Analysis of furin ectodomain shedding in epididymal fluid of mammals: demonstration that shedding of furin occurs in vivo

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Abstract

Sperm cell surface proteins and proteins of their surrounding fluids are reported to be proteolytically processed in relation to acquisition of sperm fertility during epididymal transit. Several of these proteins might be potential targets for subtilisin-like pro-protein convertase. Using immunochemistry and mass spectrometry analysis, we found that an 80 kDa form of furin (EC 3.4.21.75) is present in the fluid from the mid-caput to the distal corpus regions of the epididymis of various domestic mammals. This protein is absent from the fluid of the caudal region, suggesting that it is reabsorbed or degraded. The cDNA sequence of ovine furin was obtained and the mRNA was found throughout this organ, although in greater amounts in the mid and distal caput regions. Metabolic labeling with 35S-amino acids indicated that the protein was synthesized and released from the epithelium only in a restricted area of the mid-caput, suggesting a specific regionalized mechanism of secretion. The fluid protein is not pelleted at 100 000 g and did not react with a C-terminal antibody indicating that it is not bound to membranous materials. These findings demonstrate that a furin ectodomain shedding occurs naturally in vivo in the epididymis where this enzyme could be involved in fluid and/or sperm membrane protein processing.


Introduction

The transformation of male gametes into fully motile and fertile spermatozoa is a post-testicular event that occurs in the epididymis, a long duct connecting the testis to the vas deferens and in which spermatozoa spend from days to weeks in transit (Yanagimachi et al. 1985, Jones 1998). The epididymis can be divided into three main morphological regions, i.e. the caput, the corpus, and the cauda, each region being the site of specific secretion and reabsorption of the duct fluid compounds by epithelial cells (Dacheux et al. 2003). The different interactions between the epididymal fluid proteins and the germinal cells are key factors in sperm maturation. Among these interactions, cell surface modifications of proteins by proteolysis and inclusion of newly synthesized and secreted proteins have been clearly demonstrated, but none of the mechanisms involved has yet been characterized (Gatti et al. 2004). However, findings have indicated that pro-protein processing can occur in the fluid and therefore specialized protease(s) involved in such activity should be present.

Furin, a calcium-dependent serine endoprotease expressed ubiquitously in mammalian tissues, is capable of processing precursors of a wide range of bioactive proteins, including some members of MMPs (matrix metalloproteinases) and surface proteins of the ADAM (a disintegrin and metalloprotease) family (for reviews: Seidah & Chretien 1999, Seidah & Prat 2002, Thomas 2002, Stawowy et al. 2005). This enzyme acts by endoproteolytic cleavage after the characteristic motifs containing single or paired basic amino acid residues RX(K/R)R although it is able to cleave after motifs such as (R/K)Xₙ(R/K)R (where n could be 0, 2, 4, or 6 amino acids) and also more degenerated motifs (Seidah & Chretien 1999, Duckert et al. 2004). This glycoprotein is structurally composed of several domains, including a transmembrane domain and a cytoplasmic tail. The transmembrane
domain anchors furin in the membrane of the Golgi network, but the enzyme is also localized in endosomal vesicles and at the cell surface level of different types of epithelium and endothelium (Mayer et al. 2004). It has been reported that the furin ectodomain can be found in supernatants from cell culture and this truncated form called ‘shed’ furin exhibits functional activity although it lacks the transmembrane domain and the cytoplasmic tail (Hatsuzawa et al. 1992, Vidicaire et al. 1993, Vey et al. 1994, Plaimauer et al. 2001, Mayer et al. 2004). However, it has not been demonstrated that an in vivo circulating form of this enzyme exists in body fluids.

This report shows that the epididymal epithelial membrane-bound furin undergoes post-translational processing that results in a soluble enzyme only in distal caput and corpus epididymal fluids of mammals. This enzyme could be a potential candidate for various modifications occurring on the sperm membrane or fluid in the distal caput-corpus region, a place where sperm acquire their ability to move and fertilize the egg.

