Regional expression of prosaposin in the wild-type and saposin D-deficient mouse brain detected by an anti-mouse prosaposin-specific antibody

By Azusa YONESHIGE,[*1] Kunihiko SUZUKI,[*1] Naoya KOJIMA[*1] and Junko MATSUDA[*1,†]

(Contributed by Kunihiko SUZUKI, M.J.A.)

Abstract: Prosaposin is a precursor of saposins A, B, C, and D. Saposins are indispensable for lysosomal hydrolysis of sphingolipids. The notion that prosaposin itself is likely involved in brain development led us to generate an anti-mouse prosaposin-specific antibody that do not cross-react with any of the processed saposins. We have used it to study expression of prosaposin in the brain of wild-type (WT) and saposin D knockout mice (*Sap-D$^{-/-}$*). Immunoblot studies indicated that prosaposin, already abundant in the brain of WT, was dramatically increased in *Sap-D$^{-/-}$*. By immunohistochemistry, the brain of WT was rich in prosaposin in hippocampal CA3 pyramidal neurons, tufted cells and mitral cells in olfactory bulb, and cerebellar Purkinje cells. In *Sap-D$^{-/-}$*, immunoreactivity of prosaposin was increased in these neurons, most notably in the CA3 pyramidal neurons which contained prosaposin immuno-positive inclusion bodies in the endoplasmic reticulum. Further characterization of these prosaposin-rich neurons may provide new insights into the physiological functions of prosaposin in the nervous system.

Keywords: Sphingolipid activator protein, saposins, neurotrophic factor, hippocampus, olfactory bulb, cerebellar Purkinje cell

Introduction

Prosaposin is a highly conserved glycoprotein of about 70 kDa and is the precursor of four saposins A, B, C, and D. Prosaposin (*Psap*) gene encodes saposins A-D in tandem with N- and C-terminal and inter-saposin sequences.[1),2)] Prosaposin is synthesized in the endoplasmic reticulum (ER) and transported through the Golgi apparatus. Major fractions of prosaposin are intracellularly targeted to the lysosomes via the mannose-6-phosphate receptor (M6PR) or by sortilin, where it is sequentially processed to individual saposins by proteolytic digestion. The remainder of prosaposin is secreted to the extracellular space and re-enters the cell by endocytosis through M6PR, a low-density lipoprotein receptor-related protein (LRP), or mannose receptors.[3)–6)]

The mature four saposins A-D are heat-stable glycoproteins of approximately 80 amino acids and 15 kDa.[1),2)] The most notable *in vivo* function of each saposin is to facilitate degradation of some sphingolipids in the lysosome as a cofactor of hydrolases. It has been clarified definitively that the deficiency of each saposin causes a lysosomal storage disease similar to that produced by the absence of the corresponding hydrolase both in humans and the mouse.[1),2),7)–9)] On the other hand, there is growing evidence showing that saposins have membrane-binding and lipid transport properties other than their basic function as enzyme cofactors.[2)]

It has been known that secreted prosaposin is present at relatively high concentrations in body fluids such as milk, cerebrospinal fluid, semen, bile, and pancreatic juice.[10),11)] In contrast to the ubiquitous expression of mature saposins in tissues, unprocessed prosaposin has been detected mainly in the brain, heart, and muscle tissues.[12)–14)] These observations support often-stated notion that prosaposin may possess its own physiological functions other than being a mere precursor of saposins. For example, the function of secreted prosaposin as a neurotrophic factor has long been proposed in the nervous system[15),16)] and as a spermatogenesis related factor in the reproductive organs.[17),18)] Recently, in the prostate cancers, the new role of secreted prosaposin to control the tumor metastasis has been

[*1] Institute of Glycoscience, Tokai University, Kanagawa, Japan.

[†] Correspondence should be addressed: J. Matsuda, Institute of Glycoscience, Tokai University, Hiratsuka, Kanagawa 259-1292, Japan (e-mail: matsujun@keyaki.cc.u-tokai.ac.jp).

reported.[19],[20] However, the specific *in vivo* functions of prosaposin in the nervous system and other organs are still not well understood.

In the present work, in order to clarify the possible physiological role of unprocessed prosaposin itself, we have generated an anti-mouse prosaposin-specific antibody that does not cross-react with any processed saposins. Using this antibody, we investigated the expression pattern of prosaposin in the tissues of wild-type mice and saposin D knockout (*Sap-D⁻/⁻*) mice[9] by immunoblot and immunohistochemical analyses.

Materials and methods

Antibody. Polyclonal antibody against mouse prosaposin was generated by immunizing rabbits with three synthetic oligopeptides corresponding to 178-LYPQDHPRSQPQPKAN-193, 299-EMMD-PYEQNLVQAH-312, and 413-KEPTPPKQPAQ-PKQSALP-430. These oligopeptides were selected from the mouse *Psap* sequences (GenBank accession number: U-57999) such that do not encode any saposins. These synthetic peptides with an additional cysteine residue at the N-terminal were conjugated to keyhole limpet hemocyanin. The antiserum was passed through a Sepharose column with bound antigen peptides and the bound antibody eluted with 0.1 M glycine (pH 2.7), and neutralized immediately with 1 M Tris-HCl (pH8.0). The rabbit antiserum against recombinant mouse saposin D was a gift from the laboratory of Dr. G. Grabowski.[21]

Animals. The prosaposin, saposin A, and saposin D knockout mice (*Psap⁻/⁻*, *Sap-A⁻/⁻*, and *Sap-D⁻/⁻*) were originally generated in our laboratory by the gene targeting technology.[7],[9],[22] *Twitcher* mouse, which is the naturally occurring mouse model of human Krabbe disease due to genetic galactosylceramidase deficiency, was originally purchased from the Jackson Laboratory (Bar Harbor, ME). The *Psap⁻/⁻*, *Sap-A⁻/⁻*, and *Sap-D⁻/⁻* mice were backcrossed to the C57BL/6J strain more than 10 generations so that the genetic background of all mice used in this study was C57BL/6J. A colony was maintained in the facility for the experimental animals at the Tokai University and all experimental procedures were approved by the institutional review committee.

