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

Véronique Thimon, Maya Belghazi¹, Jean-Louis Dacheux and Jean-Luc Gatti

Equipe 'Gamètes Males et Fertilité', UMR 6175 INRA, CNRS-Université de Tours-Haras Nationaux, Station de Physiologie de la Reproduction et des Comportements, Institut National de la Recherche Agronomique, 37380 Nouzilly, France and ¹Service de Spectrométrie de Masse pour la Protéomique, UMR 6175 INRA, CNRS-Université de Tours-Haras Nationaux, Station de Physiologie de la Reproduction et des Comportements, Institut National de la Recherche Agronomique, 37380 Nouzilly, France

Correspondence should be addressed to J-L Gatti; Email: gatti@tours.inra.fr

V Thimon is now at Unité d'Ontogénie et Reproduction, Centre de Recherche du CHUL, 2705, Blv. Laurier, Sainte Foy, Québec, Canada G1V4G2

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.

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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)X_n(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 vitro* 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, Vidricaire *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-corporis 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 Français' 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, Fouchecourt *et al.* 2000, Metayer *et al.* 2002a). Spermatozoa were separated from testicular and epididymal fluids by centrifugation (10 min, 1500 g) and the supernatants were centrifuged twice at 15 000 g for 5 min and kept at –20 °C. For high-speed centrifugation, 2 ml of supernatant from zones 3 and 9 from ram and boar were submitted at >100 000 g for 2 h (rotor 50ti, L8M ultracentrifuge, 35 000 r.p.m.; Beckman-Coulter, Villepinte, France), the high speed supernatant and the pellets were separated and treated for gel electrophoresis (see further).

The *in vitro* secretion of proteins from isolated tubules was analyzed after metabolic labeling with 100 µCi of ³⁵S-protein labeling mix (NEN, Les Ulis, France) under O₂–CO₂ (95–5%) as previously described (Syntin *et al.* 1996). At the end of the incubation period, the luminal content of the tubule was collected and centrifuged (15 000 g for 30 min), and the supernatants kept at –20 °C until analysis by one-dimensional (1D) and two-dimensional (2D) SDS-PAGE and western blotting.

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 Duijnhoven *et al.* 1992) or with the rabbit polyclonal directed against the furin C-terminal peptide RGERTA-FIKDQSAL (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-α-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/µl 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/acetonitrile 98/2 (v/v) 0.1% formic acid; buffer B, water/acetonitrile 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 (RNable Method, Eurobio, les Ulis, 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

sets of primers were designed from the partial sheep sequences (A, 1-ATGCTGAGGCCTGGCCTGGTT-20; A', 549-CGGGGCCATGTGTGTCTACT-569; A'', 549-GCC-CCGGTACACACAGATGA-569; B, 1068-TGCGGCA-GAAGTGACAGAG-1086; B', 1257-TTTCCTCGGT-AAGACCCGG-1276; C, 1310-GGCCCAGAATGGACGA-CAG-1330; C', 1500-GACCTTGCCATCCACCTGGT-1520; D, 1629-CTCTGGCGAGTGGGTCTTAG-1648; D', 1805-CGAAGAGGAACGTGGTCTTC-1824; F, 1746-CCGAGAGCATCGGCTGTAAG-1764; F', 2292-ACTTC-GGACCGTCTCCTCA-2311; G, 2283-ACTACCCCT-GAAGCCTGGC-2311; G', 2426-ACCCCGTGAATAAT-TAAGTGG-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'-CTCTGGC-GAGTGGGTCTTAG-3' and reverse 5'-CTTCTGG-TGCAAGGAGAAGC-3', for either 25 or 40 cycles. β -actin primers (forward 5'-GGACTTCGAGCAGGA-GATGG-3' and reverse 5'-GCACCGTGTGGCGTA-GAGG-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 formaldehyde 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 32 P-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 radioactivity-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-propanesulfonate), 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 pI 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

Figure 1 Immunodetection of furin in mammalian epididymal fluids. Equivalent amounts of proteins from ram, boar, and horse rete testis fluid, and epididymal fluids (zones 0 to 8/9) were loaded on a 6–16% SDS-PAGE, blotted, and probed with anti-furin MAB. A strong immunoreaction at about 70 kDa was observed from the mid caput and corpus regions in all three species.

Figure 2 2D SDS-PAGE separation of furin. Ram epididymal fluid from zone 3 (about 100 µg protein) was separated by 2D electrophoresis. One gel was transferred and probed with the anti-furin antibody revealed by 4-chloro- α -naphthol and then stained with Ponceau red (A). The matching spots (arrows) found on an equivalent Coomassie blue stained gel (B) were excised from the gel to be treated for mass spectrometry analysis (see Table 1).

