The lysosomal trafficking of sphingolipid activator proteins (SAPs) is mediated by sortilin

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Most soluble lysosomal proteins bind the mannose 6-phosphate receptor (M6P-R) to be sorted to the lysosomes. However, the lysosomes of I-cell disease (ICD) patients, a condition resulting from a mutation in the phosphotransferase that adds mannose 6-phosphate to hydrolases, have near normal levels of several lysosomal proteins, including the sphingolipid activator proteins (SAPs), $G_{M2}AP$ and prosaposin. We tested the hypothesis that SAPs are targeted to the lysosomal compartment via the sortilin receptor. To test this hypothesis, a dominant-negative construct of sortilin and a sortilin small interfering RNA (siRNA) were introduced into COS-7 cells. Our results showed that both the truncated sortilin and the sortilin siRNA block the traffic of $G_{M2}AP$ and prosaposin to the lysosomal compartment. This observation was confirmed by a co-immunoprecipitation, which demonstrated that $G_{M2}AP$ and prosaposin are interactive partners of sortilin. Furthermore, a dominant-negative mutant GGA prevented the trafficking of prosaposin and $G_{M2}AP$ to lysosomes. In conclusion, our results show that the trafficking of SAPs is dependent on sortilin, demonstrating a novel lysosomal trafficking.

Keywords: lysosomes/prosaposin/sortilin/sorting/traffic

Introduction

To exit from the Golgi apparatus, soluble lysosomal proteins must bind to a trans-membrane sorting receptor containing a cytoplasmic tail that interacts with adaptor proteins (Lobel *et al*., 1989). This interaction results in the recruitment of clathrin and ARF, and the formation of cargo vesicles (Lobel *et al*., 1989). Integral lysosomal membrane proteins do not require sorting receptors since their cytoplasmic tails interact directly with multimeric adaptor proteins (Peden *et al*., 2002). Multimeric adaptor proteins are involved in several trafficking processes such as endocytosis, basolateral targeting and lysosomal targeting (Robinson and Bonifacino, 2001). Multimeric adaptors (AP-1, AP-2, AP-3 and AP-4) are composed of four polypeptide chains that form a functional complex (Robinson and Bonifacino, 2001; Peden *et al*., 2002). AP-3 has been shown to traffic integral lysosomal membrane proteins, such as LAMPs, to the lysosome (Robinson and Bonifacino, 2001).

The mannose 6-phosphate receptor (M6P-R) is a well-characterized trans-membrane protein receptor that binds and routes to the lysosomal compartment most soluble hydrolases including several cathepsins (Lobel *et al*., 1989; Nielsen *et al*., 2001). A novel class of adaptor proteins, the monomeric GGAs (Golgi-localizing, γ-adaptin ear homology domain, ARF-binding proteins), have been shown to bind the cytosolic tail of the M6P-R (Puertollano *et al*., 2001a; Zhu *et al*., 2001) and to recruit the components needed for the formation of clathrin-coated vesicles. The GGAs are monomeric adaptor proteins composed of four domains: a VHS domain that binds to the tail of the sorting receptor via a specific protein motif, a GAT domain which interacts with ARF (Puertollano *et al*., 2001b; Zhu *et al*., 2001), and the hinge and ear domains that bind clathrin and accessory proteins to form coated vesicles (Hirst *et al*., 2000). The use of dominant-negative GGA constructs lacking the hinge and ear domains abolished the recruitment of clathrin and caused the retention of M6P-R in the Golgi, preventing proper intracellular sorting of several lysosomal proteins (Puertollano *et al*., 2001a).

