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Functional processing of fertilin: evidence for a critical role of proteolysis in sperm maturation and activation

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Fertilin is a sperm surface protein with an essential role in fertilization. It is required for the migration of spermatozoa through the oviduct, for binding to the zona pellucida, and for efficient binding to the egg plasma membrane. Fertilin consists of two subunits, fertilin α and β, both of which belong to the metalloprotease–disintegrin protein family (ADAMs). Fertilin α and β are made as larger precursors that are processed proteolytically at different stages of sperm maturation in the testis and epididymis. Fertilin α is processed first, most likely by a pro-protein convertase in the secretory pathway of testicular cells. Fertilin β is processed later, while spermatozoa are in transit through the epididymis. The processing of fertilin β in the epididymis correlates with the acquisition of fertilization competence in spermatozoa, exposes an epitope that has a role in sperm–egg interactions, and triggers the relocalization of fertilin from the whole sperm head to the posterior head. These findings indicate that the proteolytic processing of fertilin and perhaps also other sperm proteins plays an important role in sperm maturation and activation in the epididymis. Further evaluation of the functional significance of proteolysis for sperm maturation should lead to new and exciting insights into the mechanism of sperm maturation, and may also uncover the cause of certain types of male infertility. The identification of the responsible proteases could provide novel targets for contraceptive drugs.

Studies of the sperm surface molecule fertilin have yielded fascinating insights into the process of sperm–egg interactions. In 1987, Primakoff and colleagues showed that a monoclonal antibody against fertilin (previously referred to as PH-30) potently inhibits sperm–egg fusion, providing the first evidence that fertilin has a critical role in fertilization (Primakoff *et al*., 1987). Cloning of the cDNAs encoding fertilin revealed a strong sequence similarity to the snake venom integrin ligands termed disintegrins (Blobel *et al*., 1992; Wolfsberg *et al*., 1993). This remarkable finding indicated that fertilin interacts with an integrin on the egg, thus providing further support for a role in sperm–egg interactions. Fertilin and an epididymal apical protein (EAP I)(Perry *et al*., 1992) also served as the founding members of the ADAM protein family, modular proteins consisting of a metalloprotease domain and a disintegrin domain (Fig. 1; for recent reviews on ADAMs, see Wolfsberg and White, 1996; Blobel, 1997; Black and White, 1998; Schlöndorff and Blobel, 1999). An essential function for fertilin in fertilization was confirmed by a targeted deletion of the fertilin gene in mice (Cho *et al*., 1998). The fertility of male mice lacking fertilin is reduced substantially, although these animals are otherwise healthy. A careful functional analysis of spermatozoa from mice lacking fertilin revealed at least three distinct severe functional deficiencies: (1) inability to migrate through the oviduct; (2) inability to bind to the zona pellucida; and (3) strongly reduced binding to the egg plasma membrane (Cho *et al*., 1998). Evidently, fertilin has critical functions in several different steps leading up to the final union of spermatozoon and egg.

Guinea-pig fertilin is a heterodimer consisting of two membrane anchored glycoproteins, fertilin α and β (Fig. 1), both of which are members of the metalloprotease–disintegrin (ADAM) protein family (Primakoff *et al*., 1987; Blobel *et al*., 1990, 1992; Wolfsberg *et al*., 1993). During sperm maturation in the testis and epididymis, fertilin is subjected to several distinct proteolytic processing events (Fig. 2; Blobel *et al*., 1990). The α-subunit of fertilin is processed first in the secretory pathway of testicular cells, before emerging on the cell surface (Lum and Blobel, 1997). The β-subunit is processed later, during the passage of spermatozoa through the epididymis. Since the processing of fertilin β occurs in a region of the epididymis where spermatozoa acquire motility and fertilization competence, that is, between the distal corpus and proximal cauda, processing has been proposed to have a role in the functional activation of fertilin (Blobel *et al*., 1990).

This review will highlight what is known about fertilin processing in the guinea-pig, and about the identity of the proteases responsible for this processing. Proteolysis applied selectively and specifically has emerged as an important mechanism for regulating biological functions. For example, membrane-anchored proteins such as tumour necrosis factor α (TNFα) or transforming growth factor α (TGFα) are released from the plasma membrane by a protease, the TNFα convertase, which also has an essential role in development (Black *et al*., 1997; Moss *et al*., 1997; Peschon *et al*., 1998). Proteolysis is important for neurogenesis, apparently modulating signalling via the cell surface receptor Notch (Chan and Jan, 1998). Furthermore,