Immunoblot analyses. Mouse tissues (brain, liver, kidney, testis, intestine, and placenta) from each genotype were dissected freshly and homogenized with a Potter-Elvehjem homogenizer. For cell fractionation, tissues were homogenized in an ice-cold 10 mM Tris-HCl (pH 7.0) containing 0.25 M sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1.5% protease inhibitor cocktail (Sigma, St. Louis, MO). The homogenate was centrifuged at $10,000 \times g$ for 15 minutes at $4\,^\circ C$ and the supernate was centrifuged at $100,000 \times g$ for 1 hour at $4\,^\circ C$. The separated pellet (membrane fraction) was suspended in a buffer of the same volume as the supernate. The supernate was designated as the cytosolic fraction. The solubilized proteins (50 µg) were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on 10–15% polyacrylamide gels (Daiichi Pure Chemicals, Tokyo) and transferred to polyvinylidene difluoride membranes (Amersham Biosciences, Piscataway, NJ). The membranes were incubated with a 1% blocking solution (BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit); Roche Molecular Biochemicals, Mannheim, Germany) in TBS buffer (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl) for 16 hours at $4\,^\circ C$, then incubated with either the rabbit polyclonal anti-mouse prosaposin antibody (3.74 µg/ml), anti-saposin D antiserum diluted 1:100, or anti-mouse prosaposin antibody pre-absorbed by a 10 mg/ml solution of the three antigen peptides for 2 hour at room temperature. The membrane was then washed with the TBS-T (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% Tween-20) buffer and incubated with peroxidase-labeled anti-rabbit IgG (40 mU/ml; Roche Molecular Biochemicals) for 1 hour at room temperature, followed by treatment with the detection solution of a BM chemiluminescence kit (Roche Molecular Biochemicals). The immunoreactive protein bands were visualized with a LAS-1000 Plus luminescence image analyzer (Fujifilm, Tokyo).

Mouse brain lysates (125 µg) were denatured in $1 \times$ glycoprotein denaturing buffer with 0.2 M 2-mercaptoethanol at $100\,^\circ C$ for 3 min. The deglycosylation was carried by glycopeptidase F (1 mU) at $37\,^\circ C$ for 16 hours following manufacture's instruction (Takara Bio Inc., Shiga). The reaction mixtures were analyzed by SDS-PAGE (50 µg protein/lane, 10% polyacrylamide gel) and probed by anti-mouse prosaposin antibody.

Real-time quantitative reverse transcriptase-PCR. Adult mouse brain tissues (n = 4) were prepared from 80-day old male mice of wild-type and *Sap-D⁻/⁻* mice. The fetal mouse brain and placental

tissues were prepared from the pregnant female mice of wild-type and *Sap-D^{-/-}* mice (n = 4). Isolated tissues were immediately frozen in liquid nitrogen and stored at −80 °C until use. Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The total RNA content was quantified by UV-spectrophotometry and 10 μg of RNA was used for analysis. The TaqMan Reverse Transcription Reagent and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) were used for RT-PCR. Mouse prosaposin RNA was quantified using AB 7500 Real time PCR System (Applied Biosystems). The oligonucleotide primers and FAM-labeled TaqMan probe for mouse prosaposin were purchased from Applied Biosystems (TaqMan® Gene Expression Assay). The mouse glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene was used as an endogeneous control to normalize the amount of total RNA in each sample. The oligonucleotide primers and VIC® labeled TaqMan probe for GAPDH were purchased from Applied Biosystems (TaqMan Rodent GAPDH Control Reagent VIC® Probe). The prosaposin expression levels were evaluated by Relative Quantification (RQ) using SDS v1.3 Software (Applied Biosystems). The relative expression ratio of prosaposin to GAPDH of each sample was calculated by $2^{-\Delta C_T}$ (CT; Threshold cycle).

Immunohistochemical study. *Tissue preparation.* Three wild-type and *Sap-D^{-/-}* male mice at 5–6 months-old were used for immunohistochemical study as previously described.[23] The mice were anesthetized with ether and perfused through the left ventricle of the heart briefly with physiological saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) and immersed in the same fixative at 4 °C overnight. Then the brain was dissected and processed for sectioning. For free-floating immunohistochemical studies, the brain was sliced in the coronal and parasagittal directions at 50 μm thickness with a Microslicer (DTK-1000, Dosaka Co., Ltd., Kyoto). For cryosections, after rinsing the brain tissues in phosphate buffered saline (PBS), it was sequentially immersed in 10%, 20% and finally 30% sucrose in PBS overnight at 4 °C. The brain was sectioned, placed in the O.C.T compound (Sakura Finetek U.S.A., Inc., Torrance, CA) and frozen on the dry ice. For sectioning, the samples were cut to 6 μm thickness with a cryostat microtome (Leica) and thaw-mounted on Matsunami adhesive silane

(MAS)-coated glass slides (MATSUNAMI, Osaka). The mounted sections were air-dried for 1 hour.

