SPINKL, a Kazal-type serine protease inhibitor-like protein purified from mouse seminal vesicle fluid, is able to inhibit sperm capacitation

Ming-Huei Lin^{1,2}, Robert Kuo-Kuang Lee^{1,3,4}, Yuh-Ming Hwu^{1,2}, Chung-Hao Lu³, Shian-Ling Chu³, Ying-Jie Chen³, Wei-Chao Chang⁵ and Sheng-Hsiang Li^{2,3}

¹Department of Obstetrics and Gynecology, Mackay Memorial Hospital, Taipei 104, Taiwan, ²Mackay Medicine, Nursing and Management College, Taipei 112, Taiwan, ³Department of Medical Research, Mackay Memorial Hospital, Tamshui 251, Taipei County, Taiwan, ⁴Department of Obstetrics and Gynecology, Taipei Medical University, Taipei 110, Taiwan, and ⁵Genomics Research Center, Academia Sinica, Taipei 115, Taiwan

Correspondence should be addressed to S-H Li; Email: lsh@ms1.mmh.org.tw

M-H Lin and R K-K Lee contributed equally to this work

Abstract

We report a secreted serine protease inhibitor Kazal-type-like (SPINKL) protein. The SPINKL protein was purified from mouse seminal vesicle secretions through a series of steps, including ion-exchange chromatography on a diethylaminoethyl-Sephacel column, gel filtration on a Sephadex G-75 column, and ion-exchange HPLC on a Q strong anion exchange column. Further analysis identified several SPINKL proteins with various N-linked carbohydrates. The SPINKL protein has six conserved cysteine residues that are nearly identical to those of members of the SPINK protein family. It was noted that the SPINKL protein showed no inhibitory activities against common serine proteases such as trypsin, chymotrypsin, subtilisin, or elastase. *Spinkl* mRNA and SPINKL proteins were found to be primarily expressed in seminal vesicles. Immunohistochemistry revealed that the SPINKL protein occurred in the luminal fluid and mucosal epithelium of the seminal vesicles and was regulated by testosterone. The SPINKL protein was able to bind onto sperm and enhance sperm motility. Also, it was able to suppress BSA-stimulated sperm capacitation and block sperm–oocyte interactions *in vitro*, suggesting that SPINKL may be a decapacitation factor.

Reproduction (2008) 136 559–571

Introduction

Protease inhibitors are widely distributed in nearly all species and play a role in balancing protease activities. There are 48 distinct families of protease inhibitors, one of which is the serine protease inhibitor Kazal (SPINK) family (Rawlings *et al.* 2004). The SPINK family has a characteristic signature (PROSITE entry accession no. PS00282) consisting of six consensus cysteines with the primary structural pattern of C₁–(X)_n–C₂–(X)₇–C₃–(X)₁₀–C₄–(X)_{2/3}–C₅–(X)_m–C₆, where the cysteine (C) residues are involved in three disulfide bonds in C₁–C₅, C₂–C₄, and C₃–C₆ linkages (Laskowski & Kato 1980).

Imbalances between SPINK proteins and proteases may cause severe diseases, for example, mutations of human SPINK1 are thought to be associated with pancreatitis (Pfutzer *et al.* 2000, Kuwata *et al.* 2001), and *Spink3* (a mouse orthologue gene) knockout mice have no pancreatic acinar cells owing to autophagic cell death and impaired regeneration (Ohmuraya *et al.* 2005).

The mouse SPINK3 protein, also called pancreatic secretory trypsin inhibitor or P12, is constitutively

expressed in the pancreas (Mills *et al.* 1987); however, its expression is indeed prominent in sex accessory tissues of adult male mice, particularly in mouse seminal vesicles (Chen *et al.* 1998). It was demonstrated to bind to mouse sperm and shows an inhibitory effect against Ca²⁺ uptake by mouse sperm (Chen *et al.* 1998).

Seminal vesicle secretions (SVSs) constitute the major portion of seminal plasma. Removal of the seminal vesicle greatly reduces fertility (Pang *et al.* 1979, Peitz & Olds-Clarke 1986). Thus, the components of SVSs may affect sperm function. In fact, several proteins purified from murine SVSs have been demonstrated to affect sperm physiology (Chen *et al.* 1998, Huang *et al.* 1999, Luo *et al.* 2001, Li *et al.* 2005). However, other components of SVSs remain to be identified. Further investigation of the proteome may enhance our understanding of normal and abnormal aspects of male reproductive physiology. In addition to SPINK3, we found a SPINK-like (SPINKL) protein in the SVS. In this study, we report its purification and characterization and examine its protease inhibitory activities and effects on sperm *in vitro*.

Results

Purification and identification of a novel SPINKL protein from mouse SVSs

Three peaks obtained from liquid column chromatography of the fresh preparation of soluble SVSs were resolved by HPLC on an anion exchange column at the final purification step (Fig. 1C, peaks a–c). Each of the representative samples at various steps of purification was resolved on a reducing SDS-PAGE gel. Each of the a-to-c peak samples yielded one broad 12–15 kDa band (Fig. 1D), which was stained with the periodic acid–Schiff (PAS) reagent and could be deglycosylated by

N-glycosidase F into two ~8 kDa proteins (Fig. 1D, lane 7). The mass analyses revealed that at least six species with molecular masses of 9033.4–9852.7 kDa in the mixed peak a-to-c samples (Fig. 2A) manifested unusual mobility on reducing SDS-PAGE. Thus, peak a-to-c proteins apparently were glycoproteins with differential N-linked carbohydrate moieties.

