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Review

The sorting and trafficking of lysosomal proteins

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Summary. For a long time lysosomes were considered terminal organelles involved in the degradation of different substrates. However, this view is rapidly changing by evidence demonstrating that these organelles and their content display specialized functions in addition to the degradation of substances. Many lysosomal proteins have been implicated in specialized cellular functions and disorders such as antigen processing, targeting of surfactant proteins, and most lysosomal storage disorders. To date, about fifty lysosomal hydrolases have been identified, and the majority of them are targeted to the lysosomes via the mannose-6-phosphate receptor (M6P-Rc). However, recent studies on the intracellular trafficking of the non-enzymic lysosomal proteins prosaposin and GM2 activator (GM2AP) demonstrated that they use an alternative receptor termed "sortilin". Existing evidence suggests that some hydrolases traffic to the lysosomes in a mannose 6-phophate-independend manner. The possibility that sortilin is implicated in the targeting of some soluble hydrolases, as well as the consequences of this process, is addressed in the present review.

Key words: Lysosomes, Trafficking, Sortilin, Mannose 6-phosphate receptor

Introduction

Although sorting and trafficking are two different cellular functions, they are temporally and spatially related. Both processes involve several steps and the participation of luminal, transmembrane and cytoplasmic proteins. During the first step of sorting a soluble lysosomal protein or "cargo" must be recognized by a specific sorting receptor. This process occurs in the lumen of the Golgi apparatus and sometimes on the surface of the plasma membrane (Dell'Angelica and Payne, 2001). However, a lysosomal protein must be tagged first with a molecule such as the mannose 6-phosphate, to allow its recognition by a sorting receptor. Protein sorting in most eukaryotic cells may also involve protein-protein interactions between the cargo and the receptor. Consequently, eukaryotic cells may have an additional repertoire of receptors that recognize amino acid sequences and/or motifs in the lysosomal cargo. Such motifs have the property to specify the sorting and final destination of the cargo. This possibility is discussed in the present review.

To exit a sorting compartment a receptor must interact with cytoplasmic coat proteins such as adaptor proteins, ARF and clathrin, that cause vesicles to bud from donor membranes (trans-Golgi network/TGN) and to traffic to acceptor membranes (late endosomes and lysosomes). In addition to the Golgi apparatus, other major sites of vesicle formation and budding include the endoplasmic reticulum and the plasma membrane. The Golgi apparatus is known to be the major site of sorting for newly synthesized proteins destined to the lysosomal compartment. Two different types of proteins are sorted to the lysosomes: transmembrane and soluble lysosomal proteins. Due to their specific characteristics these types of proteins use different sorting mechanisms. This is also addressed in this review.

Finally, a succinct description of cargo molecules is provided and special attention is given to putative motifs within their primary structure that specify sorting and/or trafficking.

Lysosomes contain saposin-like protein (SAPLIP)

Members of the SAPLIP family

The saposin-like protein family (SAPLIP) is a large and diverse group of proteins found in a variety of eukaryotic cells from plants and animals. The SAPLIP members share cysteine-rich saposin-like sequences. These conserved cysteines, form three intra-domain disulfide bonds that create a common structural framework upon which other conserved amino acids form five amphipathic α-helices (Munford et al., 1995; Kervinen et al., 1999). Saposin-like sequences have been implicated in diverse functions such solubilization of sphingolipid substrates (O'Brien and Kishimoto, 1991;

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Hiraiwa and Kishimoto, 1996; Hiraiwa et al., 1997; Ciaffoni et al., 2001) and lipid-antigen presentation (Zhou et al., 2004).

Members of the SAPLIP family include saposins A, B, C, D, NK-lysine, surfactant protein B (SP-B), acid sphingomyelinase, acyloxyacyl hydrolase (AOAH) and plant aspartic protease. Most SAPLIPs may bind to or interact with one or more membrane lipids. Yet their properties and presumed functions are different (Batenburg, 1992; Hiraiwa et al., 1992; Azuma et al., 1994; Kervinen et al., 1999).

Saposins (A-D) are derived from the proteolytic cleavage of the precursor protein prosaposin in the lysosomes. Therefore, saposins are found mainly in the lysosomal compartment where they facilitate the catabolism of glycosphingolipids with short oligosaccharide groups. The deficiency of prosaposin and saposins has been associated with two lipid storage disorders in humans, Gaucher Disease and Metachromatic Leukodystrophy (Jatzkewitz, 1973; Grabowski and Horowitz, 1997; Kotani and Sano, 1998; Sano, 1998).

AOAH is a lipase found in phagocytic cells, which cleaves fatty acyl chains from bacterial lipopolysaccharides (LPS). This enzyme plays a major role in the elimination of microorganisms phagocytosed by monocytes and macrophages (Munford and Hunter, 1992).

SPB is a 9 KDa hydrophobic protein, produced by alveolar type II cells, which enhances the diffusion of surfactant along the water-air interface in the pulmonary alveolus. Deficiency of SPB has been found in infants with congenital alveolar proteinosis (Nogee et al., 1993). ASM is a soluble lysosomal hydrolase that cleave the phosphodiester bond of sphingomyelin to ceramide and phosphocholine. It is encoded by the sphingomyelin phosphodiesterase-1 (SMPD-1) gene. Mutations in this gene cause the inherited lysosomal disorders Niemann-Pick disease type A and B (Pittis et al., 2004; Ricci et al., 2004).

NK-lysin is a basic polypeptide consisting of 78 amino acids with an antibacterial activity and the capacity to lyse tumor cells. It was originally found in porcine small intestine and is synthesized by lymphocytes (Andersson et al., 1995).

Phytepsin, is a plant aspartic protease which resides in barley grain, roots, stems, leaves and flowers. It is considered a plant homologue of mammalian lysosomal cathepsin D and yeast vacuolar protease A. Although the exact function of phytepsin is unclear, it may participate in metabolic turnover and in protein processing in barley tissues (Tormakangas et al., 1991; Runeberg-Roos et al., 1994).

Structural features of SAPLIPs

Saposin-like motifs within SAPLIPs are composed on average by 80 amino acids and have a characteristic pattern of six conserved cysteines that form three disulfide bonds. The arrangement of these cysteines was first described by Schuette et al. (1998), who found that the disulphide bonds are formed between the first and last cysteines, between the second and the second last cysteines and between the third and the fourth cysteines. These bonds renders the backbone structure of SAPLIPs remarkably stable to heat, low pH and proteolytic degradation.

NK-lysine was the first protein of this family whose 3-D structure was determined by nuclear magnetic resonance (Liepinsh et al., 1997). Similarly, the plant-specific domain of phytepsin was the first member of the SAPLIP family for which the 3-D structure has been determined by x-ray crystallography (Kervinen et al., 1999). Comparison of pro-phytepsin and NK-lysine showed high 3-D structure similarity. Both proteins contain five α-helices that form a helical cage. The internal surface of the cage is lined with hydrophobic residues while the outer surface is mainly hydrophilic. The five α-helices are oriented in an up-down manner and they interact with each other through the hydrophobic residues. The helices are stabilized by inter-helical disulfide bridges. Study of the structures of saposins A, C and D showed that they all contain four or five α-helices forming similar "helical cages" as in NK-lysine and plant aspartic protease. The crystal structure of human saposin B revealed an unusual shell-like dimer consisting of a monolayer of α-helices enclosing a large hydrophobic cavity (Ahn et al., 2003). This characteristic α-helix cage or "bundle" was referred to as "saposin fold" or "saposin-like domain", which is a common feature of all SAPLIPs. In sequence, the alignment of the N- and C- terminal regions of the phytepsin's saposin-like domain are swapped as compared to those in NK-lysine. However, this swapping does not impact on the orientation of the helices (Kervinen et al., 1999).