Confocal microscopic immunohistochemical analyses. 50 μm thick microsliced sections were incubated with 1% bovine serum albumin/phosphate-buffered saline with 0.3% Triton X-100 for 1 hour to block and increase the penetration of antibodies. They were then incubated in the primary antibodies by free-floating methods at 20 °C for overnight, and in the second antibodies for 2 hours as previously described.[23] The anti-mouse prosaposin antibody was used at a concentration of 3.74 μg/ml. Other primary antibodies used for the study were anti-calbindin D-28 k antibody [mouse monoclonal: Swant (Bellinzona, Switzerland)] and anti-parvalbumin antibody (mouse monoclonal: Swant). After incubation in mixtures of the primary antibodies, the sections were then incubated in mixtures of the species-specific secondary antibodies labeled with fluorescein isothiocyanate (FITC), Cy-3, or Cy-5 (Jakson ImmunoResearch Laboratories, Inc. West Grove, PA). All the secondary antibodies were used at 1:200 dilution. Hoechst 33258 (Dojindo, Tokyo) stain was used for DNA staining. Immunostained sections were mounted in Vectashield (Vector Laboratory, Burlingame, CA) and examined with a confocal laser scanning microscope (LSM510, Zeiss, Oberkochen, Germany). As a negative control for non-specific staining, additional sections were subjected to the immunostaining procedure with anti-mouse prosaposin antibody preabsorbed by the three antigen peptides. The experiments were repeated 3 times using three different sections from different mice.

Immunoelectron microscopic analyses. The 6 μ-thick cryosections were pretreated by 3% H_2O_2 in distilled water for 30 minutes to reduce the endogenous peroxidase activities. Sections were incubated with 1% BSA/PBS for 1 hour. They were incubated with rabbit anti-mouse prosaposin antibody at 20 °C overnight and then with the 1:200 diluted HRP-conjugated goat anti-rabbit IgG, F(ab')$_2$ specific antibodies (Jackson ImmunoResearch Laboratories, Inc.) at room temperature for 2 hours. After rinsing in PBS, they were fixed with 1% glutaraldehyde in 0.1 M PB for 10 minutes. Immunoreactivity was visualized by diaminobenzidine (DAB). Subsequently, sections were treated with 2% osmium tetroxide in 0.05 M PB for 1 hour. After dehydration in a series of graded ethanol, they were embedded in epoxy resin using the inverted gelatin capsule method.

Ultrathin sections at 70-nm thickness were cut on an ultramicrotome (LKB 2800, Reichert-Jung, Wien, Austria). Sections were stained by a lead solution to identify the localization of DAB reaction products, and examined at an accelerating voltage at 80 kV by a transmission electron microscope (JEM-1200 ExII, JEOL, Tokyo) as previously described.[24]

Results

Anti-mouse prosaposin antibody specifically recognized prosaposin. The specificity of the anti-mouse prosaposin antibody was demonstrated by immunoblot analyses of mouse whole brain homogenates. In the 15% gel SDS-PAGE, anti-prosaposin antibody recognized a major band with molecular weight about 70 kDa corresponding to the size of prosaposin, but no bands were detected at around 15 kDa corresponding to the size of mature saposins and of other partially processed forms at around 30 kDa or 45 kDa (Fig. 1A). The 70 kDa band was not detected in the brain of $Psap^{-/-}$ mice either by the anti-mouse prosaposin antibody or the anti-saposin D antibody, confirming that this 70 kDa band detected by the anti-prosaposin antibody was prosaposin (Fig. 1A). On the other hand, anti-saposin D antibody recognized not only the 70 kDa band but also the 15 kDa band, which was completely absent in $Sap\text{-}D^{-/-}$ mice, but clearly detected in $Sap\text{-}A^{-/-}$ mice (Fig. 1A). These results not only confirmed the absence of saposin D protein in $Sap\text{-}D^{-/-}$ mice but also the retention of saposin D protein in $Sap\text{-}A^{-/-}$ mice supporting the specific destruction of each saposin in our targeting strategy used previously to generate specific saposin knockout mice.[7],[9] Unexpectedly, the intensity of the 70 kDa band was dramatically increased in $Sap\text{-}D^{-/-}$ mice and it was also increased in $Sap\text{-}A^{-/-}$ and $Twitcher$ mice to a lesser degree (Fig. 1A). In 10% SDS-PAGE, anti-mouse prosaposin antibody recognized additional band of about 60 kDa. Both 70 and 60 kDa bands were not detected in prosaposin knockout mouse (Fig. 1B). To check whether the difference of two bands of 70 kDa and 60 kDa were due to the glycosylation of prosaposin, brain lysates from wild-type and $Sap\text{-}D^{-/-}$ mice were treated with glycopeptidase F, which cleaves N-glycans from glycoproteins, and visualized by immunoblot using anti-mouse prosaposin antibody (Fig. 1B). Both in the wild-type and $Sap\text{-}D^{-/-}$ mice, the major portion of 70 kDa band was glycopeptidase F sensitive and shifted to the 60 kDa

band whereas the 60 kDa band showed no changes in its size. These results indicate that the 70 kDa bands were N-glycosylated prosaposin and that the 60 kDa bands were prosaposin without N-glycosylation. Therefore, it could be concluded that the glycosylated prosaposin was dramatically increased in $Sap\text{-}D^{-/-}$ mice. By the cell fractionation experiment, prosaposin was detected both in the membrane fraction and cytosole fraction as expected from the presence of both secreted and endocytosed forms of prosaposin. Both 70 kDa and 60 kDa bands were significantly reduced with antibody preabsorbed by three antigen peptides, confirming that these two bands were specific to the antigenic peptides (Fig. 1C). Furthermore, although the reactivity was much weak, this anti-mouse prosaposin antibody also recognized the human prosaposin in peripheral leukocytes and fibroblasts (data not shown).

