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***Review***

# Prosaposin: a protein with differential sorting and multiple functions

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**Summary.** In eukaryotes the delivery of newly synthesized proteins to their final destination is dependent on a series of functionally distinct compartments, including the endoplasmic reticulum and the Golgi apparatus, which plays a role in post-translational modification, sorting and distribution of proteins. Most cargo is sorted within, and exits from, the *trans*-Golgi network (TGN). Proteins delivered to lysosomes include hydrolytic enzymes and nonenzymic activator proteins. They are directed away from the cell surface by their binding to mannose-6-phosphate receptors (MPR). However, in I-cell disease, in which the MPR pathway is disrupted, the nonenzymic sphingolipid activator protein, prosaposin, continue to traffic to lysosomes. This observation led to discovery of a new lysosomal sorting receptor, sortilin. The targeting prosaposin to the lysosomes results from the interaction of its C-terminus with sortilin. Deletion of the C-terminus did not interfere with its secretion, but abolished its transport to the lysosomes. Mutational analysis revealed that the first half of the prosaposin C-terminus contains a motif required for its binding to sortilin and its transport to the lysosomes. Prosaposin can be also secreted to the extracellular space as oligomers. Extracellular prosaposin showed to exert a variety of responses in nervous tissues including the activation of G protein-coupled receptors and ERK phosphorylation. Lastly, prosaposin has been found to be expressed in other fluids of the body such as pancreatic juice, bile, cerebrospinal fluid, milk and seminal fluid, indicating that prosaposin is not only a house keeping lysosomal protein but an essential factor in the development and maintenance of the nervous systems and other systems of the body.

**Key words:** Prosaposin, Sorting, Lysosome, Secretion, Lysosomal storage disorders

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## Introduction

The plasma membrane of eukaryotic cells continuously flows and recycles between cell surface and organelles through a vesicular system (Steinman et al., 1983). During receptor mediated and bulk endocytosis of ligands, hormones and solutes, small portions of the plasma membrane bud into coated and noncoated pits, which are internalized as vesicles and transported from the cell surface to endosomes that mature into lysosomes after receiving a set of nearly sixty acid hydrolases and five sphingolipid activator proteins (Sandhoff and Kolter, 2003).

Lysosomes are the major degradation compartments in eukaryotic cells, a function largely accomplished by the activity of acid hydrolases (Luzio et al., 2007). A direct outcome of endocytosis is the incorporation of plasmalemma into the lysosomal membrane, which is protected from the action of hydrolases by the presence of a thick sugar coat on the luminal surface (Carlsson et al., 1988; Peters and Von Figura, 1994). This coat corresponds to the carbohydrate portion of N-glycosylated lysosomal integral membrane proteins

(LIMPs) and lysosomal associated membrane proteins (LAMPs) (Carlsson et al., 1988; Peters and Von Figura, 1994). Eventually, a part of the internalized membrane recycles back to the cell surface carrying cell surface receptors that escape from degradation (Steinman et al., 1983). This recycling process enhances the ability of the cell surface to internalize ligands and other materials (Steinman et al., 1983). The input of plasmalemma into the endolysosomal system is so prominent that a subset of membrane must be destined for degradation by lysosomal hydrolases, thus preventing its accumulation in the lysosomes (Schulze et al., 2009).

To accomplish this process, some regions of the membrane evaginate into the lumen of the endosomes and multivesicular bodies to form luminal vesicles ranging between 40 and 100 nm in diameter (Sandhoff and Kolter, 2003). The convex curvature of those small vesicles expose the head groups of glycosphingolipids in the outer leaflet of the membrane making them more accessible to the lysosomal hydrolases (Wilkening et al., 1998). Due to an endosomal pH decrease, the composition of the internal membrane changes and most of the membrane-stabilizing cholesterol is removed (Umebayashi, 2003; Frederick et al., 2009). Bis (monoacylglycerol) phosphate (BMP), a negatively charged phosphate lipid that favors a strong curvature, is also increased to facilitate the membrane degradation (Umebayashi, 2003; Frederick et al., 2009). Through this digestive process, glycosphingolipids are cleaved into small molecules by lysosomal glycosidases, with the stepwise release of sialic acid, monosaccharides, sulfate, sphingosine, fatty acid and glycerol (Kolter and Sandhoff, 2005).

Lysosomal glycosidases are water-soluble enzymes with the ability to bind to negatively charged membrane or water-soluble substrates (Kolter and Sandhoff, 2005). They are also able to digest membrane-bound substrates with long sugar chains that reach the aqueous phase (Meier et al., 1991). However, glycosphingolipids with short oligosaccharide side chains (i.e., four or less sugar residues) cannot access the active sites of the glycosidases (Sandhoff and Kolter, 2003). Their degradation requires sphingolipid activator proteins and negatively charged lysosomal lipids, which disturb the membrane structure, extract the sphingolipids from the membrane and expose them to the glycosidases (Furst and Sandhoff, 1992; Wright et al., 2000). As a result, when an enzyme and/or its activator protein are mutated, the specific sphingolipid substrate accumulates in the lumen of lysosomes leading to the development of a lysosomal storage disorder (LSD) or sphingolipidosis.

## Significance of Prosaposin (PSAP) in health and disease

### *Prosaposin and sphingolipid activator proteins*

Sphingolipid activator proteins (SAPs) are soluble lysosomal nonenzymic cofactors involved in the degradation of sphingolipids. This category includes five activators, the $G_{M2}$ activator protein ($G_{M2}$AP) and the saposins A, B, C, and D. Since glycosphingolipids are unable to interact directly with the hydrolytic enzymes in a hydrosoluble milieu, SAPs act as detergents to facilitate the interaction of glycosphingolipid substrates with their respective enzymes for degradation (Burg et al., 1985; Morimoto et al., 1990; Sandhoff, 2001). According to the "liftase hypothesis", $G_{M2}$AP recognizes $G_{M2}$ gangliosides within the membrane, binds to and lifts it out of the bilayer to then present it in a water-soluble format to β-hexosaminidase A (Conzelmann and Sandhoff, 1979). Another hypothesis proposes that GM2AP binds to an already solubilized ganglioside to ensure its recognition by the β-hexosaminidase A enzyme responsible for its degradation (Hama et al., 1997; Sandhoff, 2001).

