CSIRO PUBLISHING

www.publish.csiro.au/journals/rfd *Reproduction, Fertility and Development*, 2008, **20**, 713–723

Expression and compartmentalisation of the glycolytic enzymes GAPDH and pyruvate kinase in boar spermatogenesis

Sandra Feiden[A], *Uwe Wolfrum*[B], *Gerhard Wegener*[A] *and Günter Kamp*[A,C]

[A]Institute of Zoology, Molecular Physiology Section, Johannes Gutenberg University Mainz, Becherweg 9-11, 55099 Mainz, Germany.

[B]Cell and Matrix Biology Section, Johannes Gutenberg University Mainz, Becherweg 9-11, 55099 Mainz, Germany.

[C]Corresponding author. Email: kamp@uni-mainz.de

Abstract. Boar spermatozoa contain isoforms of both glyceraldehyde 3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) and pyruvate kinase (PK, EC 2.7.1.40). The sperm-specific forms, GAPDH-S and PK-S, are tightly bound to cell structures. By immunofluorescence microscopy GAPDH-S and PK-S were localised in the principal piece of the boar sperm flagellum as well as in the acrosomal region of the sperm head and at the head–midpiece junction. The midpiece of the flagellum, however, contains isoforms of GAPDH and PK that were only recognised by antibodies against somatic GAPDH and PK, respectively, but not by the antibodies against GAPDH-S and PK-S. In sections of boar testis, GAPDH-S and PK-S were first detected in elongating spermatids when both the developing flagellum and the head were labelled with antibodies against GAPDH-S and PK-S. In contrast, antibodies against rabbit muscle GAPDH and PK labelled all developmental stages of germ cells and also neighbouring contractile cells. Thus, the structure-bound sperm-specific enzymes, GAPDH-S and PK-S, appeared only late in spermatogenesis simultaneously with the development of the structures to which they are bound. Anchoring glycolytic enzymes to structures in these mitochondria-free regions may secure ATP-production for both motility and acrosome function.

Additional keywords: glycolysis, PK, spermatozoa.

Introduction

Mammalian spermatozoa are highly-specialised cells that show a conspicuous degree of metabolic compartmentation. Aerobic ATP production is restricted to the midpiece of the flagellum where all mitochondria are concentrated. Glycolysis with lactate as product is an important pathway in mammalian spermatozoa (Mann and Lutwak-Mann 1981), especially in animals such as boar (Kamp *et al*. 2003; Marin *et al*. 2003). Boar spermatozoa lack a shuttle for the transport of high-energy phosphate from the site of aerobic ATP production to the site of ATP consumption (for review see Kaldis *et al*. 1997). Glycolysis can provide ATP locally and independent of mitochondria. There is good evidence that mammalian spermatozoa possess specific isoforms of glycolytic enzymes which are bound at the fibrous sheath, a structure that surrounds the dense fibres and the axoneme of the mitochondria-free principal piece of the flagellum (Visconti *et al*. 1996; Westhoff and Kamp 1997; Bunch *et al*. 1998; Travis *et al*. 1998; Eddy *et al*. 2003; Krisfalusi *et al*. 2006). Glycolytic ATP seems to be particularly important for the vigorous whiplash motility (hyperactivated motility) required for penetrating the zona pellucida of the egg (Fraser and Quinn 1981; Urner and Sakkas 1996; Mahadevan *et al*. 1997; Bone *et al*. 2000; Williams and Ford 2001).

In boar spermatozoa, sperm-specific glyceraldehyde 3-phosphate dehydrogenase (GAPDH-S) and pyruvate kinase (PK-S) were identified with specific antibodies (Westhoff and Kamp 1997; Feiden *et al*. 2007). These isoforms are tightly bound to the fibrous sheath and their solubilisation required tryptic digestion suggesting a covalent binding. Our findings are in line with the results of Krisfalusi *et al*. (2006), who demonstrated binding of both enzymes to isolated fibrous sheath of mouse sperm, as well as Welch *et al*. (1992, 2000, 2006), who reported that GAPDH-S from mouse, human and rat differed from the respective somatic enzyme forms in that their N-termini are elongated which, as we believe, might serve for anchoring the sperm-specific enzymes to the fibrous sheath. This view is further supported by recent evidence that PK-S of boar sperm is N-terminally elongated (Feiden *et al*. 2007).

In mouse and boar spermatozoa, sperm-specific hexokinases (HK1-S) are also bound to the fibrous sheath but, unlike GAPDH-S and PK-S, could be solubilised by the detergent Triton X-100 (Westhoff and Kamp 1997; Travis *et al*. 1998; Krisfalusi *et al*. 2006). Triosephosphate isomerase and 3-phosphoglycerate kinase appear to be hydrophobically attached to GAPDH in boar spermatozoa (Westhoff and Kamp 1997) while the main regulatory enzyme of glycolysis,

 10.1071/RD08004 1031-3613/08/060713

6-phosphofructokinase is soluble, i.e. readily extracted from boar sperm in hypotonic media (Kamp *et al*. 2007). Together, these results suggest a complex organisation of glycolytic enzymes along the fibrous sheath of mature spermatozoa in which various types of binding are involved.

Little is known about the localisation of glycolytic enzymes in the head. Travis *et al*. (1998) found hexokinase in mouse sperm head, and we have labelled 6-phosphofructokinase and pyruvate kinase at the boar acrosome (Feiden *et al*. 2007; Kamp *et al*. 2007). In contrast GAPDH-S could not be found in the head of mouse, human or rat sperm (Bunch *et al*. 1998; Welch *et al*. 2000, 2006). The transporter for glucose and fructose (GLUT8), however, was found in the acrosomal region of mouse and human sperm (Schürmann *et al*. 2002), and GLUT3 was present in membranes of boar sperm head (Medrano *et al*. 2006). One goal of the present paper was to examine whether the antiserum against purified boar GAPDH-S, which labelled the enzyme at the fibrous sheath (Westhoff and Kamp 1997), would also label the acrosome.

Another objective was to study the expression of the various forms of GAPDH and PK during spermatogenesis in order to better understand the possible functions of glycolysis in mammalian sperm. Spermatogenesis comprises a well-ordered sequence of developmental steps, in which the germ cells proliferate, differentiate and mature structurally and physiologically. Spermatogonia located in the periphery of seminiferous tubules of testis undergo mitotic and meiotic divisions thus giving rise to spermatocytes which are more medially located in the tubules. The haploid round spermatids are found near the lumen. During the following spermiogenesis their nuclei elongate and condense so that transcription is fairly depressed (elongating/condensing spermatids). Spermatids develop acrosomes and flagella and turn into elongated/condensed spermatids that are released as premature spermatozoa into the lumen. Some information is available about the expression of GAPDH-S (Bunch *et al*. 1998; Welch *et al*. 2006) and hexokinase (HK-S; Mori *et al*. 1998; Travis *et al*. 1998) in mouse and rat testes. The present paper presents immunofluorescence analyses on the presence of somatic and sperm-specific GAPDH and PK in boar testis to evaluate whether the expression of GAPDH-S and PK-S correlates with the development of acrosomes and flagella in spermatids.

Materials and methods

Samples and chemicals

Fresh ejaculates from fertile boars, diluted 10-fold with BTS (Beltsville Thawing solution), were provided by a breeders' association (64347 Griesheim, Germany). The testes were surgically removed from a sexually-mature and anaesthetised boar by a local veterinarian and immediately transferred to the laboratory on ice. Monoclonal mouse antibodies against glyceraldehyde 3-phosphate dehydrogenase from rabbit muscle (anti-rabbit muscle GAPDH) were obtained from Calbiochem (EMD Biosciences Inc., San Diego, CA, USA), goat antibodies against pyruvate kinase from rabbit muscle (anti-PK-M1) from DPC Biermann (61231 Bad Nauheim, Germany), monoclonal mouse antibodies against boar acrosin from Acris Antibodies GmbH (32120 Hiddenhausen, Germany), peroxidase-conjugated secondary antibodies from Sigma-Aldrich Inc. (Saint Louis, Missouri, USA) and Rockland Immunochemicals Inc. (Gilbertsville, PA, USA), and fluorescence-labelled secondary antibodies from Molecular Probes (2333 AA Leiden, The Netherlands). A rabbit antiserum against purified tightly-bound GAPDH from boar sperm (anti-GAPDH-S) was kindly provided by Dr D. Westhoff (see Westhoff and Kamp 1997). Specific rabbit antibodies against boar PK-S (anti-PK-S) were obtained as described by Feiden *et al*. (2007).

