Dynamic Microscopy: Reconstructing a Novel Lysosomal Trafficking Pathway

Libin Yuan¹, Flavia Lorena Carvelli² and Carlos R. Morales¹

¹Department of Anatomy and Cell Biology, McGill University, Montreal, Canada
²Instituto de Ciencias Básicas, Universidad Nacional de Cuyo, IHEM, Mendoza, Argentina

The delivery of newly synthesized lysosomal proteins to endosomes and lysosomes is dependent on several functionally distinct compartments, i.e., the endoplasmic reticulum (ER), the Golgi apparatus and small carrier vesicles. These compartments also play a role in the synthesis, post-translational modification, sorting and distribution of proteins to other destinations. In fact, most cargo is sorted within, and exits from, the trans-Golgi network (TGN). Proteins delivered to the endosomal/lysosomal system include a large and diverse class of hydrolytic enzymes and non-enzymatic activator proteins that were thought to be directed to the lysosomes by their binding to mannose-6-phosphate receptors (MPRs). Surprisingly, in I-cell disease, in which the MPR pathway is disrupted, the non-enzymatic sphingolipid activator protein, prosaposin, continues to traffic to lysosomes. The use of dynamic immunofluorescence and immunoelectron microscopy along with mutational analysis and chimeric protein technology led us to the discovery of a new lysosomal sorting receptor, sortilin. We found that prosaposin is targeted to the lysosomes through an interaction with sortilin. Deletion of the C-terminus of prosaposin abolished its transport to the lysosomes, and mutational analysis revealed that the first half of the prosaposin C-terminus (aa524-540) contains a motif required for its binding to sortilin and its transport to lysosomes. Additionally, a chimeric construct consisting of albumin and a distal segment of prosaposin, which includes its C-terminus, resulted in the routing of albumin to lysosomes. In conclusion, we have identified a specific region in the C-terminus of prosaposin, as well as amino acid residues that are critical for the binding of prosaposin to sortilin and its subsequent lysosomal trafficking.

Keywords Lysosome; Golgi apparatus; sorting; lysosomal trafficking; prosaposin; sortilin

1. Introduction

Light (LM) and electron microscope (EM) images are generally viewed as two dimensional micrographs of three dimensional structures, where the latter is deduced from the observation of similar objects and serial sections. Thus, the possibility of analyzing and detecting dynamic processes by light (LM) or electron microscopy (EM) is difficult since the specimens must be fixed with chemical agents or fixatives in order to be structurally preserved. This obstacle is particularly problematic when analyzing the sorting and transport of molecules between compartments.

The advent of the confocal microscope permitted us to achieve fast and excellent structural results. In recent years, confocal microscopy (CM) has gained popularity in life sciences. It is an optical imaging technique used to increase the resolution of the light microscope by using point illumination and a spatial pinhole to eliminate out-of-focus planes in specimens that are thicker than the focal objects, thus enabling the reconstruction of three-dimensional structures from the obtained images[1]. Because the identification of organelles is far from trivial and the resolution power lower than EM, the identification of cellular components in CM has to be done in combination with immunocytochemical and/or cytochemical procedures. In fact, at such structural level, the decisive identification of certain structures such as lysosomes and the Golgi apparatus requires additional identification criteria (i.e., enzymatic, immunocytochemical and/or structural characterization). The identification of enzymes, substrates and/or specific markers by means of immunocytochemistry has been the most effective method of CM to unequivocally recognize organelles. Nevertheless, the main problem of microscopy, i.e., the necessity of working with fixed specimens could not be resolved, except in those cases where time-lapse epifluorescence was used in conjunction with video microscopy to record life specimens [2]. In this instance, a cell must be transfected with vectors expressing fusion proteins tagged with green fluorescent protein (GFP) or other fluorochromes. Although this approach allows the tracing of proteins within the cell, the unequivocal identification of compartments is not possible.

