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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 ^{125}I-SVS VII to phospholipids and the interaction between SVS VII and phospholipid liposomes demonstrated a specific binding of this protein to both phosphatidylethanolamine 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 reproduction 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)[1] 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 motilities in SDS-PAGE (10). Recently, we demonstrated two of the minor proteins, a caltrin-like trypsin inhibitor/P12, which suppressed the Ca^{2+}-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 lectin; 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. C_4 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 Biotecx (Houston, TX). The Oligotex 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, *Taq* polymerase, T_4 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-backed silica gel TLC plates and GF/C glass microfiber were from Whatman.

Fractionation of Mouse SVS and Preparation of Spermatozoa—Out-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF134204.

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[1] The abbreviations used are: SVS, seminal vesicle secretion; BSA, bovine serum albumin; BSP, bovine seminal plasma; PAGE, polyacrylamide gel electrophoresis; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; PtdIns, phosphatidylinositol; SVA, seminal vesicle autoantigen; PBS, phosphate-buffered saline.

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 of light, 10 h of dark) at 21–22 °C and were 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 fractionated initially by 35% saturation of ammonium sulfate precipitation at pH 2.0. The precipitate was removed using centrifugation at 8000 × *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 × 120 cm) preequilibrated 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. 1*A*). The peak III fractions were resolved further using HPLC on a Waters C4 300 A column (3.9 × 300 mm, 15μ). The column was eluted with a linear gradient of 15–60% acetonitrile in 0.1% (v/v) trifluoroacetic acid at a flow rate of 1.0 ml/min for 40 min (see Fig. 1*B*). SVS VII was identified in peak 2 using SDS-PAGE.

In accordance with a method previously used (14), a modified Tyrode's buffer, which consisted of 124.7 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 5.6 mM glucose, 0.5 mM sodium pyruvate, 15 mM $NaHCO_3$, 10 mM HEPES, 100 IU/ml penicillin, and 100 μg/ml streptomycin, was adjusted to pH 7.3–7.4 by aeration with humidified air/CO_2 (19:1) in an incubator for 48 h at 37 °C before use. The epididymides were removed and immersed in the medium. After they had been carefully dissected from the connective tissue, spermatozoa were extruded from the distal portion of the tissues for 10 min at 37 °C. The cells were gently filtered through two layers of nylon gauze, layered on top of a linear gradient of 20–80% Percoll (v/v), and centrifuged at 275 × *g* for 30 min at room temperature (15, 16). Three distinct cell layers formed. The lowest layer, which contained cells with progressive motility, was washed in three volumes of the medium and collected using centrifugation at 60 × *g* for 10 min at room temperature. The sperm were resuspended and centrifuged similarly twice more. The cell pellets were resuspended, and $CaCl_2$ was added to the culture medium at a final concentration of 1.8 mM before the sperm was assayed.

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 15% gel slab (6.5 × 10.5 × 0.075 cm) by the method of Laemmli (17). The proteins on the gel were stained with Coomassie Brilliant Blue or 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.

The proteolysis of protein bands in the polyacrylamide gel was carried out following the method of Hanspal *et al*. (18). After resolution of proteins using SDS-electrophoresis on a gel slab of 15% polyacrylamide copolymerized with 0.1% gelatin, the gel was gently washed three times in 100 ml of 2.5% (w/v) Triton X-100 for 45 min each to remove SDS. The gel was washed in distilled water for 45 min and incubated for 15 h at 37 °C in a solution containing chymotrypsin (1 *N*-benzoyl-L-tyrosine ethyl ester unit/ml) or trypsin (50 *N*-benzoyl-L-arginine ethyl ester units/ml) in 0.1 M Tris-HCl and 20 mM $CaCl_2$ at pH 8.0. The gel was washed similarly as above and stained with Coomassie Brilliant Blue.

Preparation of Sperm Lipid and Phospholipid Liposomes—The total lipids of spermatozoa were extracted using a modified method of Folch *et al*. (19). The cells were suspended in 1.0 ml of 0.05 M KCl, sonicated in a Branson ultrasonic water bath (model B-52) for 5 min at room temperature, and extracted twice with 3.0 ml of chloroform/methanol (2:1, v/v). The lipid extract was dried by flushing with N_2 and redissolved in a suitable organic solvent for TLC. Meanwhile, the sperm lipid was dissolved in ethanol and injected through a 26-gauge needle into PBS (ethanol/PBS, 1:20, v/v). The suspension was repeatedly forced through the needle, sonicated for 1 h at room temperature, centrifuged at 18,000 × *g*, and used immediately in the study of the inhibitory effects on SVS VII-sperm binding.

The pure phospholipids in the chloroform solution were evaporated under N_2 and formed thin films in glass tubes. Liposomes of phospholipids were prepared following the method of Genge *et al*. (20). A sufficient volume of incubation buffer composed of 10 mM HEPES and 100 mM KCl at pH 7.4 was added to give a final concentration of 2 mg of lipid/ml. The mixture was sonicated at full power for 10 min until the lipids formed a faintly opalescent suspension.

Binding Assay—A modified method developed by Markwell (21) was followed to prepare ^{125}I-SVS VII. In brief, 10 μl of Na^{125}I (1.0 mCi) from a commercial source was mixed with 50 μg of SVS VII in 90 μl of PBS in the presence of IODO-BEADs. The radiolabeled protein was separated from free Na^{125}I through a PD-10 column preequilibrated with PBS. ^{125}I-SVS VII showed a single band and was indistinguishable from its parent protein by migration on SDS-PAGE.

Spermatozoa (2.5 × 10^6 cells/ml) and 100 nM ^{125}I-SVS VII in 10 mM HEPES containing 138 mM NaCl and 3 mM KCl at pH 7.4 were incubated at specified conditions. The cells were collected on a Whatman GF/C glass microfiber filter using rapid filtration at a pressure of 50.66 kilopascals (0.5 atm). In accordance with the method described previously (11, 22), the filter was blocked with 5% (v/v) nonfat skimmed milk in the buffer for 30 min and washed with the same ice-cold buffer before use to minimize the background. The filter was washed with six changes of 0.2 ml of the same ice-cold buffer, air-dried, and counted using a γ-counter.