The remaining SAPs result from a common precursor, prosaposin (PSAP), which is the product of a different gene than that of the $G_{M2}$AP. PSAP is proteolytically cleaved in the lysosomes to generate the smaller saposins A, B, C, and D (Kishimoto et al., 1992; Schuette et al., 2001). Saposins are approximately 80 amino acids in length, they are glycosylated, and they all form three internal disulfide bridges in their hydrophobic core (Burg et al., 1985; Kishimoto et al., 1992; Ahn et al., 2003). Saposin B, was the first identified activator protein. Saposin B activates the hydrolysis of sulfatide by arylsulfatase A, the hydrolysis of GM1 ganglioside by β-galactosidase, and globotriaosylceramide by α-galactosidase (Zhao et al., 1998; Harzer et al., 2001). While many other lipids (such as $G_{M2}$ ganglioside) interact with saposin B, this activator is not responsible for stimulating their hydrolysis (Kretz et al., 1990). Saposin C, activates the hydrolysis of glucosylceramide by β-glucosidase and galactocerebroside by β-galactosidase (Kishimoto et al., 1992). Unlike saposin B, saposin C does not interact directly with its lipid substrates, but rather binds to and activates its effector enzymes. The activation effect of saposin C has been proposed to be either due to its ability to induce a conformational change in the effector enzyme allowing for optimal catalysis or more likely by reconstituting the enzyme activity by favoring its localization to appropriate lipid surfaces (Ho and O'Brien, 1971; Kishimoto et al., 1992; Tylki-Szymanska et al., 2007). Saposin A stimulates acid β-glucosidase (glucocerebrosidase) to hydrolyze glucocerebroside (Spiegel et al., 2005). Saposin D, the final and least characterized SAP, has been implicated in the activation of both sphingomyelin and ceramide hydrolysis (Kishimoto et al., 1992; Klein et al., 1994; Matsuda et al., 2004).

### *Extracellular PSAP and saposins*

While PSAP and mature saposins are distributed to lysosomes, PSAP also exists in secretory body fluids and on the surface of a variety of cells. The first report of an extracellular PSAP was in the testis and epididymis

(Sylvester et al., 1989). Therein, PSAP was observed to be synthesized and secreted by both Sertoli and epididymal cells. Once in the lumen PSAP was readily absorbed to the spermatozoa plasma membrane (Sylvester et al., 1989; Clermont et al., 1990; Morales et al., 1996).

Interestingly, spermatozoa formed in the testis during spermatogenesis are immature and incapable of fertilization. As the spermatozoa migrate through the epididymis they encounter a host of luminal proteins, some of which mediate biochemical modifications that transform spermatozoa into a fertile state, a process termed "maturation" (Hinton, 2010). We and others documented that during maturation a selective uptake and loss of epididymal glycoproteins occur along with modifications of the lipid composition (reduction of cholesterol/phospholipid content and desulfation of glycolipids) of the plasma membrane (Cooper, 1995; Jones, 1998; Toshimori, 2003; Rejraji et al., 2006; Morales et al., 2008, 2012). Unlike the plasma membrane of somatic cells, the glycolipids of the sperm plasmalemma are based on glycerol instead of ceramide. In fact, the major sulfoglycolipid is a sulfogalactosylglycerolipid (SGG), which is suspected to be a substrate of the lysosomal enzyme Arylsulphatase A (ARSA). ARSA has also been shown to be secreted by the testis and epididymis and to bind the sperm plasma membrane (Ngernsoungnern et al., 2004). Although ARSA may modify the sulfate composition of the sperm plasma membrane and possibly SGG, this activity requires the presence of saposin B (SapB) (Matzner et al., 2009). However, the processing of prosaposin to mature saposins requires cathepsin D (CatD) (Hiraiwa et al., 1997), an aspartic-protease that is synthesized by the epididymal epithelium and secreted to the lumen of this organ (Igdoura et al., 1995b; Carvelli et al., 2010). Whether CatD is capable of generating sapB in the lumen of the epididymis, is still unknown.

Hammerstedt et al., (2001) isolated a fragment of PSAP (related to SapA and B) from the supernatant of cryopreserved rooster sperm that increase their *in vitro* capability to bind the perivitelline membrane of chicken eggs and to substantially raise their fertility after artificial insemination. Similarly, a PSAP synthetic peptide was shown to enhance fertilization in other species, including bull and horses, underscoring the importance of PSAP and SapB in reproduction (Amann et al., 1999a-c).

PSAP was also found to be synthesized and secreted in nervous tissue. In addition to its role as the saposin precursor, PSAP was reported to exist as an integral component of the surface of neuronal cells (Fu et al., 1994). A number of reports documented a variety of neurotrophic and myelinotrophic activities through a receptor-mediated mechanism (Campana et al., 1998; Hiraiwa et al., 1999).

The neurotrophic region of prosaposin has been localized to a 12-amino acid sequence within the saposin C domain and has been used to derive biologically active synthetic peptides (14-22 residues), called prosaptides. Treatment of primary and immortalized Schwann cells with prosaptide enhanced the phosphorylation of the mitogen activated kinases ERK1 and ERK2 within 5 min, and this activity was blocked with pertussis toxin. Incubation of Schwann cells with the non hydrolyzable GDP analog GDP (betaS) inhibited prosaptide induced ERK phosphorylation. Prosaptide enhanced the sulfatide content of Schwann cells, which was prevented by treatment with pertussis toxin or by the synthetic MAP kinase inhibitor PD098059. Prosaptide increased the tyrosine phosphorylation of all three isoforms of the adapter molecule Shc, which coincided with the association of p60Src and PI(3)K, and the inhibition of PI3(K) by wortmannin blocked prosaptide induced ERK phosphorylation. Taken together these results demonstrated that prosaptide stimulates a G-protein-associated receptor pathway, thus enhancing sulfatide synthesis in Schwann cells and preventing cell death in nervous tissue (Hiraiwa et al., 1997; Campana et al., 1998).

Furthermore, Meyer et al. (2013) observed that prosaptide and prosaposin could bind the G protein-coupled receptor, GPR37, and that together participated in neuroprotective and glioprotective processes. Interestingly, GPR37 has been implicated in the pathogenesis of Parkinson disease as a result of its intracellular aggregation in dopaminergic neurons leading to cell death, as opposed to its plasma membrane localization that provides cellular protection (Takahashi and Imai, 2003). Thus, the subcellular distribution of GPR37 affects cell viability and extracellular prosaposin promotes the permanence of GPR37 in the plasma membrane (Lundius et al., 2014). Studies *in vivo*, also demonstrated that PSAP can exert a variety of responses in nervous tissues such as prevention of neurodegeneration following neuroinjury and promoting the amelioration of allodynia and hyperalgesia in pain models (Hiraiwa et al., 1999). These findings demonstrated a protector role of prosaposin against neurodegenerative disorders.

Additionally to its neurotropic and neuroprotective role, the myelinotrophic action of prosaptide was investigated in developing rats. To assess myelination, sulfatide concentrations in brain and sciatic nerve were determined after subcutaneous prosaptide injection. Prosaptide significantly increased sulfatide concentrations in both brain and sciatic nerve by 250 and 150 per cent over controls, respectively. Likewise prosaposin, prosaptide promoted ERK phosphorylation in immortalized Schwann cells, substantiating that prosaptide treatment stimulates myelination in developing rat via a specific signalling pathway (Hiraiwa et al., 2001).

