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Review

The interactomics of sortilin: an ancient lysosomal receptor evolving new functions

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Summary. The delivery of soluble lysosomal proteins to the lysosomes is dependent primarily on the mannose 6-phosphate receptor (MPR). The MPR has been demonstrated to attain the early endosomes via a process that requires the interaction of its cytosolic domain with the GGA and AP-1 adaptor proteins. Additionally, the MPR can be recycled back to the trans-Golgi network (TGN) through its interaction with the retromer complex. Interestingly, in I-cell disease (ICD), in which the MPR pathway is non-functional, many soluble lysosomal proteins continue to traffic to the lysosomes. This observation led to the discovery that sortilin is responsible for the MPR-independent targeting of the sphingolipid activator proteins (SAPs) and acid sphingomyelinase (ASM). More recently, our laboratory has tested the hypothesis that sortilin is also capable of sorting a variety of cathepsins that exhibit varying degrees of MPR-independent transport. We have demonstrated that the transport of cathepsin D is partially dependent upon sortilin, that cathepsin H requires sortilin, and that cathepsins K and L attain the lysosomes in a sortilin-independent fashion. As a type-1 receptor, sortilin also has numerous cytosolic binding partners. It has been observed that like the MPR, the anterograde trafficking of sortilin and its cargo require both GGAs and AP-1. Similarly, the retrograde recycling pathway of sortilin also involves an interaction with retromer through a YXXϕ site in the cytosolic tail of sortilin. In conclusion, the cytosolic domains of sortilin and MPR possess a high degree of functional homology and both receptors share a conserved trafficking mechanism.

Key words: Sorting receptors, Lysosomes, Trafficking, Sortilin

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Introduction

The sorting of soluble lysosomal proteins in the Golgi apparatus is an essential step in their transport to the lysosomes. In mammals, the mannose 6-phosphate receptors (MPRs) are the principal sorting receptors involved in the recognition of newly synthesized soluble hydrolases in the trans-Golgi network (TGN) (Lobel et al., 1989). There exist two forms of the MPR, the 46 kDa cation-dependent (CD) MPR and the 300 kDa cation-independent (CI) MPR (Lobel et al., 1987; Stein et al., 1987). The CD-MPR has little homology (20%) to the CI-MPR other than the presence of a 13 amino acid consensus sequence in the luminal domain (Dahms et al., 1987; Hancock et al., 2002). It is accepted that both MPR genes share a common ancestor, and that the CI-MPR originated from multiple duplications of this single ancestral gene (Kornfeld, 1992). Both MPRs have been described in chicken, reptiles, amphibians and fish, and a putative MPR has been reported in invertebrates (Matzner et al., 1996; Nadimpalli et al., 1999; Nadimpalli and von Figura, 2002). However, the invertebrate MPR is not well characterized and for the moment it remains difficult to confirm the point of origin in which the ancestral MPR gene appeared during evolution (Nolan et al., 2006). Nonetheless, recent experimental evidence demonstrated that MPR is present and fully functional in ancient teleosts vertebrates which appeared during the Triassic period over 200 million years ago (Palmer, 2003; Nolan et al., 2006).

The necessity for a sorting mechanism emerged with the development of eukaryotes approximately 2.7 billion years ago after 1 to 1.5 billion years of prokaryotic evolution (Brocks et al., 1999). A critical step in the evolution of eukaryotic cells was the acquisition of membrane-bound organelles. These organelles are thought to have been acquired as a result of the incorporation of prokaryotic cells within eukaryote ancestors (Witzany, 2006). Because of this added level

of complexity in eukaryotic cells, the transport of proteins to the appropriate compartment requires sorting mechanisms. The endoplasmic reticulum and the Golgi apparatus are specific organelles devoted to the sorting of proteins destined for secretion or their transport to the lysosomes and the plasma membrane (Griffiths and Simons, 1986; Tekirian, 2002). In yeast and plant cells the vacuoles are classified as lytic and storage compartments (Hoh et al., 1995). In these cells the sorting and transport of proteins was found to occur via clathrin-coated vesicles that bud from the Golgi apparatus and fuse with a pre-vacuolar compartment, probably the equivalent of late endosomes in mammalian cells (Tse et al., 2004).

The search for yeast and plant vacuolar sorting receptors resulted in the discovery of the Vps10p (Marcusson et al., 1994) and the BP80 receptors (Kirsch et al., 1994). Both receptors exhibit the same membrane topology and analogous sorting functions, but little sequence similarity (Kirsch et al., 1994). Recently, sortilin, a mammalian sorting receptor, has been shown to be a member of the Vps10 family (Petersen et al., 1997; Hermey et al., 1999). The Vps10 family of proteins is a novel family of heterogeneous type-I transmembrane receptors which includes sortilin (100 kDa), Vps10p (160 kDa), SorLA (250 kDa), and SorCS1-3 (130kDa) (Westergaard et al., 2004). The names of Vps10 family members begin with the pre-fix "sor" as an abbreviation for "sorting receptor related" (Hermey et al., 2001). These receptors are expressed in many tissues and are targets of a variety of different ligands (Petersen et al., 1997; Mazella et al., 1998; Hermans-Borgmeyer et al., 1999; Nielsen et al., 1999; Jacobsen et al., 2001; Lefrancois et al., 2003).

In this review we will focus exclusively on sortilin and describe the interactomics of this receptor in relation to its Golgi sorting functions. Special emphasis will be given to our findings and to the description of the cytoplasmic interactive partners of sortilin. We will also describe the luminal ligands or "cargo" destined to the lysosomes. Understanding the network topology of sortilin will permit a better understanding of why certain lysosomal enzymes travel to the lysosomes in a MPR-independent fashion and help predict lysosomal storage disorders of unknown origin.