Biochemicals and enzymes were purchased from Roche Diagnostics GmbH (68305 Mannheim, Germany) and Sigma-Aldrich Inc. Jung Tissue Freezing Medium was purchased from Leica Microsystems Nussloch GmbH (69226 Nussloch, Germany), ovalbumin and Tween 20 from AppliChem GmbH (64291 Darmstadt, Germany), and cold-water fish gelatine as well as 4′-6-diamidino-2-phenylindol from Sigma-Aldrich Inc. Mounting medium Mowiol was obtained from Hoechst (65926 Frankfurt, Germany). Other chemicals were purchased from Merck KGaA (64271 Darmstadt, Germany) and Sigma-Aldrich Inc.

Electrophoresis and immunoblotting

These methods were performed as previously described (Feiden *et al*. 2007). For immunochemical detection the antibodies were diluted as follows: mouse anti-rabbit muscle GAPDH 1 : 3200; goat anti-PK-M1 1 : 12 500; anti-mouse IgG 1 : 7000; anti-goat IgG 1 : 10 000. Proteins of boar sperm were extracted with and without trypsin treatment. PK-S preparation was partially purified according to Feiden *et al*. (2007).

Preparation of sperm for immunofluorescence labelling

Samples of 5 μL boar ejaculate diluted 10-fold with BTS were smeared on a poly-L-lysine-precoated coverslip and left to dry. Sperm were then permeabilised with 0.1% (v/v) Triton X-100 in PBS at room temperature for 5 min. For immunofluorescence labelling of acrosin the permeabilisation with Triton was preceded by fixation with 2% (w/v) paraformaldehyde (PFA) in PBS (1 min at room temperature).

Preparation of boar testes

Testes of an 18-month-old breeding boar were prepared for immunofluorescence labelling. Fresh testis parenchyma opposite the epididymis was cut into 10 mm cubes. After swabbing up the blood the cubes were soaked in Tissue Freezing Medium. Frozen isopentane was then melted with a metal rod; the tissue cube was immersed in the molten isopentane and after 30 s transferred into liquid nitrogen (Wolfrum 1990). Until cryostat slicing the frozen cubes were stored in Falcon tubes at −80°C.

Cryostat slicing of tissue cubes from testis and preparation for immunofluorescence labelling

Frozen tissue cubes were cut at −20°C with a Microm HM 560 in slices of 10 μm which were then placed on poly-L-lysine-precoated coverslips and left to dry at room temperature. After microscopic examination suitable slices were fixed and permeabilised with methanol for 6 min and acetone for 1 min at −20°C and left to dry again. For labelling of acrosin, methanol was

replaced by fixation with 2% (w/v) PFA in PBS (1.5 min at room temperature) followed by acetone as before at −20°C. The slices were then encircled with a hydrophobic barrier (PAP-Pen) for immunofluorescence labelling.

Immunofluorescence labelling of GAPDH, PK and acrosin

Sperm and testis slices pretreated as described were covered first with a solution of 0.01% (v/v) Tween 20 diluted in PBS, then with 50 mmol L^{-1} NH_4Cl diluted in PBS, both for 10 min, and washed with PBS. Spermatozoa and the testis slices were then treated with blocking solution (0.5% (w/v) cold-water fish gelatine plus 0.1% (w/v) ovalbumin in PBS) for 2 h at room temperature to saturate non-specific binding sites. This was followed by overnight incubation at 4°C with primary antibodies in blocking solution. Mouse anti-rabbit muscle GAPDH was diluted 1 : 100, rabbit antiserum against purified tightly-bound GAPDH from boar sperm 1 : 3000, goat anti-PK-M1 1 : 10 000, rabbit anti-PK-S 1 : 5, mouse anti-acrosin 1 : 67. On the following day, after washing with PBS, sperm and testis slices were incubated in the dark at room temperature for 2 h with secondary antibodies that were fluorescence-labelled (anti-mouse IgG with Alexa Fluor 488 or anti-rabbit IgG with Alexa Fluor 568 or anti-goat IgG with Alexa Fluor 546; dilution 1 : 400). For labelling of sperm the secondary antibodies were diluted in PBS only, for labelling testis slices they were diluted in PBS containing 4′-6-diamidino-2-phenylindol (DAPI; 1 μg DAPI μL^{-1} PBS). Blue-fluorescent DAPI was used to stain the nuclei. Finally sperm and testis slices were washed in PBS and in bidistilled water and mounted in Mowiol 4.88. Fluorescence labelling was analysed with a DM RP microscope (Leica, 64625 Bensheim, Germany). Images were taken with a Hamamatsu ORCA ER CCD camera (Hamamatsu, 82211 Herrsching, Germany) and processed using Photoshop (Version 7.0; Adobe Systems Inc., San Jose, CA, USA).

Results

Localisation of GAPDH and PK in boar sperm

Four antibody samples were used to localise GAPDH and PK in boar sperm. In order to test the specificity of the antibodies western blot analyses were performed. Purified rabbit muscle GAPDH and PK as well as proteins extracted from boar sperm without (soluble proteins) and with trypsin treatment (tryptically-solubilised proteins) as well as a sample enriched in PK-S were run by SDS–PAGE. In the following western blot analysis anti-rabbit muscle GAPDH exclusively labelled GAPDH of rabbit muscle and boar sperm. The sperm extract obtained after trypsin treatment showed a substantial labelling of the sperm-specific form (GAPDH-S) which counts for more than 90% of total GAPDH activity in boar sperm (Westhoff and Kamp 1997). The fraction of soluble sperm GAPDH (extracted without trypsin treatment) was low, preventing significant labelling. Anti-rabbit muscle PK (anti-PK-M1) labelled not only the rabbit muscle PK but also PK-S obtained after trypsin treatment. Soluble boar sperm PK showed only a faint labelling due to its small amount compared with other soluble proteins. The specificities of the antibodies against the sperm-specific enzymes were published in Westhoff and Kamp (1997) for GAPDH-S and in Feiden *et al*. (2007) for PK-S. The specificity of anti-acrosin is given in the company's manual and could be confirmed with boar sperm extract.

For immunofluorescence microscopy ejaculated boar spermatozoa were permeabilised (with Triton X-100) on poly-L-lysine-precoated coverslips and incubated with anti-rabbit muscle GAPDH (Fig. 1*a*, *c*) or anti-PK-M1 (Fig. 1*b*, *d*), respectively. The antibody fluorescences of images with (Fig. 1*a*, *b*) or without differential interference contrast (DIC; Fig. 1*c*, *d*) indicate the presence of both GAPDH and PK in all parts of sperm, i.e. in the principal piece and the midpiece of the flagellum, as well as in the head. Controls obtained under identical conditions without primary antibodies (Fig. 1*e*, *f*) show only a minor unspecific fluorescence of head and midpiece, which was also seen without the secondary antibodies and was hence due to autofluorescence (not shown). Controls with the primary antibodies that had been preincubated with their specific antigens, rabbit muscle GAPDH or PK, respectively, were only faintly labelled (not shown).