Lastly, PSAP has been found to be expressed in other fluids of the body such as pancreatic juice, bile, cerebrospinal fluid and milk (Hineno et al., 1991). PSAP expression was also found to be increased in mammary tumours in concert with other lysosomal proteins,

including catD (Campana et al., 1999). It remains to be seen if PSAP plays a role in cell growth and/or metastasis.

In conclusion, the preceding observations indicate that PSAP is not a house keeping precursor protein but rather an essential factor in sperm maturation and in the development and maintenance of the central and peripheral nervous systems, functions that potentially can be extended to other system of the body.

### *PSAP and lysosomal storage disorders*

Lysosomal storage disorder (LSDs) is the term given to a group of more than fifty inherited diseases that are caused by the malfunction of lysosomal enzymes, sphingolipid activators, or adaptors proteins involved in enzyme targeting and lysosomal biogenesis (Meikle et al., 1999; Parkinson-Lawrence et al., 2006). The combined frequency of all LSDs is 1:7700 live births approximately, although this number is growing with the biochemical and genetic characterization of new disorders (Meikle et al., 1999).

The first reported case of LSD was in the early 1880s by physicians Warren Tay and Bernard Sachs (Fernandes Filho and Shapiro, 2004). By noting a cherry red fovea centralis of the eye, cessation of mental development, flaccid or spastic paralysis at approximately 1-2 years of age, physicians identified the main characteristics of LSD presently known as infantile Tay-Sachs disease or the B-variant of $G_{M2}$ gangliosidosis (Tay, 1884; Sachs, 1896; Kolter and Sandhoff, 1998). In the 1960s, the cause of infantile Tay-Sachs was identified as an autosomal recessive mutation in the α-subunit of β-hexosaminidase A, an enzyme involved in the lysosomal degradation of the $G_{M2}$ ganglioside (Okada and O'Brien, 1969; Sandhoff, 1969, 2001).

LSDs have also been associated with the activator

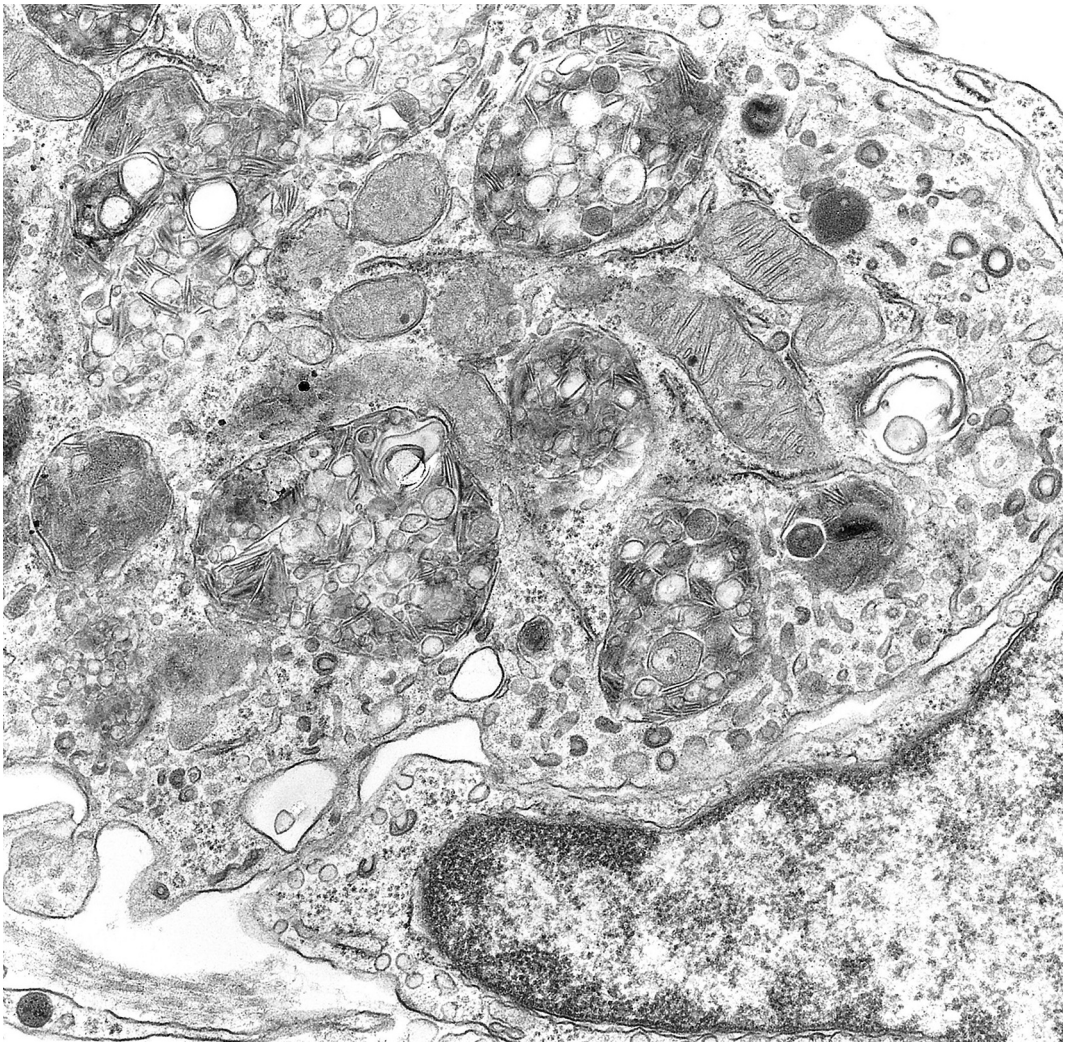

**Fig. 1.** Effect of prosaposin gene inactivation. Professional phagocytes show accumulation of large multivesicular bodies. x 60,000.