Sortilin is a member of a multiligand tye-1 receptor family

Sortilin is a 100 kDa sorting receptor that is expressed in most tissues with a particularly high expression in the brain, testis and skeletal muscle (Petersen et al., 1997). Sortilin belongs to a growing family of multiligand type-1 receptors with homology to the yeast receptor Vps10p (Petersen et al., 1997; Westergaard et al., 2004). Receptors of this family are composed of a luminal/extracellular region containing a cysteine-rich domain homologous to the yeast vacuolar sorting protein Vps10p and one trans-membrane region that precedes a short intracellular domain that contains a signal for rapid internalization and trafficking (Nielsen et al., 2001; Westergaard et al., 2004; Zeng et al., 2004). This Vps10 homology region is common to the yeast Vps10p, a vacuolar sorting receptor for carboxy-peptidase Y (Marcusson et al., 1994). The Vps10 domain of mouse sortilin and SorLA/LR11 is preceded by a furin cleavage site and by two furin cleavage sites in SorCS (Fig. 1). In the case of human sortilin it has been demonstrated that this protein is synthesized as a precursor and converted to the mature protein by furin in the late Golgi compartments (Munck Petersen et al., 1999; Nielsen et al., 2001). The furin cleavage leads to the formation of a 44-amino acid propeptide that exhibits high affinity binding to the processed sortilin. Interestingly, receptor associated protein (RAP) and neurotensin (NT), ligands of sortilin, compete with the propeptide for the binding to sortilin, but prevention of propeptide cleavage inhibits the intracellular binding of RAP and NT (Munck Petersen et al., 1999). In fact, both RAP and NT are unable to bind sortilin in the absence of maturation by furin. On the other hand, cleavage and release of the propeptide is a necessary step for the full functional activity of sortilin (Munck Petersen et al., 1999).

While sortilin is a member of the Vps10 family of proteins, it also shares important similarities with the MPR sorting receptor. In fact, chimeric constructs containing the cytosolic domain of sortilin and the luminal domain of the MPR restored lysosomal trafficking of soluble hydrolases in MPR-deficient cells (Nielsen et al., 2001). Thus, based on structural features and experimental evidence sortilin was proposed to traffic lysosomal proteins from the TGN to the endosomes (Petersen et al., 1997; Nielsen et al., 2001). Paradoxically, until very recently no lysosomal proteins were known to interact with sortilin. Our laboratory was the first to identify two bonafide ligands of sortilin, namely, prosaposin and the $G_{M2}AP$, as well as to examine the structure of the mouse sortilin gene (Lefrancois et al., 2003; Zeng et al., 2004).

Cytoplasmic partners of sortilin

In functional terms, the cytosolic tail of mouse sortilin closely resembles the cytosolic tail of the MPR. In fact, the cytosolic domain of sortilin contains motifs

Fig. 1. Sortilin Sorting Motifs. Sortilin consists of a luminal domain that possesses a single Vps10 domain, a TM domain and a short cytosolic tail of 53 amino acid residues. Known sorting motifs in the cytosolic tail are highlighted and include FLV, YSVL and an acidic-cluster dileucine (DXXLL) motif.

known to be involved in trafficking from the Golgi to the endosome and *vice versa* (Fig. 1) (Nielsen et al., 2001; Seaman, 2007; Canuel et al., 2008a). 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). Like 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 (Nielsen et al., 2001; Seaman, 2007; Canuel et al., 2008a). These results validate the novel role assigned to sortilin as a lysosomal sorting receptor. While the other members of the Vps10 receptor family also contain sorting motifs, it appears likely that only SorLA and Vps10p are implicated in lysosomal/vacuolar sorting (Cooper and Stevens, 1996; Hermey et al., 1999; Jacobsen et al., 2002; Nielsen et al., 2008).

Adaptators involved in the forward transport of sortilin

In order to be transported throughout the cell, sorting receptors must interact with adaptor proteins. Adaptor proteins are the link between the sorting receptor and various cytoplasmic elements such as clathrin, Arf1, γ-synergin, epsinR and others (Nielsen et al., 2001). Adaptor proteins are implicated in both the forward or anterograde translocation of sortilin from the TGN as well as in its retrieval or retrograde transport from the endosomes back to the TGN (Nielsen et al., 2001; Seaman, 2007; Canuel et al., 2008a). Two main categories of adaptors are implicated in the anterograde transport of sortilin and its cargo: multimeric and monomeric adaptor proteins (Dell'Angelica et al., 2000; Nielsen et al., 2001; Canuel et al., 2008a).

The multimeric adaptor protein family includes AP-1, AP-2, AP-3, and AP-4, whereas the monomeric adaptor family consists of GGAs1-3 (Boman et al., 2000; Meyer et al., 2000). The multimeric adaptor proteins consist of four different subunits: two large subunits (γ, α, δ, ε, ß), a medium subunit (μ), and a small subunit (σ). In particular AP-1A adaptor protein has been found to play a role in sorting events at the TGN involving an interaction of its μ subunit with a YXXϕ motif in the cytosolic tail of the MPR (Ohno et al., 1995; Owen and Evans, 1998; Canuel et al., 2008a). Given the similarities between the cytosolic domains of sortilin and the MPR, we recently tested the ability of AP-1 to bind to sortilin. Our results showed that residues 789-799 of sortilin mediate an interaction with the μ subunit of AP-1 (Fig. 1). Moreover, inhibition of AP-1, through the use of siRNA specific to the μ subunit, resulted in the blockage of sortilin transport and its accumulation in the TGN. This finding led us to propose that AP-1 is implicated in the anterograde trafficking of sortilin (Fig. 2) (Canuel et al., 2008a).