In this review we discuss a multi-technical approach, consisting of the use of fusion proteins and mutational analysis of the lysosomal protein prosaposin, in conjunction with CM. This approach allowed us to reconstruct a sorting and trafficking mechanism not described before. The analysis revealed that the C-terminus of prosaposin is required and necessary for its transport to lysosomes.

2. Eukaryotic Cell and Cellular Compartments

The distinction between prokaryotes (before nuclei) and eukaryotes (with nuclei) was introduced 80 years ago by the French zoologists E. Chatton[3]. Eukaryotes are all organisms that are not bacteria (prokaryotes). Eukaryotes include three large groups of multicellular organisms (animals, plants and higher fungi), plus a large group of unicellular
organisms referred as protozoa, protophytes (unicellular algae), multicellular algae, and some fungi-like organisms. Eukaryotes that belong to neither the group of animals, plants and fungi are called protists [3].

The origin of eukaryotes represented an evolutionary jump in the complexity of the cell which resulted in the presence of cellular compartments or organelles, including the need to sort proteins between them, to the plasma membrane or to the extracellular space. It is commonly thought that this new level of complexity arose approximately 2.7 billion years ago [4], and that it was the consequence of the appearance of membrane-bound organelles within the cytoplasm, believed to result from a process of endosymbiosis [3].

To cope with the resultant intricacies of functionally distinct compartments engaged in the synthesis and transport of proteins (i.e., endoplasmic reticulum, Golgi apparatus, lysosomes, etc), the eukaryotic cell evolved complex systems for the sorting and transport of proteins to their final destinations, including lysosomes [5, 6].

In mammals, the mannose 6-phosphate receptors (MPRs) are canonical sorting receptors responsible for the transport of newly synthesized soluble hydrolases destined for lysosomes [7]. Two forms of the MPRs exist, the 46 kDa cation-dependent (CD) MPR and the 300 kDa cation-independent (CI) MPR [8, 9]. Although there is only modest homology between the two MPRs (~20%), it is accepted that both MPR genes arose from a common ancestor. It has been suggested that the more complex CI-MPR originated from multiple duplications of a single common ancestral gene [10-12]. The MPRs have been described in chickens, amphibians, and reptiles. Although not well characterized, a putative MPR has also been reported in invertebrates [13-15]. However, at present, given the lack of information, it remains difficult to confirm the point of origin in which the ancestral MPR gene appeared during evolution [16]. Nonetheless, recent findings have demonstrated that the MPR is present and fully functional in ancient teleosts, which appeared during the Triassic period over 200 million years ago [16, 17]. As eukaryotes need to sort proteins at a much earlier point in time, the MPR would either need to have arisen before the Triassic period, or another more ancient sorting receptor must have existed. The quest for sorting receptors in yeast and plants resulted in the discovery of the Vps10p and the BP80 receptors [18, 19].

The Vps receptor family is a novel class of heterogeneous type-I trans-membrane receptors which includes sortilin (100 kDa), Vps10p (160 kDa), SorLA (250 kDa), and SorCS1-3 (130kDa) [20]. The pre-fix “sor” is an abbreviation for “sorting receptor related” and highlights the functional role of these proteins [21]. These receptors have a diverse pattern of expression in many tissues and are responsible for the targeting of a variety of different ligands [22-27]. As Vps10 domain containing proteins are found in a variety of simple organisms such as Dictyostelium, Neurospora, and Metarhizium, it is probable that sorting pathways involving these receptors arose before that of the MPR [28]. Sortilin, a well-studied member of the Vps10 family, is a 95-100 kDa sorting receptor that is highly expressed in brain, testis and skeletal muscle, was known to bind and internalize proteins at the cell surface [22]. However, our laboratory was the first to identify the intracellular ligands, namely, prosaposin and the G_{M2}AP, that require sortilin for their intracellular transport to the lysosomes [24, 29].