Saposin B is considered a special SAPLIP. Its structure is similar to the saposin like-structures of the monomeric members of the SAPLIP family. However, the α-helices of saposin B differ from the rest of the SAPLIP members in the sense that they are repacked into a different tertiary conformation that form a homodimer.

The sphingolipid activator protein GM_2AP is not considered a saposin-like protein. GM_2AP contains eight cysteines that form four disulfide bonds but the secondary structure of the GM_2AP differs from that of the SAPLIPs. Nonetheless, circular dichroism spectroscopy predicted that the GM_2AP has a unique hydrophobic ß-cup structure that results in the formation of a spacious cavity, largely, due to the presence of extensive ß-sheet structures. Interestingly, the GM_2AP traffics to the lysosomes following the same pathway of prosaposin (Lefrancois et al., 2003).

The lipid-binding ability of SAPLIPs

All SAPLIPs share both lipid-binding and

membrane-perturbing properties, a characteristic feature of this protein family. Surfactant protein B (SPB) interacts preferentially with anionic lipids forming monolayers capable of lowering the normal surface tension at the alveolar interface (Brockman et al., 2003). NK-lysine, a tumorolytic and antibacterial peptide of NK cells, interacts with and destabilizes lipid bi-layers. Saposin C stimulates glucosylceramidase by interacting with phophatidyl-serine-containing membranes (Vaccaro et al., 1993). Furthermore, inhibition of sphingolipid biosynthesis with organic and synthetic compounds such as fumonisin B1 (FB1) and tricyclodecan-9-yl xanthate potassium salt (D609), interferes with the lysosomal targeting of prosaposin. Since both FB1 and D609 inhibit the biosynthesis of sphingomyelin it has been postulated that this sphingolipid interact with prosaposin (Lefrancois et al., 2002). These observations indicate that lipid binding is important for intracellular trafficking of SAPLIPs.

Interestingly, the removal of sugar moiety does not influence the association of saposin D with phospholipid membranes, suggesting that deglycosylation does not affect the lipid-binding property of SAPLIPs (Tatti et al., 1999). Similarly, tunicamycin treatment did not interfere with the lipid interaction of non-glycosylated prosaposin and increased its transport to the lysosomes, suggesting that an intrinsic sequence or sequences may specify the lipid binding activity (Igdoura and Morales, 1995). On the other hand, the maintenance of disulfide bonds appears to be essential for the interaction of SAPLIPs with membrane lipids within lysosome and vacuole targeting. In fact, mutations that disrupt disulfide bridges in saposin B and C are the cause of variant forms of MLD and Gaucher disease, respectively (Holtschmidt et al., 1991; Schnabel et al., 1991; Rafi et al., 1993). In conclusion, disulfide bonds are critical for the three dimensional structure of SAPLIPs and for the formation of "helical cages". It is also possible that the hydrophobic internal surface of the helical cages provides an interface for lipid-binding. Thus, the presence of saposin-like domains may be linked to the observed lipid-binding property of all SAPLIP members (Misasi et al., 1998; Vaccaro et al., 1999).

Role of saposin-like domains in intracellular targeting of SAPLIPs

The association of SAPLIPs with membrane lipids has been well demonstrated. In addition, SAPLIPs may interact with membrane-associated receptor proteins during their Golgi-mediated intracellular. There are some direct and indirect evidence suggesting that saposin-like domains may play an important role during this process.

The AOAH lipase is a soluble lysosomal enzyme that is synthesized as a single-chain precursor in the endoplasmic reticulum (ER). In turn it is proteolytically processed into mature protein in the lysosomes. Mature AOAH consists of a small and a large subunit. Since the small subunit bears a saposin-like domain, AOAH is also considered a member of the SAPLIP family (Hagen et al., 1991). Immunofluorescence staining and pulse-chase experiments showed that both a recombinant AOAH large subunit and an AOAH variant lacking a region of 33-amino containing the saposin-like domain were not found in the lysosomes. Instead, both subunits were secreted to the extracellular space. When similar cells were transfected with wild-type recombinant AOAH, the enzyme reached the lysosomes (Staab et al., 1994). This indicated that the absence or disruption of the saposin-like domain prevents the lysosomal targeting of AOAH (Staab et al., 1994).

Studies carried out in plant cells showed that the saposin-like domain of SAPLIPs plays a role in vacuolar transport. Barley aspartic protease (phytepsin) reaches the vacuole via trafficking through the Golgi apparatus. However, deletion of the plant-specific insert (PSI) which contains a saposin-like domain results in the secretion of truncated phytepsin (Tormakangas et al., 2001).

Similarly, the selective deletion of the D domain (corresponding to saposin D), and the C-terminus disrupted the transport of prosaposin to the lysosomes, demonstrating that both regions are required for its targeting (Lefrancois et al., 2002). Conversely, chimeric proteins composed of albumin and prosaposin were only routed to the lysosomes if the fusion proteins contained the D domain and C-terminus of prosaposin (Zhao and Morales, 2000). Interestingly, the C-terminus of prosaposin also has a saposin-like domain that is significantly similar to the N-terminus of surfactant protein B (SP-B). In fact, SP-B requires the presence of the N-terminus for its transient routing to multivesicular and lamellar bodies (Lin et al., 1996).

Structural and functional studies of pro-phytepsin revealed that a putative membrane receptor-binding region may be located on the outer surface of its inter-domain (Kervinen et al., 1999). Therefore, it is likely that the saposin-like domain brings pro-phytepsin into contact with membrane microdomains, such as lipid rafts, containing the sorting receptor. In this manner, the resulting complex could be then packed into sorting vesicles destined to the vacuoles.

Acid sphingomyelinase (ASM)

Acid sphingomyelinase is a soluble lysosomal enzyme found in all mammalian cells, possessing an optimum pH of 4.5-5.5. ASM hydrolyzes sphingomyelin into ceramide and phosphocholine. Individuals who have mutations in the ASM gene develop type A and B Niemann-Pick disease (NPD). NPD is characterized by an accumulation of undigested sphingomyelin in the lysosomes (Lampert and Teller, 1967; Miranda et al., 1998). As in type A NPD patients, ASM knock out mice accumulate sphingomyelin in the lysosomes of cells of the reticulo-endothelial system, predominantly in liver, spleen, lung, bone marrow and brain. In humans NPD

leads to early childhood death (Miranda et al., 1998). Sphingomyelin storage also produces unbalanced cholesterol-sphingolipid ratios and severely perturbed raft formation and raft-associated functions in the plasma membrane.

