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Novel epididymal protease inhibitors with Kazal or WAP family domain ☆,☆☆

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Abstract

The epididymal maturation of spermatozoa is regulated by changes in the luminal ion concentration and the processing of the sperm surface membrane by several glycosidases and proteases. In the present study, we identified five novel protease inhibitors that are highly expressed in the mouse epididymis. Four of the proteins were found to belong to the Kazal protease inhibitor family and were named SPINK8, SPINK10, SPINK11, and SPINK12, whereas one of the proteins, WFDC10, contained the WAP four-disulfide core domain structure. The novel genes showed very specific segmental expression patterns. The expression of all the five genes was regulated by testis-derived factors and decreased after gonadectomy. With the exception of *Spink11*, mRNA levels could be restored by testosterone replacement. We hypothesize that the protease inhibitors discovered represent a group of epididymal genes that contribute to the regulation of sperm maturation by regulating the proteolytic processing of the sperm membrane during epididymal transit.

Keywords: Epididymis; Protease inhibitor; EST; Kazal; WAP

The spermatozoa produced in the testis are passed into the epididymal duct and transported through the duct by contractions of the surrounding smooth muscle layer [1]. These muscle contractions also cause reciprocal movement that allows mixing of the contents of the lumen and enhances contact of spermatozoa with the lining epithelium [2,3]. The epididymis is composed of three segments: caput, corpus, and cauda, and the proximal part of the caput in rodents is referred to as the initial segment. Each epididymal segment has a unique composition of cells contributing to its specialized function.

Post-testicular maturation of sperm commences in the initial segment of the epididymis, and major changes in spermatozoa occur during their passage through the initial segment and the rest of the caput [4]. The maturational changes in spermatozoa are affected by changes in the luminal ion concentration and by the processing of the sperm surface membrane by several glycosidases and proteases secreted into the epididymal lumen [5,6]. Examples of proteolytically processed proteins include the germinal form of the angiotensin I converting enzyme (*gACE*), which is released from the surface of the spermatozoa during their transit through the caput, and the disintegrin and metallopeptidase domain 2 (*Adam2*), which is degraded by two successive cleavages during the caput transit, leaving the protein to the post-acrosomal domain of the spermatozoa [7]. It is believed that the actions of the proteases are controlled by protease inhibitors present in specific areas of the epididymis. Mature spermatozoa are then stored in a functional but immotile state in the cauda epididymis [1,8].

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☆☆ GenBank Accession Nos. DQ437329 for *Spink8*, DQ437330 for *Spink10*, DQ437331 for *Spink11*, DQ437332 for *Spink12*, and DQ437333 for *Wfdc10*.
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Sperm are protected from cells of the immune system as well as from self-destruction by enzymes in the cytoplasmic droplet [9].

Several protease inhibitors have been detected in the epididymis, but the exact function of most of them remains unconfirmed. *Cystatin 8* (*Cst8*) is expressed in the epithelium of the proximal caput epididymis, where it is secreted into the epididymal lumen. Of the several proteases tested, CST8 inhibited only serine protease prohormone convertase 2 (PC2), a protease involved in prohormone processing in the neuroendocrine system [10]. CST12 is also highly expressed in the epithelial cells of the proximal caput and secreted into the lumen, where it attaches to the cytoplasmic droplet of spermatozoa [11].

EPPIN (also known as SPINLW1) is a serine protease inhibitor that contains the whey acidic protein (WAP) and Kunitz protease inhibitor domains. In humans, three differently expressed splice variants have been described: *Eppin1* is expressed in testis and epididymis, *Eppin2* only in epididymis, and *Eppin3* only in testis. EPPIN1 and 3 are secretory proteins, and immunohistochemistry with anti-EPPIN antibody shows intense staining on the acrosome, post-acrosome, midpiece and tail of ejaculated human spermatozoa [12]. Furthermore, WFDC2 (also known as HE4) is an epididymal protein that contains cysteine residues similar to WAP [13]. Recently, it was discovered that, in addition to epididymis, WFDC2 is highly expressed in serous and endometroid epithelial ovarian cancer [14].

In this study, we present the characterization of five novel protease inhibitors highly expressed in epididymis. Four of the proteins were found to belong to the Kazal protease inhibitor family, whereas one shares structure characteristics of the WAP protein family.

Table 1
Gene-specific primers used in the study

Primer	Length	Sequence	Annealing *T* (°C)
Spink8Fw1	20	acactgagctgaggctcctt	57
Spink8Rev1	22	tgtttttctcaaggtgctgttc	57
Spink8Rev2	20	tgctgccacatataggctca	57
Spink8Rev4	20	tgccctctggctttgcttgc	61
Spink10Fw3	19	gaggtcctgcattggaaca	57
Spink10Fw4	20	cggcattttggtccctgctg	61
Spink10Rev1	20	ccacagaaagtttcccttgc	57
Spink10Rev3	20	tttgggtgctgataggaagg	57
Spink10Rev4	21	tggcagcaggtgttccaatgc	61
Spink11Fw1	22	acagtaagcacagagtgctggt	57/59
Spink11Fw4	24	caagaaacattttctcaggttgcc	59
Spink11Rev1	24	tcattgaaaataaaacaggcctaa	57
Spink11Rev2	20	tctgcacaaactgggatcag	59
Spink11Rev4	21	tgggatcagcgttctggagca	61
Spink12Fw1	20	aacaccatcctgcatgttca	57
Spink12Fw4	20	tgggtgtgcctttgctttgc	59
Spink12Rev1	20	gaagcacactgctgtggaaa	57
Spink12Rev2	20	cacagcggttttggtaggtt	57
Spink12Rev4	20	ggcacctgctggcttcatgg	63
Spink12BspHFw	29	catgtcatgagccaaggaggctttcaggc	68
Spink12XhoRev	33	ccgctcgaggcatttcccttcatgtttgaaacc	68
Wfdc10Fw1	20	ccacagagaaaaagggcaag	57
Wfdc10Fw4	20	cccgcccagctttgaaggag	63
Wfdc10Rev1	20	aggaggcaggtgcttgttta	57
Wfdc10Rev2	20	gtcttggtgggcttcacaat	57
Wfdc10Rev4	20	tggccaaatcagggcagagc	61
Rpl19sense	21	ctgaaggtcaaagggaatgtg	57
Rpl19anti	21	ggacagagtcttgatgatctc	57
Hprtfw	20	aagcttgctggtgaaaagga	55
Hprtrev	20	ttgcgctcatcttaggcttt	55
hWfdc10afw	20	acaacctggccagacatagg	56
hWfdc10arev	20	tgcttgacaatctcggtgag	56
hHprtfw	20	tgaggatttggaaagggtgt	56
hHprtrev	20	aatccagcaggtcagcaaag	56
hWfdc10bafw	20	gccaaaggatggtgtcttgt	59
hWfdc10bbfw	20	cagccttctgtgggaacatt	59
hWfdc10brev	20	gagcgtgagccctaagtctg	59