However, in fibroblasts deficient in the μ subunit of AP-1, the MPR attains the endosomal compartment (Hirst et al., 2000). This finding suggested that a second group of adaptor proteins is implicated in the anterograde transport of sorting receptors and led to the discovery of the role of GGA adaptor proteins in protein sorting (Boman et al., 2000; Hirst et al., 2000). GGAs1-3 are a family of three homologous proteins that were identified in an attempt to uncover homologues of AP subunits (Boman et al., 2000; Dell'Angelica et al., 2000; Ghosh and Kornfeld, 2004). The GGAs are composed of four domains with various functions: the VHS domain, the GAT domain, the hinge domain and the EAR domain (Misra et al., 2000). The VHS domain has been shown to be responsible for interacting with the cytoplasmic tails

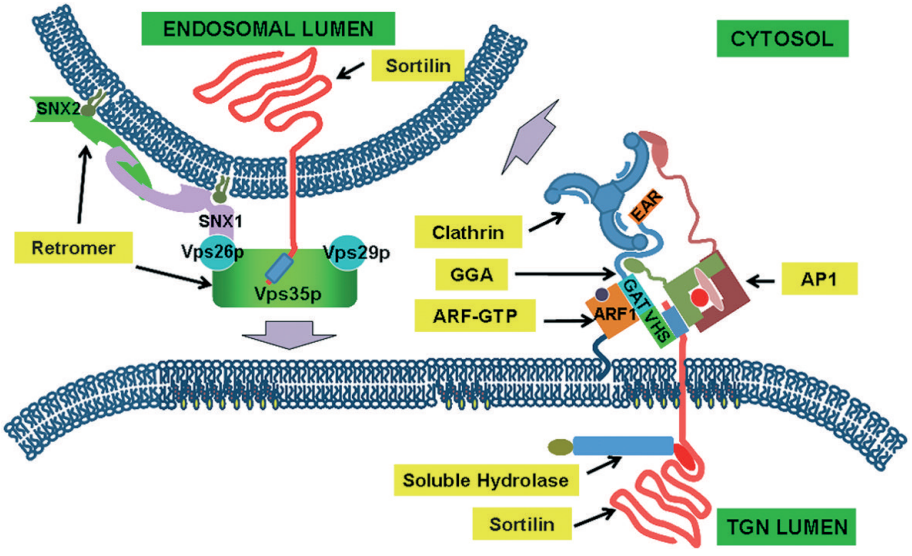

Fig. 2. Cytoplasmic Ligands of Sortilin. Diagrammatic representation of the proposed functions of GGA, AP-1 and retromer in the trafficking of sortilin. As illustrated in the lower half of the drawing, sortilin interacts with clathrin in the TGN via the monomeric GGA adaptors and/or the multimeric AP-1 adaptor complex. Once the receptor-ligand complex is incorporated into clathrin-coated-cargo vesicles, the complex is transported to the endosomes. In the endosomes, GGA and AP-1 are released and the cytoplasmic tail of sortilin is exposed, allowing interaction with the retromer complex (upper half of the drawing). Thus sortilin is recycled to the TGN for a new round of sorting.

of the sortilin and MPR sorting receptors (Nielsen et al., 2001; Puertollano et al., 2001). The acidic-cluster dileucine (DXXLL) motif, present in the cytosolic tails of both sortilin and the MPR, has been demonstrated to mediate binding to the VHS domain of GGAs (Fig. 1) (Nielsen et al., 2001; Collins et al., 2003). However, prior to interacting with the sorting receptors, GGAs must be recruited to the Golgi membrane. This has been shown to be achieved by an interaction of the GAT domain of the GGAs with Arf1 and Arf3 (Dell'Angelica et al., 2000). The hinge domain of the GGAs interacts with clathrin, whereas the EAR domain recruits coat proteins such as clathrin, γ-synergin, p56, and rabaptin-5 (Nielsen et al., 2001; Bonifacino, 2004).

In addition to interacting with sortilin, the GGAs have been proven to be essential in the transport of sortilin and its cargo from the TGN to the endosomes (Fig. 2) (Nielsen et al., 2001; Lefrancois et al., 2003). In fact, removal of the hinge and ear domains of GGA3 resulted in the abolishment of staining for sortilin and its ligands, prosaposin and $G_{M2}AP$, in punctate lysosomal structures and in the accumulation of sortilin in the perinuclear region (Lefrancois et al., 2003). Additionally, more recent work has shown that depletion of GBF1, a guanine nucleotide exchange factor (GEF) responsible for the activation of Arf1, affects the recruitment of GGAs to the Golgi apparatus and the normal processing of the sortilin ligand, prosaposin (Lefrancois and McCormick, 2007). Thus, both the GGAs and AP-1 are essential for the anterograde trafficking of sortilin from the TGN to the endosomal compartment (Nielsen et al., 2001; Lefrancois et al., 2003; Lefrancois and McCormick, 2007; Seaman, 2007; Canuel et al., 2008a).

Adaptors involved in the reverse transport of sortlilin

The failure to recycle sorting receptors leads to disruption of the lysosomal compartment, thus retrograde transport of these receptors is essential for the normal function of lysosomes (Seaman et al., 1998; Seaman, 2007). Recent evidence has shown that the pentameric retromer complex is largely responsible for mediating the recycling of the MPR, Vps10p and sortilin sorting receptors from the endosomes to the TGN (Arighi et al., 2004; Seaman, 2007; Canuel et al., 2008a). Retromer was first identified in Saccharomyces cerevisiae (Seaman et al., 1998) in which it was demonstrated to play a role in the recycling of the Vps10p receptor (Seaman et al., 1997). Mammalian retromer is comprised of Vps26, Vps29, Vps35 and sorting nexins 1 and 2 (SNX1 and SNX2) (Seaman et al., 1998; Haft et al., 2000; Collins et al., 2005). The Vps35 subunit and, quite likely, the Vps26 subunit, bind to cargo receptors. The other subunits of retromer are involved in reshaping the endosomal membrane (SNX1) and dephosphorylation of a serine residue that precedes the DXXLL motif in the cytosolic tail of the MPR (phosphoesterase Vps26) (Arighi et al., 2004; Damen et al., 2006; Seaman, 2007).

