SSLP-1, a secreted Ly-6 protein purified from mouse seminal vesicle fluid

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Abstract

The Ly-6 protein family refers to a group of glycoposphatidyl inositol-anchored membrane proteins with ten conserved cysteines. They are thought to be involved in cellular adhesion and signaling. Recently, a subfamily of secreted Ly-6 proteins has been identified. In the present study, we report a secreted Ly-6 protein, secreted seminal vesicle Ly-6 protein 1 (SSLP-1) purified from mouse seminal vesicles using a series of steps including ion-exchange chromatography on a diethylaminoethyl (DEAE)-Sephacel column, gel filtration on a Sephadex G-75 column, and ion-exchange HPLC on a sulfopropyl column. Further analysis demonstrated it to be a novel, previously unnamed, 17 kDa glycoprotein. N-glycosidase F treatment revealed a core protein with a molecular mass of 8720 Da. By Basic Local Alignment Search Tool Protein analysis, we found that SSLP-1 had ten conserved cysteine residues identical with other secreted Ly-6 proteins. The gene *Gm191*, which is located on chromosome 9, encodes SSLP-1. By Northern blotting with 21 different mouse tissues, we found that *Sslp-1* mRNA was predominantly expressed in the seminal vesicle. Immunohistochemistry revealed SSLP-1 protein in the luminal fluid and mucosal epithelium of the seminal vesicles. The amount of *Sslp-1* mRNA and SSLP-1 protein in the seminal vesicle was regulated by testosterone and correlated with the stage of animal maturation. The tissue-specific expression pattern suggests that SSLP-1 may play a physiological role in male mouse reproduction.

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Introduction

The Ly-6 (also known as Ly-6/urokinase-type plasminogen activator receptor, uPAR) protein superfamily refers to a group of proteins with ten conserved cysteine residues (LeClair *et al.* 1986, Bamezai 2004). They are classified into two subfamilies based on whether they have a glycoposphatidyl inositol (GPI)-anchored signal sequence. GPI-anchored Ly-6 receptor proteins are thought to be involved in cellular adhesion and signaling (Gumley *et al.* 1995, Bamezai 2004). The second non-GPI-anchored subfamily contains secreted Ly-6 proteins, including secreted mammalian Ly-6/uPAR-related protein (SLURP)-1 (Adermann *et al.* 1999), SLURP-2 (Tsuji *et al.* 2003), rat urinary protein (RUP)-1, RUP-2, pig protein (PIP)-1, and rat spleen protein (RSP)-1 (Southan *et al.* 2002), and seminal vesicle secretion 7 (SVS 7; Southan *et al.* 2002).

SLURP-1 in human blood and urine was the first of the secreted Ly-6 protein to be discovered (Adermann *et al.* 1999). *SLURP-1* gene mutations have been implicated in a rare skin disorder, Mal de Meleda (Fischer *et al.* 2001, Eckl *et al.* 2003, Mastrangeli *et al.* 2003, Ward *et al.* 2003). SLURP-1 protein is an epidermal neuromodulator of the $\alpha 7$ nicotinic receptor and thought to be involved in epidermal homeostasis and cutaneous inflammation (Chimienti *et al.* 2003). *SLURP-2* has been shown to be upregulated in psoriasis vulgaris, suggesting a possible pathogenetic role in that disorder (Tsuji *et al.* 2003).

RUP-1 and RUP-2 have been found in rat. While it is known that they are secreted and glycosylated proteins, their biological functions are obscure (Southan *et al.* 2002). SVS7 was originally purified from the mouse seminal vesicle and has caltrin activity inhibiting calcium uptake by epididymal sperm (Coronel *et al.*

1992). It is a phospholipid-binding protein and a sperm motility enhancer which is expressed and secreted exclusively in the seminal vesicle (Luo *et al.* 2001).

SVS contribute the major portion of the seminal plasma. Previously, several proteins purified from murine SVS have been demonstrated to have effects on sperm physiology (Chen *et al.* 1998, Huang *et al.* 1999, Luo *et al.* 2001, Li *et al.* 2005). However, other components of SVS remain to be identified. Further investigation of the proteome may enhance our understanding of normal and abnormal male reproductive physiology. Here, we report a novel secreted Ly-6 protein found in murine SVS.