proteins derived from the PSAP precursor. Deficiency of saposin B results in a variant form of metachromatic leukodystrophy (MLD) that is characterized by the absence of saposin B and normal activity of arylsulfatase A (ARSA) (Stevens et al., 1981; Gieselmann, 2003). Similar to many LSDs, MLD results in progressive neurological deterioration, accumulation of globotriaosylceramide, sulfatide and $G_{M3}$ ganglioside, demyelination and mental retardation (Holtschmidt et al., 1991; Gieselmann et al., 2003; Nalini and Christopher, 2004). Saposin C deficiency results in a variant form of a LSD, namely Gaucher's disease (Christomanou et al., 1986). In a typical Gaucher's disease, over 200 mutations have been identified in the glucosylceramide β-glucosidase enzyme responsible for the hydrolysis of glucocerebroside, leading to glucocerebroside accumulation (Beutler and Grabowski, 1991). However, in a variant form of Gaucher's disease in which saposin C is affected, there is normal glucosylceramide β-glucosidase activity with accumulation of glucocerebroside (Christomanou et al., 1986; O'Brien and Kishimoto, 1991). Although mutations of saposins A and D have not been implicated in any known LSD, a mouse model suggests that deficiency of saposin A can cause a LSD similar to globoid cell leukodystrophy (Krabbe's disease) (Matsuda et al., 2001). Clinical, phenotypical, and pathological studies of the saposin A knock-out mouse have revealed that the absence of saposin A results in symptoms similar to, but more mild than traditional globoid cell leukodystrophy caused by mutations in the galactosylceramidase gene (Suzuki and Suzuki, 1970; Matsuda et al., 2001). Interestingly, numerous differences exist between metachromatic and globoid cell leukodystrophy. While in the case of metachromatic leukodystrophy there is a pronounced sulfatide accumulation in the brain, there is no over accumulation of galactosylceramide in the brain of patients with globoid cell leukodystrophy (Jatzkewitz, 1958; Suzuki, 2003). This difference has been attributed to the early and rapid loss of myelin, the exclusive site of galactosylceramide, in globoid cell leukodystrophy (Miyatake and Suzuki, 1972; Suzuki, 2003). Mouse models of a saposin D knock-out suggest that saposin D deficiency leads to a unique condition characterized by urine and neural ceramide lipidosis (Matsuda et al. 2004). Other mutations, although rare, affecting the initiation codon of the PSAP gene results in total prosaposin deficiency. Biochemical analysis of tissue from these patients revealed accumulation of different sphingolipids including glucosylceramide, galactosylceramide, and ceramide. Clinically, total PSAP deficiency resulted in hyperkinesia, respiratory insufficiency, and hepatosplenomegaly (Harzer et al., 1989; Paton et al., 1992; Schmid et al., 1992; Bradova et al., 1993; Hulkova et al., 2001; Elleder et al., 2005). Electron microscopy studies demonstrated that the inactivation of the mouse PSAP gene leads to the accumulation of large multivesicular bodies in professional phagocytes (Morales et al., 1999) (Fig. 1).

## The routing of PSAP to the lysosomes and to the extracellular space

### *The duality of PSAP*

Prosaposin is synthesized in the ER as a 53 kDa protein and post-translationally modified to a 62-65 kDa form after the addition of high mannose. In the TGN, this protein is further glycosylated or not to a 68-70 KDa secretory form that is found in various fluids of the body (Igdoura and Morales, 1995; Rosenthal et al., 1995; Zhao and Morales, 2000) (Fig. 2).

Unlike the secreted 68-70 kDa form, the 65 kDa protein is found associated with Golgi membrane and within the lysosomal compartment (Fig. 3A) (Igdoura et al. 1996). The majority of soluble lysosomal proteins are delivered to the endosomes by two M6P receptors (MPRs). As the newly synthesized protein pass through the Golgi apparatus, a unique mannose 6-phosphate (M6P) tag is added to its *N*-linked oligosaccharide. In the *trans*-Golgi network (TGN), the MPRs recognize the

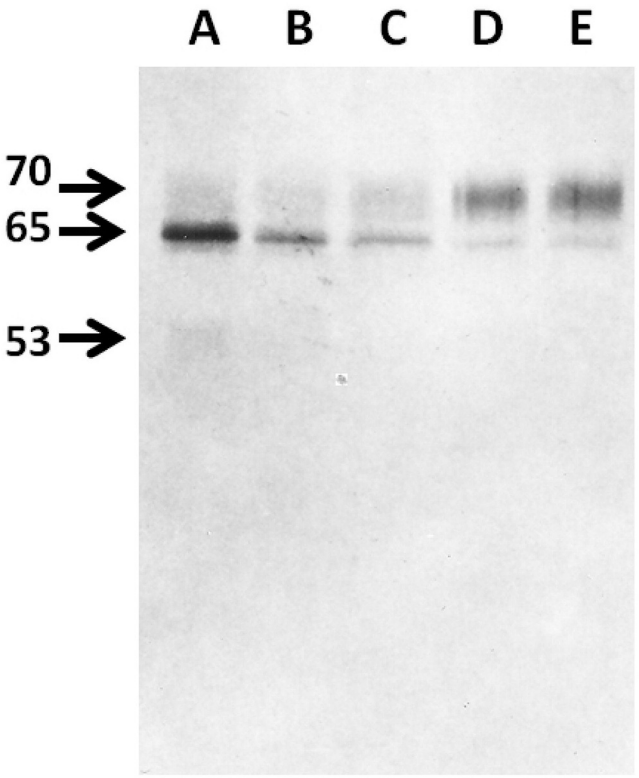


**Fig. 2.** Immunoprecipitation of prosaposin from testicular homogenates analyzed by SDS-PAGE. Lanes A, B, C, D and E represent 30, 60, 120, 180 and 240 minutes respectively after an intravenous injection of 35S-cysteine. The 70 kDa form of prosaposin is a posttranslational modification of the 65 kDa polypeptide.

M6P group and move the lysosomal protein into a clathrin-coated vesicle destined the endosome (Hille-Rehfeld, 1995).

Permeabilization of Golgi-enriched fractions with the mild detergent saponin liberated the 68-70 kDa form but not the 65 kDa protein, and excess of free M6P did not release the lysosomal PSAP from the Golgi membranes (Fig. 3A) (Igdoura et al., 1996). Furthermore, quantitative electron microscopy demonstrated that the lysosomal content of PSAP increased significantly after administration of tunicamycin (Igdoura et al., 1996). These results constituted a clear indication that the trafficking of the 65 kDa form of prosaposin to the lysosomes was independent of the MPR pathway.

One of the main mechanisms regulating the sorting of secretory proteins within the TGN is their selective aggregation in response to a mild acidic pH and an elevated $Ca^{2+}$ concentration (Chanat and Huttner, 1991). The 70 kDa form of prosaposin was found to aggregate within the permeabilized Golgi fractions in a pH and $Ca^{2+}$ dependent manner. Enriched Golgi fractions incubated at pH 5.4 and 7.4 with the mild detergent saponin released the 70 kDa form, whereas a pH of 6.4 caused retention of this protein (Fig. 3B) (Igdoura et al., 1996).

These early findings demonstrated that PSAP has two possible destinations. Initially glycosylated as a membrane associated 65 kDa protein, PSAP could be either transported to the lysosomes or further processed into a highly glycosylated 68-70 kDa form. While the 68-70 kDa protein readily aggregated within the TGN to be secreted, possibly in a regulated manner, the 65 kDa isomer was transported to the lysosomes and submitted to partial proteolysis resulting in four smaller non-enzymatic saposin molecules implicated in the hydrolysis of sphingolipids (Morimoto et al., 1988, 1989; Igdoura and Morales, 1995).

