leaving the testis are immotile and infertile and must undergo a post-testicular maturation that takes place as they pass through the epididymis (Bedford, 1979; Cooper, 1986; Amman et al. 1993; Yanagimachi, 1994; Cooper et al. 1998). Post-testicular changes in the form of the acrosome as a concomitant of sperm transit through the epididymis have been observed in many mammals.

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Major acrosomal reorganisations have been recorded in the guinea pig and chinchilla, while modest changes are observed with some rodents and some primates (Bedford, 1979). Recently, Lin & Rodger (1999) confirmed that an extremely striking reorganisation of the acrosome in the epididymis occurs in 2 Australian marsupials, the tammar wallaby (*Macropus eugenii*) and the brushtail possum (*Trichosurus vulpecula*). As the epididymal maturation of the wallaby spermatozoon involves far more structural change than is seen in any eutherians, the acrosome of this marsupial species provides an ideal model to study the post-testicular maturation of the mammalian acrosome in the epididymis.

In all animals, cell shape is primarily determined by the internal cytoskeleton, of which actin is a major component. Many dynamic behaviours of the cell, such as spreading, motility, polarisation and cytokinesis, often involve the cell surface, and depend on a precise and dynamic process of actin filament (F-actin) assembly and disassembly to form a cortical meshwork underlying the plasma membrane. Thus understanding the dynamics of F-actin is critical to our understanding of the morphogenesis of animal cells. It is now known that a number of the most dynamic protrusions at the somatic cell periphery are actin filament containing structures, and the constant assembly and disassembly of F-actin within the cell protrusions determines the structural reorganisation and functions of the cell (Arpin et al. 1994; Ballestrem et al. 1998; Carraway et al. 1998). As the acrosome is a peripheral structure on mammalian spermatozoa and its development involves a dynamic shaping process, actin proteins may be present in the acrosome and play a role in its formation.

The presence of actin in mammalian spermatozoa has been demonstrated in several species including cattle, boar, rat, mouse, guinea pig, hamster, mole, human, rabbit, fat tailed dunnart and opossum (Flaherty et al. 1986; Halenda et al. 1987; Olson & Winfrey, 1991; Castellani-Ceresa et al. 1992; Moreno-Fierros et al. 1992; Breed et al. 1993; Paranko et al. 1994). However, the precise localisation of this protein has not been conclusively demonstrated (Lora-Lamia et al. 1986; Paranko et al. 1994; Yagi & Paranko, 1995). In the prepubertal testis, F-actin is uniformly distributed throughout the cytoplasm of the undifferentiated spermatid. After the onset of spermiogenesis, F-actin becomes concentrated in the developing subacrosomal space in round spermatids and subsequently extends beneath the acrosome as the spermatid elongates (Russell et al. 1986; Fouquet & Kann, 1992). However, there is no general agreement on the occurrence and localisation of F-actin in the acrosome of mammalian spermatozoa. To date, there is no information on the possible role of actin proteins in the construction of the acrosome, or other sperm surface structures, in either testicular or epididymal spermatozoa.

Using the acrosome of the tammar wallaby as a model, the present study has generated cytochemical and histological evidence to demonstrate that actin proteins exist in the acrosome and other subcellular locations of the spermatozoa. This study has also demonstrated that the assembly and disassembly of actin filaments within the acrosome is temporally and spatially associated with the process of the acrosome shaping during epididymal maturation.

MATERIALS AND METHODS

Animals

Five male tammar wallabies (*Macropus eugenii*) were obtained from Kangaroo Island, South Australia. The animals were maintained on a diet of kangaroo pellets, grasses, lucerne hay and a fresh supply of water, in the holding facilities of the Animal Service Unit at the University of Newcastle, Australia. The use of protected animals and animal experimentation were approved by the appropriate Australian state authorities and by the Animal Care and Ethics Committee at the University of Newcastle, respectively.

