A Novel Heat-labile Phospholipid-binding Protein, SVS VII, in Mouse Seminal Vesicle as a Sperm Motility Enhancer*

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SVS VII, one of seven major proteins in mouse seminal vesicle secretion, was purified to homogeneity. Neither glycoconjugate nor free thiol group was detected in the protein. The primary structure deduced from the corresponding cDNA was confirmed using amino acid sequence determination, which supported the finding that SVS VII consists of 76 amino acid residues with five disulfide bridges. Accordingly, it has a theoretical molecular mass of 8538, which was proven using the mass spectrum of SVS VII. The CD spectrum of SVS VII in 50 mM phosphate buffer at pH 7.4 appeared as one negative band arising from the β form at 217 nm and several fine structures due to nonpeptide chromophores including a prominent band for the disulfide bond at 250 nm. This, together with the predicted secondary structures, indicated no helices but a mixture of β form, β turn, and unordered form in SVS VII. A cytochemical study illustrated the presence of the SVS VII-binding region on the entire surface of mouse sperm. The SVS VII-sperm binding was inhibited by the dispersed sperm lipids. The results of TLC overlay assay for the binding of 125I-SVS VII to phospholipids and the interaction between SVS VII and phospholipid liposomes demonstrated a specific interaction of this protein to both phosphatidyethanolamine and phosphatidylserine. The SVS VII-sperm binding greatly enhanced sperm motility but did not induce sperm capacitation. Heating the protein solution for 10 min at 90 °C unfolded the protein molecule, and the unfolded SVS VII immobilized the sperm.

The fertilization capabilities of spermatozoa are not permanent but rather transient (1). It is well known that mammalian sperm display an intriguing sense of timing to undergo some modification during their transit in the reproductive tract before encountering an egg. This involves multiple steps, and their molecular mechanisms are far from understood. Identifying the molecular event(s) associated with the cell modifications becomes a prerequisite to unravel the puzzle. In this regard, studying how the materials in the lumen of the reproductive tract affect sperm function is an important subject.

Seminal plasma of mammals is a complex biological fluid formed from the mixing of various fluids in the male reproductive tract. Factors that affect sperm motility have been reported in the seminal plasma of various mammals including boar (2–4), bull (5), mouse (6), and human (7). The fluid secreted from the seminal vesicle, an accessory reproductive gland in most male mammals, accumulates in the lumen of this reproductive gland after puberty. Upon ejaculation, seminal vesicle secretion (SVS)† is discharged to constitute the major portion of seminal plasma. It was found that extirpation of the seminal vesicle from a mouse greatly reduced fertility (8, 9), thus manifesting the importance of SVS in the sperm modification. Since rodents have proven to be good experimental animals for the molecular study of mammalian reproduction, attempts have been made to isolate the proteins involved in the cell modification from mouse SVS that contains several minor proteins and seven well defined major proteins designated SVS I–VII in decreasing order of molecular size according to their migration during SDS-PAGE (10). Recently, we demonstrated two of the minor proteins, a cultrin-like trypsin inhibitor/P12, which suppressed the Ca2+-uptake of sperm (11), and a seminal vesicle autoantigen, which served as a decapacitation factor (12, 13). Here we present the protein structure, cDNA cloning, and function of SVS VII purified from mouse SVS.

EXPERIMENTAL PROCEDURES

Materials—Bovine pancreatic chymotrypsin (type II) and trypsin (type III); chlortetracycline; phosphatidylcholine (PtdCho), lysophosphatidylcholine, and phosphatidylethanolamine (PtdEtn) from egg yolks; phosphatidic acid and lysophosphatidic acid from egg yolk lecithin; phosphatidylinositol from pig livers, phosphatidylserine (PtdSer), and sphingomyelin from bovine brains; and fatty acid-free BSA were purchased from Sigma. Goat anti-rabbit IgG conjugated with horseradish peroxidase, fluorescein-conjugated donkey anti-rabbit IgG, Percoll, and Sephadex G-50 (superfine) were procured from Amersham Pharmacia Biotech. C18, 300A column was from Waters Co. (Bedford, MA). The BCA protein assay reagent and IODO-BEADs were obtained from Pierce. The Ultraspec-II RNA isolation kit was purchased from Biotex (Houston, TX). The Oligotest mRNA minikit was from Qiagen GmbH (Hilden, Germany). The cloning systems including the cDNA synthesis kit, ZAP-cDNA synthesis kit, and ZAP-cDNA gigapack III cloning kit were obtained from Stratagene (La Jolla, CA). pGEM-T, T4 DNA polymerase, T4 DNA ligase, the Prime-a-gene labeling system, and restriction enzymes were purchased from Promega (Madison, WI). Freund’s adjuvants were from Life Technologies, Inc. Aluminum-backcoated silica gel TLC plates and GF/C glass microfiber were from Whatman.