Sperm lipids and purified lipids were chromatographed on aluminum-backed silica gel TLC plates in chloroform/methanol/water (65:25:4, v/v/v). The plates were treated according to the method developed by Desnoyers and Manjunath (23). The plates were overlaid with ^{125}I-SVS VII (100,000 cpm/ml) in a blocking buffer of PBS containing 5% nonfat skimmed milk (100 μl/cm^2), incubated for 90 min at room temperature, washed five times each for 1–2 min with cold PBS, and then dried and exposed to x-ray film for 18–36 h. The lipids on duplicate plates were sprayed with phosphomolybdic acid solution to allow us to detect phospholipids (24).

According to a modified method by Glenney (25), 10 μg of protein and 200 μg of liposomes in 200 μl of incubation buffer were gently mixed with or without 1.8 mM $CaCl_2$ for 45 min at room temperature. Liposomes became sediment at 100,000 × *g* for 30 min, and equivalent fractions of pellet and supernant were analyzed using SDS-PAGE.

Cytological Observation and Assay of Sperm Motility—The chlortetracycline staining method developed by Ward and Storey (26) was employed to score the population of mouse spermatozoa in the uncapacitated, capacitated, and acrosome-reacted stages. Briefly, a 5-μl sample of the sperm suspension was mixed on a slide with 5 μl of buffer containing 130 mM NaCl, 5 mM cysteine, 1 mM chlortetracycline, and 20 mM Tris-HCl at pH 7.8. After 30 s, 1.0 μl of buffer containing 1.25% glutaraldehyde, 130 mM NaCl, 1.8 mM $CaCl_2$, and 20 mM Tris-HCl at pH 7.8 was added. The samples were kept in a light-shielded environment until they were seen under a fluorescence microscope (AH3-RFCA; Olympus, Tokyo, Japan).

Freshly prepared spermatozoa (10^6 cells/ml) were preincubated in a blocking solution (3% nonfat skimmed milk in PBS) for 30 min at room temperature. The cells were further incubated with 25 μM SVS VII for 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 slides were incubated with the SVS VII-induced antiserum diluted 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 the blocking solution for 30 min. All of the slides were washed with PBS, covered with 50% (v/v) glycerol in PBS, and photographed with a microscope equipped with epifluorescence.

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 intensity gate 0.5, high intensity gate 1.5, nonmotile head size 29, nonmotile head intensity 76, medium average path velocity 50 μm/s, low path velocity 7.0 μm/s, slow motile cells yes, and threshold straightness (STR) greater than 80%. Ten fields were assessed for each sample.

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 Edman degradation with a gas phase sequenator (492 protein sequencer with on line 140 C analyzer, (PerkinElmer Life Sciences). DNA sequencing was carried out by an ABI PRISM 377-96 DNA sequencer 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 N_2 at

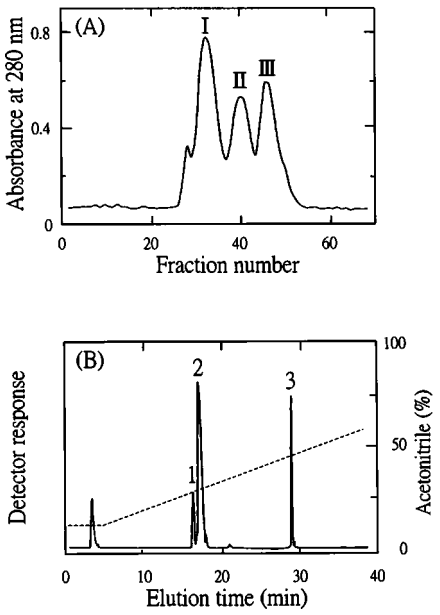

FIG. 1. **Purification of SVS VII from mouse SVS.** *A*, fractionation of the soluble proteins of mouse SVS from 35% ammonium sulfate saturation at pH 2.0 by Sephadex G-50 column chromatography. *B*, resolution of the peak III sample by reverse phase of HPLC on a C_4 column. The *broken line* indicates a linear gradient of acetonitrile (see "Experimental Procedures" for details).

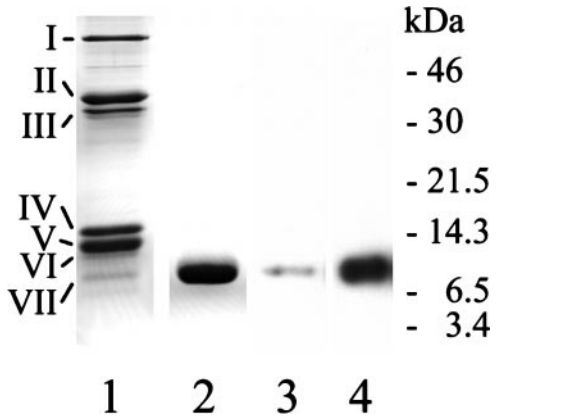

FIG. 2. **Protein identification by SDS-PAGE and specificity of SVS VII-induced antiserum.** The proteins of mouse SVS (15 μg, *lanes 1* and *3*) and peak 2 of Fig. 1*B* (5 μg, *lanes 2* and *4*) were subjected to SDS-PAGE on a 15% polyacrylamide. The gels of *lanes 1* and *2* were stained with Coomassie 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 Western blotting with the SVS VII-induced antiserum, diluted 1:2000 in the blocking solution.

room temperature. The mean residue elipticity, [θ], was estimated from the mean residue weight, which was calculated from the primary structure.