Incubating boar sperm with anti-GAPDH-S or anti-PK-S instead of anti-rabbit muscle GAPDH or anti-PK-M1 resulted in a clearly different pattern. The fluorescence labels were conspicuous in the principal piece of the flagellum, the head–midpiece junction and the acrosomal area of the sperm head but hardly noticeable in the midpiece or in the postacrosomal area of the head (Fig. 2*a*, *b*, *d*, *e*). Under otherwise identical conditions, controls without primary antibodies (Fig. 2*g*, *h*) showed only a weak unspecific red autofluorescence (mainly seen in Fig. 2*h*). Control with anti-PK-S preabsorbed by the specific antigen PK-S peptide was previously performed in immunogold experiments (Feiden *et al*. 2007). Fig. 2*c*, *f* shows the immunofluorescence of acrosin indicating the shapes of acrosomes (control in Fig. 2*i*), which were very similar to that of GAPDH-S and PK-S labelling. This suggests that the two glycolytic enzymes in sperm heads are present at the acrosome and not randomly distributed.

Expression of GAPDH-S and PK-S during boar spermatogenesis

In order to analyse the expression of GAPDH-S and PK-S during spermatogenesis slices of boar testis were fluorescence-labelled with the antiserum against purified boar GAPDH-S or with affinity-purified antibodies specific for boar PK-S. Antibodies against somatic GAPDH and PK were used to probe for the presence of other forms of GAPDH and PK as well. Acrosin and the precursor proacrosin were localised to facilitate the demarcation of spermatids (acrosome formation) from younger stages of germ cells.

First the effects of conservation of testis as well as fixation and permeabilisation of tissue slices on immunofluorescence pattern of (pro)acrosin, GAPDH and PK in the specimens were analysed. Testicular tissue after PFA fixation (3%, at room temperature for 2 h) was used as well as tissue cryosections fixed and permeabilised on poly-L-lysine-precoated coverslips. For fixation and permeabilisation of slices various media were tested; these consisted of PFA, methanol, acetone, and Triton X-100 in various combinations. Acrosin was well-labelled when cryostat slices of unfixed testis were fixed and permeabilised with 2% PFA (at

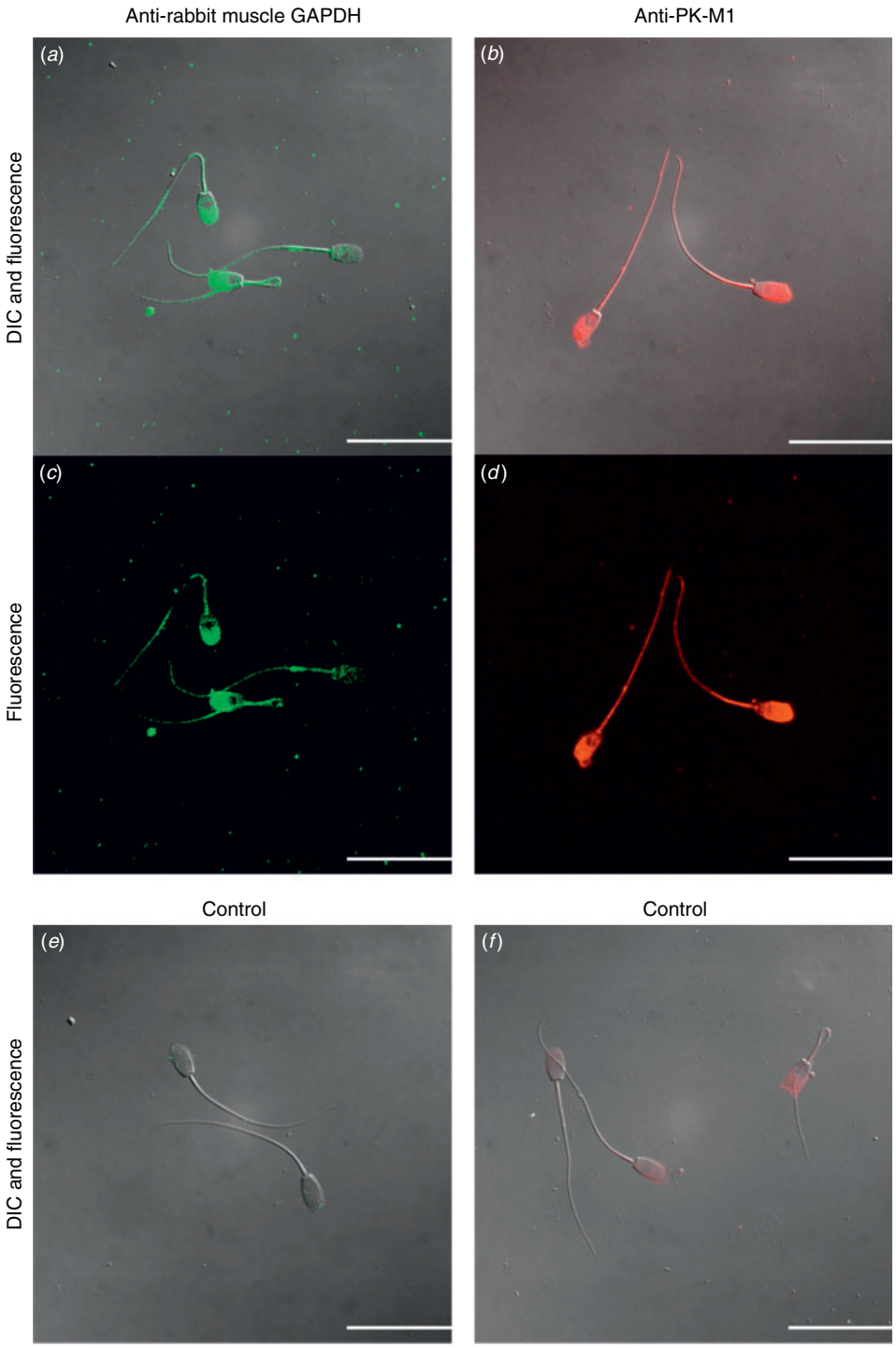

Fig. 1. Localisation of GAPDH and PK in boar spermatozoa. Immunofluorescence of boar sperm merged with differential interference contrast (DIC) images is shown in (*a*) and (*b*), fluorescence only in (*c*) and (*d*). (*a*, *c*) Monoclonal mouse anti-rabbit muscle GAPDH and (*b*, *d*) polyclonal goat anti-PK-M1 were used for labelling GAPDH and PK, respectively, in boar spermatozoa that had been permeabilised with Triton X-100. Both antibodies labelled all sperm compartments, in particular the acrosomal area. Controls without the respective primary antibodies (here merged with DIC images) showed only weak unspecific fluorescence at the (*e*, *f*) acrosome and the (*f*) midpiece. Scale bars = 16.5 μm.