### *The lysosomal transport of PSAP is mediated by sortilin*

I-cell disease (ICD), results from mutation of a single gene that causes the missorting of several lysosomal hydrolases and subsequent accumulation of undigested substrates within lysosomes. I-cell disease is an autosomal recessive mutation caused by the absence of UDP-N-acetylglucosamine N-acetylglucosaminyl-1-phosphotransferase.

I-cell disease was first identified in 1967 as a LSD and was classified as a mucolipidosis in 1970 since it causes symptoms similar to mucopolysaccharidoses and sphingolipidoses (Leroy and Demars, 1967; Spranger and Wiedemann, 1970). Findings suggested that ICD fibroblasts could internalize and utilize lysosomal enzymes produced by normal cells, whereas normal cells could not internalize enzymes produced by ICD fibroblasts (Ullrich and Von Figura, 1979). This observation led to the biochemical analysis of the differences between enzymes produced by normal cells and those from ICD cells and the discovery of the M6P-tag as a lysosomal sorting signal (Vladutiu and Rattazzi, 1979). The absence of functional phosphotransferase in the Golgi of ICD cells results in soluble hydrolases that lack a M6P-tag required for recognition and sorting by the mannose 6-phosphate receptor (MPR). Under these conditions, most soluble hydrolases are missorted to the extracellular milieu (Nolan and Sly, 1989). However, B-lymphocytes from patients with ICD have near normal lysosomal levels of several soluble lysosomal proteins (Tsuji et al., 1988). A B-lymphoblast cell line derived from patients with ICD was in fact found to appropriately target approximately 45 % of the normal levels of cathepsin D to the lysosomes (Glickman and Kornfeld, 1993). Lysosomal targeting of SAPs, PSAP and $G_{M2}AP$ was also unaffected in ICD fibroblasts. These observations resulted in the formulation of the hypothesis that an alternative lysosomal sorting pathway exists that is independent of the MPR (Lefrancois et al., 2003).

In the late nineties it became evident that Sortilin and the MPR shared similar structural features. As a consequence, sortilin (100 kDa) was proposed to traffic

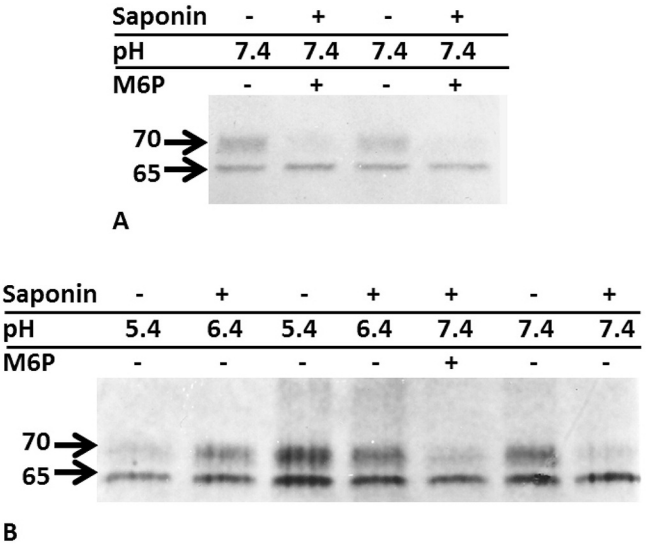


**Fig. 3. A.** Effect of Golgi permeabilization on prosaposin after intravenous injection of 35S-cysteine. Enriched Golgi fractions of rat Sertoli cells were permeabilized in presence or absence of mannose 6 phosphate (MP6), immunoprecipitated with anti prosaposin antibody, run on a gradient SDS polyacrylamide gel and fluoragraphed. Permeabilization of Golgi fractions released the 70 kDa form of prosaposin but not the 65 kDa form. Permeabilization in presence of free M6P did not release the 65 kDa form, indicating that it is not associated with M6P receptors (MPRs). **B.** Effect of pH on release of prosaposin. Golgi fractions permeabilized or not in presence or absence of M6P at pH 5.4, 6.4 and 7.4 and treated as in A (see above). Permeabilization of Golgi fractions released the 70 kDa form of prosaposin, but not the 65 kDa form. Permeabilization at pH 6.4 did not cause release of the 65 kDa form but caused retention of the 70 kDa prosaposin. The result suggests that the 70 kDa protein aggregates at pH 6.4.

lysosomal proteins from the TGN to the lysosomes (Petersen et al., 1997; Nielsen et al., 2001). Paradoxically, no lysosomal proteins were known to interact with sortilin. Our laboratory was the first to identify two *bonafide* ligands of sortilin, namely, PSAP and the $G_{M2}AP$ (Lefrancois et al., 2003; Zeng et al., 2004). In functional terms, the cytosolic tail of mouse sortilin closely resembles the cytosolic tail of the MPR. Both contain motifs known to be involved in trafficking from the Golgi to the endosome and *vice versa* (Nielsen et al., 2001; Seaman, 2007; Canuel et al., 2008). Chimeric constructs that contained the luminal domain of the MPR and the cytosolic component of sortilin, rescued the trafficking of MPR dependent proteins to the lysosomal compartment in MPR-deficient cells (Nielsen et al. 2001). Likewise the MPR cytosolic domain, the cytosolic region of sortilin was shown to bind to the same adaptor proteins, including the Golgi-localized, γ-ear containing, Arf binding proteins (GGAs), AP-1, and retromer (Lefrancois et al., 2003; Seaman, 2007; Canuel et al., 2008). In fact removal of the hinge and ear domains of GGA3 resulted in the abolishment of staining for sortilin and it ligands, prosaposin and $G_{M2}AP$, in lysosomes and in the accumulation of sortilin in the perinuclear region (Lefrancois et al., 2003). These results validated the novel role assigned to sortilin as a lysosomal sorting receptor.

To address the question that sortilin was implicated in the lysosomal transport of PSAP we generated a truncated sortilin construct lacking its cytoplasmic tail. Used as a dominant-negative competitor of endogenous sortilin, truncated sortilin could not exit the Golgi apparatus. Confocal immunomicroscopy revealed that this construct inhibited the targeting of PSAP to the lysosomes causing its retention in the Golgi apparatus. This observation was further substantiated by the abrogation sortilin expression with a specific siRNA. Lack of sortilin resulted in the mis-routing of PSAP to the extracellular space (Lefrancois et al., 2003).