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; pI 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

Table 1 LC-MS-MS sequences obtained for the Ram, Boar and Stallion epididymal furin.

Species	Peptide sequences
Ram	LTLSYNR
	CVIDILTEPK
	DMQHLLVVR
	GIVVSILDDGIEK
	MLDGEVTDAREAR
	FPQQWYLSGVTQR
	TVDGPALHAEEAFFR
	TLTSSQACVVCEEGLSLHQR
Boar	LTLSYNR
	QIVTTDLR
	GIVVSILDDGIEK
	CIIDILTEPKDIGKR
	MLDGEVTDAREAR
	GGLGSIFVWASGNNGR
	TVTACLGEPSHITR
	LAEEAFFRGVSQGR
	RGDLAIHLVSPMGR
	KTVTACLGEPSHITR
	DLNVKEAWAQGYTGR
	TLTSSQACVVCEEGLSLHQR
	TVTACLGEPSHITRLEHAQAR
	FTLVLYGTAPEGLPTPPESIGCK
Stallion	KTVTACLGEPSHITRLEHAQAR
	NHPDLAGNYDPGASFDVNDQDPDPQPR
	SLGLNPNHIIHYSASWGPEDDGKTVVDGPAR
	LTLSYNR
	QIVTTDLR
	GIVVSILDDGIEK
	MLDGEVTDAREAR
	MLDGEVTDAREAR
	GGLGSIFVWASGNNGR
	TLTSSQACVVCEEGLSLHQR
	TVTACLGEPSHITRLEHAQAR
	GIVVSILDDGIEKNHPDLAGNYDPGASFDVN...DQDPDPQPR

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.

