Original Article

Prosaposin is a novel coenzyme Q10-binding protein

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(Received 14 July, 2023; Accepted 28 October, 2023; Released online in J-STAGE as advance publication 8 November, 2023)

Coenzyme Q10 (CoQ10) is essential for mitochondrial ATP production and functions as an important antioxidant in every biomembrane and lipoprotein. Due to its hydrophobicity, a binding and transfer protein for CoQ10 is plausible, and we previously described saposin B as a CoQ10-binding and transfer protein. Here, we report that prosaposin, the precursor of saposin B, also binds CoQ10. As prosaposin is both a secretory protein and integral membrane protein, it is ubiquitous in the body. Prosaposin was isolated from human seminal plasma, and CoQ10 was extracted from hexane solution into the water phase. It was additionally found that immunoprecipitates of mouse brain cytosol generated using two different anti-prosaposin antibodies contained coenzyme Q9. Furthermore, mouse liver cytosol and mouse kidney cytosol also contained prosaposin–coenzyme Q9 complex. These results suggest that prosaposin binds CoQ10 in human cells and body fluids. The significance and role of the Psap-CoQ10 complex *in vivo* is also discussed.

***Key Words*: coenzyme Q10-binding protein, prosaposin, seminal plasma, cytosol**

Coenzyme Q (CoQ), which was first isolated as an essential component of the mitochondrial respiratory chain,[1] is present in all tissues.[2,3] CoQ is thought to function as a lipid-soluble, front-line antioxidant due to its ubiquitous presence in biomembranes and lipoproteins.[2–4] CoQ also has many homologues with isoprene chains of various lengths. For example, the isoprene chain is 10 units (CoQ10) in length in humans and guinea pigs but 9 units (CoQ9) in length in rats and mice.

CoQ is synthesized in the mitochondria, ER-Golgi system, and peroxisomes,[2,3] and therefore, CoQ must be transferred intracellularly to other organelles and extracellularly to lipoproteins. Studies using radiolabeled CoQ have confirmed this pathway. Experiments involving intravenous injection of ^{14}C-labeled CoQ10 in guinea pigs confirmed uptake by the liver, spleen, and adrenal glands, and to a lesser extent, by the kidney and heart.[5] Nakamura *et al.*[6] intravenously injected rats with ^{14}C-labeled CoQ10 and measured its content in subfractions of the heart. At 2 h after administration, radioactivity was found mostly in the cytosolic fraction, followed by the mitochondrial, lysosomal, microsomal, and nuclear fractions at 72 h. At 2 days after intraperitoneal injection of ^{3}H-labeled CoQ10, uptake into various organelles and cytosol of rat liver was confirmed.[7] Oral administration of deuterium-labeled CoQ10 in humans confirmed uptake into the blood circulation.[8] Rosenfeldt *et al.*[9] measured cardiac mitochondrial CoQ10 levels in patients undergoing cardiac surgery and found that levels were significantly higher in patients orally administered CoQ10 (300 mg/day for two weeks) compared with placebo controls and this supplementation resulted in greater ATP production in mitochondria. These results clearly demonstrate that CoQ10 is transferred intracellularly and extracellularly, resulting in its ubiquitous distribution throughout the body.

We previously identified saposin B (Sap B) in human urine as a CoQ-binding and transfer protein and suggested that prosaposin (Psap),[10,11] the precursor of Sap B, might be also a CoQ-binding protein.[10] Here, we report the direct evidence that Psap is a CoQ-binding protein. Psap is both an integral membrane protein (68 kDa, according to the difference in glycosidation) and secretory protein (73 kDa) and thus a ubiquitous protein in the body.[11] Intercellular Psap is proteolytically processed to generate saposin A, B, C, and D.[11] The highest concentrations of Psap are found in the brain and testes.[11] We isolated Psap from human seminal plasma and demonstrated that water-soluble Psap extracted CoQ10 in hexane. Furthermore, we demonstrated that immunoprecipitates of mouse brain cytosol generated using two different anti-Psap antibodies contained CoQ9. Furthermore, mouse liver cytosol and mouse kidney cytosol also contained the Psap-CoQ9 complex. These results suggest that Psap binds CoQ10 in human cells and body fluids.

Materials and Methods

The protocol of this study was approved by the Ethics Committee of Tokyo University of Technology (E23LA-004). Seminal plasma samples were obtained from healthy volunteers and stored at −20°C.

