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Identification of the Major TG_4 Cross-Linking Sites in the Androgen-Dependent SVS I Exclusively Expressed in Mouse Seminal Vesicle

Huan-Chin Tseng,[1] Han-Jia Lin,[2] Jyh-Bing Tang,[3] P.S. Sudhakar Gandhi,[1] Wei-Chao Chang,[4] and Yee-Hsiung Chen[1,3]*

[1]*Institute of Biochemical Sciences, College of Life Science, National Taiwan University, Taipei, Taiwan*
[2]*Institute of Bioscience and Biotechnology, National Taiwan Ocean University, Keelung, Taiwan*
[3]*Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan*
[4]*Genomics Research Center, Academia Sinica, Taipei, Taiwan*

ABSTRACT

SVS I was exclusively expressed in seminal vesicle in which the protein was immunolocalized primarily to the luminal epithelium of mucosal folds. The developmental profile of its mRNA expression was shown to be androgen-dependent, manifesting a positive correlation with the animal's maturation. There are 43 glutamine and 43 lysine residues in one molecule of SVS I, which is one of the seven major monomer proteins tentatively assigned on reducing SDS–PAGE during the resolution of mouse seminal vesicle secretion. Based on the fact that *SVS I*-deduced protein sequence consists of 796 amino acid residues, we produced 7 recombinant polypeptide fragments including residues 1–78/F1, residues 79–259/F2, residues 260–405/F3, residues 406–500/F4, residues 501–650/F5, residues 651–715/F6, and residues 716–796/F7, and measured the covalent incorporation of 5-(biotinamido)pentylamine ($BPNH_2$) or biotin-TVQQEL (A25 peptide) to each of F1-to-F7 by type 4 transglutaminase (TG_4) from the coagulating gland secretion. F2 was active to a greater extent than the other fragments during the $BPNH_2$-glutamine incorporation, and a relatively low extent of A25-lysine cross link was observed with all of the seven fragments. The MS analysis of $BPNH_2$-F2 conjugate identified Q^{232} and Q^{254} as the two major TG_4 cross-linking sites. This was substantiated by the result that much less $BPNH_2$ was cross-linked to any one of the three F2 mutants, including Q232G and Q254G obtained from single-site mutation, and Q232G/Q254G from double-site mutation. J. Cell. Biochem. 107: 899–907, 2009.

KEY WORDS: ANDROGEN; PROTEIN CROSS LINK; SEMINAL COAGULATION; SEMINAL VESICLE; TRANSGLUTAMINASE

Seminal vesicle and coagulating gland (anterior prostate) are major male accessory apparatus in many mammalian species. Upon ejaculation, seminal vesicle secretion (SVS) and coagulating gland secretion (CGS) are accumulated in the lumen of the respective reproductive glands. SVS makes up the major portion of plasma in the semen which is coagulated in a substantial number of mammalian species, including many myomorphic rodents, some moles, hedgehogs, marsupials, rabbits, stallions, boars, and several primates [Williams-Ashman, 1984]. The deposition of semen coagulum in animals such as rodents into the vagina at coitus results in the formation of a copulatory plug that occludes the vaginal barrel close to the uterine cervix. Despite that the physiological utilization of semen coagulum in mammalian reproduction is not yet well defined, its importance should not be overlooked, taking into consideration that extirpation of seminal vesicle and/or the coagulating gland from mice and rats prevents the

Abbreviations used: A25 peptide, Biotin-TVQQEL; $BPNH_2$, 5-(biotinamido)pentylamine; CGS, coagulating gland secretion; DTT, dithiothreitol; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GST, glutathione S-transferase; HMWC, high molecular weight complex, PBST, phosphate buffer saline containing 0.1% Tween 20; PMSF, phenylmethylsulphonyl fluoride; SVS, seminal vesicle secretion; TG_4, type IV transglutaminase.

Huan-Chin Tseng and Han-Jia Lin contributed equally to this work.

Additional Supporting Information may be found in the online version of this article.

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*Correspondence to: Prof. Yee-Hsiung Chen, Institute of Biological Chemistry, Academia Sinica, P.O. Box 23-106, Taipei 106, Taiwan. E-mail: bc304@gate.sinica.edu.tw

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copulatory plug formation resulting in greatly reduced fertility [Pang et al., 1979; Peitz and Olds-Clarke, 1986]. It is generally believed that the cross links among the SVS proteins by transglutaminase (TG; EC2.3.2.13), a Ca^{2+}-dependent enzyme that catalyzes the formation of an isopeptide bond between its protein substrate through ε-(γ-glutamyl) lysine cross-bridges [Folk, 1980], is crucial for the formation of a semisolid gelatinous mass in human semen [Aumuller and Riva, 1992; Peter et al., 1998] or the seminal clotting in rodent semen [Harris et al., 1990; Lundwall et al., 1997]. The key step to initiate the TG-catalyzed protein cross link is thought to be the transformation of a glutamine residue on one protein molecule by deamination to an activated acyl group which facilitates the nucleophilic attack by a ε-amino group of a lysine residue on the other protein molecule [Lorand and Graham, 2003].