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

ATG	GAG	CTG	AGG	CCC	TGG	TTG	TTC	TGG	GTG	GTA	GCA	ATA	GCG	GGA	GCC	TTG	ATT	CTG	CTG	GTG	GCC	GAT	GCC	CGC	GGA	GAG	AAG	84	
M	E	L	R	P	W	L	F	W	V	V	A	I	A	G	A	L	I	L	L	V	A	D	A	R	G	E	K	28	
GTC	TTC	ACC	AAC	ACC	TGG	GCC	GTG	CAC	ATT	CCT	GGA	GGC	CCA	GCC	GTC	GCT	GAC	CGT	GTG	GCG	CAC	AAG	CAT	GGC	TTC	CTC	AAC	168	
V	F	T	N	T	W	A	V	H	I	P	G	G	P	A	V	A	D	R	V	A	H	K	H	G	F	L	N	56	
CTG	GGC	CAG	ATC	TTC	GGT	GAC	TAT	TAC	CAC	TTC	TGG	CAT	CGG	GCG	GTG	ACA	AAG	CGG	TCC	CTG	TCG	CCT	CAC	CTC	CCG	CGG	CAC	252	
L	G	Q	I	F	G	D	Y	Y	H	F	W	H	R	A	V	T	K	R	S	L	S	P	H	L	P	R	H	84	
AAC	CGG	CTG	CAG	CGG	GAA	CCT	CAA	GTT	AAG	TGG	CTG	GAG	CAG	CAG	GTG	GCA	AAG	CGA	CGG	GCC	AAA	CGG	GAC	ATA	TAC	CAG	GAG	336	
N	R	L	Q	R	E	P	Q	V	K	W	L	E	Q	Q	V	A	K	R	R	A	K	R	D	I	Y	Q	E	112	
CCC	ACG	GAC	CCC	AAG	TTT	CCC	CAG	CAG	TGG	TAC	CTG	TCT	GGT	GTC	ACC	CAG	CGG	GAC	CTG	AAT	GTG	AAG	GAG	GCC	TGG	GGC	CAG	420	
P	T	D	P	K	F	P	Q	Q	W	Y	L	S	G	V	T	Q	R	D	L	N	V	K	E	A	W	G	Q	140	
GGC	TAC	ACG	GGG	CGC	GGC	ATT	GTG	GTC	TCT	ATC	CTG	GAT	GAC	GGC	ATC	GAG	AAG	AAC	CTC	CCC	GTC	TTG	GCA	GGC	AAT	TAT	GAT	504	
G	Y	T	G	R	G	I	V	V	S	I	L	D	D	G	I	E	K	N	L	P	V	L	A	G	N	Y	D	168	
CCT	GGG	GCC	AGC	TTC	GAT	GTC	AAT	GAT	CAG	GAC	CCT	GAC	CCC	CAG	CCC	CGG	GTC	ACA	CAG	ATG	AAT	GAC	AAC	AGG	CAT	GGC	ACA	588	
P	G	A	S	F	D	V	N	D	Q	D	P	D	P	Q	P	R	V	T	Q	M	N	D	N	R	H	G	T	196	
CGG	TGT	GCA	GGA	GAG	GTG	GCT	GCN	GTG	GCC	AAC	AAT	GGT	GTC	TGT	GGT	GTA	GGC	GTG	GCC	TAC	AAT	GCC	CGC	ATT	GGA	GGG	GTG	672	
R	C	A	G	E	V	A	A	V	A	N	N	G	V	C	G	V	T	G	V	A	Y	N	A	R	I	G	G	V	224
CGC	ATG	CTG	GAC	GGC	GAA	GTG	ACA	GAC	GCC	GTG	GAG	GCA	CGC	TCG	CTG	GGC	CTG	AAC	CCC	AAC	CAC	ATC	CAC	ATC	TAC	AGT	GCC	756	
R	M	L	D	G	E	V	T	D	A	V	E	A	R	S	L	G	L	N	P	N	H	I	H	I	Y	S	A	252	
AGC	TGG	GGC	CCC	GAG	GAC	GAC	GGC	AAG	ACC	GTG	GAT	GGG	CCA	GCC	CAC	CTG	GCT	GAG	GAG	GCC	TTC	TTC	CGG	GGG	GTC	AGC	CAG	840	
S	W	G	P	E	D	D	G	K	T	V	D	G	P	A	H	L	A	E	E	A	F	F	R	G	V	S	Q	280	
GGC	CGT	GGG	GGT	CTG	GGC	TCC	ATC	TTT	GTC	TGG	GCC	TCG	GGG	AAC	GGG	GGC	CGG	GAA	CAT	GAC	AGC	TGC	AAT	TGC	GAC	GGC	TAC	924	
G	R	G	G	L	G	S	I	F	V	W	A	S	G	N	G	G	R	E	H	D	S	C	N	C	D	G	Y	308	
ACC	AAC	AGC	ATC	TAC	ACG	CTG	TCC	ATC	AGC	AGC	GCC	ACA	CAG	TTC	GGC	AAC	GTG	CCC	TGG	TAC	AGT	GAG	GCC	TGC	TCG	TCC	ACA	1008	
T	N	S	I	Y	T	L	S	I	S	S	A	T	Q	F	G	N	V	P	W	Y	S	E	A	C	S	S	T	336	
CTG	GCC	ACC	ACC	TAC	AGC	AGT	GGC	AAC	CAG	AAC	GAG	AAG	CAG	ATC	GTG	ACC	ACT	GAC	CTG	CGG	CAG	AAG	TGT	ACA	GAG	TCT	CAC	1092	
L	A	T	T	A	Y	S	S	G	N	N	E	K	Q	I	V	T	T	D	L	R	Q	K	C	T	E	S	H	364	
ACG	GGC	ACC	TCT	GCG	TCT	GCC	CCC	CTG	GCA	GCA	GGC	ATC	ATT	GCT	CTC	ACC	CTG	GAG	GCC	AAT	AAG	AAC	CTC	ACC	TGG	CGG	GAC	1176	
T	G	T	S	A	S	A	P	L	A	A	G	I	I	A	L	T	L	E	A	N	K	N	L	T	W	R	D	392	
