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Purification, molecular cloning and functional characterization of swine phosphatidylethanolamine-binding protein 4 from seminal plasma

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ABSTRACT

Phosphatidylethanolamine-binding proteins (PEBPs) are found in various species and have multiple functions. In this study, we purified the swine homolog of human PEBP4 (sPEBP4) from swine seminal plasma, cloned the sPEBP4 cDNA and functionally characterized this protein. The molecular mass of the purified protein was calculated to be 25 kDa by SDS–polyacrylamide gel electrophoresis under reducing conditions. The full-length cDNA of sPEBP4 contains 815 bp with an open reading frame of 669 bp that encodes a protein 222 residues in length. sPEBP4 contains a putative phosphatidylethanolamine-binding domain between residues 79 and 195; however, this domain did not show lipid binding activity. The overall amino acid sequence identity of PEBP4s from swine, human, mouse, bovine and canine ranges between 56.1% and 82.4%. Immunohistochemical staining and western blotting analysis showed that sPEBP4 is secreted from epithelial cells in the epididymis to the seminal plasma. To explore the role of sPEBP4 in the seminal plasma, we tested the effect of sPEBP4 on swine sperm motility. Sperms suspended in phosphate-buffered saline began to swim after the addition of purified sPEBP4, but not when swine serum albumin was added, indicating that sPEBP4 promotes sperm motility.

1. Introduction

Phosphatidylethanolamine-binding proteins (PEBPs) have been identified in various organisms: bacteria, yeast, nematodes, plants, drosophila and mammals [1]. The members of the PEBP family are expressed in different tissues such as the brain and testis [2–6]. Mouse PEBP1, a member of the PEBP family, exerts inhibitory activity against several serine proteases including thrombin, neuropsin and chymotrypsin [5], whereas human PEBP1, also called Raf kinase inhibitory protein, inactivates the Raf-1 and MEK1 pathway [7,8]. Thus, PEBP homologs from different species may have respective functions.

Human PEBP4, a new member of the PEBP family, is highly expressed in cancer cells and inhibits tumor necrosis factor-induced apoptosis [9], whereas mouse PEBP4 is expressed specifically in the eye [10]. Mouse PEBP4 promotes cell migration and protects against drug-induced apoptosis by inhibiting ERK1/2 and JNK activation and up-regulating the expression of COX-2.

We have purified several proteins, such as angiotensin I-converting enzyme, dipeptidyl peptidase II and dipeptidyl peptidase IV, from swine seminal plasma [11–14]. In this study, we conducted purification, molecular cloning and functional characterization of a newly isolated protein from swine seminal plasma. This protein consists of 222 amino acids deduced from the nucleotide sequencing data. We named this protein swine PEBP4 (sPEBP4) because sPEBP4 has a similar sequence to PEBP4s from other species. The results of immunohistochemical staining of a series of sections in the male genital tracts suggested that sPEBP4 is secreted from the epididymis. Functional analysis of this protein demonstrated its ability to stimulate sperm motility.

Abbreviations: EDTA, ethylene diamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; 2-ME, 2-mercaptoethanol; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PEBP, phosphatidylethanolamine-binding protein; PVDF, polyvinylidene difluoride.