Prosaposin expression was dominant in the brain and glycosylated prosaposin was increased in Sap-D$^{-/-}$ mice. Tissue distribution of prosaposin in wild-type and $Sap\text{-}D^{-/-}$ mice was examined in adult and fetal tissues. In wild-type mice, prosaposin was expressed mainly in the brain followed by the kidney (Fig. 2A). In $Sap\text{-}D^{-/-}$ mice, in both adult and fetus, the remarkable increase of prosaposin with molecular weight of 70 kDa was demonstrated in all tissues examined, especially in the brain and the placenta (Fig. 2A). When we have done the immunoblot of fetal tissues from a $Sap\text{-}D^{+/-}$ crossing, the protein levels of prosaposin were elevated in the order of $Sap\text{-}D^{-/-}$, $Sap\text{-}D^{+/-}$, $Sap\text{-}D^{+/+}$ both in the brain and placenta, suggesting that the increase of prosaposin in $Sap\text{-}D^{-/-}$ mice might be due to the mutant prosaposin protein (Fig. 2B). In $Twitcher$ mice, the protein levels of prosaposin were not elevated in the fetal tissues (data not shown), suggesting that the elevation of prosaposin in the brain of symptomatic $Twitcher$ mice (30 days-old) (Fig. 1A) was due to the secondary response to the pathology.[25],[26]

No increase of prosaposin RNA level in Sap-D$^{-/-}$ mice. To address the question of whether the accumulation of prosaposin protein in the tissues of $Sap\text{-}D^{-/-}$ mice is due to the transcriptional regulation, we analyzed the mRNA levels of prosaposin in the brain and placenta of wild-type and $Sap\text{-}D^{-/-}$ mice. Although it is known that the mRNA that encodes proteins of the glycosphingolipid catabolism is often upregulated,[25] no increase was detected in the mRNA levels of prosaposin either in adult or fetal
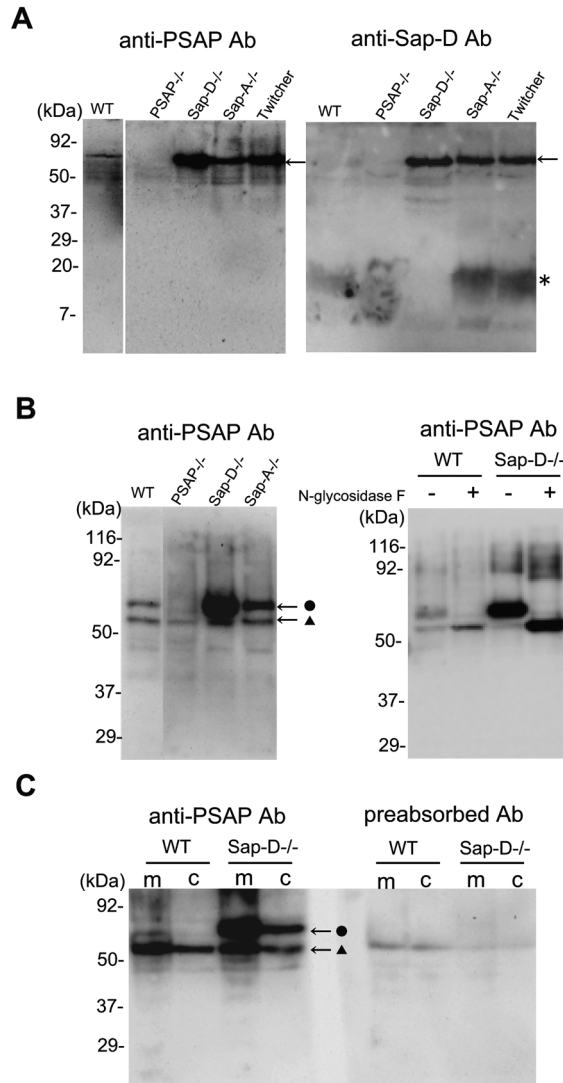
Fig. 1. Immunoblot analyses of mouse brain homogenates with the anti-mouse prosaposin antibody. **A**: Tissue lysates from mouse brain of wild-type, *Psap*^{-/-}, *Sap-D*^{-/-}, *Sap-A*^{-/-}, and *Twitcher* mice were separated by SDS-PAGE (15% gel) and probed with anti-mouse prosaposin antibody and anti-mouse saposin D antibody, respectively. Anti-mouse prosaposin antibody recognized only a band of approximately 70 kDa (arrow) corresponding to the molecular weight of prosaposin, but not a band of approximately 15 kDa (∗), corresponding to the mature saposins in wild-type mice. This 70 kDa band was completely absent in *Psap*^{-/-} mice and was increased in the *Sap-D*^{-/-}, *Sap-A*^{-/-}, and *Twitcher* mice. In contrast, anti-mouse saposin D antibody recognized not only the 70 kDa band but also the 15 kDa band, which was completely absent in *Sap-D*^{-/-} mice and clearly detected in *Sap-A*^{-/-} mice. **B**: In 10% gel, in addition to the 70 kDa band (●), anti-mouse prosaposin antibody recognized 60 kDa band (▲). The intensity of 70 kDa band was dramatically increased in *Sap-D*^{-/-} mice followed by *Sap-A*^{-/-} mice. Both bands were not detected in *Psap*^{-/-} mice. By the glycopeptidase F treatment, in both the wild-type and *Sap-D*^{-/-} mice, the majority of 70 kDa band was shifted to 60 kDa band. **C**: Both 70 kDa and 60 kDa bands were detected both in the membrane (**m**) and cytosolic (**c**) fraction and were significantly reduced with antibody preabsorbed by the three antigen peptides.

tissues between wild-type and *Sap-D*^{-/-} mice (Fig. 3). These results indicated that there is no transcriptional upregulation of prosaposin in the tissues of *Sap-D*^{-/-} mice.