### *The PSAP C-terminus contains the sortilin recognition site*

Experiments performed in our laboratory showed

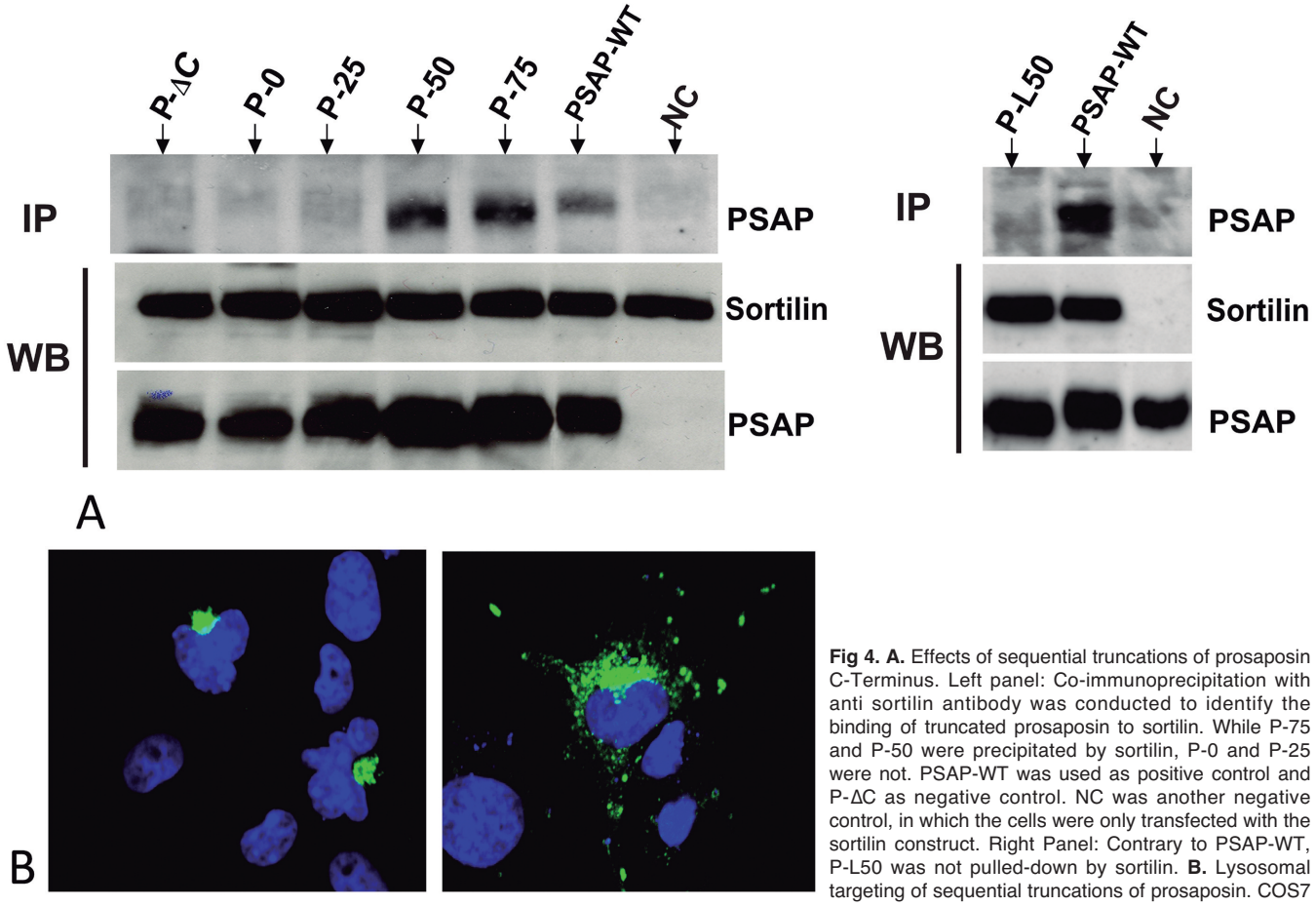


**Fig 4. A.** Effects of sequential truncations of prosaposin C-Terminus. Left panel: Co-immunoprecipitation with anti sortilin antibody was conducted to identify the binding of truncated prosaposin to sortilin. While P-75 and P-50 were precipitated by sortilin, P-0 and P-25 were not. PSAP-WT was used as positive control and P-ΔC as negative control. NC was another negative control, in which the cells were only transfected with the sortilin construct. Right Panel: Contrary to PSAP-WT, P-L50 was not pulled-down by sortilin. **B.** Lysosomal targeting of sequential truncations of prosaposin. COS7 cells transfected with the truncated constructs were examined by confocal microscopy. Prosaposin constructs appear stained in green and nuclei in blue. Left panel: representative field of P-L50, P-25, P-0 and P-ΔC constructs, showing that prosaposin staining was restricted to the Golgi apparatus. Right panel: representative field of cells transfected with PSAP-WT, P-75 and P-50 showing that the constructs labeled the Golgi and lysosomes.

that the lysosomal trafficking of prosaposin was dependent on the saposin D domain along with its highly conserved C-terminal region (Zhao and Morales, 2000). Deletion analysis revealed that unlike the N- terminus, removal of the C-terminus abolished the trafficking of prosaposin to the lysosomes (Zhao and Morales, 2000; Canuel et al., 2009). We have demonstrated that a fragment within the first half of the C-terminus of prosaposin (aa524-540) is responsible for the binding to sortilin and required for the targeting of prosaposin to the lysosomes (Yuan and Morales, 2010). To identify this fragment, we performed sequential deletion on prosaposin C-terminus. First, we predicted the secondary structure of the C-terminus using EMBOSS Garnier (Garnier et al., 1978) and found two α-helices within the C-terminus. The first helix was localized to the linker region between aa518-523, while the second between aa540-550. Based on this structure information, we generated several truncated prosaposin constructs. For the first construct P-75, we deleted the C-terminal region immediately after C551 to avoid the disruption of the predicted helixes. P-75 lacked aa552-557, which represented 25% of the C-terminus. In the second construct termed P-50, the deleted sequence spanned between aa541-557. In this case, the second helix (E540 to H550) was eliminated. In P-25, the deletion spanned between aa531-557, resulting in the elimination of two cysteine residues. The construct P-0 lacked the entire A-type domain (aa524-557). The final construct, P-ΔC, was a truncated prosaposin lacking the entire C-terminus and the linker that connects this region to saposin D (aa518-557). We also generated a construct (P-L50), which lacked aa524-540, i.e, the first half of the C-terminus. COS7 cells were co-transfected with sortilin and each of the prosaposin constructs described above. The cell lysates were subjected to co-immunoprecipitation. Anti-sortilin antibody was used to pull-down sortilin-PSAP complex and anti-myc antibody used for immuno-blotting. The results showed that sortilin pulled down PSAP-WT and truncated constructs P-75 and P-50 while

