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Review

Protein phosphorylation in mammalian spermatozoa

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Spermatozoa undergo a series of changes before and during egg binding to acquire the ability to fuse with the oocyte. These priming events are regulated by the activation of compartmentalized intracellular signalling pathways, which control the phosphorylation status of sperm proteins. Increased protein tyrosine phosphorylation is associated with capacitation, hyperactivated motility, zona pellucida binding, acrosome reaction and sperm–oocyte binding and fusion. The main tyrosine phosphorylated proteins during the course of capacitation and fertilization are localized to the flagellum, although tyrosine phosphorylation of less abundant proteins may also be regulated in the sperm head. Spermatozoa bound to the zona pellucida and fusing with the oocyte plasma membrane are characterized by a tyrosine phosphorylated flagellum. Protein phosphorylation in the flagellum is linked to hyperactivated motility in spermatozoa, but may also regulate additional functions involved in sperm–oocyte fusion. Factors involved in the appearance of phosphorylation more likely arise from the milieu surrounding the spermatozoa, but their uptake and processing are likely to be regulated differentially at specific steps within the female genital tract and during penetration of the egg vestments. One of these factors is glucose, the metabolic products of which (ATP and NADPH) appear to participate in signalling pathways by supporting a precise onset of tyrosine phosphorylation in the sperm flagellum leading to successful fertilization.

Protein phosphorylation is a post-translational modification of proteins that allows the cell to control various cellular processes. In eukaryotic cells, most phosphorylation occurs on serine or threonine residues and to a lesser extent on tyrosine residues. The phosphorylation state of phosphoproteins is controlled by the activity of protein kinases and phosphatases.

Mature spermatozoa have their own particular idiosyncrasies as highly specialized cells. They are highly compartmentalized, transcriptionally inactive and unable to synthesize new proteins. Therefore, it could be argued that the reliance of mature spermatozoa on protein phosphorylation as a means of altering their function is greater than in many other types of cell. During fertilization, sperm function is regulated by the activation of intracellular signalling systems that control protein phosphorylation. Serine/threonine and tyrosine phosphorylation occur in spermatozoa, but only a few phosphorylated proteins have been identified. Although cAMP-dependent protein kinase A plays a central role in sperm function and has been studied in detail (Visconti and Kopf, 1998), knowledge about tyrosine kinases and other seronine/threonine kinases remains limited.

The processes regulated by protein phosphorylation include capacitation, hyperactivated motility and the acrosome reaction, all of which are required for spermatozoa to reach and fuse with the oocyte. In general, protein phosphorylation in mammalian spermatozoa has been studied by examining phosphorylated proteins in sperm populations during capacitation and after exposure to inducers of the acrosome reaction using western blot analysis. This approach has led to the accumulation of information on the molecular weight of the phosphorylated proteins and their regulation during capacitation and acrosome reaction. Phosphorylation in individual spermatozoa has also been examined by immunocytochemistry, which has revealed compartmentalization of the phosphoproteins in individual spermatozoa and heterogeneity in sperm populations. Knowledge of the phosphorylation pattern in fertilizing spermatozoa and its regulation during gamete interaction remains one of the most important issues in this area of investigation. This review summarizes the accumulated knowledge concerning regulation of protein tyrosine phosphorylation in the

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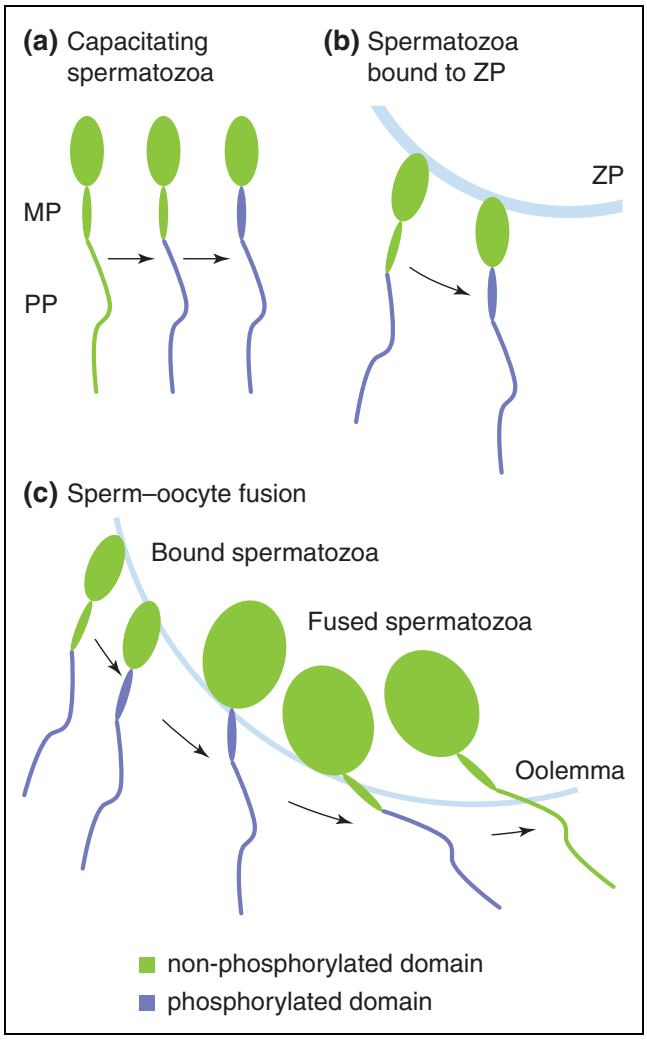

