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· *Review* ·

Proteomic changes in mammalian spermatozoa during epididymal maturation

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Abstract

Epididymal maturation is associated with the activation of a cAMP-induced tyrosine phosphorylation cascade, which is ultimately associated with the expression of capacitation-dependent sperm functions, such as hyperactivated movement and acrosomal exocytosis. As spermatozoa progress through the epididymis they first acquire the capacity to phosphorylate tyrosine on targets on the principal piece, followed by the midpiece. By the time these cells have reached the cauda epididymidis they can phosphorylate the entire tail from neck to endpiece. This particular pattern of phosphorylation is associated with the ontogeny of fully functional spermatozoa that are capable of fertilizing the oocyte. Proteomic analyses indicate that this change is associated with the phosphorylation of several mitochondrial proteins, creation of a mitochondrial membrane potential and activation of mitochondrial free radical generation. At least in rodent species, activation of sperm mitochondria appears to be a particularly important part of epididymal maturation. *(Asian J Androl 2007 July; 9: 554–564)*

Keywords: epididymis; mitochondria; spermatozoa; tyrosine phosphorylation

1 Introduction

When mammalian spermatozoa leave the testes they are still in a functionally immature state and will not become competent to fertilize the oocyte until they have engaged a process of post-testicular maturation in the epididymis. During epididymal transit, the spermatozoa experience a series of poorly characterized post-translational modifications that confer upon these cells the ability to exhibit a burst of vigorous motility upon ejaculation and subsequently undergo a further period of maturation during their ascent of the female reproductive tract. This process is known as "capacitation" [1, 2]. As a consequence of capacitation, spermatozoa are able to bind to the surface of the zona pellucida and to respond to this recognition event with the activation of acrosomal exocytosis and the concomitant remodeling of the sperm surface so that sperm-oocyte fusion can occur [3]. In addition, capacitation confers upon spermatozoa the ability to express a vigorous form of movement, hyperactivation, which generates the propulsive forces necessary for spermatozoa to penetrate the zona pellucida [3]. Given the absolute necessity of epididymal maturation for the generation of fertile spermatozoa, it is clearly important that the molecular mechanisms underpinning this functional transformation be elucidated. Success in this context will have implications both for development of reversible male contraceptive agents and the aetiology of male infertility, which frequently involves defects in aspects of sperm function, such as zona binding or zona-induced acrosomal exocytosis, that are acquired in the epididymis [4–8].

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One of the most remarkable features of the epididymis is the way in which the secretion of specific proteins is spatially restricted to very precisely defined areas of this organ. As a consequence, the microenvironment in which spermatozoa undergo their maturation is constantly changing in a carefully orchestrated sequence. Whether the constituents of the epididymal secretions are soluble [9], contained in large dense granules [10] or exosome-like vesicles [11], they must support and possibly induce the acquisition of sperm function. In addition, by this point in their life history spermatozoa are largely devoid of cytoplasm and heavily dependent on the extracellular milieu provided by the epididymis for protection from various kinds of attack, including infection and oxidative stress. Elucidating the way in which constituents of the epididymal secretions interact with spermatozoa to promote and protect their functional maturation is, therefore, an area of great scientific interest. The approach we have taken to resolve these mechanisms is to focus on the male gamete, to explain the biological changes that are taking place in this cell during epididymal maturation and, from a knowledge of these changes, to deduce how the constituents of the epididymal secretions might be driving the maturation process to completion.

2 Acquisition of sperm function

To determine where specific sperm functions are acquired in the epididymis, spermatozoa were recovered from seven epididymal zones, as defined by Takano [12], and assessed for biological activity [13] (Figure 1). Using this strategy, the capacities of spermatozoa to exhibit coordinated movement, to hyperactivate, to bind to the zona pellucida and to acrosome-react after stimulation with cAMP (achieved through a combination of dibutryl cAMP [dbcAMP] and pentoxifylline) were carefully monitored during epididymal transit.

Analysis of the ability of murine spermatozoa to exhibit coordinated movement using computer-aided sperm analysis revealed a progressive increase in functionality that was initiated in the distal caput (zones 2 and 3) and fully acquired by the proximal corpus epididymidis (zone 4a) regardless of which aspect of sperm kinematics was analyzed (Figure 2A). However, the ability of spermatozoa to exhibit hyperactivated movement in response to a cAMP stimulus was not expressed until the proximal corpus epididymidis and then progressively increased to reach full expression in the distal cauda (zone 5b; Figure 2B). Therefore, the competence for

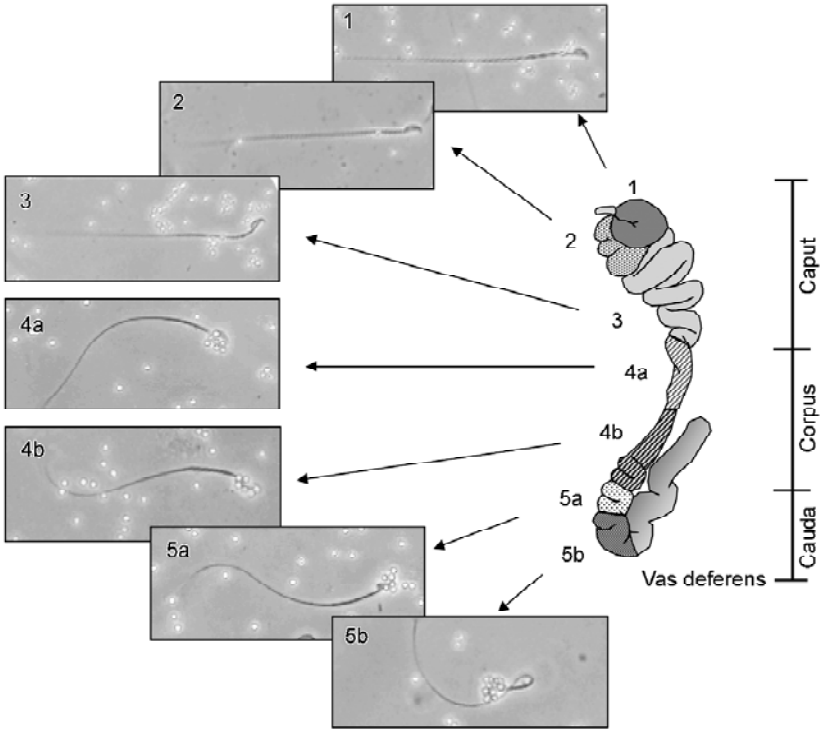

Figure 1. Analysis of the ability of murine spermatozoa recovered from different regions of the epididymis to bind acid-solubilized zona glycoproteins attached to microbeads (Dynabeads M450). The results show that at least some capacitated spermatozoa have acquired the ability to bind to the zona pellucida once they have arrived in the proximal corpus (zone 4a).

coordinated progressive movement appears to be acquired before a majority of the spermatozoa gain the ability to exhibit hyperactivated movement in response to cAMP. Because hyperactivation is dependent on capacitation, and specifically a cellular response to cAMP, it was of interest to examine the ontogeny of other sperm functions that are driven by cAMP, including the abilities to bind to the zona pellucida and to undergo acrosomal exocytosis.