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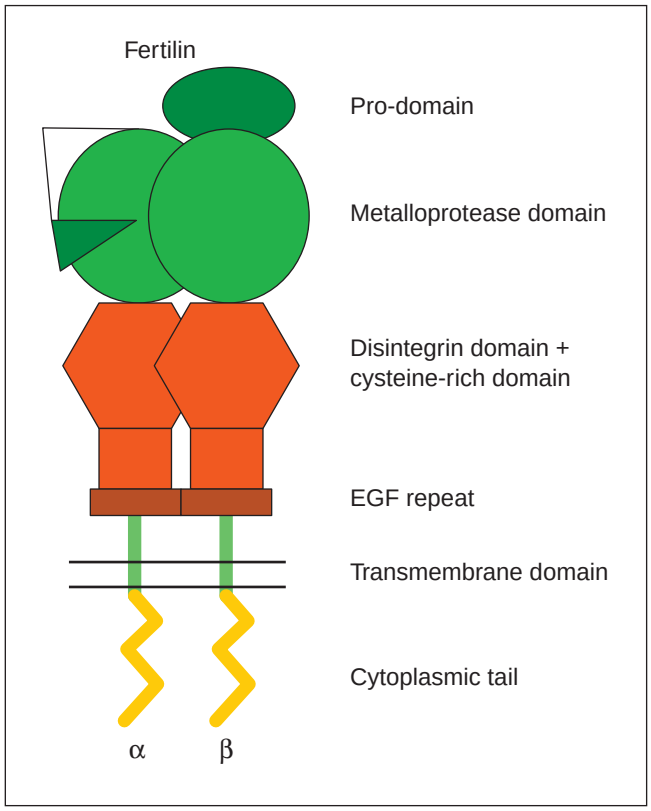

Fig. 1. Model of the fertilin heterodimer. Fertilin consists of an α and β subunit, both of which are founding members of the metalloprotease-disintegrin (ADAM) protein family (Wolfsberg and White, 1996; Blobel, 1997; Black and White, 1998; Schlöndorff and Blobel, 1999). ADAMs are modular proteins consisting of an N-terminal pro-domain, followed by a metalloprotease domain, a disintegrin domain and a cysteine rich region, an EGF repeat, transmembrane domain and cytoplasmic tail. The metalloprotease domain of fertilin α contains the catalytic site consensus sequence HEXXH, indicating that it has catalytic activity. The β subunit contains a metalloprotease domain without a catalytic site. The metalloprotease domain is otherwise remarkably conserved compared with the metalloprotease domain of fertilin α. To date, approximately 30 ADAMs have been identified in different cells and tissues, and about half of these have a catalytic site (like fertilin α), while the other half (like fertilin β) do not. In ADAMs with a catalytically active metalloprotease domain, the pro-domain is thought to function as an inhibitor which must be released for the protease to become active (Roghani *et al*., 1999). Therefore, the pro-domain of fertilin α is depicted as an inhibitory wedge in the catalytic site of the metalloprotease domain, whereas the pro-domain of fertilin β is shown above the metalloprotease domain.

proteolysis can expose and activate cryptic functional epitopes (Giannelli *et al*., 1997). Thus, there are several precedents to support the notion that proteolysis of fertilin may be linked to the regulation of its function. Since other proteins besides fertilin are also processed during the epididymal passage, it is tempting to speculate that specific and regulated proteolysis of sperm surface proteins may be required to prepare spermatozoa for its interaction with the egg.

Discovery of fertilin

Fertilin was discovered by Diana Myles and Paul Primakoff in a screen for monoclonal antibodies that bind to sperm membrane proteins on guinea-pig spermatozoa (Myles *et al*., 1981). This screen was designed to generate markers for the different plasma membrane domains on mammalian spermatozoa, both with the goal of studying how these domains are established and maintained, and to evaluate the function of sperm surface proteins by testing the effect of the monoclonal antibodies on *in vitro* fertilization assays (Myles and Primakoff, 1984; Primakoff and Myles, 1983; Myles *et al*., 1987). A monoclonal antibody (mAb), named PH-30 because it binds to a protein on the posterior head of mature spermatozoa, attracted considerable attention due to its ability to block sperm–egg membrane fusion (Primakoff *et al*., 1987). This finding indicated that the protein recognized by the PH-30 mAb, initially referred to as PH-30, and later renamed fertilin (Myles *et al*., 1994), has a role in fertilization. A second antibody (PH-1) against fertilin did not affect sperm–egg fusion, even though both antibodies bound to the protein on live spermatozoa, indicating that simple steric inhibition is not responsible for the effect of the function-blocking monoclonal antibody (Primakoff *et al*., 1987). Instead, this result indicated that the epitope in fertilin recognized by function blocking mAb PH-30 is important for the role of fertilin in sperm–egg fusion. Furthermore, the PH-30 monoclonal antibody bound only to mature fertilization-competent epididymal spermatozoa, but not to testicular spermatozoa or spermatogenic cells (Fig. 3). Yet, if a polyclonal antibody was used instead of the PH-30 mAb, fertilin could be readily detected on the surface of testicular spermatozoa (Blobel *et al*., 1990; Phelps *et al*., 1990). Evidently, an epitope linked to sperm–egg fusion through the function blocking PH-30 antibody is exposed only on fertilization-competent spermatozoa, but not on testicular spermatozoa.

Proteolytic processing steps of fertilin during sperm maturation

A potential explanation for the selective binding of the function blocking PH-30 monoclonal antibody to mature spermatozoa emerged once the different forms of fertilin were analysed at various stages of sperm maturation (Blobel *et al*., 1990). Western blots of testicular cells and testicular spermatozoa, and of epididymal spermatozoa taken from different stages of sperm maturation, uncovered the intricate processing pattern of both fertilin subunits during sperm maturation in the testis and epididymis (see below, and Fig. 2). Western blot analysis also showed that both the function-blocking PH-30 monoclonal antibody and the PH-1 control mAb bind to the β subunit of fertilin. Because the β subunit of fertilin is processed in the epididymis, whereas fertilin α is processed in the testis, the epitope recognized by the PH-30 epitope is probably exposed in the epididymis by proteolysis. This finding indicated that the functional epitope could be exposed by removing the fertilin β pro-domain in the epididymis, or by removing another protein that may cover the epitope.