ATG	CAG	CAC	TTG	GTG	GTC	CGG	ACC	TCA	AAG	CCA	GCC	CAC	CTC	AAC	GCC	AAC	GAC	TGG	GCC	ACC	AAC	GGT	GTG	GGC	CGC	AAA	GTG	1260	
M	Q	H	L	V	V	R	T	S	K	P	A	H	L	N	A	N	D	W	A	T	N	G	V	G	R	K	V	420	
AGC	CAT	TCG	TAC	GGC	TAT	GGG	CTG	TTG	GAC	GCC	GGC	GCC	ATG	GTG	GCT	CTG	GCC	CAG	AAT	TGG	ACG	ACA	GTG	GCC	CCT	CAG	CGG	1344	
S	H	S	Y	G	Y	G	L	L	D	A	G	A	M	V	A	L	A	Q	N	W	T	T	V	A	P	Q	R	448	
AAA	TGC	GTC	ATC	GAC	ATC	CTC	ACT	GAG	CCC	AAG	GAC	ATT	GGG	AAG	CGG	CTG	GAG	GTG	CGG	AAG	ACT	GTG	ACC	GCC	TGC	CTG	GGG	1428	
K	C	V	I	D	I	L	T	E	P	K	D	I	G	K	R	L	E	V	R	K	T	V	T	A	C	L	G	476	
CAG	CCC	AGC	CAC	ATC	ACG	CGG	CTG	GAG	CAC	GCT	CAG	GCG	CGG	CTC	ACC	CTG	TCC	TAC	AAC	CGC	CGC	GGT	GAC	CTT	GCC	ATC	CAC	1512	
Q	P	S	H	I	T	R	L	E	H	A	Q	A	R	L	T	L	S	Y	N	R	R	G	D	L	A	I	H	504	
CTG	GTC	AGC	CCC	ATG	GGC	ACC	CGC	TCC	ACC	CTG	CTG	GCT	GCC	AGG	CCA	CAC	GAC	TAC	TCT	S	GCA	GAT	GGG	TTT	AAC	GAC	TGG	GCC	1596
L	V	S	P	M	G	C	R	S	C	T	L	L	A	A	R	C	H	D	Y	S	A	D	G	F	N	D	W	A	532
TTC	ATG	ACG	ACC	CAT	TCC	TGG	GAC	GAG	GAT	CCC	TCT	GGC	GAG	TGG	GTC	TTA	GAG	ATT	GAA	AAC	ACC	AGT	GAA	GCC	AAA	AAC	TAT	1680	
F	M	T	T	H	S	W	D	E	D	P	S	G	E	W	V	L	E	I	E	N	T	S	E	A	K	N	Y	560	
GGG	ACG	CTG	ACC	AAG	TTC	ACC	CTC	GTG	CTT	TAT	GGC	ACG	GCC	CCT	GAG	GGT	TTG	CCC	ACA	CCC	CCC	GAG	AGC	ATC	GGC	TGT	AAG	1764	
G	T	L	T	K	F	T	L	V	L	Y	G	T	A	P	E	G	L	P	T	P	P	P	E	S	I	G	C	K	588
ACC	CTC	ACT	TCC	AGC	CAG	GCC	TGT	GTG	GTG	TGC	GAG	GAA	GGC	TTC	TCC	TTG	CAC	CAG	AAG	AAC	TGC	GTC	CAG	CAC	TGT	CCC	CCA	1848	
T	L	T	S	S	Q	A	C	V	V	C	E	E	G	F	S	L	H	Q	K	N	C	V	Q	H	C	P	P	616	
GGC	TTC	GCT	CCC	CAA	GTC	CTG	GAC	ACA	CAC	TAC	AGC	ACA	GAG	AAC	GAC	GTG	GAG	ATC	ATC	CGG	GCC	AGC	GTC	TGC	ACG	CCC	TGC	1932	
G	F	A	P	Q	V	L	D	T	H	Y	S	T	E	N	D	V	E	I	I	R	A	S	V	C	T	P	C	644	
CAC	ACC	TCG	TGC	GCC	ACG	TGC	CAG	GGC	CCC	GCC	CCC	ACA	GAC	TGC	CTC	AGC	TGC	CCC	AGC	CAC	GCC	TCC	CTG	GAC	CTC	GTG	GAG	2016	
H	T	S	C	A	T	C	Q	G	P	A	P	T	D	C	L	S	C	P	S	H	A	S	L	D	L	V	E	672	
CGG	ACG	TGC	TCG	CGC	CAG	AGC	CAG	AGC	AGC	CGC	GAG	TCG	CAC	CAG	CAG	CAG	CCG	CCA	CCG	CCG	CCG	CCC	GTG	GAG	GTG	GCG	TCG	2100	
R	T	C	S	R	Q	S	Q	S	S	R	E	S	H	Q	Q	Q	Q	P	P	P	P	P	V	E	V	A	S	700	
GAG	CCG	CGG	CTG	CGG	GCG	GAC	CTG	CTG	CCC	TCG	CAC	CTG	CCC	GAG	GTG	GTG	GCC	GGC	CTC	AGC	TGC	GCC	TTC	ATT	GTG	CTG	GTC	2184	
E	P	R	L	R	A	D	L	L	P	S	H	L	P	E	V	V	A	G	L	S	C	A	F	I	V	L	V	728	
TTC	GTC	ACC	GTC	TTC	CTG	GTC	CTG	CAG	CTG	CGC	TCG	GGC	TTC	AGC	TTC	CGA	GGG	GTG	AAA	GTG	TAC	ACC	ATG	GAC	CGT	GGC	CTC	2268	
F	V	T	V	F	L	V	L	Q	L	R	S	G	F	S	F	R	G	V	K	V	Y	T	M	D	R	G	L	756	
ATC	TCC	TAC	AAG	GGA	CTA	CCC	CCT	GAA	GCC	TGG	CAG	GAG	GAG	TGC	CCG	TCC	GAC	TCG	GAG	GAG	GAC	GAG	GGC	CGG	GGC	GAG	AGG	2352	
I	S	Y	K	G	L	P	P	E	A	W	Q	E	E	C	P	S	D	S	E	E	D	E	G	R	G	E	R	784	
ACC	GCC	TTT	ATC	AAA	GAC	CAG	AGC	GCC	CTT	2382																			
T	A	F	I	K	D	O	S	A	L	794																			