Materials and Methods

Animals and hormone treatment

Specific pathogen-free outbred ICR mice were purchased from BioLASCO Taiwan Co., Ltd (Taipei, Taiwan). The animals were bred based on the technology derived from Charles River Laboratories (Wilmington, MA, USA), maintained in the animal center at the Department of Medical Research, Mackay Memorial Hospital and treated according to the institutional guidelines for the care and use of experimental animals. They were housed under controlled lighting (14 h light:10 h darkness) at 21–22 °C and provided with water and NIH-31 laboratory mouse chow *ad libitum*.

Normal 10-week-old adult mice were used to purify the protein. To investigate the androgenic effects, adult male mice ($n=24$) were used. Twenty were castrated at the age of 5 weeks; the remaining four were kept as controls. When the mice were 8 weeks old, five of the castrates were given s.c. injection of testosterone propionate (Sigma) in corn oil (5 mg/kg body weight) for eight consecutive days. The 15 castrated control animals received corn oil only. The four normal controls were not given any treatment. The mice were killed by cervical dislocation and the seminal vesicles removed 12 h after the last injection. To examine the differential expression of mRNAs and proteins in the above treatment, the total RNA of tissues was purified for the Northern blot analysis; total protein extracts were used for the Western blot analysis, and paraffin-embedded tissue sections were used for immunohistochemistry analysis.

To examine the developmental profile of the protein, 22 mice at various ages ranging from 3 to 16 weeks were killed, the seminal vesicles removed, and the amount of mRNA transcripts and proteins were measured. The distribution of the mRNA was assessed by Northern blot analysis in various reproductive tissues of adult mice, including three males, three females, and two pregnant females.

Protein purification

Normal adult mice (10 weeks old) were sacrificed by cervical dislocation. The seminal vesicles collected from ~300 mice were carefully dissected to free them from the adjacent coagulating glands, and the secretions were squeezed directly 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 DEAE-Sephacel (Amersham) column (12 × 2.6 cm) pre-equilibrated with 10 mmol/l Tris-HCl at pH 8.0. After non-retarded fractions were washed out, the column was eluted with 0.5 mol/l NaCl in the same buffer at a flow rate of 18 ml/h; fractions of 4 ml per tube were collected and the absorbance of each fraction at 280 nm was recorded (Fig. 1A). Fraction I was concentrated and subjected to elution in a Sephadex G-75 (Sigma) column (2.6 × 120 cm) pre-equilibrated with 50 mmol/l Tris-HCl, 150 mmol/l NaCl, at pH 7.4. The column was eluted with the same buffer at a flow rate of 10 ml/h; fractions (2 ml) were collected and the absorbance recorded (Fig. 1B). Peak 3 from this step was further subjected to ion-exchange-HPLC on a Protein PAK SP 5PW (Waters, Milford, MA, USA) column (7.5 cm × 7.5 mm). The column was eluted with a linear gradient of 0–100% (w/v), 1.0 mol/l NaCl in 20 mmol/l sodium acetate at pH 6.0 at a flow rate of 1.0 ml/min for 60 min (Fig. 1C).

Protein analysis

Protein components were resolved by 15% SDS-PAGE. The *N*-glycoconjugate of a glycoprotein was removed using the method of Tarentino & Plummer (1994). The protein was boiled in 1.0% (w/v) SDS and incubated with *N*-glycosidase F (40 U/mg protein; TaKaRa, Shiga, Japan) in 20 mmol/l sodium phosphate at pH 7.2 in the presence of 50 mmol/l EDTA, 0.5% (v/v) Nonidet P-40, and 10 mmol/l sodium azide for 16 h at 37 °C. Periodic acid-Schiff (PAS) staining of glycoproteins was performed by using the Gelcode glycoprotein staining kit (Pierce, Rockford, IL, USA). The protein concentration was determined using the bicinchoninic acid protein assay (Smith *et al.* 1985). The amino acid sequence was determined using automated Edman degradation with a 492-protein sequencer with an online 140 C analyzer (Applied Biosystems, Foster City, CA, USA). Database searching was performed using the Basic Local Alignment Search Tool (BLAST) algorithms against the non-redundant database up to May 2006 (<http://www.ncbi.nlm.nih.gov/BLAST>). The multiple protein sequence alignments were performed by ClustalW (<http://www.ebi.ac.uk/clustalw>).