Fractionation of Mouse SVS and Preparation of Spermatozoa—Out-
bred CD-1 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. Animals were 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 dark) at 21–22 °C. The animal room was provided with water and NIH 31 laboratory mouse chow ad libitum. Adult mice (8–12 weeks) were humanely killed by cervical dislocation.

The seminal vesicles were carefully dissected to free them from the adjacent coagulating glands, and the secretions collected from 50 mice were expressed directly into 50 ml of ice-cold 5% acetic acid. After stirring for 30 min at 4 °C, the supernatant was collected and the precipitate solubilized initially by 35% saturation of ammonium sulfamate precipitation at pH 2.0. The precipitate was removed using centrifugation at 8000 x g for 20 min, and the supernatant was dialyzed against 0.5% acetic acid and lyophilized. The dry sample was redissolved in a minimum volume of PBS and loaded onto a Sephadex G-50 column (1.5 x 120 cm) equilibrated with PBS. The column was washed with PBS at a flow rate of 6 ml/h. Fractions (2 ml) were collected, and their absorbance at 280 nm was recorded (see Fig. 1A). The peak III fractions were resolved further using HPLC on a Waters C4 300 A column (3.9 x 300 mm, 15μ). The column was eluted with a gradient of 15–60% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min for 40 min (see Fig. 1B). SVS VII was identified in peak 2 using SDS-PAGE.

Protein Blotting and Proteolysis in the Polyacrylamide—Antisera against SVS VII were raised in New Zealand White rabbits. Proteins were resolved using SDS-PAGE, on a 10% gel slab (6.5 x 7.8 cm) by the method of Laemmli (17). The proteins on the gel were stained with Coomassie Brilliant Blue and transferred to a nitrocellulose membrane. After transfer, the protein blots were immunodetected using Western blot procedures; the SVS VII-induced antisera was the primary antibody, and goat anti-rabbit IgG conjugated with horseradish peroxidase was the secondary antibody.

Sperm motility was determined using computer-assisted sperm assays with a sperm motility analyzer (IVOS version 10; Hamilton-Thorne Research, Beverly, MA). A 7.0-μl sample was placed in a 10-μm-deep Makler chamber at 37 °C. The analyzer was set as follows: negative phase-contrast optics and recording at 60 frames/s, minimum contrast 40, minimum cell size 4 pixels, low size gate 0.2, high size gate 1.5, low path velocity 7.0 μm/s, high path velocity 200 μm/s, contrast 40, minimum cell size 4 pixels, low size gate 0.2, high size gate 1.5, low intensity gate 0.5, high intensity gate 1.5, nonmotile head size 4 pixels, another 30 min. At the end of 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 protein incubation in the test specimen was continued by incubating 1:250 in the blocking solution for 30 min. The slides were washed three times with PBS to remove excess antibodies before they were incubated with fluorescein-conjugated donkey anti-rabbit IgG diluted to 1:100 in PBS. The slides were then washed again three times for 5 min each.

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**Analytical Method**—The concentration of SVS VII was determined using the BCA protein assay (27) according to the manufacturer’s instructions. The amino acid sequence was determined using automated gas-phase sequencer (482 protein sequence with on line 140 C analyzer, (PerkinElmer Life Sciences). DNA sequencing was carried out by an ABI PRISM 377-96 DNA sequence using the ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (PerkinElmer Life Sciences).

**Spectral Measurements**—The CD spectra were measured with a Jasco J-700 spectropolarimeter under constant flushing with N₂ at 150 μM Chlortetraacycline (CTC) concentration.
Structure and Function of SVS VII

6915

against SVS VII showed high immunoreactivity to the antigen (Fig. 2, lane 4). Among the protein components of mouse SVS, the antiserum only immunoreacted with SVS VII (Fig. 2, lane 3), thus showing the high specificity of the SVS VII antibody in the antiserum. Therefore, we used the antiserum for the immunodetection of SVS VII throughout the study.