Both human and murine ASMs are products of conserved genes that share 82% identity. The human ASM gene encodes a 629 amino acid precursor protein that contains a saposin-like domain and a phosphodiesterase domain. The precursor protein is then modified by the addition of high mannose oligosaccharide residues (Newrzella and Stoffel, 1996). The mannosylated precursor traffics to the lysosomes (L-ASM) or is secreted in the extracellular space (S-ASM). Sequence analysis of the N-terminus revealed that L-ASM starts with the amino acid sequence GHPARLH whereas S-ASM begins with HPLSPQGHPARLH due to different N-terminal proteolytic processing. This differential processing has been implicated in the targeting of ASM to its final destination (Schissel et al., 1998).

ASM contains six potential N-linked glycosylation sites five of which are glycosylated. Elimination of any of these sites results in decreased enzyme activity due to lack of structural stability and/or misfolding (Newrzella and Stoffel, 1996). L-ASM was found to bind to a mannose-6 phosphate receptor affinity column and to elute with free mannose-6 phosphate. This finding indicated that L-ASM is transported to the lysosomes by the mannose-6 phosphate receptor. Although lysosomal ASM activity was found at a reduced level in fibroblasts from patients with ICD, endocytosis of radiolabeled extracellular ASM precursor by fibroblasts was not prevented by the addition of free mannose-6 phosphate (Hurwitz et al., 1994). This indicates the possibility of an alternative pathway independent of the mannose 6-phosphate receptor. Interestingly, the ASM N-terminus contains a saposin-like domain. This finding raises the possibility that the saposin-like domain may be involved in lipid binding and trafficking of ASM to the lysosomes. It is also plausible that the ASM saposin-like domain is involved in a protein-protein interaction with an alternative sorting receptor to the mannose-6 phosphate receptor as in the case of prosaposin.

The targeting of soluble lysosomal hydrolases

Mannose-6 phosphate (M6P) dependent transport of hydrolases

Soluble lysosomal enzymes contain a N-terminal signal peptide which directs the ribosome to the endoplasmic reticulum (ER) membrane and initiates transport of the growing polypeptide across the ER membrane (Lodish, 1999). Following core glycosylation in the ER, hydrolases are transported to the Golgi apparatus where they acquire a phoshomannosyl recognition marker (M6P) that mediates their translocation to lysosomes. The marker is added in two steps: First, a N-acetylglucosamine-1-phospho-transferase transfers N-acetylglucosamine-1-phosphate from UDP-GlcNAc to one or more mannose residues on the lysosomal protein to give rise to a phosphodiester intermediate (Little et al., 1986, 1987). Subsequently, a N-acetylglucosamine-1-phosphodiester α-N-acetyl-glucosaminidase removes the N-acetylglucosamine residue to generate an active phosphomonoester (Reitman and Kornfeld, 1981a,b; Waheed et al., 1982). The initial phosphorylation event may occur in a pre-Golgi compartment (Pohlmann et al, 1982), and the conversion of diester to active phosphomonoester may take place in the TGN. Human N-acetylglucosamine-1-phosphodiester α-N-acetyl-glucosaminidase, also termed "uncovering enzyme" (UCE), is a type I membrane protein consisting of a 24-residue signal peptide, a 423-residue luminal domain, a 27-residue transmembrane domain and a 41-residue cytoplasmic tail. Upstream of the signal peptide is a furin cleavage site (Kornfeld et al., 1999). Pro-UCE has little or no enzymatic activity and the removal of propeptide by furin in the TGN has been shown to be essential for the generation of the active enzyme (Do et al., 2002). Sialylation of UCE in the TGN of mouse L cells further confirms its localization within this compartment (Rohrer and Kornfeld, 2001).

Phosphotransferase and I-cell disease

The initial interest on N-acetylglucosamine-1-phosphotransferase started from studies of patients with I-cell disease (mucolipidosis type II). I-cell disease is an inherited lysosomal storage disorder described by Leroy and Demars (1967). A unique feature of this disorder is the presence of phase-dense intracytoplasmic inclusions in fibroblasts. These cells are termed "inclusion cells". Based on this morphological appearance the disorder was designated I-cell disease. Spranger and Wiedermann (1970) subsequently classified this disorder as mucolipidosis type II (ML II) since I-cell disease patients exhibit clinical characteristics of mucopolysaccharidoses and sphingolipidoses. I-cell disease is an autosomal recessive disorder caused by the deficiency of UDP-N-acetylglucosamine N-acetyl-glucosaminyl-1-phosphotransferase. This enzyme is the product of the GNPTA gene, which has been mapped to chromosome band 4q21-q23. Cells from patients with I-cell disease express extremely low or undetectable levels of phosphotransferase leading to massive storage of carbohydrates and lipids in the lysosomes. In addition, a large number of lysosomal enzymes (soluble hydrolases) are present in excess in extracellular fluids such as serum and urine and in the media of cultured fibroblasts obtained from ML II patients (Leroy and Spranger, 1970). However, when fibroblasts from I-cell disease patients were grown in the presence of sucrose, the phosphotransferase activity was restored to almost normal level and the activities of hydrolases in the lysosomes increased. It appears that sucrose stabilizes the defective phosphotransferase (Okada et al., 1987).

Another intriguing finding was that I-cell disease fibroblasts were able to internalize and use lysosomal enzymes produced by normal cells, whereas normal cells and cells with other kind of lysosomal disorders were incapable of internalizing lysosomal enzymes secreted by I-cell disease fibroblasts (Ullrich and von Figura, 1979). The biochemical comparison of lysosomal enzymes from normal individuals with those from patients with I-cell disease led to the discovery of mannose 6-phosphate as the lysosomal sorting signal (Vladutiu and Rattazzi, 1979). In I-cell disease, the deficiency of phosphotransferase prevents the formation of the mannose-6-phosphate recognition marker as the lysosomal enzymes are modified in the Golgi apparatus before being transported to the lysosome. Thus, in absence of mannose 6-phosphate most soluble hydrolases cannot be transported to the lysosomes for normal processing and use.

Mannose-6 phosphate (M6P) receptors

When fibroblasts from patients with I-cell disease were grown in media containing lysosomal enzymes with mannose 6-phosphate residues, these cells acquired near normal intracellular content of exogenous lysosomal enzymes. This finding showed that the plasma membrane has M6P receptors (M6P-Rc) involved in the internalization of phosphorylated hydrolases. Two distinct type I integral membrane glycoproteins that exhibited high affinity for mannose 6-phosphate residues were identified. Both appear to transport hydrolases from TGN to endosomes and/or lysosomes. One of these receptors is a 46 KDa protein originally purified based on its ligand binding ability in the presence of divalent cations, particularly Mn^{+2}. This receptor is known as the cation-dependent M6P receptor. Sequence analysis of the bovine CD-M6P-Rc revealed that it is comprised of a 28-residue N-terminal signal sequence, a 159-residue luminal domain, a single 25-residue transmembrane region and a 67-residue C-terminal cytoplasmic domain. It is highly conserved from mouse to human (93% homology) and has identical sequences within the cytoplasmic tail across different species except the chicken. The receptor was shown to have five potential Asn-linked glycosylation sites, two of which are linked to complex oligosaccharides (Dahms et al., 1987), and two are occupied by high mannose residues.