Because rodents have proven to be good experimental animals for the molecular study of mammalian reproduction, attempts have been made with some progress on the systematic analysis of the mouse SVS proteins that had been shown to consist of several minor proteins such as SVA [Huang et al., 1999, 2000, 2005], P12 [Chen et al., 1998; Luo et al., 2004; Lin et al., 2006b], Ceacam 10 [Li et al., 2005], and seven well-resolved monomer proteins designated SVS I-VII in the decreasing order of M_r values (95,000–8,000) according to their mobility on reduced SDS–PAGE [Chen et al., 1987; Luo et al., 2001]. Although TG of guinea pig liver (TG_2) had been shown to cross-link mouse SVS I-III [Lundwall et al., 1997; Lin et al., 2002], in reality this is not the enzyme involved in seminal coagulation during natural coitus. Rather, the activity of TG_4, which has been recently purified from mouse CGS [Tseng et al., 2008], is responsible for this reproductive event. Here, we present data to support that: (i) the androgen-dependent SVS I is exclusively expressed in the luminal epithelium of seminal vesicle; (ii) Gln^{232} and Gln^{254} are the major TG_4-reactive residues on SVS I and (iii) TG_4 is much stronger than TG_2 to cross-link the glutamine residues in SVS I.

MATERIALS AND METHODS

The following materials were obtained from commercial sources: nickel agarose beads, goat anti-rabbit IgG conjugated with alkaline phosphatase or horseradish peroxidase, Ready-To-Go™ RT-PCR beads, and ECL kit (Amersham Corp., Buckinghamshire, UK and Amersham Pharmacia Biotech, Uppsala, Sweden); Protein PAK SP 5PW column (Waters, Milford, MA); BCA protein assay kit, 5-(biotinamido)pentylamine (EZ-Link) (Pierce Chemical Co, Rockford, IL); phenylmethylsulphonyl fluoride, silanated glass slides, isopropyl β-D-1-thiogalactopyranoside (Sigma Chemical Co., St. Louis, MO); flat-bottom high binding 96-well enzyme immunoassay microtiter plates (catalog no.442404) (NUNU, Roskilde, Denmark); pET-21-a expression vector (Novagen, Darmstadt, Germany); streptavidin-β-galactosidase, O-nitrophenyl-β-D-galactopyranoside (CALBIOCHEM, San Diego, CA); Ultraspec-IIRNA isolation kit (Biotex, Houston, TX); Western Blue Stabilized Substrate for Alkaline Phosphatase, pGEM-T-easy vector (Promega, Madison, WI). Quik Change® II XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). Ultraspec-II and RNA isolation kit (Biotex). All other chemicals were reagent grade.

ANIMALS, PREPARATION OF SVS AND CGS, AND PURIFICATION OF TG_4

Outbred ICR mice were purchased from Charles River Laboratories (Wilmington, MA) and were maintained and bred in the animal center at the College of Medicine, National Taiwan University. The animals were treated according to the institutional guidelines for the care and use of experimental animals. They were housed under controlled lighting (14L:10D) at 21–22°C and were provided with water and NIH-31 laboratory mouse chow ad libitum. Normal adult mice (8–12 weeks old) were sacrificed by cervical dislocation. SVS and CGS were squeezed individually into ice-cold 10 mM Tris-HCl, pH 8.0 in the presence of 1 mM PMSF and centrifuged at 8,000*g* for 20 min at 4°C to remove the precipitate. TG_4 was purified from CGS according to the previous method [Tseng et al., 2008].

PREPARATION OF ANTISERUM, PROTEIN BLOTTING, AND IMMUNOCHEMICAL STAINING

Mouse SVS proteins were identified as the monomer proteins by reducing SDS-PAGE on a 12% gel slab (6.5 cm × 10.5 cm × 0.075 cm) [Laemmli, 1970]. The proteins on the gel were stained with Coomassie Brilliant blue to reveal the protein bands of SVS I-VII. The 91-kDa SVS I band excised from the polyacrylamide gel was homogenized and mixed with complete adjuvant. The preparation was used to boost the New Zealand White rabbits. After 2 weeks the complete adjuvant was replaced with incomplete adjuvant in the SVS I preparation and was used to boost the immunized animals twice at an interval of 2 weeks time. Meanwhile, the protein bands on the gel were transferred to a nitrocellulose membrane. After transfer, the protein blots were detected by Western blotting using the antiserum against SVS I diluted to 1:10,000 in a blocking solution (5% non-fat skimmed milk in PBS) as the primary antibody, and goat anti-rabbit IgG conjugated with horseradish peroxidase diluted to 1:10,000 in the blocking solution as the secondary antibody. The bands detected by Western blotting were visualized using an ECL kit according to the manufacturer's instruction.

The immunochemical staining of SVS I in the sections (6 μm) of mouse seminal vesicle generally followed a previously described method [Li et al., 2005]. Antiserum against SVS I was diluted to 1:500 in blocking solution and goat anti-rabbit IgG conjugated with alkaline phosphatase used at a dilution of 1:1,000 in blocking solution served as the secondary antibody. To visualize the enzyme activity, the slides were incubated with Western Blue, a Stabilized Substrate for Alkaline Phosphatase for 15 min and then photographed using a bright field microscope (BX 61, Olympus, Tokyo, Japan).