SVS VII dissolved in 50% acetonitrile containing 1% acetic acid at a final concentration of 10 μM was analyzed in the ESI source of a Finnigan LCQ ion trap mass spectrometer (Finnigan MAT Instruments Inc., San Jose, CA). Data were acquired using Bioworks software.

RNA Isolation, cDNA Construction, Cloning, and Northern Blotting—Total cellular RNA was prepared from fresh tissue homogenates, using the Ultraspec-II RNA isolation kit. The polyadenylated fraction of total RNA was isolated using the procedures recommended for the Oligotex mRNA minikit. Preparation of double-stranded cDNAs, construction of orientation-specific cDNAs in Uni-ZAP XR vectors with the *Eco*RI/*Xho*I terminus, and packaging of the constructed vectors into phages generally followed the instructions of Stratagene. The recombinant phages were transfected into the bacterial host XL1-Blue MRF′ strain. The cDNA library efficiency was 2.5×10^5 plaque-forming units/μg of cDNA. Plaques from the cDNA library in the vectors were induced by isopropyl-1-thio-β-D-galactopyranoside and immunochemically screened using the antiserum against SVS VII. Randomly chosen positives from different pools were purified. They were excised into phagemids using ExAssist interference-resistant helper phages and transformed into bacterial SOLR™ strain for DNA sequencing.

In Northern blotting, total RNA samples were analyzed by separation in a denaturing 1.5% agarose/formaldehyde gel (28) followed by capillary transfer to a nylon membrane and hybridization to a ^{32}P-labeled nucleotide probe. ^{32}P-labeled random-primed probe for the glyceraldehyde-3-phosphate dehydrogenase gene was prepared with a Promega Prime-a-gene labeling kit using a cDNA segment of the mouse glyceraldehyde-3-phosphate dehydrogenase gene inserted into a pGEM-T vector as a template. According to the method developed by Lee *et al.* (29), incorporation of [α-^{32}P]dATP to DNA was carried out using PCR, which was designed to amplify the whole reading frame of SVS VII cDNA in the constructed vector.

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. 1*B* 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 against SVS VII 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.

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 polyadenylated region (Fig. 3*A*). Automated Edman degradation of SVS VII for 18 cycles gave reliable data, which indicated Leu as the N-terminal residue and the amino acid sequence, LICNSCEKSRDSRCTMSQ, which was identical with the corresponding cDNA-deduced peptide sequence in all positions. Apparently, 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 6.0 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 8538 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).

(A)

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1    CC AAC ATG AAT TCA GTG ACG AAA ATC AGC ACA CTG CTC ATC GTG ATT TTA TCC TTT CTC
           Met Asn Ser Val Thr Lys Ile Ser Thr Leu Leu Ile Val Ile Leu Ser Phe Leu
                      -20                                       -10

60   TGT TTT GTG GAG GGT CTG ATC TGT AAT TCA TGT GAA AAG TCA CGA GAT TCA AGA TGT ACA
     Cys Phe Val Glu Gly Leu Ile Cys Asn Ser Cys Glu Lys Ser Arg Asp Ser Arg Cys Thr
                         1                                  10
                        Leu Ile Cys Asn Ser Cys Glu Lys Ser Arg Asp Ser Arg Cys Thr
                        ------------------------------------------------------------

120  ATG TCA CAA AGC AGA TGC GTT GCA AAA CCT GGT GAA TCA TGC AGT ACC GTA TCA CAC TTT
     Met Ser Gln Ser Arg Cys Val Ala Lys Pro Gly Glu Ser Cys Ser Thr Val Ser His Phe
                    20                                      30
     Met Ser Gln (from intact protein)
     ------------

180  GTT GGA ACA AAG CAT GTG TAC TCA AAG CAA ATG TGC TCG CCT CAG TGC AAG GAA AAA CAG
     Val Gly Thr Lys His Val Tyr Ser Lys Gln Met Cys Ser Pro Gln Cys Lys Glu Lys Gln
                    40                                      50

240  CTT AAC ACT GGA AAG AAG TTG ATT TAC ATA ATG TGC TGC GAA AAA AAC TTG TGT AAT AGC
     Leu Asn Thr Gly Lys Lys Leu Ile Tyr Ile Met Cys Cys Glu Lys Asn Leu Cys Asn Ser
                    60                                  *   *       70            *       *
                                                     (Phe Gly)                (Met)    (Phe)

300  TTC TAG ATGGAGAAGAATCACTCGAAGATCATCTTCTGTTCAAGATCCAAACTTACAAATGAATCAAGATGGGGAAC
     Phe

     TTGCTTTCCTACTCGATTTCCATGATGGCCTGAGATCCCTGAGATAGCAACAGAGGCTGTGTCCTCGTTTCAGTGAAAG
     ACTTCTTGACCACACCATTAGAGCACTGCAAAAAAAAAAAAAAA
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(B)

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          10          20          30          40          50
          :           :           :           :           :
LICNSCEKSRDSRCTMSQSRCVAKPGESCSTVSHFVGTKHVYSKQMCSPQCKEKQLN
BBBBBB...TTTTbbbbbbbbbb.TTttt.BBBBBB...bbbbbbbb.TThhhhhh.