Zona binding was assessed by monitoring the ability of spermatozoa to bind either intact zonae pellucidae or microbeads coated with acid-solubilized zona proteins (Figure 1). Whatever method was used to monitor this aspect of sperm function, the results clearly demonstrate that zona binding is a capacitation-dependent process driven by cAMP [10] and that although subpopulations of spermatozoa capable of binding zona glycoproteins could be identified as early as zone 4a (Figure 1), this response was optimal in caudal epididymal spermatozoa that had acquired the ability to capacitate [10].

Analysis of acrosomal exocytosis in murine spermatozoa in response to cAMP revealed a very similar pattern to hyperactivation and zona binding. Therefore, the ability of the cAMP-treated spermatozoa to respond to A23187 with acrosomal exocytosis began to rise in the proximal corpus and then increased dramatically during the passage of spermatozoa through the distal corpus and caudal regions of the epididymis (Figure 2C).

Overall, this analysis of sperm function in the mouse indicates that the competence for movement was fully acquired as spermatozoa entered the corpus epididymidis. However, other sperm functions, including hyperactivation, zona binding and acrosomal exocytosis did not reach optimal levels until the spermatozoa had reached the cauda and vas deferens.

Because the acquisition of functions such as hyperactivation, zona binding and acrosomal exocytosis are driven by cAMP, and one of the major mechanisms by which mammalian spermatozoa respond to cAMP is through the induction of tyrosine phosphorylation [14–17], we next undertook an analysis of the patterns of cAMP-induced tyrosine phosphorylation as spermatozoa engaged in the process of epididymal maturation [13].

3 Epididymal maturation, tyrosine phosphorylation and the acrosome reaction

Immunofluorescence analysis revealed a profound effect of epididymal maturation on the patterns of tyrosine phosphorylation, as revealed in Figures 3 and 4. In the caput epididymidis, the acrosomal domain was phosphorylated through mechanisms that were quite independent of cAMP (Figures 3A and 4). This fluore-

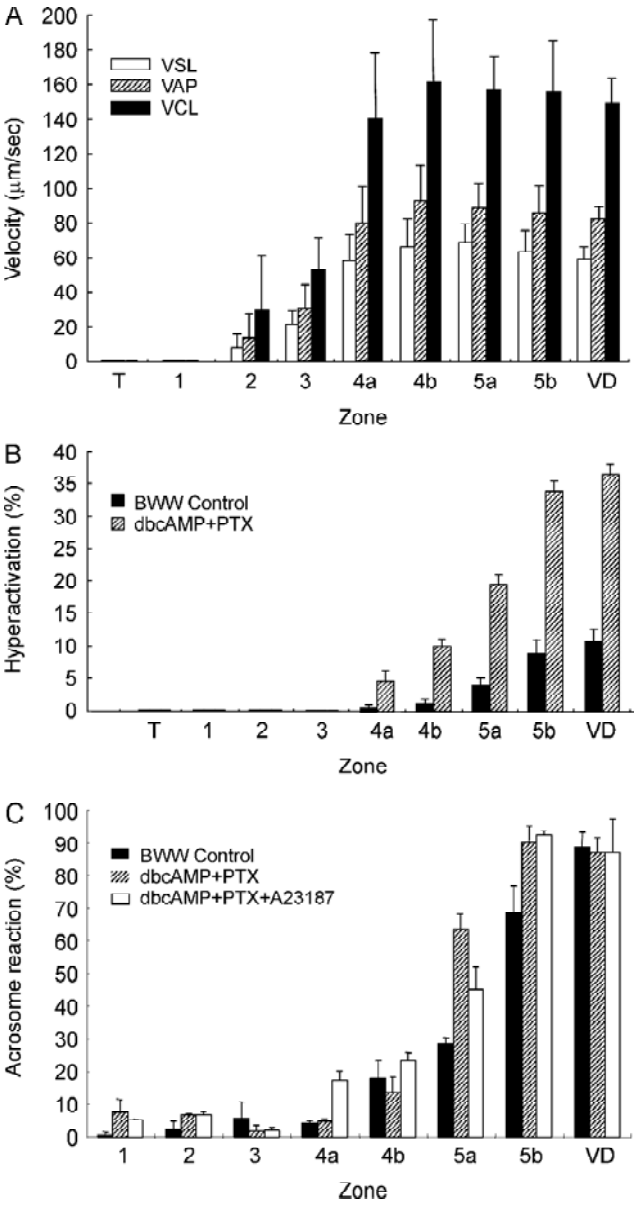

Figure 2. Analysis of the functional attributes of murine spermatozoa at different stages of epididymal maturation. (A): Computer-aided sperm analysis of sperm movement characteristics after stimulation with dibutryl cAMP (dbcAMP) and pentoxifylline (PTX), showing the initiation of motility in the caput epididymis and expression of full motility by the time spermatozoa have reached the proximal corpus (zone 4a). (B): Expression of hyperactivated movement after 45-min incubation in the presence of dbcAMP and PTX; maximal activity was expressed in spermatozoa recovered from the distal cauda (zone 5b) and vas deferens (VD). (C): The capacity of the spermatozoa to acrosome-react; as with hyperactivation, maximal activity was expressed in spermatozoa recovered from the distal cauda (zone 5b) and VD after exposure to dbcAMP and PTX. 1–5b, epididymal zones as described in Figure 1. Reproduced with permission from the Society for the Study of Reproduction, Lin *et al.* [13].