Immunohistochemical localization of prosaposin in the brain of wild-type and Sap-D^{-/-} **mice.** We next examined the *in situ* expression of prosaposin in the brain of wild-type and *Sap-D*^{-/-}
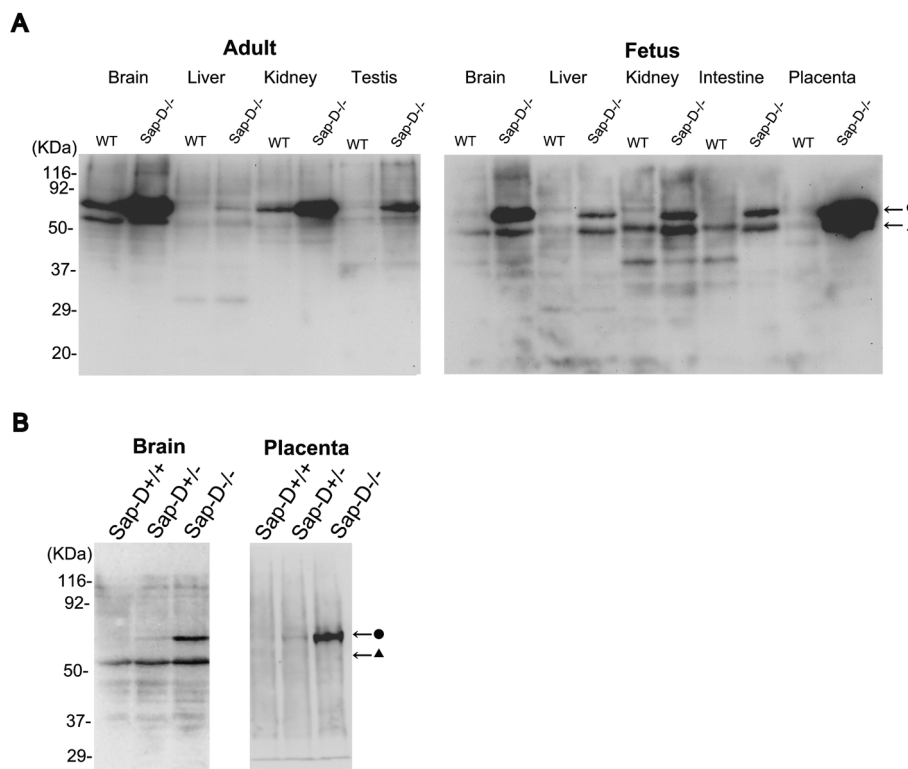
Fig. 2. Tissue distribution of prosaposin and its elevation in *Sap-D*$^{-/-}$ mice. **A:** In tissues of wild-type mice, prosaposin was expressed mainly in the brain followed by the kidney. In *Sap-D*$^{-/-}$ mice, the protein levels of prosaposin, mainly of the 70 kDa size, were increased in all tissues examined both in adults and fetuses, most dramatically in the brain and placenta. **B:** The protein levels of prosaposin were already elevated fetuses of the *Sap-D*$^{-/-}$ mice both in the brain and placenta, compared to wild-type or heterozygous littermates.

mice by immunohistochemical analyses using the anti-mouse prosaposin antibody and anti-mouse cal-bindin D-28K antibody. No immunoreactivity was observed by the incubation with pre-immune serum or with antibody preabsorbed with excess antigen peptides supporting the reliability and specificity of the anti-mouse prosaposin antibody (data not shown). In the brain of wild-type mice, the immunoreactivity of prosaposin was relatively weak, but regionally observed in the cytoplasm of certain types of neurons. In the hippocampus, pyramidal neurons especially in the CA3 area were prosaposin immune-positive (Fig. 4A). In the olfactory bulb, the immunoreactivity of prosaposin was clearly observed in tufted cells and mitral cells (Fig. 4B). In the cerebral cortex, neurons in layer II to VI were weakly prosaposin immune-positive (Fig. 4C). In the cerebellum, Purkinje cell bodies and granular cell layer were prosaposin immune-positive (Fig. 4D).

In the brain of *Sap-D*$^{-/-}$ mice, over all, the im-munoreactivity of prosaposin was increased and clearly detected even at 40 days, when *Sap-D*$^{-/-}$ mice do not yet show any clinical, pathological, and biochemical changes. In the hippocampus of *Sap-D*$^{-/-}$ mice, immunoreactivity of prosaposin was dramatically increased in the pyramidal neurons, especially in the CA3 area (Fig. 4E). In the CA3 pyramidal neurons, the prosaposin immune-positive perinuclear inclusion bodies were detected (Fig. 5A, B). These were periodic acid-Schiff (PAS) positive in paraffin sections suggesting that these were glycoproteins (Fig. 5C). These inclusion bodies were rarely observed in other pyramidal neurons in CA1 or CA2 area (Fig. 5A, B). In the olfactory bulb, the immunoreactivity of prosaposin was clearly observed in tufted cells and mitral cells (Fig. 4F). In the cerebral cortex, neurons in layer II to VI were strongly prosaposin immunoreactive and some neurons in Layer III and V had prosaposin immune-positive granular staining (Fig. 5G). In the cerebellum, Purkinje cells
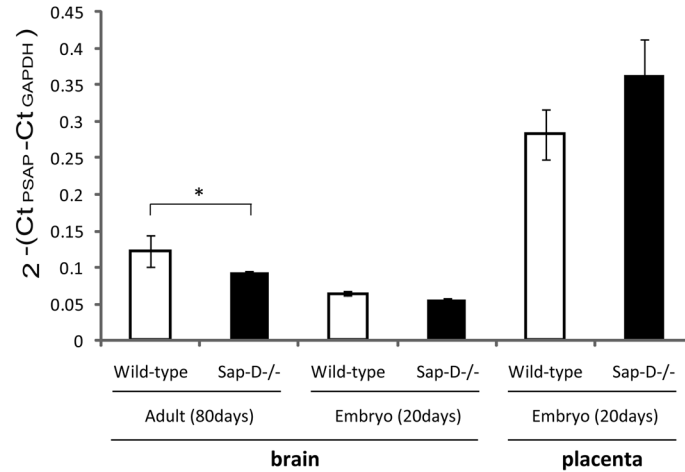
Fig. 3. qRT-PCR analyses of prosaposin RNA in wild-type and *Sap-D*^{-/-} mice. qRT-PCR analyses showed no significant difference in prosaposin mRNA levels between wild-type and *Sap-D*^{-/-} mice, both in the brain and placenta. Prosaposin mRNA amounts were normalized to GAPDH mRNA for each sample. The data were presented as means ± S.E. *: $P < 0.05$ compared to the value of wild-type mice.