PREPARATION OF THE POLYPEPTIDE FRAGMENTS OF SVS I AND THE RECOMBINANT F2 VARIANTS

Based on the *SVS I* cDNA and its deduced protein sequence (Fig. 2), we divided the protein components into seven polypeptide fragments, including F1/residues 1–78, F2/residues79–259, F3/residues 260–405, F4/residues 406–500, F5/residues 501–650, F6/residues 651–715, and F7/residues 716–796 encoded respectively by

nucleotides 73–306, 307–849, 850–1,287, 1,288–1,572, 1,573–2,022, 2,023–2,217, and 2,218–2,463. We amplified the seven polynucleotides from the *SVS 1* cDNA as a template, using the primer pairs of 5′-GCTAGCAAGCACTCCAGGTGGGCA and 5′-GGATCCGCGGCTTCACGTAAAGGAAGCAT for F1, 5′-GCTAGC-CGTGTTCTGATCTACTTTGGT and 5′-GGATCCGC GGAGAACTGTG-GAAACTGCTCTGT for F2, 5′-GCTAGCACCTACAAGCCCTATGC-TGAA and 5′-GGATCCGCGTCTCCATAGAGCACAGTGTT for F3, 5′-GCTAGC TGGAACTTCTTCTTCAAGCTGCACT and 5′-GGATCC-GCGC GAGGGCTCGACGAGAGAGGACAGG for F4, 5′-GCTAGC TG-TATCTTTGAAATGCCCCTTAGG and 5′-GGATCCGC CAGAGTCT-GACGGAAGTGGAAGGC for F5, 5′-GCTAGCCCCAAGTACCTGCTT-TTTAGC and 5′-GGATCCGCGTAAAGGCTACCGTGGAAACG for F6, and 5′-GCTAGC AACCAGAACCACCACTGGGCT and 5′-GGATCC GCTCAGCACACACACTGGGCATGAGTTGGAGAGGCTGTATATCT-ACG for F7, where the restriction sites of *Nhe*I and *Bam*HI are boxed and 5 sets of ACG complementary to CGT/Arg that replaced the rare codon CGG/Arg of *E. Coli* in the original cDNA sequence are underlined (Fig. 2). Each amplified fragment was ligated to pET21a expression vector *via Nhe*I and *Bam*HI site. Thus, each fragment possessed a his_6-tag at the C-terminal region. The constructed plasmids were used to transform *E. coli* strain BL21 that was induced by 0.4 mM IPTG at 37°C for 5 h. The inclusion body was dissolved in 6.0 M urea and the his_6-tagged protein fragments were purified by Ni-Sepharose 6 Fast Flow (GE Healthcare Bioscience, NJ) according to the manufacturer's instruction. The purity of each polypeptide was identified by 15% reducing SDS–PAGE.

Site-directed mutagenesis for the F2 cDNA in pET21a expression vector followed the instruction of Quik Change^{B} II XL Site-Directed Mutagenesis kit (Stratagene). The mutated polypeptides are indicated by one-letter-code for mutation of amino acids. Two mutants including Q232G and Q254G were raised from single-site mutation whereas Q232G/Q254G was raised from double-site mutation. CAG encoding Q^{232} and Q^{254} was replaced by GGA for glycine in the amplification for the mutant cDNAs. Expression of the constructed plasmids and purification of the recombinant variants followed according to the procedures mentioned above.

TISSUE RNA ISOLATION AND NORTHERN BLOT ANALYSIS

Total cellular RNA was prepared from the seminal vesicles of adult mice using an Ultraspec-II RNA isolation kit (Biotex). A PCR-amplified fragment of *SVS I* cDNA (nucleotides 975–1,133), which was inserted in pGEM-T-easy, and a cDNA fragment of the mouse GAPDH gene (1,233 bp), which was inserted into pGEM3 vector, were used as a template to prepare a ^{32}P-labeled cDNA probe using a random-priming kit (Promega). RNA samples (20 μg) were subjected to 1.0% agarose-formaldehyde gel electrophoresis and then blotted onto nylon membranes by capillary transfer as previously described [Maniatis et al., 1989]. After incubation with the prehybridization buffer (50% deionized formamide, 6× SSC, 5× Denhardt solution, 1.0% SDS, and 100 μg/ml of sheared salmon sperm DNA) for 2 h at 50°C, the membranes were hybridized with the first labeled probe overnight at 50°C. Following hybridization, the membranes were washed using standard procedures. RNA messages on one filter membrane were observed after autoradiography and the probes were removed completely from the membranes as previously described [Maniatis et al., 1989]. The same membrane was then hybridized with the second labeled probe. Thus, hybridization with *SVS I* or *GAPDH* cDNA probe was performed on the same filter membrane.

SOLID-PHASE MICROTITER ASSAY

The method of Slaughter et al. [1992] was modified to assay the TG substrate activity of F1-to-F7. Individual wells of a microtiter plate were coated with 100 μl each of the recombinant polypeptide (25 μg/ml) in 50 mM carbonate buffer, pH 9.6. After the unbound protein substrate was discarded, the well was blocked with 0.3% GST in 50 mM Tris-HCl [Tseng et al., 2008], pH 8.5 for 60 min, washed three times with 350 μl of the Tris buffer containing 150 mM NaCl. The above blocking buffer (100 μl) containing 0.5 mM $BPNH_2$ or 0.5 mM A25 peptide was added in the presence of TG_2 or TG_4 (0.5 μg/100 μl). The reaction was stopped by washing the microtiter plates with 350 μl of 200 mM EDTA, pH 8.0 followed by three washes with PBS containing 0.1% Tween 20 (PBST). Streptavidin-β-D-galactosidase (0.5 mg/ml) diluted 1:500 with PBST was added (100 μl/well) and incubated for 60 min at room temperature. The plate was washed four times with 350 μl of PBST prior to the addition of 150 μl of 0.1% O-nitrophenyl-β-D-galactopyranoside in 50 mM sodium phosphate, pH 7.2 containing 1.5 mM $MgCl_2$. The substrate hydrolysis was allowed to proceed for 15 min and then stopped by the addition of 150 μl of 1.0 M Na_2CO_3. The absorbance at 420 nm (OD_{420}) due to the formation of O-nitrophenol was determined. Meanwhile, the immobile protein coated on each well was detected by ELISA using anti-his-tag prepared from mouse and the anti-mouse IgG conjugated with horse radish peroxidase. Its amount was proportional to the absorbance at 450 nm (OD_{450}) arising from the enzymatic oxidation of substrate (3,3′,5,5′) tetramethyl benzidine.

