Original article

Spectrin and ankyrin like proteins in spermatids and spermatozoa of the hamster and some other mammals

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Summary — The presence of spectrin and ankyrin-like proteins was investigated during the differentiation and maturation of spermatozoa in mammalian species which have previously been studied for actin and calmodulin. These actin-binding proteins were characterized by immunoblotting and localized by immunoelectron microscopy. Neither spectrin nor ankyrin could be detected in the F-actin rich subacrosomal layer of spermatids in any species. In hamster and mouse maturing spermatids and spermatozoa, spectrin was mainly evidenced around the fibrous sheath of the flagellum whereas ankyrin was detected only in the neck. In rabbit spermatozoa, spectrin was evidenced in the outermost cytoplasmic layer of the post-acrosomal region and a light ankyrin labeling appeared in the neck. In rat, monkey and human sperm cells, these 2 proteins were not demonstrated. These results showed that as for actin there was no uniform pattern of distribution of spectrin and ankyrin among the 6 species studied.

actin-binding proteins / hamster / spermatozoa / differentiation

Résumé — Spectrine et ankyrine dans les spermatides et spermatozoïdes du hamster et d'autres mammifères. La présence de spectrine et d'ankyrine a été recherchée au cours de la différenciation et la maturation du spermatozoïde de mammifère chez des espèces antérieurement étudiées pour l'actine et la calmoduline. Ces protéines de liaison de l'actine ont été caractérisées par immuno-transfert et mises en évidence par immunocytochimie ultrastructurale. Aucun marquage de spectrine et d'ankyrine n'a été détecté dans la couche subacrosomique riche en actine F des spermatides quelle que soit l'espèce étudiée. Dans les spermatides en maturation et les spermatozoïdes de hamster et de souris la spectrine a été localisée principalement autour de la gaine fibreuse du flagelle tandis que l'ankyrine a été seulement détectée dans le cou. Dans les spermatozoïdes de lapin, la spectrine a été localisée dans le cytoplasme périphérique de la région post-acrosomique et un faible marquage d'ankyrine a été observé dans le cou. Dans les spermatozoïdes du rat, du singe et

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de l'homme ces deux protéines n'ont pas été démontrées. Ces résultats suggèrent que, comme pour l'actine, il n'existe pas un modèle uniforme de distribution de la spectrine et de l'ankyrine dans les spermatozoïdes des six espèces étudiées.

protéines de liaison de l'actine / hamster / spermatozoïde / différenciation

INTRODUCTION

Filamentous actin is one of the most prominent cytoskeletal components of eucaryotic cells. In the erythroid cell model, actin filaments are cross-linked and linked to the plasma membrane by actin binding proteins. First, spectrin *via* a linkage to ankyrin binds short actin filaments to the plasma membrane. Second, spectrin acts to form a 2-dimensional meshwork with these actin oligomers. Third, these associations are regulated by other proteins including protein 4.1, adducin, protein 4.9 and calmodulin (Bennett, 1990).

In addition, in many non erythroid cells, spectrin and ankyrin are universal actin binding proteins, as demonstrated for example in brain tissue (brain spectrin is called fodrin) and enterocytes (Niggli and Burger, 1987; Mangeat, 1988; Carraway and Carothers-Carraway, 1989).

In sperm cells of many mammals, actin has been extensively investigated (for a review, see Oko *et al*, 1991; Fouquet and Kann, 1992). The presence of filamentous actin in the subacrosomal layer of spermatids is the most striking feature during the greater part of spermiogenesis. In testicular and/or epididymal spermatozoa, F-actin is depolymerized to G-actin which is redistributed in a species-specific pattern (Fouquet and Kann, 1992). Up to now there have been only a few reports as regards actin binding proteins in mammalian spermatozoa. Spectrin was demonstrated by immunofluorescence microscopy in human spermatozoa (Virtanen *et al*, 1984) and mouse germ cells (Damjanov *et al*, 1986) as well as in rabbit sperm using im-