Fig. 1. Protein tyrosine phosphorylation localized to the mouse sperm flagellum during capacitation and interaction with the oocyte. (a) During capacitation, tyrosine phosphorylation appears first in the principal piece (PP) and then in the midpiece (MP) in about 20% of spermatozoa, whereas 15% of spermatozoa remain phosphorylated in the principal piece only. (b) After binding to the zona pellucida (ZP), tyrosine phosphorylation is stimulated in the midpiece, resulting in nearly 100% of spermatozoa phosphorylated in the whole flagellum. (c) After binding to the oolemma, tyrosine phosphorylation is also stimulated in the midpiece. Soon after fusion, the spermatozoa undergoing decondensation present tyrosine phosphorylation in the whole flagellum or restricted to the principal piece. Tyrosine phosphorylation limited to the principal piece in fused spermatozoa may be explained by a process of dephosphorylation of the flagellum after fusion, culminating in the dephosphorylation of the entire flagellum.

different sperm compartments during capacitation and interaction of spermatozoa with the oocyte.

Protein tyrosine phosphorylation in the flagellum

It is necessary to differentiate the localization of the tyrosine phosphorylated proteins in spermatozoa to understand the link between the different phosphorylated proteins and the corresponding regulated sperm function. Except in boars (Petrunkina *et al.*, 2001; Tardif *et al.*, 2001), the flagellum appears to be the principal sperm compartment presenting tyrosine phosphorylated proteins. Immunocytochemistry has been used to localize tyrosine phosphorylated proteins to the flagellum in human (Naz *et al.*, 1991; Carrera *et al.*, 1996; Leclerc *et al.*, 1997), monkey (Mahony and Gwathmey, 1999), hamster (Si and Okuno, 1999), rat (Lewis and Aitken, 2001), and mouse (Urner *et al.*, 2001a) spermatozoa.

Protein tyrosine phosphorylation during capacitation and interaction with the oocyte

Protein tyrosine phosphorylation increases in spermatozoa during capacitation in a number of species including mice, humans, cattle, pigs, hamsters and cats (Leyton and Saling, 1989; Visconti *et al.*, 1995a; Leclerc *et al.*, 1997; Galantino-Homer *et al.*, 1997; Kalab *et al.*, 1998; Pukazhenthi *et al.*, 1998; Si and Okuno, 1999). Protein tyrosine phosphorylation appears to be a necessary prerequisite for a spermatozoon to fertilize an egg (Visconti *et al.*, 1995a; Urner *et al.*, 2001).

Immunofluorescence was used to localize tyrosine phosphorylated proteins to the flagellum during capacitation, zona pellucida binding, oolemma binding and gamete fusion (Urner *et al.*, 2001). A summary of the changes in tyrosine phosphorylation in the mouse flagellum (Fig. 1) shows that the proportion of spermatozoa presenting phosphorylated proteins in the whole flagellum increases with capacitation, and phosphorylation in the principal piece is a prerequisite for phosphorylation in the midpiece (Fig. 1a). A similar increase has been observed in human spermatozoa but was restricted to the principal piece (Sakkas *et al.*, in press). In both mice and men, approximately 50% of spermatozoa remain non-phosphorylated after capacitation, indicating that there are subpopulations of spermatozoa that exhibit a different susceptibility to undergo tyrosine phosphorylation. The possibility that the phosphorylation-positive spermatozoa have a competitive advantage to fertilize an egg remains to be determined. This finding also has implications for certain types of infertility in males, as the proportion of spermatozoa in an ejaculate that are unable to undergo the correct sequence of phosphorylation to achieve capacitation may render them unable to fertilize an egg.