Extraction and collection of spermatozoa

The animals were killed with an overdose of sodium pentobarbitone (>30 mg/kg body weight, Birbac Australia, Peakhurst, NSW) intravenously via a lateral tail vein. The testis and epididymis were excised and separated. The whole epididymis was carefully dissected into 3 segments: the caput, corpus and cauda epididymidis according to the previous classification (Lin & Rodger, 1999; see Fig. 1). The testis and different segments of the epididymis were cut into small pieces in approximately 10 ml phosphate buffered saline (PBS, 145 mM NaCl and 20 mM sodium phosphate, pH 7.4) in plastic cell culture dishes, and then incubated for 30 min at 37 °C in 5% CO_2 in air with humidity at 100% to allow spermatozoa to diffuse into PBS. Supernatants containing spermatozoa from the testis and different segments of the epididymis were carefully collected. Subsequently, the spermatozoa were obtained via gentle centrifugation (850 g for 5 min) for further treatments or examinations.

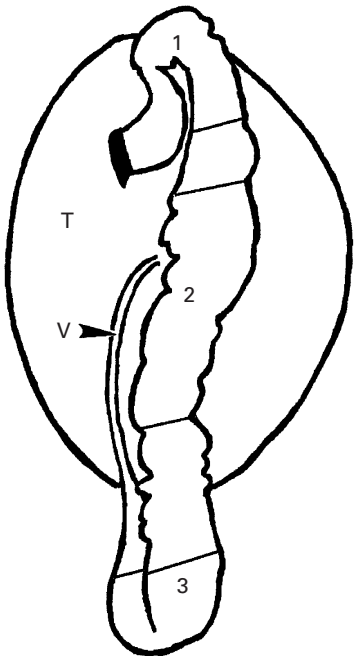

Fig. 1. Drawing of the tammar wallaby epididymis showing the dissected epididymal segments for this study: 1, caput epididymidis; 2, corpus epididymidis; 3, cauda epididymidis; T, testis; V, vas deferens.

Preparation for scanning electron microscopy

Tissues of the testis and epididymis were fixed in 2.5% (v/v) glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer overnight at 4 °C. The samples were then treated with 1% osmium tetroxide for 4 h. After dehydration through serial concentrations of acetone and critical point drying, the tissues were coated with gold and examined in a JSM 840 scanning electron microscope (JEOL, Tokyo, Japan) operated at 15 kV.

Fluorescence of globular-actin (*G-actin*)

DNase I binds to G-actin with an affinity of about 5×10^8 M^{-1} and has been used to detect and measure G-actin in previous studies (Knowles & McCulloch, 1992). Spermatozoa extracted from the testis and the caput, corpus and caudal epididymidis were fixed in 4% (w/v) paraformaldehyde and 0.1% (v/v) glutaraldehyde in PBS for 15 min at 4 °C. After 3 washes in PBS (via centrifugation at 850 g for 2 min), the spermatozoa were permeabilised in cold acetone (−20 °C) for 10 min, and then attached on poly-L-lysine coated slides by air drying. Following rehydration by 3 washes with PBS, the spermatozoa were stained with 1:50 DNase I–tetramethylrhodamine conjugate (Rh-DNase I, stock solution of 5 mg/ml, Molecular Probes, Eugene, OR, USA) in PBS for 30 min at room temperature. After 3 washes with PBS, the samples were mounted with Mowiol medium (Calbiochem, La Jolla, CA, USA). Fluorescent micrographs were taken under a Zeiss Axiovert S 100 inverted phase contrast microscope through a Zeiss No. 15 (Rhodamine) filter system with green excitation at a wavelength of 546 nm. Control samples underwent the same process as described above, however the step of staining with DNase I–tetramethylrhodamine was excluded.

Fluorescence of filamentous actin (*F-actin*)

Phalloidin has been found to bind only to polymeric and oligomeric forms of actin (F-actin) and not to monomeric actin (G-actin) (Estes et al. 1981; Cooper, 1987). Phalloidin-FITC conjugate was therefore used to identify F-actin in this study. In brief, spermatozoa from the testis and epididymis were fixed in 4% (w/v) paraformaldehyde in PBS for 60 min at 4 °C. Following 3 washes (5 min each) in PBS, the spermatozoa were permeabilised in cold methanol and acetone (−20 °C) for 10 min each, and then attached onto poly-L-lysine coated slides by air drying. After rehydration by 3 washes (5 min each) in PBS, the spermatozoa were stained with phalloidin conjugated with fluorescein isothiocyanate (phalloidin-FITC, 50 μg/ml in PBS with 1% DMSO, Sigma Aldrich, Missouri, USA) for 40 min at RT. After 3 washes (5 min each) in PBS, fluorescent micrographs were taken under a Zeiss Axiovert S 100 inverted phase contrast microscope through a Zeiss No. 9 (FITC) filter system with a blue excitation of wavelengths between 450 and 490 nm. Control samples were processed as described above excluding the step of staining with phalloidin-FITC.