munolectron microscopy (Camatini *et al*, 1991). In addition, calmodulin has been characterized in spermatozoa of many mammals and its distribution has also been studied during spermiogenesis (Kann *et al*, 1991). In rabbit sperm head a colocalization for actin, spectrin and calmodulin has been claimed (Camatini *et al*, 1991). Similar information is still lacking in spermatozoa of other species although a colocalization for actin and calmodulin has been recently evidenced in the perinuclear structures of spermatids (Kann *et al*, 1991). Since the function of actin in mammalian spermatids and spermatozoa remains speculative, it would be useful to characterize various actin binding proteins in these cells. Therefore, in this work, the presence of spectrin and ankyrin was investigated during spermiogenesis and sperm epididymal transit in various mammalian species previously studied for actin distribution (Fouquet *et al*, 1989, 1990; Fouquet and Kann, 1992).

MATERIALS AND METHODS

Testes and epididymides of adult hamsters, mice, rats, rabbits and monkeys (*Macaca fascicularis*) were removed under anesthesia. Testicular biopsies and ejaculates from human donors were also used.

Preparation of sperm extracts

Cauda epididymidis spermatozoa were collected as previously described (Fouquet *et al*, 1990). The sperm suspensions were washed twice by centrifugation (2 000 g, 5 min) in PBS containing

4 mM ethylene diamine tetraacetic acid (EDTA) and protease inhibitors: 4 µg/ml diisopropyl fluorophosphate (DFP), 1 µg/ml leupeptin, antipain and pepstatin A, 2 µg/ml aprotinin and 0.5 mM phenyl-methyl-sulfonyl fluoride (PMSF). Pellets of intact sperm (10^9 spermatozoa containing less than 0.1% contaminating cells) were homogenized in 400 µl of 10 mM imidazole buffer pH 7.3, 75 mM KCl, 2 mM $MgCl_2$, 1 mM NaN_3 , 0.5% Triton x 100 and the protease inhibitors at the concentration indicated above. The suspensions were centrifuged for 5 min at 9 000 g in a microfuge. The pellets were mixed in 300 µl of 2 mM EDTA 0.5% sodium dodecyl sulfate (SDS) in the presence of protease inhibitor cocktail and were sonicated 3 x 3 s, incubated 5 min at 25 °C and centrifuged for 5 min in a microfuge (9 000 g). The supernatants were removed and mixed with 2 x sample buffer of Laemmli (1970) and boiled for 5 min.

Antibodies preparation

The purified polyclonal antibody raised against pig brain-spectrin and the purified polyclonal antibody raised against human erythroid cell ankyrin have been previously characterized (Regnoul *et al*, 1985; Kordeli *et al*, 1986). The antispectrin was reactive mainly with α (240 kDa) and faintly with β (235 kDa) subunits of brain spectrin.

SDS-PAGE and immunoblotting

The sperm extracts, 50 µg protein per sample as determined by the procedure of Bradford (1976), were electrophoresed on a 4–10% polyacrylamide gel as described by Laemmli (1970). Immunoblot analysis was performed using conditions described by Frappier *et al* (1991) after electrophoretic transfer of proteins to nitrocellulose.

Tissue preparation for immunocytochemistry

Pieces of testes, epididymides and pellets of human ejaculated sperm were routinely fixed in 1% glutaraldehyde buffered with 0.1 M phosphate pH 7.3 for 60 min at room temperature. After overnight washing in the same buffer containing 0.1 M glycine to block free aldehyde groups, the samples were embedded in Lowicryl K4M according to Carlemalm *et al* (1982) or Altman *et al* (1984). Thin sections were collected on uncoated 200 mesh nickel grids.