3. Prosaposin Overview. Evidence for a MPR Independent Trafficking

It is well established that to reach the lysosomes, a soluble lysosomal protein must interact with a trans-membrane sorting receptor containing a cytoplasmic domain that binds adaptor proteins capable of recruiting clathrin [7, 30]. The mannose 6-phosphate receptors (MPRs) bind and route most soluble lysosomal hydrolases [7]. The recognition of soluble proteases by the MPRs occurs in the trans-Golgi network (TGN), and depends upon the tagging of M6P residues to the newly synthesized enzyme by a specific phosphotransferase [31-33]. Transport to the lysosomes is achieved through the binding of monomeric adaptor proteins (GGAs) to an acidic cluster di-leucine motif in the cytoplasmic tail of the MPRs [34, 35]. Mutation of the phosphotransferase that adds the M6P tag to lysosomal proteases results in 1-cell disease (ICD). ICD fibroblasts are characterized by blockage of the MPR trafficking pathway and accumulation of storage material in lysosomes [31]. Interestingly, the lysosomes of ICD fibroblasts contain near normal levels of several soluble proteases, as well as soluble non-enzymatic sphingolipid activator proteins (SAPs) such as prosaposin and GM2AP [36]. This observation indicated that SAPs traffic to lysosomes by an MPR independent mechanism.

Prosaposin is a glycoprotein with different functions and destinations. However, the main function ascribed to this protein is being the precursor of four non-enzymatic sphingolipid activator proteins, or saposins, required for the hydrolysis of sphingolipids. Mutations in the prosaposin gene result in several lysosomal storage disorders including a variant form of metachromatic leukodystrophy (MLD) and a variant form of Gaucher’s disease [37]. Prosaposin is synthesized in the ER as a 53 kDa protein and post-translationally modified to a 65 kDa form after the addition of high mannose [38-40]. In the TGN, this protein is further glycosylated to a 70 kDa secretory form that is found in various fluids. Unlike the 70 kDa form, the 65 kDa protein is associated with the membrane of the Golgi apparatus, where it is sorted and targeted to lysosomes [41]. In fact, permeabilization of Golgi-enriched fractions with mild detergents liberated the 70 kDa form but not the 65 kDa protein, and excess free M6P did not release lysosomal prosaposin from Golgi membranes [41]. Furthermore, quantitative electron microscopy demonstrated that the lysosomal content of prosaposin increased significantly after administration of tunicamycin [41]. These results also indicated that the trafficking of the 65 kDa form of prosaposin to lysosomes was independent of the MPR pathway. In addition, strong
evidence from a number of laboratories suggested that prosaposin was targeted to lysosomes in a MPR-independent manner [36, 41, 42]. Evidence for MPR-independent transport of prosaposin included experiments in cultured cells incubated with fumonisin B1, an inhibitor of sphingolipid synthesis that competes with sphinganine as a substrate of ceramide synthase [43]. This treatment produced a dramatic decrease in the immunogold labeling of lysosomes with anti-prosaposin antibody [43]. To examine if the MPR-mediated pathway was affected by this treatment, cells treated or not with fumonisin B1 were labeled with anti-cathepsin A antibody. The results showed no significant differences in the immunogold labeling of the lysosomal compartment of treated or untreated cells, indicating that the effect of fumonisin B1 on the transport of prosaposin to the lysosomes was specific [43]. The effects of DL-threo-1-phenyl-2-decanoyl-amino-3-morpholino-1-propanol-HCL (PDMP), a compound that selectively inhibits the synthesis of glycosphingolipids, but not of sphingomyelin and/or ceramide, and the effect of tricyclodecan-9-yl xanthate potassium (D609), which specifically blocks the formation of sphingomyelin [44], were also examined. The results showed that only D609 blocked the transport of prosaposin to lysosomes, suggesting that sphingomyelin is also implicated in the trafficking of prosaposin to the lysosomes [43]. Taken together, these observations suggest that the 65 kDa lysosomal isomer remains associated with an alternative receptor possibly on discreet membrane microdomains such as lipid rafts.