An additional consequence of the proteolytic processing of fertilin is its redistribution on the sperm surface (Fig. 3; Myles *et al*., 1987; Phelps *et al*., 1990; Hunnicutt *et al*., 1997). Fertilin is distributed over the whole surface of testicular spermatozoa.

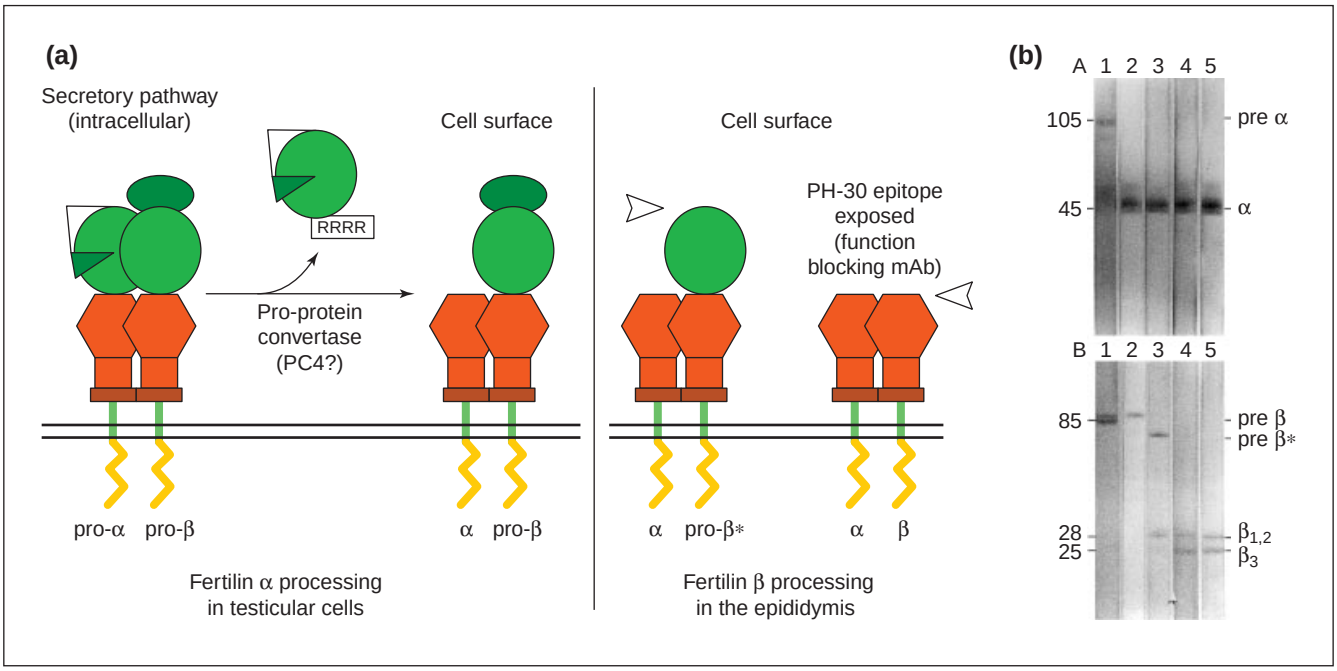

Fig. 2. Proteolytic processing of fertilin during sperm maturation in the testis and epididymis. (a) In testicular spermatogenic cells (most likely spermatids; Carroll *et al*., 1995), fertilin is assembled into a heterodimeric complex consisting of the α and β subunits (Blobel *et al*., 1990). Fertilin α is processed in the secretory pathway of testicular cells before emerging on the cell surface (Lum and Blobel, 1997). The fertilin α metalloprotease is cleaved after four arginine residues (RRRR), a consensus sequence for a pro-protein convertase such as furin (Lum and Blobel, 1997). It is possible that the fertilin α metalloprotease domain is released by the pro-protein convertase PC4, which is mainly expressed in the testis (Mbikay *et al*., 1997). The fate of the metalloprotease domain after cleavage remains to be determined. Fertilin β is then processed in two steps during sperm maturation in the epididymis, first to a form lacking a pro-domain (fertilin pro-β*), and then to a form that comigrates with mature fertilin and lacks the pro- and metalloprotease domain (Blobel *et al*., 1990; Hunnicutt *et al*., 1997). The appearance of mature fertilin β correlates with the acquisition of fertilization competence in the distal corpus epididymidis (Blobel *et al*., 1990). Finally, processing of fertilin β exposes an epitope that is recognized by the function-blocking monoclonal antibody PH-30 (see Fig. 3), indicating a link between the processing and functional activation of fertilin. (b) Western blot analysis of the different forms of fertilin α and β found at different stages of sperm maturation (Fig. 3a,b from Blobel *et al*. (1990), reproduced with kind permission of Rockefeller University Press). Panel A was probed with antibodies against fertilin α, whereas panel B was probed with antibodies against fertilin β. Lane 1, testicular cells; lane 2, testicular spermatozoa; lane 3, distal corpus epididymidal spermatozoa; lane 4, proximal cauda epididymidal spermatozoa; lane 5, distal cauda epididymidal spermatozoa. The reason for the different migration of fertilin β1,2 and β3 is not understood at present. No heterogeneity was observed in the N-terminal sequence of fertilin β that was purified from distal cauda epididymidal spermatozoa and, therefore, included fertilin $\beta_{1,2}$ and β_3 (Blobel *et al*., 1992). It is possible that fertilin is subjected to additional processing at its C-terminus in the cauda epididymidis.