Immunogold procedure

This was carried out as previously described (Kann and Fouquet, 1989). The sections were pre-treated in 20 mM Tris buffer pH 7.8, 150 mM NaCl (TBS) containing 0.2% BSA (fraction V, IBF) and 10 mM glycine for 30 min. They were incubated with primary antibodies at a final concentration of 20–40 µg/ml of TBS–0.2% BSA. After a rinse in a stream of TBS–BSA they were incubated with secondary antibodies conjugated to either 10 or 15 nm gold particles (Janssen or Sigma) diluted 1 : 15. Controls either omitting the primary antibodies or using these antibodies pre-adsorbed with a 20-fold molar excess of antigens were also performed to assess the specificity of the immunostaining. The presence of gold particles in these control sections allowed to detect either background or unspecific staining. For the species-specific localization of actin in spermatozoa, different antibodies were used according to Fouquet and Kann (1992) in the hamster a polyclonal antibody recognizing β and γ cytoplasmic actin isoforms (Chailley *et al*, 1986), in the rabbit a monoclonal IgM antibody (code 350, Amersham) recognizing cytoplasmic actin isoforms. For double labelling of actin and calmodulin in rabbit sperm, a polyclonal anticalmodulin (Kann *et al*, 1991) was also used.

Staging and cell identification. The steps of spermiogenesis were classified according to Clermont (1954) for the hamster and Oakberg (1956) for the mouse.

RESULTS

Hamster

Identification of spectrin and ankyrin in sperm extracts

To ascertain the presence of spectrin and ankyrin in spermatozoa, protein extracts were subjected to SDS-PAGE and immunoblotting. The antibody directed against pig brain spectrin recognized in sperm protein extracts one protein band of 240 kDa (fig 1A, lane c) which co-migrated with purified brain spectrin (fig 1A, lane b) and one

Fig 1. A. Detection of brain spectrin-like protein in hamster spermatozoa. Lane a, molecular weight marker proteins (pig brain spectrin, 240–235 kDa; myosin heavy chain, 200 kDa; phosphorylase a, 95 kDa; bovine serum albumin, 67 kDa; actin, 43 kDa). Lane b, immunoblot of purified pig brain spectrin (1 µg). Lane c, immunoblot of sperm extracts. **B.** Immunoblot of ankyrin like proteins: lane a, extracts of human ghost erythrocytes; lane b, sperm extracts; lane c, purified pig brain ankyrin (0.5 µg).

band of 150 kDa. The human erythrocyte ankyrin antibody recognized 2 protein bands of 215 and 186 kDa in ghost erythrocytes extracts (fig 1B, lane a) and one protein band of 215/220 kDa in sperm extracts which co-migrated with purified brain ankyrin (fig 1B, lanes b and c). As expected, these 2 cytoskeletal proteins were found in the Triton x 100 insoluble fraction of sperm extracts.

Immunostaining of spectrin and ankyrin-like proteins

In round spermatids (steps 1–7) spectrin and ankyrin were not detected. In elongating spermatids (steps 8–13), spectrin labelling was observed around the acrosome at the level of the closely associated outer acrosomal membrane and plasma membrane (fig 2A). The Sertoli ectoplasmic specializations surrounding the acrosomal region of these spermatids were not stained. During the maturation phase of spermatids (steps 14–16), the periacrosomal spectrin labelling was predominantly detected on the dorsal (convex) face of spermatid heads. Subplasmalemmal gold particles were not observed in testes sections incubated with brain-spectrin preadsorbed antibody (control: fig 2B). In late spermatids (step 17), the subplasmalemmal spectrin labelling was progressively restricted to the apical part of the acrosome and was still detectable in epididymal spermatozoa. In step 16, spermatid labelling became apparent both for ankyrin in the neck (fig 3) and spectrin in the principal piece of the flagellum (fig 4). In these cells as well as in testicular and epididymal spermatozoa, gold particles detecting ankyrin-like material were found around the connecting piece and the centriole associated body (fig 3, 5A). The specificity of this immunostaining was demonstrated using ankyrin preadsorbed antibody (fig 5B). Spectrin labelling of the principal piece of the tail was localized over the fibrous sheath and/or between the fibrous sheath and the plasma membrane (figs 4, 6, 7, 8). In all step 16 spermatids, the spectrin labelling was restricted to the proximal part of the principal piece near the annulus (fig 4). This spectrin labelling spread progressively in step 17 spermatids (*ie* testicular spermatozoa) (fig 6) and epididymal spermatozoa (fig 7) about halfway along the principal piece. However, spectrin was not detected in the distal part of the principal