Recently, a conserved WLM sequence in the cytosolic tail of the MPR was implicated in the interaction of this receptor with retromer. Mutation of the WLM motif resulted in the failure of the MPR to be recycled and in its rapid degradation (Seaman, 2007). A similar sequence in sortilin, FLV, was proposed to mediate the interaction of sortilin and the Vps35 subunit of the retromer complex (Seaman, 2007). However, Yeast Two-Hybrid analysis revealed that amino acid residues 789-799 are essential for the binding of sortilin to Vps35. The FLV motif in fact occurs outside of the identified region (Canuel et al., 2008a). Rather, the stretch of amino acids we identified contained another common sorting motif, YXXϕ (Fig. 1). Additionally, our results showed that mutating either Y14A or L17A inhibited the interaction of the cytosolic tail of sortilin with Vps35. Moreover, attenuation of Vps26 expression caused depletion of sortilin in the perinuclear Golgi region. Together these results indicated that in the absence of a functional retromer complex, sortilin accumulated in the endosomal system and was depleted from the Golgi complex (Fig. 2) (Canuel et al., 2008a).

Nevertheless, it seems that retromer is not the only player implicated in mediating recycling of sorting receptors from the endosomal compartment to the TGN. Inhibition of the soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) proteins syntaxin 5 and 16 by siRNA abrogated recycling of the MPR from endosomes and resulted in the increased degradation of this receptor. It was therefore postulated that syntaxin 5 and 16 are important mediators of MPR recycling. Transport protein phosphofurin acidic cluster sorting protein (PACS-1), tail-interacting protein 47 (TIP-47), and the intracellular adaptor epsinR have also been demonstrated to help regulate recycling from the endosomal compartment (Nielsen et al., 2001; Saint-Pol et al., 2004; Scott et al., 2006). Given that the cytosolic tail of mouse sortilin closely resembles that of the MPR (Nielsen et al., 2001; Seaman, 2007; Canuel et al., 2008a), it is likely some of these accessory proteins also regulate the retrograde transport of sortilin.

Luminal cargo of sortilin

Although the MPR is the primary sorting receptor implicated in lysosomal targeting, it is clear that an alternative receptor is also involved in the lysosomal sorting of some soluble proteins. Evidence for this hypothesis originally came from fibroblasts of patients with ICD, a lysosomal storage disorder in which the MPR sorting pathway is disabled due to a loss of function mutation of the UDP-N-acetylglucoasmine-1-phosphotransferase gene. Under this condition, in which formation of M6P residues is inhibited, soluble proteins such as the SAPs and some soluble hydrolases continue to traffic to the lysosomes in a MPR-independent

manner (Rijnboutt et al., 1991b; Mahuran, 1998).

Activator proteins

SAPs are a category of soluble lysosomal co-factors involved in the non-enzymatic degradation of sphingolipids in lysosomes. This family of proteins includes 5 activators, the G_{M2} activator protein ($G_{M2}AP$) and prosaposin which is proteolytically cleaved in the lysosomes to saposins A, B, C, and D. SAPs act as biological detergents to facilitate the interaction of glycosphingolipid substrates with their respective enzymes for degradation, since glycosphingolipids are unable to interact directly with hydrolytic enzymes in a hydrosoluble milieu (O'Brien and Kishimoto, 1991; Furst and Sandhoff, 1992; Lefrancois et al., 2003).

We previously tested the hypothesis that sortilin is implicated in the lysosomal transport of SAPs using a sortilin construct that lacked its cytosolic domain. This truncated-sortilin construct was shown to act as a dominant-negative competitor of endogenous sortilin and induce the retention of sortilin in the Golgi apparatus. Confocal immunomicroscopy analysis also revealed that the truncated sortilin construct inhibited the targeting of SAPs to the lysosomes and caused their retention in the Golgi apparatus. This result suggested that sortilin is required for the lysosomal transport of prosaposin and $G_{M2}AP$. This discovery was confirmed by examining the fate of prosaposin in the absence of a functional sortilin pathway. Abrogation of sortilin using a siRNA resulted in the mis-routing of prosaposin to the extracellular milieu. Consequently our findings disclosed a novel mechanism of lysosomal trafficking mediated by sortilin (Fig. 2) (Lefrancois et al., 2003).

Soluble hydrolases

Acid sphingomyelinase (ASM) also showed reduced activity in the lysosomes of patients with ICD, suggesting that the lysosomal targeting of ASM is not entirely reliant on the MPR (Hurwitz et al., 1994). ASM is a soluble lysosomal enzyme involved in the hydrolysis of sphingomyelin to ceramide and phosphocholine (Lansmann et al., 2003). Although it is accepted that ASM is secreted and endocytosed by the MPR, the lysosomal targeting of ASM cannot be entirely attributed to the MPR, suggesting that this hydrolase is also sorted in an MPR-independent manner (Hurwitz et al., 1994).