Materials and methods

Characterization of EST clones and full-length cDNAs. The protease inhibitors were identified through exploration of the UniGene RIKEN epididymal EST library deposited at the NCBI database (http://www.ncbi.nlm.nih.gov). The primers (Fw1 and Rev1) used to obtain full-length cDNA by RT-PCR were designed on the basis of the sequences present in the database (Table 1). The cDNAs were cloned into the pCR 4-TOPO vector (Invitrogen, Carlsbad, CA) and sequenced with an ABI PRISM 377-XL DNA Sequencer by using the ABI PRISM BigDye Terminators v3.0 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and the M13Fw and M13Rev primers from the vector sequence. The cDNA sequence was confirmed and the intron–exon structure obtained by comparing the sequence with the mouse genomic DNA sequence available in the NCBI database (http://www.ncbi.nlm.nih.gov/genome/guide/mouse/).

Bioinformatics. The peptide sequences and putative functions of the novel genes were deduced using the Expasy Translate tool (http://us.expasy.org), InterProScan (http://www.ebi.ac.uk/Tools/) and the protein–protein blast (http://www.ncbi.nlm.nih.gov/BLAST/). SignalP (http://www.cbs.dtu.dk), PSORTII, and WoLFPSORT (http://psort.nibb.ac.jp) were used to identify putative signal peptides and the intracellular localization of the protein. The phylogenetic trees of the protein families were generated using MEGA 3.1 [15], the sequences were aligned by ClustalW using Blosum matrix, and the tree was constructed using the Neighbor-Joining method.

5′-and 3′-RACE. The 5′- and 3′-RACE approaches were used to obtain the full-length cDNAs. The GeneRacer Kit (Invitrogen, Carlsbad, CA) was used according to the manufacturer's protocol. Rev4 primers were used for 5′-RACE and Fw4 primers for 3′-RACE (Table 1).

Tissue collection and RNA extraction. Male and female mice of the FVB/N strain were used in the study. The mice were specifically pathogen-free, and they were housed under controlled environmental conditions (12 h light and 12 h darkness, temperature 21 ± 1 °C). The animals were fed complete pelleted chow and allowed tap water ad libitum. The mice were killed by cervical dislocation or CO_2 asphyxiation. All mice were handled in accordance with the institutional animal care policies of the University of Turku (Turku, Finland).

For the analysis of the tissue distribution of gene expression, seven mice (2–3 months old) were euthanized and harvested for pre-selected tissues (brain, hypothalamus, heart, muscle, spleen, pancreas, liver, kidney, adrenal gland, seminal vesicle, testis, vas deferens, prostate, lung, intestine, mammary gland, ovary, uterus, and the initial segment, caput, corpus, and cauda epididymis). For the analysis of the androgen regulation of gene expression, 16 sexually mature male mice were gonadectomized under avertin anesthesia. The mice were divided into four groups (4 mice/group) and euthanized 1, 4, and 7 days after gonadectomy, and their epididymides were collected. The fourth group of mice was treated with supraphysiological doses of testosterone for 7 days, starting immediately after gonadectomy. One-cm-long silastic tubes (inner diameter 1.98 mm,

outer diameter 3.18 mm) were filled with testosterone powder (Fluka Chemie AG, Buchs, Switzerland), and the ends of the tubes were sealed with silastic adhesive (Elasitol RTV-1 Silicone Rubber, Wacker–Chemie GmbH, Munich, Germany) [16]. The tubes were placed subcutaneously into the mice for testosterone replacement therapy. Human epididymis was provided by Prof Anders Bjartell (University Hospital of Malmö, Sweden). All collected tissues were frozen in liquid nitrogen and stored at −70 °C. RNA extraction was carried out using the single-step method [17].

Quantitative (Q) RT-PCR. The tissue distribution and testis-dependent regulation of the novel genes were analyzed by quantitative (Q) RT-PCR with gene-specific primer pairs (Table 1). The analyses were performed using the DNA Engine Opticon system (MJ Reaserch, Inc, Waltham, MA) with continuous fluorescence detection. One μg of RNA (pooled from 3–5 animals) was treated with deoxyribonuclease I (Invitrogen), and the PCR reaction was performed using the QuantiTect SYBR Green RT-PCR Kit (Qiagen, Valencia, CA). For each reaction 50 ng RNA was used, and triplicate reactions were run for all samples and standards. The expression of the genes analyzed in the different tissues was normalized to the ribosomal protein L19 (*Rpl19*) expression, while the androgen dependence analysis included both *Rpl19* and hypoxanthine guanine phosphoribosyl transferase (*Hprt*) as reference genes. Human *Hprt* was used as control when analyzing the expression of the human orthologues for *Wfdc10*.

In situ hybridization. Five micrometer thick paraffin sections of adult mouse epididymides were used for in situ hybridization. The cDNA of the gene in the pCR 4-TOPO vector was used as a template for sense and antisense [^{35}S]-αUTP-labelled probes generated by in vitro transcription with T3 and T7 RNA polymerases using the Riboprobe system II kit (Promega, Madison, WI). After deparaffinisation and rehydration, the slides were denatured in 0.2 N HCl and 2 × SSC at 70 °C, followed by fixation in 4% paraformaldehyde (PFA) and proteinase K treatment (1 mg/l). Thereafter, the sections were post-fixed in 4% PFA, acetylated with 0.25% and 0.5% acetic anhydride in 0.1 M triethanolamine, dehydrated and air-dried. The slides were stored at −72 °C until hybridized.