The second receptor is a 215-300 KDa protein, also known as the insulin-like growth factor II (IGF II) receptor. The ligand binding ability of this receptor is independent of the presence of cations. Thus, this receptor is referred as the cation-independent M6P receptor (CI-M6P-Rc). The CI-M6P-Rc is a multifunctional receptor that binds lysosomal proteins bearing M6P recognition markers as well as the IGF-II (Morgan et al., 1987). The IGF-II/CI-M6P-Rc consists of a N-terminal signal peptide of 44-residues, a luminal region of 15 homologous repeating domains, a single transmembrane region and a 163-residues C-terminal cytoplasmic tail (Morgan et al., 1987). Biochemical studies showed that the luminal region contains two distinct M6P binding sites (repeating domains 3 and 9) and a single IGF-II binding site (repeating domain 11) (Waheed et al., 1988). Although the binding sites for M6P and IGF-II are different, IGF-II can inhibit lysosomal enzyme binding and vice-versa (MacDonald et al., 1988).

The luminal domain of CD-M6P-Rc, unlike the transmembrane and cytoplasmic regions, shares sequence similarity with each of the 15 repeating domains of the CI-M6P-Rc. A structure-based sequence alignment generated between the CD-M6P-Rc and domains 3 and 9 of the IGF-II/CI-M6P-Rc by maintaining the positions of the cysteine residues involved in disulfide bond, showed that these domains posses conserved residues that are responsible for ligand binding (Hancock et al., 2002). Due to their common function in targeting lysosomal proteins and the similarity of their amino acid sequence, it has been postulated that the two receptors evolved from a common ancestral gene with the CI-M6P-Rc arising from the duplication of the ancestral gene (Klier et al., 1991) .

Crystallographic analysis revealed that the CD-M6P-Rc contains a single M6P binding site and that is present primarily as a non-covalent homodimer at the membrane (Dahms and Hancock, 2002). The quaternary structure of the CI- M6P-Rc is still unclear. Available information suggests that this receptor may dimerize in the membrane although it has been shown to be in monomeric stage in detergent solutions (York et al, 1999).

Both receptors bind M6P with essentially the same affinity ($7\text{-}8x10^{-6}$M), however, the CI-M6P-Rc binds diphosphorylated oligosaccharide with a much higher affinity ($2x10^{-9}$M) than the CD-M6P-Rc (Tong et al., 1989; Tong and Kornfeld, 1989).

Role of M6P receptors in lysosomal targeting

In vitro the CI-M6P-Rc is the dominant lysosomal targeting receptor of soluble hydrolases as it is able to compensate for the loss of the CD-M6P-Rc. In fact, cells deficient in the CI-M6P-Rc only sort 30-40% of newly synthesized enzymes (Gabel et al., 1983). Cells that express both M6P-receptors do not secrete large amounts of lysosomal enzymes when anti-CD-M6P-Rc antibodies are added. However, addition of anti-CI-M6P-Rc antibodies induces lysosomal enzyme secretion. Transfection of CI-M6P-Rc deficient cell lines with a CI-M6P-Rc cDNA results in complete or almost complete correction of the hyper-secretion of lysosomal enzymes. Transfection of a CD-M6P-Rc cDNA to the same cell line partially corrected the hyper-secretion of lysosomal enzymes, reinforcing that the CD-M6P-Rc is less efficient than the CI-M6P-Rc in lysosomal protein targeting (Kyle et al., 1988; Lobel et al., 1989).

Final destination of the Mannose 6- Phosphate Receptors

In vivo, the M6P-Rc lysosomal protein complex is transported from the Golgi apparatus to an acidified, pre-lysosomal compartment where the low pH of the compartment induces the dissociation of the complex. The released lysosomal proteins are retained within this compartment while the M6P receptors either return to the trans Golgi network to repeat the process or move to the plasma membrane where they function to internalize exogenous ligands (Lodish et al., 1999).

M6P independent transport of hydrolases and non-enzymic lysosomal proteins

Mannose 6-phosphate Independent transport

In the yeast Saccharomyces cerevisiae, the sorting and delivery of vacuolar hydrolases is similar to that of lysosomal proteins in mammalian cells. Yeast mutants defective in vacuole acidification missort newly synthesized enzymes just as mammalian cell mutants defective in endosome acidification (Banta et al., 1988). The precursor of carboxypeptidase Y (CPY), a soluble vacuole protein, has been shown by site-directed mutagenesis to contain a signal peptide and a N-terminal region necessary for vacuole targeting. Overexpression of CPY resulted in missorting and secretion of newly synthesized enzyme, suggesting that CPY is transported to the vacuole by a sorting receptor (Rothman et al., 1986; Stevens et al., 1986; Johnson et al., 1987).

The receptor for Golgi-vacuole transport of CPY, was found to be a type I integral membrane protein Vps10p (Marcusson et al., 1994). More recently, Vps10p was found to bind CPY via its N-terminal domain (Jorgensen et al., 1999). Although Vps10p bears no homology to the M6P receptors its function in the yeast corresponds to the function of the M6P receptors in mammalian cells (Jorgensen et al., 1999). Unlike the M6P receptors, Vps10p sorts CPY via peptide sequence recognition (Valls et al., 1990; Winther et al., 1991). A recent study showed that the cytoplasmic tail of Vps10p mediated lysosomal sorting in mammalian cells by means of its interaction with GGA (Golgi-localizing γ-adaptin ear homology domain ADP-ribosylation factor binding protein). GGA is a novel ubiquitous coat protein mediating the formation of intracellular transport-intermediates and selection of cargo (Nielsen et al., 2001). Interestingly, the missorting and secretion of CPY due to its overexpression in yeast was not accompanied by missorting of other vacuolar enzymes, suggesting the existence of alternative sorting receptors (Stevens et al., 1986).

Mannose 6-phosphate independent sorting in mammalian cells

Although M6P receptors play a major role in the intracellular transport of newly synthesized lysosomal enzymes in mammalian cells, several lines of evidence suggest the existence of an alternative mechanism of lysosomal targeting. B-lymphocytes from patients with I-cell disease maintain near normal cellular levels of lysosomal enzymes despite their inability to add mannose 6-phosphate to newly synthesized hydrolases (Tsuji et al., 1988). An I-cell disease (ICD) B lymphoblastoid cell line targets about 45% of the lysosomal protease cathepsin D to dense lysosome in the absence of detectable mannose 6-phosphate residues (Glickman and Kornfeld, 1993). The secretory protein pepsinogen (closely related to cathepsin D) is mostly excluded from dense lysosomes, indicating that the lymphoblast-targeting pathway of cathepsin D is specific. Analysis of a number of cathepsinD/pepsinogen chimeras indicates that extensive polypeptide determinants in the cathepsin D C-terminal region confer efficient lysosomal sorting when introduced into a pepsinogen sequence. These results also indicate that a specific protein recognition event underlies the mannose 6-phosphate independent lysosomal sorting in ICD lymphoblast (Glickman and Kornfeld, 1993).