  60          70
  :           :
TGKKLIYIMCCEKNLCNSF
TThhhhhhhhhhh...tt.
```

FIG. 3. **The primary structure and the predicted secondary structure of SVS VII.** *A*, the nucleotide sequences of a 500-base pair SVS VII cDNA were determined to deduce amino acid sequences. The initial and stop codons of the open reading frame are *underlined*. The deduced protein sequence and the amino acid sequences determined directly from the protein analysis agree in all positions. The latter is indicated by a *dashed underline*. The cleavage point for the generation of mature peptide is indicated by an *arrow*. The cDNA-deduced amino acid sequences of SVS VII are identical to all positions of the protein sequences of a mouse caltrin, except that Cys^{67}, Cys^{68}, Cys^{73}, and Ser^{75} of SVS VII, which are denoted by *asterisks*, disagree with Phe^{67}, Gly^{68}, Met^{73}, and Phe^{75} of the caltrin molecule, which are listed in *parentheses*. *B*, the secondary structure of SVS VII was predicted using Chou and Fasman algorithm. *B* or *b*, strong or weak β form former; *T* or *t*, strong or weak β turn former; *h*, weak helix former.

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. 3*B*). 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 β-form, β-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

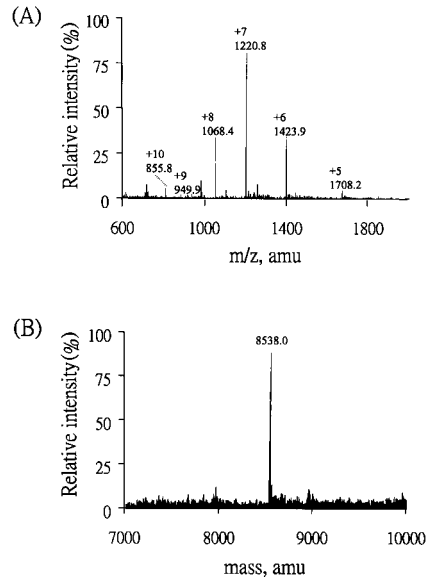

FIG. 4. **Electrospray mass spectrum of SVS VII.** SVS VII dissolved in 50% acetonitrile in the presence of 1% acetic acid was analyzed in the ESI source mass spectrometer. *A*, original spectrum. *B*, computer deconvolution of the peaks in *A*.

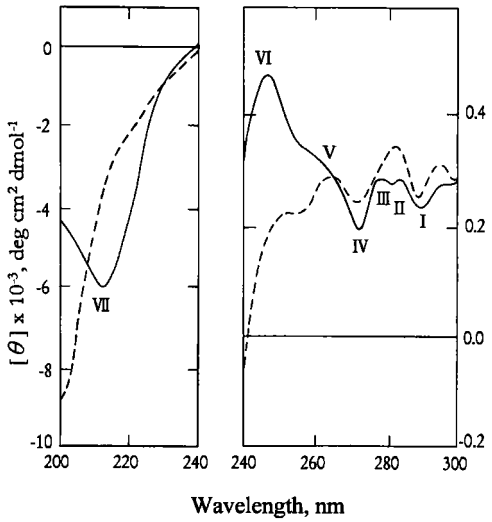

FIG. 5. **Circular dichroism of SVS VII.** The protein was in 50 mM phosphate buffer at pH 7.4 at room temperature (*solid line*). The protein solution was heated for 10 min at 90 °C, cooled for 10 min at 4 °C, and kept at room temperature for 10 min before the optical measurement (*dashed line*).

IV were assigned to cystine residue(s). The description of bands I–VI to specific residues may be fortuitous. The CD data from site-specific mutagenesis for the protein may support the assignment in the future.

The secondary structure of SVS VII was stable over a wide range of pH values. Even at pH 3 or 10, the profiles of CD band VII as well as the CD fine structures in the near-UV region remained virtually the same as in neutral solution (not shown). After heating the protein solution for 10 min at 90 °C, the protein conformation changed remarkably as evidenced by the disappearance of CD bands VI and VII of native protein and the appearance of a strong negative band below 200 nm (*cf. solid* and *dashed lines* of Fig. 5), which was the characteristic CD of a protein in a completely unordered form. The protein sample after heat treatment is referred to as SVS VII_h hereafter.

The mouse SVS proteins resolved in a slab gel of polyacrylamide copolymerized with gelatin were digested with protease. The protein band of SVS VII remained in the gel after either 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 antiserum 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 antiserum 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 ^{125}I-SVS VII-sperm binding. The radiolabeled ^{125}I-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 VII_h in the assay. Apparently, after heat treatment, the protein did not lose its sperm binding ability. The ^{125}I-SVS VII-sperm binding was slightly reduced by the presence of 1.8 mM Ca^{2+}. Increased levels of Ca^{2+} 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. 8*A*). 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. 8*A*, *lane 1*). Based on the R_f 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. 