Lipid analysis. CoQ homologue concentrations were determined using an HPLC-ECD system, as reported previously,[12] with minor modifications. Briefly, samples were added to a 9-fold volume of HPLC-grade 2-propanol (Fisher Chemicals, Fairlawn, NJ), vigorously mixed, and centrifuged. Supernatants thus obtained were injected onto the HPLC-ECD system. Mobile phase: 50 mM $NaClO_4$ in methanol/2-propanol (7/3, v/v); flow rate: 1.0 ml/min; analytical column: KANTO RP-18 (L) GP, 5 μm × 150 mm × 4.6 mm (Kanto Chemical, Tokyo, Japan); post-reduction column: RC-10, 15 mm × 4 mm (IRICA, Kyoto, Japan); detector: ECD (600 mV) NANOSPACE SI-1 (Shiseido, Tokyo, Japan).

Purification of Psap. Seminal plasma (43 ml) from healthy volunteers was pooled, protease inhibitors (1 μg/ml tosyl-phenylalanine chloromethyl ketone, 1 μg/ml pepstatin, 1 μg/ml tosyl-lysine chloromethyl ketone, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) were added, and the samples were then centrifuged at 2,330 × *g* for 10 min at 4°C and stored at −20°C. The samples were mixed with saturated ammonium sulfate solution and stirred for 30 min in a cold room to precipitate proteins, then centrifuged at 2,330 × *g* for 10 min at 4°C. The resulting precipitate was collected and dissolved in 200 ml of DEAE buffer (20 mM NaPi, 50 mM NaCl, pH 7.0) containing the same protease inhibitors.

Dissolved protein samples were dialyzed in a cellulose tube

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doi: 10.3164/jcbn.23-57

(Sanko Juneau, Tokyo, Japan) against 3 L of the abovementioned DEAE buffer overnight in a cold room and then dialyzed against fresh DEAE buffer for 60 min. The samples were mixed with protease inhibitors, and desalting was confirmed by chromatography for protein purification (AKTA go; Wakenyaku, Kyoto, Japan).

Next, each desalted protein sample was applied to a DEAE Sepharose column (100 ml) pre-equilibrated with start buffer (20 mM phosphate buffer containing 50 mM NaCl, pH 7.0). Proteins were eluted at a flow rate of 7.0 ml/min using elution buffer (1.0 M NaCl in start buffer, pH 7.0) and a step-wise gradient of NaCl. Psap was detected by Western blotting using Sap B monoclonal antibody and goat anti-mouse IgG-HRP as the primary and secondary antibodies, respectively. Psap-rich fractions were concentrated and subjected to gel filtration (Hiload 26/60 Superdex 200 pg) (Amersham Biosciences). Proteins were eluted using 50 mM phosphate buffer containing 150 mM NaCl (pH 7.0) at a flow rate of 1.0 ml/min. Psap level was by Western blotting as described above, and Psap-rich fractions were pooled and mixed with the abovementioned protease inhibitors. Concentrated fractions from Superdex 200 pg gel filtration were then subjected to affinity chromatography using and anti-Sap B antibody. The column was prepared by AfFigel Hz (Bio-Rad Japan, Tokyo, Japan) and pre-equilibrated with start buffer (20 mM phosphate buffer, pH 7.0). Proteins were eluted using elution buffer (0.1 M glycine, 0.5 M NaCl, pH 2.7) at a flow rate of 1.0 ml/min with a step-wise gradient of glycine. The level of Psap in each fraction was then determined, and Psap-rich fractions were pooled and concentrated using an ultrafiltration membrane (Amicon 50K; Millipore, Tokyo, Japan).

SDS-PAGE, silver staining, and Western blotting. Samples were separated by electrophoresis on an SDS-polyacrylamide gel (12% acrylamide). After electrophoresis, the gel was stained using a silver staining kit (ATTO, Tokyo, Japan). For Western blotting analysis, proteins were transferred onto PVDF membranes. The membranes were incubated with mouse anti-Sap B IgG for 1 h at room temperature. Proteins were visualized with HRP-conjugated secondary antibodies (Bio-Rad Japan).

Lipid-binding assay. Psap (0.5 μM) in 10 mM phosphate buffer (pH 7.4) containing 150 mM NaCl was vigorously mixed for 5 min with hexane containing 10 mM CoQ10, followed by centrifugation at 3,000 × *g* for 20 min. Hexane was removed, and the aqueous layer was further centrifuged in Eppendorf tubes at 3,000 × *g* for 10 min to separate contaminating hexane. The concentration of CoQ10 in the aqueous layer was determined by HPLC-ECD. Psap-free phosphate buffer was used as a blank.