Concomitant with the processing of fertilin while spermatozoa are in transit through the epididymis, fertilin migrates to the posterior sperm head (Hunnicutt *et al*., 1997). This migration of fertilin to the posterior head can also be induced by gentle trypsinization of isolated testicular spermatozoa (Phelps *et al*., 1990; see Fig. 4). Western blot analysis revealed that trypsinization of testicular spermatozoa converts the fertilin β precursor into a form that resembles mature fertilin (Blobel *et al*., 1990). Although the purpose of relocalizing fertilin to the posterior head remains to be determined, an intriguing possibility is that it serves to increase the local concentration or surface density of fertilin which, in turn, may be important for its function in sperm–egg interactions.

Cloning of fertilin and insights into its function

Exciting additional clues about the function of fertilin emerged once the cDNA for both subunits had been cloned and sequenced (Blobel *et al*., 1992; Wolfsberg *et al*., 1993). The most revealing insight was the discovery that the N-terminus of mature fertilin β is related to snake venom integrin ligands termed disintegrins. Furthermore, a potential fusion peptide, resembling the fusion peptide in viral fusion proteins, was found in the α-subunit. While the initial N-terminal protein sequence information for the mature fertilin α-subunit indicated that it lacks an intact disintegrin domain (Blobel *et al*., 1992), a re-evaluation of the N-terminus of mature fertilin α showed clearly that this subunit also contains an intact disintegrin domain (Lum and Blobel, 1997). Thus, mature, processed fertilin is a heterodimeric complex of two membrane anchored glycoproteins which both contain an N-terminal disintegrin domain (Fig. 2), raising the possibility that one or both subunits can interact with an integrin on the egg.

This hypothesis has since been tested using several different approaches (for review, see Snell and White, 1996; Myles and Primakoff, 1997; Evans, 1999; Primakoff and Myles, 2000).