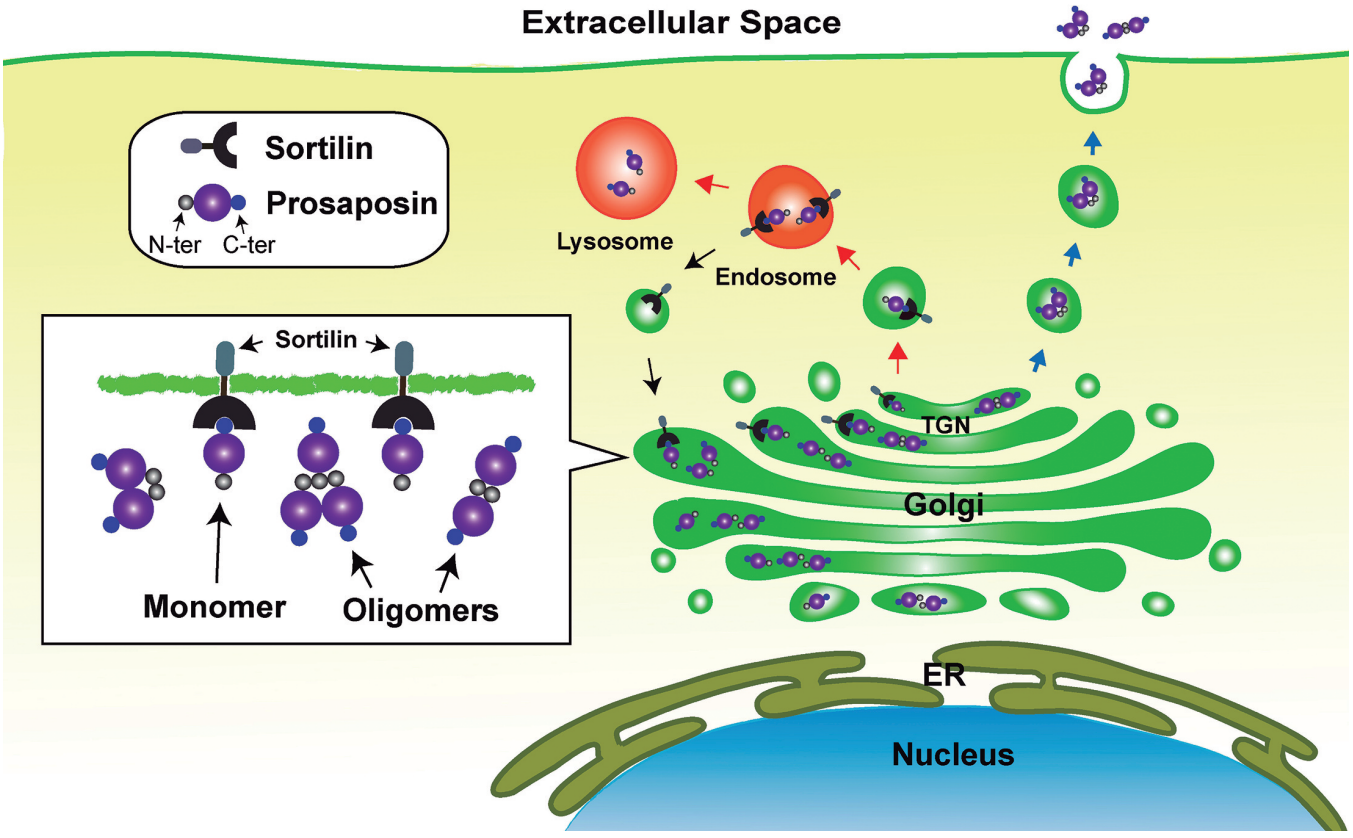


**Fig. 5.** Proposed model of PSAP sorting in the TGN. In the ER, newly synthesized PSAP molecules can be found as monomers and dimers. In the proximal compartment of the Golgi apparatus the molecules are linked to high-mannose oligosaccharides. Before reaching the distal regions of the TGN monomeric PSAP binds sortilin. This interaction is accomplished via the C-terminus of PSAP. The PSAP-sortilin complex enters the lysosomal trafficking pathway, escaping terminal glycosylation. Once in the endosomes, sortilin releases PSAP and recycles back to the TGN. The PSAP molecules that do not bind sortilin remain as dimers or form larger oligomers. Due to their large size and changes in structure, prosaposin oligomers are not able to bind sortilin. Instead, they reach the terminal regions of the TGN, where they are linked to complex oligosaccharides and directed to the secretory pathway.

it failed to precipitate P-25, P-0, P-ΔC and P-L50 (Fig. 4A) (Yuan and Morales, 2010).

To further verify if the truncations affected the transport of PSAP to the lysosomes, COS7 cells transfected with each construct were examined by confocal microscopy after immunofluorescence labeling. The results demonstrated that the wild type construct (PSAP-WT) exhibited Golgi and vesicular staining and overlaid with both anti-Golgin 97 (Golgi marker) and anti-LAMP-1 (lysosomal marker) staining. The distribution of P-75 and P-50 was similar to that of PSAP-WT. However, P-ΔC, P-0, and P-25 localized only in the perinuclear region and overlaid with anti-Golgin 97 but not with anti-LAMP-1 staining. The results demonstrated that the elimination of the terminal half of the C-terminus (aa541-557), did not affect the transport of PSAP to the lysosomes. However, elimination of it first half (aa524-540), containing 17 amino acids located between the two α-helices, abolished the transport of PSAP to the lysosomes (Fig. 4B) (Yuan and Morales, 2010).

### *The binding of PSAP to sortilin is pH dependent*

It is well know that ligand-receptor binding and dissociation are pH-dependant processes (Mellman et al., 1986). While mild acidic compartments, such as the lumen of the TGN (pH 6.0), favor the binding of soluble hydrolases to the MPRs, the strong acidic compartments of the endosomes and lysosomes (pH 5.0 or lower) induce their release from the MPRs (Coffey and De Duve, 1968; Van Weert et al., 1995; Schindler et al., 1996; Demaurex et al., 1998). We proposed that the binding of PSAP to sortilin was also pH-dependent. To test this hypothesis, we performed an *in vivo* pH-dependent assay. COS7 cells were co-transfected with wild-type PSAP and sortilin constructs. The transfected cells were homogenized in lysis buffer at pH 4.0, 5.0, 5.5, 6.0, 6.5, or 7.0. All samples subjected to immuno-precipitation contained equal concentration of lysate, and the washing buffer for each sample had the same pH as the initial lysis buffer to keep the sample in a stable pH condition. The complexes pulled down by anti-PSAP antibody were resolved on a 10% acrylamide gel. After transferring to a nitrocellulose membrane, the samples were immunoblotted for prosaposin and sortilin using anti-prosaposin and anti-sortilin antibodies respectively. The results showed that the presence and/or intensity of the sortilin bands varied at different pHs. The strongest sortilin bands were observed at pH 6.0, 6.5 and 7.0 whereas a substantial decrease of intensity was detected at pH 5.5, and no sortilin band was observed at pH5.0. This result indicated that the binding of prosaposin to sortilin was pH-dependent and suggested that the pH of the TGN (pH 6.0) (Demaurex et al. 1998) favoured the association between PSAP and sortilin while the pH of endosomes (pH 5.5) (Coffey and De Duve 1968; Van Weert et al. 1995) favoured the dissociation between PSAP and sortilin.