In fibroblast from ICD patients most hydrolases are not targeted to the lysosomes, and as a consequence, they are missorted to the extracellular space (Lightbody et al., 1971). However, not all lysosomal proteins were aberrantly secreted since prosaposin and the GM2AP were found in the lysosomes of the defective cells (Rijnboutt et al., 1991).

Studies of intracellular trafficking of prosaposin and GM_2AP (the cofactor of ß-hexosaminidase A) in cultured cells transfected with a dominant-negative truncated human sortilin demonstrated that both prosaposin and GM_2AP use sortilin instead of mannose 6-phosphate receptor to reach the lysosomes (Lefrancois et al., 2003; Hassan et al., 2004). In addition, a sortilin siRNA blocked the trafficking of prosaposin and GM_2AP to the lysosomal compartment and induced the retention of these proteins in the Golgi apparatus. These results substantiate that the trafficking of prosaposin and GM_2AP to lysosomes is dependent on sortilin (Lefrancois et al., 2005).

Similarly to prosaposin, immunocytochemical analysis of tunicamycin treated cells demonstrated that glycosylation is not essential for the targeting of the AOAH precursor to the lysosomes (Staab et al., 1994). This finding suggests that the lysosomal targeting of the AOAH precursor is independent of the mannose 6-phosphate receptor and opens the question as to whether sortilin may be responsible for the targeting of some hydrolases in addition to the sphingolipid activator proteins prosaposin and GM_2AP.

The Vp10p superfamily of receptors

4. Sortilin, a Novel Sorting Receptor

Sortilin is a member of the type I Vps10p

superfamily. The Vps10 proteins constitute a family of heterogeneous type I transmembrane receptors. They are expressed in many tissues and target a variety of ligands (Hermey et al., 1999; Jacobsen et al., 2001). In mammalian cells the VpS10p family consists of 5 members: SorLA (250KDa) (Jacobsen et al., 1996), sortilin (100KDa) (Petersen et al., 1997), SorCS1, SorCS2 and SorCS3 (130KDa) (Hermey et al., 1999). These receptors are characterized by a luminal domain homologous to the yeast vacuolar sorting protein VPS10p and by a short cytoplasmic region harboring signals for rapid internalization and intracellular sorting (Nielsen et al., 2001). Consequently, they are also known as Vps10p domain containing receptors.

The human Vps10p genes

Analysis of the human Vps10p domain containing receptor genes has shown that they contain several short exons separated by large introns, some of which extend over more than 50 KB. The three SorCS genes encompass more than 500 KB of genomic DNA and represent some of the largest known human genes. The function of the large introns is unknown. However, they may be involved in splicing. The first coding exon of all human Vps10p receptor proteins has a high G/C content, indicative of CpG islands. A CG island is a short stretch of DNA in which the frequency of CGs is higher than in other regions. The letter "p" of a CpG island, indicates that "C" and "G" are connected by a phosphodiester bond. Methylation of CpG islands might be involved in the developmental or tissue-specific regulation of the Vps10p receptors (Hampe et al., 2001).

Common structural features of Vps10p receptors

All members of the Vps10p receptor family are synthesized as pro-receptors containing one or two consensus sequences for cleavage by furin [RX(R/K) R] in their N-terminal region. In the case of sortilin, the furin cleavage occurs during the passage of the receptor through the trans-Golgi network (Petersen et al., 1999). Affinity chromatography showed that the pro-peptide cleavage is necessary and sufficient for activation of RAP (receptor associated protein) binding to sortilin. Moreover, sortilin pro-peptide and the sortilin ligands neurotensin and RAP, bind to the same site on the luminal domain of the fully processed receptor. The pro-peptide therefore appears to provide a static hindrance, preventing the ligands from gaining access to the binding site in uncleaved pro-receptors (Munck Petersen et al., 1999). Another feature of the Vps10p family is a conserved region containing 10 cysteines in the C-terminus of the Vps10p domain. In fact, this is the most conserved region of all members of this family. The cysteines form 5 disulfide bridges in sortilin and SorLA. In sortilin, the deletion of this segment abolishes the binding to RAP, providing evidence that the cysteine module constitutes the major binding site in the Vps10p domain (Westergaard et al., 2004).

Vps10p receptors bind unrelated ligands

Most members of the Vps10p family bind more than one ligand and exhibit multifunction. SorLA is a highly conserved "putative" sorting receptor located mainly in the TGN. From the N-terminus, the luminal region comprises a Vps10p domain, a cluster of LDLR (low density lipoprotein receptor related protein), class-A repeats and six fibronectin type III repeats. SorLA binds neurotensin (NT) and heat activator (HA) via its Vps10p domain, and apolipoprotein E and lipoprotein lipase (LpL) via its cluster of class-A repeats (Jacobsen et al., 1996; Lintzel et al., 2002; Gliemann et al., 2004). SorLA is structurally and functionally related to sortilin and to receptors of the LDLR family, and might be involved in protein sorting and signal transduction (Lintzel et al., 2002). SorLA is also implicated in the generation of Alzheimer's disease as well as in atherosclerotic plaque formation (Wolozin, 2004; Zhu et al., 2004). Its mouse homologue, mSorLA, exhibits a unique pattern of expression in the developing brain, suggesting a potential function in the formation of this organ.

Sortilin, also named neurotensin receptor 3 (NTR3), was originally purified from brain extracts (Zsurger et al., 1994). Sortilin contains a single Vps10p domain within its luminal region. It binds receptor-associated protein (RAP), neurotensin and prosaposin (Zsurger et al., 1994; Lefrancois et al., 2003). Thus, sortilin functions as a lysosomal sorting receptor and participates in signal transduction in cooperation with the NTR1. A large pool of sortilin is localized within the Golgi apparatus and intracellular vesicles. However, in response to insulin, sortilin is translocated with the glucose transporter Glut4 to the plasma membrane (Lin et al., 1997; Morris et al., 1998). Recent studies showed that sortilin mediates the uptake and degradation of lipoprotein lipase (LpL) (Nielsen et al., 1999).

SorCS1 belongs to a sub-group of the mammalian Vps10p family containing a luminal N-terminus where the Vps10p region is contiguous to a leucine-rich domain implicated in protein-protein interaction. Two other members of this subgroup are SorCS2 and SorCS3. No specific ligand has been reported to bind these receptors, except that mature SorCS1 binds its own pro-peptide with low affinity. These receptors are abundant in the developing and mature brain, and they may play a role in the central nervous system (Hermey et al., 2004).

Structure and function of the cytoplasmic tail of Vps10p receptors

Each Vps10p receptor carries a short cytoplasmic tail of 50 to 80 amino acids comprising typical motifs for interaction with cytoplasmic adaptor molecules. Functional sorting sites, such as dileucine motifs, acidic clusters and tyrosine-based motifs involved in endocytosis and intracellular transport have been found

in sortilin and SorLA (Nielsen et al., 2001; Jacobsen et al., 2002). The sortilin cytoplasmic domain contains several potential signal sequences that conform to established consensus motifs, known to be involved in adaptor protein binding, endocytosis, basolateral targeting and Golgi-endosome sorting. Furthermore, the sortilin cytoplasmic tail was shown to bind the VHS domain of GGA2, suggesting that sortilin cytoplasmic domain conveys signals for Golgi localization and Golgi-lysosome transport (Nielsen et al., 2001).