Figure 1 Purification of the peak-a protein from mouse seminal vesicle secretions. (A) Fractionation of soluble proteins by ion-exchange chromatography on a DEAE-Sephacel column. (B) Resolution of fraction I from (A) by gel filtration on a Sephadex G-75 column. (C) Resolution of peak 3 fraction from (B) by ion-exchange HPLC on a SP column.

Mass spectrometry

The protein band on the SDS-PAGE gel was excised, washed in a solution containing acetonitrile and 100 mmol/l NH_4HCO_3 (1:1, v/v), and subjected to in-gel digestion with trypsin overnight at 37 °C. The tryptic peptides were extracted with a solution of 50% (v/v) acetonitrile and 1% (v/v) formic acid, lyophilized, resuspended in 0.1% (v/v) formic acid, and analyzed with a QSTAR XL Hybrid liquid chromatographic mass spectrometry (LC/MS/MS; Applied Biosystems) equipped with an UltiMate TM Capillary/Nano LC system (LC Packings, Sunnyvale, CA, USA). Peptide sequences were identified by using Mascot software (Matrix Science, London, UK) analysis, which searched the NCBI non-redundant sequence database using as a query the mass spectra we had obtained.

Western blotting

Antisera against SSLP-1 were raised in New Zealand White rabbits. Proteins were resolved using SDS-PAGE on a 15% gel slab (8.2 × 7.3 × 0.075 cm) by the method by Laemmli (1970). The proteins on the gel were stained with Coomassie Brilliant Blue or transferred to a nitrocellulose membrane using electroblotting at 35 V at 4 °C for 18 h in a solution of 25 mmol/l Tris-HCl, 197 mmol/l glycine, and 13.3% (v/v) methanol. Membranes were blocked with 5% (w/v) skim milk in PBS for 2 h, and incubated with anti-SSLP-1 antiserum (1:10 000) in the blocking solution for 1 h at room temperature. After gently agitated in four changes of PBS for 15 min each, horseradish peroxidase-conjugated goat anti-rabbit IgG (Zymed Laboratories, South San Francisco, CA, USA) diluted to 1:10 000 was added to the blocking solution for 1 h. Immunoreactive bands were revealed using an enhanced ECL substrate according to the manufacturer's instructions (NEN Life Science Products, Boston, MA, USA).

Immunohistochemical staining

Mouse seminal vesicles were fixed in 10% (v/v) formaldehyde solution and embedded in paraffin, after which 8 µm serial cross-sections were mounted on silanated glass slides (Sigma). Deparaffinized sections were immersed in 0.3% (v/v) H_2O_2 solution to block endogenous peroxidase activity, blocked with 10% (v/v) normal goat serum in PBS for 1 h at room temperature and incubated with anti-SSLP-1 antiserum diluted to 1:1000 in the blocking solution for 1 h. The slides were gently agitated in three changes of washing solution for 10 min each and 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 as mentioned above and incubated with horseradish peroxidase-conjugated streptavidin (~1 µg/ml; Zymed Laboratories) in blocking solution for 1 h at room temperature. Protein signals were examined after the slides were incubated for 10 min with 3-amino-9-ethylcarbazole staining solution (Zymed Laboratories). The slides were washed in three changes of water for 3 min each and counterstained with hematoxylin (Vector Laboratories, Burlingame, CA, USA) for 10 s. The sections were photographed using an Olympus microscope (BX40; Olympus, Tokyo, Japan) equipped with an Olympus DP70 video camera.