PREPARATION OF $BPNH_2$-CONJUGATED F2

The $BPNH_2$-conjugated F2 was prepared by a modified method [Ruoppolo et al., 2003]. Incubation of 22.4 μg F2 or its mutant, with 0.12 μg $BPNH_2$ in the presence of TG_4 in 100 μl of 50 mM Tris-HCl containing 10 mM $CaCl_2$ and 10 mM DDT, pH 8.0, was carried out at 37°C for 2 h. The reaction mixture was directly analyzed by RP-HPLC, using a C_4 column (3.9 mm × 300 mm, 15 μ). The eluting system consisted of 0.1% TFA (eluent A) and 0.1% TFA in acetonitrile (eluent B). The proteins were eluted as a single fraction by means of a linear gradient of eluent B in eluent A, from 5% to 95% in 40 min at a flow rate of 1.0 ml/min. The eluate was monitored at 220 nm. The purified proteins were lyophilized, re-dissolved in 2.0 ml PBS, and subjected to affinity chromatography on a prepacked monomeric avidin column which was washed, saturated, and equilibrated according to the instruction of manufacturer (Pierce). The unbound fraction was eluted in PBS, whereas the $BPNH_2$-conjugated F2 was selectively eluted with 0.3% formic acid.

ANALYTICAL METHODS

Protein concentrations were determined using the BCA protein assay [Smith et al., 1985] according to the manufacturer's instruction. The $BPNH_2$-conjugated F2 in 50 μl of 50 mM ammonium bicarbonate, pH 8.5, was incubated in the presence of

5 μg trypsin at 37°C for 18 h. The protease-digested sample was separated by a nano-chromatographic system with an Agilent 1100 Series binary HPLC pump (Agilent Technologies, Palo Alto, CA), a Famos autosampler (LC Packings, San Francisco, CA), a self-packed pre-column (150 μm ID × 20 mm) and a reverse phase C_{18} column (Michrom Bioresources, CA: 75 μm ID × 300 mm; Particle size, 5 μm; pore size, 100 A). Chromatographic separation was achieved by using 0.1% formic acid in water as mobile phase A and 0.1% formic acid in 80% acetonitrile as mobile phase B in a 60 min running cycle, with a split flow rate of around 300 μL/min. MS experiments were performed with a LTQ-FTICR MS (Thermo Electron, CA) equipped with an electrospray ion source (New Objective, MA). The full-scan mass survey (m/z 320–1,800) was executed by FTICR MS with a mass resolution of 100,000 at m/z 400. The parent ions were selected for MS/MS analysis when their intensities were above a minimum threshold of 1,000 counts. Single ions were rejected for MS/MS sequencing.

The RAW files of MS profile were processed to MGF files with Mascot Daemon and searched by MASCOT (version 2.1, Matrix Science, London, UK) software platform based on the Swiss-Prot protein database. With regard to the MASCOT parameter settings, the peptide tolerance was 15 ppm with 2^+ and 3^+ peptide charges, and the MS/MS tolerance was 0.5 Da. Trypsin cleavage and specific modification were chosen. The significance threshold for the identification was set to $P < 0.01$.

The DNA insert in the pGEM-T-easy vector or phagemid was sequenced by the dideoxynucleotide chain termination method using a primer designed for each individual fragment. Each base was determined not less than three times by an ABI PRISM 377-96 DNA sequencer using an ABI PRISM BigDye™ terminator cycle sequencing ready reaction kit (Applied Biosystem, Foster City, CA).

STATISTICAL ANALYSIS

Data are presented as the mean ± SD. Differences were analyzed by the Bonferroni post hoc test followed by oneway ANOVA using InStat software (GraphPad, San Diego, CA). A P value of <0.05 was considered to be significant.

RESULTS

PREDOMINANT SVS I EXPRESSION IN THE LUMINAL EPITHELIUM OF SEMINAL VESICLE

We examined the distribution of *SVS I* mRNA in the tissue homogenates of sexual glands, including the seminal vesicle, epididymis, testis, coagulating gland, vas deferens, prostate, ovary, and vagina. Despite the RNA message being detected only in the seminal vesicle, none appeared in the other sexual glands even when followed by over autoradiography (Fig. S1). Virtually no *SVS I* mRNA was found either in lung, kidney, brain, spleen, liver, pancreas, or heart when equal amounts of total RNA from the homogenate of these organs were compared with those of the seminal vesicle (data not shown). Further, the level of *SVS I* mRNA in the seminal vesicle of mice at different age groups was compared. The RNA message initially appeared at a considerable level in 3 weeks old animals and thereafter, the amount of transcript increased rapidly and reached a maximum in the 4th week (Fig. 1A).