Although tyrosine phosphorylation during capacitation is not required for spermatozoa to bind to the zona pellucida in an *in vitro* system (Pukazhenthi *et al.*, 1998), most mouse spermatozoa binding to the zona pellucida display a tyrosine phosphorylated principal piece, indicating that this pattern of phosphorylation is

beneficial for binding. Binding to the zona pellucida maintains the principal piece phosphorylation and promotes tyrosine phosphorylation in the midpiece (Fig. 1b). Similarly, most of the spermatozoa bound to the oolemma display a tyrosine phosphorylated principal piece, whereas tyrosine phosphorylation in the midpiece is stimulated and maintained until fusion with the oocyte. Nearly all fused spermatozoa display a tyrosine phosphorylated flagellum that is subsequently dephosphorylated (Fig. 1c). In the absence of glucose, both sperm–oocyte fusion and tyrosine phosphorylation in the midpiece are inhibited, indicating that tyrosine phosphorylation in the whole flagellum is mandatory for fusion (Urner *et al.*, 2001).

Significance of protein tyrosine phosphorylation in the flagellum

Tyrosine phosphorylated proteins in the flagellum are linked to sperm hyperactivated motility (Mahony and Gwathmey, 1999; Nassar *et al.*, 1999; Si and Okuno, 1999), which is a motility pattern required for spermatozoa to penetrate the cumulus and the zona pellucida of the oocyte. Exposure of spermatozoa to drugs that increase intracellular cAMP enhances both hyperactivated motility and the proportion of spermatozoa presenting tyrosine phosphorylation in the flagellum in human (Nassar *et al.*, 1999), monkey (Mahony and Gwathmey, 1999) and rat (Lewis and Aitken, 2001) spermatozoa. Glucose, which is required for hyperactivated motility (Cooper, 1984; Williams and Ford, 2001) and zona pellucida penetration (Urner and Sakkas, 1996a), promotes tyrosine phosphorylation in the flagellum in mouse spermatozoa (Urner *et al.*, 2001).

Hyperactivated motility and the associated tyrosine phosphorylation in the flagellum are initiated during *in vitro* capacitation, and their maintenance in spermatozoa bound to the zona pellucida is of great importance (Stauss *et al.*, 1995) and explains why the zona pellucida promotes the tyrosine phosphorylation of proteins in the flagellum. After penetration through the zona pellucida, the fertilizing spermatozoon binds to the oolemma and membrane fusion occurs subsequently between the equatorial segment of the sperm head and the microvilli of the oocyte. In addition, the sperm flagellum appears to fuse at multiple sites with the oocyte plasma membrane (Simerly *et al.*, 1993). This process requires calcium (Yanagimachi, 1978) and glucose (Urner and Sakkas, 1996b; Redkar and Olds-Clarke, 1999), but the molecular events occurring during binding and fusion have yet to be elucidated. In contrast to the events during sperm penetration through the zona pellucida, hyperactivated motility is not required for spermatozoa to fuse with the oolemma. It is possible that the tyrosine phosphorylated proteins in the flagellum are linked to another sperm function important for both zona pellucida and oocyte penetration and that a common pathway is activated in spermatozoa interacting with the zona pellucida and the oolemma. The identification of the tyrosine phosphorylated proteins in the mouse midpiece would provide further insight into the specific role of this pattern of phosphorylation and how it influences membrane fusion occurring between the sperm equatorial segment and the oolemma.

Tyrosine phosphorylated proteins in the flagellum

In human spermatozoa, the A-kinase anchoring proteins (AKAPs) localized to the fibrous sheath AKAP82, its precursor pro-AKAP82, and FSP95 are the most prominent tyrosine phosphorylated proteins during capacitation (Carrera *et al.*, 1996; Mandal *et al.*, 1999), indicating that their affinity for the regulatory subunit of protein kinase A or other molecules is regulated by their phosphorylation status. An 86 kDa calcium-binding and tyrosine phosphorylation-regulated protein (CABYR) is localized to the principal piece of human spermatozoa (Naaby-Hansen *et al.*, 2002). Tyrosine phosphorylated CABYR acquires the capacity to bind calcium during capacitation and may therefore play a role in calcium sequestration and release in the principal piece, establishing a link between tyrosine phosphorylation and calcium, which is itself involved in regulating sperm-hyperactivated motility (Ho and Suarez, 2001). Although both AKAPs and CABYR are associated with the fibrous sheath, soluble proteins may also be associated with motility, as exemplified by the 55 kDa tyrosine phosphorylated soluble protein linked to motility in bovine spermatozoa (Vijayaraghavan *et al.*, 1997a).