RESULTS

Shaping of the wallaby acrosome in the epididymis

The final shaping of the wallaby acrosome in the epididymis has been investigated previously by transmission and scanning electron microscopic studies (Setiadi et al. 1997; Lin & Rodger, 1999). The scanning electron microscopic (SEM) images in Figure 2 showed that on leaving the testis, the testicular spermatozoa displayed a thumbtack or T shape with the nucleus perpendicular to the midpiece. The acrosome of the wallaby spermatozoa was an elongated 'scoop' shape projecting from the dorsal surface of the nucleus (Fig. 2*a*). In the caput epididymidis, the acrosome started to condense, and

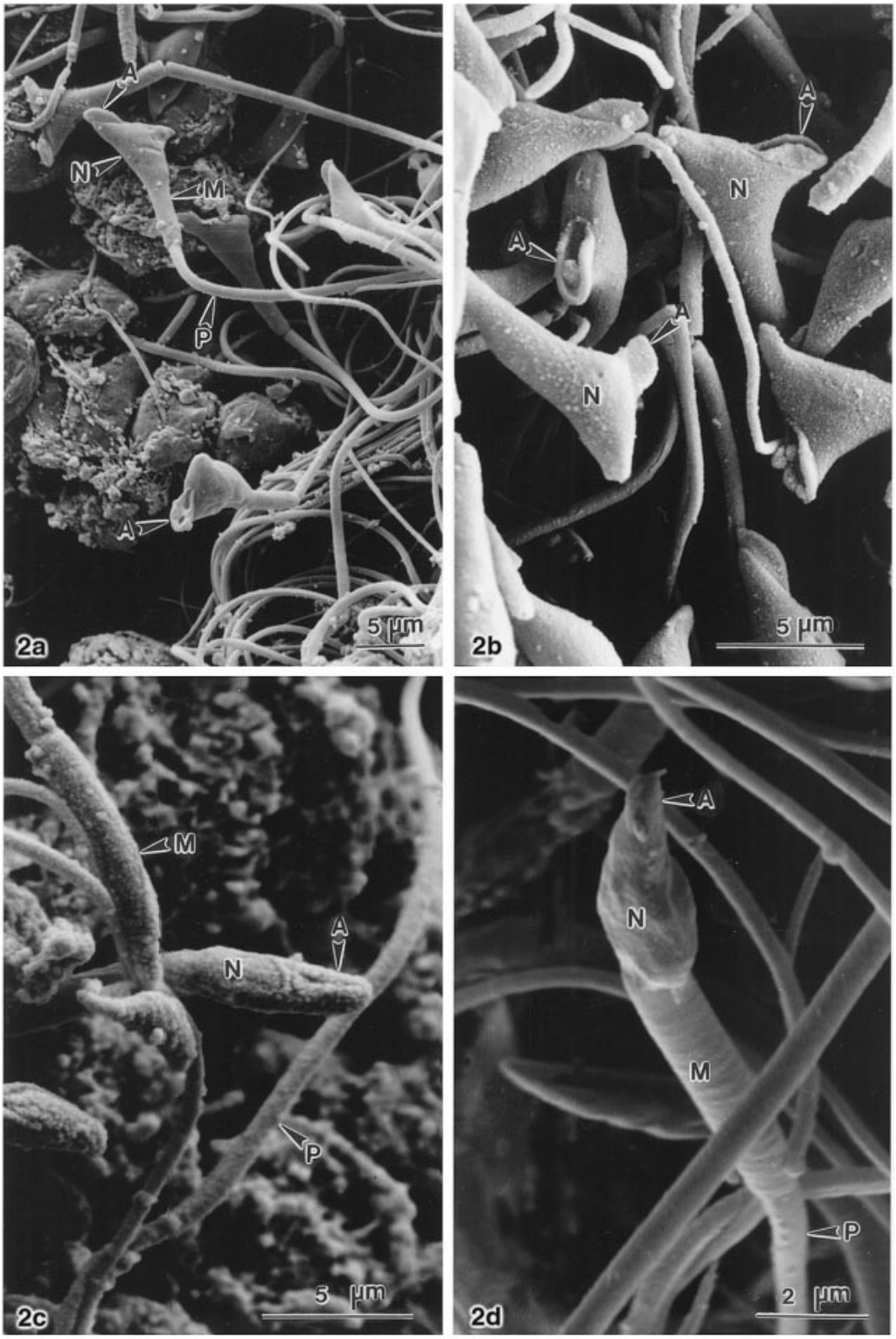

Fig. 2. Scanning electron micrographs of tammar wallaby spermatozoa, at different maturational stages, in the testis and epididymis. (*a*) Testicular spermatozoa in the lumen of the seminiferous tubule. Note that the immature acrosome (A) is in a scoop shape projecting from the anterior end of the dorsal surface of the nucleus (N). The acrosome scoop contains a remnant of Sertoli cell cytoplasm. The nucleus is perpendicular to the midpiece (M) of the sperm portraying the characteristic immature 'T' shape. P, principal piece. (*b*) Spermatozoa in the caput epididymidis. Note that the lateral projections of the acrosome (A) have started to condense, and the nucleus (N) is still perpendicular to the midpiece. Some acrosomal scoops (A) still contain Sertoli cell cytoplasm remnants. (*c*) Spermatozoa in the corpus epididymidis. The acrosome (A) has further condensed and the lateral projections have fused along its longitudinal axis. The nucleus (N) of a spermatozoon is nearly parallel with the midpiece (M). P, principal piece. (*d*) Streamlined spermatozoon from the caudal epididymidis. The acrosome (A) has condensed into a compact 'button' shaped organelle located within the depression on the anterior end of the nucleus (N). M, midpiece; P, principal piece.