Materials and Methods

Collection of fluids and spermatozoa

Experiments on animals were conducted according to the French laws on animal experimentation (Authorization 37-081). Epididymides and testes were removed from adult Ile de France rams (at least seven different animals), large white boars (three animals) and ‘selle Franc¸ais’ horses (two different animals) either by castration of anesthetized animals or after slaughter. Luminal fluids were collected from the testis and different zones of the epididymis identified by numbers from 0 to 9 (caput, 0–3/4; corpus, 4–6/7; cauda, 7–9) as previously described (Edwards et al. 1976, Fouche court et al. 2000, Metayer et al. 2002a). Spermatozoa were separated from testicular and epididymal fluids by centrifugation (10 min, 1500 g) and when needed 5% dried skimmed milk (polyclonals). The proteins were transferred to nitrocellulose (0.8 A/cm2 for 2 h) for immunodetection. Membranes were blocked for 1 h with TBS (tris buffered saline) supplemented with 0.5% (w/v) Tween (TBST; monoclonal) and when needed 5% dried skimmed milk (polyclonals). The western blots were incubated either with the mouse MAB MON-148 (Alexis Biochemical Corp., San Diego, CA, USA) directed against the subtilisin-like catalytic domain of human furin amino acid residues 16–189 (van Duinjhoven et al. 1992) or with the rabbit polyclonal directed against the furin C-terminal peptide RGERTASAL (PA1-062; Affinity BioReagents, Golden, CO, USA). The antibodies were diluted in the ratio of 1:1000 and blots incubated 2 h at 37 °C or overnight at 4 °C under agitation. Blots were washed and then incubated for 1 h at 37 °C with a goat anti-mouse or a goat anti-rabbit antibody conjugated with peroxidase (dilution in the ratio of 1:5000; Sigma). After washing thrice with TBST, the peroxidase was detected either on blots with 4-chloro-a-naphthol or visualized by a digital imaging camera with a chemoluminescent substrate (as indicated).

Furin spots excised from 2D SDS-PAGE were reduced and alkylated with iodoacetamide and incubated overnight at 37 °C in a microtube with 12.5 ng/ul trypsin (Sequencing grade, Sigma, France). The solution was then dried, reconstituted with 0.1% formic acid and sonicated for 10 min. The peptides generated were sequenced by nano-LC-MS/MS (Q-TOF-Global equipped with a nano-ESI source; Waters Micromass, St Quentin-en-Yvelines, France) in data-dependent acquisition mode using the three most intense parent ions. The peptides were loaded on a C18 column (Nano Ease Atlantis dC18, 3 µm × 75 µm × 150 mm, Waters) and eluted with 5–60% linear gradient at a flow rate of 180 nl/min in 30 min (buffer A, water/acetoni trile 98/2 (v/v) 0.1% formic acid; buffer B, water/acetoni trile 20/80 (v/v) 0.1% formic acid). MS/MS data analysis was performed with the Mascot software (www.matrixscience.com) using the NCBI non-redundant database or blasted manually against the translated sequence obtained for the ovine furin.

RNA extraction and reverse transcriptase PCR (RT-PCR)

Total RNA samples were prepared from frozen samples (200 mg) of liver, kidney, lung, heart, testis, and specified zones of the epididymis (RNAbile Method, Eurobio, les Ullis, France). The reverse transcriptase assay was performed on 3 µg total RNA using the Superscript Reverse transcriptase H (Invitrogen, Cergy Pontoise, France) and oligo(dt) primers. The epididymal furin sequence was obtained by successive PCRs with 30 pmol of specific primers from the bovine furin precursor sequence (EMBL; European Molecular Biology Laboratory, X75956). New