To test whether sortilin was also implicated in the MPR-independent trafficking of ASM, co-immunoprecipitation was conducted and demonstrated that ASM was capable of interacting with sortilin. Sortilin was also shown to be, in part, responsible for the lysosomal translocation of ASM using the same truncated-sortilin construct utilized in the study of SAPs (Ni and Morales, 2006). The truncated-sortilin construct caused a decrease in the lysosomal immunostaining of ASM. However, the lysosomal transport of ASM was completely abolished in cells transfected with a truncated GGA construct that lacked the hinge and ear domains, thereby inhibiting both the sortilin and MPR pathways. Similarly, the presence of ASM in lysosomes was inhibited in ICD fibroblasts that were transfected with truncated-sortilin. Thus, ASM was the first soluble lysosomal protein demonstrated to utilize both the MPR and sortilin for its lysosomal trafficking (Ni and Morales, 2006).

Interestingly, both prosaposin and ASM (Ponting, 1994) are members of the SAPLIP family of lysosomal proteins which also includes acyloxyacyl hydrolase (AOAH) and the plant aspartic protease phytepsin. This family of proteins is characterized by the presence of a saposin-like domain that is hypothesized to be implicated in the lysosomal targeting of all SAPLIPs (Staab et al., 1994; Lin et al., 1996b). The saposin-like domain consists of 5 α-helices that surround a hydrophobic core that is believed to interact with sphingolipids in the luminal leaflet of Golgi membranes (Kervinen et al., 1999). The N-terminus of the surfactant protein B (SP-B) also contains a saposin-like domain and is therefore also classified as a member of the SAPLIP family. Unlike other SAPLIPs, SP-B is considered a secretory protein. However, to reach the extracellular space SP-B must first be routed to multivesicular bodies (MVBs) and deletion of its N-terminal region abrogates its transport to MVBs (Lin et al., 1996a). Based on the fact that both prosaposin and ASM are targeted to the lysosomes via sortilin, it is tempting to speculate that other members of the SAPLIP family may use the same receptor. Thus, future studies should address this question.

Several biochemical studies have suggested that additional soluble lysosomal proteins exhibit MPR-independent sorting. In addition to SAPs, hepatocytes, Kupffer cells and lymphocytes from ICD patients have near normal levels of several soluble lysosomal proteases (Rijnboutt et al., 1991a; Glickman and Kornfeld, 1993). One specific cathepsin, cathepsin D, which has been previously suggested to traffic to the lysosomes via the MPR, is found at approximately 45% of its normal amounts in the lysosomes of ICD B lymphoblastoid cell lines (Rijnboutt et al., 1991a). This result indicated the existence of a trafficking mechanism independent of the MPR pathway for at least this protease. Another cathepsin with proposed MPR-independent sorting and trafficking is cathepsin H. Primary cultures of rat hepatocytes metabolically labeled with ^{32}P and ^{35}S were used to examine the subcellular localization and different pro-forms of cathepsin H. It was determined that only the pro-form of cathepsin H obtained an M6P tag on its high mannose oligosaccharide, and that regardless of this tag, pro-cathepsin H was secreted into the medium with no correlate targeting to the lysosomes. Therefore in at least rat hepatocytes, cathepsin H is targeted from the TGN in

an MPR-independent manner (Tanaka et al., 2000).

Using an approach similar to that used in the investigation in SAP trafficking, the lysosomal targeting of cathepsins D and H was recently investigated. We tested the hypothesis that sortilin is responsible for the MPR-independent targeting of cathepsins D and H. Indeed, both cathepsins D and H were demonstrated to be interactive partners of sortilin (Fig. 3). Truncated-sortilin, bound to cathepsins D and H (Fig. 3) caused their retention within the Golgi apparatus, indicating that the transport of cathepsins D and H to the lysosomes was disrupted by inhibition of the sortilin pathway. In the case of cathepsin D, the truncated-sortilin construct not only competed with the endogenous sortilin, but with the MPR as well since cathepsin D was excluded from the lysosomes. Our results also suggested that fluid-phase endocytosis does not play a major role in the uptake of cathepsins D or H since the dominant-negative sortilin completely blocked the transport of cathepsin D and H to the lysosomes. On the other hand, the effect of the inactivation of sortilin by siRNA on cathepsin D differed from the experiment using truncated sortilin. While the truncated-sortilin construct inhibited the exit of cathepsin D from the Golgi apparatus, the sortilin siRNA did not. This result indicated that in the absence of a functional sortilin pathway, the MPR continued to traffic cathepsin D to the lysosomes. Therefore, our findings suggested that while sortilin may be the sole receptor required for the lysosomal translocation of cathepsin H in COS-7 cells, cathepsin D requires both the MPR and sortilin (Canuel et al., 2008b).

Two other soluble proteases suspected to be capable of trafficking to the lysosomes independently of the MPR are cathepsins K and L. Evidence for the MPR-independent sorting of cathepsin K arises from experiments in which M6P was exogenously added to CHO cells expressing cathepsin K. While it was predicted that the exogenous M6P should out-compete cathepsin K for binding to the MPR, the subcellular localization of cathepsin K was unaffected. This result implied that an MPR-independent pathway exists and that cathepsin K may use this alternative pathway (Gottesman, 1978). Different lines of evidence suggested that cathepsin L, a lysosomal cysteine protease, is also sorted from the TGN in a manner that is independent of the MPR. While cathepsin L carries an M6P tag in its