Protein tyrosine phosphorylation in the sperm head

Protein tyrosine phosphorylation during capacitation

Tyrosine phosphorylation in the sperm head represents a minor pattern of phosphorylation in mouse spermatozoa. Non-capacitated spermatozoa are all non-phosphorylated, except for about 5–10% which display protein tyrosine phosphorylation restricted to the acrosomal region (Leyton and Saling, 1989; Urner *et al.*, 2001). In contrast to tyrosine phosphorylation in the flagellum, the proportion of spermatozoa displaying a tyrosine-phosphorylated acrosome does not increase with capacitation (Urner *et al.*, 2001).

The only tyrosine-phosphorylated protein detected by western analysis in non-capacitated mouse spermatozoa is hexokinase, indicating that this enzyme is probably the tyrosine-phosphorylated protein localized to the acrosome in uncapacitated spermatozoa by immunofluorescence. In addition, both the phosphorylation status of hexokinase and the proportion of tyrosine phosphorylated acrosome are constant during capacitation. In mice, the germ-cell specific hexokinase (Kalab *et al.*, 1994) is the major membrane tyrosine

phosphorylated protein (116/95 kDa, reducing/non-reducing condition). Although hexokinase was localized to the head, midpiece and principal piece (Travis *et al.*, 1998), the localization and the role of the phosphorylated form of the enzyme have not been determined.

The significance of tyrosine phosphorylation in the head during capacitation is unclear, but does not appear to be influenced by capacitation. In rats, tyrosine phosphorylation in the acrosomal region is characteristic of immature spermatozoa from the caput epididymidis (Lewis and Aitken, 2001). This pattern of phosphorylation decreases from 100 to 10–20% with maturation in the rat epididymis and the acrosome-positive spermatozoa that persist in the cauda epididymidis have been interpreted as representative of remaining immature spermatozoa.

Protein tyrosine phosphorylation upon zona pellucida interaction

A tyrosine phosphorylation step is necessary for the acrosome reaction induced by the zona pellucida, as inhibition of tyrosine phosphorylation prevents the acrosome reaction (Leyton *et al.*, 1992; Pukazhenthi *et al.*, 1998). In addition, exposure to progesterone, which induces the acrosome reaction, promotes an increase in tyrosine phosphorylation in the head of human spermatozoa (Tesarik *et al.*, 1993). Solubilized zona pellucida has been used to observe an increase in tyrosine phosphorylation of a 95 kDa protein in mouse spermatozoa (Leyton and Saling, 1989; Leyton *et al.*, 1992). This protein, which is not hexokinase, localizes to the head surface in the acrosomal region and becomes tyrosine phosphorylated upon binding to the zona pellucida (Leyton *et al.*, 1995). In humans, tyrosine phosphorylation of a protein of approximately 95 kDa has also been observed after exposure to solubilized and recombinant ZP3 (Burks *et al.*, 1995; Brewis *et al.*, 1998). In contrast to the above studies, a high percentage of mouse spermatozoa bound to intact zona pellucida do not present any sign of tyrosine phosphorylation in the acrosomal region (Urner *et al.*, 2001). This discrepancy might be explained if the tyrosine phosphorylated protein localized to the head is considered as a less abundant protein in which changes in phosphorylation are not detected easily by conventional immunofluorescence. In boars, the tyrosine phosphorylated plasma membrane proteins bound to the zona pellucida were found to be minor phosphorylated proteins compared with the tyrosine phosphorylated proteins of the whole sperm cells, even though they may still play a key role in sperm–zona pellucida interaction (Flesch *et al.*, 1999, 2001).

Protein tyrosine kinases in the sperm head

The 95 kDa sperm membrane protein that becomes tyrosine phosphorylated upon binding to the zona pellucida in mice possesses the characteristics of a receptor protein tyrosine kinase (Leyton *et al.*, 1992). This protein displays intrinsic tyrosine kinase activity and contains phosphotyrosine that increases upon binding to ZP3; because of this property it has been referred to as zona receptor kinase (ZRK). A similar protein, Hu9, has been described in humans (Burks *et al.*, 1995) but the *Hu9* cDNA used in this study encoded for the human proto-oncogene c-mer instead of the zona receptor kinase, bringing into question the existence of a human homologue of mouse ZRK (Bork, 1996).

Receptor protein tyrosine kinases (RPTK) are transmembrane proteins presenting an extracellular binding domain and an intracellular tyrosine kinase domain. Upon extracellular ligand binding, kinase is activated and phosphorylates tyrosine residues on the RPTK itself (autophosphorylation) or on other proteins. After autophosphorylation of RPTK, intracellular signalling proteins such as phosphoinositide 3-kinase and phospholipase Cγ may be recruited to the phosphorylated domain of RPTK and activated. The targeting of a phospholipase Cγ to the plasma membrane and its tyrosine phosphorylation-dependent activation in mouse spermatozoa (Tomes *et al.*, 1996), and phosphoinositide 3-kinase activity operating downstream of tyrosine phosphorylation in human spermatozoa (Fisher *et al.*, 1998) indirectly indicate that a receptor protein tyrosine kinase is operative in spermatozoa.