The 54-residue cytoplasmic tail of SorLA comprises a putative internalization motif (FANSHY), an acidic cluster (DDLGEDDED), and a C-terminal patch of hydrophobic residues (VPMVIA), preceded by two acidic residues (DD). GGA1 and 2 were shown to bind SorLA with differential requirements via three critical residues, two acidic residues and one hydrophobic amino acid in the C-terminal segment of its cytoplasmic tail (Jacobsen et al., 2002). Unlike sortilin and the mannose 6-phosphate receptor, the GGA binding segment in SorLA contains neither an acidic cluster nor a dileucine motiff, which suggests that key residues in SorLA and sortilin conform to a new motif, ΨXX∅ for interaction with GGA and ΨΨΞΞ/Ψ∅∅ for interaction with GGA2, defining minimum requirements for GGA binding to cytoplasmic receptor domains (Jacobsen et al., 2002).

Sortilin: A multifunctional protein

Sortilin, was originally cloned and purified from mouse and human brain extracts by affinity chromatography with a RAP column. In human, the gene maps to chromosome 1p and encodes a polypeptide of 833 amino acids. Human sortilin is a 100 KDa, non-G-coupled type I transmembrane protein consisting of a 44-amino acid N-terminal pro-peptide, followed by a furin cleavage signal ($R^{41}WRR^{44}$), a large luminal domain, a single transmembrane region and a short cytoplasmic tail (Petersen et al., 1997). The major pool of sortilin accumulates in the trans-Golgi-network and in vesicles. Sortilin co-localizes with CI-M6P-Rc (Petersen et al., 1997), while about 10% is expressed in plasma membrane. The plasma membrane expression of sortilin is increased after neurotensin-induced sequestration of neurotensin receptor 1 (NTR1).

Sortilin is synthesized as a precursor and converted to mature protein after the removal of the pro-peptide by furin before or during its passage through the Golgi apparatus. The pro-peptide exhibits high affinity binding to the fully processed sortilin, and the binding is competed by RAP and NT. Interestingly, both RAP and NT are unable to bind sortilin in the absence of maturation by furin. Studies by Westergaard et al. (2004) have shown that the pro-peptide has at least two functions: 1) sortilin depends on its pro-peptide for proper protein folding and normal passage through the biosynthetic pathway (Munck Petersen et al., 1999); 2) the pro-peptide acts as a safeguard protecting the cells against formation of death-signaling intracellular complexes (Westergaard et al., 2004). In contrast other Vps10p receptors, such as SorLA and SorCS3, do not need their pro-peptide for normal processing (Westergaard et al., 2004). Finally, unlike other Vps10p members, the luminal domain of sortilin exclusively comprised a single Vps10p domain (Petersen et al., 1997).

Sortilin appears to be a multifunctional protein since it is capable to bind different ligands such as neurotensin, RAP, prosaposin and LpL. Sortilin is also engaged in intracellular sorting as well as in endocytosis and signal transduction. For instance, sortilin mediates rapid endocytosis of lipoprotein lipase, neurotensin (NT) and the precursor of nerve growth factor (pro-NGF). It also forms a complex with G protein-coupled neurotensin receptor-1 on the plasma membrane modulating NT signaling, and is able to target lysosomal proteins such as prosaposin and GM_2AP to the lysosomes (Fig. 1). Sortilin is essential to pro-NGF induction of neuronal death via complex formation with $p75^{NTR}$ on the cell membrane. A recent study on the hydrophobic protein conotoxin-TxVI suggests that sortilin interacts with TxVI in the ER and facilitates its export from ER to the Golgi apparatus (Conticello et al., 2003).

The cytoplasmic domain of sortilin lacks characteristic sequence for signal transduction, but shares a homologous sequence with the CI-M6P-Rc, which binds GGAs. Cellular trafficking of chimeric receptors containing the luminal and transmembrane domains of the M6P-Rc followed by the sortilin cytoplasmic tail showed that the sorting signal ΨXX∅ and the dileucine motif of sortilin mediate the rapid endocytosis of the chimeric protein, which subsequently was found in the TGN. The CI-M6P-Rc/sortilin chimera was as efficient as the CI-M6P-Rc itself for the transport of newly synthesized lysosomal proteins to the lysosomes (Nielsen et al., 2001). Furthermore, using the cytoplasmic tail of sortilin as bait, yeast-two-hybrid experiment showed that sortilin binds the VHS domain of the cytosolic sorting protein GGA2 (Takatsu et al., 2001).

In conclusion, the structural features of the luminal and cytoplasmic domains of sortilin indicate that this protein functions as a sorting and trafficking receptor for lysosomal soluble proteins. Nonetheless, some researchers believe that sortilin is also a neuropeptide receptor, which unlike other receptors, does not belong to the family of G-protein-coupled receptors.

Role of GGAs in lysosomal trafficking

Interaction of GGAs with ADP-ribosylation proteins (ARFs)

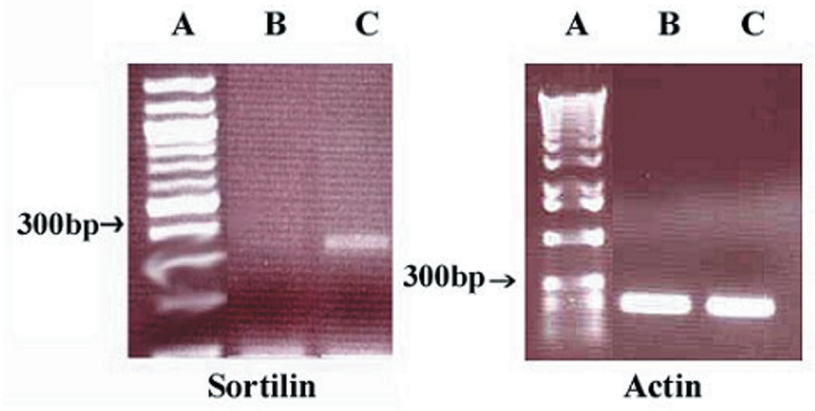
The GGA (Golgi-localizing, γ-adaptin ear homology domain, ARF-binding protein) proteins constitute a novel multidomain protein family implicated in trafficking between TGN and endosomes (Ghosh and

Kornfeld, 2004). Using a yeast two-hybrid screen of human cDNA libraries with activated ARF3 as the bait, two proteins, GGA1 and GGA2 were discovered simultaneously by several labs (Boman et al., 2000; Dell'Angelica et al., 2000; Hirst et al., 2000; Poussu et al., 2000). GGA3 was identified in a BLAST search of the GeneBank database. GGA1 and GGA2 were also found in yeast (Boman, 2001). GGA proteins are conserved throughout eukaryotes and all identified GGAs range between 65 and 80 KD in size (Boman, 2001).