Table 2
UniGene cluster codes and GenBank accession number codes for the cDNAs submitted

Gene	Cluster ID	Accession Nos.
Spink8	Mm.99613	DQ437329
Spink10	Mm.312675	DQ437330
Spink11	Mm.117440	DQ437331
Spink12	Mm.99782	DQ437332
Wfdc10	Mm.189007	DQ437333

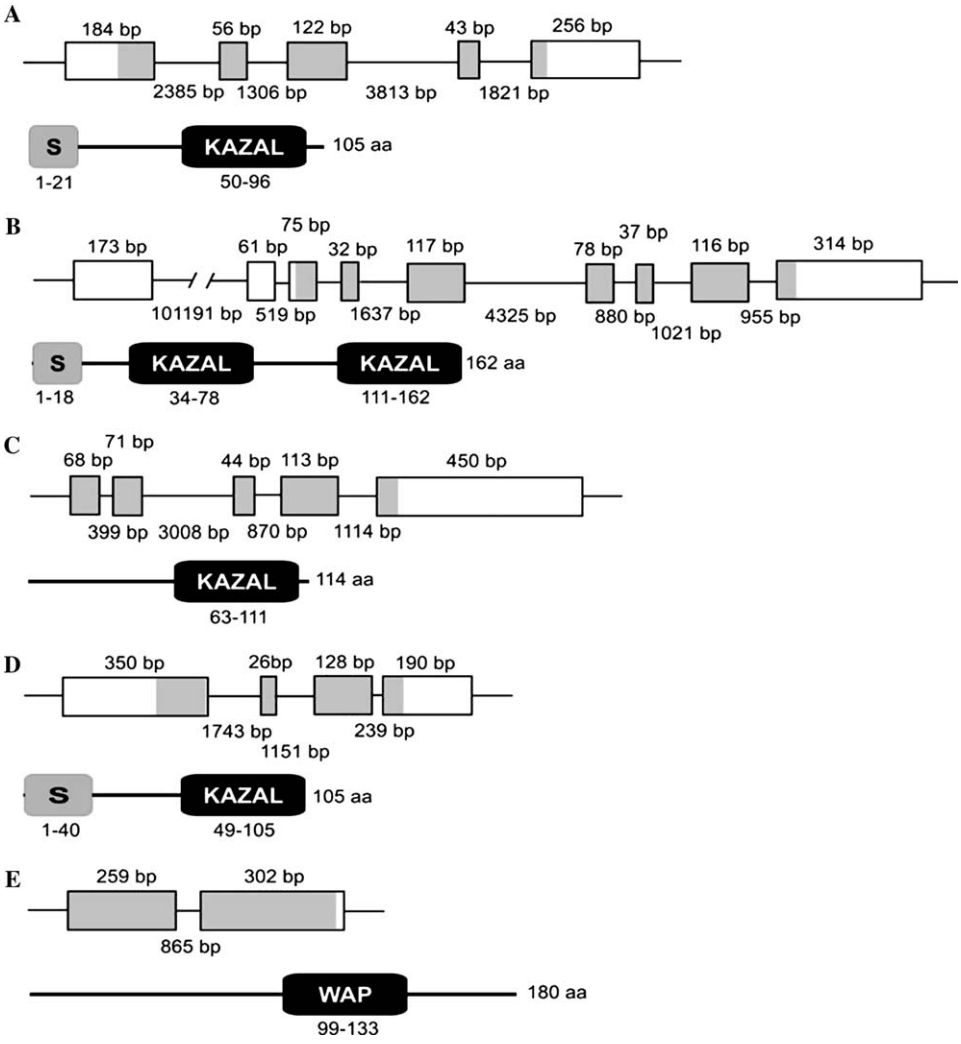

Fig. 1. Gene structures of the novel protease inhibitors and the proteins encoded by the genes. (A) *Spink8* is composed of five exons, and the protein (below) has a putative signal peptide sequence (S) and one Kazal domain. (B) The *Spink10* gene has nine exons, and the protein contains two Kazal domains in addition to a putative signal peptide sequence. (C) *Spink11* is composed of five exons, and the protein contains a single Kazal domain. (D) *Spink12* contains four exons, and similarly to SPINK8, the protein has a putative signal peptide and one Kazal domain. (E) *Wfdc10* is composed of only two exons, and the peptide contains a single WAP domain. The coding regions in the exons are indicated with grey.

After overnight hybridization at 55 °C, the slides were washed in descending concentrations of SSC solution, treated with RNase A solution (10 mg/l) (Boehringer Mannheim, Ingelheim, Germany), washed with 2 × SSC, dehydrated and air-dried. The slides were then dipped in NTB2 autoradiography emulsion (Eastman Kodak, Rochester, NY) and exposed in the dark at 4 °C for 1 to 5 days. The slides were developed with Dektol developer (Eastman Kodak), fixed with Kodak Fixer (Eastman Kodak) and counterstained with Hoechst 33258 (Sigma, St. Louis, MO), after which they were mounted with DAKO fluorescent mounting medium (DAKO Corporation, Carpinteria, CA).