its lateral scoop projections became shorter, thicker and folded inwards (Fig. 2*b*), while the sperm nucleus was still perpendicular to the midpiece. As the spermatozoa passed through the corpus epididymidis, the acrosome fused midway along the longitudinal axis via a complex process of infolding (Fig. 2*c*), as the nucleus of the spermatozoa became almost parallel to the midpiece. In the cauda epididymidis, the acrosome had condensed into its mature form as a compact 'button' shaped structure (Fig. 2*d*), whereas the spermatozoa had developed a streamlined shape with the nucleus fully parallel with the midpiece, thus adopting its characteristic mature form as found in ejaculated spermatozoa.

G-actin in the testicular and epididymal spermatozoa of the tammar wallaby

DNase I fluorescence revealed that there was a pool of G-actin existing in the wallaby testicular and epididymal spermatozoa, particularly in the acrosome, midpiece and principal piece, but not found in the nuclear area (Fig. 3*b*, *d*, *f*). In testicular spermatozoa, the DNase I fluorescence labelled the 'scoop' shaped acrosome which was located at the anterior end of the dorsal surface of the nucleus (Fig. 3*b*). The caput epididymal spermatozoa showed a similar pattern of DNase I labelling as that seen in the testicular spermatozoa, as fluorescence was readily visible in the 'scoop' shaped acrosome (Fig. 3*d*). Since the corpus epididymis housed spermatozoa at varying maturational stages, the DNase I labelling on the acrosome could be seen in both the T shaped and the more streamlined spermatozoa. In the T shaped spermatozoa, the DNase I labelling was observed in the acrosomal lateral projections, while in streamlined spermatozoa, the labelling was found in the 'button-like' acrosome at the depression on the anterior third of the dorsal surface of the nucleus (Fig. 3*f*). In caudal epididymal spermatozoa, intensive DNase I labelling was seen on the fully condensed, compact button-like acrosome (Fig. 3*h*). No autofluorescence was found in any control samples.