8*B*) showed that ^{125}I-SVS VII bound to purified PtdSer and PtdEtn but did not interact with phosphatidic acid, PtdCho/PtdCho plasmalogen, phosphatidylinositol, lysophosphatidic acid, sphingomyelin, or lysophosphatidylcholine. Among the sperm lipids, ^{125}I-SVS VII bound to PtdEtn and PtdSer but did not interact with the other phospholipids (Fig. 8*B*, *lane 1*). PtdSer is in a relative small amount of sperm phospholipid (43). This may account for the weak radioactivity of ^{125}I-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 VII_h, or Ca^{2+}. On the contrary, PtdCho liposomes did not settle in the presence of the proteins or Ca^{2+} 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 Ca^{2+} partially retarded the cosedimentation of each protein with PtdSer lipo-

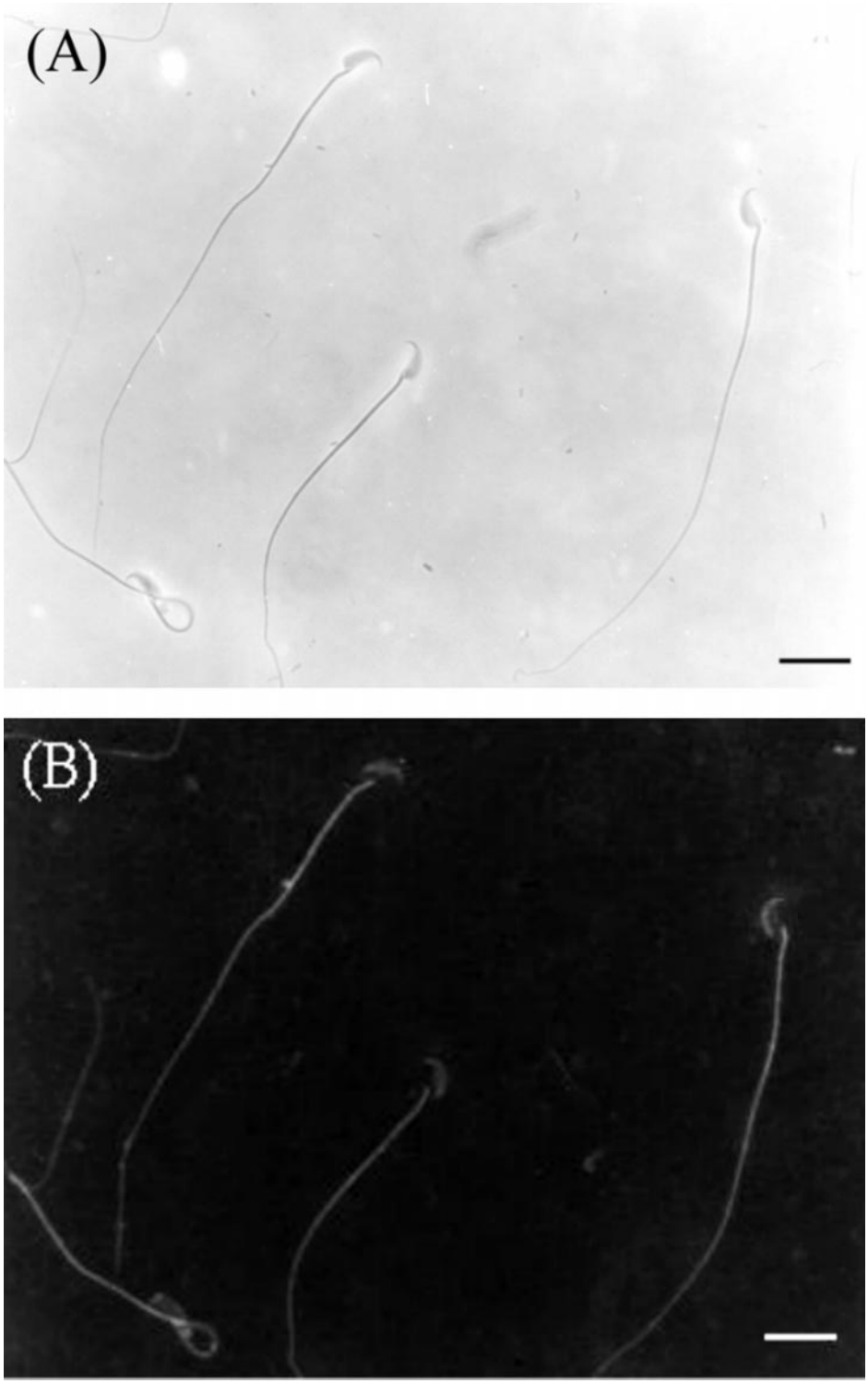

FIG. 6. **Demonstration of the SVS VII binding zone on the spermatozoa.** Fresh cells were incubated with SVS VII as described under "Experimental Procedures." The SVS VII-binding zone on the cells was immunolocalized by the indirect fluorescence method using the SVS VII antiserum and fluorescein-conjugated anti-rabbit IgG. The slides were observed by a light microscope (*A*) or a fluorescence microscope (*B*). *Bar*, 10 μm.

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 VII_h 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 VII_h 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-

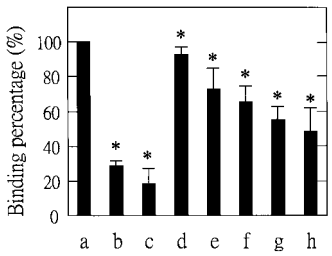

FIG. 7. **The specific binding of SVS VII to sperm lipid.** Spermatozoa (2.5×10^6 cells/ml) in HEPES buffer containing 1% (w/v) nonfat skimming milk were incubated for 1 h at room temperature in the presence of 100 nM ^{125}I-SVS VII and a 100-fold excess of unlabeled SVS VII (*column b*) or SVS VII_h (*column c*). The ^{125}I-SVS VII-sperm binding was also assayed in the presence of 1.8 mM Ca^{2+} (*column d*), 6 mM Ca^{2+} (*column e*), or the dispersed sperm lipids prepared from 2.5×10^8 cells (*column f*), 5×10^8 cells (*column g*), and 1.25×10^9 cells (*column h*). Results are the percentage of ^{125}I-SVS VII-sperm binding of the control (*column a*) expressed as means ± S.D. *, $p < 0.01$ in the paired statistical comparison with the corresponding control values are evaluated using one-way analysis of variance.

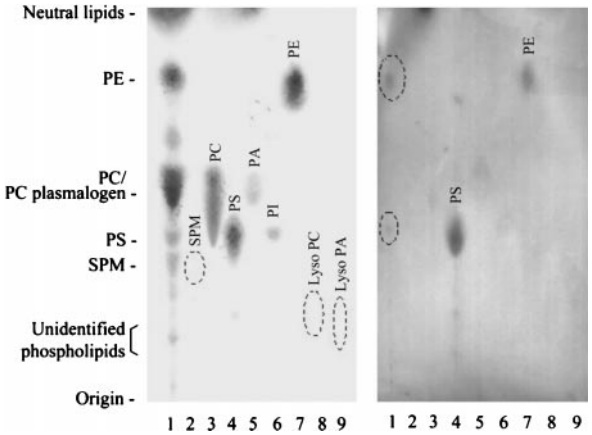

FIG. 8. **Binding of ^{125}I-SVS VII to phospholipids separated by TLC.** Lipids extracted from 2.5×10^7 spermatozoa (*lane 1*) and 30 μg of each of the pure phospholipids (*lanes 2–9*) were chromatographed on silica gel TLC plates. *A*, phospholipids were detected with phosphomolybdic acid spray. *B*, autoradiograms for the binding of radiolabeled SVS VII to separated phospholipids were obtained after a TLC overlay binding technique described under "Experimental Procedures." *lyso PA*, lysophosphatidic acid; *lyso PC*, lysophosphatidylcholine; *PA*, phosphatidic acid; *PC*, phosphatidylcholine; *PE*, phosphatidylethanolamine; *PS*, phosphatidylserine.

tions. As shown in Fig. 10*A*, both 45 μM BSA and 40 μM SVS VII in the cell culture relative to the motility of control cells greatly enhanced the sperm motility at any incubation time. SVS VII_h at a concentration of 40 μM in the culture medium retained the ability to enhance the sperm motility before 10 min of incubation, but the cells became immobile and stuck in clusters thereafter. At 90 min of incubation, more than 90% of the control cells remained uncapacitated, and almost no acrosome-reacted cells appeared (Fig. 10*B*, *panel a*). The addition of SVS VII to the incubation medium showed very slight effects on the cellular stage (Fig. 10*B*, *panel b*). The population of capacitated cells increased remarkably after similar incubation in the presence of BSA. Around 50% of the BSA-treated cells were capacitated, but acrosome-reacted cells constituted less than 15% of the total (Fig. 10*B*, *panel c*). The BSA-induced capacitating sperm were not influenced as the cells were exposed to BSA and SVS VII together (Fig. 10*B*, *panel d*).