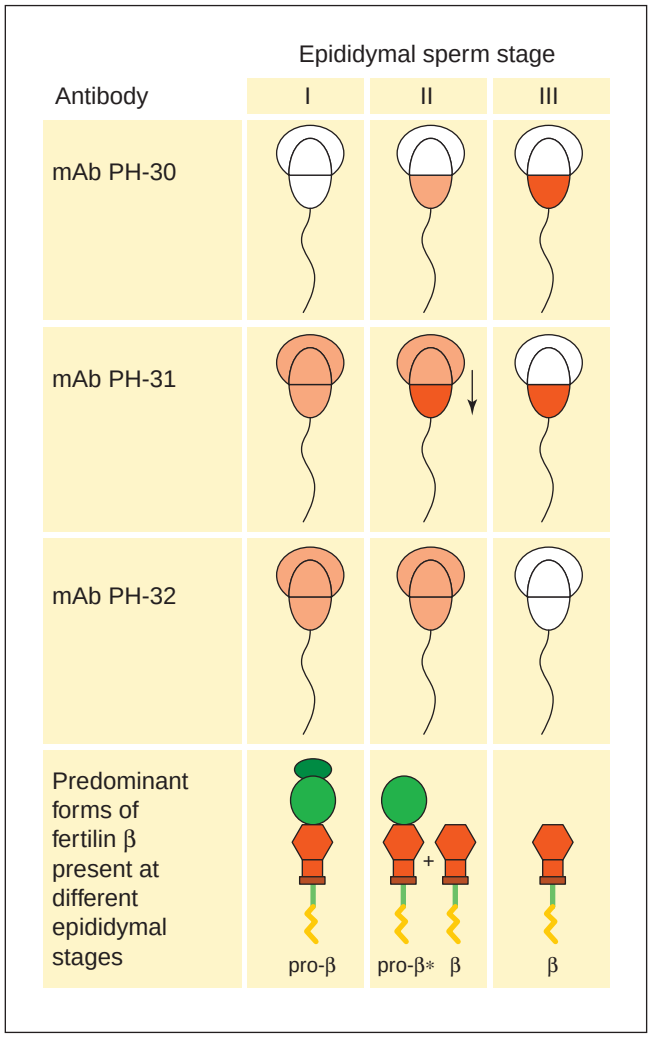

Fig. 3. Changes in the localization of fertilin on the sperm head correlate with the proteolytic exposure and removal of epitopes. The immunofluorescent staining pattern of different monoclonal antibodies against fertilin β on guinea-pig spermatozoa is shown. The epididymal stages are defined by Hunnicutt *et al.* (1997). Stage I corresponds approximately to the caput epididymidis, although the guinea-pig epididymis does not readily follow the classical subdivisions into caput, corpus and cauda epididymidis (Hunnicutt *et al.*, 1997). With respect to fertilin processing, spermatozoa taken from stage I of the epididymis resemble testicular spermatozoa, in that fertilin β exists mainly in its pro-form, which includes the pro- and metalloprotease domain (see bottom panel). Fertilin β processing begins in sections II and III (corresponding approximately to what is termed the distal corpus epididymidis by Blobel *et al.* (1990), and is complete by section IV (corresponding approximately to the proximal cauda epididymidis described by Blobel *et al.* (1990) and Hunnicutt *et al.* (1997). Immunofluorescence analysis shows that the epitope recognized by mAb PH-30 on fertilin β is first exposed in section II (Hunnicutt *et al.*, 1997), which is the time of the appearance of processed forms of fertilin lacking either the pro-domain or the pro- and metalloprotease domains. The PH-30 mAb binds only to the posterior head, even though processing most likely begins in the whole sperm head. Hunnicutt and colleagues proposed that the first step of fertilin pro-β processing to pro-β* induces relocalization from the whole head to the posterior head, but does not expose the PH-30 epitope. The final processing of fertilin pro-β* to mature fertilin β would then only occur once a given fertilin heterodimer enters the posterior head domain. The PH-31 mAb allows visualization of fertilin at all stages of sperm maturation in the epididymis. The PH-32 mAb does not bind to stage IV epididymal spermatozoa, indicating that it recognizes the metalloprotease or pro-domain of fertilin β, which has been removed by stage IV. The bottom panel shows the forms of fertilin β that can be detected by western blot with mAb PH-31 at the various epididymal stages. Note that several other proteins also change their localization on the sperm head during epididymal maturation (Myles and Primakoff, 1984; Myles *et al.*, 1987; Phelps and Myles, 1987; Hunnicutt *et al.*, 1997).

Essentially, peptide inhibition studies support the hypothesis that fertilin interacts with a receptor on the egg (Myles *et al.*, 1994; Almeida *et al.*, 1995; Evans *et al.*, 1995, 1997a,b, 1998; Gichuhi *et al.*, 1997). Myles and colleagues provided the first evidence that peptides that mimic a predicted integrin binding sequence in guinea-pig fertilin β, defined as a sequence that is found in lieu of the snake venom integrin binding sequence RGD, block sperm–egg fusion in a concentration-dependent manner (Myles *et al.*, 1994). Scrambled control peptides do not block sperm–egg fusion. Furthermore, antibodies against the α6 integrin (Almeida *et al.*, 1995) or against the β1 integrin (Evans *et al.*, 1995) block fertilization, and a fertilin binding sequence peptide can be crosslinked to an α6 integrin (Chen and Sampson, 1999). The binding of spermatozoa from mice lacking fertilin β to zona pellucida-free eggs is decreased eight-fold, providing direct evidence for a role of fertilin in sperm–egg membrane binding (Cho *et al.*, 1998). It should be noted that, although several lines of evidence argue for a role of fertilin as an integrin ligand, definitive proof of such an interaction has not yet been reported. (A critical discussion of the role of fertilin in integrin binding can be found in a recent review of ADAMs (Primakoff and Myles, 2000). The possibility must be considered that, in addition to or instead of binding to integrins, fertilin may also interact with other proteins on the egg surface. Spermatozoa lacking fertilin are also unable to bind to the zona pellucida, and are unable to migrate through the oviduct, indicating that fertilin has critical functions at other steps in fertilization besides binding to the egg plasma membrane (Cho *et al.*, 1998).

With respect to a role as a *bona fide* membrane fusion protein, all evidence linking fertilin to the process of membrane fusion is also consistent with a role for fertilin in a step that is a prerequisite for membrane fusion to occur. Spermatozoa lacking fertilin β are able to fuse with eggs quite efficiently once they have bound to the egg plasma membrane, even though the amounts of fertilin α are markedly decreased (Cho *et al.*, 1998). Therefore it seems unlikely that fertilin is essential to mediate the process of sperm and egg membrane fusion, although it remains possible that a small amount of fertilin α is sufficient to mediate fusion.

Processing of fertilin α during sperm maturation in the testis

Biochemical analysis using affinity purified antibodies against the α or β subunit of fertilin demonstrated that both subunits are made as larger precursors that assemble into a heterodimer

before beginning their transport through the secretory pathway (Blobel *et al.*, 1990). The fertilin β subunit is synthesized in pachytene spermatocytes, but does not appear on the sperm surface until the late elongating spermatid stage (Carroll *et al.*, 1995). This finding raises the question of whether transport of fertilin out of the endoplasmic reticulum and to the cell surface is regulated either by limited availability of the α subunit before the elongating spermatid stage or by retaining an assembled heterodimer intracellularly by a yet to be determined mechanism.

On isolated testicular spermatozoa, almost all detectable fertilin α has been processed to a form that resembles mature fertilin α on epididymal spermatozoa (Blobel *et al.*, 1990). No further processing of fertilin α is detectable during epididymal maturation. Although the exact role of this processing step in the functional maturation of fertilin is not clear, one of its consequences is the release of the fertilin α metalloprotease domain (see below and Fig. 2). The disintegrin domain, cysteine-rich region and EGF repeat remain membrane-anchored (Lum and Blobel, 1997). The fertilin α protease domain has a catalytic site consensus sequence (HEXXH) and is predicted to be catalytically active. With respect to the release of the metalloprotease domain from its membrane anchor, fertilin α is an exception among the ADAM family of metalloproteases. In most cases, the metalloprotease domain remains membrane-anchored, and only the pro-domain is removed (Black *et al.*, 1997; Moss *et al.*, 1997; Lum *et al.*, 1998; Loechel *et al.*, 1999; Roghani *et al.*, 1999). It is possible that the soluble metalloprotease domain of fertilin α has a function in sperm maturation or in fertilization. It remains to be determined whether the fertilin α metalloprotease is secreted from spermatozoa as a soluble, active molecule, in which case it might process proteins in the extracellular matrix or on the sperm surface, or whether it is sorted to and stored in the acrosome together with other hydrolytic enzymes.