The peak a-to-c bands on the SDS-PAGE gel were excised and digested in-gel with trypsin, and the resulting tryptic peptides were purified and subjected to liquid chromatography–mass spectrometry (LC/MS/MS) analysis. A representative MS/MS spectrum of a peptide is shown (Fig. 2B). The results showed that each of the a-to-c peak

Figure 1 Purification of peak a-to-c proteins from the mouse SVSs. (A) Fractionation of soluble SVSs by ion-exchange chromatography on a DEAE-Sephacel column. (B) Resolution of fraction IV from (A) by gel filtration on a Sephadex G-75 column. (C) Resolution of the peak 3 fraction from (B) by HPLC on a Q HyperD 10 column. (D) A representative sample at various purification steps was resolved by 15% SDS-PAGE. Lane 1, 15 µg seminal vesicle fluid; lane 2, 15 µg fraction IV of DEAE-Sephacel chromatography; lane 3, 10 µg peak 3 of gel filtration; lane 4, 2 µg peak a of the HPLC; lane 5, 5 µg peak b of the HPLC; lane 6, 3 µg peak c of the HPLC; lane 7, 5 µg peak b digested by N-glycosidase. (E) Specificity of antiserum. The SDS-PAGE on (D) was electroblotted onto a nitrocellulose membrane and immunodetected by antiserum raised against protein mixtures of peaks a–c of the HPLC. (F) The SPINKL protein sequence is shown. Open boxes denote the potential N-linked glycosylation sites. The amino acid sequences determined directly from Edman sequencing of the first 15 amino acids agreed at all positions except that Asn¹⁵ and Cys¹⁶ were not identified. The tryptic peptides matched by the LC/MS/MS analyses are underlined. Arrows indicate cleavage points for generating mature protein.

Figure 2 Mass spectrometric analyses of the SPINKL protein. (A) Determination of the molecular mass by MALDI-TOF. (B) MS/MS spectrum of a peptide derived from the SPINKL protein is shown. The amino acid sequence of the peptide is given. The mass of each b- and y-ion is indicated.

samples had significant homology to a hypothetical protein containing a SPINK domain (accession no. IPI00229083) with the tryptic peptides matching 41–43% of the putative protein sequences (Fig. 1F).

Automated Edman degradation of each peak of the a-to-c samples for 15 cycles gave reliable data, which were assembled into the N-terminal sequences. Two amino acids were detected in each cycle during the protein analysis of each of the peaks a–c. The actual yields of the two sequences in an individual cycle were such that the ratios of the major sequence to the minor ones were estimated to be 1.7–2.9:1. Assembly of the major and minor sequences produced peptide sequences of VNI-FAGPENVIKEPX and AGPENVIKEPXXXTMY respectively

with the latter being the major one. The two N-terminal peptides were completely confirmed in the hypothetical protein consisting of 90 amino acid residues in all positions, except that X (asparagine or cysteine) was not identified in the protein sequence (Fig. 1F). This asparagine residue is the only potential site for N-linked carbohydrates in this protein. Post-translational cleavage at the peptide bond between Ser and Val or between Phe and Ala in the signal peptide of the hypothetical protein sequence gave rise to the peak a-to-c proteins. As a result, peaks a–c share two protein cores with different N-linked glycans. They were estimated to be 4–5 mg/ml in the seminal vesicle fluid. Thereafter, we combined them to produce antibodies. Among SVS protein components on

the SDS-PAGE gel, the antibody against SPINKL immunoreacted only to a broad 12–15 kDa band corresponding to the antigen, thus indicating the high specificity of the antibody (Fig. 1E).

A BLASTP search of the non-redundant protein database using the hypothetical protein sequence as the query revealed that this protein is similar to a group of SPINK proteins, with the highest identity (64%) found to be with mouse SPINK11. Thus, we named it a serine protease inhibitor Kazal-type-like protein, abbreviated to SPINKL protein. A computer-annotated UniProtKB/TrEMBL entry (Q8CEK3) was previously assigned according to the translation of a coding sequence corresponding to *Spinkl* mRNA. We have submitted the MS and Edman data of SPINKL to SwissProt to update this entry. From the entry, we found that the SPINKL protein was encoded by the *9530002K18Rik* gene and was mapped to chromosome 18 B3. Interestingly, *Spink3* and *Spink11* also have the same chromosome location, even though *Spink11* is immediately adjacent to *9530002K18Rik*.

Alignment of the SPINKL protein with several murine SPINK proteins retrieved from SwissProt revealed that the SPINKL protein nearly completely matched the putative Kazal signature except for an additional residue inserted between C₂ and C₃, indicating that the SPINKL protein is potentially a member of the SPINK protein family (Fig. 3).

Serine protease inhibitor activity assay for the SPINKL protein

To examine the specificity of the SPINKL protein, we analyzed its inhibitory activity towards common serine proteases, including trypsin, chymotrypsin, subtilisin, and elastase. The SPINKL protein showed no activity toward the serine proteases assayed (data not shown). To confirm whether protein purification caused SPINKL protein dysfunction, we purified SPINK3 from mouse SVSs for which we used an organic solvent in even harsher conditions than those for SPINKL (Lai *et al.* 1991). Activity assays demonstrated that SPINK3 had strong trypsin inhibitory activity (Fig. 4A); however, no activity was found even at a higher molar ratio of the SPINKL protein to trypsin (Fig. 4B). Thus, the SPINKL protein might have no inhibitory activity towards serine proteases.