Gel electrophoresis and mass spectrometry

Methods for preparing gels and samples have been previously described (Syntin et al. 1996). For non-reducing conditions, β-mercaptoethanol was omitted from sample buffer. The proteins were transferred to nitrocellulose (0.8 A/cm2 for 2 h) for immunodetection. Membranes were blocked for 1 h with TBS (tris buffered saline) supplemented with 0.5% (w/v) Tween (TBST; monoclonal) and when needed 5% dried skimmed milk (polyclonals). The western blots were incubated either with the mouse MAB MON-148 (Alexis Biochemical Corp., San Diego, CA, USA) directed against the subtilisin-like catalytic domain of human furin amino acid residues 16–189 (van Duinjhoven et al. 1992) or with the rabbit polyclonal directed against the furin C-terminal peptide RGERTASAL (PA1-062; Affinity BioReagents, Golden, CO, USA). The antibodies were diluted in the ratio of 1:1000 and blots incubated 2 h at 37 °C or overnight at 4 °C under agitation. Blots were washed and then incubated for 1 h at 37 °C with a goat anti-mouse or a goat anti-rabbit antibody conjugated with peroxidase (dilution in the ratio of 1:5000; Sigma). After washing thrice with TBST, the peroxidase was detected either on blots with 4-chloro-a-naphthol or visualized by a digital imaging camera with a chemoluminescent substrate (as indicated).

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sets of primers were designed from the partial sheep sequences (A, 1-ATGCTGAGCCCTGGCCTGGTG-20; A', 549-CGGGGCCATGTGGTCTACT-569; A", 549-GCC-CGGTGACACAGATGA-569; B, 1068-TGGCGCA-GAAATGACAGAG-1086; B', 1257-TTTCTACTGCTT-AAGACCCG-1276; C, 1310-GCCCGCAAGTGAGACGA-CAG-1330; C', 1500-GACTTGGCTACCCACCTGGCTGGTGTCTTCTCCTTCTCCTCA-1520; D, 1629-CTCTGGCGAGTGGGTCTTAG-1648; D', 1805-GAGAGGGAACGTGGTCTTC-1824; F, 1746-CGGGGCCATGTGTGTCTACT-1764; F', 2292-AGCCGCACAGATGA-2311; G, 2283-ACTACCCCCTTGACTTTCTCTGC-2283; G', 2426-AACCAGTGAAACATTAATGACAGAG-2448). Complete coverage was obtained by using primer sets A–A', A"–B', B–C', D–D', C–D', F–F', and G–G' respectively. PCR was performed at the temperature specified for the primer set and a final elongation step at 72 °C for 5 min. Aliquots (5 μl) of each reaction mixture were analyzed on a 1.5% ethidium bromide stained agarose gel. PCRs on tissues were performed with the following pairs of primers: forward 5'-CTCTGGCGAGTGGGTCTTAG-3' and reverse 5'-CTTCTGGTGCAAGGGAACGGAG-3', for either 25 or 40 cycles. β-actin primers (forward 5'-GGACCTTGCCATCCACCTGGT-3' and reverse 5'-GGAGGACCGTCCTCCTCA-3') were used as PCR controls and to equilibrate the quantity of mRNA.

**Northern blotting**

For each sample, 20 μg total RNA were separated by electrophoresis on 1% agarose formamide gel. The RNA was transferred to a nylon membrane (Hybond N+, Amersham, Les Ulis, France) by overnight capillary blotting in 20× SSC and cross-linked by exposure for 30 s under u.v. light. The membrane was stored at room temperature until pre-hybridization. A cDNA probe for furin was made by RT-PCR from zone 3 and labeled with 32P-dCTP (Megaprime II, Amersham). Hybridization was performed overnight at 42 °C with a membrane pre-hybridized with 100 μg/ml salmon sperm DNA (2 h at 42 °C). After incubation, the membrane was then washed once in 1× SSC–0.5% SDS (20 min at room temperature) and then thrice in 0.2× SSC–0.5% SDS (20 min at 68 °C). The transcript was visualized after exposure on radiation-sensitive screen or film.

**Protein deglycosylation**

Fluid from zones 3/4 was boiled in the presence of 1% (v/v) β-mercaptoethanol, and 0.2% (w/v) SDS for 5 min and then incubated for 2 h at 37 °C in the presence of 2% (w/v) CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulphonate), 5 mM EDTA, and 5 U N-glycosidase F (Roche) for 2 h at 37 °C. Negative controls were prepared by replacing the N-glycosidase F by an equal volume of H2O. The reaction was stopped by heating the sample for 5 min at 95 °C.