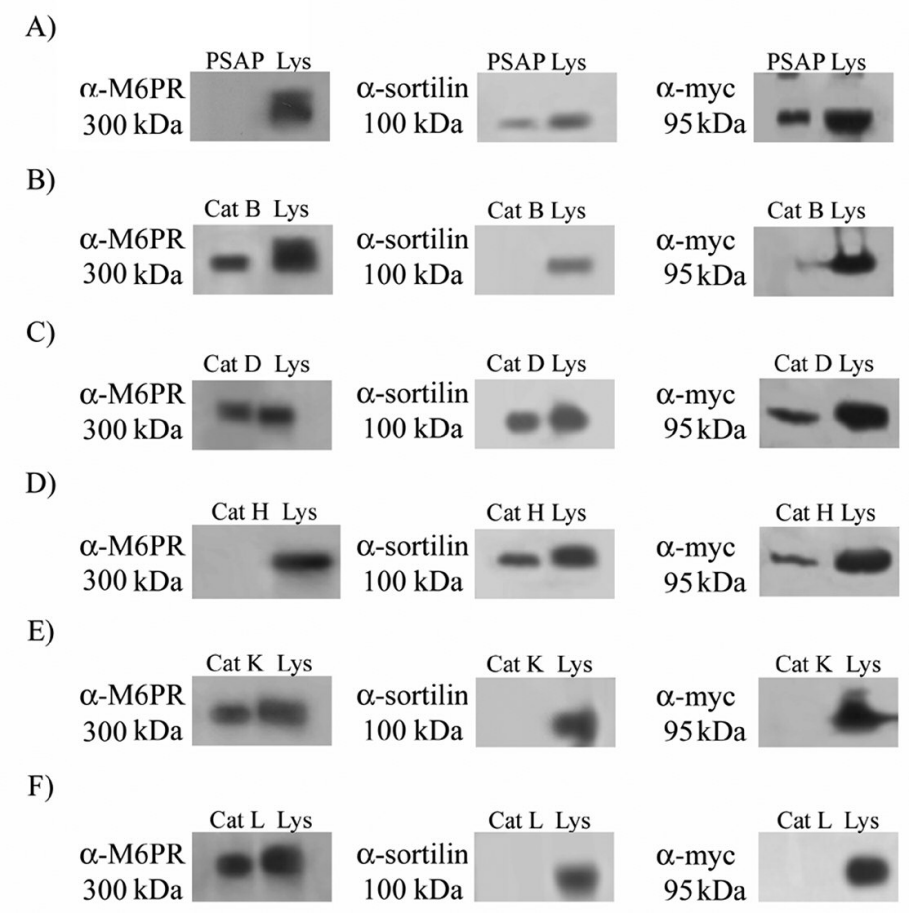

Fig. 3. The interaction of soluble lysosomal proteins with sorting receptors in COS-7. Co-immunoprecipitation was conducted in COS-7 cells to identify the sorting receptors capable of binding to cathepsins D, H, K, and L. Prosaposin was used as a positive control to test the binding to sortilin, whereas cathepsin B was used as a positive control in the binding to the MPR. The MPR (300 kDa) was precipitated by cathepsins B, D, K, and L (**B, C, E, and F**), while prosaposin and cathepsin H did not precipitate the MPR (**A and D**). However, endogenous sortilin (100 kDa) was immunoprecipitated by anti-prosaposin and anti-cathepsins D and H antibodies (**A, C, and D**). Comparable results were observed using cells expressing the truncated-sortilin-myc construct (**A, C, and D**). Similarly, both the endogenous and truncated forms of sortilin remained in the cell lysate and were not immunoprecipitated by anti-cathepsin B, K, or L antibodies (**B, E, and F**).

proform, it is synthesized at high levels, but has very low affinity for the MPR (Johnson et al., 1987). Further to this, mutagenic analysis of vacuolar sorting determinants suggested that the nature of the cathepsin L vacuolar sorting signal was proteinaceous and not due to oligosaccharide chains (Valls et al., 1987; Nielsen et al., 2007). These results further confirmed the existence of a transport pathway from the Golgi apparatus to the endosomes that was independent of the MPR for certain soluble lysosomal proteins.

However, our investigation into the ability of cathepsins K and L to traffic via a sortilin pathway revealed that unlike cathepsins D and H, they did not immunoprecipitate sortilin (Fig. 3). Cathepsins K and L however did interact with and immunoprecipitate the MPR (Fig. 3). Moreover, in cells that were transfected with truncated-sortilin, the localization of cathepsins K and L to lysosomal structures labeled with LAMP-1 was unaltered when compared to untransfected cells (Figs. 4, 5). This result implied that cathepsins K and L do not use sortilin to be transported to the lysosomes. Since both cathepsins have been shown to attain the lysosomes independently of the MPR, the MPR-independent trafficking of cathepsins K and L must be accounted for by fluid-phase endocytosis or another sorting receptor. Potential sorting receptors might include another member of the Vsp10 family, possibly SorLA. Like sortilin, the cytoplasmic tail of SorLA has been shown to interact with the adaptor proteins necessary for vesicle formation, including GGAs, AP-1, AP-2, and components of the retromer complex (SNX1 and Vps35) (Nielsen et al., 2007). SorLA expression has been mapped mainly to the nervous system, in cortical neurons, hippocampus, the cerebellum, and the spinal cord, as well as in non-neural tissue such as the testis, ovary, lymph nodes, distal kidney tubules, and vascular smooth muscle cells (Jacobsen et al., 1996; Yamazaki et al., 1996; Hermans-Borgmeyer et al., 1998; Kanaki et al., 1999; Riedel et al., 2002). In accordance with its widespread expression pattern, sorLA has been demonstrated to bind and internalize numerous ligands including neurotensin, platelet-derived growth factor-bb, and lipoprotein lipase, apolipoprotein E, and amyloid precursor protein (APP) (Jacobsen et al., 2001; Taira et