Recombinant protein production. The cDNA of the protein-coding region of *Spink12*, excluding the signal peptide, was amplified by RT-PCR using Spink12BspHFw and Spink12XhoRev as primers (Table 1). After cleavage with *Bsp*HI and *Xho*I (New England Biolabs, Ipswich, MA), the transcript was cloned into the multiple cloning site of pTriEx-2 (EMD Biosciences, Inc, Darmstadt, Germany). The recombinant SPINK12

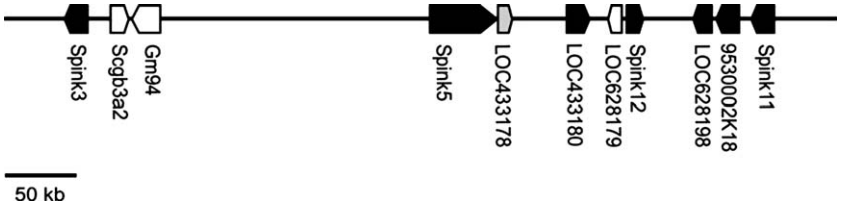

Fig. 2. Cluster of Kazal protease inhibitors on chromosome 18. The cluster contains *Spink11*, *Spink12*, previously characterized *Spink3* and *Spink5* and three novel genes containing a Kazal domain (black). LOC433178 (grey) has sequence similarity with the Kazal protease inhibitors but was not found to contain the functional domain.

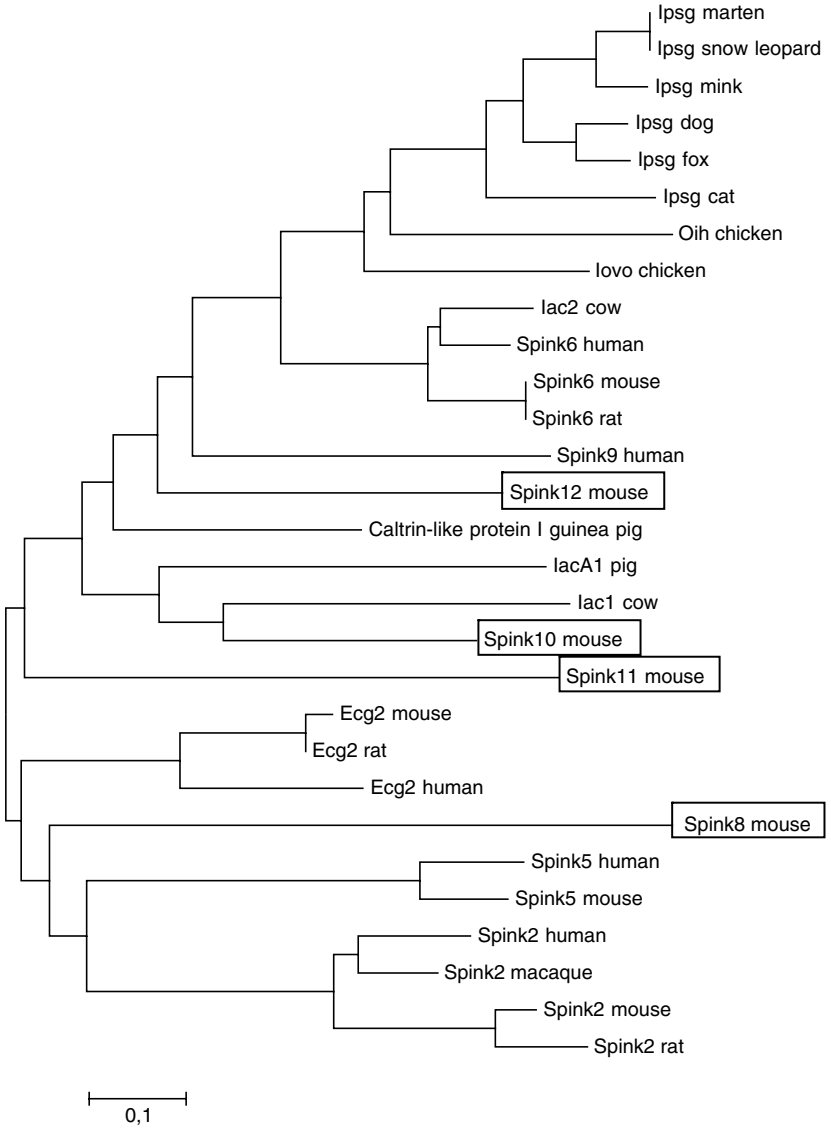

Fig. 3. Phylogenetic tree of Kazal protease inhibitors showing homology of the novel Spinks with the other members of the family.

protein with C-terminal His-tag was produced and purified by Ni-affinity chromatography.

Protease inhibitor assay. The Protease Assay Kit (EMD Biosciences, Inc, Darmstadt, Germany) was used to test the function of the SPINK12-His protein. Sixty nanograms trypsin and 0.6–30 μg SPINK12-His were added to the assay buffer with FTC-casein as a substrate. The samples were incubated at 37 °C overnight, and the uncleaved FTC-casein was precipitated by 5% TCA. The TCA precipitate was pelleted by centrifugation, and the absorbance of the supernatant at 490 nm was measured with a Victor2 multilabel counter (Perkin-Elmer, Turku, Finland).

Results

Identification of the genes for novel protease inhibitors

To identify novel epididymal genes, we analyzed EST clusters in the UniGene mouse epididymal library at the NCBI database, as previously reported by us [18]. The clusters representing four or more ESTs from the epididymis were included in the analysis, as this is likely to indicate moderate to high epididymal expression levels. Among the clusters, five novel putative protease inhibitors were identified. The translated peptide sequences were submitted to InterProScan for identification of protein domains, motifs and family signatures. The search against various databases revealed that four of the novel sequences showed characteristics typical of Kazal protease inhibitors, while one contained a WAP four-disulfide core domain (Fig. 1). We concluded that four of the novel genes belong to the Kazal family, and they were therefore named as *Spink8*, *Spink10*, *Spink11*, and *Spink12* (serine peptidase inhibitor, Kazal type), and the names were approved by the Mouse Genome Informatics nomenclature committee. The gene with a WAP four-disulfide core domain was found to contain the partial sequence of *Wfdc10* published recently by Clauss et al. [19]. The EST cluster codes representing the novel genes and the GenBank accession numbers of the cDNAs are shown in Table 2.