Mouse tissue cytosol preparation. The liver, kidney, and brain were removed from normal mice (male C57BL-6J, 240 days old) and homogenized with 9 volumes of 0.25 M sucrose buffer (1 M Tris-HCl, 500 mM EDTA, pH 7.4) containing the abovementioned protease inhibitors. The resulting suspension was centrifuged at 600 × *g* for 10 min, and the supernatant was centrifuged at 8,000 × *g* for 10 min. The final supernatant was centrifuged at 100,000 × *g* for 1 h, and the supernatant was used as the cytosol fraction.

Immunoprecipitation. Two types of Psap polyclonal antibodies were used. One antibody was obtained from Dr. Matsuda (Tokai University), and the other was purchased from Santa Cruz Biotechnology (Dallas, TX). The antibodies were generated by immunizing rabbits with synthetic oligopeptides corresponding to 178-LYPQDHPRSQPQPKAN-193, 299-EMMDPYEQNLVQAH-312, 413-KEPTPPKQPAQPKQSALP-430,[13] and 301-MDPYEQNLVQAHNVILCQTCQFVMNKFSELIVNNATEELLVKGLSNACALLPDPARTKCQEVVGTFGPSLLDIFIHEVNPSSLCG-385 (Santa Cruz, technical note sc-32876) of the 557-amino acid mouse Psap (UniProt: Q61207), respectively.

Samples were placed in protein LoBind tubes (Eppendorf, Hamburg, Germany) and incubated with 5 μg of anti-Psap IgG or normal rabbit IgG for 1 h at room temperature. We tested two different types of anti-Psap IgG. After incubation, 10 μl of protein G beads (Amersham Biosciences, Buckinghamshire, UK) were added to the samples, followed by further incubation for 1 h. The beads were then washed three times with PBS. CoQ9 was extracted from the beads using 2-propanol and analyzed by HPLC-ECD. The beads were mixed with SDS-PAGE sample buffer, and Psap was analyzed by Western blotting.

Statistical analyses. Data were analyzed using the Student *t* test, and values are indicated as the mean ± SD. Significant differences between the two groups are denoted by ** and *** ($p<0.01$ and 0.001, respectively). Statistical analysis was performed using BellCurve for Excel (Social Survey Research Information Co., Ltd., Tokyo, Japan).

Results

Purification of Psap. We first isolated Psap from human seminal plasma using ammonium sulfate precipitation, anion exchange chromatography, gel filtration chromatography, and affinity chromatography using an anti-Sap B antibody, as described in the Materials and Methods. The chromatographic results are shown in Fig. 1. SDS-polyacrylamide gel electrophoreses revealed that purification of the protein proceeded progressively, and a single protein band was detected at a molecular weight of 73 kDa (Fig. 2). The secretory form of Psap is 73 kDa,[11] identical to the molecular weight of the purified protein.

Psap is a precursor of saposins A, B, C, and D, and each saposin has a similar amino acid sequence.[11] Therefore, each has 4 similar peptides. Sap B creates a shell-like dimer possessing a hydrophobic cavity for lipid binding.[14] Thus, it is not surprising that Psap creates a similar hydrophobic cavity for CoQ10 binding via the Sap B domain and one of the other saposin peptides. Similar degrees of CoQ10 binding by Psap and Sap B were obtained in the same CoQ10 extraction assay; molar ratios of CoQ10 binding by Psap and Sap B were 0.12 (Fig. 3) and 0.08,[10] respectively.

Presence of Psap-CoQ9 complex in mouse cells. We immunoprecipitated mouse brain cytosol using two types of anti-Psap antibodies against normal rabbit IgG in order to confirm the cellular presence of the Psap-CoQ9 complex. As shown in Fig. 4, the two types of anti-Psap IgG, but not normal IgG, precipitated both Psap and CoQ9. The Psap-CoQ9 complex was also found in liver cytosol and kidney cytosol (Fig. 5), suggesting that Psap is a CoQ-binding protein.