Figure 3 Multiple protein sequence alignment of SPINK family proteins. The Kazal inhibitor domains derived from SPINK2 (SwissProt: Q8BMY7), SPINK3 (SwissProt: P09036), SPINK4 (SwissProt: Q149Z4), SPINK7 (SwissProt: Q6IE32), and SPINK11 (GenBank: XP_915755.1) were aligned with SPINKL (SwissProt: Q8CEK3). Dashes represent gaps introduced to optimize the alignment. The shaded areas indicate matching residues. C₁–C₆ stand for the six conserved cysteine residues of the SPINK protein family.

Predominant SPINKL expression in the luminal epithelium of seminal vesicles

To study the tissue distribution, we examined the expression of *Spinkl* transcripts in reproductive tissues, including the testes, epididymides, vas deferens, seminal vesicles, coagulating glands, prostate, uterus, oviducts, and ovaries. A ~0.6 kb band corresponding to *Spinkl* mRNA was exclusively detected in seminal vesicles (Fig. 5A). Several characteristic forms of the SPINKL protein were detected by Western blotting (Fig. 5B). When an equal amount of total RNA from the homogenate of a non-reproductive organ was compared with that of the seminal vesicle, no *Spinkl* mRNA was found in the brain, heart, lungs, liver, spleen, kidneys, stomach, small intestines, muscle, skin, thymus, placenta, or bladder (data not shown).

We examined SPINKL expression in the seminal vesicles of mice at different ages and found that it first appeared at a low level in 3-week-old mice, increased rapidly from 4 weeks onward, and reached its highest level in 8-week-old mice (data not shown). SPINKL was mainly immunolocalized to the luminal fluid and the epithelium of the mucosal folds of seminal vesicles of adult mice (Fig. 5C and D). The smooth muscle layer contained almost none. The strong immunohistochemical staining in the lumen supports the view that the SPINKL protein is secreted from the luminal epithelium.

To evaluate the effect of testosterone on *Spinkl* gene expression in seminal vesicles, we compared the findings in castrated mice. The relative levels of *Spinkl* mRNA in seminal vesicles were significantly lower in castrated mice treated only with vehicle compared with castrated mice treated with testosterone (data not shown). The immunohistochemical results revealed that the SPINKL protein intensity in the mucosal epithelium of castrated mice treated with only corn oil (Fig. 5E) was obviously lower than that in normal adults or castrated mice treated with testosterone (Fig. 5D and F). Taken together, these results suggest that *Spinkl* gene expression in the seminal vesicles is modulated by androgen.

Enhancement of sperm motility by SPINKL in vitro

We used indirect fluorescence staining to assess the binding of SPINKL to epididymal sperm. Weak fluorescence was seen on the epididymal sperm after they had

Figure 4 The inhibitory effect of the SPINKL protein on trypsin. No inhibitory activity of the SPINKL protein (■) against trypsin was found (A and B), in contrast with the positive control of SPINK3 (□), a known serine protease inhibitor towards trypsin (A). Data represent the mean of three repeated experiments and error bars represent the s.d.

been consecutively treated with the SPINKL antibody and the fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG, indicating that a small amount of the SPINKL protein exists on the sperm surface (Fig. 6A and B). In contrast, when spermatozoa were preincubated with SPINKL proteins, the FITC fluorescence was prominent on the middle piece, relatively weak on the tail, and not found on the head (Fig. 6C and D). Thus, spermatozoa have a SPINKL-binding zone on their surface. Moreover, when the ejaculated sperm on the slides were immunostained with the antiserum pretreated with SPINKL protein, no fluorescence signal was detected (Fig. 6E and F). However, the SPINKL protein was immunodetected on the surface of the ejaculated sperm despite a high fluorescence background due to a large amount of SPINKL protein in semen that was difficult to remove completely during the sperm preparation (Fig. 6G and H).

Most freshly prepared epididymal sperm in mKSOM medium without BSA were mobile with a visibly beating tail. When BSA was added to the medium, we observed high percentages of motile sperm. Supplementing the medium with SPINKL did not seem to influence the motile percentages. Replacement of BSA with SPINKL in the medium also revealed the similar motility percentages (Fig. 7A). Furthermore, we observed more-vigorous movement in medium with SPINKL only. Therefore, we analyzed the detailed motility parameters under various protein treatments. Obviously, the medium with 0.2 mg/ml of SPINKL revealed a relatively higher percentage of progressive

motility, an increase in the linearity (LIN), and a higher movement velocity. Values of straight line velocity (VSL), curvilinear velocity (VCL), and average path velocity (VAP) were significantly higher than those in the medium with BSA (Fig. 7B–F). Increasing the concentration of SPINKL to 0.6 mg/ml had the similar results (not shown). Thus, the SPINKL protein has the ability to enhance sperm motility.

Inhibition of BSA-induced sperm capacitation by SPINKL

To examine the effect of SPINKL on epididymal sperm capacitation, we assessed the protein tyrosine phosphorylation pattern of epididymal sperm after incubation with BSA or/and SPINKL. As shown in Fig. 8, only limited sperm proteins were phosphorylated in the control medium without BSA and SPINKL (lane 1). Addition of SPINKL to the medium showed a similar pattern to that of the control group (lane 3). Addition of BSA to the medium induced sperm capacitation accompanied by tyrosine phosphorylation of a group of proteins with a pattern similar to previous studies (Visconti *et al.* 1995a, Travis *et al.* 2001; lane 2). However, the extent of BSA-induced protein tyrosine phosphorylation was gradually suppressed by the increased concentration of SPINKL (lane 4 and 5). Obviously, the characteristic capacitation-specific protein tyrosine phosphorylation pattern induced by BSA could be prevented by SPINKL.