The recombinant phages of the cDNA library were screened using the antiserum against SVS VII, and the positives were excised into phagemids. The SVS VII cDNA in the phagemid was sequenced to establish the gene structure that included a 5′-untranslated region of 5 base pairs, an open reading frame of 297 base pairs, which encoded 99 amino acid residues, and a 3′-untranslated region of 183 base pairs, which ended with a TAA stop codon. Automated Edman degradation of SVS VII for 18 cycles gave reliable data, which indicated that during the post-translational cleavage occurred at the Gly-Leu peptide bond in the signal peptide that had 23 amino acid residues to produce a mature protein of 76 amino acid residues containing 10 cysteines.

**Protein Characterization and Gross Conformation of SVS VII**—We found that SVS VII was not reactive to Ellman’s reagent (31), indicative of the absence of a free thiol group on the protein molecule. The protein also did not react with Ellman’s reagent when the experiment was performed in the presence of 5.0 M urea. Apparently, there were no free cysteines that were partially or fully buried in the protein molecules. In addition, the SVS VII band in the polyacrylamide gel did not stain with periodic acid–Shiff reagent, which revealed that it was not a glycoprotein. Accordingly, the theoretical molecular mass was estimated to be 8588 from the cDNA-deduced protein sequence. This was proven in the electrospray mass spectral profile of SVS VII (Fig. 4).

The CD spectrum of SVS VII in 50 mM phosphate buffer at pH 7.4 had at least seven bands in the wavelength region of 200–300 nm. Bands I–VI in the near-UV region arose from nonpeptide chromophores (Fig. 5, right side), and band VII in the UV region was mainly due to peptide chromophores (Fig. 5, left side). Band VII was negative with a minimum at 217 nm. In addition, a positive band would appear as the CD profile extended below 200 nm. The spectral profile had some resemblance to that of the β form of protein conformation (32–35).

**RESULTS**

**Purification of SVS VII and Establishment of the Protein Sequence**—SVS VII was purified from SVS through a series of isolation steps. The peak 2 sample in Fig. 1B shows that a single 8-kDa band has the same mobility as SVS VII on SDS-PAGE (cf. lanes 1 and 2 in Fig. 2), which indicates that the protein was purified to homogeneity and was distinct from the peak 1 sample, which gave a single 6-kDa band that corresponds with P12 reported previously (30). The antiserum was raised against SVS VII and was used for the immunoblotting analysis. The antiserum showed high immunoaffinity to the antigen (Fig. 2, lane 4). Among the protein components of mouse SVS, the antiserum only immunoreacted with SVS VII (Fig. 2, lane 3), thus showing the high specificity of the SVS VII antibody in the antiserum. Therefore, we used the antiserum for the immunodetection of SVS VII throughout the study.

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**FIG. 1. Purification of SVS VII from mouse SVS.** A, fractionation of the soluble proteins of mouse SVS from 35% ammonium sulfate saturation at pH 2.0 by Sephadex G-50 column chromatography. B, resolution of the peak III sample by reverse phase of HPLC on a C₄ column. The broken line indicates a linear gradient of acetonitrile (see “Experimental Procedures” for details).

**FIG. 2. Protein identification by SDS-PAGE and specificity of SVS VII-induced antiserum.** The proteins of mouse SVS (15 µg, lanes 1 and 3) and peak 2 of Fig. 1B (5 µg, lanes 2 and 4) were subjected to SDS-PAGE on a 15% polyacrylamide gel. The gels of lanes 1 and 2 were stained with Coomassie Brilliant Blue to reveal the protein bands. The proteins in the gels of lanes 3 and 4 were transferred to nitrocellulose membranes and immunodetected using the SVS VII-induced antiserum, diluted 1:2000 in the blocking solution.
Using the criteria of the Chou and Fasman algorithm (36, 37) or the GOR algorithm (38), we predicted the potentials for the formation of secondary structures in SVS VII (Fig. 3B). The helical formation by weak helix formers along the two peptide segments of residues 51–56 and 60–70 was overpredicted in view of our CD results. The predicted secondary structure, together with the position, sign and magnitude of band VII of SVS VII (Fig. 5), strongly supported the presence of some ordered structure other than the helix, probably a mixture of \(\beta\)-form, \(\beta\)-turn, and unordered form in the SVS VII molecule.

There were two tyrosines, two phenylalanines, and five cystines but no tryptophan in the SVS VII molecule. According to the fine CD structures of nonpeptide chromophores of a protein near UV (39, 40), bands I–III arose from tyrosine residue(s), and band V, which appeared as a shoulder between the positive band VI and the negative band IV, may be attributable to phenylalanine residues, since the CD spectrum of this amino acid in a protein is usually weak. Band VI was the most prominent band among the CD fine structures of SVS VII. Based on the study of Beychok and Breslow (40), bands VI and...
the chymotrypsin- or trypsin-mediated proteolysis. According to previous methods (41, 42), we measured no inhibitory effects of SVS VII on the proteolytic activity of either chymotrypsin or trypsin (data not shown), suggesting that the cross-linkages of five disulfide bridges constrained this rather small protein molecule to hamper the proteolytic digestion or/and the release of peptide segment(s) that remained in the protein core after proteolytic degradation.