and the granular cell layer were strongly prosaposin immune-positive (Fig. 5H). These results suggest that there may be a neuron type-specific regulation of prosaposin expression in the central nervous system.

Prosaposin were abnormally trapped in the ER of Sap-D^{-/-} mice.   To define the cellular localization of prosaposin immune-positive inclusion bodies in the hippocampal CA3 pyramidal neurons of *Sap-D*^{-/-} mice, the immunoelectron microscopic analyses were carried out. In the hippocampal CA3 pyramidal neurons, prosaposin immuno-positive products were observed in the ER, but not in the Golgi apparatus (Fig. 5D, E). In most of the CA3 pyramidal neurons, balloon-like ER with the prosaposin immune-positive products were observed, which may correspond to the inclusion bodies (Fig. 5F). By the confocal microscopic analyses, these inclusion bodies were colocalized with the ER marker protein of BiP/ GRP78 (data not shown). These findings suggested that the processing of prosaposin in the ER to Golgi apparatus were impaired in *Sap-D*^{-/-} mice.

Discussion

Because the prosaposin is proteolytically processed to four saposins efficiently within the acidic compartments,[1),2)] it can be assumed that, the unprocessed prosaposin play no role in the membrane digestion in the lysosome. If the prosaposin functions just as the precursor of four saposins, why the unpro-

cessed prosaposin is secreted or rich in the nervous system? There are reports that the secreted form of prosaposin could act as a glycolipid transfer protein, since several gangliosides were found to bind with high affinity to prosaposin.[27),28)] It has also been proposed that the prosaposin has the function to promote neurite outgrowth and nerve regeneration, and prevent cell death, when supplied exogenously.[15),29)–31)] Furthermore, the expression of prosaposin markedly increases in response to brain injury such as ischemia, or sciatic nerve crush injury.[32),33)] Several reports have identified the saposin C domain of prosaposin responsible for its *in vitro* and *in vivo* neurotrophic activity.[16),34)]