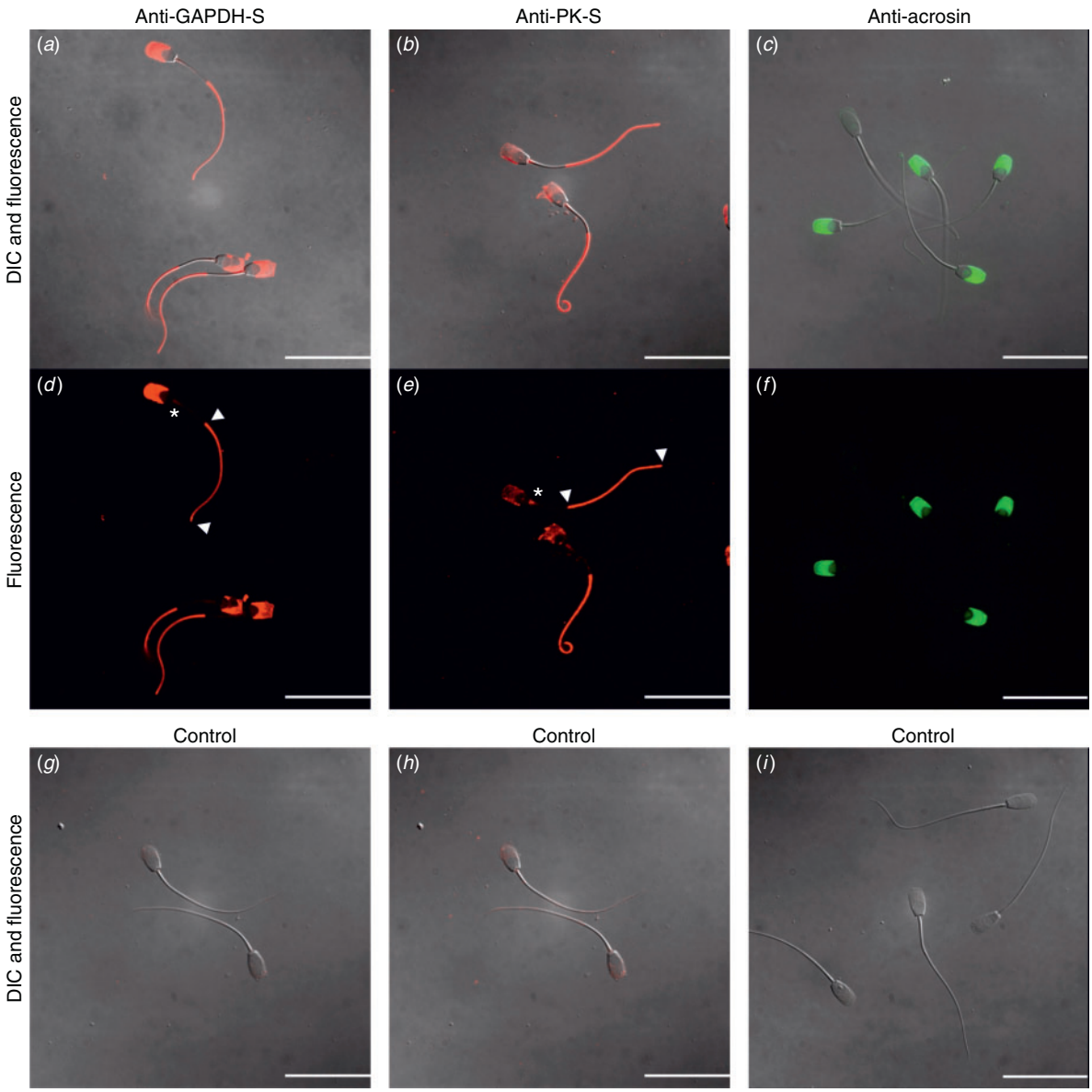

Fig. 2. Localisation of the sperm-specific isoforms GAPDH-S and PK-S and of acrosin in boar spermatozoa. Immunofluorescence of boar sperm merged with differential interference contrast (DIC) images is shown in (*a*), (*b*) and (*c*), fluorescence only in (*d*), (*e*) and (*f*). (*a*, *d*) A rabbit antiserum against purified boar GAPDH-S (anti-GAPDH-S) and (*b*, *e*) immunopurified rabbit anti-PK-S were used for labelling GAPDH-S and PK-S, respectively, in boar spermatozoa that had been permeabilised with Triton X-100. With both antibodies the acrosomal area, the head-midpiece junction (marked by asterisks (∗) in (*d*) and (*e*)) and the flagellar principal piece (between the two white triangles (▽) in (*d*) and (*e*), respectively) were specifically labelled, while the midpiece of boar sperm was hardly fluorescent. (*g*, *h*) The controls without primary antibodies (here merged with DIC images) showed only weak fluorescence, mainly in the acrosomal area. (*c*, *f*) Localisation of acrosin with monoclonal mouse anti-boar acrosin required fixation of sperm with PFA before permeabilisation with Triton. Thus the area of the acrosome could be clearly defined (control without anti-acrosin is shown in (*i*), fluorescence merged with DIC) to confirm the labelling of GAPDH-S and PK-S at the acrosome. The unlabelled sperm at the top on the left of (*c*) and (*f*) indicates a damaged acrosome. Scale bars = 16.5 μm.

room temperature for 1.5 min) and acetone (at −20°C for 1 min) before applying antibodies (Fig. 3). Fluorescent spots appeared first in the area of round spermatids (rs, Fig. 3*a*) indicating developing acrosomal caps. Towards the lumen of tubules, where elongated spermatids (es, Fig. 3*a*) as well as sperm are located, acrosomes were identified due to their typical shape (Fig. 2*c*, *f*). No area of the tubules showed fluorescence in control slices not exposed to primary antibodies (Fig. 3*c*), but the neighbouring Leydig cells displayed autofluorescence, as has been previously reported (Millette *et al*. 1984; Hedger and Eddy 1986). We

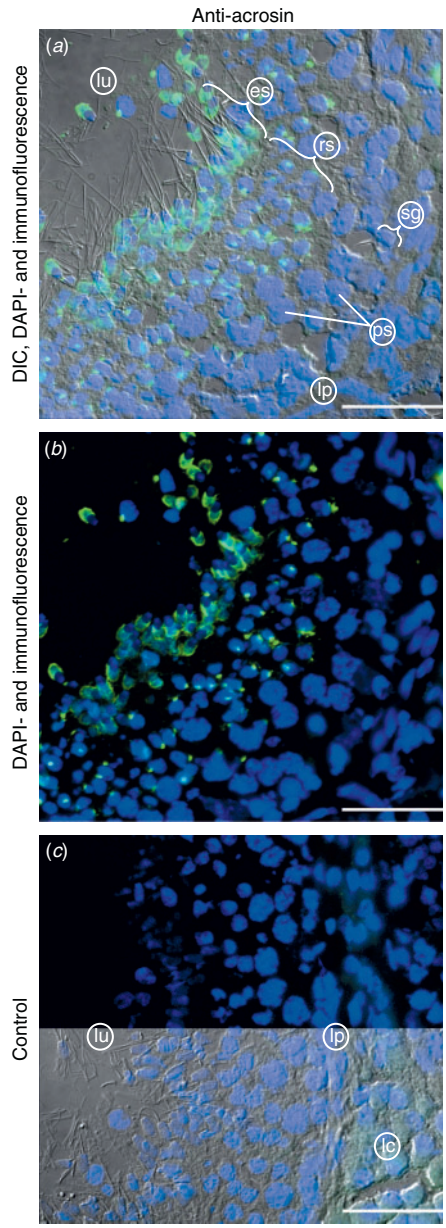

Fig. 3. Localisation of acrosin in boar testis. Immuno- (acrosin = green) and DAPI-fluorescence (blue) from a slice of boar testis merged with the differential interference contrast (DIC) image are shown in (*a*), the merged fluorescence images only in (*b*). Monoclonal mouse anti-boar acrosin was used to localise proacrosin and acrosin in a boar testis slice that had been fixed with PFA and acetone on a coverslip before immunofluorescence labelling. Anti-acrosin labelled spots in the area of round spermatids (rs) as well as the acrosomes in elongated spermatids (es) and of sperm in the lumen (lu) of testicular tubules, whereas younger stages of germ cells (sg, spermatogonia; ps, primary spermatocytes) as well as the lamina propria (lp) were not labelled (marking of tissue areas is shown in (*a*)). In (*c*), the control without primary antibodies is shown (upper half, blue DAPI-fluorescence merged with unspecific green fluorescence; lower half, DAPI-fluorescence and unspecific green fluorescence merged with the DIC image). There was no labelling in the tubule area, but the adjacent Leydig cells (lc) showed unspecific green fluorescence. Scale bars = 26.2 μm in (*a*) and (*b*), and 26 μm in (*c*).

have confirmed these reports in control slices that were neither exposed to primary nor to secondary antibodies (not shown).