**Results**

**Immunodetection of furin in mammalian epididymal fluids**

Equivalent amounts of fluid proteins obtained from the rete testis and different epididymal zones (0–9) from rams, boars, and stallions were separated by SDS-PAGE and transferred to nitrocellulose. The membrane probed with the anti-furin MAB showed the presence of one strong reactive band at about 80 kDa in the distal caput and corpus fluids in all three species (Fig. 1). This immunoreactive protein was absent in the proximal caput and cauda, except in the stallions where faint reactivity could be detected. A less intense immunoreaction could be also detected in zones 1/2 in some rams but never in the testis, efferent duct, or zones 0/1. We also extracted the proteins from ovine sperm obtained from the same zones, but when probed with the anti-furin antibody these extracts did not show any reactive band (not shown), indicating that the fluid furin did not derive from sperm.

Ram epididymal fluid from zone 3 was further separated by 2D electrophoresis and one gel was blotted, probed with anti-furin antibody, and revealed with 4-chloro-α-naphthol before staining with Ponceau red (Fig. 2A). Several reactive spots with pl around 6.5–7 were found, suggesting that the protein is post-translationally modified. A similar gel was stained with Coomassie brilliant blue and the equivalent spots to those immunoreactive on western blot were localized (Fig. 2B, arrows). Porcine and equine epididymal fluids from zone 3 were treated the same way (data not shown). All these matching spots from the different species were excised from the gel and treated for analysis by mass...
spectrometry. The sequence obtained from the tryptic fragments by LC-MS-MS confirmed that the spots in the three species were furin (Table 1).

**Sequence and tissue expression of ram furin**

We determined the complete cDNA sequence of ovine furin by successive PCRs (Fig. 3; EMBL acc no. AM084381). The derived 794 amino acid sequence (theoretical Mw: 86 898; pl 6.06) showed up to 97% identity with the bovine furin sequence and more than 90% identity to the other mammalian (human, rat, mouse) furin sequences available on the database.

This sequence presented the canonical regions of furin as four furin-like repeats including two cysteine-rich regions (regions 586–618 and 643–675), the peptidase region (129–301) including the classical triad Asp-His-Ser forming the active center (149, 194, and 366 respectively) and the p-domain specific of the eukaryotic subtilisin-like pro-protein convertases (444–574) (Seidah & Chretien 1999, Thomas 2002).

From this sheep sequence, specific primers were designed and used to study furin expression. Furin amplicon was found as expected in the kidneys, heart, lungs, and liver, although the amounts of messenger were variable even after 40 PCR cycles (Fig. 4A). The messenger was present in the testes and the different parts of the epididymis, as shown after 40 cycles of PCR, although at a lower number of cycles (25) differences in quantity were visible, with preferential expression in the caput (Fig. 4B). The difference was further confirmed by northern blotting (Fig. 4C). Using the amplicon from zone 3 as a probe, a >4 kb radioactive band was observed in the different zones but radioactivity was much higher in zones 2–4 although the same quantities of mRNA were used as shown by the 28S and 18S mRNA staining in the different gel lanes (Fig. 4C, bottom).

**Epithelial cell-derived epididymal fluid furin**

Furin mRNA was present throughout the epididymal zones, although in different amounts, but the protein was visible only in the distal caput and corpus. We then investigated whether this protein was secreted in the different zones or in a more restricted area. Isolated tubules from the different zones were incubated in the presence of 35S-methionine and cysteine, and after 6 h the luminal fluid was perfused and separated by 1D and 2D gel (Syntin et al. 1996). The 1D SDS-PAGE probed with the anti-furin antibody indicated that the protein was present from zones 2 to 6 in this animal (data not shown). Two gels were then prepared for each immunoreactive zone. One gel was silver stained and the second was blotted and revealed with