Discussion

Difference between Psap and Sap B. What is the difference between the two CoQ-binding proteins? Sap B is present *in vivo* primarily in the intracellular space,[11] but Psap is present in both the intracellular and extracellular spaces, such as bile, pancreatic juice, gastric juice, saliva, milk, semen, cerebrospinal fluid, and plasma,[11,15] as mature Psap is secreted. Therefore, it would be interesting to study the role of Psap in absorbing exogenous CoQ10 in the future.

Effect of Psap knockdown and overexpression. Psap knockdown and overexpression resulted in a decrease and increase in CoQ10, respectively,[16] as expected. Furthermore, knockdown of Psap in Caco-2 cells reportedly decreases cellular levels of ATP together with CoQ10 and induces the loss of tight junction barriers.[17] Interestingly, long-term CoQ10 deficiency induces a decrease in Psap levels in HepG2 cells.[18] These data suggest that Psap and CoQ are affected by each other.

Psap deficiency. Psap deficiency was shown to cause severe leukodystrophy and widespread storage of multiple sphingolipids in mice.[19] Psap deficiency in humans also results in sphingolipi-

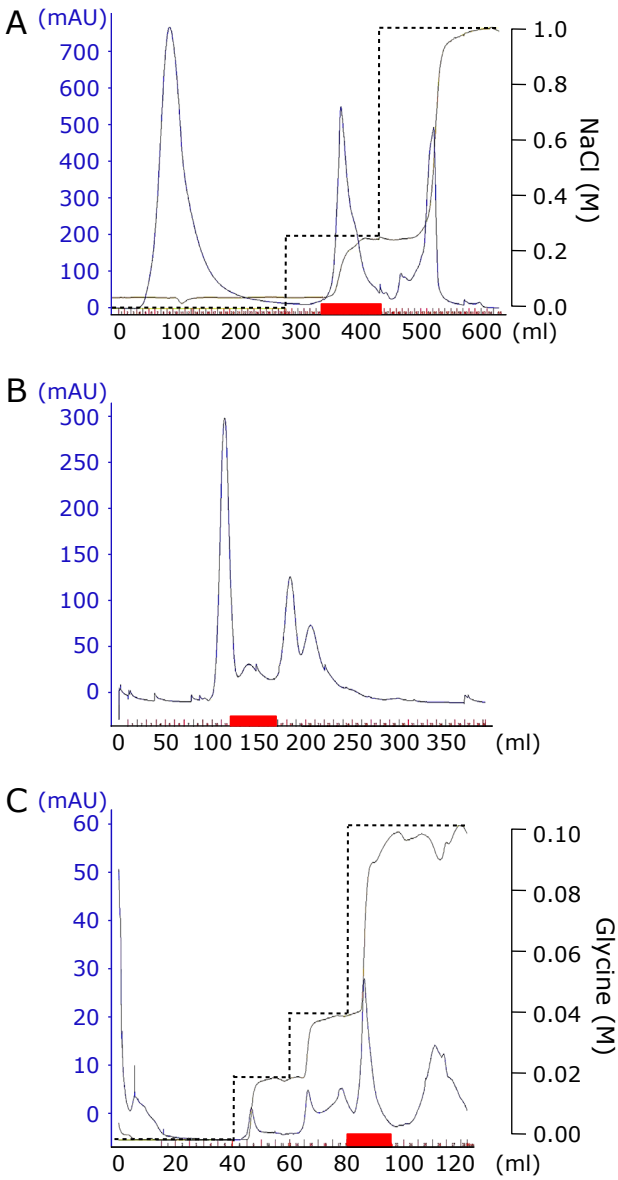

Fig. 1. Chromatographic profiles of eluates from (A) DEAE Sepharose, (B) Hiload 26/60, and (C) Sap B antibody-affinity chromatography columns utilized in the purification of Psap from human seminal plasma. Solid and dotted lines indicate protein concentration estimated from absorbance at 280 nm and the concentration of NaCl (A) and glycine (C) in the eluates, respectively. The thick red solid line indicates the fractions collected.