Occurrence of F-actin in the testicular and epididymal spermatozoa of the tammar wallaby

Immunofluorescence of phalloidin-FITC in Figure 4 showed that the distribution of F-actin was temporally and spatially associated with the process of acrosomal shaping in the wallaby epididymis. In the testicular spermatozoa, an extremely low level of phalloidin-FITC fluorescence, if any, was detected on the 'scoop'-shaped acrosome and other subcellular locations of the spermatozoon (Fig. 4*b*). As the spermatozoa entered the caput epididymidis, however, very intense phalloidin-FITC fluorescence was found on the folding acrosome, the posterior end of the nucleus, the midpiece and principal piece, but not on the major area of the nucleus (Fig. 4*d*). A similar labelling pattern was also seen in the corpus epididymal spermatozoa whose acrosome was still in the process of infolding and fusion of its lateral projections (Fig. 4*f*). As the spermatozoa completed their acrosome shaping in the cauda epididymidis, phalloidin-FITC fluorescence disappeared from the 'button'-shaped acrosome, but remained on the midpiece and principal piece (Fig. 4*h*).

DISCUSSION

Our SEM studies further confirmed that the post-testicular acrosomal shaping involved a complex infolding and fusion of the anterior and lateral projections of the scoop-shaped acrosome. This shaping process commenced as spermatozoa entered the distal caput epididymidis and was completed by the time spermatozoa reached the proximal caudal region of the epididymis resulting in a button-like structure occupying the depression on the anterior end of the sperm nucleus. This cytohistochemical study has revealed that the occurrence of actin filaments (F-actin) in the acrosome of tammar wallaby spermatozoa was temporally and spatially associated with the process of acrosomal shaping in the epididymis, through a pool of monomeric actin (G-actin) that existed in the acrosome throughout all stages of epididymal maturation. In the tammar wallaby, F-actin was not detected in the acrosome of testicular spermatozoa, but was found in the infolding and condensing acrosome of caput and corpus epididymal spermatozoa. When spermatozoa completed acrosome shaping in the cauda epididymidis, F-actin disappeared from the acrosomal area. These findings suggested that the post-testicular shaping of the acrosome might depend on a precise succession of assembly and disassembly of F-actin within the acrosome as the spermatozoa transit through the epididymis. The strong correlation between the occurrence of F-actin and the events of acrosomal shaping in the epididymis suggested that actin filaments might play a significant role in the acrosomal transformation, as they are commonly involved in morphological changes in somatic cells (Ballestrem et al. 1998; Carraway et al. 1998).

Via DNase I fluorescence, this study discovered that a pool of monomeric G-actin existed within the