Proteases that process fertilin α

While the substrates and function of the fertilin α metalloprotease domain remain to be elucidated, the subcellular localization of fertilin α processing is quite well-defined. Fertilin α contains a tetrabasic sequence (RRRR) between the metalloprotease domain and the disintegrin domain, a consensus cleavage site of pro-protein convertases such as furin (Fig. 2). N-terminal sequence analysis of mature fertilin confirmed that this site is indeed used to remove the metalloprotease domain (Lum and Blobel, 1997). Two additional findings confirm that fertilin α is cleaved late in the secretory pathway. First, when fertilin α is treated with endoglycosidase H (endo H), only the precursor form of fertilin α is deglycosylated, whereas processed fertilin α lacking the metalloprotease is largely resistant to treatment with endo H. Since most *N*-linked carbohydrates become resistant to endo H treatment after transit through the medial Golgi, fertilin is probably processed in or after the medial Golgi. The second finding is that only mature, processed fertilin can be labelled on the sperm surface with a non-membrane permeable biotinylation reagent, whereas profertilin α is not labelled. Taken together, these findings indicate that fertilin α is processed by a pro-protein convertase in the trans-Golgi network just before or immediately after the protein emerges on the cell surface (Lum and Blobel, 1997).

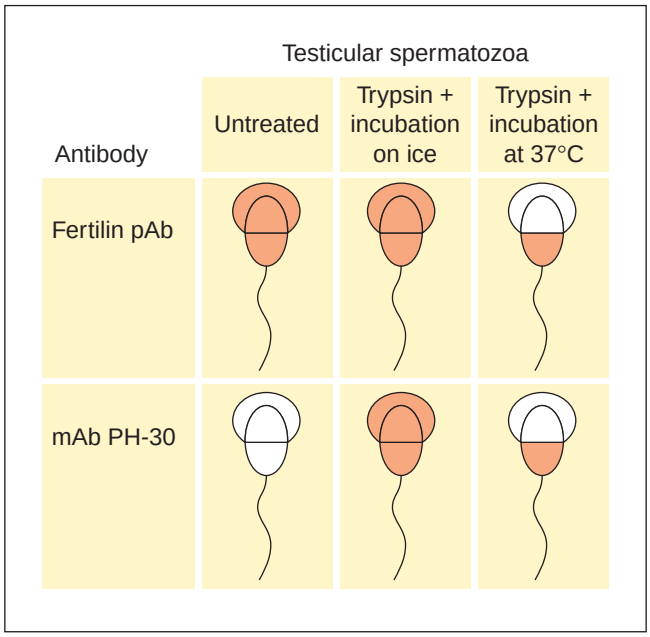

Fig. 4. Changes in the localization of fertilin on the sperm head can be induced by gentle trypsinization of testicular spermatozoa. Trypsinization of testicular spermatozoa can mimic certain aspects of sperm maturation in the epididymis (Blobel *et al.*, 1990; Phelps *et al.*, 1990; Hunnicutt *et al.*, 1997). The top panel shows a diagram of testicular spermatozoa stained with a polyclonal antiserum against fertilin, which binds to the whole sperm head. Trypsinization followed by incubation on ice does not affect the localization of fertilin (Blobel *et al.*, 1990; Phelps *et al.*, 1990). However, if gently trypsinized spermatozoa are incubated at 37°C, fertilin relocalizes to the posterior sperm head, as would occur normally in the epididymis. Although the mAb PH-30 does not bind to testicular spermatozoa, binding can be induced by trypsinization on ice (Blobel *et al.*, 1990; Phelps *et al.*, 1990). Western blot analysis confirmed that incubation with relatively large amounts of trypsin completely converts fertilin pro-β to mature fertilin (Blobel *et al.*, 1990). After incubation of gently trypsinized spermatozoa at 37°C, mAb PH-30 is found localized to the posterior head, consistent with the relocalization of fertilin observed with the polyclonal antibody (Phelps *et al.*, 1990). Gentle trypsinization results in intermediates of fertilin β processing that resemble at least some of the forms observed in the epididymis (Lum and Blobel, 1997). The finding that gentle trysinization can trigger relocalization when spermatozoa are incubated at 37°C is presumably due to partial processing to fertilin pro-β*, but not mature fertilin (Hunnicutt *et al.*, 1997). The treatment of testicular spermatozoa with large amounts of trypsin that lead to complete conversion of fertilin pro-β to mature fertilin β do not trigger relocalization at 37°C (C. Blobel, unpublished).

The pro-protein convertase PC4 is highly expressed in the testis and also has an essential role in fertilization (Mbikay *et al.*, 1997). A targeted deletion of PC4 leads to male infertility, with a significantly reduced ability of spermatozoa to fuse with the egg. The morphology and motility of spermatozoa from mice lacking PC4 is normal. Although the precise cleavage specificity of PC4 has not yet been reported, it is possible that fertilin α is one of the substrates for PC4. If this hypothesis is correct, then a lack of fertilin α processing in mice lacking PC4 might explain, at least in part, how PC4 affects sperm function.