Analyses of human fibroblasts from patients with I-cell disease (ICD) showed that sphingolipid activator proteins (SAPs) use an alternative targeting mechanism to reach the lysosomes (Rijnboutt *et al*., 1991). ICD results from a mutation in the phosphotransferase that adds mannose 6-phosphate to hydrolases that must be delivered to the lysosomes via the M6P-R (Reitman *et al*., 1981). SAPs are five non-enzymatic cofactors required for the lysosomal degradation of glycosphingolipids with short oligosaccharide chains (Bierfreund *et al*., 2000). Four of them (saposins A, B, C and D) are small homologous glycoproteins derived from a common precursor protein (prosaposin) encoded by a single gene (Hiraiwa *et al*., 1997). Saposins increase the catalytic rate of lysosomal hydrolases by forming water-soluble complexes with specific sphingolipids, hence lysosomal hydrolases can cleave sugar residues to yield ceramide, which is deacylated to sphingosine. The fifth activator, the G_{M2} activator protein ($G_{M2}AP$), is the product of a separate gene and is an essential cofactor of β-hexosaminidase A in the degradation of G_{M2} to G_{M3} ganglioside (Yamanaka *et al*., 1994). The lysosomal trafficking of prosaposin is dependent on the D domain along with its highly conserved C-terminal region (Zhao and Morales, 2000). Deletion of either of these regions causes the misrouting of prosaposin to the secretory pathway (Zhao and Morales, 2000). Sphingomyelin, present in the inner leaflet of the Golgi membrane, is also required to target prosaposin to the lysosomes (Lefrancois *et al*., 2002). Depletion of this
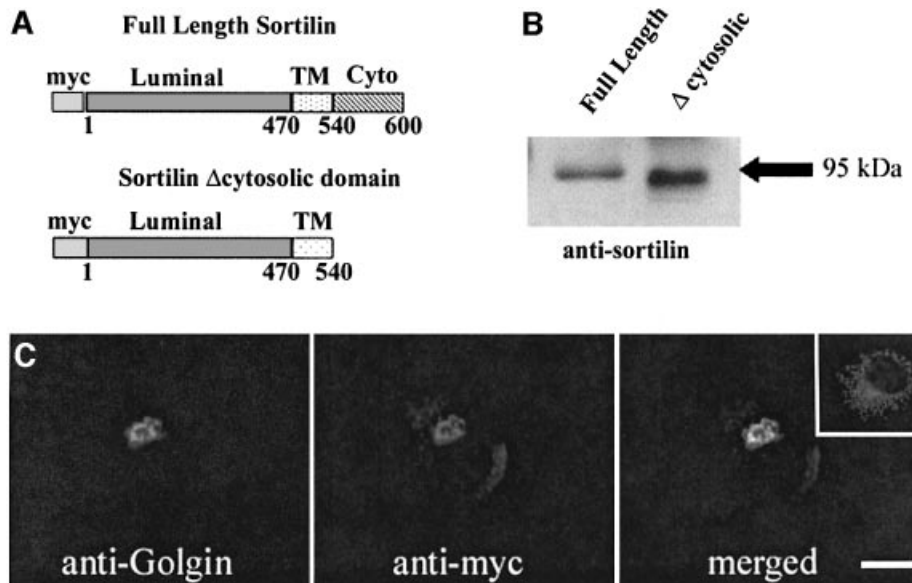
Fig. 1. Expression and localization of truncated sortilin. Myc-tagged full-length and a truncated sortilin construct that lacked its cytosolic domain (**A**) (residues 540–600) were *in vitro* translated and run on a 12.5% SDS–polyacrylamide gel. The full-length construct ran to the standard 95 kDa (**B**, lane 1) while the truncated construct was slightly smaller (B, lane 2). The truncated construct was transfected into COS-7 cells and localized in the perinuclear region (**C**, anti-myc). This staining pattern was merged to the Golgi staining with anti-Golgin, indicating that the truncated sortilin was being retained in the Golgi apparatus (merged). The inset shows the immunofluorescent staining of full-length sortilin. Scale bar = 10 μm.
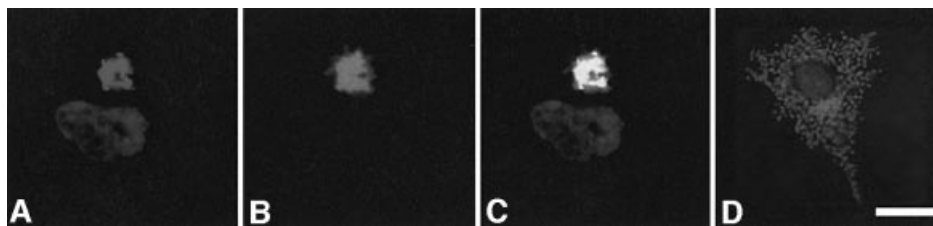

Fig. 2. Effect of dominant-negative sortilin lacking its cytoplasmic domain in COS-7 cells immunostained with anti-prosaposin antibody. COS-7 cells were immunostained with anti-prosaposin antibody following transfection with truncated sortilin (**A**). COS-7 cells were also stained for the truncated sortilin construct (**B**). The merged image shows prosaposin in a perinuclear staining pattern in the same compartment as the truncated sortilin construct (**C**). Immunostaining for endogenous prosaposin demonstrates its normal distribution in non-transfected COS-7 cells (**D**). Scale bar = 20 μm.