RNA isolation and Northern blot analysis

Total RNA was extracted from tissue homogenates using an Ultraspec-II RNA isolation kit (Biotecx Laboratories, Inc., Houston, TX, USA). A PCR-amplified fragment of *Sslp-1* cDNA (479 bp) and a cDNA fragment of the

mouse *Gapd* gene (557 bp) were used as a template to prepare a ^{32}P -labeled cDNA probe using a Promega random-priming kit (Promega). RNA samples (20 μg) were subjected to denaturation by 1.0% (w/v) agarose-formaldehyde gel electrophoresis and blotted onto nylon membranes by capillary transfer as previously described (Maniatis *et al.* 1989). Blotted membranes were first incubated with pre-hybridization buffer (50% deionized formamide, $6\times$ SSC, $5\times$ Denhardt's solution, 1.0% SDS, and 100 $\mu\text{g}/\text{ml}$ sheared salmon sperm DNA) for 2 h at 45°C and hybridized with labeled *Sslp-1* probes overnight at 45°C . Following hybridization, membranes were washed using standard procedures. The mRNA on a filter membrane was examined after autoradiography and probes were removed from the membrane as previously described (Maniatis *et al.* 1989). The same membrane was then hybridized with labeled *Gapd* probes. Thus, hybridization with *Sslp-1* and *Gapd* probes was performed on the same filter membrane. To quantitate the relative *Sslp-1* gene expression, blots were exposed to a Fuji phosphorimager screen and quantified with Fuji Science Lab software (Fuji Laboratories, Japan). As a control for loading differences, the *Sslp-1* mRNA level was normalized to the level of the *Gapd* mRNA on the same blot.

Statistical analysis

Data are presented as mean $\pm$ s.d. Differences in mRNA expression 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.

Results

Purification and identification of a novel secreted Ly-6 protein from the mouse SVS

Three peaks obtained from liquid column chromatography of the fresh preparation of soluble SVS were resolved by ion-exchange HPLC on a sulfopropyl (SP) column at the final purification step (Fig. 1C, peaks a–c). Each of the representative samples at various steps of purification was resolved on reducing SDS-PAGE gel (Fig. 2A). Peak-a yielded one broad ~ 17 kDa band that could be deglycosylated by exhaustive digestion with *N*-glycosidase F to a sharp ~ 9 kDa protein (Fig. 2A, lane 5). The peak-a protein stained with PAS reagent, but its deglycosylated form did not (Fig. 2B), which implies that peak-a apparently was a glycoprotein with *N*-linked carbohydrate moieties. The *N*-linked glycans seemed to be the only glycosylation, since the PAS reagent did not stain the deglycosylated protein (cf. lanes 4 and 8 of Fig. 2B).

The antibody against peak-a protein immunoreacted to a broad ~ 17 kDa protein band in the SVS, to the

Figure 2 Glycoprotein nature and antiserum specificity of the peak-a protein. (A) A representative sample at various purification steps was resolved by 15% SDS-PAGE. Lane 1, 15 μg seminal vesicle secretion; lane 2, 15 μg fraction I of DEAE-Sephacel chromatography; lane 3, 10 μg peak 3 of gel filtration; lane 4, 1.5 μg peak-a of HPLC. Lane 5, 2 μg deglycosylated peak-a. (B) Demonstration of glycosylation. Each protein was identified by SDS-PAGE on a 15% polyacrylamide gel slab. The proteins in the gel were stained with Coomassie Blue (lanes 1–4) and PAS reagent (lanes 5–8). Lanes 1 and 5, 2 μg horseradish peroxidase as a positive control; lanes 2 and 6, 2 μg soybean trypsin inhibitor as a negative control; lanes 3 and 7, 2 μg peak-a; lanes 4 and 8, 2 μg deglycosylated peak-a protein. (C) Specificity of antiserum. The SDS-PAGE gel on (A) was electroblotted onto a nitrocellulose membrane and immunodetected by antiserum raised against peak-a protein.

purified peak-a protein, and to a single ~ 9 kDa deglycosylated peak-a protein (Fig. 2C). Therefore, extensive *N*-glycosylation seems to account for the higher molecular mass of the peak-a protein (17 kDa compared with ~ 9 kDa).