scence remained high throughout the caput and proximal corpus and then decreased in the distal corpus to reach very low levels in the cauda epididymidis. Because this pattern of tyrosine phosphorylation was the mirror image of the ability of epididymal spermatozoa to undergo the acrosome reaction, we reasoned that it might be involved in the suppression of this secretory response. Although a highly significant inverse correlation was observed between the ability of spermatozoa to acrosome-react and the tyrosine phosphorylation of this subcellular compartment ($P < 0.001$), this relationship appeared to be purely correlative [13]. Therefore, using a double labeling procedure that allowed us to assess simultaneously the tyrosine phosphorylation status of murine spermatozoa and their ability to acrosome-react, we examined the interaction of individual cells recovered from the proximal corpus epididymidis with the surface of the zona pellucida. When the subpopulation of capacitated spermatozoa that had bound to the zona pellucida were examined, approximately 50% had acrosome-reacted, and more than half of these cells still exhibited tyrosine phosphorylation in the acrosomal domain. We concluded from this analysis that tyrosine dephosphorylation of the acrosomal domain during epididymal transit is not an essential prerequisite for the acrosome reaction to occur [13].

4 Epididymal maturation, tyrosine phosphorylation and hyperactivation

In contrast to the acrosome reaction, the induction of hyperactivation did appear to be causally linked to changes in the pattern of tyrosine phosphorylation during epididymal maturation. Exposure to cAMP induced a clear tyrosine phosphorylation response in the principal piece of the sperm tail that was already significant in the distal caput and proximal corpus, at which point spermatozoa had no capacity for hyperactivated movement (Figures 2 and 3). However, as the spermatozoa entered the proximal corpus, a variable proportion were able to phosphorylate the midpiece as well as the principal piece. This proportion increased to reach maximal levels in the cauda epididymidis, coincident with the full acquisition of sperm functionality, including hyperactivated movement [10] (Figures 3 and 4). Because the suppression of tyrosine phosphorylation in caudal epididymidal spermatozoa is an extremely effective means of blocking capacitation-dependent sperm functions [10, 13], we conclude that epididymal maturation confers upon spermatozoa the ability to tyrosine-phosphorylate the entire tail (midpiece and principal piece) and that this change is an important prerequisite for the expression of full fertilizing potential.

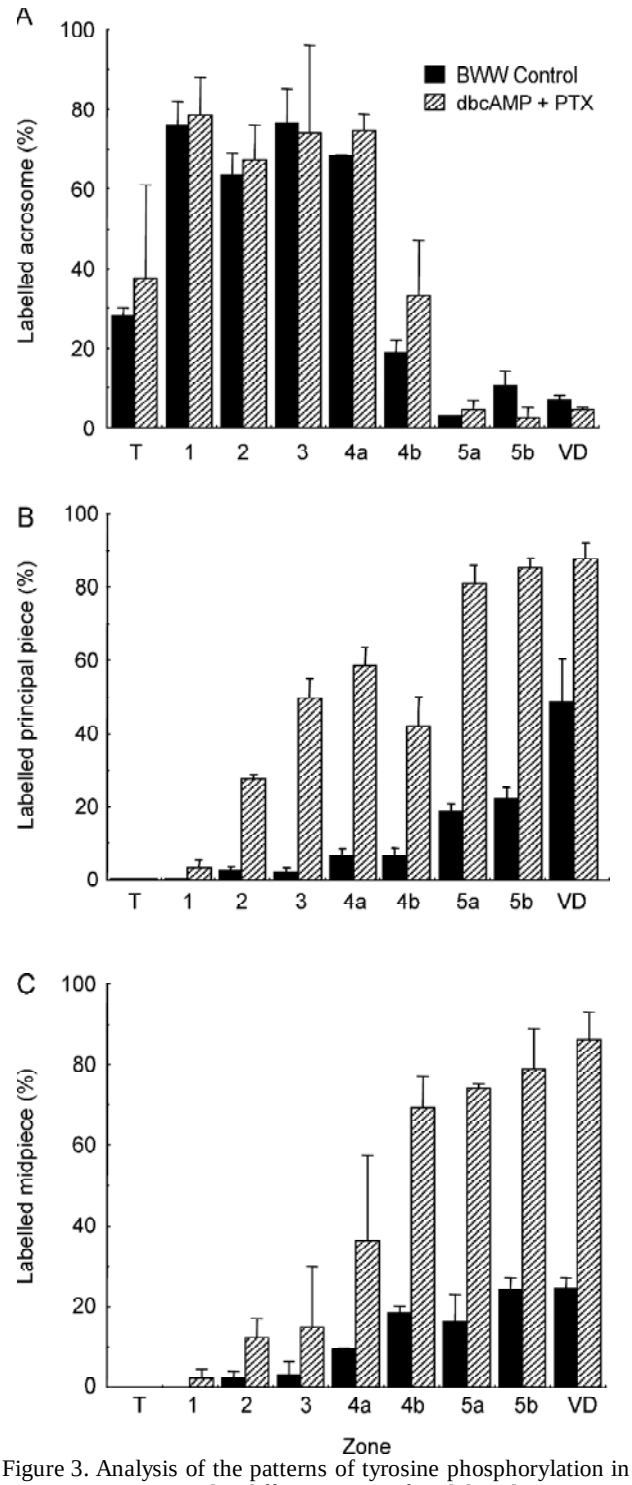

Figure 3. Analysis of the patterns of tyrosine phosphorylation in spermatozoa recovered at different stages of epididymal maturation. (A): Labeling over the acrosome, (B): labeling of the principal piece of the tail and (C) labeling of the tail midpiece. Spermatozoa were incubated in medium BWW containing 1.7 mmol/L calcium with or without supplementation with dbcAMP and pentoxifylline (PTX) for 90 min. T, testes; 1–5b, epididymal zones as described in Figure 1; VD,vas deferens. Reproduced with permission from the Society for the Study of Reproduction, Lin *et al*. [13].