Lack of fertilin α processing could, for example, prevent the release of the protease domain so that it would be unable to reach its substrates. Alternatively, fertilin α processing in the testis may be a prerequisite for subsequent processing of fertilin β in the epididymis. It will be interesting to determine whether fertilin α processing is indeed affected in PC4 knockout mice, or whether another member of the pro-protein convertase family is responsible for processing fertilin α.

Pro-protein convertases have also been implicated in the processing and activation of other ADAMs (Loechel *et al*., 1998, 1999; Lum *et al*., 1998; Roghani *et al*., 1999). In most cases, a pro-protein convertase consensus sequence directs removal of the pro-domain in the trans-Golgi network, leaving the metalloprotease domain membrane anchored. In the case of the metalloprotease-disintegrin MDC9, the pro-domain is apparently necessary for proper protein folding and for keeping the protease inactive, at least until the pro-domain is removed (Roghani *et al*., 1999). Thus, ADAMs with a catalytic site are apparently made as inactive zymogens that need to be activated. Once antibodies against the fertilin α metalloprotease domain become available, it will be possible to determine where and when the pro-domain of the fertilin α metalloprotease is removed, and whether pro-domain removal is necessary for protease activity. There is no indication that the fertilin α pro-domain is removed before the protease domain itself is clipped off its membrane anchor in the secretory pathway (Fig. 2), indicating that the protease is only activated after being released from the membrane.

Proteases that cleave fertilin β

Fertilin β processing begins once fertilin α processing has been completed and spermatozoa leave the testis to migrate through the epididymis (Blobel *et al*., 1990). At least two steps in fertilin β processing can be distinguished (Fig. 2). The first is a cleavage from pro-fertilin β to pro-fertilin β*. The metalloprotease domain of fertilin β does not have a catalytic site and, therefore, fertilin β is not predicted to be catalytically active. Nevertheless, this first processing step resembles the processing of other ADAMs between the pro- and metalloprotease domains. In the second step, pro-fertilin β* is further processed to yield mature fertilin β, consisting of a membrane-anchored disintegrin domain and cysteine-rich region, but lacking the metalloprotease-like domain. While pro-fertilin β* is still detectable in the distal corpus epididymidis, in the distal cauda epididymidis almost all of the pro-fertilin β has been converted to mature fertilin. Taken together, the correlation between proteolytic processing of fertilin β, the exposure of an epitope recognized by a function-blocking antibody, the relocalization of fertilin to the posterior sperm head, and the acquisition of fertilization competence between the distal corpus and proximal cauda epididymidis indicate a potential role of proteolysis in activating fertilin.

How can we learn more about the proteases that process and presumably activate fertilin? In principle, proteases that attack a sperm surface protein such as fertilin in the epididymis could derive from the epididymis, from spermatozoa, or from a combination of both sources. One approach towards isolating biologically relevant proteases is purification from a cell extract on the basis of substrate cleavage specificity and inhibitor profile (for example, see Black *et al*., 1997; Moss *et al*., 1997). However, the epididymis is not an abundant source of material for isolating proteases on the basis of their ability to cleave fertilin or other sperm proteins correctly. Therefore, it was intriguing to observe that the fertilin β precursor (pro-β) is processed spontaneously when testicular spermatozoa are lysed or disrupted by freezing and thawing in the absence of protease inhibitors (Lum and Blobel, 1997). Even though testicular spermatozoa have never been in contact with the epididymis, the resulting fertilin β band pattern closely resembled that of fertilin β on spermatozoa taken from the distal corpus epididymal spermatozoa and lysed in the presence of protease inhibitors.

A more detailed analysis of the processing of fertilin by testicular sperm proteases *in vitro* provided additional evidence for their potential role in fertilin maturation in the epididymis (Lum and Blobel, 1997). First, a time course of fertilin conversion by testicular sperm proteases *in vitro* yielded only fertilin cleavage products that were indistinguishable by western blot analysis from the fertilin intermediates observed in epididymal spermatozoa isolated from different parts of the epididymis. No additional intermediates were observed, even at the earliest time points. Second, partial proteolysis of pro-fertilin β with several commercially available proteases in each case produced a characteristic pattern of fertilin intermediates that was distinct from the maturation pattern seen in the epididymis *in vivo*. (In this second experiment, it was important to use the added proteases at concentrations that lead to partial digestion of pro-fertilin β, mainly because intermediate processing products that are distinct from those observed in the epididymis are most clearly apparent under these conditions.) The observation that distinct fertilin β band patterns were generated by different proteases (Lum and Blobel, 1997) further established that an *in vitro* cleavage assay might be useful to identify candidate fertilin β-converting enzymes. Taken together, these results raised the possibility that testicular sperm proteases are either involved in processing fertilin β in the epididymis, or have very similar cleavage specificity to that the proteases that process fertilin in the epididymis.

Further characterization indicated that the fertilin β-processing activities in testicular sperm proteases are serine proteases (Lum and Blobel, 1997). This finding is consistent with the known cleavage of fertilin β in the epididymis, which occurs after an arginine residue (Blobel *et al*., 1992).