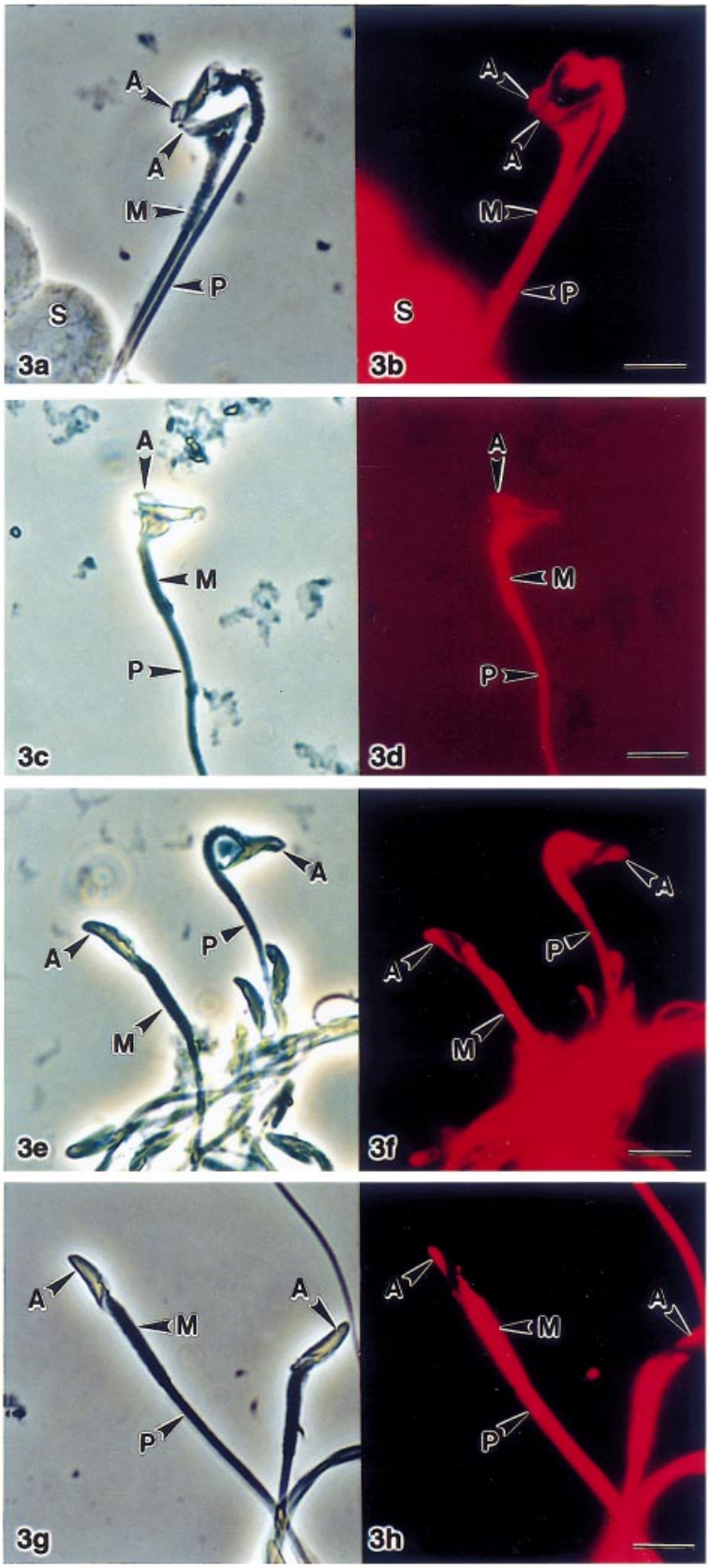

Fig. 3. Phase-contrast and fluorescent micrographs showing G-actin locations in the testicular and epididymal spermatozoa of the tammar wallaby. (*a*, *c*, *e*, *g*) Phase-contrast micrographs of spermatozoa extracted from the testis and caput, corpus and cauda epididymidis respectively. Note the spermatozoa have changed from a T shape to become streamlined, while the acrosome has changed from a scoop shape to a compact 'button' like structure. (*b*) Rh-DNase I fluorescent image of testicular spermatozoa indicating that the G-actin is located on the acrosome (A), midpiece (M) and principal piece (P), but not in the nuclear area. Intense labelling is seen in the cytoplasm of Sertoli cells (S). A similar pattern of the G-actin locations is evident in the spermatozoa from the caput (*d*), corpus (*f*) and cauda (*h*) epididymidis.