Complementary DNA clones were obtained by RT-PCR, and sequencing revealed that *Spink8* contains one exon more than predicted by the cDNA present in the NCBI database. 5′-RACE analysis allowed us to extend the sequence of *Spink11* by 159 bp, and 3′-RACE yielded the poly(A)-tail to all of the cDNAs and additional 2, 170, 7, and 2 nucleotides of the sequence for *Spink8*, *10*, *11*, and *12* cDNAs, respectively. As the 5′-RACE approach did not give any additional sequence to the other four genes, and as the poly(A)-tails of all the cDNAs had been obtained, the cDNA sequences were considered to be complete.

Chromosomal location and phylogenetic relations of the genes

Spink8, which consists of five exons (Fig. 1), is located on chromosome 9F2. *Spink10* is located on chromosome 18E1 in close proximity to another Kazal protease inhibitor, the esophagus cancer-related gene-2 (Ecg2), that has significant sequence similarity to *Spink10*. The distribution of the eight

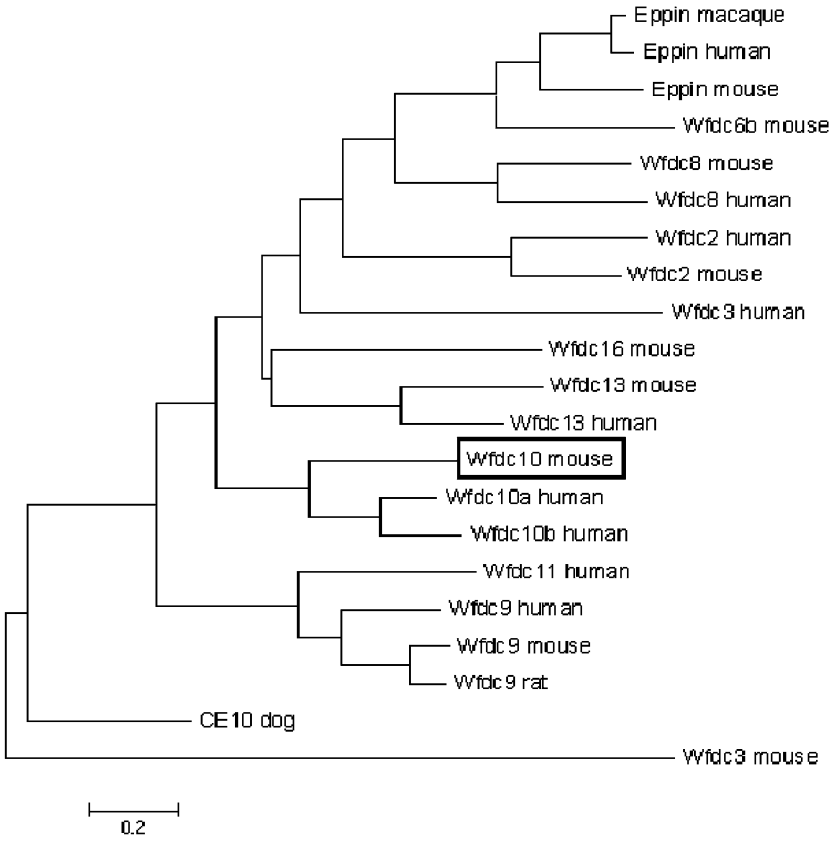

Fig. 4. Phylogenetic tree of WAP protease inhibitors showing that *mWfdc10* is the orthologue of *hWfdc10a* and *hWfdc10b.*

exons of *Spink10* is shown in Fig. 1. *Spink11* and *Spink12* are located on chromosome 18B3 in a cluster of Kazal protease inhibitors. In addition to *Spink11* and *Spink12*, the cluster contains three other novel protease inhibitors and two previously known Kazal protease inhibitors, namely *Spink3* and *Spink5* (Fig. 2). The gene closest to *Spink11* is RIKEN cDNA 9530002K18Rik, which has 60% homology with *Spink11* in the nucleotide sequence. The protein encoded by the gene is predicted to be shorter than SPINK11 but otherwise very similar. Thus, the two genes might have evolved through duplication of a single ancestor gene. *Wfdc10* consists of two exons and is located on chromosome 2H3 in a cluster of WFDC-type proteinase inhibitors. Spint4, a Kunitz protease inhibitor previously described by us [20], is also located among the WFDC genes.

The phylogenetic relations of the novel Spinks to the closely related members of the Kazal family are shown in Fig. 3. The novel mouse *Wfdc10* was found to have two human homologues: *Wfdc10a* and *Wfdc10b* (Fig. 4). Based on SignalP prediction, SPINK8, SPINK10, and SPINK12 might have N-terminal signal peptides, the locations of which are shown in Fig. 1.

Tissue distribution and epididymal expression

Q-RT-PCR and in situ hybridization analyses showed that all the five genes were highly expressed in the epididymis with differential segment specificities. *Spink8* is highly expressed in the cauda, corpus and caput, excluding the initial segment, and less intensively in the vas deferens (Table 3 and Fig. 5). *Spink10* is expressed maximally in the cauda epididymis, and its expression decreases towards the caput epididymis, being very weak in the segments II and III of the caput (according to Abou-Haila and Fain-Maurel [21]). Detectable expression was also identified in the brain. The expression of *Spink11* starts in the proximal caput epididymis and increases towards the segments IV and V, after which the expression signal disappears where the tubule reaches the corpus region, and no expression can be detected in the cauda, either. Similar expression of *Spink11* is visible in the seminal vesicles. Similarly, the expression of *Spink12* begins in the proximal caput, but in contrast to *Spink11*, its expression gradually decreases towards the corpus epididymis. *Wfdc10* shows maximal expression in the proximal caput epididymis, after which its expression decreases towards the corpus, increasing again in the distal corpus and cauda epididymides.