DISCUSSION

Previously, Lardy and co-workers (45) purified an 8-kDa caltrin 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 caltrin revealed a very high degree of similarity with 71 identical residues, except that Cys^{67}, Cys^{68}, Cys^{73}, and Ser^{75} of SVS VII were replaced by Phe^{67}, Gly^{68}, Met^{73}, and Phe^{75} of caltrin (Fig. 3*A*). Since SVS VII and caltrin were secreted from the same reproductive gland

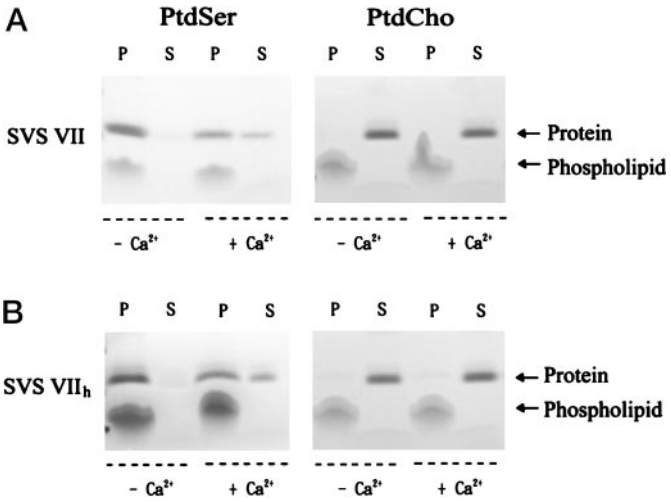

FIG. 9. **Interaction of SVS VII or SVS VII_h with various phospholipid liposomes.** Each liposome (200 μg) was mixed with 10 μg of each protein in 200 μl of 10 mM HEPES buffer containing 100 mM KCl and 1.8 mM $CaCl_2$ ($+Ca^{2+}$) or in the absence of Ca^{2+} ($-Ca^{2+}$) and incubated for 45 min at room temperature. After centrifugation, the protein in the supernatant (*S*) and pellet (*P*) fraction was determined using SDS-PAGE.

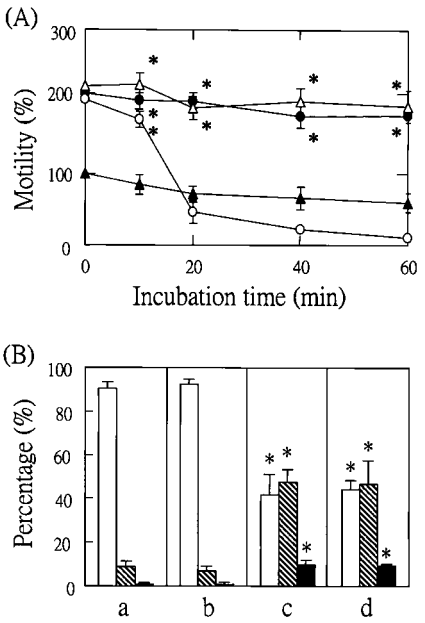

FIG. 10. **Analysis of sperm motility and capacitation under the influence of SVS VII and BSA.** *A*, freshly prepared mouse spermatozoa in modified Tyrode's solution (10^6 cells/ml) in the presence of 1.8 mM $CaCl_2$ was incubated for 0–60 min in the presence of 40 μM SVS VII (●), 40 μM SVS VII_h (○), or 45 μM BSA (△) at 37 °C. The cell motility determined at each specified incubation was expressed as a percentage of control cell motility (▲) measured at zero time incubation. Points are means ± S.D. for 10 determinations. *B*, the chlortetracycline fluorescence method described under "Experimental Procedures" was exploited to score the population of uncapacitated cells (*open bars*), capacitated cells (*striped bars*), and acrosome-reacted cells (*solid bars*) after the cell incubation alone (*panel a*) or in the presence of 40 μM SVS VII (*panel b*), 45 μM BSA (*panel c*), or a combination of 40 μM SVS VII and 45 μM BSA (*panel d*) for 90 min at 37 °C. The data represent the means of eight individual trials counting 200 cells/treatment/trial. The *error bars* represent the S.D. of the mean. *, $p < 0.01$ in the paired statistical comparison with the corresponding control values are evaluated using one-way analysis of variance.

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 antiserum against SVS VII in the Western blot analysis.

Of the 10 cysteines in the SVS VII molecule, six residues, namely Cys^3, Cys^6, Cys^{14}, Cys^{21}, Cys^{29}, and Cys^{47} were sterically restricted in the predicted β form/β turn (Fig. 3*B*). The chirality of a disulfide bond is relevant to its skewed conformation. Heating SVS VII caused a great diminution of the CD band VI due to the chiral disulfide bond and the disappearance of CD band VII arising from β form/β turn at the same time (Fig. 5), manifesting that the configuration of the disulfide conformers in the protein molecule is important to confine the ordered structure and *vice versa*.

Cytochemical observations revealed that sperm surface has SVS VII-binding sites that cover the entire cell surface (Fig. 6). The demonstration that the dispersed sperm lipids were able to inhibit SVS VII-sperm binding (Fig. 7) supports the notion that the SVS VII-binding sites are lipids in nature. In addition, the binding assay indicated that the active conformation of SVS VII for the binding to sperm could be maintained, although the protein unfolded during heat treatment. ^{125}I-SVS VII blotting of the membrane phospholipid of spermatozoa on TLC plates (Fig. 8*B*) suggests that PtdEtn and PtdSer might constitute the major SVS VII-binding sites on the cell surface. The ability of SVS VII to bind to these two phospholipids was also confirmed by both the ligand blotting of purified lipids on the TLC plate (Fig. 8*B*) and the interaction of SVS VII with PtdSer liposomes (Fig. 9). Together, these two phospholipids constitute less than 15% of the total lipid in the plasma membrane of mouse spermatozoa (43). SVS VII itself is a sperm motility enhancer, but in its unfolded form, on the other hand, it immobilizes sperm. PtdSer liposomes alone can undergo fusion with cell membrane (46, 47) and enhance sperm motility (48, 49) under physiological conditions. More studies are needed to unravel the complexity of interaction among PtdSer liposomes, SVS VII, and sperm.