We then used the chlortetracycline (CTC)-staining method to morphologically assess sperm capacitation. The majority of sperm remained uncapacitated in the mKSOM medium without BSA and SPINKL. The medium supplement with 0.2 or 0.6 mg/ml SPINKL did not augment the population of capacitated sperm. The population of capacitated sperm increased remarkably (~58%) after supplement of 3 mg/ml of BSA. However, SPINKL inhibited BSA-induced sperm capacitation with significant decreases after addition of 0.2 or 0.6 mg/ml SPINKL to the BSA-containing medium (Fig. 9A). Even an ~82% decrease was found in the incubation with 1 mg/ml of SPINKL (not shown). These observations were correspondent to that inhibition of BSA-induced tyrosine phosphorylation by SPINKL (Fig. 8).

Next, we examined the acrosome reaction induced by calcium ionophore A23187. The spontaneous acrosome reaction was found in the *in vitro* capacitated sperm (Mortimer *et al.* 1989, Klemm & Engel 1991). We also detected the spontaneous acrosome-reacted sperm (20–30%) in the mKSOM incubation medium regardless with or without BSA or/and SPINKL (Fig. 9B). The concentration of vehicle used, 0.2% DMSO, did not increase the percentage of acrosome-reacted sperm (not shown). SPINKL-treated sperm had no increased acrosome reaction when compared with the control medium. However, BSA-treated sperm showed remarkable

Figure 5 Distribution of *Spinkl* messages and SPINKL proteins among reproductive tissues. (A) Total RNA (20 µg) prepared from the homogenates of each sexual tissue was analyzed by northern blotting. T, testes; E, epididymides; V, vas deferens; S, seminal vesicle; C, coagulating glands; P, prostate; O, ovaries; Od, oviducts; U, uterus. (B) Total protein (50 µg) of each male sexual tissue was analyzed by Western blotting. (C–F) Immunolocalization of the SPINKL protein to the luminal fluid and epithelium of seminal vesicle mucosal folds. (C) Tissue slices from 9-week-old mice were stained with the antiserum pretreated with SPINKL protein, biotin-conjugated goat-anti-rabbit IgG, and horseradish peroxidase-conjugated streptavidin (red). (D) Specimens were stained as in (C) except that the antibody was replaced with anti-SPINKL antiserum. The slices from castrated mice treated with corn oil (E) or testosterone (F) were also stained as in (D). For contrast, specimens were further stained with hematoxylin (blue). Photographs were taken under bright-field illumination: MF, mucosal fold; SM, smooth muscle; LF, luminal fluid. Bar=200 µm.

enhancement of acrosome reaction after A23187 induction. By contrast, these acrosome reactions were significantly inhibited when the sperm were incubated with BSA and SPINKL. About 32 and 42% decreases were seen after treatment with 0.2 and 0.6 mg/ml of SPINKL respectively (Fig. 9B). Only capacitated sperm can be induced to undergo acrosome reaction. Thus, the results further show that SPINKL is able to inhibit sperm capacitation induced by BSA.

We subsequently used *in vitro* fertilization (IVF) to test the effect of SPINKL on sperm fertility. The epididymal sperm had a lower ~20% fertilization rate, which may result from spontaneous acrosome-reacted sperm, in the medium without any proteins. Addition of SPINKL to the medium did not seem to influence the sperm fertilization rate. As usual, BSA-capacitated epididymal spermatozoa largely fertilized the cumulus-intact eggs. By contrast, SPINKL significantly impaired the fertilization rate of BSA-capacitated epididymal spermatozoa with 36 and 52% decreases when treatment with 0.2 and 0.6 mg/ml SPINKL respectively (Fig. 10). Therefore, the SPINKL protein disturbed the IVF process between spermatozoa and oocytes.

Discussion

In this study, we demonstrate that the *9530002K18Rik* gene encodes the polypeptide SPINKL protein. The SPINKL protein has the characteristic signature of Kazal inhibitors, but exhibited no inhibitory activities against common serine proteases, such as trypsin, chymotrypsin, subtilisin, and elastase. It is a secretory glycoprotein, predominantly expressed in mouse seminal vesicles, as indicated by its intraluminal location and the presence of a signal peptide in its sequence.

The evidence that the SPINKL protein is a secreted member of the SPINK family includes its purification from SVSs and its expression in the lumen and apical region of the mucosal epithelium (Fig. 5). Edman sequencing indicated that the SPINKL protein has a 23- or 27-amino acid signal peptide (Fig. 1F). Removal of the hydrophobic leader sequence left a protein core consisting of 63 or 67 amino acid residues with a molecular mass of 7379 or 7853 kDa respectively. However, mass data revealed several SPINK products in the SVS with molecular masses of 9033–9852 kDa (Fig. 2A).

The results of PAS staining and *N*-glycosidase F treatment demonstrated that the SPINKL protein is a

Figure 6 Demonstration of the binding of SPINKL to sperm. Epididymal sperm incubated with (C and D) or without SPINKL (A and B) and ejaculated sperm (E–H) without incubation with the exogenous SPINKL were smeared onto slides. For immunolocalization of SPINKL on sperm, the slides were incubated with anti-SPINKL antiserum (A–D, H, and G) or the antiserum pretreated with SPINKL protein (E and F), and then were treated with FITC-conjugated goat anti-rabbit IgG (A, C, E, and G) and counterstained with Hoechst dye to localize the nuclei for contrast (D, B, F, and H). The white arrows indicate staining signals on the middle piece of sperm. Bar = 10 μ m.