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2. Materials and methods

2.1. Purification of swine seminal proteins

All purification steps were performed at 4 °C unless otherwise specified, and all buffers prepared contained 1 mM ethylene diamine tetraacetic acid (EDTA) and phenylmethylsulfonyl fluoride. Swine seminal plasma (~500 ml), which was kindly provided by Okayama Prefecture Center for Animal Husbandry (Okayama, Japan), was dialyzed overnight against 20 mM Tris–HCl buffer, pH 7.5. After centrifugation of the dialyzate at 8000*g* for 45 min, the supernatant was loaded onto a Q Sepharose column (6 × 10 cm; GE Healthcare, Piscataway, NJ, USA) equilibrated with 20 mM Tris–HCl buffer, pH 7.5. After the column had been washed extensively with the equilibration buffer, the elution was conducted with a linear gradient from 0 to 0.5 M NaCl. The first peak of the fractions (10 ml/collection tube) were collected and concentrated with solid ammonium sulfate (80% saturation). The concentrated fractions were dialyzed overnight against 20 mM phosphate buffer, pH 6.8. The dialyzate was applied onto a Matrex Gel Red A column (2.5 × 20 cm; Amicon, Lexinton, MA, USA) equilibrated with 20 mM phosphate buffer, pH 6.8, and the second peak pass-through fractions (5 ml/collection tube) were eluted with a linear gradient from 0 to 0.5 M NaCl and collected. After overnight dialysis against phosphate-buffered saline (PBS), pH 8.0, containing 1.5 M ammonium sulfate, the dialyzate was applied at a flow rate of 0.2 ml/min onto a Phenyl Superose HR5/5 column (Pharmacia, Uppsala, Sweden) equilibrated with PBS, pH 8.0, containing 1.5 M ammonium sulfate. The first peak flow-through fractions (1 ml/collection tube) were collected, dialyzed overnight against PBS and used in subsequent experiments.

2.2. N-Terminal amino acid sequencing

The N-terminal amino acid sequencing of the purified protein was performed as described previously [14]. Briefly, the purified protein was applied onto 10% SDS–polyacrylamide gel electrophoresis (PAGE), and the protein on the gel was blotted onto a polyvinylidene difluoride (PVDF) membrane. The protein bands that had transferred to the membrane were excised and sequenced using an Applied Biosystems Model 492cLC Protein Sequencer equipped with an online phenylthiohydantoin analyzer (ABI 140D Microgradient Delivery System, Foster City, CA, USA).

2.3. cDNA cloning and sequencing

Total RNA was extracted from the swine male genital tract tissues, which were purchased from a local slaughterhouse (Second Central Municipal Wholesale Market, Kyoto, Japan), and reversely transcribed using the Transcriptor first-strand cDNA synthesis kit (Roche Diagnostics, Tokyo, Japan) according to the instruction manual. To obtain the whole cDNA of the purified protein, we performed PCR using the above first-strand cDNA as a template and a pair of appropriate forward and reverse primers. In this procedure, one forward primer was in the SMART PCR cDNA synthesis kit (BD Biosciences Clontech, Franklin Lakes, NJ, USA), and two kinds of mixed forward primers, 5′-CARTTYATGACNCARTAYTAYCA-3′ and 5′-GCNCARTTYTGGAGRCAYTGG-3′, were designed on the basis of our directly determined amino acid sequences, Gln–Phe–Met–Thr–Gln–Tyr–Tyr and Ala–Gln–Phe–Trp–Arg–His–Trp, respectively. In the primer sequence, "R" stands for G/A, "Y" stands for C/T and "N" stands for A/G/C/T. Oligo-dT and two primers, 5′-CCA GACCGAGGGCGGTGAGCACTGTAGA-3′ and 5′-GCCCTTGATATCTGT CACCAG-3′, were used as reverse primers. DNA sequencing of the obtained cDNA was conducted by a PE Applied Biosystems DNA sequencer PRISM 310 at the Central Research Laboratory located at the Shiga University of Medical Science.

2.4. Anti-sPEBP4 antibody production

Antibody preparation was carried out at the Research Center for Animal Life Science located at the Shiga University of Medical Science under the institutional guidelines for animal use. Briefly, purified sPEBP4 protein (200 μg) was used to immunize a rabbit and the antisera from the rabbit were purified using a cyanogen bromide-activated Sepharose 4B column (GE Healthcare) coupled with sPEBP4 to obtain an anti-sPEBP4 polyclonal antibody.

2.5. Polyacrylamide gel electrophoresis and western blot analysis

Proteins purified from swine seminal plasma were separated by SDS–PAGE in the presence or absence of 2-mercaptoethanol (ME), and stained with Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA, USA). For western blot analysis, proteins from male genital tissues were extracted with lysis buffer (50 mM Tris–HCl containing 0.15 M NaCl, 0.1% SDS, 1% sodium chloride, 1% Nonidet P-40, 1 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride, pH 7.4), separated by SDS–PAGE, and then transferred onto PVDF membranes. The membranes were probed with an anti-sPEBP4 antibody (1:1000). After treating the membranes with anti-rabbit IgG horseradish peroxidase-linked secondary antibody (1:1000), bound antibodies were detected using enhanced chemiluminescence (GE Healthcare) and imaged on an LAS-4000 luminescent image analyzer (Fujifilm Life Science, Stamford, CT, USA).