Characteristics of SVS VII-Sperm Binding—Fig. 6 displays the sperm micrographs examined using the indirect fluorescence staining technique. No fluorescence appeared on the epididymal spermatozoa after they immunoreacted successively with the SVS VII antisera and fluorescein isothiocyanate-conjugated anti-rabbit IgG, manifesting the lack of SVS VII on the cell surface. When spermatozoa were preincubated with 25 μM SVS VII in the blocking solution at room temperature for 30 min, the fluorescein fluorescence was visible around acrosome, middle piece, and principal tail. No fluorescence was seen when the antisera was replaced with the normal serum. Apparently, sperm had SVS VII-binding sites that covered the entire cell surface.

Fig. 7 shows the data from one representative determination for 125I-SVS VII-sperm binding. The radiolabeled125I-SVS VII bound to the cell surface was greatly inhibited by a 100-fold excess of the unlabeled SVS VII, indicating the specificity of SVS VII-sperm binding. A similar situation was also observed by the replacement of unlabeled SVS VII with SVS VIIh in the assay. Apparently, after heat treatment, the protein did not lose its sperm binding ability. The 125I-SVS VII-sperm binding was slightly reduced by the presence of 1.8 mM Ca2+. Increased levels of Ca2+ to 6.0 mM during the incubation period occurred in more than 75% of the total SVS VII bound to sperm. The presence of the dispersed sperm lipids during the cell incubation also suppressed the binding of SVS VII to the cell surface; thus, SVS VII bound to the sperm decreased as the quantity of dispersed sperm lipids increased.

The lipid extract of epididymal spermatozoa and purified phospholipids were chromatographed on silica gel-coated aluminum plates (Fig. 8A). As in a previous report (23), the mouse sperm phospholipids were well separated into six major components and several minor components in the developing solvent employed except that PtdCho and PtdCho plasmalogens migrated together (Fig. 8A, lane 1). Based on the RI values of purified lipids, the minor components remained unidentified, and five of the main components were identified as neutral lipid, PtdEtn, PtdCho/PtdCho plasmalogen, PtdSer, and sphingomyelin. The results of a TLC overlay binding assay (Fig. 8B) showed that 125I-SVS VII bound to purified PtdSer and PtdEtn but did not interact with phosphatidic acid, PtdCho/PtdCho plasmalogen, phosphatidylinositol, lysophosphatidic acid, sphingomyelin, or lysophosphatidylethanolamine. Among the sperm lipids, 125I-SVS VII bound to PtdEtn and PtdSer but did not interact with the other phospholipids (Fig. 8B, lane 1). PtdSer is in a relative small amount of sperm phospholipid (43). This may account for the weak radioactivity of 125I-SVS VII bound to sperm PtdSer on the TLC plate.

We found that PtdSer liposomes in 10 mM HEPES and 100 mM KCl at pH 7.4 slowly settled to form solid phase aggregates during their incubation in the presence of SVS VII, SVS VIIh, or Ca2+. On the contrary, PtdCho liposomes did not settle in the presence of the proteins or Ca2+ during incubation. As shown in Fig. 9, both proteins appeared only in the pellet fractions of PtdSer liposomes after centrifugation of the incubation mixture, suggesting that they bound to PtdSer liposomes. Incubation in the presence of 1.8 mM Ca2+ partially retarded the cosedimentation of each protein with PtdSer lipos-
somes, revealing that Ca$^{2+}$ might be able to release the protein bound to PtdSer liposomes, which can chelate Ca$^{2+}$ (44). SVS VII as well as SVS VIIh did not bind to PtdCho liposomes as evidenced from the observation that they did not cosediment with the phospholipid vesicles and appeared only in the supernatant fraction after incubation.

**Enhancement of Sperm Motility by SVS VII**—We examined the distribution of SVS VII and its RNA message in the tissue homogenates of reproductive glands, such as the seminal vesicle, epididymis, testis, coagulating gland, vas deferens, uterus, ovary, prostate, vagina, and nonreproductive organs, including lungs, kidney, brain, spleen, liver, pancreas, and heart. SVS VII was immunodetected in the seminal vesicle only, and the mRNA was abundant in the seminal vesicle and a trace in coagulating gland and vas deferens but was not detected in the other tissues (not shown).