4. Identification of the Region Involved in the Trafficking of Prosaposin

The Golgi apparatus is not only responsible for accomplishing the molecular sorting of the 70 kDa form, but also for decoding the lysosomal sorting signal from the amino acid backbone of the 65 kDa form of prosaposin. The identification of the prosaposin region involved in this interaction was found in our laboratory by mutational deletion of each of the four known saposin functional domains and the highly conserved N- and C- termini of prosaposin [40, 45]. The truncated cDNAs were subcloned into expression vectors and transfected into COS-7 cells. The myc-tagged truncated proteins were detected by immunofluorescence using anti-myc antibody, followed by a secondary FITC conjugated goat anti-mouse antibody [40]. We found that deletion of the C-terminus did not interfere with the routing of prosaposin to the extracellular compartment but abolished its transport to lysosomes (Figure 1). However, deletion of each of the saposin regions and N-terminal domain did not affect the lysosomal or secretory routing of prosaposin [40].
5. Role of the C-terminus in the Lysosomal Transport of Prosaposin: Use of Chimeric Proteins

To determine whether the C-terminus alone was sufficient for the intracellular targeting of prosaposin, a chimeric construct encoding the full-length albumin plus the C-terminus of prosaposin (Alb/COOH) was engineered and subcloned into an expression vector, and transfected into COS-7 cells. A wild type albumin cDNA was also prepared as a control (Alb). After transfection, cells were immunostained with anti-myc antibody, followed by a secondary FITC conjugated goat anti-mouse antibody. The cells were simultaneously stained with the lysosomal marker LysoTracker. The results showed that the C-terminus alone was insufficient to target the chimeric construct to lysosomes [40]. Based on these results we postulated that one or more saposin domains are required, along with the C-terminus, to direct
albumin to the lysosomes. To test this hypothesis, a chimeric protein was engineered by fusing an albumin cDNA with a cDNA sequence encoding the domain D and the C-terminus of prosaposin (Alb/D/COOH). In addition, an albumin fusion protein containing domains C and D plus the C-terminus of prosaposin (Alb/C/D/COOH; data not shown) was also constructed. In these cases, the anti-myc antibody yielded a punctuate reaction (Figure 2) which overlaid with LysoTracker staining. This observation indicated that the C-terminus and at least one saposin domain was required to direct albumin to the lysosomal compartment [40].

![Figure 2](image_url)

**Fig 2.** Targeting of myc-albumin-prosaposin chimeric constructs as visualized by dynamic microscopy. COS-7 cells expressing the secretory protein albumin showed a perinuclear reaction but not lysosomal staining, indicating that the construct followed the secretory pathway (see lower drawing). Albumin linked to prosaposin C-terminus (Albumin+PSAP-C-term) yielded a prominent Golgi reaction and a weak lysosomal staining, suggesting that the C-terminus alone was inefficient to target the chimeric construct to lysosomes. Albumin linked to prosaposin D domain followed by its C-terminus (Albumin+PSAP-D&C-term) produced a strong lysosomal reaction, demonstrating that the D domain plus the C-terminus were required and necessary for the transport of the chimeric protein to lysosomes (see lower drawing).

### 6. Demonstration that Sortilin Mediates the Sorting of Prosaposin

The ability of prosaposin to interact directly with sortilin was assessed using an *in vitro* Co-IP assay. Prosaposin, cathepsin B, full-length sortilin, and truncated sortilin lacking the cytoplasmic domain were translated *in vitro* using a cell-free system. Prosaposin and cathepsin B were then incubated with full-length or truncated sortilin and immunoprecipitated with specific antibodies. The results demonstrated that anti-prosaposin immunoprecipitated both full-length and truncated sortilin. Anti-cathepsin B, however, did not immunoprecipitate sortilin. Similar results were obtained using culture cells transfected with the same expression vectors. Therefore, sortilin was demonstrated to interact directly with prosaposin [24].