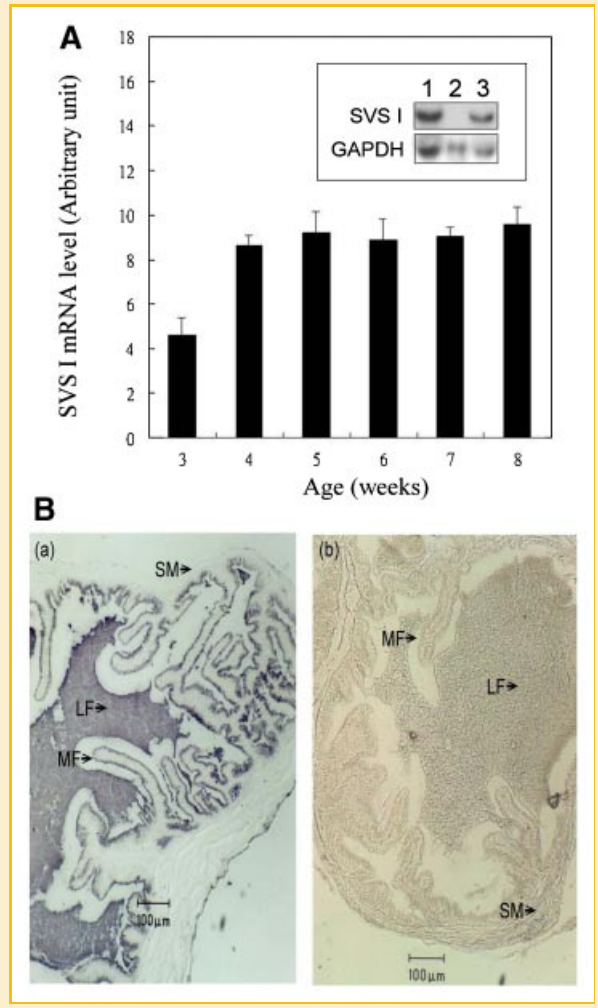

Fig. 1. Androgen dependent SVS I and its RNA message exclusively expressed in seminal vesicles of adult mice. A: Developmental profile of *SVS I* mRNA in seminal vesicle. *SVS I* mRNA and GAPDH mRNA in the total RNA (15 μg) prepared from mouse seminal vesicle homogenates were detected by Northern blot as described in the text. The relative amount of *SVS I* mRNA in the tissue of different age was determined by densitometer scanning of the autoradiogram and adjusted with respect to the GAPDH mRNA level. Data represents the means values of three experiments, and error bars represent SD. The inset displays *SVS I* mRNA and GAPDH mRNA in the seminal vesicle of normal adult (lane 1) and adult mice castrated before 3 weeks that received just corn oil (lane 2) or testosterone propionate (lane 3) for 8 consecutive days. B: Immunolocalization of SVS I to the luminal epithelium of seminal vesicle. Tissue slices were histochemically stained for SVS I with the antiserum against the protein, biotin-conjugated goat anti-rabbit IgG, and alkaline phosphatase-conjugated streptavidin (a). The specimens were stained as in (a) except that the antiserum was replaced by normal serum (b). Photographs were taken with brightfield illumination: MF, mucosal fold; SM, smooth muscle; LF, luminal fluid. The signals of SVS I, demonstrated by staining of alkaline phosphatase activity, are in dark blue. Bar = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Considering the fact that the seminal vesicle growth is androgen-dependent, we determined how androgen influenced the *SVS I* expression in the seminal vesicles of adult mice that had been castrated prior to 3 weeks (Fig. 1A, inset). *SVS I* mRNA was undetectable in the total RNA prepared from the control castrates

Fig. 2. Rare low t-RNA codons of *E. coli* in the reading frame of *SVS I* cDNA The nucleotide sequences of a 2,463-bp reading frame in *SVS I* cDNA (GenBank sequence databank under accession no. Ay283179) are in capital LETTERS. The deduced protein sequences are given in one-letter code numbered from the post-translational cleavage site indicated by an upward arrow. The amino acid residues encoded by the rare low t-RNA codons of *E. Coli* are shown in dashed squares for AGG/Arg, AGA/Arg, CGC/Arg, GGA/Gly, ATA/Ile, CTA/Leu, and CCC/Pro, and those for CGG/Arg in solid squares. The horizontal arrows denote the primer pairs used for the amplification of cDNAs of F1-to-F7. The seven highly conserved peptide segments in F3, each comprising 18 amino acid residues are aligned and the invariant residues are shown in gray box. The secondary structures designated in two lines below the amino acid residues of F3 were predicted from the Chou-Fasman algorithm [Chou and Fasman, 1978] and the Garnier-Osguthorpe-Robson algorithm [Garnier et al., 1978], respectively: B and b, strong or weak β form former; T or t, strong or weak turn former; H or h, strong or weak helix former.

that had received a daily injection of mere corn oil when compared with that of the normal adults. Conversely, induction of *SVS I* mRNA was apparent in the castrates treated with testosterone (5 mg/kg/day) for 8 consecutive days.

We prepared the rabbit antiserum against SVS I (see Materials and Methods Section). Among the seven major mouse SVS proteins, SVS I–SVS VII resolved on a reducing SDS–PAGE, the antiserum reacted with a major protein band corresponding to SVS I and a minor 53-kDa band (Fig. S2) which was demonstrated to have ^{318}YREFPMPISK^{327} of SVS I as the N-terminal sequences, signifying that it was a partial SVS I. None of the other SVS proteins were recognized by the antiserum, thus indicating its high specificity to SVS I. Therefore, the antiserum was used for the immunochemical staining. As shown in Figure 1B, SVS I was immunolocalized primarily to the luminal epithelium of the mucosal folds and not to the smooth muscle layer in the seminal vesicle slices of adult mice. The strong immunochemical staining in the lumen supports the view that SVS I accumulates in the lumen after its secretion from the epithelium.