### Table 1

<table>
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<tr>
<th>Species</th>
<th>Peptide sequences</th>
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<td>DMDQHILVVR</td>
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<tr>
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<tr>
<td>Boar</td>
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Sequences of peptides were obtained by homology with bovine furin precursor (Q28193), rat furin precursor (P23377), and human furin precursor (P09958) and the sheep sequence from Fig. 3.
Figure 3 Sheep furin cDNA sequence. Nucleotide and predicted amino acid sequences of the cDNA that encodes sheep furin (EMBL acc no. AM084381). The underlined sequences are those obtained by LC-MS-MS from the ram epididymal fluid furin.
mammals (Dacheux et al. 2005, Gatti et al. 2005), it was important to determine whether the fluid furin was attached to membrane or secretion vesicles or free in the fluid as a shed extracellular domain. The fluid from zones 3 and 9 from ram and boar were high-speed centrifuged at >100,000 g for 2 h in order to pellet all membranous materials and vesicles. Visible pellets were found in cauda fluids of both species and in zone 3 fluid of boar but not of ram. The supernatant of each samples and the resuspended pellet (or the solution used for washing the bottom of the tube of zone 3 from ram) were loaded on gels, transferred to nitrocellulose and probed with the monoclonal MON-148 or the anti-C-terminal polyclonal (Fig. 6). For both ram and boar the >100,000 g centrifugation did not change the immunoreactivity of the fluid at 80 kDa and only low reactivity was found in the pellet (note that the pellet is concentrated 25 times for ram and 5 times for boar from the initial volume). In boar, a 43 kDa reactive band became visible in the pellet that was only barely visible in the fluid. No clear reactive band could be found in the cauda fluid and pellets from ram and boar (data not shown). When similar blots were probed with the polyclonal antibody against the C-terminal only a low intensity band could be observed at 50–55 kDa in ram fluid but not in pellet, while in boar only immunoreactive bands at about 14 kDa could be observed in the fluid and at 43 kDa in the pellet. Only the 14 kDa bands were clearly visible in boar cauda fluids before and after high-speed centrifugation but not in pellet, and no other bands could be observed (data not shown).

The fluid furin is a shed domain truncated C-terminally

Since furin is normally an intravesicular protein that could be found at the cell surface and as it is known that vesicular systems are present in the epididymal fluid of mammals (Dacheux et al. 2005, Gatti et al. 2005), it was important to determine whether the fluid furin was attached to membrane or secretion vesicles or free in the fluid as a shed extracellular domain. The fluid from zones 3 and 9 from ram and boar were high-speed centrifuged at >100,000 g for 2 h in order to pellet all membranous materials and vesicles. Visible pellets were found in cauda fluids of both species and in zone 3 fluid of boar but not of ram. The supernatant of each samples and the resuspended pellet (or the solution used for washing the bottom of the tube of zone 3 from ram) were loaded on gels, transferred to nitrocellulose and probed with the monoclonal MON-148 or the anti-C-terminal polyclonal (Fig. 6). For both ram and boar the >100,000 g centrifugation did not change the immunoreactivity of the fluid at 80 kDa and only low reactivity was found in the pellet (note that the pellet is concentrated 25 times for ram and 5 times for boar from the initial volume). In boar, a 43 kDa reactive band became visible in the pellet that was only barely visible in the fluid. No clear reactive band could be found in the cauda fluid and pellets from ram and boar (data not shown). When similar blots were probed with the polyclonal antibody against the C-terminal only a low intensity band could be observed at 50–55 kDa in ram fluid but not in pellet, while in boar only immunoreactive bands at about 14 kDa could be observed in the fluid and at 43 kDa in the pellet. Only the 14 kDa bands were clearly visible in boar cauda fluids before and after high-speed centrifugation but not in pellet, and no other bands could be observed (data not shown).

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Figure 4 Tissue expression of furin mRNA. (A) Complementary cDNA obtained after 40 PCR cycles for furin in the Kidneys (Kd), Heart (H), Lung (Lu), and Liver (Li). Actin PCR (25 cycles) showed the related amounts of cDNA in the different samples. (B) Furin mRNA was also amplified by 40 PCR cycles using cDNA prepared from the testis and the different epididymal zones. When only 25 cycles were used, furin amplicon was not observed in the testis and was less amplified in the cauda than in the caput and corpus. Actin PCR (25 cycles) was used to equilibrate cDNA of the different tissues. (C) Total RNA samples from the different epididymal zones. When only 25 cycles were used, furin mRNA quantities were greater in zones 2 to 4. 18S and 28S mRNAs were used as internal controls. (D) Lung (Lu), and Liver (Li). Actin PCR (25 cycles) was used to detect actin mRNA in the different zones. The fluid furin is a shed domain truncated C-terminally.
Shed furin domain is monomeric and N-glycosylated