Figs 2–4. Hamster spermatids. **2A.** Spectrin labelling in the head of a step 11 elongating spermatid. **2B.** Control using pig brain spectrin pre-adsorbed antibody. Ac, acrosome; subacrosomal layer (➤); Sertoli ectoplasmic specializations (⇔). x 27 000. **3–4.** Step 16 maturing spermatids. **3.** Ankyrin labelling around the segmented columns and the centriole associated body (ca). **4.** Spectrin labelling of the proximal part of the principal piece of the tail. x 40 000.

piece which is devoid of outer dense fibers. The neck and the middle piece of the flagellum were never labelled (fig 8). No staining of the tail could be observed when spectrin pre-adsorbed antibody was used (fig 9). The localization of spectrin as described above and its extent along the principal piece of the flagellum was similar to that observed for actin (compare figs 10 and 11).

Other species

Ankyrin and spectrin-like proteins were undetectable in round and elongating mouse spermatids. However, in late spermatids (step 16) and epididymal spermatozoa, ankyrin immunostaining was detected around the neck (fig 12). In late spermatids, light spectrin labelling also occurred over the fibrous sheath in the proximal part of the tail principal piece. In epididymal spermatozoa (fig 13) this labelling extended backward as described in hamster sperm.

In rabbit testicular and epididymal spermatozoa, a weak ankyrin labelling was also detected in the neck (not shown) whereas spectrin was detected only in the post-acrosomal region. This spectrin labelling was observed in the peripheral layer of the cytoplasm of the post-acrosomal region possibly including the postacrosomal lamina, although the perinuclear substance was unlabelled (fig 14). The post-acrosomal region of rabbit sperm head also contained calmodulin and actin but in the post-acrosomal lamina and perinuclear substance respectively (fig 15).

In rat, monkey and human spermatids and spermatozoa, ankyrin was undetectable. This was equally true for spectrin although some gold particles were occasionally observed over the fibrous sheath of the tail principal piece in monkey epididymal spermatozoa.

DISCUSSION

The purpose of this study was to determine if spectrin and ankyrin, 2 ubiquitous actin-binding proteins were present in mammalian spermatids and spermatozoa. The comparison of the localization of these proteins with that of actin and calmodulin could indicate potential sites of interaction between these various proteins during the differentiation and maturation of sperm cells. In hamster sperm extracts, as in brain extracts, the antispectrin antibody recognized a 240 kDa polypeptide (α spectrin subunit like) and a 150 kDa polypeptide known to arise from Ca^{2+} dependent proteolytic cleavage of the α spectrin subunit (Harris and Morrow, 1990). On the other hand, in sperm extracts, the ankyrin antibody recognized a 215 kDa ankyrin isoform as in ghost erythrocytes (Kordeli *et al*, 1990) and brain extracts (Kordeli *et al*, 1986). Since no-cross-reactivity with other polypeptides could be observed with these antibodies, they were used for immunocytochemical localization of spectrin and ankyrin in sperm, in combination with appropriate controls.

Figs 5–11. Hamster spermatozoa. **5.** Ankyrin labelling (A) and its negative control (B) using ankyrin preadsorbed antibody. **6–8.** Spectrin labelling of the principal piece of the tail. In testicular spermatozoa (fig 6) the midpart of the principal piece is not yet labelled (*) whereas in epididymal spermatozoa (fig 7) it is labelled (*). **8.** The neck and middle piece of epididymal spermatozoa are not labelled. **9.** Negative control for spectrin. **10–11** show spectrin and actin labelling respectively in epididymal spermatozoa. Some background gold particles are indicated in figures 8 and 9 (→). **5A–6,** x 40 000. **5B, 7–11,** x 27 000.