PRODUCTION OF RECOMBINANT POLYPEPTIDE SEGMENTS OF SVS I

Based on the nucleotide sequence of *SVS I* cDNA and its deduced protein sequence (Fig. 2) previously reported by Lundwall et al. [2003], we designed our strategy for the production of recombinant polypeptides. We inserted the cDNA for the whole SVS I molecule into the plasmid of pET 21a but failed to express the constructed vector in *E. coli* strain BL 21. This may be attributed to the presence of rare low t-RNA codons of *E. coli* as reported by Kane [1995]. He found that the expression of a vector containing even single rare codons as in the case of AGG/AGA for Arg in *E. coli* during the production phase might result either in reduced quantity or quality of the protein synthesized. Hence, with the high content of 66 rare low t-RNA codons in the reading frame of *SVS I* gene, which translates a putative protein containing 820 amino acid residues it was not possible to express the protein. We tackled the problem by an alternative approach. Within the central part of SVS I comprising residues 260–405 referred to as F3, there are seven copies in tandem of a highly conserved sequence of 18 amino acid residues including the invariant sequence F-PISKGGP-Q (Fig. 2). Taking into account the distribution of rare codon CGG/Arg in the reading frame of *SVS I* gene, we divided the N-terminal flank of F3 into two polypeptide segments, F1/residues 1–78, and F2/residues 79–259, and the C-terminal flank of F3 into four segments, F4/residues 406–500, F5/residues 501–650, F6/residues 651–715, and F7/residues 716–796. To express each of F1-to-F7 in *E. coli* strain BL21-codon plus (DE3)-RP, which contains extra t-RNA genes for Arg (AGA/AGG) and Pro (CCC), we replaced CGG that encodes Arg^{76} in F1, Arg^{497} in F4, Arg^{647} in F5, Arg^{709} in F6 and Arg^{783} in F7 by CGT which encodes Arg but not a rare codon, and constructed the expression vector by inserting the corresponding cDNAs for each polypeptide segment into the plasmid pET21a. Although the inclusion body occurred in the expression system, we succeeded in purifying each of the seven his-tagged polypeptides to homogeneity from the soluble portion of the inclusion body dissolved in 6.0 M urea by Ni^{2+}-affinity chromatography (Fig. S3).

DETERMINATION OF THE TG_4–CATALYZED SITE IN SVS I

To test the substrate activity of TG_2 or TG_4 on F1-to-F7, we applied a method of solid-phase assay to measure each enzyme-catalyzed cross links of the biotinylated substrate, $BPNH_2$ as an acyl acceptor or A25 peptide as an acyl donor, into each polypeptide segment coated on microtiter plates. Each immobile polypeptide fragments and its incorporated biotinylated substrate were quantified by ELISA (see Materials and Methods Section). The ratio of the amount of incorporated biotinylated substrate to that of the immobile SVS I fragments reflected the TG substrate activity of each immobile peptide. As shown in Figure 3, TG_2 and TG_4 showed unequal cross-linking activities for the covalent incorporation of both biotinylated substrates. In the $BPNH_2$-glutamine incorporation, TG_4 was more active than TG_2 to cross-link any one of F1-to-F7. In particular, there was a striking difference between TG_2 and TG_4 in their cross-linking activity for F2 which showed the strongest protein substrate activity, whereas the other SVS I fragments yielded rather a weak activity for either enzyme: TG_4 showed around eight times more

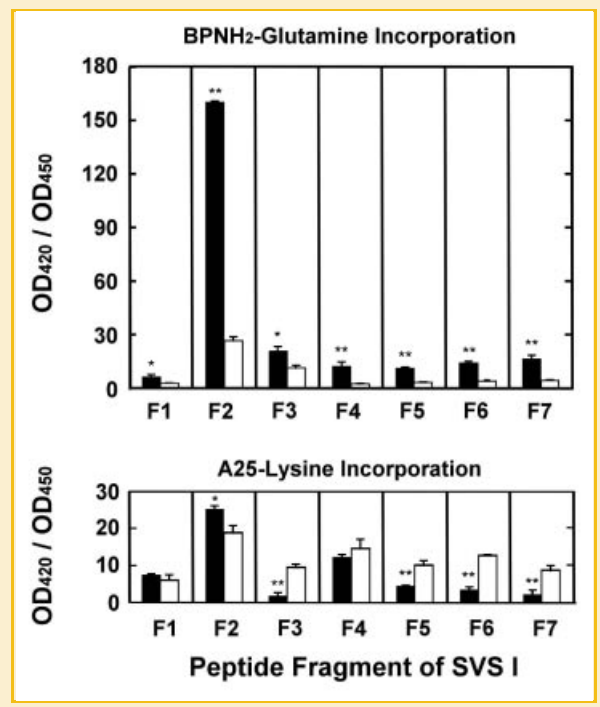

Fig. 3. TG_4 is unequal to TG_2 for the cross link of SVS I Each of the his_6-tagged F1-to-F7 was coated on micotiter plates and the incorporation of $BPNH_2$ or A25 to the immobile proteins by TG_2 (open bar) or TG_4 (solid bar) was measured as described in Materials and Methods Section. Each biotinylated substrate incorparated on the solid-phase protein substrate was examined by ELISA using Streptavidin-β-D-galactosidase which hydrolyzed its substrate O-nitrophenyl-β-D-galactopyranoside to give OD_{420}. The immobile protein was detected by ELISA using anti-his-tag from mouse and the anti-mouse IgG conjugated with horse radish peroxidase. Its amount was proportional to OD_{450} arising from the enzymatic oxidation of substrate (3,3′,5,5′) tetramethyl benzidine. The covalent incorporation of $BPNH_2$ (upper figure) and A25 (lower figure) into an immobile protein was reflected by a ratio of OD_{420}/OD_{450}. The data were the average of three determinations and error bars the SD. $^{*}P < 0.01$and $^{**}P < 0.001$ are the paired statistical comparison in each column using one-way ANOVA.

activity than TG_2 during the cross link of glutamine residues on F2. In contrast to $BPNH_2$-glutamine incorporation, a relatively low extent of A25-lysine incorporation was found in all the seven immobile polypeptides by either enzyme. Nevertheless, TG_4 seemed more active than TG_2 for the A25-F2 incorporation, but was weaker than TG_2 for the A25 incorporation to other SVS I fragments.