Fluids from zones 3/4 were run on mono-dimensional gel under non-reducing or reducing conditions in order to ascertain the size of the shed domain (Fig. 7). In the presence or the absence of β-mercaptoethanol, the protein migrated at the same apparent molecular weight, suggesting that it is monomeric and no part of the pro-domain remains linked by disulfide bonds to the shed ectodomain. It has been shown that furin is a N-glycosylated protein, when the fluid from regions 3/4 was treated with N-glycosidase (PNGase F), a shift of about 7–10 kDa on the apparent MW of furin was observed showing the presence of N-glycosylation and indicating that the shed domain amino acid sequence is about 70 kDa in mass.

Discussion

Furin (EC3.4.21.75, also called PACE or paired basic amino acid residue-cleaving enzyme) is an endoprotease that belongs to the family of mammalian subtilisin-like pro-protein convertases. This family comprises nine members subdivided into three classes according to their tissue distribution. For example, furin, PACE4, PC5/PC6, and PC7/PC8 are widely distributed in the body tissues, PC1/PC3 and PC2 are mostly in neuroendocrine tissues, while PC4 is a mammalian testicular enzyme (for reviews: Steiner 1998, Seidah & Chretien 1999, Bergeron et al. 2000, Taylor et al. 2003). PCs are processing enzymes that act to mature a wide variety of proteins, such as hormones, growth factors, proteases, bacterial toxins, and virion coat glycoproteins, by cleaving their pro-domains (Chretien et al. 1995, Denault & Leduc 1996, Nakayama 1997). These different enzymes may have a certain level of redundancy but may also have specific functions in certain organs (Roebroek et al. 2004). Furin mRNA has previously been found in the testis (Torii et al. 1993) but not, to our knowledge,
reported in the epididymal tissue where it is present throughout this organ but with different levels of expression according to the region. We also clearly demonstrated by immunoblotting and mass spectrometry that this protease is transiently present in the luminal fluid of the mammalian epididymis, as the result of shedding after synthesis by the epithelium occurring in a very restricted area comprising the mid- and distal caput.

Furin shares its common mosaic structure with the other family members: i.e. a signal peptide, a pro-domain, a catalytic domain, a ‘P’ domain, and a carboxy-terminal domain that anchors the protein in membrane. The sequence obtained for the sheep furin was in complete agreement with this description. In general, the catalytic domain is the most conserved sequence among the subtilisin-like proteases while the C-terminal domain is less conserved, although it plays an important role in the intracellular routing of the enzyme (Seidah & Chretien 1999, Thomas 2002). For example, the furin cytoplasmic tail contains phosphorylation sites and their level of phosphorylation allows the protein to pass from the Golgi to the plasma membrane via the secretory granules and then to return to the Golgi via the endocytic pathway. The presence of furin at the cell surface during this cycle has been documented and its involvement in cell surface modifications of proteins demonstrated (Steiner 1998, Mayer et al. 2004, Koo et al. 2006). Several studies using different in vitro cell systems overexpressing this enzyme have reported the existence of truncated forms of furin in culture medium as a result of a proteolytic process (Vidicaira et al. 1993, Vey et al. 1994, Plaimauer et al. 2001). Furin is normally synthesized in a zymogenic form as a 100 kDa precursor and is activated and sorted after an intramolecular autoproteolytic cleavage of its pro-peptide, leaving a 90 kDa protein in cells (Creemers et al. 1995). Once released in the culture medium, the mature active ectodomain was found at 75–80 kDa, in agreement with an initial site of cleavage after Arg683 leaving a 10–15 kDa tail in the cell (Plaimauer et al. 2001). The presence of only one mRNA transcript in the epididymis indicates that the mature protein must be at least 90 kDa (from the translated cDNA sequence) without the mass of post-translational processing such as glycosylation and sialylation (Hatusuzawa et al. 1992). The fluid protein is about 80 kDa under denaturing and non-denaturing conditions and N-glycosylations represent 7–10 kDa of this mass. Thus the final difference in mass observed of about 20 kDa fits quite well with the removal of the transmembrane and intracellular domains and strongly suggests that the extracellular domain is released in the fluid upon a proteolytic shedding. This conclusion is also sustained by our different results which showed that the 80 kDa fluid furin is (i) not bound to membraneous materials, (ii) not reactive with the anti-C-terminal polyclonal antibody, and (iii) none of the peptides obtained by mass spectrometry in the three species are situated within the N-terminal or C-terminal part of the enzyme.