Figure 7 Effects of SPINKL on sperm motility. Epididymal spermatozoa were incubated in mKSOM medium (containing 3 mg/ml BSA; ○), or the medium was supplemented with 0.2 mg/ml SPINKL (■), or BSA was removed from the medium and replaced with 0.2 mg/ml SPINKL (□) at 37 °C in an atmosphere of 5% (v/v) CO₂ for 30–150 min. Motility parameters at each specified incubation time were determined. Points are the mean $\pm$ s.d. of three independent determinations. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ in comparisons to the mKSOM medium group.

glycoprotein. The protein molecule contains only one potential N-glycosylation site (Fig. 1F). The results of Edman sequencing suggested that it is an N-glycosylated site. In contrast to these findings, SPINK3 is not a glycoprotein (our unpublished data).

There are several similarities between SPINKL and SPINK3, including the same gene locus in chromosome 18, gene regulation by testosterone, tissue-specific expression and localization in the mouse seminal vesicles, and conserved cysteine positions that are a feature of the SPINK protein family (Chen *et al.* 1998 and herein). Nonetheless, they differ in terms of glycosylation nature, protease inhibitory activities, and low sequence identities.

The growth and differentiation of seminal vesicles are closely associated with androgens (Chai 1956, Morley & Wright 1972). Many gene expressions in this tissue have been demonstrated to be regulated by androgen (Chen *et al.* 1987, 1998, Yu *et al.* 1993, Li *et al.* 2005, 2006). In this study, regulation of the *Spinkl* gene was also the case (Fig. 5). Androgen and its receptor form a complex that interacts with androgen response elements (AREs) in androgen-responsive genes and regulates their expression. We employed the MatInspector program (Cartharius *et al.* 2005) to predict the potential AREs in the promoter to a part of the intron 1 region of 9530002K18Rik corresponding to nucleotides 70 000 to 72 486 of GenBank accession no. AC124181. We found one conserved ARE in the promoter and another one in the intron 1 region. Thus, it is possible that androgen acts directly on *Spinkl* to regulate its gene expression.

The SPINKL protein can enhance sperm motility, especially the movement velocity and the LIN of cell tracks as they swim (Fig. 7). The molecular mechanism of

how it enhances sperm motility deserves our further studies. It is noted that SPINKL protein inhibits BSA-induced *in vitro* capacitation and thus blocks sperm–oocyte interactions (Figs 8–10). Therefore, SPINKL may

Figure 8 Effects of SPINKL on protein tyrosine phosphorylation during murine sperm capacitation. Epididymal spermatozoa were incubated in the presence of 3 mg/ml of BSA or/and different concentrations of SPINKL at 37 °C for 90 min (as described at the bottom). The soluble fraction of the sperm lysate was resolved by SDS-PAGE, electro-transferred onto a nitrocellulose membrane, and immunoblotted with anti-phosphotyrosine antibodies.

Figure 9 Effects of SPINKL on murine sperm capacitation and acrosome reaction. (A) Epididymal spermatozoa were incubated in the presence of 3 mg/ml of BSA or/and different concentrations of SPINKL at 37 °C for 90 min (as described at the bottom), as described in the Materials and Methods section. The CTC fluorescence staining method was conducted to score the population of capacitated sperm (gray bars). A minimum of 200 sperm per trial was scored. (B) The acrosome reaction induced by calcium ionophore A23187 in sperm treated with BSA or/and SPINKL. After epididymal sperm were capacitated in the presence of BSA or/and SPINKL, they were either not treated at all (blank bars) or were treated with A23187 (gray bars). The acrosomal status was estimated by PNA staining. A minimum of 200 spermatozoa per group were evaluated. Data represent the mean $\pm$ s.d. of three independent experiments. ** $P < 0.01$ and *** $P < 0.001$ in comparisons with the BSA only group.

inhibit premature sperm capacitation and serve as a decapacitation factor.

Several murine decapacitation factors have been identified, including a 40 kDa glycoprotein (Fraser 1998), a phosphatidylethanolamine-binding protein (Nixon *et al.* 2006), and two secreted seminal vesicle proteins, SVA (Huang *et al.* 2000) and SVS2 (Kawano & Yoshida 2007). The seminal vesicles secrete primarily basic proteins (Gerhardt *et al.* 1985). However, SPINKL and SVA are the acidic seminal vesicle-secreted proteins. Although they all inhibit BSA-stimulated sperm capacitation *in vitro*, their effects on sperm motility are very different. SPINKL enhances sperm motility, while SVA inhibits motility (Huang *et al.* 1999). The differences remain to be determined.

Although proteases have been implicated in the reproduction, only a few are associated with the process of capacitation (Zheng *et al.* 2001, Kawakami *et al.* 2004). As for serine protease inhibitors, there also seem

few reports to point out the direct action on the process of sperm capacitation. A protease inhibitor of seminal vesicle origin was shown to block sperm's zona binding and acrosome reaction, thus has been suggested to be a decapacitation factor (Boettger-Tong *et al.* 1992). SPINKL may also be a decapacitation factor but with no protease inhibitor activity. It is of interest to note that SPINK3, another seminal vesicle protease inhibitor, possesses trypsin inhibitor activity but has no effect on capacitation (Lin *et al.* 2006). Thus, for a serine protease, the inhibitor activity and the role as a decapacitation factor may be not necessarily related.