Immunoelectron microscopy analyses showed that human GGAs localized to electron-dense coats associated with TGN membranes, suggesting a function in the TGN region. The three human GGAs showed overlapping but subtle differences in subcellular staining. In addition to their shared TGN localization, GGA2 showed a diffuse cytosolic distribution, GGA3 showed a granular cytoplasmic pattern (Boman et al., 2000; Hirst et al., 2000).

Structure of GGA proteins

Alignment of amino acid sequences of human and yeast GGAs demonstrated that GGAs consist of four domains. The N-terminal 150 residues constitute the "VHS" domain. This domain was also found in three proteins: Vps27, Hrs and STAM. The crystal structure of



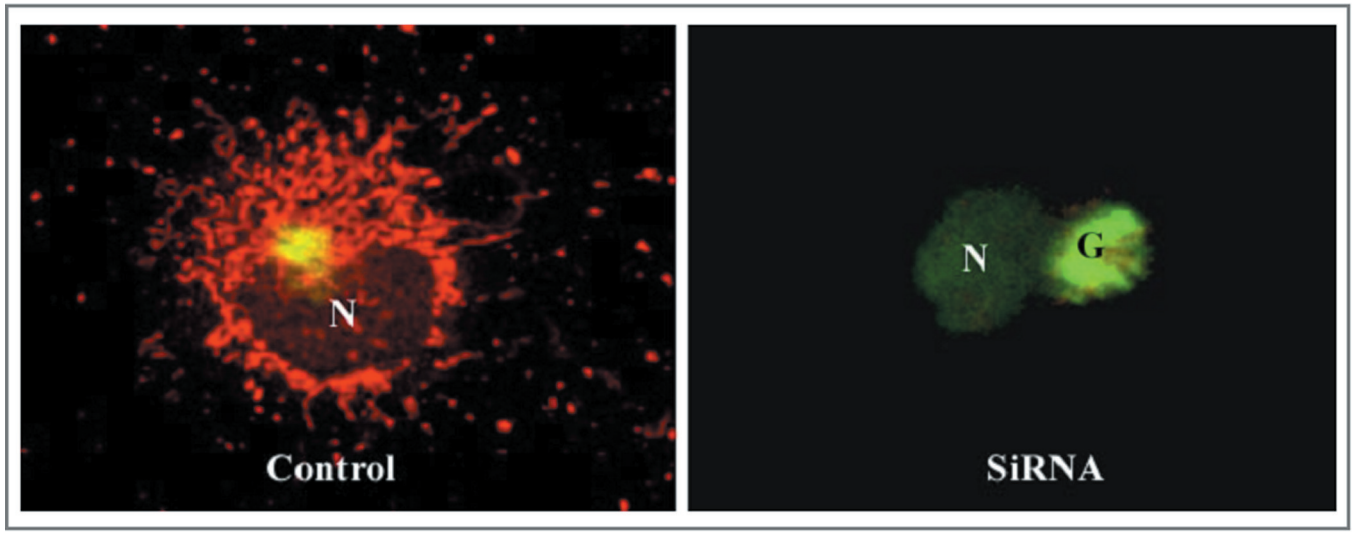


Fig. 1. Effect of stably knockdown of sortilin in COS-7 cells. RT-PCR with specific primers demonstrates that sortilin mRNA levels decreased in cells transfected with a hairpin siRNA vector (upper left panel, lane B). A, markers; C, RT-PCR of COS-7 cells transfected with empty vector. The upper right panel is an RT-PCR with ß-actin primers. Note that transfections have no effect on actin mRNA levels. Similary, confocal double-immunostaining with anti-sortilin and anti-Golgin antibodies demonstrates that sortilin siRNA eliminates the lysosomal red granular fluorescence and Golgi yellow fluorescence due to overlay with anti-Golgin staining (lower right panel) observed in control COS-cells (lower left panel). The control is a COS-7 cell transfected with an empty vector. N, nucleus; G, Golgi-region.

the VHS domain from another protein, Tom1, suggests that this region is involved in protein-protein or protein-membrane interactions (Misra et al., 2000). The most highly conserved domain is the 170 residues long "GAT" domain. With approximately 65% identity among GGAs, the GAT domain mediates the interactions with ARF1 and ARF3. The name of this domain derives from the sequence homology between GGA and Tom1 (Dell'Angelica et al., 2000). The "hinge" domain, of variable lengths among GGAs, contains one or more clathrin-binding motifs. Two conserved clathrin-box motifs are present in the GGA2 hinge (LIDLE and LLDLL), whereas only one motif has been found in the GGA1 hinge domain (Dell'Angelica et al., 2000). The C-terminal "GAE" (or "EAR") domain is composed of 120 residues and has homology to the ear domain of γ-adaptin. The GAE domain interacts with accessory proteins such as r-synergin, p56, Rabaptin-5 and enthoprotein (Bonifacino, 2004), and it has been shown to interact with clathrin (Puertollano et al., 2001b).

Function of GGAs in the Yeast

In yeasts, GGA1 and GGA2 share 50% amino acid identity. While deletion of either GGA alone causes minor or no defects, deletion of both GGAs lead to trafficking defects of carboxypeptidase Y (CPY) and carboxypeptidase S (CPS) (Mullins and Bonifacino, 2001). Furthermore, a chimeric Pep12p protein normally delivered from the TGN to late endosomes, is missorted into early endosomes in yeast strains lacking both GGA1 and GGA2. These data suggests that GGAs are key components of a specific pathway from the TGN to late endosomes (Black and Pelham, 2000).