Using dynamic CM, sortilin was demonstrated to be responsible for mediating the transport of prosaposin to lysosomes [24]. Firstly, this was determined by using truncated sortilin as a dominant-negative competitor. Truncated sortilin, lacking the cytosolic domain that is required to bind and recruit adaptor proteins, was overexpressed in COS-7 cells. This construct abolished the ability of prosaposin to traffic to lysosomes and resulted in its retention in the Golgi apparatus (Figure 3). Under this condition, prosaposin was secreted into the extracellular medium, suggesting that upon saturation of sortilin, prosaposin entered a default secretory pathway. The effect of the truncated sortilin construct was specific, as trafficking of cathepsin B, a ligand of the MPR, was unaffected (Figure 3). Secondly, to validate these findings, the lysosomal trafficking of prosaposin was examined in TM4 cells deficient in sortilin. This was achieved by the knockdown of sortilin using a specific siRNA. As expected, in sortilin-deficient cells prosaposin was not routed to the lysosomal compartment (Figure 4). Pulse-chase analysis revealed that in these cells, prosaposin secretion was augmented [24, 26].
**Fig 3.** Effect of sortilin truncation on the targeting of prosaposin as visualized by dynamic microscopy. Expression of wild type sortilin (WT, upper drawing) yielded a strong prosaposin lysosomal staining. Removal of the cytoplasmic tail of sortilin (Truncated Sortilin, upper drawing) produced a Golgi reaction but not a lysosomal staining, indicating that prosaposin was directed to the secretory pathway (see lower drawing). As expected, truncated sortilin had no effect on the targeting of cathepsin B to lysosomes since this hydrolase uses the MPR.

**Fig 4.** Effect of sortilin silencing on the targeting of prosaposin as visualized by dynamic microscopy. To remove the ability of sortilin to sort proteins to lysosomes, TM4 cells were transfected with a sortilin specific siRNA probe. Unlike the control, the siRNA abolished the lysosomal staining of prosaposin (red fluorescence). Anti-Golgin antibody was used to stain the Golgi apparatus (green fluorescence). The merging of both fluorochromes produced a yellow pattern in the Golgi region of the control cells and a green reaction in the treated cells.
7. Identification of the Prosaposin Domain that Interacts with Sortilin

In an attempt to identify the domain that binds sortilin, we deleted specific segments of the prosaposin C-terminus [46]. To engineer the constructs, we analyzed the predicted secondary structure of the C-terminus using EMBOSS Garnier [47]. The EMBOSS output file predicted the existence of two α-helices within the C-terminus. The first helix was localized to the linker region between aa518-523, and the second between aa540-550. The PredictProtein software suggested that two pairs of cysteine residues (C528-C536 and C545-C551) may form two disulfide bonds that stabilize the tertiary structure of the C-terminus [48]. Thus, to generate the first construct, termed P-75, and to avoid disruption of the predicted helices and disulfide bonds, we deleted the C-terminal region located immediately after C551. In the second construct, termed P-50, we deleted the region spanning between aa541-557 and eliminated the second helix (E540 to H550). In the third construct, termed P-25, the deletion spanned between aa531-557, resulting in the elimination of two cysteine residues. We also generated a construct, termed P-0, which 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 region that spans between the C-terminus and saposin D (aa518-557). Subsequently, COS-7 cells were co-transfected with sortilin and each of the prosaposin constructs described above. The cells were homogenized in lysis buffer (pH 6.0) and subjected to immunoprecipitation. The complexes were pulled down with anti-sortilin antibody and resolved on a 10% acrylamide gel. Immunoblotting with anti-myc antibody showed that sortilin pulled down the wild type prosaposin (PSAP-WT) and truncated constructs P75 and P50, while it failed to precipitate P-25, P-0 and P-ΔC. These results demonstrated that the critical domain for the binding of prosaposin to sortilin was located within the first half of the C-terminus of prosaposin (aa524-540). To confirm that the external portion of the C-terminus was not involved in this process, we generated a construct lacking the first half of the A-type domain, termed P-L50. In this construct, the external region of the A-type domain was attached to the linker region. As expected, P-L50 was not pulled down by sortilin [46].