Table 3
Tissue distribution of the novel protease inhibitors

Tissue	Spink8	Spink10	Spink11	Spink12	Wfdc10
IS	8	3	4	2	4
Caput	50	1	100	100	65
Corpus	100	27	2	1	40
Cauda	74	100	0	1	100
Testis	0	3	0	1	4
Prostate	0	1	0	0	1
Sem ves	1	1	72	1	1
Vas def	47	1	1	1	9
Uterus	0	0	0	0	1
Ovary	0	0	0	0	0
Mammary	0	1	0	0	0
Brain	0	14	0	2	3
Hypot	0	1	0	1	1
Lung	0	3	0	1	2
Heart	0	4	0	4	4
Muscle	0	6	0	5	6
Liver	0	1	0	2	2
Pancreas	0	2	0	1	2
Spleen	0	1	0	0	2
Intestine	0	1	0	0	0
Kidney	0	1	0	0	0
Adrenal	0	3	0	0	1

The value in the tissue with the highest expression level was adjusted to 100, and the expression levels in the other tissues are shown relative to that. Levels of expression more than 10% of the expression level in the tissue with maximal expression are highlighted.

Testis-dependent expression

The data on *Wfdc10* indicate typical androgen-dependent regulation of the gene (Fig. 6). Its expression decreased after gonadectomy and was restored by androgen replacement. The expression of *Spink8*, *Spink10*, and *Spink12* is also likely to be regulated by androgens, but interestingly, their expression patterns are more complex. Their expression first increased one day after gonadectomy, most probably as a response to the inflammatory reaction and tissue destruction caused by the operation. Moreover, the expression of *Spink8*, *Spink11*, and *Spink12* seemed to increase in the caput four days after gonadectomy. Their expression increased after supraphysiological testosterone replacement, indicating androgen regulation. Surprisingly, the expression of *Spink11* was not restored by seven days of testosterone treatment, suggesting that it could be regulated by testicular factors other than androgens.

Expression of human Wfdc10

Two copies of *Wfdc10* have been identified in the human genome: *hWfdc10a* and *hWfdc10b*. Moreover, two splice variants of *Wfdc10b* are known. Exon three is spliced out in isoform *hWfdc10bb*, and the coding region starts in exon one, whereas in isoform *hWfdc10ba* the translation starts in exon three. The peptide encoded by variant b lacks the signal peptide, which is encoded by the sequence in exon three, and variant b is therefore probably not a secretory protein like variant a. However, both isoforms contain the WAP four-disulfide core domain sequence. Both *Wfdc10a* and *Wfdc10b* show maximal mRNA expression in the corpus epididymis, while *Wfdc10a* is also expressed at a low level in the caput epididymis and variant *Wfdc10bb* in the cauda epididymis (Fig. 7).