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.

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 testis and different epididymal zones were hybridized with a cDNA furin probe, a transcript was observed in all epididymal zones but quantities were greater in zones 2 to 4. 18S and 28S mRNAs were used to visualize mRNA quantities from the gel. pb, bp.

the anti-furin antibody, then both the gel and the membrane were dried and exposed to autoradiographic films (Fig. 5). The furin immunoreactive spots were found by immunoblotting from zones 2 to 6 and these spots could be matched with radioactive spots only from zones 2 to 4 (Fig. 5). Immunoreactive spots could be seen in zone 2, but the radioactive and silver stain spots were barely visible, indicating a low level of secretion and accumulation (Fig. 5, Z2 arrows). In zones 3 and 4, the secreted spots were well individualized and could be easily matched with the strong immunoreactive and silver stained spots (Fig. 5 Z3 and Z4 boxes). The secretion was lower in zone 4 than in zone 3. In zone 6, the protein was still present in the fluid, as shown both by silver staining and immunoblotting, but no ^{35}S -labeled protein could be seen (Fig. 5, Z6), suggesting that it is only weakly secreted and that the immunoreactive protein was a remnant from secretion of the previous zones transported by the fluid.

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

Figure 5 Analysis of furin secretion. Ram epididymal fluid from was obtained from Z2 to Z6 after metabolic labeling of the tubule secretion by ^{35}S -cysteine and methionine. Equivalent amounts of fluid proteins from each zone were separated by 2D gel electrophoresis. One gel was transferred and probed with the anti-furin antibody, revealed by 4-chloro- α -naphthol and then stained with Ponceau red (WB + PR). The strong dark spots indicated the furin position. This blot was dried and the radioactive proteins revealed by autoradiography (^{35}S) to match the immunoreactive spots with the secretions (boxes). An equivalent gel was silver stained (silver) to visualize the proteins present in the fluid and match the furin spots with the immunoreactive and radioactive spots (boxes).

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 $>100\,000\text{ g}$ 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 $>100\,000\text{ g}$ 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).

Figure 6 The furin ectodomain is shed and C-terminally truncated. Fluid from epididymal zone 3 of ram and boar (2 ml pooled from three different animals for both species) were centrifuged at 15 000 *g* then at 100 000 *g* for 2 h. The supernatants labeled 15 000 and 100 000 *g* respectively as well the pellet or the material collected from the bottom of the tube (labeled pellet) were separated on a 6–16% SDS-PAGE, transferred onto nitrocellulose, and probed either with the mouse monoclonal 148 (Mon-148) or the rabbit polyclonal anti C-terminal (polyclonal anti-Ct). The secondary peroxidase antibody was revealed with 4-chloro- α -naphthol and the blot photographed for the Mon-148 and imaged with a digital device using a sensitive chemoluminescent substrate for the rabbit polyclonal.

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

Figure 7 The shed furin ectodomain is monomeric and glycosylated. Ram epididymal fluid from zones 3 to 4 was run under reducing (Red) or non-reducing (NoRed) conditions and the nitrocellulose probed with the MAB Mon-148. No important difference in migration was observed. (B) The same fluid was run under reducing conditions with (+) or without (–) treatment with PNGase F in order to remove the *N*-glycosylation. The treatment resulted in a molecular weight shift of about 7–10 kDa as calculated from the standard molecular weight migration.

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 carboxyl-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 endocytotic 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 (Vidricaire *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 (Hatsuzawa *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 membranous 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 membranous 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 (TACE (tumor necrosis factor- α -converting enzyme), ADAM10, ADAM9, etc.) or an unknown serine protease (Pang *et al.* 2001). Interestingly, several ADAMs (ADAM7, ADAM28, etc.) are expressed in an epididymal region-specific manner (caput region) and the activation of these proteases may require the action of furin or other PCs (Howard *et al.* 2000, Oh *et al.* 2005) and may in turn play a role in the shedding of these proteins.

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 (decanoyl-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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