Non-receptor protein tyrosine kinases may also be present in the sperm head, as c-yes, which is a member of the nonreceptor Src family of tyrosine kinases, has been detected in the human sperm head (Leclerc and Goupil, 2002). The activity of the c-yes kinase depends on cAMP, indicating that tyrosine phosphorylation of proteins in the sperm head results from the cross-talk between the cAMP pathway and tyrosine-kinases, as it does in the flagellum.

Regulation of protein tyrosine phosphorylation

Tyrosine phosphorylation occurs spontaneously in spermatozoa *in vitro*, provided they are diluted in culture medium supporting capacitation (Urner *et al.*, 2001) and freed of seminal plasma (Tomes *et al.*, 1998). *In vivo*, the appearance of capacitation-associated tyrosine phosphorylation is probably regulated by sperm binding to oviductal epithelial cells before fertilization (Petrunkina *et al.*, 2001). The oocyte itself triggers changes in tyrosine phosphorylation that are linked to the acrosome reaction and other functions related to gamete fusion. The sequence of events responsible for capacitation, including removal of cholesterol from the sperm plasma membrane, increase in bicarbonate uptake, activation of adenylate cyclase, and the cross-talk between the cAMP pathway and protein tyrosine phosphorylation have been described in reviews by Visconti and Kopf (1998) and Visconti *et al.* (2002). This

section describes recent contributions to our knowledge on protein kinases and glucose metabolism in regulating tyrosine phosphorylation.

cAMP-dependent protein kinase A

The cAMP-dependent protein kinase A (PKA) plays a central role in sperm capacitation, motility and the acrosome reaction (Bielfeld *et al.*, 1994; Visconti *et al.*, 1995b; Harrison *et al.*, 2000; Skalhegg *et al.*, 2002). PKA regulates protein tyrosine phosphorylation in spermatozoa by either direct or indirect effects on tyrosine kinase or phosphatases (Visconti *et al.*, 2002). PKA is a tetrameric enzyme composed of two regulatory and two catalytic subunits. Two isoforms of the regulatory subunit (RI and RII) are present in spermatozoa (Vijayaraghavan *et al.*, 1997b; Visconti *et al.*, 1997). The activity of PKA is dependent on the amount of cAMP, which is in turn regulated by adenylate cyclase and phosphodiesterase. The binding of cAMP to the regulatory subunit promotes the dissociation and activation of the catalytic subunits that catalyse phosphorylation on serine/threonine residues.

PKA is anchored to specific sperm compartments through binding of its regulatory subunit to the AKAPs. In general, AKAPs are implicated in the regulation of protein phosphorylation by the tethering of protein kinases and phosphatases in close proximity to their target proteins within specific cell compartments. Different types of AKAP have been found in spermatozoa (for review, see Moss and Gerton, 2001). AKAP82 (Carrera *et al.*, 1994; Johnson *et al.*, 1997) and FSP95 (Mandal *et al.*, 1999) are both localized to the fibrous sheath of the principal piece and anchor the RII subunit of PKA. AKAP110, localized to the principal piece and the acrosomal region (Vijayaraghavan *et al.*, 1999), presents unique characteristics in spermatozoa. In addition to binding to the regulatory subunit of PKA, the sperm AKAP110 interacts with other signalling proteins (such as ropporin) that may regulate motility independently of PKA (Carr *et al.*, 2001). Two other AKAPS have been identified in the midpiece: S-AKAP84, which is associated with mitochondria (Lin *et al.*, 1995) and AKAP220, which is anchored to cytoskeletal structures and binds both the RII and RI subunits (Reinton *et al.*, 2000). The localization of AKAPs in all sperm compartments indicates that they participate indirectly (through scaffolding of PKA or other signalling molecules) in the regulation of tyrosine phosphorylation in both the flagellum and the sperm head. In addition to participating in the regulation of protein phosphorylation in spermatozoa, AKAPs are themselves phosphorylated on tyrosine residues in humans (AKAP82, FSP95; Carrera *et al.*, 1996; Mandal *et al.*, 1999) and on serine/threonine residues in mice (AKAP 82; Johnson *et al.*, 1997) during capacitation, indicating that their functions are modified by their phosphorylation status.