5 Mechanisms for the induction of tyrosine phosphorylation

Given the importance of tyrosine phosphorylation of the sperm tail in the functional differentiation of spermatozoa during epididymal transit, it was clearly important to understand the cellular mechanisms responsible for the activation of this signal transduction cascade. The cascade is clearly initiated by cAMP and mediated by a protein kinase A (PKA)-activated tyrosine kinase. A kinase that fits this description is pp60c src (SRC), a non-receptor tyrosine kinase. Recent studies have established that a kinase of the appropriate size is present in the murine sperm tail and cross-reacts with an anti-SRC antibody [18]. Although this kinase is present in murine spermatozoa at all stages of epididymal maturation, it only associates with PKA in the sperm tails of mature, capacitated spermatozoa (Figure 5). Using an antibody against activated SRC (phosphotyrosine residue at position 416), it was found that the induction of sperm capacitation in mature epididymal spermatozoa led to the activation of this kinase in the sperm tail [18]. Furthermore, C-terminal SRC kinase (CSK), a physiological inhibitor of SRC, was also phosphorylated by PKA in the sperm tail during capacitation. Significantly, phosphorylation of CSK is known to inhibit the activity of this SRC-inhibitor. Therefore, activated PKA achieves an optimal phosphorylation response through the activation of its target kinase, SRC, and simultaneous inhibition of its natural inhibitor, CSK [18]. The relationship between PKA-activated SRC and hyperactivated movement has been confirmed in the mouse using inhibitors against both PKA (H89) and SRC-family tyrosine kinases (SU6656) [18].

The identification of SRC as a critical regulator of the tyrosine phosphorylation during mouse sperm capacitation raises important questions about the status of this kinase in immature spermatozoa. After all, in immature sperm from the caput epididymidis both SRC and

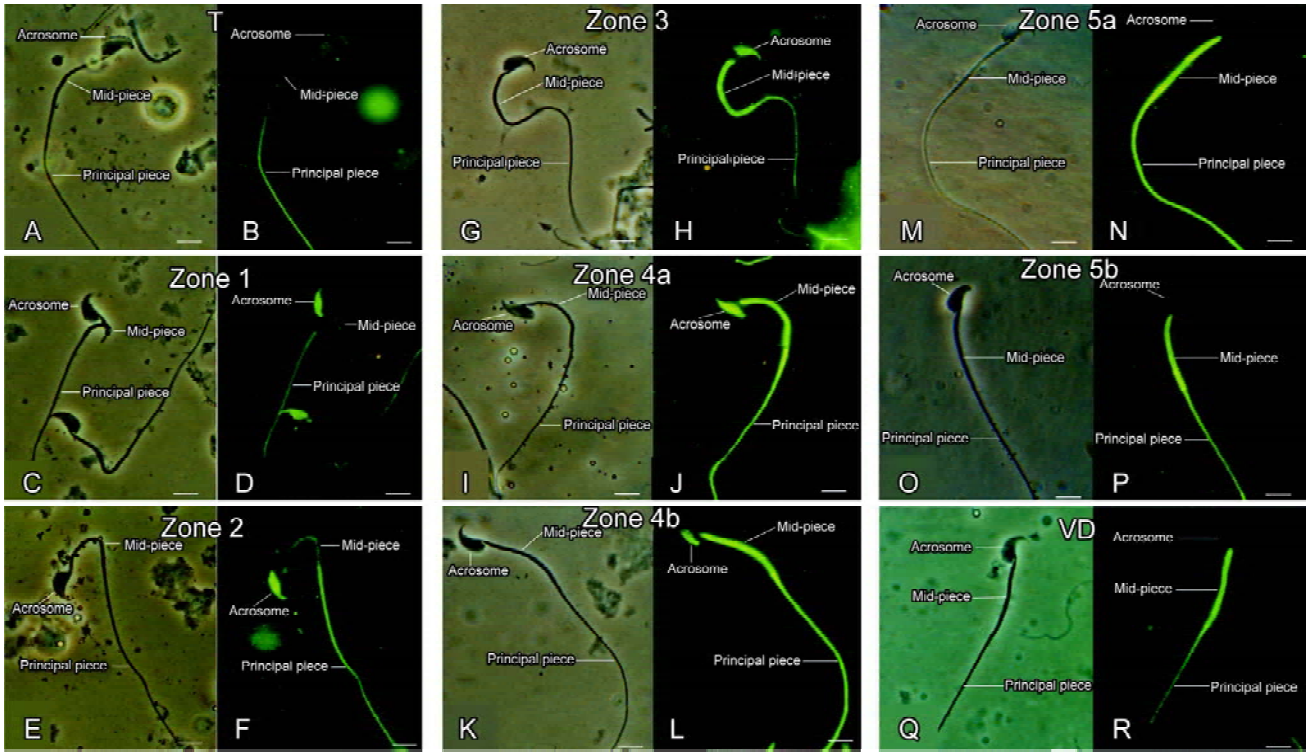

Figure 4. Phase-contrast and immunofluorescence micrographs of mouse spermatozoa showing dynamic changes in the subcellular locations of phosphotyrosine expression after dbcAMP/pentoxifylline (PTX) stimulation. In testicular spermatozoa (A and B), weak phosphotyrosine labeling is seen only on the principal piece of a small number of cells. In zones 1 and 2 of the proximal caput region, tyrosine phosphorylation is observed in the acrosome and principal piece, with little or no labeling in the midpiece (C–F). In zones 3, 4a and 4b phosphotyrosine expression is still observed in the sperm head and principal piece but at this point in epididymal maturation spermatozoa are also capable of exhibiting a strong tyrosine phosphorylation response in the midpiece (G–L). A majority of spermatozoa recovered from the cauda epididymal zones 5a and 5b (M–P) and vas deferens (Q and R) respond to cAMP with intense phosphorylation of the principal piece and midpiece of the sperm tail but exhibit complete dephosphorylation of the acrosome. Scale bar = 5 μm. Left panel, phase contrast; right panel, immunofluorescence. Reproduced with permission from the Society for the Study of Reproduction, Lin *et al.* [13].

PKA are present [18]. Moreover, using a combination of dbcAMP and pentoxifylline we can artificially deliver a cAMP stimulus to the PKA located in these cells. Under such circumstances, one would expect to see the efficient induction of a PKA-activated tyrosine phosphorylation response along the length of the sperm tail at all stages of epididymal maturation. However, as illustrated in Figure 4, this is clearly not the case. Only in mature caudal spermatozoa do we see the efficient induction of tyrosine phosphorylation along the length of the sperm tail and an optimal hyperactivation response. So, why are the caput cells inhibited in this context?