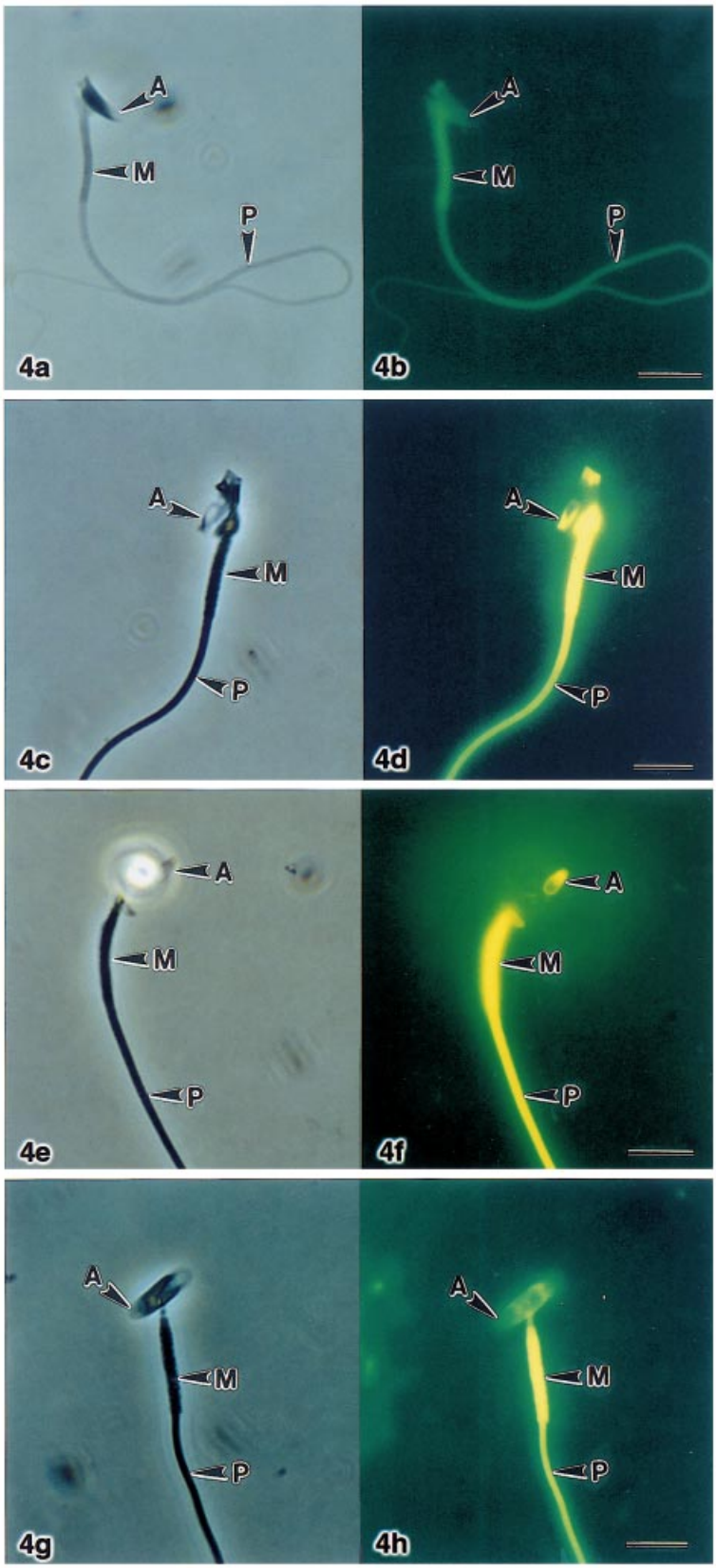

Fig. 4. Phase-contrast and fluorescence micrographs showing F-actin locations in the testicular and epididymal spermatozoa of the tammar wallaby. (*a*, *c*, *e*, *g*) Phase-contrast images of sperm extracted from the testis and the caput, corpus and cauda epididymidis respectively. (*b*) Phalloidin-FITC fluorescence was present at an extremely low level in testicular spermatozoon. (*d*) Intense labelling in the 'scoop' shaped acrosome (A) of a caput epididymal spermatozoon. Strong phalloidin-FITC labelling is also seen on the midpiece (M) and principal piece (P). (*f*) Intense phalloidin-FITC labelling of the infolding acrosome (A) in a corpus epididymal spermatozoon. Labelling of the midpiece (M) and principal piece (P) is also observed. (*h*) Spermatozoon (the streamlined nucleus orientation has been altered by the treatment for staining) from the cauda epididymidis showing no phalloidin-FITC labelling on the 'button' shaped acrosome (A). Strong labelling is seen on the midpiece (M) and principal piece (P).

acrosome of tammar wallaby testicular spermatozoa along with the acrosomes of the caput, corpus, and caudal epididymal spermatozoa. This pool of unpolymerised G-actin provided the means necessary for polymerisation into active F-actin (Wegner, 1976; Orlova & Egelman, 1993; Carraway et al. 1998). There has been no common sperm F-actin pattern reported among the studied species, with the sperm F-actin location even varying between laboratories (Halenda et al. 1987; Castellani-Ceresa et al. 1992; Moreno-Fierros et al. 1992). One of the difficulties was that the sperm F-actin might only appear at certain developmental stages and disappear when its function was completed, as we found in the tammar wallaby acrosome. Since G-actin consistently occurred at the sites of F-actin polymerisation, an exploration of the G-actin subcellular locations would provide a strong basis, and facilitate the investigation of F-actin.