For labelling the two glycolytic enzymes, replacing PFA fixation by methanol (see methods) proved advantageous. Using monoclonal anti-rabbit muscle GAPDH, fluorescence was scattered widely, with the highest intensity found in the area of spermatogonia (Fig. 4*a*, *c*) and at the lamina propria (lp, Fig. 4*a*), where contractile cells are localised. Control slices not exposed to primary antibodies were hardly labelled, but as in Fig. 3*c* showed slight autofluorescence of Leydig cells (lc, Fig. 4*e*). Anti-GAPDH-S brought about significant fluorescence only in the area of elongated spermatids and sperm (Fig. 4*b*, *d*). In particular, the flagella of these cells showed intensive red fluorescence, whereas the fluorescence at the head was weaker and more diffuse. Controls without primary antibodies showed no unspecific fluorescence (Fig. 4*f*). Even the autofluorescence of Leydig cells was hardly noticeable because exposure time for detection of the anti-GAPDH-S signal was shorter than with anti-rabbit muscle GAPDH or anti-PK-M1 and anti-PK-S (see below).

The labelling pattern for PK (Fig. 5) was similar to that for GAPDH. Anti-rabbit muscle PK (anti-PK-M1) labelled most of the different cell types inside and outside of seminiferous tubules (Fig. 5*a*, *c*). In contrast, PK-S was detected by anti-PK-S only in elongated spermatids and sperm (Fig. 5*b*, *d*). The controls without primary antibodies showed little unspecific fluorescence inside the tubules, but the Leydig cells showed autofluorescence (Fig. 5*e*, *f*).

Discussion

Compartmentalisation of GAPDH and PK in ejaculated boar spermatozoa

Using two different antibodies against GAPDH forms it has been shown that the sperm-specific form (GAPDH-S) is localised not only in the flagellum along the fibrous sheath but also at the acrosome of boar spermatozoa. The midpiece was not labelled with anti-GAPDH-S but showed a weak labelling with anti-rabbit muscle GAPDH, suggesting the presence of a form other than GAPDH-S. The amount of this form, however, might be small because more than 90% of total GAPDH activity is tightly bound (Westhoff and Kamp 1997). The tightly-bound GAPDH is certainly equivalent to sperm-specific forms of GAPDH-S that were demonstrated at the fibrous sheath in various species such as mouse (Bunch *et al*. 1998; Krisfalusi *et al*. 2006), man (Welch *et al*. 2000), and rat (Welch *et al*. 2006). The localisation of GAPDH-S at the fibrous sheath is uniform in all mammalian species studied so far (see references above) but the presence of GAPDH or GAPDH-S at the acrosome is controversial. In earlier work Westhoff and Kamp (1997) had studied only the flagellum of boar sperm but Bunch *et al*. (1998) and Welch *et al*. (2000, 2006) did not observe fluorescence labelling in the acrosomal area of mouse, human or rat sperm. Whether this discrepancy is species-related or due to experimental differences needs to be tested. Variations in sperm fixation and permeabilisation can alter access to and binding of antigens even if the same antibodies were used. Moreover, the antiserum that we used was most likely directed against sequences at the surface of the native protein, whereas Bunch *et al*. (1998) as well as Welch *et al*. (2000, 2006)

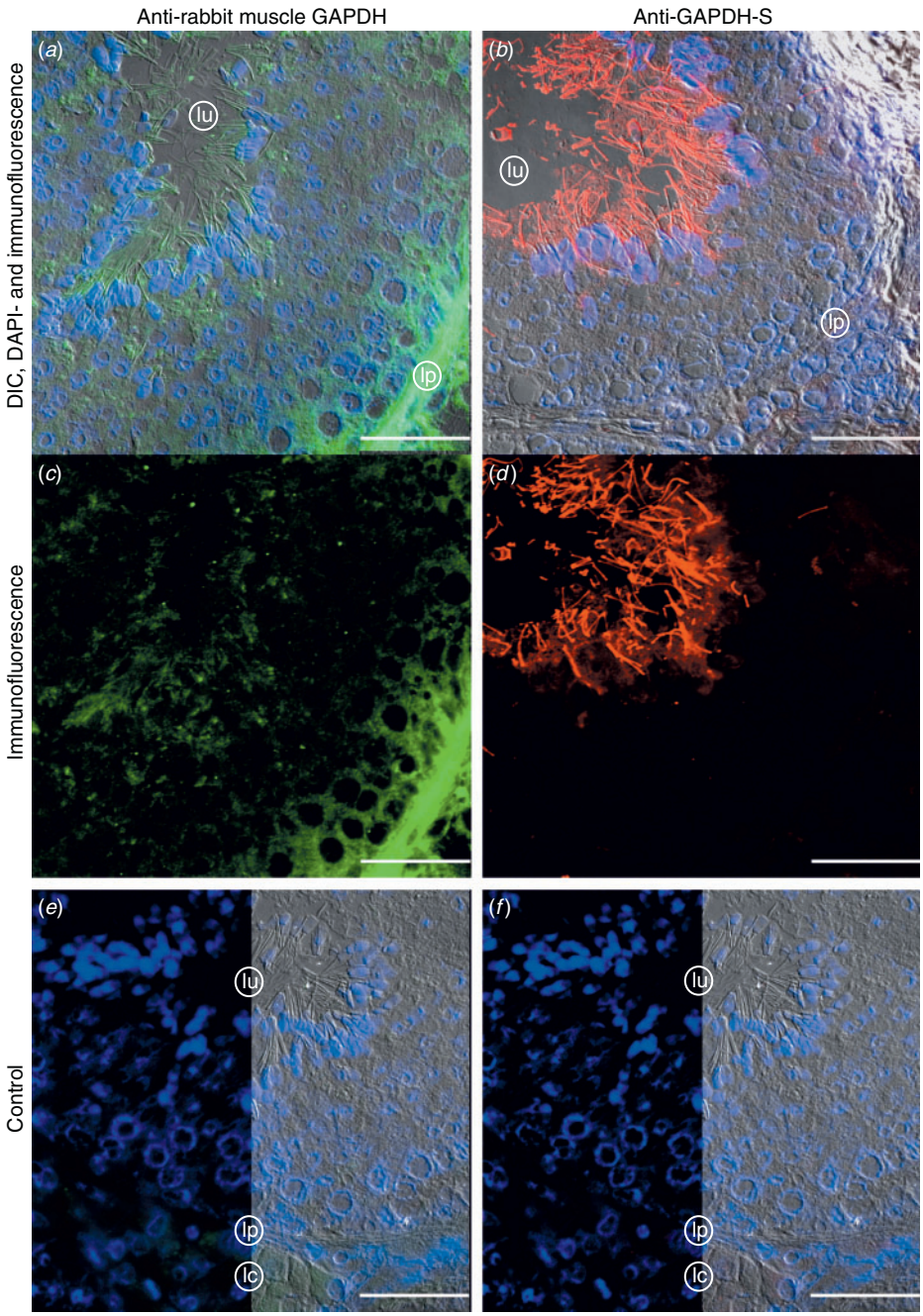

Fig. 4. Expression of GAPDH-S during spermatogenesis in boar testis is first seen in elongated spermatids. In (*a*) and (*b*), immuno- (green = anti-muscle GAPDH and red = anti-GAPDH-S, respectively) and DAPI-fluorescence (blue) from slices of boar testis merged with differential interference contrast (DIC) images are shown. In (*c*) and (*d*), only the fluorescence of GAPDH isoforms are shown. (*a*, *c*) Monoclonal mouse anti-rabbit muscle GAPDH and (*b*, *d*) a rabbit antiserum against purified boar GAPDH-S (anti-GAPDH-S) were used for labelling GAPDH in testis slices that had been fixed with methanol and acetone on a coverslip. Labelling with anti-rabbit muscle GAPDH was spread over all developmental stages with the highest intensities seen in the area of spermatogonia and also at the lamina propria (lp in (*a*)), whereas with anti-GAPDH-S fluorescence was strongest in the luminal areas (lu = lumen in (*b*)) of testicular tubules and restricted to the elongating/elongated spermatids and sperm. Controls without primary antibodies show no unspecific fluorescence inside the tubules but weak unspecific fluorescence from Leydig cells (lc in (*e*) and (*f*)). The left halves of (*e*) and (*f*) show the blue DAPI-fluorescence merged with the unspecific green and red fluorescence, respectively. In the right halves DAPI- and unspecific fluorescence are merged with the respective DIC images. Scale bars = 26 μm.