While the identification of a protease activity on testicular spermatozoa that is a candidate fertilin β convertase is intriguing, several important issues remain to be resolved. It is important that the subcellular localization of the fertilin convertase on testicular spermatozoa and the means by which it may be activated are determined. Fertilin is on the sperm surface, so any protease that processes fertilin should either be present on the sperm surface, or should gain access to the sperm surface in the epididymis. As processing of pro-fertilin β *in vitro* requires that spermatozoa are lysed or otherwise disrupted, the protease activity may derive from an intracellular source, such as the acrosome. For an acrosomal protease to function in sperm maturation *in vivo,* it would need to be released in the epididymis to gain access to fertilin. Alternatively, the protease activity observed *in vitro* may reside on the cell surface but require activation, perhaps by releasing other proteases from an intracellular compartment. Again, the physiological activation of a fertilin β convertase on the sperm surface

would normally occur in the epididymis, but would also somehow be triggered by lysing spermatozoa. A potential intracellular source of fertilin β convertase or the activators thereof is the cytoplasmic droplet, a cytoplasmic remnant attached to the sperm tail that contains Golgi vesicles and other intracellular vesicles (Oko *et al*., 1993). The contents of the cytoplasmic droplet are gradually released in the epididymis, where they could have a role in sperm maturation. Finally, fertilin processing *in vitro* can only mimic processing *in vivo* up to the proximal cauda epididymidis stage of sperm maturation. Since the pro domain of fertilin β is completely removed on distal cauda epididymidal spermatozoa, other proteases may be responsible for completing fertilin processing *in vivo,* or it may be that the *in vitro* assay does not activate fertilin β convertases maximally on testicular spermatozoa.

Processing of other sperm proteins in the epididymis

Besides fertilin β, several other proteins are known to be processed during sperm maturation in the epididymis (Phelps *et al*., 1990; Hunnicutt *et al*., 1997), such as PH-20 and its putative rat orthologue 2B1 (Jones *et al*., 1996), the rat posterior tail protein CE9 (Petruszak *et al*., 1991), a rat sperm mannosidase (Tulsiani *et al*., 1995), and other ADAMs with a potential role in fertilization (Linder *et al*., 1995; Yuan *et al*., 1997; Frayne *et al*., 1998). For several of these proteins, proteolytic processing also coincides with a change in localization on the sperm surface. Beyond these specific examples, the pattern of glycoproteins or proteins that can be labelled on the cell surface changes during epididymal maturation (for example, see Brooks and Tiver, 1984; Young *et al*., 1985; Dacheux *et al*., 1989), indicating that processing affects several sperm surface proteins. In addition, proteins secreted from the epididymis are apparently added to the sperm surface in transit through the epididymis (for example, see Okamura *et al*., 1992; Topfer-Petersen *et al*., 1998). With respect to the assay described above, it would be interesting to determine whether other known sperm surface proteins can also be processed by testicular sperm proteases in a manner that resembles processing in the epididymis.

Establishment of the functional significance of processing

Given that processing in the epididymis affects several different sperm surface proteins, it is important to evaluate the functional significance of this processing. One approach would be to analyse spermatozoa from infertile males (human patients, but also animals, including mice possessing mutations or targeted deletions that result in infertility) by searching for processing defects of fertilin or other sperm proteins. With respect to fertilin processing, cases with a normal sperm count and motility but where spermatozoa fail to bind to the zona pellucida or the egg plasma membrane might initially be focussed on. While fertilin α has been found in several species (Perry *et al*., 1995; Wolfsberg *et al*., 1995; Hardy and Holland, 1996; McLaughlin *et al*., 1997; Waters and White, 1997), a functional fertilin α is apparently not expressed in man or some higher primates (Jury *et al*., 1998). Therefore, in humans, this type of analysis may have to concentrate on fertilin β, or on other human sperm proteins that are processed during sperm maturation and for which antibodies are available. An alternative approach that would not require specific antibodies would be to compare glycoproteins (purified on a lectin column), or surface biotinylated proteins isolated from normal spermatozoa or from spermatozoa of infertile patients or animals, and to look for potential differences in processing. If some patients have a defect in one or more of the proteolytic processing events that normally occur during sperm maturation, this would further support the idea that processing is important for function.

The fertilization competence of human spermatozoa would have to be restored by treatment with exogenous proteases to prove that a defect in proteolytic process causes infertility. There are certain caveats about this approach, including the fact that it may be difficult to find defects in sperm surface proteolysis if there is a redundant system of proteases. Conversely, it may not be possible to restore function by adding an exogenous protease if a very specific cleavage site has to be processed for activity (unless a candidate spermatozoa or epididymal protease is available for this purpose). Nevertheless, while cleavage site specificity may be an important criterion for purifying the correct protease from a cell extract, it may not be important for rescuing function so long as a protease added exogenously cleaves in a similar position to that used *in vivo*. While trypsin does not have the same specificity as the endogenous protease that processes fertilin, it nevertheless can convert pro-fertilin into a band that co-migrates with mature fertilin if added at high enough concentrations (Blobel *et al*., 1990).

Conclusion

In summary, there is intriguing circumstantial evidence to indicate that proteolytic processing of fertilin is important for activating its function. Furthermore, other sperm proteins are also processed during epididymal maturation, indicating that proteolysis is used as a more general means to activate sperm surface proteins. Taken together, these observations indicate that a quest for the responsible proteases should be a worthwhile, albeit challenging, endeavour. Identification of the responsible enzymes would provide targets for the design of specific protease inhibitors as male contraceptives. Furthermore, a better understanding of the role of proteolytic processing in sperm maturation in the epididymis may shed light on the potential causes of male infertility, and may lead to novel treatment options for these patients.

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