To examine the effect of the sequential deletions on the transport of prosaposin to lysosomes, COS-7 cells were transfected with each prosaposin construct and examined by dynamic CM (Figure 5). The wild-type and truncated prosaposin constructs were stained in green with anti-myc antibody. The TGN and lysosomes were stained in red with an anti-Golgin 97 or with an anti-LAMP1 antibody, respectively. The nuclei were counterstained in blue with Hoechst 33342. The results demonstrated that wild-type prosaposin exhibited perinuclear and granular staining and overlaid with both TGN and lysosomal staining. The distribution of P75 and P50 was similar to that of PSAP-WT. However, P-ΔC, P0, and P-25 localized only in the perinuclear region and overlaid with anti-Golgin97, but not with anti-LAMP-1 staining. Statistical analysis showed that the percentage of overlaid granules of P-ΔC, P-0, P-25 and P-L50 significantly decreased as compared to PSAP-WT (P<0.01), while P-50 and P-75 did not (P>0.05) (Figure 6).

In conclusion, the analysis of the effect of sequential deletions in the C-terminus of prosaposin has allowed us to identify the putative sortilin binding site. Our findings suggest that this region is located within the first half of the A-type domain on the C-terminus, which is located between aa524 and 540. According to the predicted secondary structure of the C-terminus, this region may contain a β-sheet and several turns, which may be stabilized by proline and tryptophan residues. In fact 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 [46].
Effect of sequential truncations of prosaposin C-terminus as visualized by dynamic microscopy. COS7 cells were transfected with truncated constructs and examined by confocal microscopy. Truncated prosaposin was stained green with chicken anti-myc antibody, while TGN or lysosomes stained red with anti-Golgin97 or anti-LAMP-1 antibodies. Nuclei appear in blue. Anti-myc staining of cells transfected with PSAP-WT, P-75 and P-50 constructs labeled the perinuclear region and cytoplasmic vesicular structures and overlaid with anti-Golgin staining respectively. P-L50, P-25, P-0 and P-ΔC (not shown) labeled the perinuclear region but not the lysosomes and overlaid only with anti-Golgin staining, indicating that the recombinant proteins were redirected to the extracellular space.
8. Role of the TGN in the sorting of Prosaposin to lysosomes and to the Extracellular Space

The Trans-Golgi Network (TGN) is a key sorting station for intracellular and extracellular trafficking and thus, it plays a pivotal role in directing proteins to the secretory or to the lysosomal transport pathways [49]. However, understanding the sorting mechanism of proteins with dual destinations such as prosaposin can be challenging. In fact, prosaposin, a glycoprotein with multiple functions, is either transported to lysosomes to mediate sphingolipid degradation or secreted into the extracellular space to act as trophic factor [41].

Recently, we showed that the primary structure of prosaposin was essential for its interaction with sortilin in the TGN and for its lysosomal transport [50]. The identification of the prosaposin region involved in the interaction with sortilin was found in our laboratory by mutational deletion of each of the four known saposin functional domains (A, B, C and D) and the highly conserved N- and C- termini of prosaposin. Using the same mutants we found that, while deletion of the C-terminus blocked the lysosomal targeting of prosaposin without disturbing its release to the extracellular space, deletion of the N-terminus or of any saposin domain did not affect the lysosomal trafficking or secretion of prosaposin [40].