### *The routing of PSAP to the extracellular space*

In eukaryotes the delivery of newly synthesized proteins to the lysosomes or to the extracellular space is dependent on a series of functionally distinct compartments, including the endoplasmic reticulum, the Golgi apparatus and carrier vesicles. The sorting mechanism of proteins with multiple destinations such as PSAP is sketchy. Nevertheless, it is known that 65 kDa PSAP is transported to the lysosomes while 68-70 kDa PSAP is sorted to secretory pathway in the Golgi apparatus. The intracellular trafficking of PSAP to different destinations has raised the question of how subsets of PSAP molecules escape the selective lysosomal transport to a non-selective secretory pathway.

A recent report suggested that secreted PSAP form oligomers in the extracellular space (Grassel and Hasilik, 1992; Hiraiwa et al., 1993). We investigated PSAP oligomerization using non-reducing SDS-PAGE (Yuan and Morales, 2011). We found that: 1) PSAP oligomerized covalently and 2) oligomerization took place within the cell. Since PSAP monomer was never found in the culture media, we tested the hypothesis that monomeric PSAP was the sortilin ligand. Our results showed that when COS7 cells were cotransfected with sortilin and PSAP, only the PSAP monomer was immunoprecipitated by the anti-sortilin antibody, suggesting that PSAP was bound to sortilin in its monomeric form.

We also examined the effect of pH on PSAP oligomerization. The results showed that PSAP contains multiple forms, including monomer and oligomers at all pH levels. However, when pH was changed from neutral to acidic, the percentage of monomer decreased from 38.8% (pH 7.0) to 31.3% (pH 6.5), 22.4% (pH 6.0), and 17.8% (pH 5.5) and then increased to 23.8% (pH 5.0) and 31.5% (pH 4.0). Conversely, the dimer presented reversed changes that almost mirrored the values of the monomers. Thus, the percentage of dimer increased from 31.1% (pH 7.0) to 35.3% (pH 6.5), 42.2% (pH 6.0), and 47.2% (pH 5.5) and then decreased to 43.4% (pH 5.0) and 37.3% (pH 4.0). The effects of pH on the percentage of trimer, tetramer and other oligomers were minimal. The biphasic variation of monomers and dimers suggested that pH values ranging from neutral to mildly acidic favoured dimerization. We have also noticed that increased levels of dimers between pH 7.0 to 5.5 mirrored the decrease of monomers. This observation may have physiological relevance since the lumen of the TGN is more acidic than other regions of the Golgi apparatus (Demaurex et al., 1998). Thus, the increased dimers may facilitate the aggregation of molecules within the TGN resulting in their dissociation from the PSAP-sortilin complex and their entry to the secretory pathway.

A relevant issue was which of the PSAP domains was responsible for covalent oligomerization. To answer this question we used truncated PSAP constructs (Zhao

and Morales, 2000). Among those constructs, ΔA, ΔB, ΔC and ΔD lacked a single saposin domain A, B, C, or D. ΔN-ter and ΔC-ter lacked the N-terminus and C-terminus, respectively. The constructs, including wild-type PSAP, were transfected to COS7 cells and the cell lysates were examined by SDS-PAGE. While half aliquot of the cell lysate was resolved by reducing SDS-PAGE, the other half aliquot was analyzed by non-reducing SDS-PAGE. In the non-reducing gel, ΔN-ter, ΔA, ΔB, ΔC and ΔD were all detected with the specific antibody as single bands at sizes ranging between 60 and 70 kDa. This indicated that deletion of the N-terminus and of any saposin domains inhibited oligomerization. However, deletion of the PSAP C-terminus did not prevent oligomerization, indicating that this region was not responsible for this process. Since the C-terminus contains the sortilin binding site and is required for the lysosomal trafficking of prosaposin (Zhao and Morales 2000; Yuan and Morales 2010), our results suggest that oligomerization is not required for the lysosomal transport of PSAP.

## Conclusion

It is accepted that soluble lysosomal proteins are sorted within the TGN by a sorting receptor and that the ligand-receptor complex is targeted to the endosomes by accessory proteins that interact with the sorting receptor (Hirst and Robinson, 1998; Rouille et al., 2000). We have demonstrated that unlike the majority of soluble hydrolases, which are transported via the MPRs, prosaposin is translocated to the endosomes via the alternative receptor sortilin (Lefrancois et al., 2003). Preliminary evidence suggests that the C-terminus of prosaposin is required for the transport of prosaposin to the lysosomes (Zhao and Morales, 2000). In a previous comparative study on the structure of human, mouse and rat prosaposin we found that the D-domain and the C-terminus were the most conserved regions among the three species with more than 90% of sequence identity (Zhao et al., 1998). The high level of conservation suggests that this region carries a critical function. Interestingly, the C-terminus of prosaposin contains a saposin-like motif significantly similar to the one found in the N-terminus of the surfactant B associated protein (Patthy, 1991) which has been implicated in the transport of this protein to the lamellar bodies of type II pneumocytes (Lin et al., 1996).

We have shown that the deletion of this region and the substitution of critical hydrophobic residues abolished the binding of prosaposin to sortilin and the targeting of prosaposin to the lysosomes (Yuan and Morales, 2010). We also have demonstrated that monomeric, endo H sensitive PSAP is the form of the protein that binds sortilin and traffics it to lysosomes, and that oligomerized endo H resistant PSAP is the form of the protein that is delivered to the secretory pathway (Yuan and Morales, 2011). Based on accepted knowledge on the structure and function of sortilin, and on our observations on PSAP, we proposed a sorting model for PSAP. According to our model, newly synthesized prosaposin enters the Golgi apparatus as both dimers and monomers where they are partially glycosylated. A subset of monomers is sequestered by sortilin in the mid-Golgi compartment and/or in the proximal region of the TGN where the ligand-receptor complex is routed to lysosomes (Lefrancois et al., 2003). The remaining PSAP molecules reach the edges of the TGN where they are fully glycosylated with complex oligosaccharides. Alternatively, due to space constraints within the tunnel of the sortilin β-propeller (Quistgaard et al., 2009), the oligomers may not be able to bind sortilin. Once in the TGN, PSAP oligomers aggregate within secretory vesicles, which deliver them into the extracellular space (Fig. 5).

Finally, collective findings suggest that in addition to its function in lysosomal degradation of sphingolipids, PSAP may play a potential role in the extracellular space with prospective clinical implications.

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