6 Energy status of epididymal spermatozoa

One potential reason for the lack of protein phosphorylation and, hence, function in immature cells from the caput epididymidis is a lack of ATP. Previous studies from our group have shown that tyrosine phosphorylation in caput epididymidal cells is profoundly influenced by the presence of calcium. If calcium is present in the medium in physiological amounts (1.7 mmol/L) then tyrosine phosphorylation in response to cAMP stimulation is profoundly suppressed. In caudal cells, the induction of tyrosine phosphorylation by cAMP is functional in the presence of extracellular calcium, even though the presence of this cation curtails the magnitude of this response [13, 19]. The suppression of calcium-sensitive tyrosine kinases, such has c-yes, have been suggested to account for the negative effect of this cation on tyrosine phosphorylation in mammalian spermatozoa [20]. However, c-yes is present in the sperm head rather than the tail where a majority of the tyrosine phosphorylation is observed. An alternative explanation is that immature caput epididymidal spermatozoa are unable to regulate their intracellular calcium levels effectively. As a consequence, when such cells are placed in calcium-containing medium the intracellular concentration of this cation increases dramatically [21]. This rise in intracellular calcium activates plasma membrane ATPases to remove the excess cytosolic calcium. Under these circumstances, ATP is consumed by these immature cells faster than it can be generated, and ATP levels fall. Because the intracellular availability of ATP is a major rate-limiting factor in the control of kinase activity, tyrosine phosphorylation is suppressed.

In light of these considerations, it becomes important to establish why caput epididymidal spermatozoa are

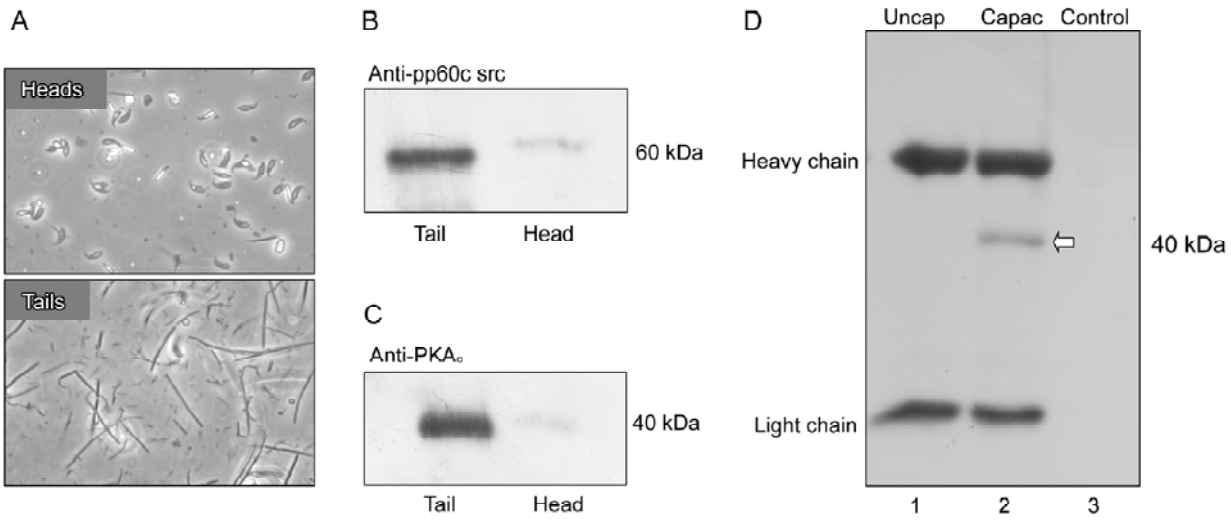

Figure 5. Significance of pp60c src (SRC) in the induction of tyrosine phosphorylation in murine sperm tails during capacitation. (A): Back-flushed murine spermatozoa were sonicated and Percoll-purified to obtain populations consisting of pure (> 95%) sperm heads or sperm tails. (B): Approximately 2 μg of these fractions were then lysed and subjected to 10% SDS PAGE after which Western-blot analysis was performed using anti-SRC antibody. (C): To demonstrate an association between SRC and PKA_c, the above fractions were incubated with beads coated with anti-pp60c-src antibodies and the precipitated proteins probed with an antibody against PKA_c. (D): The importance of sperm capacitation in this association between SRC and PKA_c was also confirmed in experiments involving the inmmunoprecitation of SRC-containing complexes from capacitated (incubated with 1 mmol/L PTX and 1 mmol/L dbcAMP for 45 min) and uncapacitated cells (freshly isolated from the cauda epididymis with no incubation) followed by the interrogation of these immunoprecipitates with anti-PKA_c antibodies. The western blot illustrates uncapacitated spermatozoa (Uncap, lane 1), capacitated spermatozoa (Capac, lane 2) and a control incubation (lane 3) in which beads were incubated with sperm lysates in the absence of antibody. Arrows indicate the location of band of appropriate size cross reacting with the PKA_c antibody. Reproduced with permission from the Company of Biologists, Baker *et al.* [18].

so deficient in their ability to generate the ATP needed to support SRC kinase activity. Proteomic analyses of the post-translational processing of sperm proteins during epididymal maturation suggest that one of the reasons for this deficiency is the impairment of mitochondrial function in immature spermatozoa [22].

A recent advance in proteomics research has been our ability to compare complex electrophoretic profiles using two-dimensional difference gel electrophoresis (2D-DIGE). 2D-DIGE enhances traditional 2D-PAGE by pre-labeling protein mixtures with spectrally resolvable cyanine dyes that are matched for size and molecular mass to minimize any dye-induced changes in electrophoretic behavior [23]. The power of these dyes (Cy2, 3 and 5) comes from their high fluorescence extinction coefficients and the fact that each possesses unique excitation and emission spectra, allowing three differentially labeled protein populations to be analyzed within the same gel. This greatly reduces the number of samples that have to be run in order to determine whether there are significant differences in the position or intensity of any given protein in the course of a proteomic comparison.