2.6. Immunohistochemical staining of various swine male genital tracts

Immunohistochemical staining of the sections in the adult swine male genital tract tissues, including the testis, epididymis, seminal vesicle and prostate, was carried out as described previously [15]. Briefly, these tissue specimens were fixed in 10% neutral buffered formalin to be embedded in paraffin, and 5-mm-thick paraffin sections were cut. Immunostaining was performed with an anti-sPEBP4 antibody and a Histofine SAB-PO kit (Nichirei, Tokyo, Japan) according to the manufacturer's instructions. Their images were taken using a Nikon Microphot-FXA microscope (Tokyo, Japan).

2.7. Phospholipid binding assay

Detection of phospholipid binding was performed by an enzyme-linked immunosorbent assay (ELISA) using commercially available phospholipids: phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine, as described previously [16]. Briefly, the wells of a 96-well plate were coated with 50 μg/ml phosphatidylserine, phosphatidylcholine or phosphatidylethanolamine overnight at 4 °C. After blocking with 10% fetal calf serum, 2 μM sPEBP or GST-hPEBP1 was added in doublet in each well. The plate was subsequently incubated for 1 h at room temperature. The binding activity was assessed using an anti-sPEBP4 or anti-GST antibody (Santa Cruz Biotechnology), followed by the detection of the antibody binding using a HRP-conjugated second antibody. 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (Roche Diagnostics, Tokyo, Japan) was used as a substrate. The absorbance at 405 nm was then measured by a Multiscan JX photometer (Thermo Lab Systems, Yokohama, Japan).

2.8. Sperm motility assay

Swine sperms from the epididymis cauda were washed three times with PBS and suspended in PBS. The sperm solution (100 μl) was gently mixed with 1.5 μg of purified sPEBP4 or purified swine serum albumin and transferred onto a glass plate. The motility of the sperms was observed by an Olympus inverted microscope IX-71 (Tokyo, Japan) system with a camcorder.

3. Results

3.1. Protein purification from swine seminal plasma

Protein purification was performed from approximately 500 ml of swine seminal plasma by Q Sepharose, Matrex Red A and Phenyl Superose HR5/5 columns (Fig. 1A–C). We analyzed the eluate of the final step included in the flow-through fractions from 3 to 13 (Fig. 1C). Proteins in these fractions were separated by SDS–PAGE in the presence or absence of 2-ME and the gel was stained with Coomassie Brilliant Blue. The resulting SDS–PAGE showed only a single band under both conditions (Fig. 1D), indicating that one protein existed in the collected eluate. The molecular weight (*Mr*) of the protein estimated from the SDS–PAGE analysis was 24 and 25 kDa in the absence and presence of 2-ME, respectively.

3.2. Amino acid and nucleotide sequence of the purified protein

The amino acid sequence of the purified protein was determined by automated N-terminal sequencing. The first 29 amino acids of the protein were GDEEDSDPCV[10] YEALSDSDAV[20] LCKGLKVFY[29] (Fig. 2A, double-underlined). The internal amino acid sequence of the protein was further determined using a set of overlapping peptides generated by cleavage at different peptide bonds by bromocyan and the lysyl endopeptidase (Fig. 2A, underlined). Using this approach we were able to directly determine 78% of the amino acid sequence of the protein based on the whole amino acid sequence deduced from the result of the nucleotide sequencing experiment as described below.