The phospholipid-binding proteins in the reproductive tract have received attention recently. Several bovine seminal plasma (BSP) proteins, which are the major secretory products of the seminal vesicle, have been purified and identified (50, 51). They belong to a family of closely related acidic proteins, designated BSP-A1, BSP-A2, BSP-A3, and BSP-30 kDa. It has been suggested that they have some role in the membrane modification of spermatozoa that occurs during capacitation and/or acrosome reaction through the interaction with the membrane phospholipids (52–54). In mouse SVS, we demonstrated the presence of the seminal vesicle autoantigen (SVA). SVA is 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 reproductive 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 into the uterus after coitus of the rodent. Lardy *et al.* (45) reported the ability of mouse caltrin to affect the Ca^{2+} 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.

REFERENCES

1. Barros, C. (1977) *Rev. Microsc. Electron* **4,** 107–113
2. Nichol, R., Hunter, R. H., de Lamirande, E., Gagnon, C., and Cooke, G. M. (1997) *J. Reprod. Fertil.* **110,** 79–86
3. Iwamoto, T., Tsang, A., Luterman, M., Dickson, J., de Lamirande, E., Okuno, M., Mohri, H., and Gagnon, C. (1992) *Mol. Reprod. Dev.* **31,** 55–62
4. Jeng, H., Liu, K. M., and Chang, W. C. (1993) *Biochem. Biophys. Res. Commun.* **191,** 435–440
5. Al-Somai, N., Vishwanath, R., Shannon, P., and Molan, P. C. (1994) *Reprod. Fertil. Dev.* **6,** 165–171
6. Peitz, B. (1988) *J. Reprod. Fertil.* **83,** 169–176
7. Robert, M., and Gagnon, C. (1996) *Biol. Reprod.* **55,** 813–821
8. Pang, S. F., Chow, P. H., and Wong, T. M. (1979) *J. Reprod. Fertil.* **56,** 129–132
9. Peitz, B., and Olds-Clarke, P. (1986) *Biol. Reprod.* **35,** 608–617
10. Chen, Y. H., Pentecost, B. T., McLachlan, J. A., and Teng, C. T. (1987) *Mol. Endocrinol.* **1,** 707–716
11. Chen, L. Y., Lin, Y. H., Lai, M. L., and Chen, Y. H. (1998) *Biol. Reprod.* **59,** 1498–1505
12. Huang, Y. H., Chu, S. T., and Chen, Y. H. (1999) *Biochem. J.* **343,** 241–248
13. Huang, Y. H., Chu, S. T., and Chen, Y. H. (2000) *Biol. Reprod.* **63,** 1572–1576
14. Bellv'e, A. R., Zheng, W., and Martinova, Y. S. (1993) *Methods Enzymol.* **225,** 84–113
15. Ruiz-Romero, J., Antich, M., and Bassas, L. (1995) *Andrologia* **27,** 149–153
16. Miyake, M., Coney, P., Iritani, A., and Kling, O. R. (1989) *Gamete Res.* **24,** 49–57
17. Laemmli, U. K. (1970) *Nature* **227,** 680–685
18. Hanspal, J. S., Bushell, G. R., and Ghosh, P. (1983) *Anal. Biochem.* **123,** 288–293
19. Folch, J., Lees, M., and Sloane, Stanley, G. H. (1957) *J. Biol. Chem.* **226,** 497–509
20. Genge, B. R., Wu, L. N., and Wuthier, R. E. (1989) *J. Biol. Chem.* **264,** 10917–10921
21. Markwell, M. A. (1982) *Anal. Biochem.* **125,** 427–432
22. Thaler, C. D., and Cardullo, R. A. (1996) *J. Biol. Chem.* **271,** 23289–23297
23. Desnoyers, L., and Manjunath, P. (1992) *J. Biol. Chem.* **267,** 10149–10155
24. Usui, T. (1963) *J. Biochem.* (*Tokyo*) **54,** 283–286
25. Glenney, J. (1986) *J. Biol. Chem.* **261,** 7247–7252
26. Ward, C. R., and Storey, B. T. (1984) *Dev. Biol.* **104,** 287–296
27. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) *Anal. Biochem.* **150,** 76–85
28. Lehrach, H., Diamond, D., Wozney, J. M., and Boedtker, H. (1977) *Biochemistry* **16,** 4743–4751