Mitogen-activated protein kinases

The mitogen-activated protein kinases (MAPK), also known as extracellular signal-regulated kinases (ERK), are serine/threonine kinases involved in signal transduction of several extracellular messengers. Their activity is regulated by a phosphorylation cascade initially triggered by the GTP-binding protein Ras or protein kinase C. MAPK-kinase-kinase (Raf), which is the first enzyme of this cascade, phosphorylates MAPK-kinases (MEK) on serine/threonine, which in turn phosphorylates MAPK on threonine and tyrosine residues. MAPK requires phosphorylation on both threonine and tyrosine to become active.

The MAPK pathway has been identified in spermatozoa and appears to play a role in capacitation (de Lamirande and Gagnon, 2002) and the acrosome reaction (Luconi *et al.*, 1998; du Plessis *et al.*, 2001). The zona pellucida- and lysophosphatidylcholine-induced acrosome reactions are prevented when MAPK activity is inhibited (du Plessis *et al.*, 2001; de Lamirande and Gagnon, 2002). Progesterone promotes the phosphorylation–activation of the MAPK isoform ERK2 in human spermatozoa and its redistribution from the post-acrosomal region to the equatorial segment (Luconi *et al.*, 1998). The MAPK isoform ERK2 (Luconi *et al.*, 1998), the adaptor protein Shc (Morte *et al.*, 1998) and Ras (Naz *et al.*, 1992) have all been localized to the sperm head, indicating that this pathway is required for controlling protein phosphorylation in the sperm head.

Although the proteins phosphorylated by MAPK in spermatozoa remain to be identified, de Lamirande and Gagnon (2002) reported a 75 and 80 kDa protein presenting a phosphorylated serine/threonine–proline motif recognized by the MPM-2 antibody, which is consistent with a MAPK-phosphorylated epitope. MPM2-reactive proteins in the 77–85 kDa range have been detected in the rabbit sperm midpiece and connective piece in different species (Pinto-Correia *et al.*, 1994; Long *et al.*, 1997) and may be components of the centrosome in the connecting piece (Simerly *et al.*, 1999). Therefore, centrosomal proteins may be considered as candidate substrates of MAPK in spermatozoa. In addition, MAPK may phosphorylate proteins that influence protein tyrosine phosphorylation indirectly, as inhibition of MAPK prevents protein tyrosine phosphorylation associated with capacitation (de Lamirande and Gagnon, 2002).

Protein phosphatases

Tyrosine phosphorylation is stimulated indirectly by activation of phosphorylation on serine/threonine (by PKA or MAPK) and, therefore, it is conceivable that dephosphorylation by phosphatases of these residues inhibits tyrosine phosphorylation. Serine/threonine-specific protein phosphatase (PP) activities have been

identified in spermatozoa from different species and are involved in the regulation of sperm motility. The isoform PP1γ2 has been detected in primate spermatozoa (Smith *et al*., 1996). PP1 colocalizes with AKAP220 to cytoskeleton structures, indicating that it is anchored via AKAP220 in the midpiece (Reinton *et al*., 2000). The activity of PP1 is regulated by the PP1 inhibitor 2 (I2) and glycogen synthase kinase 3 (GSK-3), which are both present in spermatozoa (Smith *et al*., 1996). PP1 is inactive when bound to non-phosphorylated I2 and becomes activated when I2 is phosphorylated by GSK-3, implying that PP1-dependent dephosphorylation of sperm proteins parallels GSK-3 activity, which may be itself regulated by phosphorylation, as has been reported in somatic cells. The calcium/calmodulin-dependent phosphatase calcineurin (PP2B) has been detected in dog spermatozoa (Tash *et al*., 1988) and appears to participate in dephosphorylation of tyrosine phosphorylated substrates in human spermatozoa (Carrera *et al*., 1996).

Glucose metabolism

Glucose participates in the regulation of protein tyrosine phosphorylation during capacitation and gamete interaction *in vitro*. In the absence of glucose, tyrosine phosphorylation in the principal piece is delayed and phosphorylation in the midpiece is inhibited as a consequence of this delay or because of a local inhibition in the midpiece. The absence of glucose and the delayed tyrosine phosphorylation during capacitation do not affect fertilization, as long as glucose is added during gamete mixing. However, the absence of glucose during gamete interaction prevents fertilization, indicating that a precise timing in the induction of tyrosine phosphorylation once the spermatozoa bind to the oocyte is critical for successful fertilization. The observation that high and low concentrations of glucose are necessary to support gamete fusion (Urner and Sakkas, 1996b) and protein tyrosine phosphorylation during capacitation (Travis *et al*., 2001a), respectively, emphasizes the predominant role of glucose during gamete interaction.