lipid with specific inhibitors causes the misrouting of prosaposin to the secretory pathway (Lefrancois *et al*., 1999). Based on these results, we have hypothesized that the D domain of prosaposin interacts with sphingomyelin to bring this protein into close contact with the Golgi membrane and to facilitate the binding of its C-terminal region to an unknown sorting receptor (Lefrancois *et al*., 2002). Little is known about the trafficking mechanism of $G_{M2}AP$ other than that it is M6P-R independent (Glombitza *et al*., 1997).

Recent evidence suggests that the trans-membrane Golgi protein, sortilin, may be implicated in the transport of proteins to the lysosome. Chimeric constructs containing the luminal domain of the M6P-R linked to the transmembrane and cytosolic portion of sortilin restored the lysosomal trafficking of soluble hydrolases in M6P-R-deficient cells (Nielsen *et al*., 2001). While the M6P-R binds the majority of soluble hydrolases, no lysosomal proteins are known to interact with sortilin (Nielsen *et al*., 2001). Interestingly, the cytosolic portion of sortilin and M6P-R contain an 'acidic cluster–dileucine motif' that interacts with the monomeric adaptor proteins (Nielsen *et al*., 2001). In this report, we are testing the hypothesis that sortilin is the trafficking receptor of prosaposin and

$G_{M2}AP$. Although it has been suggested that prosaposin is targeted to the lysosomal compartment via secretion and endocytosis through the lipoprotein receptor-related protein (LRP) receptor (Hiesberger *et al*., 1998), we present evidence for a direct trafficking of both prosaposin and $G_{M2}AP$ from the *trans*-Golgi network (TGN) to the lysosomal compartment via a sortilin-mediated mechanism.

Results

Expression and localization of truncated sortilin
Deletion of the acidic cluster–dileucine signal from the cytoplasmic tail of the M6P-R abolished its interaction with the VHS domain of GGA proteins and its subsequent sorting to the lysosomes. Thus, we used a similar approach to investigate the role of sortilin in the transport of prosaposin and $G_{M2}AP$. To this effect, a full-length (wild-type) and a truncated sortilin lacking the acidic cluster–dileucine signal (Δcytosolic) (Figure 1A) were *in vitro* translated, run on a 12.5% acrylamide gel and transferred to nitrocellulose paper. The membrane was blotted and reacted with an anti-sortilin antibody. The full-length sortilin produced a band at ~95 kDa while the truncated
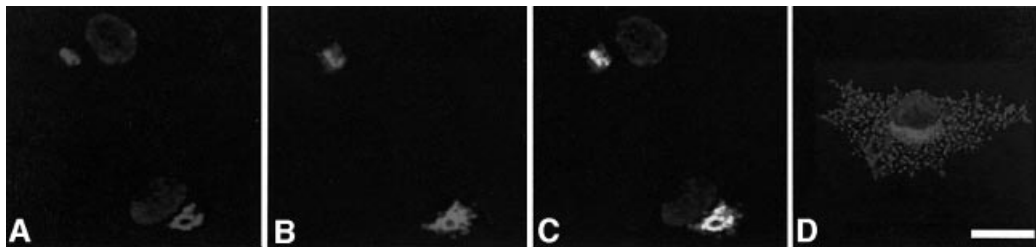
Fig. 3. Effect of dominant-negative sortilin lacking its cytoplasmic domain in COS-7 cells immunostained with anti-G_{M2}AP antibody. COS-7 cells were immunostained with anti-G_{M2}AP antibody following a transfection with truncated sortilin (**A**). COS-7 cells were also stained for the truncated sortilin construct (**B**). The merged image shows G_{M2}AP in a perinuclear staining pattern in the same compartment as the truncated sortilin construct (**C**). Immunostaining for endogenous G_{M2}AP demonstrates its normal distribution in non-transfected COS-7 cells (**D**). Scale bar = 20 μm.