We then obtained the whole cDNA of the protein by RT-PCR as described in the Section 2. The nucleotide sequence of the cDNA and the deduced amino acid sequence are shown in Fig. 2A. The cDNA was considered to be composed of 815 bp: 18 bp of a 5′ non-coding sequence, 669 bp of a predicted coding sequence, a stop codon, 108 bp of a 3′ non-coding sequence and 20 bp of a poly(A) tail. A potential initiation codon (ATG) began at nucleotide 19. The nucleotide sequence (CCATGG) surrounding this initiation codon was in agreement with the consensus sequence (NNATGG) for the start of translation in eukaryotes [17]. The total number of amino acid residues of this protein predicted from the coding region is 222. Searches in NCBI BLAST and EMBL-EBI FASTA databases

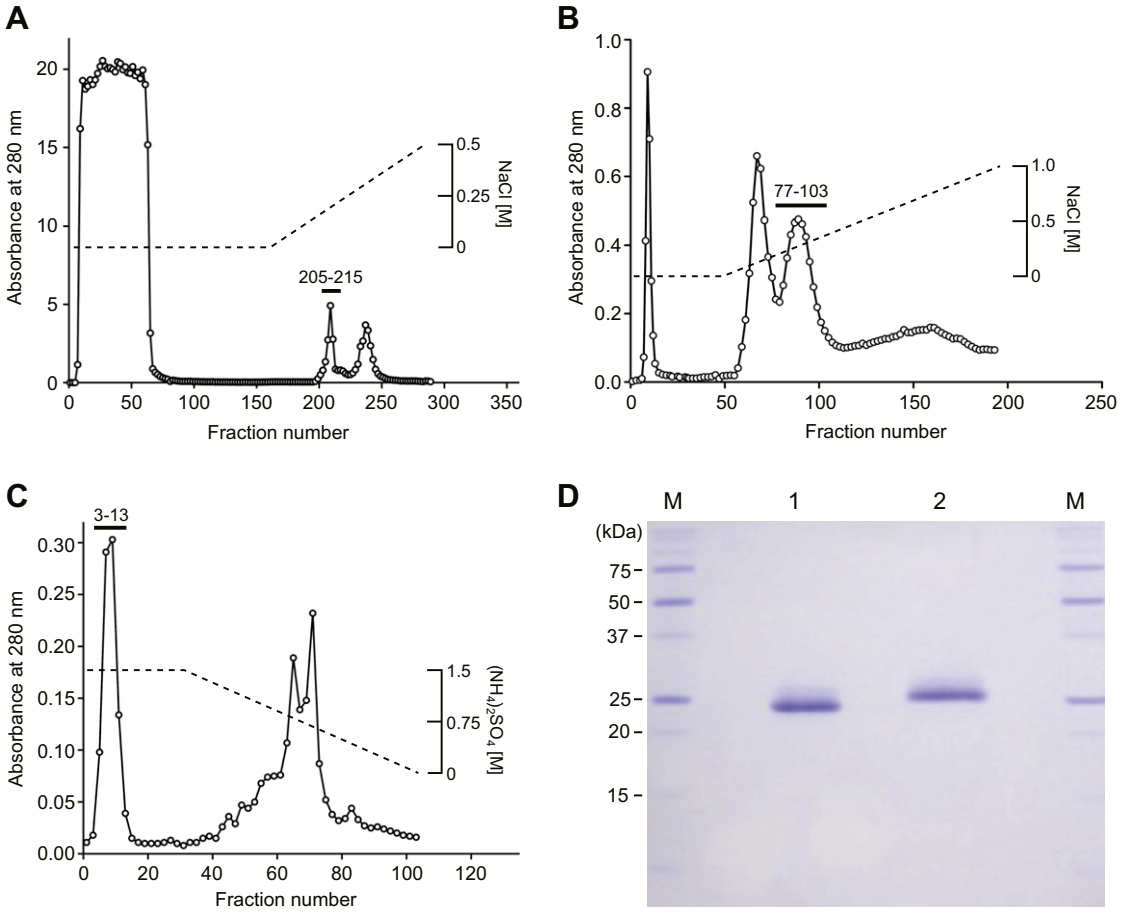

Fig. 1. Protein purification from swine seminal plasma. (A) Q Sepharose column chromatography. Dialyzed swine seminal plasma was applied onto this column. Fractions (numbered from 205 to 215, indicated by the bar) were collected, concentrated with solid ammonium sulfate and dialyzed. (B) Red A Sepharose column chromatography. The prepared sample described in (A) was applied onto this column. Fractions (numbered from 77 to 103, indicated by the bar) were collected, concentrated and dialyzed. (C) Phenyl Superose HR5/5 column chromatography. The prepared sample described in (B) was applied onto this column. Fractions (numbered from 3 to 13, indicated by the bar) were collected and dialyzed. (D) Coomassie Brilliant Blue staining. The sample obtained in (C) was separated by SDS–PAGE on a 12.5% polyacrylamide gel in the absence of 2-ME (lane 1; non-reducing condition) or in the presence of 2-ME (lane 2; reducing condition), and stained with Coomassie Brilliant Blue R-250. M, molecular marker.