Several signal transduction pathways have been demonstrated to be associated with sperm capacitation, including the extracellular signal-regulated kinase pathway (Luconi *et al.* 1998, de Lamirande & Gagnon 2002), the cyclic AMP/protein kinase A pathway (Visconti *et al.* 1995b, Breitbart 2003), and the reactive oxygen species affected pathway (de Lamirande & O'Flaherty 2008). We can speculate that SPINKL may affect the capacitation-related signal transduction machinery of sperm. To uncover its mechanism of action, we may start from searching the binding partners of SPINKL on the sperm plasma membrane by chemical cross-linking reagents or examining the activity of various kinases associated with capacitation. Whether the components of the pathways mentioned above are involved in the inhibition of capacitation by SPINKL remains to be investigated.

In conclusion, we have purified and identified a novel protein, SPINKL, which has the characteristic signature structure of the Kazal domain of SPINK, and we demonstrated its tissue-specific expression in mouse seminal vesicles. It is interesting to note that the SPINKL protein has no inhibitory activities against common serine proteases. The amount of the SPINKL protein in seminal vesicles is modulated by androgen and is correlated with

Figure 10 Influence of the SPINKL on *in vitro* fertilization. Epididymal spermatozoa were capacitated in mKSOM medium with BSA or/and SPINKL for 90 min and subsequently inseminated with cumulus-intact oocytes for 6 h. Fertilized eggs that showed two pronuclei were identified by Hoechst 33 258 staining. Data represent the mean $\pm$ s.d. from four independent experiments. ** $P < 0.01$ and *** $P < 0.001$ in comparisons relative to the fertilization rate of the medium with BSA only control.

the stage of animal maturation. SPINKL is a sperm-binding protein and is able to enhance sperm motility. Furthermore, it is able to inhibit BSA-induced sperm capacitation and impair sperm–oocyte interactions *in vitro*.

Materials and Methods

Animals

Specific pathogen-free outbred ICR mice were bred and maintained in the Animal Center of the Department of Medical Research, Mackay Memorial Hospital. Animals were treated according to institutional guidelines for the care and use of experimental animals. They were housed under controlled lighting (14h light:10h darkness) at 21–22 °C and were provided with water and NIH-31 laboratory chow *ad libitum*. To study the developmental profile and tissue distribution of the protein, we followed our previously described methods (Li *et al.* 2005, 2006). To investigate the androgenic effects, male mice were castrated and administered testosterone as previously described (Li *et al.* 2005, 2006).

Protein purification

Normal adult mice (10–12 weeks old) were killed by cervical dislocation. The seminal vesicles of 50 mice per separation were carefully dissected to free them from the adjacent coagulating glands, and the secretions were directly squeezed into 50 ml of ice-cold 10 mmol/l Tris–HCl in the presence of 1 mmol/l phenylmethyl sulfonyl fluoride at pH 8.0. After centrifugation at 10 000 *g* for 15 min, the supernatant was resolved by ion-exchange chromatography on a diethylaminoethyl (DEAE)-Sephacel (Amersham Pharmacia Biotech, Uppsala, Sweden) column (12×2.6 cm) into four fractions (Fig. 1A), as described previously (Li *et al.* 2005, 2006). Fraction IV collected from three separations was concentrated and subjected to a Sephadex G-75 (Sigma Chemical Co.) column (2.6×120 cm) pre-equilibrated with 50 mmol/l Tris–HCl and 150 mmol/l NaCl (pH 7.4). The column was eluted in the same buffer at a flow rate of 10 ml/h; fractions (2 ml) were collected, and the absorbance records are shown in Fig. 1B. Among them, peak 3 was further subjected to ion exchange HPLC on a Q HyperD 10 (Beckman, Fullerton, CA, USA) column (4.6 mm×10 cm). The column was eluted with a linear gradient of 0–15% (w/v) 1.0 mol/l NaCl in 25 mmol/l Tris–HCl (pH 8.2) at a flow rate of 1.0 ml/min for 40 min (Fig. 1C). SPINK3 was purified following a previously described method (Lai *et al.* 1991).

Mass spectrometry

The molecular mass was determined on an Ultraflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonik, Bremen, Germany). A sample was dissolved in 0.1% (v/v) trifluoroacetic acid (TFA) to a final concentration of 100 µmol/l. A sample of 1 µl was mixed with 1 µl matrix (50 mmol/l 2,5-dihydroxybenzoic acid in 50% (v/v) acetonitrile), then spotted onto a plate for mass analyses. For protein identification, protein bands on the SDS-PAGE gel were excised, washed in a solution containing acetonitrile and 100 mmol/l NH₄HCO₃ (1:1, v/v), and subjected

to in-gel digestion with trypsin overnight at 37 °C. The tryptic peptides were then extracted with a solution of 50% (v/v) acetonitrile and 1% (v/v) TFA, lyophilized, resuspended in 0.1% (v/v) TFA, and analyzed by LC/MS/MS equipped with Agilent 1100 Series HPLC (Agilent Technologies, Palo Alto, CA, USA) and an LTQ FT hybrid mass spectrometer (Thermo Electron, San Jose, CA, USA). The MS/MS data were used for protein identification using the MASCOT search engine (<http://www.matrixscience.com>) based on the International Protein Index databases (<http://www.ebi.ac.uk/IPI>).