Interestingly, secreted prosaposin forms oligomers in the extracellular space [51, 52]. However, we found that oligomerization takes place within the cell. When cell lysates were resolved by SDS-PAGE under non-reducing conditions at low voltage and long running time, prosaposin was separated into six bands, including a monomeric form of 70 kDa and five oligomers ranging from 130 kDa to 250 kDa and higher. Our results demonstrated that the majority of prosaposin was found in the form of monomers and dimers. Oligomers larger than tetramers represented less than 5% of the protein, and all oligomers, except dimers, were unstable and gradually self-dissociated into monomers and dimers. Prosaposin oligomerization was not affected by boiling or SDS but reduced by DTT, indicating that prosaposin oligomers were covalently stabilized through intermolecular disulphide bridges.

Although, it was well known that each saposin domain contains six conserved cysteines, they only form intramolecular disulphide bonds [53-55]. Therefore, it was unlikely that these cysteines were involved in the formation of oligomers. Nevertheless, our experiments showed that deletion of each saposin domain abolished oligomerization. Given that the crystal structure of the prosaposin A-type domains (found in the N-terminal and C-terminal region of the molecule) was unknown, we assessed the role of the cysteines within the C- and N-termini. We found that elimination of the C-terminus did not affect prosaposin oligomerization. Conversely, deletion of the N-terminus inhibited oligomerization, suggesting that the cysteines found within this region could be responsible for the formation of intermolecular disulphide linkages [56].
It is well known that the intraluminal pH in the different regions of the Golgi apparatus and in the endolysosomal compartment is important for intracellular trafficking, since the binding and dissociation between a soluble hydrolase and their sorting receptor are pH-dependant [46, 56]. We have reported that the binding of prosaposin to sortilin occurs at pH 6.0 or higher, with dissociation occurring at pH 5.5 or lower [46]. A previous report showed that prosaposin formed large oligomers at neutral pH (7.0) and dimers at acidic pH (5.2), suggesting that neutral pH favors the formation of PSAP multimers [51]. However, the study did not investigate the status of prosaposin oligomerization at various pHs and the experiments were performed with culture medium which mainly contains prosaposin oligomers. In contrast, our experiments were conducted with cell lysates that have all forms of intracellular prosaposin, from monomer to large multimers. Our results demonstrated that neither neutral nor acidic pH changed the formation of multimers and that while pH values of 7.0 and 5.5 amplified the ratio of dimer/monomer, pH values of 5.0 and 4.0 had an opposite effect. We also demonstrated that dimerization was optimal at pH 5.5, which was in agreement with a previous study [51]. 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 [57]. Thus, the increased dimers may facilitate the aggregation of molecules within the TGN, resulting in their dissociation from sortilin and their release to the extracellular space [56].

9. Role of Glycosylation in the sorting Prosaposin

To study the role of glycosylation in oligomerization and secretion of prosaposin, we used Endo H and PNGase F to treat wild-type and truncated proteins (ΔA, ΔB, ΔC, ΔD, ΔN-terminus and ΔC-terminus). While Endo H cleaved high-mannose and some hybrid oligosaccharides, PNGase F cleaved high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins [58]. Our results showed that both Endo H and PNGase F treatments generated 60 kDa bands of the wild type prosaposin corresponding to the 55 kDa newly synthesized endogenous prosaposin, suggesting that the 65 kDa isomer was the prosaposin form transported to lysosomes. Prosaposin without the C-terminus was also Endo H sensitive, indicating that deletion of this segment did not interfere with the secretion of the protein [40]. Intriguingly, deletion of N-terminus or of any saposin domain caused the appearance of Endo H resistant proteins in the cell lysates. Prosaposin without the N-terminus resulted in both Endo H resistant and Endo H sensitive proteins. Deletion of domains A, C or D domains yielded smeared bands after Endo H treatment, with larger sizes than those of the corresponding bands from PNGase F treatment. These results indicated that these three truncations were partially Endo H resistant. Endo H treatment of prosaposin without the domain B generated a robust band slightly higher than the band generated by PNGase F treatment and weak bands with sizes similar to those from the untreated lysate. Taken together, our results suggested that domain B contains the most complex saccharide chains in the prosaposin molecule. This observation was consistent with previous structural studies on saposin oligosaccharide moieties showing that: 1) saposin A contains a fucosylated trimannosyl core structure partially resistant to Endo H treatment [59, 60]; 2) saposin B contains complex saccharide chains resistant to Endo H treatment [61]; 3) saposin C has both Endo H sensitive oligomannose-type oligosaccharides and Endo H resistant oligosaccharides [59]; 4) saposin D has mainly Endo H sensitive oligomannose-type oligosaccharides [59]; 5) the N-terminus and C-terminus do not contain glycosylation sites [45].