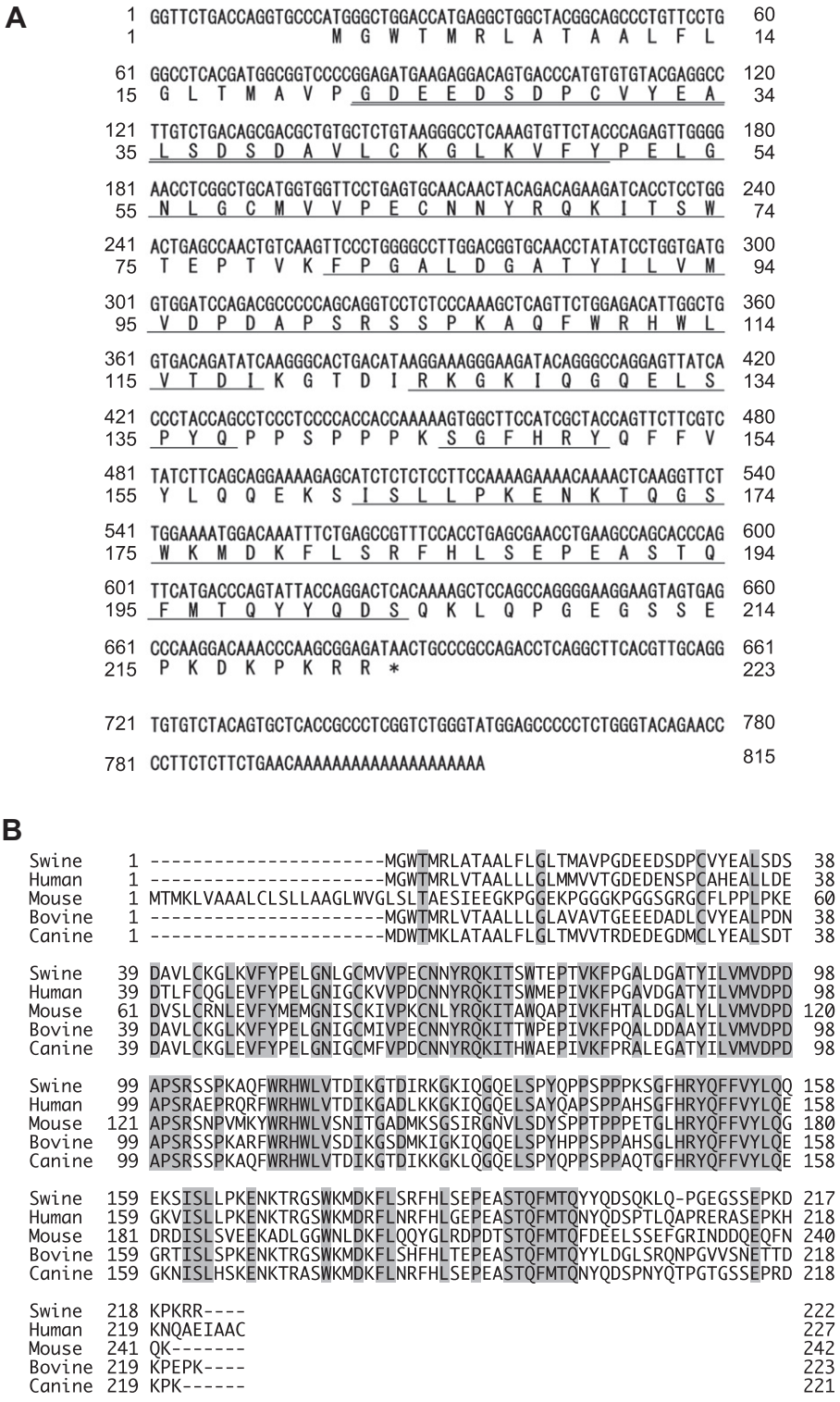

Fig. 2. (A) Nucleotide sequence and deduced amino acid sequence of the purified protein. Single-letter notions are used for amino acids. The first 29 amino acid sequence of the purified protein from swine seminal plasma determined by Edman degradation is double-underlined. The amino acid sequences of derived fragments by the BrCN- and lysyl endopeptidase-digestion are shown underlined. An asterisk indicates the stop codon. (B) Comparison of the amino acid sequence of sPEBP4 with those of human, mouse, bovine and canine PEBP4. The amino acid sequence of sPEBP4 is aligned against sequences from PEBP4 of human, mouse, bovine and canine (Accession numbers: NP_659399, NP_082836, NP_001029612 and XP_851204, respectively). Conserved amino acid residues are shown in gray boxes.

revealed that the sequence of the purified protein showed significant similarity to that of PEBP4 in several species (Fig. 2B). Thus, the purified protein was designated swine PEBP4 (sPEBP4). The levels of amino acid homology were 77.2% (swine versus human), 56.1% (swine versus mouse), 77.6% (swine versus bovine) and 82.4% (swine versus canine). The signal sequence-like region of sPEBP4 was considered to be residues 1 to 21, because the N-terminal-directed amino acid sequencing started at Gly[22]. A calculated molecular mass of sPEBP4 without the signal sequence-like region was 22,900, essentially consistent with the

results of SDS–PAGE shown in Fig. 1D. The nucleotide sequence determined in this study has been submitted to the DDBJ/GenBank/EBI Data Bank (GenBank ID: AB506709).

3.3. Localization of sPEBP4 in male genital tracts