To aid in identifying the glycoprotein, the band of the deglycosylated peak-a protein on the SDS-PAGE gel was excised and digested in-gel by trypsin, with the resulting tryptic peptides purified and subjected to LC/MS/MS analysis. The results suggested that peak-a protein had significant homology to an unnamed protein (accession no. gi74190429), with the tryptic peptides matching 38% of the putative protein sequences (Fig. 3). To further verify its authenticity, automated Edman degradation of the native peak-a protein was performed for 15 cycles. This yielded reliable peptide sequences, indicating that leucine was the *N*-terminal residue and that the amino acid sequence LTXVSXGRLXSSGI was identical with the unnamed protein sequence in nearly all positions except for X (cysteine or asparagines; Fig. 3). Apparently, the signal peptidase acting at the Ala–Leu peptide bond in the signal peptide cleaved 21 amino

Figure 3 Identification of the SSLP-1 glycoprotein. The unnamed protein sequence is shown (accession no. gi74190429). 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 in all positions except that Cys³, Cys⁶, and Asn¹⁰ from the unnamed protein were not identified in protein sequencing. The matched tryptic peptides in LC/MS/MS analysis are underlined. An arrow indicates the cleavage point for the generation of mature protein.

acid residues to produce a mature protein of 78 amino acid residues with a molecular mass of 8720 Da, matching the size of the deglycosylated band on the SDS-PAGE gel (Fig. 2A, lane 5).

BLASTP – searching the non-redundant protein database using the unnamed protein sequence as the query, we found that this protein had 30–49% identities with a group of proteins with Ly-6/uPAR domains, including bovine protein 1 (BOP-1) protein precursor (Swiss-Prot: P83107), rat RSP-1 (Swiss-Prot: Q9QXN2), mouse PIP-1 like (NCBI: XP_486210), pig PIP-1 (Swiss-Prot: P83106), rat RUP1 (Swiss-Prot: P81827), rat RUP2 (Swiss-Prot: P81828), rat RUP-3 (Swiss-Prot: P83121), and rat SVS7 (NCBI: XP_343370), as well as the acrosomal vesicle protein SP-10 of many species, including humans (NCBI: NP_064499), rats (NCBI: NP_068515), and mice (Swiss-Prot: P50289). It is to note that SLURP-1, the renowned secreted Ly-6 protein, did not appear on the list owing to the fact that there was only 15% amino acid identity with the query protein. Since the query protein was derived from SVS and contains the Ly-6 protein domain, we named it secreted seminal vesicle Ly-6 protein, abbreviated as SSLP-1. A computer-annotated UniProtKB/TrEMBL entry Q3UN54 was assigned according to the translation of *Sslp-1* mRNA during the course of this investigation. Our MS and Edman data of SSLP-1 have been submitted to SWISS-PROT to update the entry Q3UN54.

We compared SSLP-1 amino acid sequence with known secreted Ly-6 proteins, such as rat RUP-1, RUP-2, mouse SVS7, and mouse SLURP-1 and found that they all had ten conserved cysteine positions and a C-terminal CCXXXXXCN motif, indicating that SSLP-1 is a member of the secreted Ly-6 protein subfamily (Fig. 4).

To determine the genomic structure, we searched the GenBank database by BLASTN using the cDNA sequence corresponding to the *Sslp-1* gene and found that it mapped to chromosome 9. Further examination revealed that the message was encoded by the *Gm191* gene (UniGene entry: Mm.118804) and matched completely a mouse genomic contig sequence in GenBank (accession no. NT_084796). The gene spans

Figure 4 Multiple protein sequence alignment of the secreted Ly-6-related protein. Dashes represent gaps introduced to optimize the alignment. The shaded areas indicate matching residues. Asterisks mark the ten conserved cysteine residues of the secreted Ly-6 protein family.

~2.834 kb and consists of three exons separated by two introns.

Predominant SSLP-1 expression in the luminal epithelium of the seminal vesicle

To study the tissue distribution by Northern blotting, we examined the expression of *Sslp-1* transcript in the tissue homogenates of reproductive tissues, including the seminal vesicle, epididymis, testis, coagulating gland, vas deferens, prostate, uterus, and ovary. A ~0.7 kb band corresponding to *Sslp-1* mRNA was detected in the seminal vesicles exclusively (Fig. 5). When an equal amount of total RNA from the homogenate of a non-reproductive organ was compared with that of the seminal vesicle, very little to no *Sslp-1* mRNA was found in the brain, heart, lung, liver, spleen, kidney, stomach, small intestine, muscle, skin, thymus, bladder, or placenta (not shown).