Even though wild-type prosaposin and prosaposin without the C-terminus formed oligomers, their fully glycosylated forms were not retained within the cell. Conversely, deletion of the N-terminus or of any saposin domain disturbed oligomerization and caused retention of Endo H resistant proteins in the cell lysates. This observation suggested that prosaposin oligomers were secreted out of the cell as proteins linked to complex oligosaccharides, and that inhibition of oligomerization blocked, to a certain extent, the secretion of prosaposin. Since our results supported the assumption that prosaposin oligomerization was required for secretion, we investigated the state of prosaposin in the culture medium by immunoprecipitation and non-reducing SDS-PAGE. As expected, we detected prosaposin dimers, but no monomers. This result validated the view that PSAP is mainly secreted as oligomers. In support of this observation, our co-immunoprecipitation studies demonstrated that monomeric prosaposin is the only form capable of binding sortilin and to traffic to lysosomes (Figure 7).
Prosaposin sorting model. Our proposed model shows that 65 kDa prosaposin must bind sortilin to be transported from TGN to lysosomes and that a subset of prosaposin (70 kDa) is fully glycosylated in the TGN and secreted out of the cell. According to the model, newly synthesized prosaposin is partially glycosylated in the Golgi apparatus. Before reaching the distal regions of the TGN for terminal glycosylation, monomeric prosaposin binds sortilin. This interaction is accomplished via its C-terminal region. Subsequently, the prosaposin-sortilin complex enters the lysosomal trafficking pathway, escaping terminal glycosylation. In endosomes, sortilin releases prosaposin and recycles back to the TGN. The prosaposin molecules that do not bind sortilin oligomerize. Due to their large size or due to changes in structure, prosaposin oligomers are not able to bind sortilin. Instead, they reach the terminal regions of the TGN, where they are fully glycosylated and directed to the secretory pathway.

10. Conclusion

In conclusion, we identified a specific region within the C-terminus of prosaposin, as well as the amino acid residues, that are critical for the binding of prosaposin to sortilin and its subsequent lysosomal trafficking. Although the final studies presented in this chapter were biochemical in nature, they demonstrated for the first time that monomeric, Endo H sensitive prosaposin was the form of the protein that binds sortilin and traffics it to lysosomes, and that oligomerized Endo H resistant prosaposin was delivered to the secretory pathway. These results are in agreement with our confocal microscope studies and support the notion that oligomerization plays a critical role in the sorting of prosaposin to the secretory pathway. Since the dual destination of prosaposin may be shared by other proteins, our discovery may represent a general mechanism used by the cell to sort proteins, and the model emerged from our investigation may be useful for future comparative studies.

References


S.A. Igdoura, A. Rasky, C.R. Morales, Trafficking of sulfated glycoprotein-1 (prosaposin) to lysosomes or to the extracellular space in rat Sertoli cells, Cell and tissue research 283 (1996) 385-394.


L. Yuan, C.R. Morales, A stretch of 17 amino acids in the prosaposin C terminus is critical for its binding to sortilin and targeting to lysosomes, J Histochem Cytochem 58 (2010) 287-300.


L. Yuan, C.R. Morales, Prosaposin sorting is mediated by oligomerization, Experimental cell research 317 (2011) 2456-2467.