In nonmuscle somatic cells, extensive depolymerisation and repolymerisation of F-actin is thought to be a continuous process with F-actin disappearing and reappearing at different times as it is needed for specific functions (Korn et al. 1987; Theriot & Mitchison, 1991; Orlova & Egelman, 1993; Small et al. 1998). This phenomenon was also observed in the acrosome of tammar wallaby spermatozoa during its post-testicular shaping. The acrosomal F-actin only appeared as the wallaby acrosome changed its shape in the caput and corpus epididymidis, and was not present in the acrosome before its shaping in the testis nor after the completion of acrosomal morphogenesis in the cauda epididymidis. The time frame of the F-actin occurrence implied that F-actin might play a role on the shrinking of the acrosomal scoop or in the condensation of the acrosomal lateral projections, as it facilitates the reorganisation of cellular projections and protrusions in other animal cells (Arpin et al. 1994; Ballestrem et al. 1998; Carraway et al. 1998; Rosenblatt & Mitchison, 1998).

The occurrence of F-actin in the acrosome of wallaby caput and corpus epididymal spermatozoa might also function to support the delicate acrosomal projections, a role played by a remnant of Sertoli cell cytoplasm retained in the acrosomal scoop before the occurrence of F-actin. Our previous TEM study (Lin et al. 1997) had found that the acrosomal scoop contains a remnant of Sertoli cell cytoplasm when tammar wallaby spermatozoa are released into the lumen of the seminiferous tubule. Although in SEM this remnant appeared as a small droplet sitting within the scoop (see Fig. 2*a*), TEM cross sections revealed that the Sertoli cell cytoplasmic remnant fully filled the space of the acrosome scoop and was firmly attached to the inner surface of the lateral projections of the immature acrosome. In the present study, no actin filaments were found in the acrosome of testicular spermatozoa implying that the acrosomal lateral projections, which made up the sides of the scoop, were supported by the Sertoli cell cytoplasmic remnant. When the acrosomal lateral projections condensed and started to fuse together in the caput and corpus epididymidis, the Sertoli cell cytoplasmic droplet lost contact with the scoop projections and appeared to be eliminated from the shrinking scoop. The occurrence of F-actin in the acrosome at this stage suggests that F-actin may stabilise the position and shape of the acrosome during post-testicular shaping of spermatozoa, though the precise role of the actin filaments in acrosome differentiation is unclear.

Most available information on the actin filament and its control mechanisms has been obtained from studies of somatic cells and there is little information for developing sperm cells. It is not unreasonable to suppose that the F-actin organisation, especially its regulation, in the acrosome of epididymal spermatozoa might be different from that in the cytoplasm of somatic cells since the cells are structurally and functionally different from each other. Nearly all information about actin filament effects on cell morphology derives from studies of an 'outward growth' of the somatic cell, such as cell protrusions and extensions. In contrast, the final shaping of mammalian acrosome is an 'inward growth' process, in which elongated acrosomal projections are condensed into a smaller and more compact structure. In addition, it has been suggested that the existence of an autoregulatory pathway for the expression of actin and other F-actin-associated proteins is linked to the status of actin polymerisation in somatic cells (Bershadsky et al. 1995). In spermatozoa, it is long known that DNA expression and RNA transcription are inactivated during the late stages of spermiogenesis in most studied mammals (Monesi, 1970). There is no histological or biochemical evidence of DNA expression in epididymal spermatozoa, since the sperm nucleus has condensed and organelles for protein synthesis discarded before spermatozoa enter the epididymis. Consequently, the proposed mechanism of F-actin feedback control of DNA expression and RNA transcription for somatic cells may not exist in epididymal spermatozoa. Actin binding proteins and other regulators, such as the Rho family of small G-proteins, are most probably stored as proteins in the acrosome if they exist in the epididymal spermatozoa.

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