Further, we exploited the proteomic strategy of Ruoppolo et al. [2003] to identify the TG_4-reactive glutamine residues in F2. $BPNH_2$ and F2 were cross-linked in a reaction buffer in the presence of TG_4. After removal of the free $BPNH_2$ from the reaction mixture, the protein solution was digested with trypsin and the biotinylated protease digests were isolated by chromatography on an avidin affinity column. The biotinylated sample was analyzed by LC/MS/MS. The fragmentation process in the MS analysis generated a complex set of fragment ions mainly belonging to the b and y series. The sequence of two biotinylated peptides, FYNSPEELA[Q_{Bn}]K (Fig. 4a) and TTE[Q_{Bn}]FPQFSADPNSSSVDK (Fig. 4b) could be assembled from the tandem mass spectra, where Q_{Bn} denoted the biotinylated glutamine residue and ADPNSSVDK originated from the plasmid pET21a. These two peptide sequences completely aligned with ^{223}F-K^{233} and ^{251}T-S^{259} in F2, supporting Q^{232} and Q^{254} among 796 amino acid residues of SVS I as the two major cross-linking sites for the TG_4 catalysis.

We prepared and purified three F2 mutants, including Q232G and Q254G from single-site mutation, and Q232G/Q254G from double-site mutation (see Materials and Methods Section). Their $BPNH_2$-glutamine incorporation by TG_4 was measured by solid-phase assay mentioned above. As shown in Figure 5, The TG_4 substrate activity decreased in the order of wild-type F2 (100%) > Q254G (53%) > Q232G (48%) > Q232G/Q254G (29%), where the relative $BPNH_2$-glutamine incorporation is listed in the parentheses. These data substantiate the conclusion from the MS analysis.

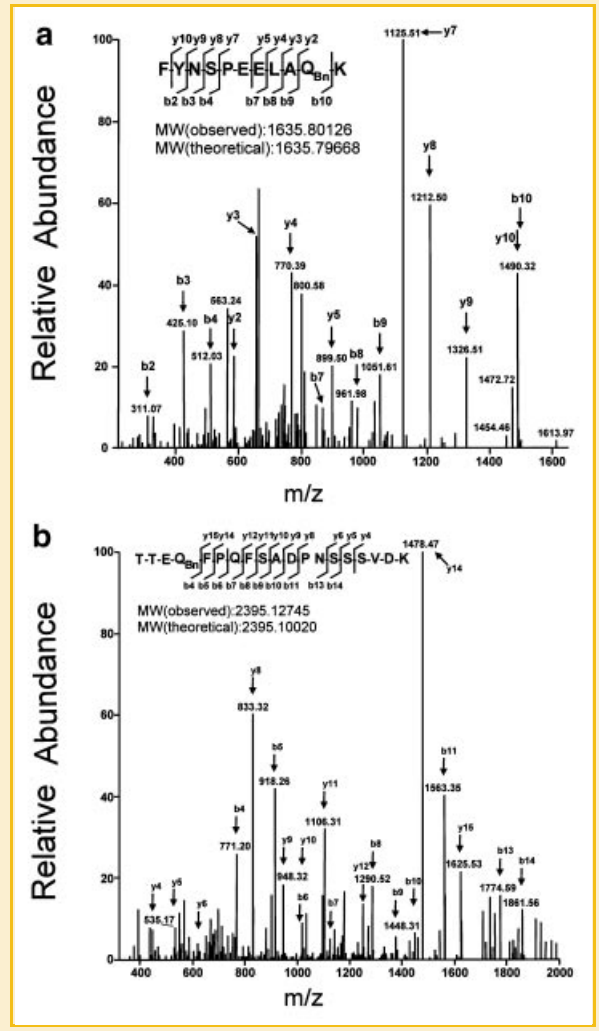

Fig. 4. Identification of the biotinylated glutamine residues in F2 being cross-linked to $BPNH_2$ by TG_4 The $BPNH_2$-conjugated F2 was prepared and further digested with trypsin (see Materials and Methods Section for details). The tryptic digests were analyzed by capillary column chromatography coupled with ESI-MS/MS. Predicted masses of ions type b and y are shown above and below the peptide sequence, respectively. The observed molecular weight and the theoretical molecular weight of each biotinylated peptide are denoted on the figure for comparison. Q_{Bn} represents the $BPNH_2$-conjugated glutamine residue.