Most spermatozoa freshly retrieved from mouse caudal epididymis in modified Tyrode’s buffer were motile with tail beating even after incubation for 120 min at 37 °C. Since BSA has often been used to study sperm capacitation in vitro, we compared BSA with SVS VII and SVS VIIh in the effects on the sperm motility and capacitation. These two kinds of sperm function were assayed after the cell incubation in the modified Tyrode’s solution containing 1.8 mM CaCl$_2$ at several condi-
Previously, Lardy and co-workers (45) purified an 8-kDa caltein from mouse SVS and established its primary structure of 75 amino acid residues containing seven cysteines. Aligning the protein sequence of SVS VII with that of caltein revealed a very high degree of similarity with 71 identical residues, except that Cys<sup>67</sup>, Cys<sup>68</sup>, Cys<sup>73</sup>, and Ser<sup>75</sup> of SVS VII were replaced by Phe<sup>67</sup>, Gly<sup>68</sup>, Met<sup>73</sup>, and Phe<sup>75</sup> of caltein (Fig. 3A). Since SVS VII and caltein were secreted from the same reproductive gland...
and have very similar molecular size, they were assumed to be identical molecules. The SVS VII primary structure established from our present work is reliable, considering our demonstration that the molecular mass of SVS VII determined from the electrospray mass spectrometry conformed to the theoretical value estimated from the cDNA-deduced protein sequence consisting of 76 amino acid residues in which five disulfide bonds were considered. Several lines of evidence suggested the incorrect assignment for the phenylthiohydantoin-derivatives stated by Lardy et al. (45), which were different from the corresponding amino acid residues in SVS VII, after the automated Edman degradation of caltrin. First, a molecular mass of 8470 for caltrin would have appeared in the profile of the mass spectrum, taking into account their claim that a free thiol group may be present in the protein molecule. However, our results shown in Fig. 4 do not prove its presence. Second, their suggestion of dimer formation through the intermolecular disulfide bond was not proven. Among the protein components of mouse SVS that were resolved using a nonreducing SDS-PAGE, we did not find a 16-kDa band. We only found a 8-kDa band that was immunoreactive to the antisem against SVS VII in the Western blot analysis.

Of the 10 cysteines in the SVS VII molecule, six residues, namely Cys³, Cys⁶, Cys¹⁴, Cys²¹, Cys²⁹, and Cys⁴⁲ were sterically restricted in the predicted β form/α turn (Fig. 3B). The chirality of a disulfide bond is relevant to its skewed conformation. Heating SVS VII caused a great diminution of the CD chirality of a disulfide bond is relevant to its skewed conformation. Heating SVS VII caused a great diminution of the CD

A 19-kDa phospholipid-binding glycoprotein that shows no significant similarities to the protein sequences of BSP proteins and exhibits the ability to suppress mouse sperm motility (12, 13). In comparison with the specificity of SVS VII-phospholipid binding, SVA did not interact with PtdSer but bound to the choline-containing phospholipids such as PtdCho/PtdCho plasmalogen and sphingomyelin. Together, these phosphocholine-containing lipids make up more than 70% of total lipid in the plasma membrane of mouse spermatozoa (43). The primary structure of SVS VII showed no significant similarities to protein sequences of both BSP proteins and SVA. Furthermore, SVS VII was not related to any other phospholipid-binding proteins such as perforin (55), phosphocholine-binding protein (56), factor V (57), factor VIII (58, 59), factor IX (60), P65 (61), pulmonary surfactant protein (62), C-reactive proteins (63), apolipoproteins A-I, A-II, and A-IV (64), or lipid transfer proteins (65–67). Therefore, SVS VII represented a novel phospholipid-binding protein. Interestingly, the differences in specificity between SVA-lipid and SVS VII-lipid binding result in their extremely different effects on the sperm function, although they are secreted from the same gland.

SVS VII was exclusively secreted from mouse seminal vesicle, and the SVS VII-sperm binding that took place upon ejaculation enhanced the sperm motility without leading to sperm capacitation. Apparently, the SVS VII effects did not cause the maturation of spermatozoa at any time earlier than the sperm-egg encounter but helped the ejaculated spermatozoa pass through the cervix after coitus of the rodent. Lardy et al. (45) reported the ability of mouse caltrin to affect the Ca²⁺uptake of guinea pig sperm. Determination of whether this kind of event is relevant to the stimulation of mouse sperm motility by SVS VII awaits the completion of future studies.

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