Spermatozoa are, in many ways, perfect cells for this kind of analysis. They can be isolated in a highly purified form, exhibit a protein profile of limited diversity, are not subject to variation because of contemporaneous gene expression and can be either obtained in, or driven into, different functional states. Using this technology in combination with mass spectrometry, we have compared the protein profiles of spermatozoa from the caput and cauda epididymidis of the rat and identified a number of significant protein changes [22]. For example, epididymal maturation in the rat is associated with a fivefold increase in the amount of phosphatidyl ethanolamine binding protein (PEBP), a protein that was subsequently shown to possess "decapacitation" activity and, therefore, is critical for the biological silencing of spermatozoa stored in the cauda epididymis [22, 24]. Of particular significance in terms of the present discussion was a twofold increase in the intensity of the β-subunit F1-ATPase. To understand the basis for this change in the F1-ATPase signal a follow-up analysis was performed that examined the profile of proteins that become serine phosphorylated during epididymal maturation. This analysis revealed a major protein spot within the molecular mass range, 55–73 kDa, that was serine phosphorylated during epididymal maturation (Figure 6). When this area was excised from the gel and subjected to MALDI-TOF analysis, one unambiguous protein identification was obtained: the β-subunit of mitochondrial F1-ATPase [22]. Phosphorylation of the F1-ATPase has been reported previously [25], although no biological significance has been assigned to this change, other than an increased rate of elimination.

Further weight was added to the above data, when the proteins undergoing threonine phosphorylation during rat epididymal maturation were examined. In this case, maturation was found to be associated with a dramatic increase in the expression of phosphothreonine-containing proteins. MALDI-TOF analysis of the phosphorylated proteins generated seven confident identifications; namely, glucose regulating protein (GRP78), heat shock protein (HSP70, testes specific), actin, β-tubulin, lactic acid dehydrogenase (LDHC4) and then two mitochondrial proteins, aconitase and the β-subunit F1-ATPase. Immunocytochemical analyses confirmed that phosphothreonine localization was confined to the midpiece (where the mitochondria are located) and principal piece (M. A. Baker, R. Witherdin and R. J. Aitken, unpublished observations)

7 Biochemical evidence of mitochondrial activation

The data presented above strongly suggest that one of the key targets for phosphorylation during epididymal maturation is the mitochondria. If this is the case, we should expect to see biochemical evidence of mitochondrial activation during epididymal maturation. One piece of evidence to support this contention comes from an analysis of reactive oxygen species (ROS) generation by spermatozoa.

It is generally acknowledged that there are two sources of ROS in mammalian spermatozoa: the sperm plasma membrane and the sperm mitochondria [26, 27]. When caudal epididymidal murine [28, 29] or rat [29] spermatozoa are released into an ROS detection system comprising luminol and horse radish peroxidase, a rapid burst of redox activity is observed (Figure 7A, B). Although several putative NAD(P)H oxidase inhibitors, such as DPI, pCMBS and capsaicin, could inhibit this response [29] the fact that a mitochondrial uncoupler CCCP is also inhibitory suggests this oxidative burst involves electron leakage from the mitochondria (R. J. Aitken, Y. H. Lee and A. J. Koppers, unpublished observations). Moreover, the fact that the response could be elevated on treatment with rotenone [29] (Figure 7C) and was massively stimulated by the complex III inhibitor, antimycin, (A. J. Koppers and R. J. Aitken, unpublished observations) suggest that the electron leakage is occurring at complexes I and III of the mitochondrial electron transport chain.

Importantly, this spontaneous mitochondrial generation of ROS is not observed when caput epididymidal spermatozoa are examined, even in the presence of anti-