29. Lee, C. C., Wu, X. W., Gibbs, R. A., Cook, R. G., Muzny, D. M., and Caskey,

C. T. (1988) *Science* **239,** 1288–1291
30. Lai, M. L., Chen, S. W., and Chen, Y. H. (1991) *Arch. Biochem. Biophys.* **290,** 265–271
31. Ellman, G. L., Courtney, K. D., Valentino, A. J., and Featherstone, R. M. (1961) *Biochem. Pharmacol.* **7,** 88–95
32. Chen, Y. H., Yang, J. T., and Martinez, H. M. (1972) *Biochemistry* **11,** 4120–4131
33. Chen, Y. H., Yang, J. T., and Chau, K. H. (1974) *Biochemistry* **13,** 3350–3359
34. Chiang, C. T., Wu, C. S., and Yang, J. T. (1978) *Anal. Biochem.* **91,** 13–31
35. Perczel, A., Park, K., and Fasman, G. D. (1992) *Anal. Biochem.* **203,** 83–93
36. Chou, P. Y., and Fasman, G. D. (1974) *Biochemistry* **13,** 222–245
37. Chou, P. Y., and Fasman, G. D. (1978) *Annu. Rev. Biochem.* **47,** 251–276
38. Garneir, J., Osguthorpe, D. J., and Robson, B. (1978) *J. Mol. Biol.* **120,** 97–120
39. Chen, Y. H., Tai, J. C., Huang, W. J., Lai, M. Z., Hung, M. C., Lai, M. D., and Yang, J. T. (1982) *Biochemistry* **21,** 2592–2600
40. Beychok, S., and Breslow, E. (1968) *J. Biol. Chem.* **243,** 151–154
41. DelMar, E. G., Largman, C., Brodrick, J. W., and Geokas, M. C. (1979) *Anal. Biochem.* **99,** 316–320
42. Lottenberg, R., Christensen, U., Jackson, C. M., and Coleman, P. L. (1981) *Methods Enzymol.* **80,** 341–361
43. Clegg, E. D., and Foote, R. H. (1973) *J. Reprod. Fertil.* **34,** 379–383
44. Ohnishi, S., and Ito, T. (1974) *Biochemistry* **13,** 881–887
45. Coronel, C. E., Winnica, D. E., Novella, M. L., and Lardy, H. A. (1992) *J. Biol. Chem.* **267,** 20909–20915
46. Martin, O. C., and Pagano, R. E. (1987) *J. Biol. Chem.* **262,** 5890–5898
47. Hoekstra, D. (1982) *Biochemistry* **21,** 2833–2840
48. Wilhelm, K. M., Graham, J. K., and Squires, E. L. (1996) *Cryobiology* **33,** 320–329
49. Butler, W. J., and Robert, T. K. (1975) *J. Reprod. Fertil.* **43,** 183–187
50. Manjunath, P., and Sairam, M. R. (1987) *Biochem. J.* **241,** 685–692
51. Manjunath, P., Chandonnet, L., Leblond, E., and Desnoyers, L. (1994) *Biol. Reprod.* **50,** 27–37
52. Thérien, I., Bleau, G., and Manjunath, P. (1995) *Biol. Reprod.* **52,** 1372–1379
53. Thérien, I., Soubeyrand, S., and Manjunath, P. (1997) *Biol. Reprod.* **57,** 1080–1088
54. Thérien, I., Moreau, R., and Manjunath, P. (1999) *Biol. Reprod.* **61,** 590–598
55. Lichtenheld, M. G., Olsen, K. J., Lu, P., Lowrey, D. M., Hameed, A., Hengartner, H., and Podack, E. R. (1988) *Nature* **335,** 448–451
56. Nagpurkar, A., Saxena, U., and Mookerjea, S. (1983) *J. Biol. Chem.* **258,** 10518–10523
57. Jenny, R. J., Pittman, D. D., Toole, J. J., Kriz, R. W., Aldape, R. A., Hewick, R. M., Kaufman, R. J., and Mann, K. G. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 4846–4850
58. Gilbert, G. E., Furie, B. C., and Furie, B. (1990) *J. Biol. Chem.* **265,** 815–822
59. Vehar, G. A., Keyt, B., Eaton, D., Rodriguez, H., O'Brien, D. P., Rotblat, F., Oppermann, H., Keck, R., Wood, W. I., Harkins, R. N., Tuddenham, E. G., Lawn, R. M., and Capon, D. J. (1984) *Nature* **312,** 337–342
60. Bloom, J. W. (1989) *Thromb. Res.* **54,** 261–268
61. Perin, M. S., Fried, V. A., Mignery, G. A., Jahn, R., and Südhof, T. C. (1990) *Nature* **345,** 260–263
62. Hawgood, S. (1989) *Am. J. Physiol.* **257,** L13–L22
63. Liu, T. Y., Syin, C., Nguyen, N. Y., Suzuki, A., Boykins, R. A., Lei, K.-J., and Goldman, N. (1987) *J. Protein Chem.* **6,** 263–272
64. Brewer, H. B., Jr., Ronan, R., Meng, M., and Bishop, C. (1986) *Methods Enzymol.* **128,** 223–246
65. Zilversmit, D. B. (1983) *Methods Enzymol.* **98,** 565–573
66. Wirtz, K. W. A. (1982) *Lipid-Protein Interactions* (Jost, P. C., and Griffith, O. H., eds), pp. 151–231, Wiley Press (Interscience), New York
67. Vahouny, G. V., Chanderhan, R., Kharroubi, A., Noland, B. J., Pastuszyn, A., and Scallen, T. J. (1987) *Adv. Lipid Res.* **22,** 83–113