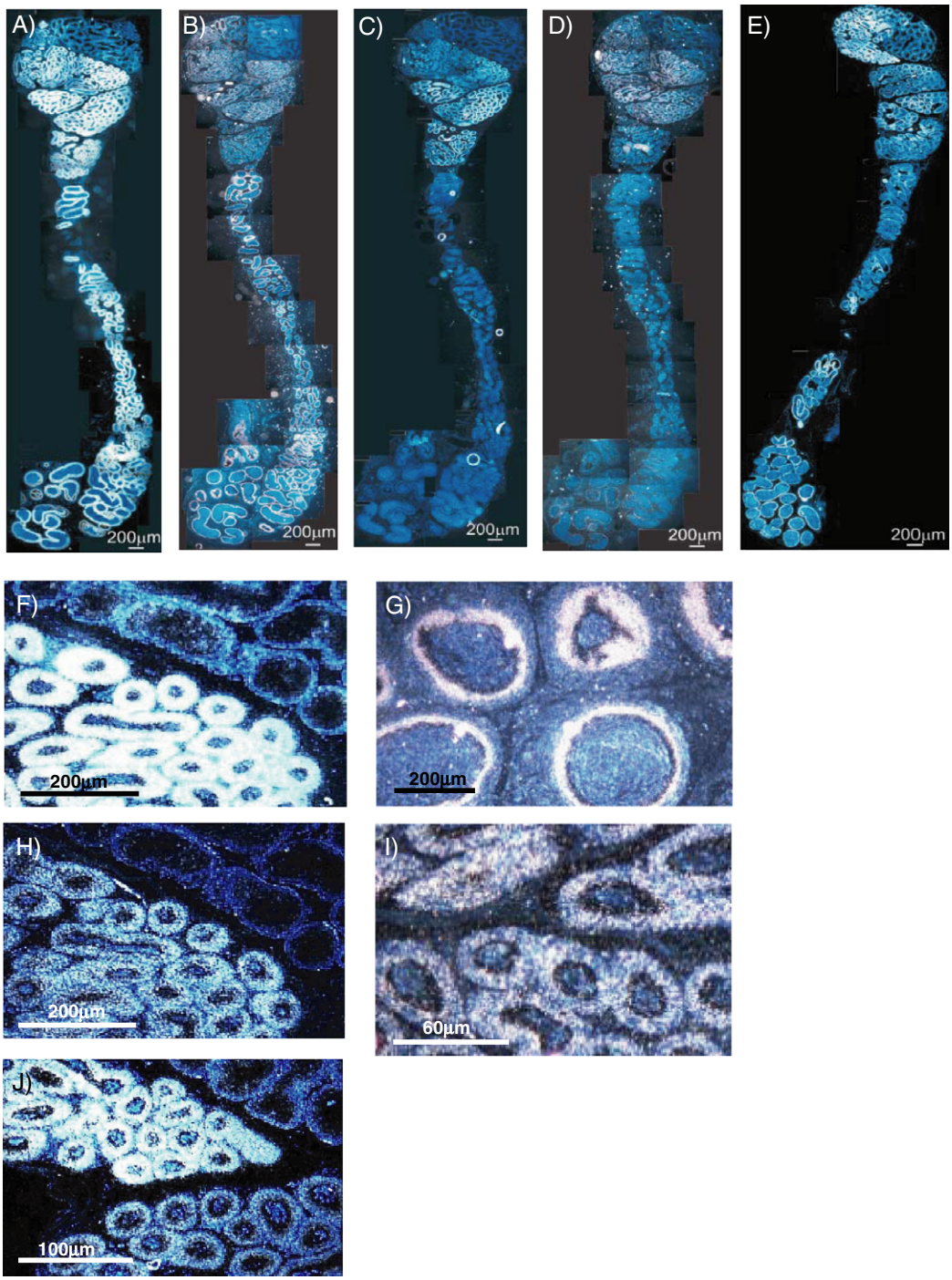

Fig. 5. Region-specific expression of the genes. The region-specific expression of *Spink8* (A), *Spink10* (B), *Spink11* (C), *Spink12* (D), and *Wfdc10* (E) in the epididymis was detected by *in situ* hybridization. (F–J) Higher resolution images of *in situ* hybridization. (F) *Spink8* expression cannot be detected in the initial segment, whereas a strong epithelial signal is visible in the adjacent lobe. (G) *Spink10* expression in the epithelium of cauda epididymis. (H) Similarly to *Spink8*, *Spink11* cannot be detected in the initial segment, whereas clear expression is seen in the adjacent lobe. (I) *Spink12* expression in the epithelium of caput epididymis. (J) There is marked variation in the expression levels of *Wfdc10* in the different lobes of caput epididymis. In the dark field images, the hybridization signal appears white. The slides were counterstained with Hoechst, which is seen as blue in the image.

Protease inhibitor assay

Of the novel protease inhibitors discovered, the properties of SPINK12 were analyzed. Recombinant SPINK12 was able to significantly inhibit the activity of trypsin *in vitro* (Fig. 8). A significant decrease in activity was noted when 30 μg of protease inhibitor was used. However, even a 500-fold amount of protease inhibitor was not able to completely block trypsin activity. It is possible that the activity of the protease inhibitor is reduced because the protein was isolated from inclusion bodies, or because His-tag disturbs the folding of the protein.

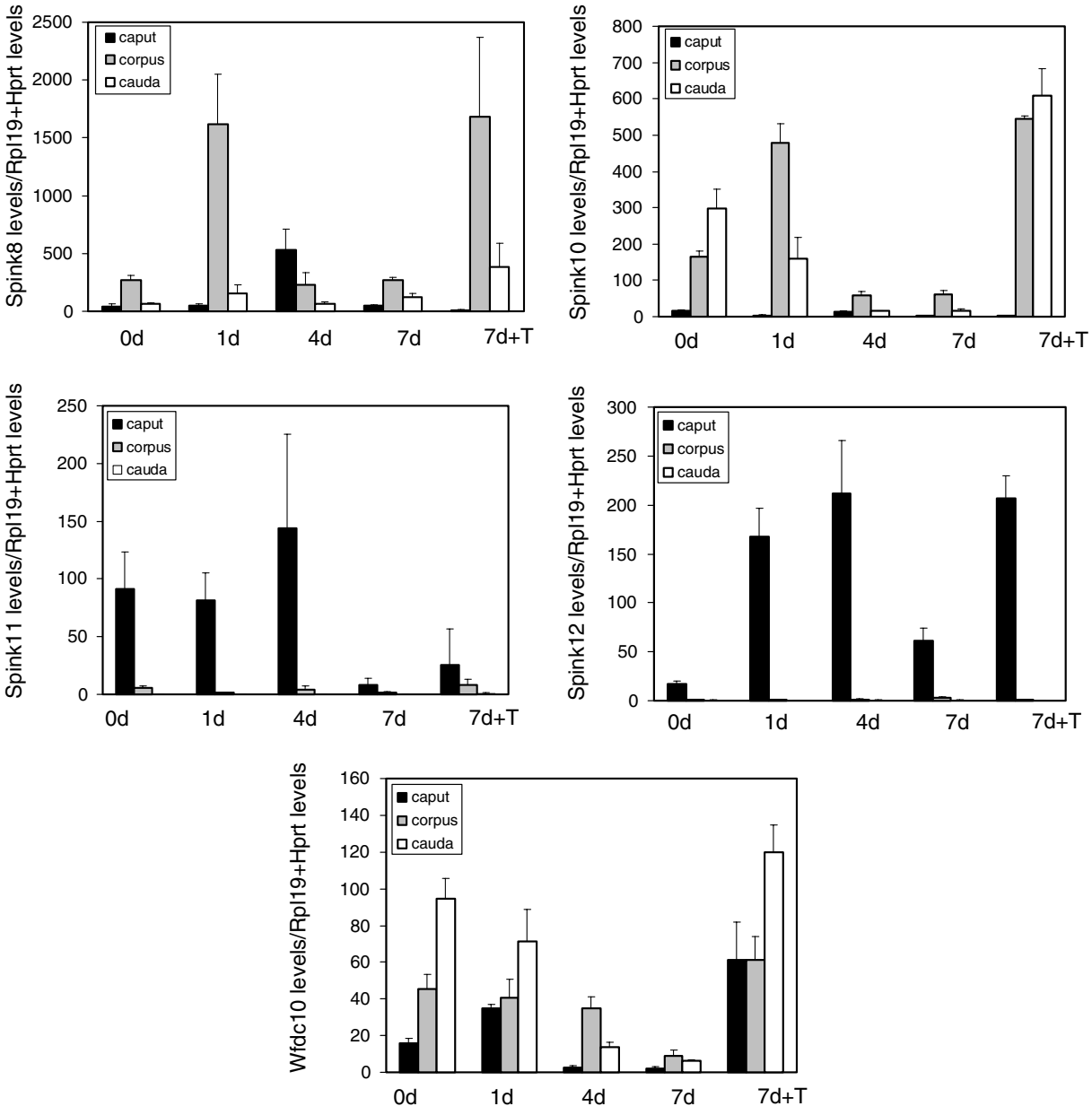

Fig. 6. Quantitative RT-PCR analysis of the regulation of the expression of *Spink8*, *Spink10*, *Spink11*, *Spink12*, and *Wfdc10*. Expression was analyzed in the mouse epididymis at baseline (0 day) and one to seven days (1, 4, and 7 day) after gonadectomy and in mice that had received testosterone replacement therapy immediately after gonadectomy for seven days (7 day + T).

Second, trypsin is a highly potent protease, and several of the proteases present in epididymal fluid probably have weaker activity.