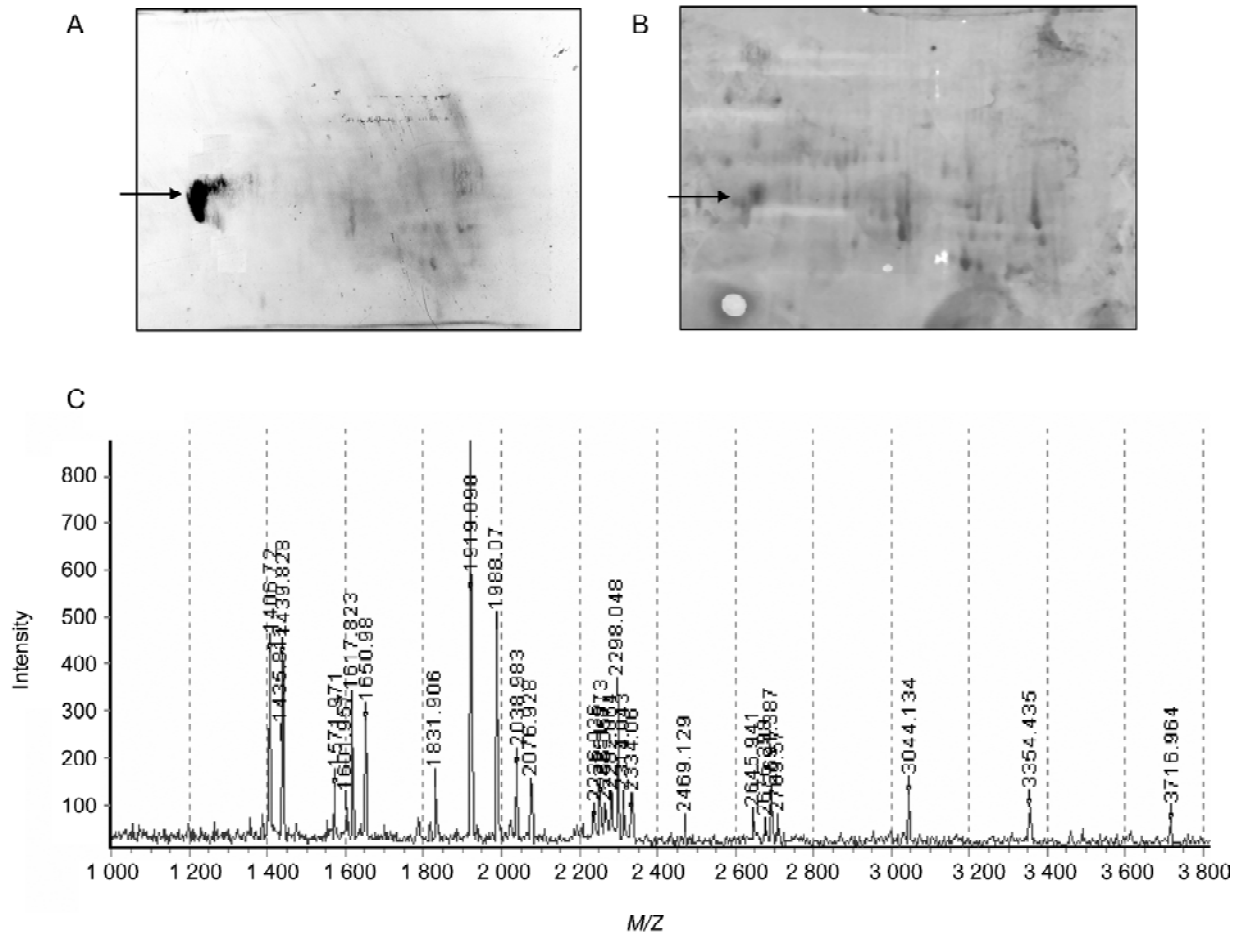

Figure 6. Analysis of phosphoserine containing proteins in relation to epididymal maturation. (A): Using a semi-transfer technique, an anti-phosphoserine Western blot was performed on caudal epididymal spermatozoa while (B): the gel was imaged after staining with Sypro-ruby. The protein serine-phosphorylated during epididymal maturation could be directly cut out of the gel, without ambiguous dectection of the spot. (C): A MALDI-TOF analysis of the spot revealed the protein identification to be the β-subunit of the mitochondrial ATPase [25]. Reproduced with permission from John Wiley & Sons, Inc., Baker *et al.* [22].

mycin (Figure 7D). Therefore, these results are in keeping with the proteomics data in suggesting that caput epididymidal spermatozoa are characterized by silent mitochondria. The fact that Percoll centrifugation can significantly increase the redox activity recorded from such caput epididymal cells suggests that this region of the epididymis elaborates an inhibitor of mitochondrial function [29]. Recent unpublished data from our laboratory corroborate these findings by demonstrating that caudal epididymidal spermatozoa actively maintain a membrane potential as indicated by the fluorescent sensor, JC-1 (Y. H. Lee and R. J. Aitken, unpublished observations). In contrast, caput epididymidal spermatozoa possess no such membrane potential although one can be generated in these cells by thorough washing of the spermatozoa.

Taken together, these data strongly suggest that epididymal maturation is associated with the derepression of sperm mitochondrial function. It is naturally tempting to speculate that this mitochondrial awakening is functionally significant. Specifically, the presence of active ATP-generating mitochondria might facilitate the tyrosine phosphorylation events associated with sperm capacitation, particularly in the context of hyperactivation, which, in the mouse at least, is tightly associated with tyrosine phosphorylation events in the midpiece, where the mitochondria are located. In keeping with this hypothesis, we have recently demonstrated that uncoupling of murine sperm mitochondria with CCCP is associated with the significant suppression of sperm hyperactivation (Y. H. Lee, M. Lin and R. J Aitken, unpublished observations).

Historically, the notion that mitochondria might play

a significant role in sperm hyperactivation has not been seriously considered because of the phenotype of knockout mice lacking the sperm specific form of glyceraldehde 3-phosphate dehydrogenase (GAPDH). Mice lacking this gene generate morphologically normal spermatozoa but are infertile as a result of an almost complete lack of movement. The mitochondria of these spermatozoa are purportedly normal and yet the cells cannot exhibit forward progressive movement even when supplied with appropriate substrates for ATP production. Because the only discernable defect in these spermatozoa is a lack of glycolytic activity, it has been reasonably concluded that glycolysis, and glycolysis alone, provides the energy for sperm movement [30]. However, in Perl *et al.* [31], the phenotype of another mouse is described that contradicts the generality of this conclusion. In his case [31], the gene knocked out was transaldolase. Transaldolase is an enzyme of the non-oxidative phase of the pentose phosphate pathway involved in the generation of NADPH and ribose 5-phosphate. Homozygous knockout animals exhibited an infertility phenotype associated with poor sperm motility that could be rescued by intra-cytoplasmic sperm injection but not *in vitro* fertilization or treatment with N-acetyl cysteine. The spermatozoa of these mice possessed defective mitochondria lacking membrane potential as well as reduced levels of NADPH and GSH, impaired ROS generation, low cytoplasmic and mitochondrial calcium levels and intracellular acidification. Perl *et al.* [31] concluded that mitochondria are directly, or indirectly, essential for the expression of normal fertilizing potential. The apparent discrepancy between these models is difficult to resolve at this stage. It is possible that spermatozoa from the GAPDH knockout mouse suffer from additional undetected abnormalities in the sperm tail. It is also possible that glycolysis is indirectly impaired in the transaldolase knockout mouse as a consequence of intracellular factors such as acidification or loss of glycolytic substrates (glyceraldehyde 3-phosphate and fructose-6-phosphate) resulting from the impaired metabolism of sedoheptulose 7-phosphate by transaldolase. It is also possible that although glycolysis is necessary to initiate basic progressive motility, it is the modulation of this movement by the sperm mitochondria to induce a state of hyperactivation that generates a functional cell capable of fertilizing the oocyte.

The importance of glycolysis for progressive movement is highlighted by the fact that the enzymes that regulate this process are associated with the fibrous sheath in the principal piece of the tail. This would explain why increasing cAMP readily induces tyrosine phosphorylation in the principal piece of the tail in caput cells because, even at this early stage of maturation, glycolysis is active