Protein analysis

Removal of the N-glycoconjugate from a glycoprotein was performed using N-glycosidase F (New England BioLabs, Beverly, MA, USA). PAS staining of the glycoproteins was performed using the Gelcode glycoprotein staining kit (Pierce, Rockford, IL, USA). The protein concentration was determined using a bicinchoninic acid protein assay (Smith *et al.* 1985). The amino acid sequence was determined using automated Edman degradation with a 492-protein sequencer and an online 140 C analyzer (Applied Biosystems, Foster City, CA, USA). A database search was performed using the Basic Local Alignment Search Tool (BLAST) algorithms against the non-redundant database up to October 2006 (<http://www.ncbi.nlm.nih.gov/BLAST>). Multiple protein sequence alignments were performed with ClustalW (<http://www.ebi.ac.uk/clustalw>). AREs in a promoter were analyzed by MatInspector (<http://www.genomatix.de>; Cartharius *et al.* 2005) against the matrix family library version 6.2 (October 2006).

Assay of inhibitory activity

The inhibitory activities of the SPINKL protein towards serine protease, trypsin, α-chymotrypsin, subtilisin Carlsberg, and elastase (Sigma) were assayed by measuring the absorbance of p-nitroaniline produced by these proteases at 410 nm following previously described methods (Hergenhahn *et al.* 1987, Somprasong *et al.* 2006).

Antibody production and Western blotting

Antisera against SPINKL were produced in two New Zealand white rabbits. Preimmune serum was collected before the immunization. For the immunization, the purified SPINKL protein in normal saline (0.4 mg/ml) was emulsified with Freund's complete adjuvant (1:1, v/v). In total, 2 ml of the mixture was injected subcutaneously in multiple sites in one rabbit. Rabbits were boosted twice every 3 weeks with the mixture of the same amount of purified protein and Freund's incomplete adjuvant (1:1, v/v). Antiserum was collected in 10 days after the last injection.

One hundred micrograms of the purified SPINKL proteins were conjugated to AminoLink beads according to the manufacturer's instructions (Pierce). Antisera against SPINKL were adsorbed by the conjugated beads to remove the specific antibody to SPINKL. The treated antiserum was used as the control antiserum.

Proteins were resolved using SDS-PAGE on a 15% gel slab (8.2×7.3×0.075 cm) and were stained with Coomassie

brilliant blue or transferred to a nitrocellulose membrane for immunostaining according to previously described methods (Towbin *et al.* 1979). Membranes were blocked with 5% (w/v) skim milk in PBS (blocking solution) for 2 h, and then incubated with anti-SPINKL antiserum (1:10 000) in blocking solution for 1 h at room temperature. After gentle agitation in four changes of PBS for 15 min each, they were immunoreacted with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Amersham Pharmacia Biotech) diluted to 1:10 000 in the blocking solution for 1 h. Immunoreactive bands were revealed using an enhanced ECL substrate according to the manufacturer's instructions (Pierce).

Immunohistochemical staining

Immunostaining was performed according to our previously described methods (Li *et al.* 2005, 2006). In brief, tissues slides were blocked with 10% (v/v) normal goat serum in PBS (blocking solution) for 1 h at room temperature and then incubated with anti-SPINKL antiserum diluted 1:1000 in the blocking solution at 4 °C for 16 h. After washing, the slides were treated with biotin-conjugated goat anti-rabbit IgG (~1 µg/ml; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) in the blocking solution for 1 h at room temperature. The slides were washed again and then incubated with HRP-conjugated streptavidin (~1 µg/ml; Zymed Laboratories, South San Francisco, CA, USA) in the blocking solution for 40 min at room temperature. Protein signals were detected by 3-amino-9-ethylcarbazole staining (Zymed Laboratories). The slides were then counterstained with hematoxylin (Vector Laboratories, Burlingame, CA, USA) and photographed using an Olympus BX 40 microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP-70 digital camera.

RNA isolation and northern blot analysis

Total RNA was extracted from tissue homogenates using an RNeasy Mini kit (Qiagen). PCR-amplified fragments of *Spinkl* cDNA (454 bp) or glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) cDNA (557 bp) were used as the templates to prepare the ³²P-labeled cDNA probe using a random-priming kit (Promega). RNA samples (20 µg) were subjected to 1.0% (w/v) agarose-formaldehyde gel electrophoresis, blotted onto nylon membranes by capillary transfer and hybridized with a probe as previously described (Maniatis *et al.* 1989). In brief, blotted membranes were first hybridized with *Spinkl* probes overnight at 45 °C, and then the free probes were washed away. Messenger RNA signals on the membranes were examined by phosphorimaging. Subsequently, membranes were examined with the *Gapdh* probes in the same way after removal of the *Spinkl* probes. Thus, hybridization with the *Spinkl* and *Gapdh* probes was performed on the same membranes.

Immunostaining of sperm

Freshly prepared epididymal spermatozoa (~10⁶ cells/ml) were blocked in PBS containing 10% (v/v) normal goat serum (blocking solution) for 30 min at room temperature. The cells were further incubated with or without 0.5 µmol/l SPINKL for

1 h. At the end of the incubation, the cells were centrifuged and the cell pellets were washed with PBS to remove the unbound ligands. The cells were air-dried on a glass slide and washed twice with PBS. The slides were incubated with the anti-SPINKL antiserum (1:1000) in the blocking solution at 4 °C for 16 h. The slides were washed thrice with PBS to remove any excess antibodies before they were incubated with FITC-conjugated goat anti-rabbit IgG (Vector Laboratories) diluted to 1:3000 in blocking solution for 1 h. All slides were then washed with PBS, counterstained with 5 µg/ml Hoechst 33 258, and mounted in ~100 µl of ProLong Gold antifade medium (Invitrogen Molecular Probes). Slides were examined by laser scanning confocal microscopy using a Leica TCS SP (Leica-Microsystems, Heidelberg, Germany) equipped with an argon/krypton ion laser and u.v. laser with the appropriate filter spectra adjusted to detect the FITC and Hoechst fluorescence.