DISCUSSION

SVS I-VII are monomer proteins that had been tentatively assigned on reducing SDS-PAGE during the analysis of mouse SVS proteins [Chen et al., 1987; Luo et al., 2001]. SVS I as well as SVS II and SVS III are all expressed in the luminal epithelium of seminal vesicle and secreted into the lumen of this accessory sexual gland. They are the three main protein substrates in SVS that can be cross-linked by TG_4 [Tseng et al., 2008]. *SVS II* and *SVS III* are divergent from a common evolutionary origin [Lin et al., 2005]. They reside in the H3 locus of mouse chromosome 2, whereas *SVS I* residing in the B2.6 locus of chromosome 6 may be divergent from a different evolutionary origin. SVS I expressed in the luminal epithelium of seminal vesicle is secreted into the lumen of this accessory sexual gland (Fig. 1). According to the study of Lundwall et al. [2003], mouse SVS I is homologous with amiloride-binding protein 1, a diamine oxidase carrying an active-site redox cofactor topaquinone which belongs to one class of the copper amine oxidase superfamily. Due to mutation at the active site, they suggested that it might lack the diamine oxidase enzyme activity. Nevertheless, it is one of the major proteins involved in the semen coagulation. As yet, purification of SVS I from mouse SVS in quantity has not been achieved by conventional techniques available for protein study in suitable solution. This hinders the characterization of transglutaminase-activated sites in this protein molecule by direct protein analysis. We tackled this problem by an alternative approach and used TG_4 instead of TG_2 to identify the enzyme cross-linking sites in the SVS I molecule. We prepared seven polypeptide fragments of SVS I, F1-to-F7 (Fig. 2), and demonstrated Q^{232} and Q^{254} in F2 as the two major activated sites for the TG_4 catalysis (Figs. 3–5).

There are 29 glutamine and 24 lysine residues in one molecule of SVS III consisting of 265 amino acid residue in which the 7 glutamines and 5 lysines in the region of residues 116–147 are in a cluster of five tandem repeats of the short peptide of QXK(S/T),

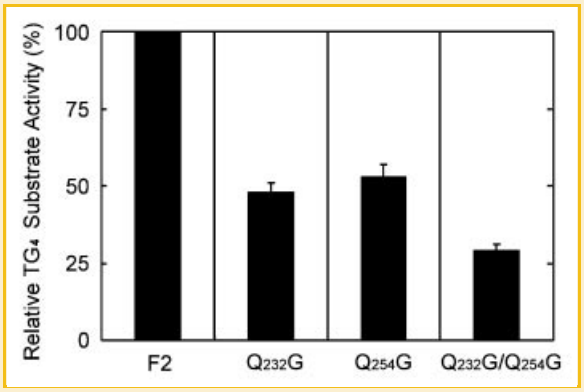

Fig. 5. Incorporation of $BPNH_2$ to immobile F2 variants by TG_4 The covalent incorporation of $BPNH_2$ to immobile F2 and its variants, namely Q232G, Q254G, and Q232G/Q254G was determined by solid-phase assay as described in Figure 3. The BPNH2-glutamine incorporation of each F2 variant was calculated relative to that of wild type F2, which was referred to as 100%. Error bars represent the SD of the mean values for three determinations.

where X represents an aliphatic amino acid residue. SVS II has 375 amino acid residues containing 64 glutamines and 39 lysines in which 31 glutamines and 22 lysines in the region of residues 82–250 appears in 20 segments of QXK(S/T) including in the sequence QVKSSGS. The central part of SVS II was proposed to have an internal repetitive structure in an anti-parallel arrangement [Harris et al., 1990], and that of SVS III was predicted to have a higher β-sheet potential [Lin et al., 2002]. Relative to SVS II and SVS III, SVS I is a large molecule containing 43 glutamines and 43 lysines with the distribution of 2 glutamines and 7 lysines in F1, 13 glutaimnes and 9 lysines in F2, 11 glutaimnes and 15 lysines in F3, 3 glutaimnes and 3 lysines in F4, 7 glutaimnes and 6 lysines in F5, 4 glutaimnes and 3 lysines in F6, and 3 glutaimnes and no lysine in F7 (Fig. 2). Similar to the central part of SVS II and SVS III, F3 of SVS I was predicted to have a higher β-sheet potential (Fig. 2), despite that each of the seven highly conserved peptide fragments in F3 contains the invariant peptide sequence F-PISKGGP-Q that completely differs from the tandem-repeated sequence in SVS II and SVS III. The peptide sequence of QXK(S/T) is characteristic of a transglutaminase acyl donor as well as an acyl acceptor [Schrode and Folk, 1979; Gorman and Folk, 1980], and such a dense QXK(S/T) in the central part of SVS II and SVS III has been demonstrated as the TG_4 cross-linking sites in these two protein molecules [Lin et al., 2002; Tseng et al., 2008], whereas neither the glutamines nor the lysines in F3 of SVS I can be cross-linked by the same enzyme. This rules out the tandem repeat region in the central part of SVS I as the major TG_4 cross-linking sites of this protein molecule. We demonstrated unequal cross-linking activities of TG_2 and TG_4 on the protein substrate of SVS I (Fig. 3). Since TG_4 is the actual enzyme involved in seminal coagulation under natural coitus, our study on the TG_4 cross-linking sites of SVS I makes sense in mammalian reproduction. Lin et al. [2006a] have clearly demonstrated that the monomer forms of SVS I-III are absent in mouse SVS but they are cross-linked by interpolypeptide disulfide bridges to various forms of high molecular weight complexes (HWMC), including the mono-polypeptide dimmers of SVS I, SVS II, SVS III; the hetero-polypeptide complexes formed by two SVS II plus one SVS III, one SVS I plus one SVS III, two SVS II plus two SVS III, one SVS I plus one SVS II plus one SVS III, one SVS I plus one SVSP55; and even larger complexes with Mr values greater than 212 kDa formed by unknown stochiometric ratios of SVS I, SVS II, and SVS III. It awaits future study to establish how TG_4 catalyzes its cross-linking sites in each HMWC for semen coagulation.

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