To determine the sPEBP4 secretion area in swine male genital tracts, we performed immunohistochemical staining with sections of the genital tracts including the testis, epididymis caput, epididymis corpus, epididymis cauda, prostate and seminal vesicles. For this experiment, we originally produced an anti-sPEBP4 antibody, which clearly detected sPEBP4 in swine seminal plasma as a 24-kDa single band in western blotting analysis when seminal plasma proteins were separated by SDS–PAGE under non-reducing conditions (Supplementary Fig. S1). The epididymis cauda as well as the secretion fluid were strongly stained by the anti-sPEBP4 antibody (Fig. 3A). In particular, the lumen side of villous epithelial cells in the epididymis cauda, not the connective tissue side, was stained strongly (Fig. 3A'). Epididymis caput and corpus were moderately immunostained, but the testis, prostate and seminal vesicles were hardly stained (Fig. 3B, C and data not shown). To confirm these results, western blot analysis using the lysates from a series of sections in swine male genital tracts was carried out. The bands for sPEBP4 were detected in the lanes of the epididymis caput (lane 2), corpus (lane 3) and cauda (lane 4) as well as seminal plasma (lane 7) (Fig. 3D). However, the band was not visible in the lane of the testis (lane 1), prostate (lane 5) or seminal vesicle (lane 6). These results suggest that sPEBP4 is secreted from epithelial cells in the epididymis, mainly at the cauda.

3.4. Phospholipid binding activity of sPEBP4

We next examined whether sPEBP4 is able to bind to phosphatidylethanolamine, because sPEBP4 has a putative phosphatidylethanolamine-binding domain (residues 79–195) predicted by the FASTA database, and another member of the PEBP family, human PEBP1, is reported to be capable of binding to phospholipids [18]. The phospholipid binding assay showed that the binding of sPEBP4 to each phospholipid was significantly less than that of GST-human PEBP1 to phosphatidylethanolamine, indicating that sPEBP4 has very low or no binding activity towards the tested phospholipids, including phosphatidylethanolamine (Fig. 4).