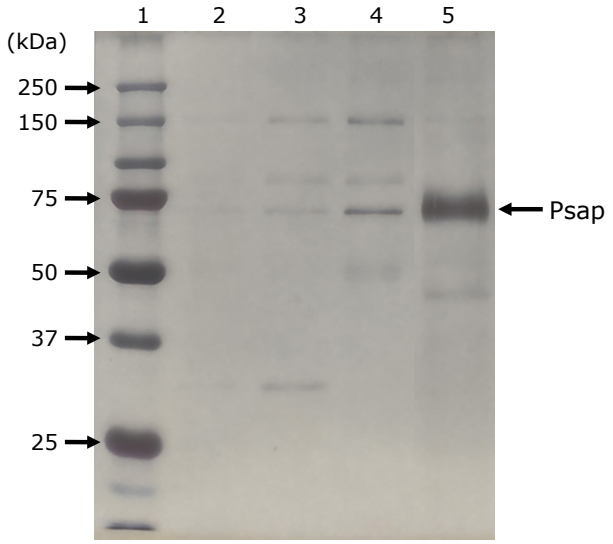

Fig. 2. SDS-polyacrylamide gel electrophoresis of the proteins in each collected fraction. Proteins were silver stained. Lane 1: Molecular weight markers; Lane 2: Seminal plasma; Lane 3: Fractions obtained from DEAE column chromatography; Lane 4: Fractions obtained from gel filtration; Lane 5: Fractions obtained from affinity chromatography.

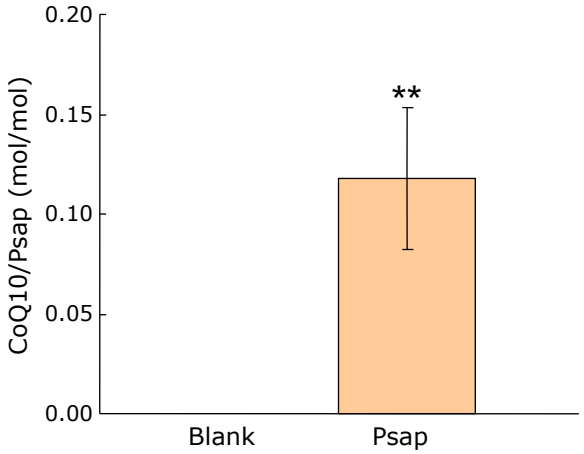

Fig. 3. Binding of CoQ with Psap. CoQ10 (10 mM) in hexane was vigorously mixed for 5 min with 2 ml of 0.5 μM Psap in 50 mM phosphate buffer containing 150 mM NaCl (pH 7.4). The aqueous phase was separated from hexane by centrifugation. The average CoQ10 content in the aqueous layer was plotted. Psap-free phosphate buffer was used as a blank. Experiments were repeated 3 times, and the means ± SD are shown.

dosis,[20] especially in the skin.[21] Psap deficiency in mice also results in abnormalities in the prostate gland,[22] progressive deafness and altered Cochlear innervation,[23] and vestibular dysfunction.[24] The neurolysosomal pathology of human Psap deficiency suggests Psap has a neurotrophic function,[25] which is consistent with the results of an *in vitro* study that identified Psap as a neurotrophic factor.[26] How would Psap protein function as a neurotrophic factor? We believe the connection with an essential nutrient such as CoQ10 is essential. Interestingly, oral supplementation with CoQ10 was shown to suppress *Bak* expression in the cochlea, reduce cochlear cell death, and prevent age-related hearing loss.[27]

Plasma levels of Psap. We previously observed an increase in plasma (serum) levels of Psap under oxidative stress conditions, such as in centenarians and patients with post-cardiac arrest syndrome,[28,29] ALS, [30] or Parkinson's disease (unpublished observation). Therefore, it was unexpected that Psap levels in patients with sepsis were significantly lower than those in healthy controls.[31] As the brain and kidney are the major organs expressing the highest levels of Psap and sepsis disrupts kidney function,[11,31] our results suggest that sepsis patients have impaired secretion of Psap.

Conclusion

We isolated Psap from human seminal plasma and demonstrated that Psap binds CoQ10. An immunoprecipitation study using mouse cell cytosol showed the presence of Psap-CoQ9