The ejaculated sperm were collected from semen in the uterine cavity of three female mice with plugged vaginas. After extensive washing with PBS, the ejaculated sperm without incubation with the exogenous SPINKL protein were smeared on slides for immunolocalization of SPINKL as mentioned above.

Analysis of sperm motility

Sperm (~10⁶ cells/ml) from the cauda epididymis of 12- to 14-week-old males were capacitated in 150 µl of a modified simple-optimized medium (mKSOM; Summers *et al.* 2000), but with a modified 3 mg/ml of BSA under mineral oil at 37 °C in an atmosphere of 5% (v/v) CO₂ in humidified air. To analyze the effect of SPINKL on sperm motility, the medium was supplemented with 0.2 mg/ml SPINKL or the BSA was replaced with 0.2 mg/ml SPINKL. Sperm motility was determined using a computer-assisted sperm assay with a sperm motility analyzer (IVOS version 10; Hamilton-Thorne Research, Beverly, MA, USA). A 10 µl sample was placed in a 10 µm deep Makler chamber at 37 °C for analyses at 30 min intervals up to 150 min. Motility parameters, including movement velocities of VCL, VAP, and VSL, the percentages of sperm motility (VAP > 7 µm/s) and progressive motility (VAP > 50 µm/s and STR > 80%, where STR = VSL/VAP), and the LIN (LIN = VSL/VCL), which measures the departure of the cell track from a straight line, were measured. The analyzer settings were described previously (Li *et al.* 2005). For each assay, five to ten fields with a range of 350–400 sperm were analyzed.

Evaluation of sperm capacitation and acrosome reaction

Sperm capacitation in the molecular basis was examined by detecting the capacitation accompanied increase in the protein tyrosine phosphorylation of a subset of proteins of molecular weight 40 000–120 000 according to the previously described method (Visconti *et al.* 1995a). In brief, about 4 × 10⁷ spermatozoa/ml were incubated in a modified Krebs–Ringer bicarbonate medium (Lee & Storey 1986) with or without BSA, as the positive or negative control respectively, or the medium supplemented with SPINKL, or BSA replaced with SPINKL at 37 °C in an atmosphere of 5% (v/v) CO₂ in humidified air for 90 min. Then, the soluble fraction of sperm protein extracts was

subjected to SDS-PAGE on an 8% gel slab. The proteins on the gel were electrotransferred onto a nitrocellulose paper. Western blot analyses were performed by using anti-phosphotyrosine antibody according to a previous method (Visconti *et al.* 1995a).

CTC fluorescence-staining method, as previously described (Ward & Storey 1984, Lee & Storey 1985), was conducted to morphologically assess sperm capacitation. In this experiment, we used the mKSOM medium instead of original modified Tyrode medium. Freshly prepared epididymal spermatozoa (10^6 cells/ml) were capacitated in 150 μ l mKSOM medium with or without BSA (3 mg/ml), as the positive or negative control respectively at 37 °C in an atmosphere of 5% (v/v) CO₂ in humidified air for 90 min. The medium was supplemented with SPINKL or the BSA was replaced with SPINKL to analyze the effects of SPINKL on sperm capacitation *in vitro*. The CTC staining of sperm was carried out following the original method and examined using a fluorescence microscope (BX 40, Olympus).

For analyzing the sperm acrosome reaction, the capacitated sperm described above was treated with 10 μ mol/l A23187 in DMSO (0.2%) at 37 °C for 30 min. Sperm were smeared on the slide and fixed with methanol for 30 s. The sperm acrosomal status was assessed by staining with 5 μ g/ml of Alexa Fluor 488-conjugated peanut agglutinin lectin (PNA; Invitrogen Molecular Probes) at dark for 10 min. The slides were immediately examined with a fluorescence microscope (BX 40, Olympus).

IVF

Epididymal sperm (2×10^5 cells/ml) in 150 μ l mKSOM medium under mineral oil with or without BSA or/and SPINKL were capacitated for 90 min at 37 °C in an atmosphere of 5% (v/v) CO₂ in humidified air. Oocyte–cumulus complexes collected by superovulation treatment (Gates & Bozarth 1978) were added to the same medium. After 6 h of insemination, the oocytes were washed with mKSOM medium, fixed on the slide with 4% (w/v) paraformaldehyde, and stained in 5 μ g/ml Hoechst 33258 for 3 min. The slides were observed with a fluorescence microscope (BX 40, Olympus). Two pronuclei embryos were scored as fertilized.

Statistical analysis

Data are presented as the mean $\pm$ s.d. Differences were analyzed by the Bonferroni *post hoc* test followed by one-way ANOVA using InStat software (GraphPad, San Diego, CA, USA). A *P* value of <0.05 was considered to be significant.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Funding

This work was supported by grants from the National Science Council (NSC95-2311-B-195-001 and NSC96-2311-B-195-001) and Mackay Memorial Hospital (MMH 9568 and MMH 9631), Taipei, Taiwan, ROC.

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Received 14 August 2007

First decision 10 October 2007

Revised manuscript received 8 August 2008

Accepted 20 August 2008