There is no doubt that inherited deficiency of prosaposin can be an indispensable tool to elucidate the function of prosaposin itself.[1)] In humans, four different genotypes (all homozygous) that lead to a complete loss of functional prosaposin and consequently of all four saposins have been reported to date.[35)–39)] In all cases so far studied in detail, patients showed a rapidly progressive, severe neurological disease with an accumulation of multiple sphingolipids in the brain and other organs leading to early death. A mouse model of prosaposin deficiency (*Psap*^{-/-}) we generated earlier showed clinical, pathological and biochemical abnormalities similar to those of human patients.[22),40)] A significant number of *Psap*^{-/-} mice die either *in utero* or within 1–2 days after birth, and most of those which survive these periods do
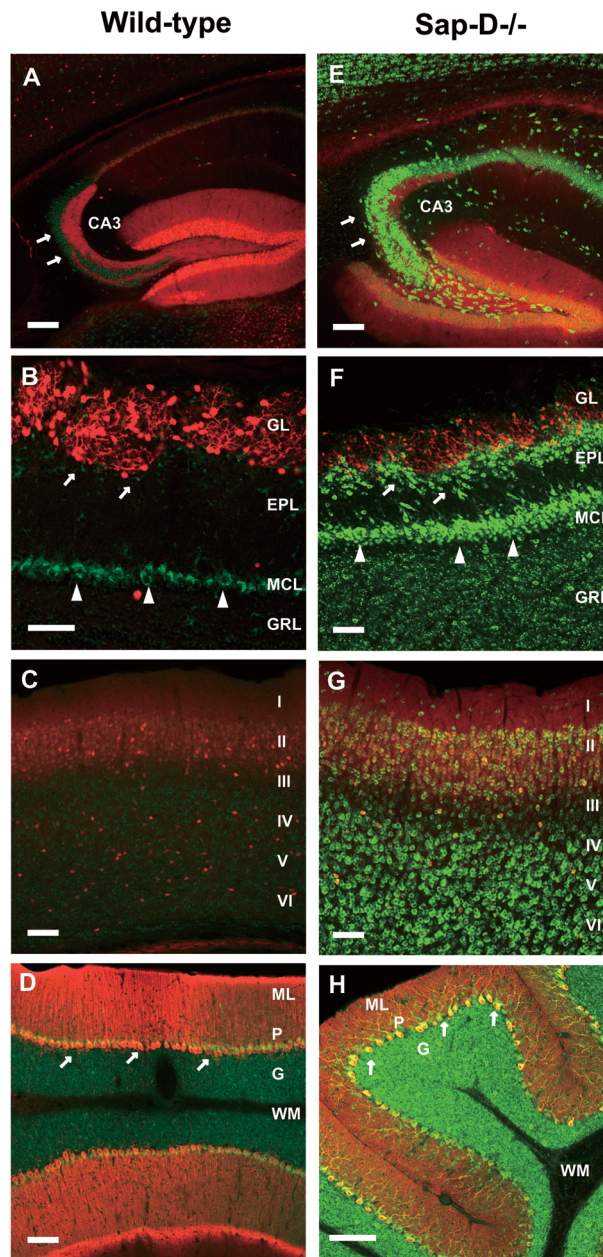
Fig. 4. Immunohistochemical localization of prosaposin in the brain of wild-type and *Sap-D*$^{-/-}$ mice. Confocal microscopic analyses using anti-mouse prosaposin antibody (green) and calbindin (red) in wild-type (**A-D**) and *Sap-D*$^{-/-}$ mice (**E-H**). Overall, immunoreactivity of prosaposin was weak in wild-type mice and was dramatically increased in the brain of *Sap-D*$^{-/-}$ mice. In the hippocampus (**A, E**), the cytoplasm of pyramidal neurons, especially of those in the hippocampal CA3 area (arrow), was prosaposin immunoreactive. In *Sap-D*$^{-/-}$ mice, it was dramatically increased in the pyramidal neurons, especially in the CA3 area. In the CA3 pyramidal neurons, the prosaposin immunoreactive inclusion bodies were observed. In the olfactory bulb (**B, F**), the immunoreactivity of prosaposin was prominently observed in tufted cells (arrow) and mitral cells (arrow head) in wild-type and *Sap-D*$^{-/-}$ mice. In the cerebral cortex (**C, G**), neurons in layer II to VI were weakly prosaposin immunoreactive in wild-type mice, but the neurons of layer III and V in *Sap-D*$^{-/-}$ mice were strongly positive. In the cerebellum (**C, H**), Purkinje cells (arrow) and granular layer were immune-positive in wild-type and *Sap-D*$^{-/-}$ mice. GL: glomerular layer, MCL: mitral cell layer, GRL: granular cell layer, EPL: external plexiformlayer, ML: molecular layer, P: Purkinje cell layer, G: glanular cell layer. WM: white matter. The lines indicate scales of 100 μm.
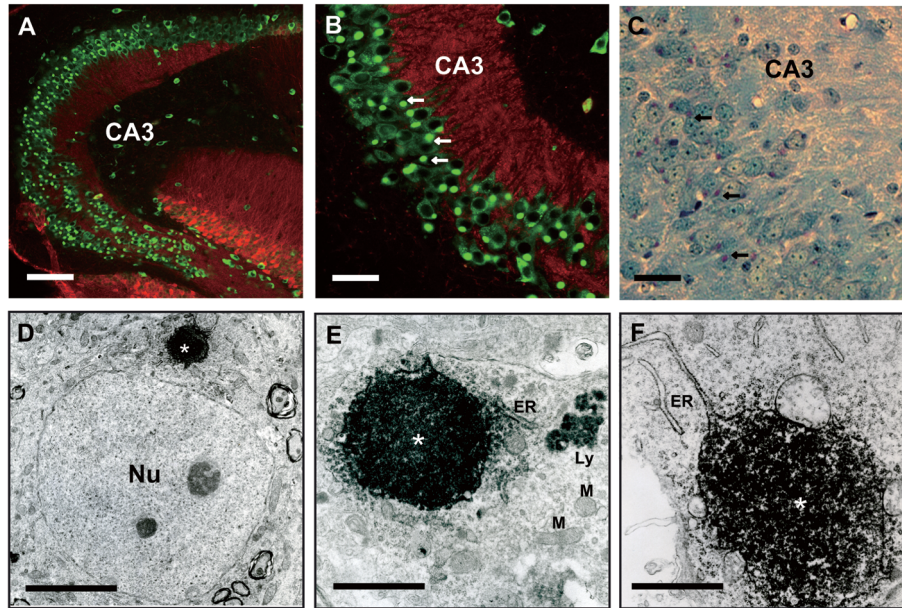
Fig. 5. The prosaposin immuno-positive inclusion bodies in the CA3 pyramidal neuron of *Sap-D$^{-/-}$* mice were localized in the ER. In the hippocampus of *Sap-D$^{-/-}$* mice, prosaposin immuno-positive inclusion bodies were characteristically observed in the CA3 pyramidal neuron (**A, B**), corresponding to the periodic acid-Schiff (PAS) positive materials in paraffin embedded sections (arrows). In the immune-electronmicroscopic analyses (**D-F**), electron dense prosaposin immuno-positive reactions were observed (✽) in the ER, and some ER showed balloon-like enlargements due to the retention of the prosaposin immunoreactive products (✽) in **E** and **F**. This finding suggests impaired intracellular transport of prosaposin in the *Sap-D$^{-/-}$* mice. ER: endoplasmic reticulum, Ly: lysosome, M: mitochondria, Nu: nucleus. The lines indicate scales of 50 µm in **A**, 25 µm in **B, C**, 2 µm in **D**, 1 µm in **E, F**.

not live beyond 30 days of life. If the prosaposin itself has neurotrophic activities, human patients of prosaposin deficiency or *Psap$^{-/-}$* mice would be expected to present neuropathological changes in the organization of cerebral architecture or the vulnerability to the brain or nerve injury. However, the neuropathological phenotypes in the human and mouse prosaposin deficiencies are too complex due to the combined deficiency of all four saposins to dissect out the function of prosaposin itself.[41]