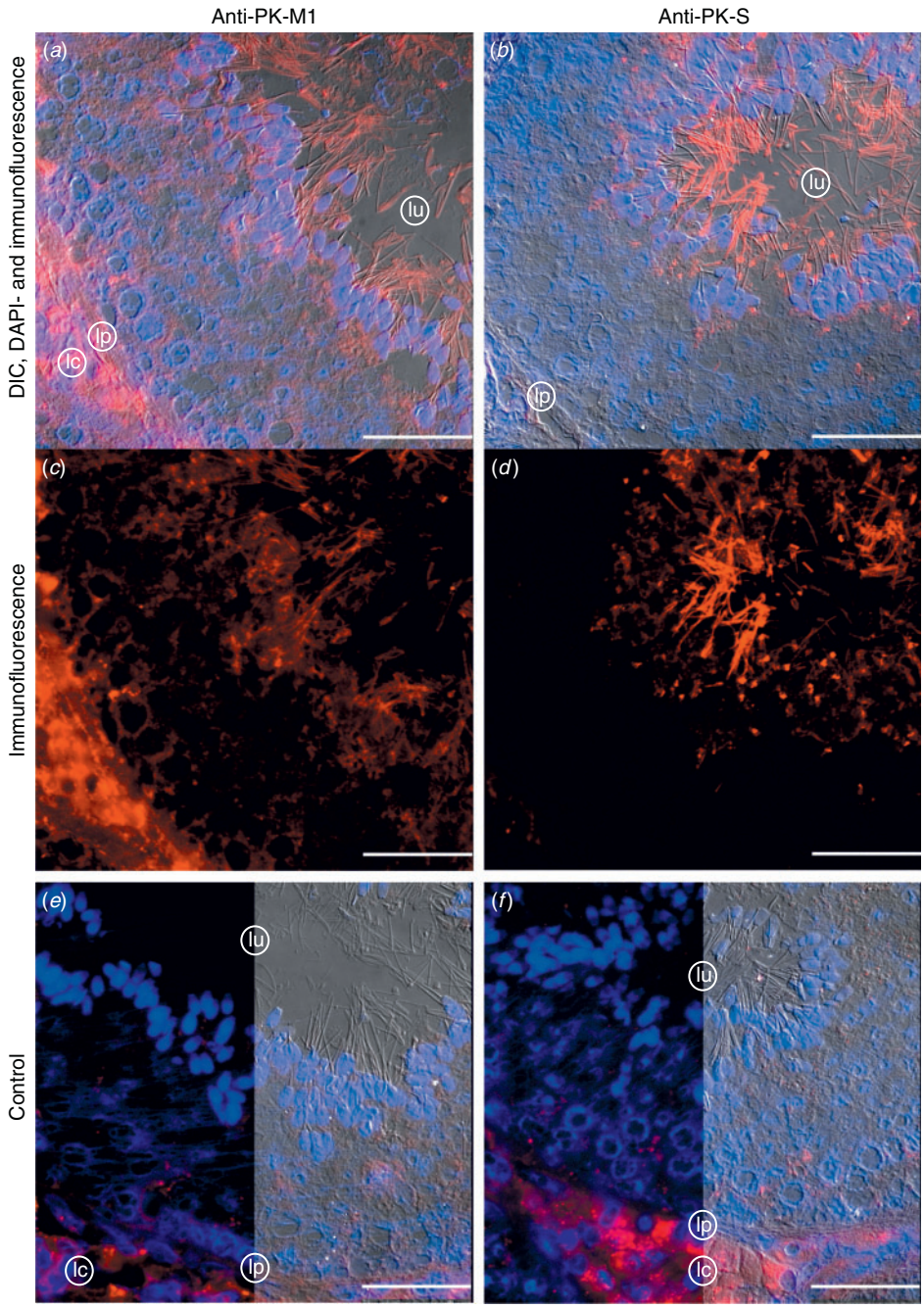

Fig. 5. Expression of PK-S during spermatogenesis in boar testis is first seen in elongated spermatids. In (*a*) and (*b*), immuno- (red) and DAPI-fluorescence (blue) from slices of boar testis merged with differential interference contrast (DIC) images are shown. In (*c*) and (*d*), only the fluorescence of PK isoforms are shown. (*a*, *c*) Polyclonal goat anti-PK-M1 and (*b*, *d*) immunopurified rabbit anti-PK-S were used for labelling PK in boar testis slices which were treated as in Fig. 4. Labelling with these antibodies led to a fluorescence pattern similar to that obtained with the different antibodies against GAPDH (Fig. 4). Most structures are labelled with anti-PK-M1 as opposed to the pattern with anti-PK-S where fluorescence is limited to flagella and head areas of elongated spermatids and sperm. (*e*, *f*) In controls without primary antibodies only minor unspecific fluorescence could be seen inside the tubules, but the autofluorescence of the Leydig cells was prominent. The left halves of (*e*) and (*f*) show blue DAPI-fluorescence merged with unspecific red fluorescence while in the right halves DAPI- and unspecific fluorescence are merged with the respective DIC images. lc, Leydig cells; lp, lamina propria; lu, lumen. Scale bars = 26.2 μm in (*b*) and (*d*), and 26 μm in (*a*), (*c*), (*e*) and (*f*).

raised antisera each against one single GAPDH(-S)-peptide. If the chosen peptide sequences were not accessible to the antibodies *in situ*, owing to protein folding or a shielding micro-environment, the enzyme could not have been labelled.

Affinity-purified anti-PK-S resulted in similar images as with the antiserum against GAPDH-S (Fig. 2; see also Feiden *et al*. 2007) indicating that GAPDH-S and PK-S are the isoforms present not only at the fibrous sheath but also in the acrosomal area (Fig. 2*c*, *f*). The presence of both enzymes at the boar acrosome can be regarded as further evidence for glycolytic activity at the acrosome.

Expression of GAPDH-S, PK-S and proacrosin during boar spermatogenesis

As already known (cf. Gupta 2005), proacrosin was first detected in early spermatids. Not surprisingly, antibodies against the somatic forms of GAPDH and PK labelled cells outside of the seminiferous tubules. Some of the cells are contractile and may have a high glycolytic potential (e.g. lamina propria; Fig. 4*a*, *c*; and Fig. 5*a*, *c*). In Leydig cells GAPDH and PK might also be present, but here the interpretation of the fluorescence is hindered due to the well-known autofluorescence of these cells (Millette *et al*. 1984; Hedger and Eddy 1986). With the exception of the Leydig cells none of the structures showed significant fluorescence in controls that had not been exposed to primary antibodies (Figs 4*e* and 5*e*). An intensive fluorescence was seen in the area of spermatogonia and elongated spermatids while the area between (spermatocytes) showed only weak fluorescence. The strong labelling of the spermatogonia, in particular with anti-rabbit muscle GAPDH, suggests that these cells have a higher glycolytic potential than spermatocytes and round spermatids as had been proposed by Nakamura *et al*. (1984) and Bajpai *et al*. (1998). In contrast to spermatids, the spermatogonia and spermatocytes were neither labelled by anti-GAPDH-S (Fig. 4*b*, *d*) nor by anti-PK-S (Fig. 5*b*, *d*), which indicates that the shift towards expression of the sperm-specific enzyme forms occurs in spermatids.

Our results for boar are in line with reports that GAPDH-S in mouse and rat first appear in condensing (elongating) spermatids (Bunch *et al*. 1998; Welch *et al*. 2006). Expression of GAPDH-S appears to be translationally controlled. While mRNA of GAPDH-S was already found in round spermatids, the GAPDH-S protein could only be labelled in condensing spermatids. The same pattern most likely applies to HK1-S in mice (Mori *et al*. 1998). The report of HK1-S in a fraction of partially-purified round spermatids from mouse testis could be due to contamination as the fraction was only 85% pure (Travis *et al*. 1998). Also the gene for the testis-specific form of 3-phosphoglycerate kinase-2 (PGK-2) was first transcribed in mouse spermatocytes whereas mRNA for PGK-1 was present in spermatogonia yet had disappeared in spermatids (Goto *et al*. 1990; McCarrey *et al*. 1992).