SSLP-1 protein was mainly immunolocalized to the luminal fluid and the epithelium of the mucosal folds of the seminal vesicles of adult mice (Fig. 6A and B). The

Figure 5 Distribution of *Sslp-1* messages among reproductive tissues. Total RNA (20 µg) prepared from the homogenates of each sexual tissue was analyzed by Northern blotting. T, testis; E, epididymis; V, vas deferens; S, seminal vesicle; C, coagulating gland; P, prostate; O, ovary; Od, oviduct; and U, uterus.

Figure 6 Immunolocalization of SSLP-1 to the luminal fluid and epithelium of seminal vesicle mucosal folds. (A) Tissue slices from 9-week-old mice were stained with normal serum, biotin-conjugated goat-anti-rabbit IgG, and horseradish peroxidase-conjugated streptavidin (red). (B) The specimens were stained as in (A) except that the antibody was replaced with anti-SSLP-1 antiserum. The slices from castrated mice treated with corn oil (C) or testosterone (D) were also stained as in (B). For contrast, the specimens were further stained with hematoxylin (blue). Photographs were taken with bright-field illumination: MF, mucosal fold; SM, smooth muscle; and LF, luminal fluid. Bar = 50 μ m.

smooth muscle layer contained almost none. The strong immunohistochemical staining in the lumen supports the view that SSLP-1 is secreted from the luminal epithelium.

Developmental profiles of *Sslp-1* mRNA and protein in seminal vesicles

The amounts of *Sslp-1* mRNA in the seminal vesicles of mice at different ages were compared. The RNA message first appeared at a relatively low level in 3-week-old mice. Thereafter, the amount of transcript began increasing rapidly at 4 weeks and reached a maximum in 8-week-old mice (Fig. 7). Similar to the profile of *Sslp-1* mRNA, SSLP-1 protein first appeared at a low level in 3-week-old mice, increased rapidly from 4 weeks, and reached its highest level in 8-week-old mice (not shown).

Effect of testosterone on *Sslp-1* gene expression

The lower levels of SSLP-1 protein in seminal vesicles of pre-puberal animals indicated that *Sslp-1* gene expression might be under androgen control. To evaluate the effect of testosterone on the *Sslp-1* gene expression in seminal vesicle, we compared the findings in castrated and uncastrated mice. The relative levels of *Sslp-1* mRNA in the seminal vesicles were significantly lower in castrates treated with vehicle only, compared with castrates treated with testosterone (Fig. 8A). Likewise, even with more protein loading in castrates treated with vehicle only, the amount remained lower than in normal adults or castrates treated with testosterone (Fig. 8B). On immunohistochemistry, SSLP-1 protein intensity in the

mucosal epithelium of castrates treated with corn oil only (Fig. 6C) was obviously lower than that in normal adults or castrates treated with testosterone (Fig. 6B and D). Taken together, these results suggest that *Sslp-1* gene expression in the seminal vesicle is modulated by androgen.

Discussion

In this study, we have demonstrated that *Gm191* gene encoded polypeptide SSLP-1, indeed, is a novel secreted Ly-6 protein expressed predominantly in the mouse

Figure 7 Developmental profile of *Sslp-1* mRNA in the seminal vesicle. *Sslp-1* mRNA and *Gapd* mRNA in total RNA (20 μ g) prepared from mouse seminal vesicles at different ages were analyzed by Northern blotting. The relative amounts of *Sslp-1* mRNA were normalized to the level of the *Gapd* mRNA on the same blot. Data represent the mean of three repeated experiments and error bars represent the s.d. # P < 0.01, * P < 0.001 in pairwise comparison relative to the mRNA level from the 3-week-old mice.

Figure 8 Effect of androgen on *Sslp-1* mRNA and protein expression in the seminal vesicle of adult mice. (A) Northern blotting was conducted to analyze the *Sslp-1* mRNA expression in total homogenates (20 µg) from seminal vesicles of normal adult mice (N), adults castrated 3 weeks previously and treated only with corn oil (C), and adults castrated 3 weeks previously and treated with testosterone propionate in corn oil for 8 consecutive days (T). The relative amounts of *Sslp-1* mRNA were determined as described in Fig. 7. Data represent the mean of three repeated experiments and error bars represent the s.d. * $P < 0.01$ in pairwise comparison relative to the mRNA level from castrates treated with testosterone. (B) Western blot analysis was performed for SSLP-1 protein in tissue homogenates from seminal vesicle samples as in (A). The applied protein amounts were 80 µg for N and T and 100 µg for C.

seminal vesicle. Unlike the GPI-anchored members of the Ly-6/uPAR protein superfamily, the secreted Ly-6 proteins are secreted as indicated by their intraluminal location and the presence of a signal peptide in the sequence.