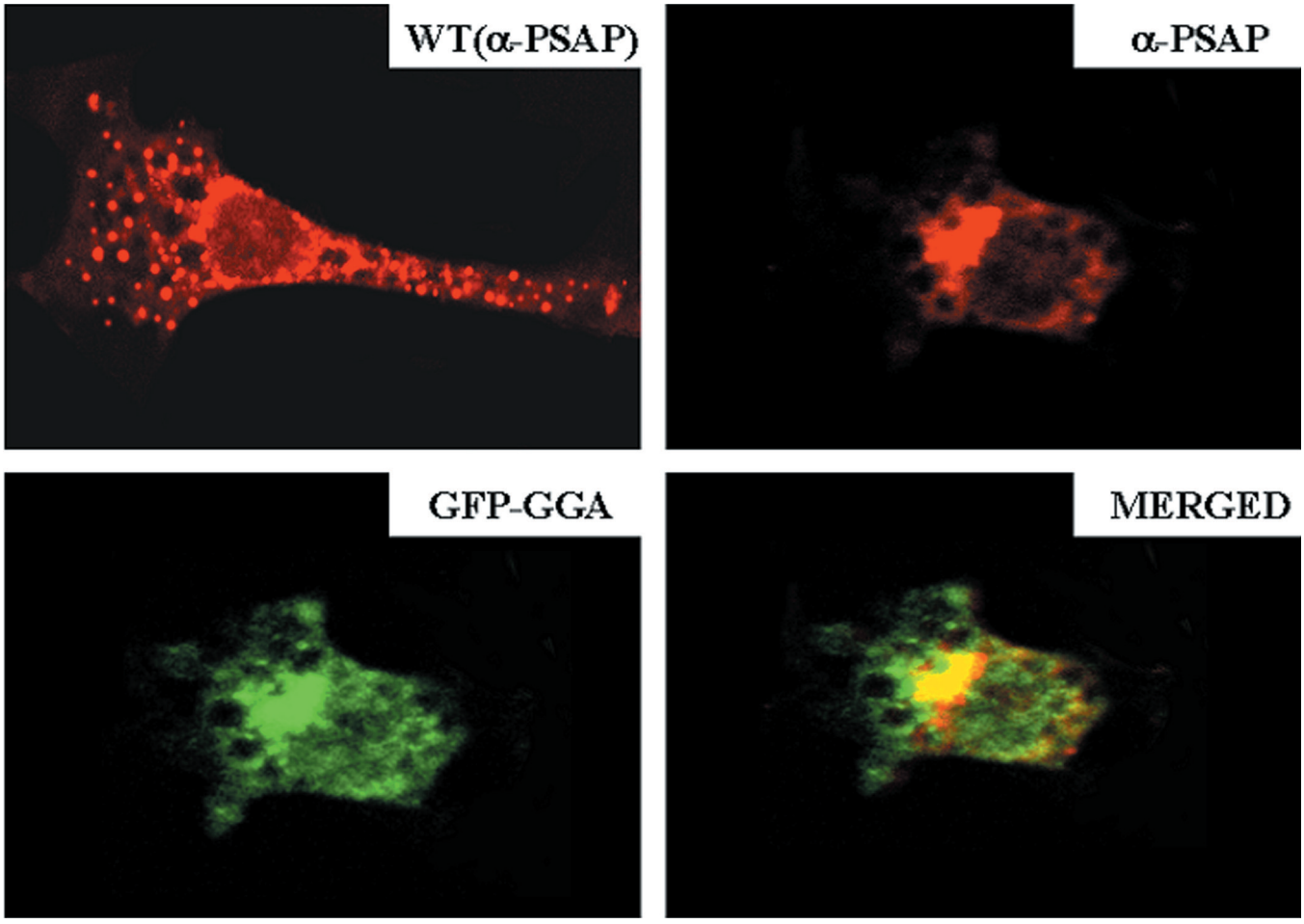


Fig. 2. Expression of a dominant-negative truncated GGA protein linked to the green fluorescent protein (GFP-GGA) causes retention of prosaposin in the perinuclear region of TM4 cells. Upper left panel is a wild type TM4 cell. Note that anti-prosaposin staining (red) is observed within granular structures (lysosomes). Upper right panel shows that prosaposin is retained in the Golgi region of a cell transfected with dominant negative GFP-GGA. Observed that GFP-GGA is localized in the same region (lower left panel). The lower right panel is the merged image of the two preceding panels.

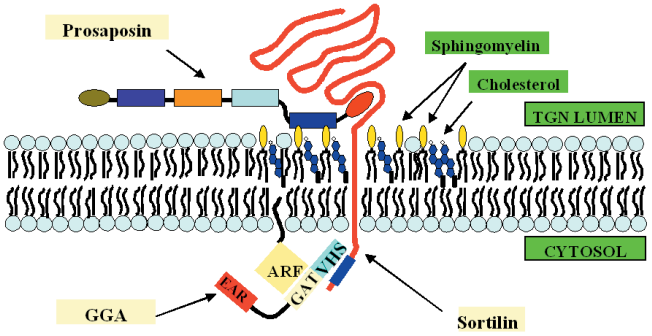


Fig. 3. Schematic diagram illustrating the mechanism of transport of sortilin (sorting receptor). Sortilin binds prosaposin and other sphingolipid activator proteins present within the TGN lumen and uses monomeric GGA to bind ARF and recruit clathrin (not shown in this drawing) in order to leave this compartment. Note that prosaposin also interacts with sphingomyelin.

GGAs interacting proteins

In mammalian cells, the VHS domain of GGAs interacts with the dileucine-sorting motif present in the cytoplasmic tail of CI-M6P-Rc and CD-M6P-Rc (Puertollano et al., 2001a,b). Similarly, GGAs bind to the acidic-cluster-dileucine motif (ACLL) of the cytoplasmic tail of sortilin (Fig. 2) (Nielsen et al., 2001; Takatsu et al., 2001) as well as the lipoprotein receptor-related protein 3 (LRP3) (Takatsu et al., 2001). SorLA, a sorting receptor of the Vps10p family, binds GGAs 1 and 2 through a signal methionine and a pair of preceding acidic residues (Jacobsen et al., 2002).

The GAT domain of GGAs binds specifically GTP-bound ARF proteins found in TGN membranes. This indicates that GGAs are effectors of ARF (Collins et al., 2003). A number of experiments showed that the localization of GGAs at the TGN is due to its interaction with ARF since the VHS domain alone was unable to recruit GGAs to the TGN membrane. However, ARF is absent from clathrin-coated vesicles containing GGAs. Therefore, it is likely that ARF binds and recruits GGAs to the TGN membrane to subsequently hands-off GGAs to cargos (Dell'Angelica et al., 2000; Hirst et al., 2000; Poussu et al., 2000; Puertollano et al., 2001b). GGAs and cargo are then incorporated into a transport intermediate that excludes ARF (Hirsch et al., 2003).

Finally, while the hinge domain of GGAs interacts with clathrin in vitro and promotes the recruitment of clathrin to membranes, the ear domain may strengthen this interaction. Nevertheless, the function of the ear domain is still unclear (Page et al., 1999). In consequence, the GGAs are monomeric, ARF-dependent clathrin adaptors involved in ARF and clathrin interactions required for the trafficking of cargo to the lysosomes (Fig. 2).

Conclusions

The majority of soluble hydrolases are targeted to the lysosomes by the mannose-6-phosphate receptor (M6P-Rc) through a specific mannose-6-phosphate tag. To be sorted from the Golgi apparatus, the M6P-Rc must bind first to Golgi associated, γ-adaptin homologous, ARF binding proteins (GGAs). GGAs are a group of monomeric adaptor proteins responsible for bridging sorting receptors and clathrin in order to be segregated into cargo vesicles. However, it is well established that the lysosomes of I-cell disease (ICD) patients resulting from a mutation in the phosphotransferase that adds the mannose-6-phosphate tag to hydrolases, have near normal levels of several lysosomal proteins, including the non-enzymic lysosomal sphingolipid activator proteins prosaposin and GM2 activator protein (GM2AP). Recently, our laboratory identified a novel lysosomal targeting receptor, sortilin, involved in the alternative sorting of prosaposin and GM2AP. Sortilin was shown to have a GGA binding motif similar to the M6P-Rc (Fig. 3). Interestingly, a dominant-negative GGA construct unable to bind clathrin, prevented the trafficking of prosaposin and GM2AP to the lysosomes. Similarly, we have demonstrated that a dominant negative sortilin lacking the GGA binding domain retained prosaposin and GM2AP in the Golgi apparatus. Although sortilin is involved in the sorting and trafficking of sphingolipid activator proteins it is also possible that this receptor may be implicated in the targeting of some hydrolases as well.

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