Reports on the expression of PK forms in mammalian spermatogenesis are so far restricted to the mRNA level. Production of PK-M1-mRNA in mouse testes was shown to increase in elongating spermatids while mRNA for PK-M2 decreased during spermatogenesis (de Luis and del Mazo 1998). This was interpreted as an indication that PK-M1 was synthesised in spermatids but the authors could not take into account the novel protein PK-S as this was described only in Feiden *et al.* (2007). In northern blots de Luis and del Mazo (1998) used two primers, one specific for PK-M2 cDNA, the other complementary to a shared region of PK-M1/M2 cDNA that is most likely also present in PK-S. Our findings suggest that the reported increase in PK-M1 mRNA may actually reflect the synthesis of mRNA for PK-S. Since the polyclonal anti-rabbit muscle PK cross-reacted with boar PK-M2 (Feiden *et al*. 2007) it probably labelled PK-M2 as well as PK-M1, which would be consistent with the results of de Luis and del Mazo (1998). Mature sperm seem to have lost PK-M2 (Feiden *et al*. 2007).

Rationale for the synthesis of sperm-specific glycolytic enzymes in spermiogenesis and for glycolytic activity at the acrosome of mature sperm

As discussed above there is increasing evidence that sperm-specific glycolytic enzymes are produced in spermatids, when the nucleus becomes condensed and inactive. Therefore mRNA for glycolytic enzymes might be already present in spermatocytes but translation may be delayed until the acrosome and the fibrous sheath are formed. There is no doubt that mature mammalian spermatozoa are glycolytically active (Mann and Lutwak-Mann 1981; Kamp *et al*. 2003; Marin *et al*. 2003; Miki *et al*. 2004; Mukai and Okuno 2004; Rodriguez-Gil 2006). Even in the presence of oxygen, boar sperm *in vitro* produce more lactate than CO_2 (Kamp *et al*. 2003) indicating that more pyruvate is reduced to lactate than is oxidised by mitochondria. This could be due to the facts that mitochondria are restricted to the midpiece of the flagellum and that the transport of high-energy phosphate from mitochondria to the axonemal dynein-ATPases in the principal piece via a phosphocreatine–creatine kinase shuttle is not feasible in boar and bull sperm (both lack this shuttle; cf. Kamp *et al*. 1996). In several other mammalian sperm the cycle is poorly developed (cf. Kaldis *et al*. 1997). Local production of ATP (and lactate) via glycolysis could significantly attenuate the problem of providing ATP for the dynein-ATPases in the principal piece of the flagellum. This would explain, at least in part, why glycolytic enzymes (such as GAPDH-S and PK-S) are tightly-bound to the fibrous sheath that is present only in the mitochondrial-free principal piece.

Glycolysis could also be vital in the acrosomal area of boar sperm for local supply of ATP to drive ion pumps (e.g. Ca^{2+}-, H^+- and Na^+/K^+-ATPases) and phosphorylation reactions that are involved in acrosome integrity and initiation and control of the acrosome reaction (Nelson and Harvey 1999; Sun-Wada *et al*. 2002; Feng *et al*. 2006; Jimenez-Gonzalez *et al*. 2006; Thundathil *et al*. 2006). If these hypotheses hold good, glycolytic capacity of mammalian sperm would be important for both reaching the egg (motility, especially hyperactivated motility) and entering it (hyperactivated motility and initiation of the acrosomal reaction).

Acknowledgements

We thank Ms. Gabi Stern-Schneider for her excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (Ka

583/7–4), and Sandra Feiden was the recipient of a scholarship (LGFG) from Rheinland-Pfalz.

References

Bajpai, M., Gupta, G., and Setty, B. S. (1998). Changes in carbohydrate metabolism of testicular germ cells during meiosis in the rat. *Eur. J. Endocrinol.* **138**, 322–327. doi:10.1530/EJE.0.1380322

Bone, W., Jones, N. G., Kamp, G., Yeung, C. H., and Cooper, T. G. (2000). Effect of ornidazole on fertility of male rats: inhibition of a glycolysis-related motility pattern and zona binding required for fertilization *in vitro*. *J. Reprod. Fertil.* **118**, 127–135. doi:10.1530/REPROD/118.1.127

Bunch, D. O., Welch, J. E., Magyar, P. L., Eddy, E. M., and O'Brien, D. A. (1998). Glyceraldehyde 3-phosphate dehydrogenase-S protein distribution during mouse spermatogenesis. *Biol. Reprod.* **58**, 834–841. doi:10.1095/BIOLREPROD58.3.834

de Luis, O., and del Mazo, J. (1998). Gene expression of mouse M1 and M2 pyruvate kinase isoenzymes correlates with differential poly[A] tract extension of their mRNAs during the development of spermatogenesis. *Biochim. Biophys. Acta* **1396**, 294–305.

Eddy, E. M., Toshimori, K., and O'Brien, D. A. (2003). Fibrous sheath of mammalian spermatozoa. *Microsc. Res. Tech.* **61**, 103–115. doi:10.1002/JEMT.10320

Feiden, S., Stypa, H., Wolfrum, U., Wegener, G., and Kamp, G. (2007). A novel pyruvate kinase (PK-S) from boar spermatozoa is localized at the fibrous sheath and the acrosome. *Reproduction* **134**, 81–95. doi:10.1530/REP-06-0250

Feng, H. L., Hershlag, A., Han, Y. B., and Zheng, L. J. (2006). Localizations of intracellular calcium and Ca^{2+}-ATPase in hamster spermatogenic cells and spermatozoa. *Microsc. Res. Tech.* **69**, 618–623. doi:10.1002/JEMT.20329

Fraser, L. R., and Quinn, P. J. (1981). A glycolytic product is obligatory for initiation of the sperm acrosome reaction and whiplash motility required for fertilization in the mouse. *J. Reprod. Fertil.* **61**, 25–35.

Goto, M., Koji, T., Mizuno, K., Tamaru, M., Koikeda, S., Nakane, P. K., Mori, N., Masamune, Y., and Nakanishi, Y. (1990). Transcription switch of two phosphoglycerate kinase genes during spermatogenesis as determined with mouse testis sections *in situ*. *Exp. Cell Res.* **186**, 273–278. doi:10.1016/0014-4827(90)90306-U

Gupta, G. S. (2005). 'Proteomics of Spermatogenesis.' (Springer Science + Business Media Inc.: New York.)

Hedger, M. P., and Eddy, E. M. (1986). Monoclonal antibodies against rat Leydig cell surface antigens. *Biol. Reprod.* **35**, 1309–1319. doi:10.1095/BIOLREPROD35.5.1309

Jimenez-Gonzalez, C., Michelangeli, F., Harper, C. V., Barratt, C. L., and Publicover, S. J. (2006). Calcium signalling in human spermatozoa: a specialized 'toolkit' of channels, transporters and stores. *Hum. Reprod. Update* **12**, 253–267. doi:10.1093/HUMUPD/DMI050

Kaldis, P., Kamp, G., Piendl, T., and Wallimann, T. (1997). Functions of creatine kinase isoenzymes in spermatozoa. *Adv. Dev. Biol.* **5**, 275–312.