The utilization of glucose depends on its availability in the environment, its uptake by specific transporters and its intracellular metabolism. Spermatozoa are compartmentalized with regard to glucose utilization, indicating a specific regulation in the different sperm domains during the course of fertilization (Fig. 2). The high affinity glucose transporter GLUT3 was localized mainly to the midpiece (Angulo *et al*., 1998; Rigau *et al*., 2002) and the recently identified GLUT8 has been localized to the acrosomal region (Schürmann *et al*., 2002).

The glucose metabolic pathways detected in mouse spermatozoa are glycolysis and pentose phosphate pathways (Urner and Sakkas, 1999). Hexokinase, which phosphorylates glucose to glucose 6-phosphate, is present in the sperm head, midpiece and principal piece (Travis *et al*., 1998). Glycolysis is associated predominantly with the sperm principal piece (Westhoff and Kamp, 1997; Bunch *et al*., 1998; Welch *et al*., 2000) and the pentose phosphate pathway with the sperm midpiece (Zimmermann and Geyer, 1981) and the sperm head (D. Sakkas, G. Leppens-Luisier and F. Urner, unpublished). The oxidative phosphorylation takes place in the mitochondria localized to the sperm midpiece.

Glucose has to be metabolized by spermatozoa for zona pellucida penetration, sperm–oocyte fusion and to ensure that tyrosine phosphorylation occurs during capacitation (Urner and Sakkas, 1996a,b; Travis *et al*., 2001). Glucose is provided to spermatozoa by the female reproductive tract fluid *in vivo* or by the culture medium *in vitro*, but studies have indicated that stores of glycogen are endogenous sources of glucose in spermatozoa (Ballester *et al*., 2000; Rigau *et al*., 2002), allowing spermatozoa to accommodate glucose-free conditions.

ATP produced by glycolysis or oxidative respiration is absolutely required for phosphorylation itself and cAMP synthesis. Although Travis *et al.* (2001a) postulated that the ATP produced by the glycolytic pathway was strictly required for protein tyrosine phosphorylation, the observation that NADPH can modulate protein tyrosine phosphorylation in human (Aitken *et al*., 1995; de Lamirande *et al*., 1998) and mouse spermatozoa (Urner *et al*., 2001) indicates that the pentose phosphate pathway is also involved. Delay rather than a total inhibition of tyrosine phosphorylation indicates that ATP and NADPH are generated by spermatozoa in the absence of glucose but to a lesser extent than in its presence. In the absence of glucose, the main sources of ATP are mitochondria in the sperm midpiece. Because the phosphorylcreatine shuttle transporting phosphate from the sperm midpiece to the principal piece is poorly developed in mammalian spermatozoa (Kamp *et al*., 1996), the principal piece may be partially deprived of ATP, explaining the delayed protein phosphorylation in this compartment. The maintenance of sperm motility in the absence of glucose indicates that ATP may be available to the dynein ATPases of the axoneme in the principal piece in the absence of metabolism through glycolysis. In contrast, delayed tyrosine phosphorylation in the midpiece is not the result of ATP deprivation but may be the consequence of low NADPH generation. In the absence of glucose, NADPH may be produced by alternate cytosolic pathways such as the malic enzyme or the NADP-dependent isocitrate dehydrogenase, which may not be as efficient as the pentose phosphate pathways in generating this nucleotide.

NADPH is involved in oxido-reductive reactions and is required for NADPH-dependent enzymes such as NADPH-oxidase and nitric oxide synthase. The influence of superoxide and hydrogen peroxide on sperm function and tyrosine phosphorylation (Aitken *et al*.,