doi: 10.3164/jcbn.23-57

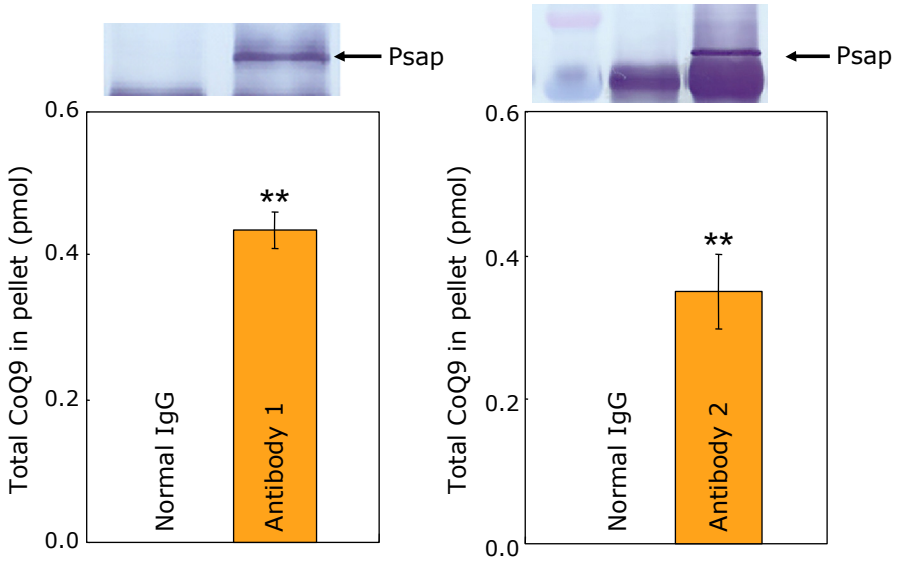

Fig. 4. Presence of Psap-CoQ9 complex in mouse brain cytosol. Solid and open bars show the CoQ9 content in immunoprecipitates obtained using polyclonal anti-Psap IgG and normal IgG, respectively. Values are the means ± SD ($n = 3$). Significant differences between the two groups are denoted by ** and *** ($p<0.01$ and 0.001, respectively), as determined using the Student t test. Upper panel shows the results of Western blotting with polyclonal anti-Psap antibody.

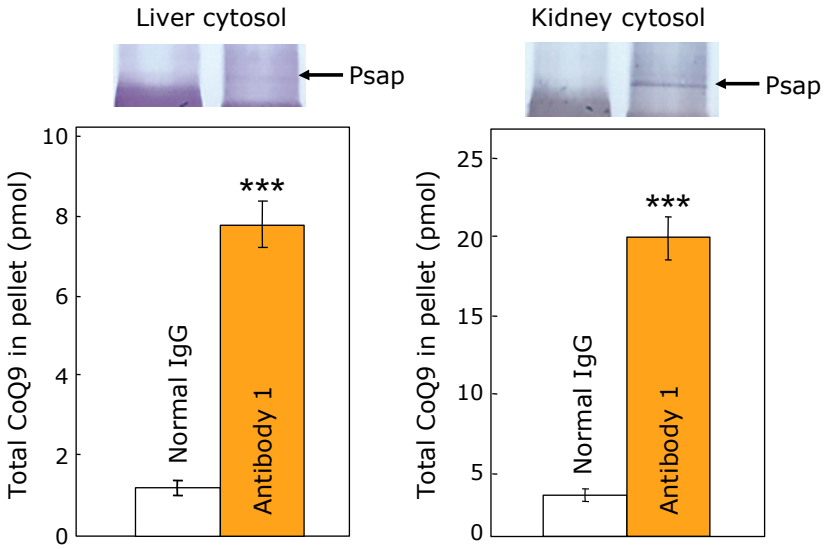

Fig. 5. Presence of Psap-CoQ9 complex in mouse liver cytosol and mouse kidney cytosol. Solid and open bars show the CoQ9 content in immunoprecipitates obtained using polyclonal anti-Psap IgG and normal IgG, respectively. Values are means ± SD ($n = 3$). Significant differences between the two groups are denoted by ** and *** ($p<0.01$ and 0.001, respectively), as determined using the Student t test. Upper panel shows the results of Western blotting with polyclonal anti-Psap antibody.

complex. Therefore, we conclude that Psap is a newly described CoQ10-binding protein. We also discussed the role of the Psap-CoQ10 complex *in vivo*.

Author Contributions

Study concept and design: YY; acquisition of data: MH, AF and MK; interpretation of data: MH, AF and YY; drafting of manuscript: YY and MK. All authors approved the final version of this manuscript to be published.

Acknowledgments

We thank the donors of seminal plasma. We also thank Dr. J. Matsuda (Tokai University) for supplying the anti-Psap antibody. We thank Mr. T. Nagata for help in earlier works.

Abbreviations

CoQ	coenzyme Q
Psap	prosaposin
Sap B	saposin B

Conflict of Interest

No potential conflicts of interest were disclosed.

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doi: 10.3164/jcbn.23-57