In boar, we observed that a 14 and 18 kDa immuno-reactive C-terminal are present in the fluid and accumulate in the cauda, but this immunoreactive compounds are not linked to vesicles or membranes. This compounds may be secreted within membraneous apical blebs that are rapidly destroyed after their secretion in the fluid (Hermo & Jacks 2002, Dacheux et al. 2005). It has not yet been clearly established whether the C-terminal cleavage of furin occurs intracellularly, leading to the secretion of the truncated form, or whether it occurs once the mature protein is on the cell surface. In the former case, it is suggested that furin itself or a related PC is involved in this process (Denault et al. 2002), although the cleavage site is not a canonical sequence for these enzymes. Moreover, the fact that mutation or deletion of the cleavage site severely reduces ectodomain release without suppressing it suggests that there is no definite cleavage site (Plaimauer et al. 2001). Alternatively, this might indicate that different enzymes could be involved in the ectodomain shedding process, such as the described membrane metalloproteases, from the ADAM family.

Shedding of furin in the epididymis occurs in a region where several proteins from the sperm surface and from their surrounding media are processed. For example, we have shown that the germinal form of angiotensin-I converting enzyme (ACE) located at the sperm surface is removed from these cells as they pass through the anterior caput epididymis. We recently reported that this release is dependent upon a serine protease activity that is present in or activated by the fluid from this area (Gatti et al. 1999, Metayer et al. 2002b, Thimon et al. 2005). The shedding of germinal ACE (gACE) and furin thus occurs at very close sites in the epididymis after a similar proteolytic cleavage after an Arg situated at about 25–30 amino acids from the transmembrane domain. Serine protease activity has also been reported to be involved in the processing of sperm surface proteins such as fertilin and some other ADAM proteins (Frayne et al. 1998, Blobel 2000, 2005) and hyaluronidase 2B1-pH20 (Jones et al. 1996, Morin et al. 2005). These proteins have been shown to be processed when sperm cross the caput or corpus epididymis and to be involved in sperm fertility. We have also reported that several members of the matrix metalloproteases present in the epididymal fluid were activated from pro- to active forms after their transformation within the fluid (Metayer et al. 2002a), and it has recently been demonstrated that an anti-microbial protein in human epididymal fluid and seminal plasma is processed in vivo and in vitro by furin (von Horsten et al. 2002).
We investigated the enzymatic activity of the shed furin in the fluid with a specific fluorogenic substrate (Angliker et al. 1995). Although the activity measured in the crude epididymal fluids was very sensitive to the reported furin peptidyl inhibitor (decanoxy-Arg-Val-Lys-Arg-chloromethylketone), we observed that the substrate was also cleaved by other peptidyl proteases present in the fluid such as ACE, neprilysin, and dipeptidyl-peptidase IV (Gatti et al. 2005, Thimon 2005). Classical chromatographic purification could not provide evidence that the fluid furin was active since furin could not be completely separated ACE (Thimon 2005). Moreover, the epididymal fluid is known to contain a large number of protease inhibitors (including PC2 cystatin-like inhibitor; Cornwall et al. 2003), which may also interfere with this type of measurement.

The role of furin on sperm fertility remains to be clearly established. Unfortunately, furin-knockout (KO) mice are not viable and cannot be used to evaluate the role of furin in fertility, and this would require a mouse model with a conditional KO in the epididymis.

In conclusion, we have provided the first demonstration that a furin-shed ectodomain is present in a body fluid, the epididymal fluid of mammals, where it might be involved in maturation of diverse pro-proteins present on sperm and in the fluid. The epididymis, which is a closed environment, represents an interesting model to study the extracellular activity of such shed ectodomain but also to study the shedding mechanism, which may also occur in other organs but may be more difficult to study in vivo.

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