Discussion

In the present study, we introduce five novel epididymal protease inhibitors. About two per cent of all genes encode for proteolytic enzymes, which are essential for several physiological events. However, their action must be strictly controlled by protease inhibitors [22]. Thus, there is a fine balance between proteases and protease inhibitors in many biological processes, and any mechanism that disturbs this homeostasis may have substantial pathological consequences [23].

Several proteases can be found in epididymal fluid. According to Metayer et al. [24], the two major groups of epididymal proteases are matrix metalloproteases and serine proteases, including acrosin. The main function of proteases is believed to be the modification of sperm surface proteins during epididymal sperm transit and maturation. The protease inhibitors previously characterized in the epididymis and especially the present discovery of the five novel protease inhibitors indicate that, apart from proteases, their inhibitors also represent a significant group of epididymal genes with putative involvement in

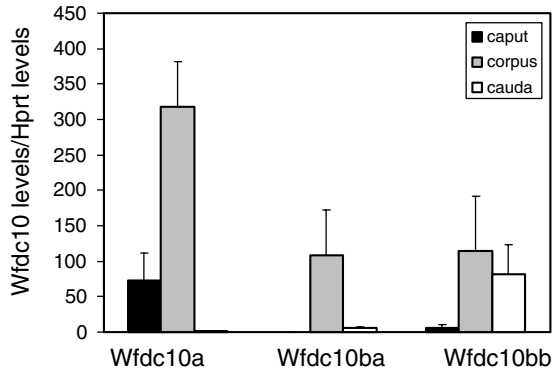

Fig. 7. Quantitative RT-PCR analysis of the expression of human *Wfdc10a* and the two splice variants of *Wfdc10b* (*Wfdc10ba* and *Wfdc10bb*) in the human epididymis.

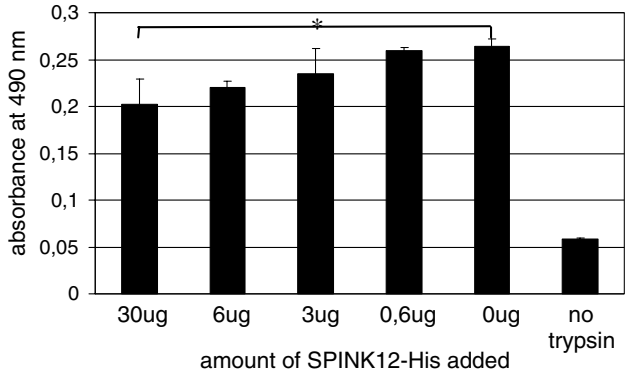

Fig. 8. Functional assay of the SPINK12-His recombinant protein. Sixty nanograms of trypsin was incubated with a synthetic substrate, FTC-casein. Addition of SPINK12-His protein to the protease assay decreased the activity of trypsin. (*) A significant decrease in trypsin activity was noted when 30 μg of SPINK12-His was used ($P = 0.012$).

the regulation of the main epididymal function, sperm maturation.

We have identified four novel Kazal protease inhibitors that are highly expressed in the mouse epididymis. While the inhibitory properties of several of them remain to be characterized, SPINK12, which was used as a model, significantly inhibited trypsin activity *in vitro*. Several members of the Kazal protease inhibitor family have been previously characterized, but only *Spink2* expression has been detected in the epididymis [25]. Another Kazal family member identified in the male reproductive tract is SPINK3, which was identified as a prostatic secretory protein [26]. Its expression was also detected in the pancreas, the coagulating gland and the seminal vesicles, and similarly to the novel epididymal Spinks, its expression in the male reproductive tissues is regulated by androgens [26]. Human *Spink2* (previously known as human acrosin-trypsin inhibitor HUSI-II) has maximal expression in the testis and is expressed at lower levels in the epididymis and the seminal vesicles [25]. Gene expression profiling of infertile men diagnosed with azoospermia revealed that they had a four-fold decrease in *Spink2* expression compared to fertile men [27].

The fifth novel protease inhibitor described in this study belongs to the family of Wfdc (WAP four-disulfide core domain) proteins. Protease inhibitors with WAP motifs have a variety of functions: some caltrins are inhibitors of calcium transport by spermatozoa [28], SPAI is a sodium/potassium ATPase inhibitor in pigs [29], anti-bacterial activity has been reported in *Wfdc15* (SWAM1), which is expressed in the epididymis and kidney, and the expression of this gene is induced in pneumonic lungs [30]. *Wfdc10* was found to be located in the large cluster of WFDC genes discovered by Clauss et al. [19]. Interestingly, homologous loci of the WFDC gene cluster have been found in human chromosome 20, rat chromosome 2 and dog chromosome 24 [19]. As shown by the phylogenetic tree and by Clauss et al. [19] in their cluster analysis, *mWfdc10* has two homologous genes in the human genome. The duplication of the gene in the human genome has occurred after the split of the lineages leading to murines and primates. No genes orthologous to the novel Spinks were identified in any other mammalian species. It seems unlikely that these genes would be specific only for the mouse, but the findings rather suggest that several members of the Kazal family remain to be discovered.

The novel protease inhibitors characterized in this study show quite variable expression in the epididymis. Some are highly expressed in the proximal segments of the epididymis, while others show high levels of expression in the distal parts. The differences in expression patterns seem logical based on the putative functions of the genes. Obviously, the action of the protease needs to be suppressed soon after the proteolytic processing of a certain component is completed, to avoid damage to the surrounding cells. On the other hand, some proteases act in the more distal parts of the epididymis, and their function should not be inhibited until they reach the distal corpus or cauda. Similarly to many of the genes believed to be involved in sperm maturation, the expression of the five protease inhibitors decreased after gonadectomy [6], and the expression of all except *Spink11* could be restored by testosterone replacement. Disturbance of organ homeostasis, such as gonadectomy, may cause changes in the expression of the genes capable of protecting cells. Possibly for that reason, the expression of some of the novel protease inhibitors also first increased after gonadectomy.

In summary, we have identified five novel protease inhibitors that are highly expressed in the mouse epididymis. The novel protease inhibitors are putative regulators of the proteolytic processing of the sperm surface proteins, and their discovery will thus help to understand the mechanisms responsible for epididymal sperm maturation.

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