3.5. Effect of sPEBP4 on sperm motility

We finally investigated the functional significance of sPEBP4 secreted into seminal plasma, focusing on its effect on sperm motility. When washed sperms were suspended in PBS and incubated with sPEBP4 or swine serum albumin as a control, sperms incubated with sPEBP4 actively moved (Supplementary Movie 1), but sperms incubated with albumin did not (Supplementary Movie 2). This result indicates that sPEBP4 promotes sperm motility.

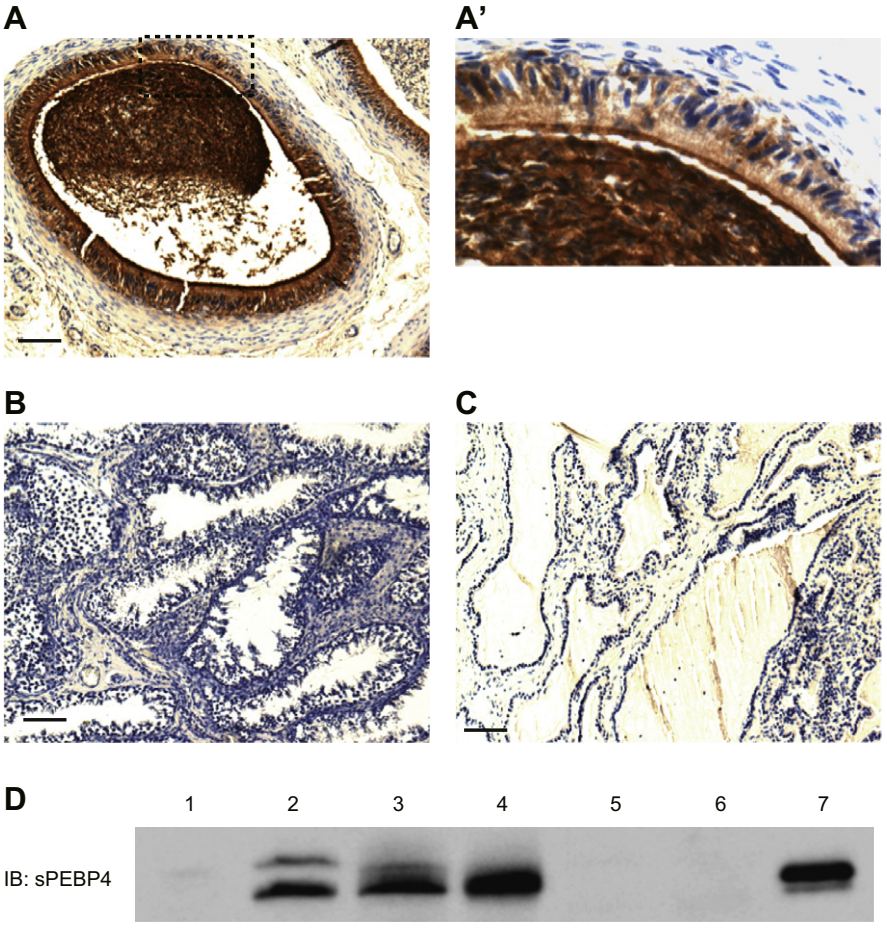

Fig. 3. Expression of sPEBP4 in swine male genital tracts. (A–C) Immunohistochemical staining of sPEBP4 in various regions of the genital tract tissues. Fixed sections of the tissues were immunostained with an anti-sPEBP4 antibody. (A) Epididymis cauda; (A′) an enlarged image of the boxed area in (A); (B) testis; (C) prostate. Scale bars: 50 μm. (D) Western blot analysis of various regions of the male genital tract tissues. Tissue lysates of various regions, testis (lane 1), epididymis caput (lane 2), epididymis corpus (lane 3), epididymis cauda (lane 4), prostate (lane 5), seminal vesicle (lane 6) and seminal plasma (lane 7), were immunoblotted with an anti-sPEBP4 antibody.