To recapitulate, the evidence that SSLP-1 is a secreted member of the Ly-6 protein family includes its purification from SVS and its expression in the lumen and apical region of the mucosal epithelium (Fig. 6). Edman sequencing indicated that SSLP-1 has a 21 amino acid signal peptide (Fig. 3). Removal of the hydrophobic leader sequence gives a protein core consisting of 78 amino acid residues with a molecular mass of 8720 Da, which is close to the molecular size of the deglycosylated protein as determined by SDS-PAGE (Fig. 2B).

The results of PAS staining and *N*-glycosidase F treatment demonstrate that SSLP-1 is a glycoprotein. The protein contains two potential *N*-glycosylation sites (Fig. 3), Asn¹⁰ and Asn⁵⁴, each being part of consensus Asn-Xaa-(Ser/Thr) (Marshall 1972, Gavel & von Heijne 1990) in the protein molecule. The results of Edman sequencing suggest that Asn¹⁰ is one *N*-glycosylated site. In contrast with these findings for SSLP-1, SVS7 (Luo *et al.* 2001), and SLURP-1 (Chimienti *et al.* 2003) are not glycoproteins, while RUP-1 and RUP-2 have only one predicted *N*-glycosylation site, and the evidence suggesting that they are glycosylated is indirect (Southan *et al.* 2002). The function of glycosylation in these secreted proteins remains to be determined.

The *Gm191* gene encoding SSLP-1 is the fourth highly expressed gene in the mouse seminal vesicle expressed sequence tag (EST) database (dbEST Library ID.9952). UniGene's EST profileviewer also indicates that it is expressed in male genital tissues and the bladder. However, the BLASTN search of the EST database found its expression in the seminal vesicle, prostate, and bladder. On the other hand, our Northern blotting detected *Sslp-1* mRNA only in the seminal vesicle. There was essentially none in the prostate (Fig. 5) and bladder (data not shown). In contrast to our findings for *Sslp-1*, another Ly-6 gene *Svs7* encoding for a secreted protein had far lower expression levels in the mouse seminal vesicle when blasting the EST database using the *Svs7* cDNA sequence as the query.

There are several similarities between SSLP-1 and SVS7, including the same gene locus in chromosome 9, tissue-specific expression and localization in the mouse seminal vesicle, and conserved cysteine positions that are a feature of the secreted Ly-6 protein subfamily. However, they differ in terms of glycosylation and have low sequence identities. These differences suggest that they may have different functions, although this remains to be determined.

The growth and differentiation of the seminal vesicle are closely associated with androgens (Chai 1956, Morley & Wright 1972). Castration caused atrophy of adult seminal vesicles and, in this study, an apparent decrease in seminal vesicle *Sslp-1* mRNA and protein levels. Testosterone administration for castrated animals largely restored the seminal vesicles to a hypertrophied state with a considerable amount of *Sslp-1* mRNA and protein, comparable to that of non-castrated animals (Figs 6 and 8). Similar results have also been demonstrated for other androgen-dependent genes in the seminal vesicles (Higgins *et al.* 1976, Chen *et al.* 1987, 1998, Huang *et al.* 1999, Li *et al.* 2005). With the limited promoter sequence of the *Sslp-1* gene, we could not find an androgen-response element that would allow direct regulation of transcription. This needs to be investigated further to determine whether the *Sslp-1* gene promoter contains androgen-response elements. Alternatively, rather than being under direct androgen control, *Sslp-1*

transcription could be regulated by other transcription factors that are themselves under androgen control.

In conclusion, we have identified a novel secreted Ly-6 protein and demonstrated its tissue-specific expression in the mouse seminal vesicle. The amount of *Sslp-1* mRNA and protein in the seminal vesicle is modulated by androgen and correlates with the stage of animal maturation, suggesting that SSLP-1 may play a role in male reproduction. Its exact function and role in male reproduction remain to be determined.

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