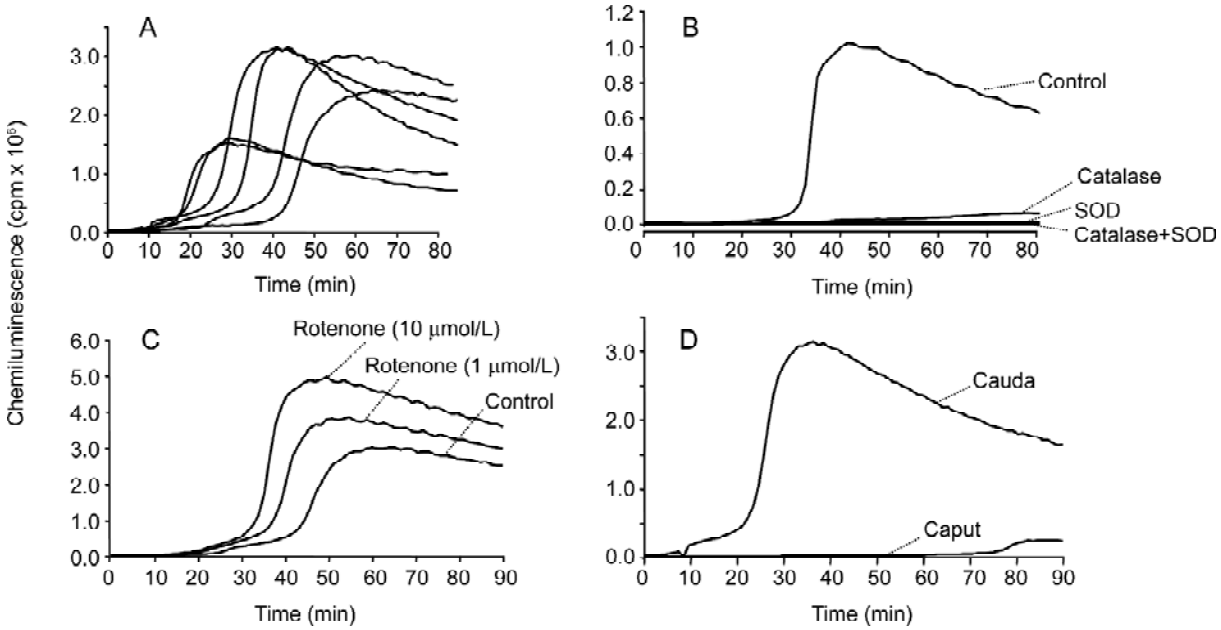

Figure 7. Analysis of spontaneous reactive oxygen species (ROS) generation by rat spermatozoa during epididymal maturation using luminol and peroxidase as the probe [29]. (A): Spontaneous ROS generation by caudal epididymal spermatozoa from six individual animals showing significant inter-individual variation in the kinetics of this response. (B): Spontaneous ROS generation significantly suppressed by catalase and superoxide dismutase (SOD). (C): Rotenone, a complex I inhibitor, enhanced ROS generation by caudal epididymal spermatozoa. (D): Spontaneous ROS generation is a characteristic of caudal but not caput epididymal spermatozoa. Reproduced with permission from Elsevier press, Aitken *et al.* [29].

(albeit less than in caudal sperm [32]) and able to supply ATP to the local tyrosine kinase machinery. However, tyrosine phosphorylation in the midpiece is delayed, reaching maximal levels in the corpus and cauda epididymidis by which time the mitochondria appear fully functional. Because tyrosine phosphorylation of the midpiece appears to be intimately associated with the induction of hyperactivated motility in the mouse [13], it could be argued that the epididymal activation of mitochondrial function plays a key role in capacitation by providing the ATP needed to drive the phosphorylation of targets in the midpiece associated with the expression of hyperactivated movement. At the very least, this concept offers an explanation for the associations between mitochondrial maturation in the epididymis, changing patterns of tyrosine phosphorylation in the sperm tail and the expression of different patterns of sperm movement in the mouse. However, extrapolation of these concepts to other species is fraught with difficulties because there appear to be considerable interspecies differences in the extent to which spermatozoa rely upon oxidative phosphorylation.

8 The control of zona binding

Although the changes in sperm movement associated with epididymal maturation might be linked with changes in the patterns of phosphotyrosine expression in the sperm tail, this explanation cannot apply to sperm–zona interaction, even though the latter is capacitation-dependent and tyrosine phosphorylation-dependent [10]. The ability of murine epididymal spermatozoa to bind to the zona pellucida is first observed in the proximal corpus epididymidis at about the time these cells acquire the potential for movement. However, unlike motility, sperm–zona interaction is dependent on the ability of the spermatozoa to undergo capacitation because non-capacitated cells cannot recognize the surface of the zona pellucida [10]. This dependence on capacitation is associated with the appearance of tyrosine phosphorylated molecular chaperones (endoplasmin and heat shock protein 60) on the sperm surface overlying the anterior acrosome, the exact location where sperm-zona interaction is initiated [10]. On the basis of these results, we have proposed that the activation of these chaperones is involved in the assembly and presentation of an oligomeric zona-receptor complex on the sperm surface [10]. Intriguingly, when the ontogeny of these particular molecular chaperones was traced, they were found to be associated with previously unreported “dense bodies” that appeared in the epididymal lumen at exactly the same region of this organ (proximal corpus) where spermatozoa first acquire the potential to engage in sperm-zona interaction [33]. We hypothesize that the migration of molecular chaperones (and possibly other molecules) from these epididymal bodies to the sperm surface completes the molecular machinery necessary to effect the subsequent orchestrated presentation of zona receptor molecules on the sperm surface in association with lipid rafts [34]. As with the control of hyperactivation reviewed above, caution should be exercised in extrapolating these results to other species. An exhaustive search for molecular chaperones, phosphorylated or otherwise, on the surface of capacitated human spermatozoa has failed to demonstrate similar mechanisms operating in our own species (L. Mitchell, B. Nixon and R. J. Aitken, unpublished observations). Clearly, the fine details of post-testicular sperm maturation show considerable interspecies variation.

9 Conclusion

Epididymal maturation of murine spermatozoa is associated with changes in the pattern of tyrosine phosphorylation that appear to relate to the potential of these cells to exhibit hyperactivated movement. One of the key elements of this process is a cAMP-PKA activated phosphorylation of targets in the sperm midpiece, which only occurs in functionally competent mature spermatozoa. We hypothesize that this pattern of tyrosine phosphorylation is associated with the derepression of mitochondrial function in maturing epididymal spermatozoa, which allows these organelles to contribute ATP to the local tyrosine phosphorylation machinery. If this is the case, elucidating the mechanisms responsible for controlling sperm mitochondria during epididymal transit assumes considerable importance.

Tyrosine phosphorylation of the chaperone proteins on the sperm head is associated with the capacity for zona recognition and, again, this property is only exhibited by mature epididymal spermatozoa. Acquisition of the competence to bind to the zona pellucida is temporally associated with the exposure of spermatozoa to large chaperone laden granules in the epididymal lumen. However, whether this relationship is causative, and if so, the nature of the underlying causative mechanisms, are questions that still remain to be answered.

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