In the present work, we generated an anti-mouse prosaposin-specific antibody by immunizing the rabbit with three oligopeptides, selected from the areas of the prosaposin sequence outside of the saposin-encoding domains. The aim of this work is to have the antibody that can overcome the problem of cross-reactivity with mature saposins we encountered when we used the anti-saposin antibodies to detect the *in situ* expression of unprocessed prosaposin by the immunohistochemical or immunocytochemical analyses.[42),43)] By the immunoblot analy-

ses using this antibody, this antibody was confirmed to recognize only unprocessed prosaposin but not to cross-react with any saposins. The tissue distribution of prosaposin in wild-type mice confirmed the previous reports showing the abundance of prosaposin in the brain.[12),13)] Eventually, we found the dramatic increase of prosaposin, especially its glycosylated form in all the tissues examined in *Sap-D$^{-/-}$* mice, most prominently in the brain and placenta. The *Sap-D$^{-/-}$* mice were generated by introduction of the C509S mutation into the saposin D domain of *Psap* gene, which disrupts one of the three disulfide bonds of saposin D. The *Sap-D$^{-/-}$* mice accumulate ceramides with hydroxylated fatty acids mainly in the brain and in the kidney.[9)] Since the mRNA levels of prosaposin were not elevated in any tissues from *Sap-D$^{-/-}$* mice, and since the anti-prosaposin immune-positive inclusion bodies were detected in the ER, it was speculated that the C509S mutation in the saposin D domain might result in the misfolding of prosaposin and lead to partial failure of lysosomal target-

ing and ER trapping of prosaposin. It is also possible that the ER quality control system of misfolded proteins via cytoplasmic peptide: N-glycanase (PNGase) and ER-associated degradation (ERAD) is impaired in *Sap-D*^{-/-} mice, which might lead to the accumulation of misfolded prosaposin in the ER.[44] On the other hand, there is a report showing that C-terminus of prosaposin and saposin D domain may be required for lysosomal targeting.[4] The combined deficiency of saposins C and D mouse model also showed the ER trapping of prosaposin.[45] The ER-trapped prosaposin in *Sap-D*^{-/-} mice may suggest the importance of saposin D domain for the proper transport of prosaposin from the ER to Golgi apparatus and to its final destination of the lysosome. A few important points remain for future studies; (1) the findings of a major portion of the mutated prosaposin being trapped in ER and the major portion of the accumulating prosaposin in the *Sap-D*^{-/-} mice being in the glycosylated form appears to be contradictory in view of the known intracellular glycosylation process of newly synthesized glycoproteins or ER quality control process for misfolded proteins, (2) the ER-trapped prosaposin may trigger the ER stress together with activation of the unfolded protein response, which cause neuronal cell death, and (3) altered processing of prosaposin could cause the decrease of secreted prosaposin. Cells from *Sap-D*^{-/-} mice could be useful to study the mechanism of intracellular processing of prosaposin. Interestingly, the recent report showed that the secretion and maturation of prosaposin was blocked in the embryonic neural progenitor cells.[46] It indicates the importance of temporal and spatial post-translational regulation of prosaposin in the neurons.

By the immunohistochemical analyses using this anti-mouse prosaposin antibody, we investigated the *in situ* expression pattern of unprocessed prosaposin in the brain and revealed the regional expression of prosaposin in wild-type and *Sap-D*^{-/-} mice. Based on the immunoblot findings that showed accumulation of prosaposin in the brain of presymptomatic *Sap-D*^{-/-} mice, it is expected that cells which produce more prosaposin in the normal state show more intense prosaposin immunoreactivity in *Sap-D*^{-/-} mice. We found it is indeed the case. In the hippocampus, pyramidal neurons in the CA3 region express more prosaposin than CA1 region. Interestingly, only the CA3 pyramidal neurons of *Sap-D*^{-/-}mice showed characteristic prosaposin immune-positive inclusion bodies suggesting the regional function of prosaposin in these cells. The pyramidal cell layer of the hippocampus has been divided into three regions designated CA1, CA2, and CA3 based on the size and appearance of the neurons. In addition to the morphological differences, there is a clear-cut connectional differences. The CA3 pyramidal neurons receive mossy fiber inputs from the dentate gyrus and the CA2 and CA1 pyramidal neurons do not. The CA3 pyramidal cells project to other levels of CA3 as well as to CA1.[47] There is a report showing that stress transiently reduces hippocampal prosaposin levels, not via an increase of glucocorticoid hormone.[48] In the olfactory bulb, mitral cells and tufted cells express more prosaposin than the other type of cells. Both neurons carry the output from the olfactory bulb projecting axons to the olfactory cortex.[49] In the cerebellum, Purkinje cells most abundantly express prosaposin. Purkinje cells are some of the largest neurons and a class of GABAergic neurons. They are the single output system of the cerebellar cortex, sending long inhibitory projections to the deep cerebellar nuclei.[50] Purkinje cells are affected in a variety of neurodegenerative diseases in humans and in spontaneous or experimental mouse mutants. Purkinje cells degeneration is also a conspicuous finding in *Sap-D*^{-/-}, combined deficiency of saposins C and D, and other prosaposin related transgenic mice, suggesting the important role of prosaposin in the Purkinje cells.[9),34),45] One of the common features of these neurons seems that they are relatively large, long projecting principal neurons. Further characterization of these neurons rich in prosaposin, may provide new insights into the physiological and pathophysiological functions of prosaposin itself. We conclude that this novel prosaposin specific antibody could be the useful tool to reveal the *in vivo* function of prosaposin itself not only in the central nervous system, but also in the other organs.

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