Figs 12–13. Mouse epididymal spermatozoa. **12.** Ankyrin labelling around the neck. x 40 000. **13.** Spectrin labelling over the fibrous sheath of the tail. x 27 000. **Figs 14–15.** Rabbit epididymal spermatozoa. **14.** Spectrin labelling of the outermost cytoplasmic layer of the post-acrosomal region (▶) and some background particles (↗). **15.** Codetection of calmodulin in the post-acrosomal lamina (▶) with 16 nm gold particles and actin with 8 nm gold particles in the innermost cytoplasmic layer of the post-acrosomal region (▶, *ie* postnuclear layer of the perinuclear substance). x 27 000.

As previously demonstrated, actin (Fouquet *et al*, 1990; Fouquet and Kann, 1992) and calmodulin (Kann *et al*, 1991) colocalized in the subacrosomal layer of the spermatids during the greater part of spermiogenesis in all species studied. However, spectrin and ankyrin were not detected in this perinuclear area in any species, neither in the present work nor in previous in-

vestigations (Virtanen *et al*, 1984; Damjanov *et al*, 1986; Camatini *et al*, 1991). Apart from some periacrosomal spectrin labelling in hamster maturing spermatids, similar results were observed in hamster and mouse late spermatids and spermatozoa: ankyrin labelling of the neck and spectrin labelling around the fibrous sheath of the flagellum. Interestingly, in these two

species, the spectrin labelling colocalized with that of actin (Fouquet *et al*, 1990; Fouquet and Kann, 1992). Up to now, mature mouse sperm had been considered devoid of spectrin (Damjanov *et al*, 1986). On the other hand, the unique presence of ankyrin in the neck did not correlate with that of actin. In rabbit sperm, spectrin was detected in the outermost cytoplasmic layer of the post-acrosomal region possibly including the post-acrosomal lamina whereas calmodulin and actin were located in the post-acrosomal lamina and perinuclear substance respectively. These results are in agreement with previously reported locations for actin (Camatini *et al*, 1987; Flaherty *et al*, 1988; Fouquet and Kann, 1992) and calmodulin (Camatini *et al*, 1991; Kann *et al*, 1991) but the presence of spectrin was also reported along all head membranes (Camatini *et al*, 1991) in areas devoid of actin. Thus, there is no true colocalization of actin, spectrin, ankyrin and calmodulin in rabbit sperm head. The significance, if any, of the unique ankyrin and actin co-distribution in the neck remains puzzling. In human spermatozoa we were unable to demonstrate the presence of actin (Fouquet and Kann, 1992) and spectrin and ankyrin (present results). This contrasts with the previously reported presence, in this species of spectrin and actin in the acrosomal cap and principal piece of the tail (Virtanen *et al*, 1984). Likewise, in rat and monkey spermatozoa actin (Fouquet and Kann, 1992) and spectrin and ankyrin were undetectable.

The comparison of previous results with those presented here did not indicate any uniform location of actin, spectrin and ankyrin in mammalian sperm. It should be noted, however, that different isoforms of spectrin (Mangeat, 1988) and ankyrin (Kordeli *et al*, 1990) may be present in various cell types. Therefore, possible sperm and/or species specific-isoforms of these proteins may not have been recognized by

the antibodies used here and in other studies. Thus, the present results must be regarded as preliminary. Even if sperm spectrin and ankyrin were not detected in all their locations, the comparison of species remains valid since a uniform immunocytochemical methodology was used. As for actin (Camatini *et al*, 1992; Fouquet and Kann, 1992) it remains to demonstrate the function of spectrin and ankyrin in mammalian sperm. This contrasts with the unique presence of calmodulin in the post-acrosomal lamina which has been proposed to play a major role in egg activation (Fouquet *et al*, 1991). Even if actin and actin binding proteins seem to be minor proteins in the organization of spermatozoa, their presence and role during spermatid differentiation should be explored. Thus, for example, in all species, actin binding proteins possibly associated with F-actin in the subacrosomal layer of spermatids are still unknown. Apart from the constant presence of calmodulin (Kann *et al*, 1991) neither spectrin and ankyrin (present results) nor vinculin and α -actinin (Scully *et al*, 1987; Pfeiffer and Vogl, 1991; our unpublished observations) could be detected in the subacrosomal layer. Therefore the regulation and function of this unique perinuclear cytoskeletal layer in spermatids remains to be determined.

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