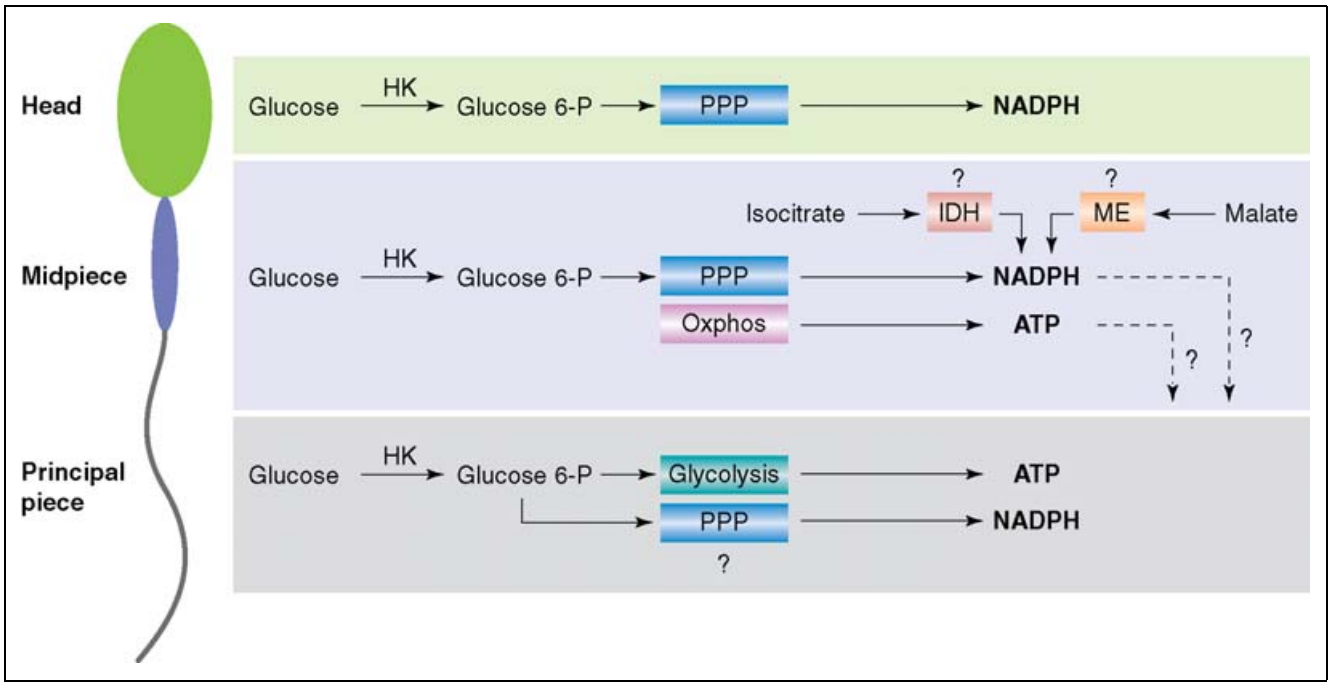

Fig. 2. Compartmentalization of glucose metabolism in the sperm flagellum. The uptake of glucose occurs through specific membrane transporters. In the presence of glucose, ATP is generated through glycolysis in the principal piece and through oxidative phosphorylation (Oxphos) in the midpiece. NADPH is generated by the pentose phosphate pathway (PPP) in the midpiece and the sperm head. Availability of NADPH in the principal piece through transport from the midpiece or local PPP has not been demonstrated. In the absence of glucose, ATP generated from oxidative phosphorylation may be transported to the principal piece. NADPH may be produced in the midpiece by the cytosolic NADP-dependent isocitrate dehydrogenase (IDH) or malic enzyme (ME). HK: hexokinase.

1995; Leclerc *et al.*, 1997) indicates that an NADPH-oxidase is involved, although superoxide may also be produced by the mitochondrial electron transport chain (Vernet *et al.*, 2001). Nitric oxide synthase, catalysing nitric oxide synthesis, is present in mouse and human spermatozoa (Herrero *et al.*, 1997; Revelli *et al.*, 1999) and plays a physiological role in sperm function (Zini *et al.*, 1995; Francavilla *et al.*, 2000) as well as in the regulation of tyrosine phosphorylation (Herrero *et al.*, 1999). On account of its calcium and NADPH requirement, it is possible that nitric oxide synthase links glucose metabolism and the regulation of tyrosine phosphorylation in spermatozoa.

Conclusion

The passage of spermatozoa in the male and female reproductive tract requires precise timing so as to synchronize the fertilization competence of the spermatozoon with ovulation. A specific sequence of events must occur to achieve the correct priming of the fertilizing spermatozoa. Several studies have indicated that the spermatozoa achieve this by a specific sequence of phosphorylation in the flagellum. The role of phosphorylation in the sperm head remains unclear. Molecules involved in the sequence of phosphorylation are more likely to arise from the milieu surrounding the spermatozoa, but their uptake and processing are probably regulated in different ways at specific steps within the female genital tract and during penetration of the egg vestments. The sperm functions known to be associated with changes in protein tyrosine phosphorylation are capacitation, hyperactivated motility, acrosome reaction and sperm–oocyte fusion. These events are regulated by the activation of compartmentalized intracellular signalling systems. One such signalling mechanism may be related to the recently reported effects of caveolins on the sperm membrane (Travis *et al.*, 2001b). The metabolic products of glucose (ATP and NADPH) appear to participate in these signalling pathways by supporting a precise onset of tyrosine phosphorylation in the sperm flagellum leading to successful fertilization. A greater understanding of the compartmentalized signals controlling phosphorylation in spermatozoa will provide insight into how spermatozoa are primed to enter the egg and may improve understanding of aspects of human infertility.

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