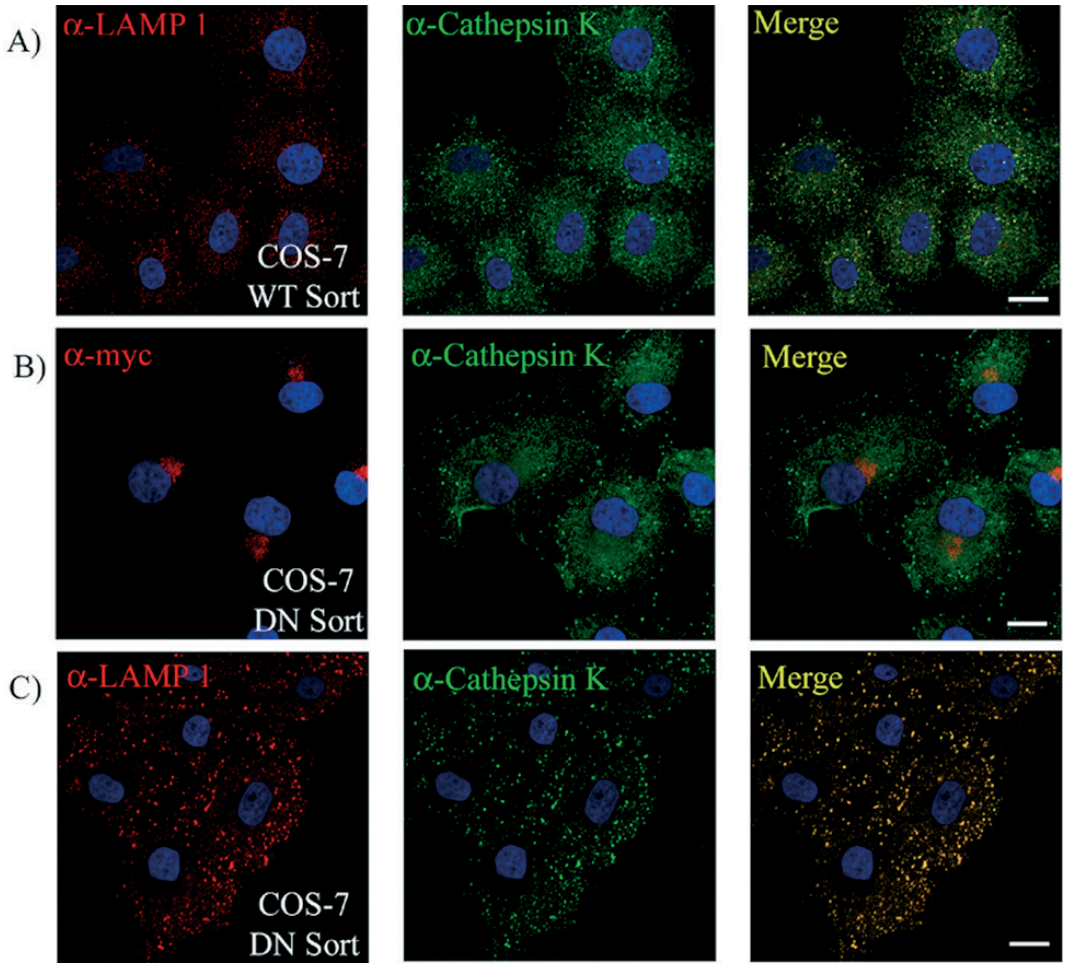

Fig. 4. Lysosomal targeting of cathepsin K. Untransfected **(A)** and truncated-sortilin-myc transfected COS-7 **(B and C)** were fixed in 4 % para-formaldehyde and stained with anti-cathepsin K and anti-myc or anti-LAMP-1 antibodies. In untransfected cells, cathepsin K (green) was found to localize to punctate structures that stained with LAMP-1 (red) **(A)**. The pattern of cathepsin K staining (green) was unaltered in cells expressing truncated sortilin (red) **(B and C)**. Nuclei are stained with Hoechst 33342 and are seen in blue. Scale bar: 8 μm.

al., 2001; Gliemann et al., 2004; Andersen et al., 2005). Therefore, SorLA may be another ancient lysosomal sorting receptor evolving new functions that require experimental verification.

Conclusion

While it is clear that the cytoplasmic domains of sortilin and the MPR possess a high degree of structural and functional similarities characterized by the utilization of similar adaptor proteins to shuttle between the TGN and endosome, it is equally clear that they transport different cargo proteins. The explanation for the existence of different sorting pathways may be evolutionary. Interestingly, the sortilin sorting pathway may represent an evolutionarily older mechanism for lysosomal transport than the MPR pathway. The Vps10 family of proteins, of which sortilin is a member, is characterized by luminal Vps10 domains that were first identified as a sorting motif in yeast (Marcusson et al.,

Table 1. Lysosomal storage disorders.

Lysosomal Storage Disorder	Deficiency	References
AB-variant of G_{M2} gangliosidosis	$G_{M2}AP$	Stevens et al., 1981
Variant of metachromatic leukodystrophy	Saposin B	Christomanou et al., 1986
Variant of Gaucher's	Saposin C	Ramirez-Montealegre et al., 2006
Neuronal ceroid lipofuscinoses (CNCL)	Cathepsin D	Helfrich, 2003
Pycnodysostosis	Cathepsin K	Dhami et al., 2006
Niemann-Pick types A and B	Acid sphingomyelinase	Dhami et al., 2006