Kamp, G., Busselmann, G., and Lauterwein, J. (1996). Spermatozoa: models for studying regulatory aspects of energy metabolism. *Experientia* **52**, 487–494. doi:10.1007/BF01919321

Kamp, G., Busselmann, G., Jones, N., Wiesner, B., and Lauterwein, J. (2003). Energy metabolism and intracellular pH in boar spermatozoa. *Reproduction* **126**, 517–525. doi:10.1530/REP.0.1260517

Kamp, G., Schmidt, H., Stypa, H., Feiden, S., Mahling, C., and Wegener, G. (2007). Regulatory properties of 6-phosphofructokinase and control of glycolysis in boar spermatozoa. *Reproduction* **133**, 29–40. doi:10.1530/REP-06-0082

Krisfalusi, M., Miki, K., Magyar, P. L., and O'Brien, D. A. (2006). Multiple glycolytic enzymes are tightly bound to the fibrous sheath of mouse spermatozoa. *Biol. Reprod.* **75**, 270–278. doi:10.1095/BIOLREPROD.105.049684

Mahadevan, M. M., Miller, M. M., and Moutos, D. M. (1997). Absence of glucose decreases human fertilization and sperm movement characteristics *in vitro*. *Hum. Reprod.* **12**, 119–123. doi:10.1093/HUMREP/12.1.119

Mann, T., and Lutwak-Mann, C. (1981). 'Male Reproductive Function and Semen.' (Springer-Verlag: Berlin, Heidelberg, New York.)

Marin, S., Chiang, K., Bassilian, S., Lee, W. N. P., Boros, L. G., Fernandez-Novell, J. M., Centelles, J. J., Medrano, A., Rodriguez-Gil, J. E., and Cascante, M. (2003). Metabolic strategy of boar spermatozoa revealed by a metabolomic characterization. *FEBS Lett.* **554**, 342–346. doi:10.1016/S0014-5793(03)01185-2

McCarrey, J. R., Berg, W. M., Paragioudakis, S. J., Zhang, P. L., Dilworth, D. D., Arnold, B. L., and Rossi, J. J. (1992). Differential transcription of Pgk genes during spermatogenesis in the mouse. *Dev. Biol.* **154**, 160–168. doi:10.1016/0012-1606(92)90056-M

Medrano, A., Garcia-Gil, N., Ramio, L., Montserrat Rivera, M., Fernandez-Novell, J. M., *et al*. (2006). Hexose-specificity of hexokinase and ADP-dependence of pyruvate kinase play important roles in the control of monosaccharide utilization in freshly-diluted boar spermatozoa. *Mol. Reprod. Dev.* **73**, 1179–1194. doi:10.1002/MRD.20480

Miki, K., Qu, W., Goulding, E. H., Willis, W. D., Bunch, D. O., Strader, L. F., Perreault, S. D., Eddy, E. M., and O'Brien, D. A. (2004). Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. *Proc. Natl. Acad. Sci. USA* **101**, 16 501–16 506. doi:10.1073/PNAS.0407708101

Millette, C. F., Laufer, M. R., Owens, N., and Scott, B. K. (1984). Serological and biochemical identification of a plasma membrane antigen specific to Leydig cells. *Biol. Reprod.* **31**, 1037–1048. doi:10.1095/BIOLREPROD31.5.1037

Mori, C., Nakamura, N., Welch, J. E., Gotoh, H., Goulding, E. H., Fujioka, M., and Eddy, E. M. (1998). Mouse spermatogenic cell-specific type 1 hexokinase (mHk1-s) transcripts are expressed by alternative splicing from the mHk1 gene and the HK1-S protein is localized mainly in the sperm tail. *Mol. Reprod. Dev.* **49**, 374–385. doi:10.1002/(SICI)1098-2795(199804)49:4<374::AID-MRD4>3.0.CO;2-K

Mukai, C., and Okuno, M. (2004). Glycolysis plays a major role for adenosine triphosphate supplementation in mouse sperm flagellar movement. *Biol. Reprod.* **71**, 540–547. doi:10.1095/BIOLREPROD.103.026054

Nakamura, M., Okinaga, S., and Arai, K. (1984). Metabolism of pachytene primary spermatocytes from rat testes: pyruvate maintenance of adenosine triphosphate level. *Biol. Reprod.* **30**, 1187–1197. doi:10.1095/BIOLREPROD30.5.1187

Nelson, N., and Harvey, W. R. (1999). Vacuolar and plasma membrane proton-adenosinetriphosphatases. *Physiol. Rev.* **79**, 361–385.

Rodriguez-Gil, J. E. (2006). Mammalian sperm energy resources management and survival during conservation in refrigeration. *Reprod. Domest. Anim.* **41**(Suppl. 2), 11–20. doi:10.1111/J.1439-0531.2006.00765.X

Schürmann, A., Axer, H., Scheepers, A., Doege, H., and Joost, H. G. (2002). The glucose transport facilitator GLUT8 is predominantly associated with the acrosomal region of mature spermatozoa. *Cell Tissue Res.* **307**, 237–242. doi:10.1007/S00441-001-0499-2

Sun-Wada, G. H., Imai-Senga, Y., Yamamoto, A., Murata, Y., Hirata, T., Wada, Y., and Futai, M. (2002). A proton pump ATPase with testis-specific E1-subunit isoform required for acrosome acidification. *J. Biol. Chem.* **277**, 18 098–18 105. doi:10.1074/JBC.M111567200

Thundathil, J. C., Anzar, M., and Buhr, M. M. (2006). Na^+/K^+ ATPase as a signaling molecule during bovine sperm capacitation. *Biol. Reprod.* **75**, 308–317. doi:10.1095/BIOLREPROD.105.047852

Travis, A. J., Foster, J. A., Rosenbaum, N. A., Visconti, P. E., Gerton, G. L., Kopf, G. S., and Moss, S. B. (1998). Targeting of a germ cell-specific type 1 hexokinase lacking a porin-binding domain to the mitochondria

as well as to the head and fibrous sheath of murine spermatozoa. *Mol. Biol. Cell* **9**, 263–276.

Urner, F., and Sakkas, D. (1996). Glucose participates in sperm–oocyte fusion in the mouse. *Biol. Reprod.* **55**, 917–922. doi:10.1095/BIOLREPROD55.4.917

Visconti, P. E., Olds-Clarke, P., Moss, S. B., Kalab, P., Travis, A. J., de las Heras, M., and Kopf, G. S. (1996). Properties and localization of a tyrosine-phosphorylated form of hexokinase in mouse sperm. *Mol. Reprod. Dev.* **43**, 82–93. doi:10.1002/(SICI)1098-2795(199601)43:1<82::AID-MRD11>3.0.CO;2-6

Welch, J. E., Schatte, E. C., O'Brien, D. A., and Eddy, E. M. (1992). Expression of a glyceraldehyde 3-phosphate dehydrogenase gene specific to mouse spermatogenic cells. *Biol. Reprod.* **46**, 869–878. doi:10.1095/BIOLREPROD46.5.869

Welch, J. E., Brown, P. L., O'Brien, D. A., Magyar, P. L., Bunch, D. O., Mori, C., and Eddy, E. M. (2000). Human glyceraldehyde 3-phosphate dehydrogenase-2 gene is expressed specifically in spermatogenic cells. *J. Androl.* **21**, 328–338.

Welch, J. E., Barbee, R. R., Magyar, P. L., Bunch, D. O., and O'Brien, D. A. (2006). Expression of the spermatogenic cell-specific glyceraldehyde 3-phosphate dehydrogenase (GAPDS) in rat testis. *Mol. Reprod. Dev.* **73**, 1052–1060. doi:10.1002/MRD.20235

Westhoff, D., and Kamp, G. (1997). Glyceraldehyde 3-phosphate dehydrogenase is bound to the fibrous sheath of mammalian spermatozoa. *J. Cell Sci.* **110**, 1821–1829.

Williams, A. C., and Ford, W. C. (2001). The role of glucose in supporting motility and capacitation in human spermatozoa. *J. Androl.* **22**, 680–695.

Wolfrum, U. (1990). Actin filaments: the main components of the scolopale in insect sensilla. *Cell Tissue Res.* **261**, 85–96. doi:10.1007/BF00329441

Manuscript received 8 January 2008, accepted 18 May 2008