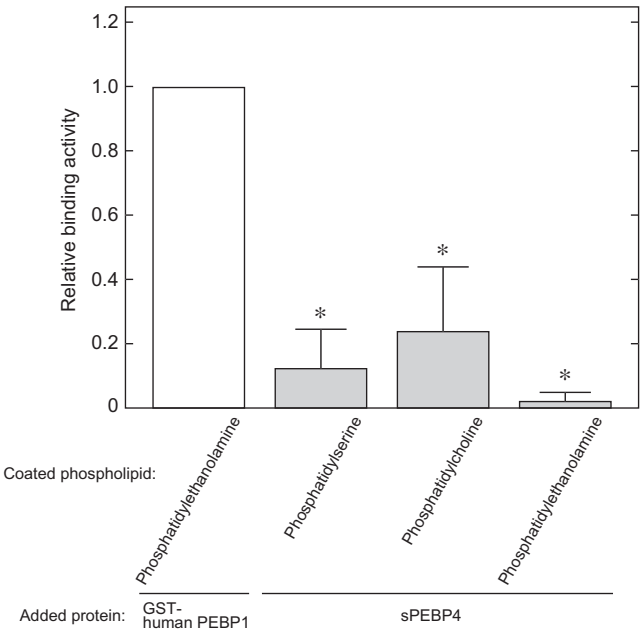

Fig. 4. Binding assay of sPEBP4 to phospholipids. The 96-well plate was coated with phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine, and then sPEBP4 or GST-human PEBP1 was added into the wells. The binding activity was measured by ELISA. The relative binding activities were calculated by comparison with the value of GST-human PEBP1 binding activity to PE, which was set as 1. The relative activities represent the average of three experiments. The error bars indicate standard deviations. *, $p < 0.01$ vs. GST-human PEBP1.

4. Discussion

Purification and molecular cloning of sPEBP4 from swine seminal plasma was successfully completed for the first time. The protein consists of 222 amino acids as determined directly by N-terminal amino acid sequencing and nucleotide sequencing of its cDNA. sPEBP4 appears to have a signal sequence (residues 1–21) and is thus secreted; although PEBP1 and PEBP2 contain no signal peptide-like amino acid sequence and localize within the cytoplasm of cells [5,6]. Unlike human PEBP, sPEBP4 did not bind to phospholipids, including phosphatidylethanolamine, despite the existence of a putative phosphatidylethanolamine-binding domain. The precise reason for the different phosphatidylethanolamine-binding ability among PEBP4 homologs remains unresolved. It is likely that only a few amino acid differences between the domains from these proteins may affect the conformation and lead to the core binding motif localizing to the interior of the protein and thus unable to function as a lipid binding protein.

Functional analysis of sPEBP4 revealed that it promotes sperm motility. The molecular mechanism that stimulates mobility was not investigated in this study. In a previous study, mouse PEBP4, which localizes primarily to the endoplasmic reticulum/Golgi apparatus, promotes cellular migration and invasion by inhibiting the activation of MAP kinases [10]. Since sPEBP4 is, however, secreted into seminal plasma and is incapable of binding to phospholipid components of the plasma membrane, it is difficult to envisage that this protein functions inside the sperm. Thus, we speculate that sPEBP4 may be associated with molecules located on the surface of the sperm plasma membrane, and that this association may transduce a signal into the sperm to activate signaling pathways that enhance sperm motility. A future study will be necessary to further elucidate the mechanism for the sPEBP4-mediated increase in sperm motility.

There is a report that approximately 10% of couples are troubled with infertility [19]. Since it is roughly estimated that male infertility results in half of these couples, it is important to study sperm motility, which is one of the major factors that affect fertilization. The results of this study that showed that sPEBP4 dramatically promotes sperm motility may be helpful in the development of new therapeutics in the field of reproductive medicine. As mentioned in the Introduction section, there is the possibility that homologs in PEBP may have respective functions. Consequently, confirmation that human PEBP4 is also present in seminal plasma and functions the same way as sPEBP4 remains to be addressed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.016.

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