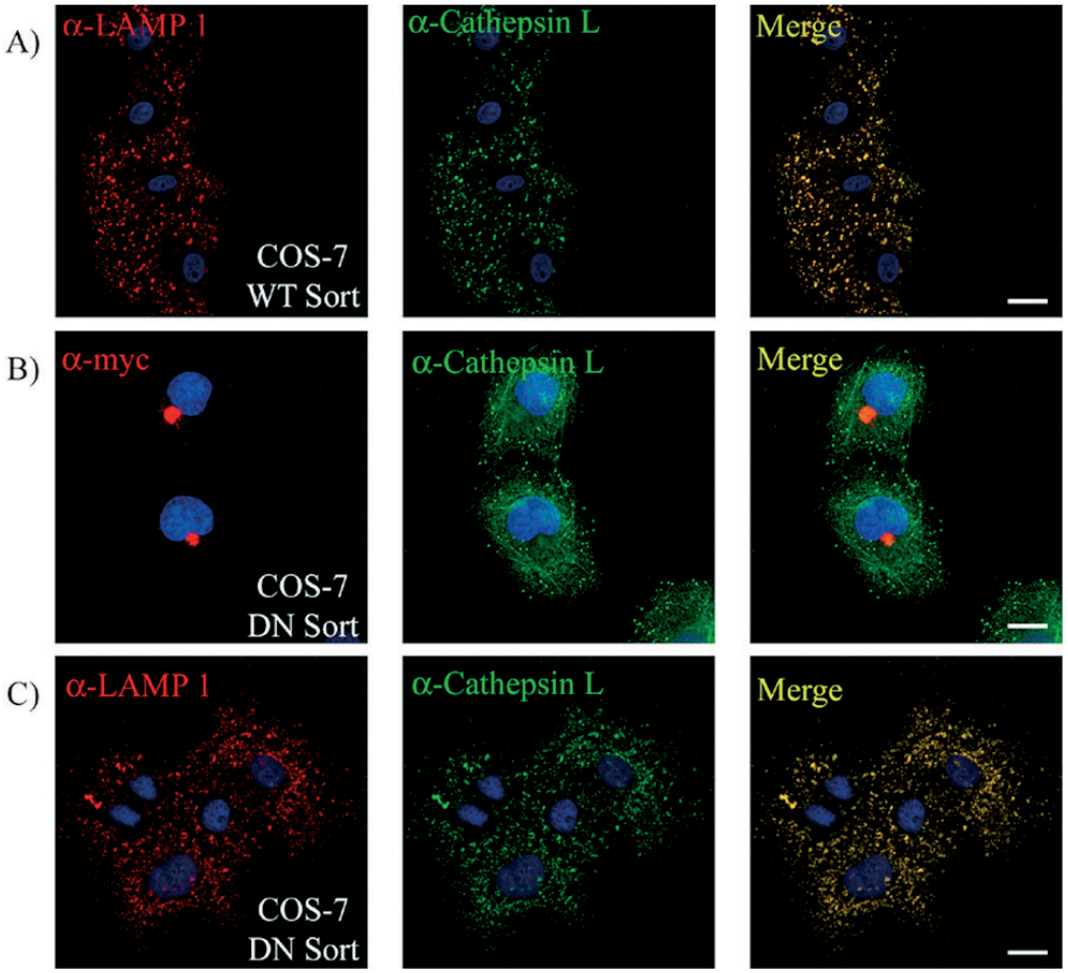

Fig. 5. Lysosomal targeting of cathepsin L. Untransfected **(A)** and truncated-sortilin-myc transfected COS-7 **(B and C)** were fixed in 4 % para-formaldehyde and stained with anti-cathepsin L and anti-myc or anti-LAMP-1 antibodies. In untransfected cells, cathepsin L (green) was found to localize to the perinuclear Golgi region as well as to punctate structures that stained with LAMP-1 (red) (**A**). The distribution of cathepsin L (green) was unaltered in cells expressing truncated sortilin (red) (**B and C**). Nuclei are stained with Hoechst 33342 and are seen in blue. Scale bar: 8 μm.

1994; Hampe et al., 2001). Vps10 domain containing proteins are found in a variety of simple organisms including *Dictyostelium, Neurospora,* and *Metarhizium* (Petersen et al., 1997).

While the yeast Vps10p receptor is often described as a yeast analogue of the MPR, it in fact possesses more similarities to sortilin than the MPR. Both sortilin and the Vps10p receptor contain Vps10 domains (1 in sortilin and 2 in the Vps10p), while the MPR has no Vps10 domain (Tauris et al., 1998). Although it has been well established that the MPR recognizes its ligands through an added oligosaccharide side-chain (the M6P tag), both the Vps10p and sortilin receptors interact with ligands through proteinaceous interactions (Kornfeld and Kornfeld, 1985; Baldwin et al., 1993; Marcusson et al., 1994). While the residues implicated in the interaction of Vps10 domains and cargo proteins have not yet been characterized, it should be noted that all of the known lysosomal cargo proteins possess prominent α-helices (Guncar et al., 1998; Zhao et al., 1998; Wright et al., 2000; Nelson and Alkon, 2007).

Together, this information would suggest that the MPR pathway is a more specific mechanism for sorting soluble lysosomal proteins. The appearance of this new pathway may also explain why soluble lysosomal proteins differentially traffic via these two different receptors. However, in spite of the development of the MPR sorting mechanism, sortilin continues to be a unique sorting receptor that is evolving new functions essential in embryonic development, neural function and apoptosis (Hermans-Borgmeyer et al., 1999; Mazella and Vincent, 2006; Canuel et al., 2008b), while still retaining its capacity to sort certain soluble proteins (Fig. 2) whose deficiences are implicated in lysosomal storage disorders (Table 1) (Kolter and Sandhoff, 1998; Lefrancois et al., 2003; Canuel et al., 2008b).

In conclusion, the disclosure of unidentified sorting receptors and pathways will allow us to better understand why certain lysosomal proteins continue to traffic to the lysosomes in cells with a disabled MPR pathway. These discoveries will undoubtedly have future implications in lysosomal enzyme therapy and in the understanding of lysosomal storage disorders of unknown etiology.

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