Fig. 4. Effect of dominant-negative sortilin lacking its cytoplasmic domain in COS-7 cells immunostained with anti-cathepsin B antibody. While anti-cathepsin B antibody produced a punctate staining in the cytoplasm of non-transfected cells (not shown), the truncated construct (**B** and **C**) had no effects on the staining pattern of anti-cathepsin B antibody (**A** and **C**). Scale bar = 20 μm.

sortilin was slightly lower (Figure 1B). COS-7 cells were then transiently transfected with the truncated construct to examine its intracellular localization. The cells were stained for the construct with an anti-myc antibody and for a Golgi marker, anti-Golgin. The construct was retained in the Golgi apparatus and localized to the same compartment as the Golgi marker (Figure 1C, merged). Non-transfected COS-7 cells were stained for full-length sortilin (Figure 1C, inset). The staining pattern for full-length sortilin was not restricted to the perinuclear region, but extended into punctate structures.

Truncated sortilin abolished the transport of prosaposin and G_{M2}AP

In this experiment, COS-7 cells were transfected with a dominant-negative sortilin construct lacking the cytoplasmic region implicated in the binding of GGAs (Nielsen *et al.*, 2001). The construct was linked to a myc tag to discriminate between transfected and non-transfected cells and to distinguish truncated from endogenous sortilin. The cells were immunostained with an anti-prosaposin (Figure 2A, C and D), anti-G_{M2}AP (Figure 3A, C and D) or anti-cathepsin B (Figure 4A and C) antibody, followed by incubation with a secondary antibody conjugated to Alexa 568. To detect the construct, the same culture cells were immunostained with an anti-myc antibody followed by a secondary antibody conjugated to Alexa 488. Non-transfected cells (Figure 2D and 3D) showed the characteristic anti-prosaposin and anti-G_{M2}AP immunostaining in the perinuclear region as well as in cytoplasmic punctate structures. The transfected cells (Figures 2 and 3) displayed a perinuclear immunostaining with the anti-prosaposin, anti-G_{M2}AP and the anti-myc antibodies. However, no punctate structures were seen in transfected cells, suggesting that overexpression of truncated sortilin competed for the binding of prosaposin and abolished its

transport to the lysosomal compartment. The immunostaining pattern of the anti-cathepsin B antibody was not different in the non-transfected (not shown) and transfected cells (Figure 4).

Sortilin inhibition prevents the normal sorting of prosaposin

To confirm the involvement of sortilin in the sorting of prosaposin, sortilin mRNA was targeted with six small interfering RNA (siRNA) constructs. Two siRNAs were able to reduce the amount of sortilin mRNA isolated from COS-7 cells (Figure 5A). The level of β-actin mRNA was similar in all samples (Figure 5B). Based on this result, we transfected siRNA 1 into COS-7 cells to examine its effect on the expression of the protein. Western blot analysis revealed that siRNA 1 reduced the amount of sortilin (Figure 5C). We also used siRNA 1, in conjunction with immunofluorescence, to examine its effect on the expression of sortilin and the sorting of prosaposin and G_{M2}AP to lysosomes. Compared with control cells (Figure 5D and E insets), the prosaposin and G_{M2}AP staining was greatly reduced and showed no punctate staining. The Golgi apparatus was stained with anti-Golgin antibody. To determine the fate of activator proteins after sortilin inhibition, COS-7 were targeted with the siRNA 1 construct, pulsed with [^{35}S]methionine/cysteine and chased with Dulbecco's modified Eagle's medium (DMEM) containing 5 mM cold methionine/cysteine. Cell lysates (not shown) and media were immunoprecipitated 30, 60 and 120 min after the initial labeling with anti-prosaposin antibody, resolved by SDS–PAGE and visualized by autoradiography. The results showed that ablation of sortilin increases the secretion of prosaposin into the culture media (Figure 5G).

Co-immunoprecipitation shows association of sortilin and SAPs

Full-length sortilin, truncated sortilin, prosaposin and G_{M2}AP were *in vitro* translated and used in a co-immunoprecipitation (Co-IP) assay. The binding of sortilin and truncated sortilin to prosaposin or G_{M2}AP was tested using an anti-prosaposin or anti-G_{M2}AP antibody and protein G-conjugated Sepharose beads. Prosaposin was able to pull-down full-length (Figure 6A, lane 1) and truncated sortilin (95 kDa band) (Figure 6A, lane 2). This demonstrated that the luminal domain of sortilin binds prosaposin, and hence substantiated the functional studies in COS-7 cells transfected with the dominant-negative form of sortilin. Similarly, G_{M2}AP was
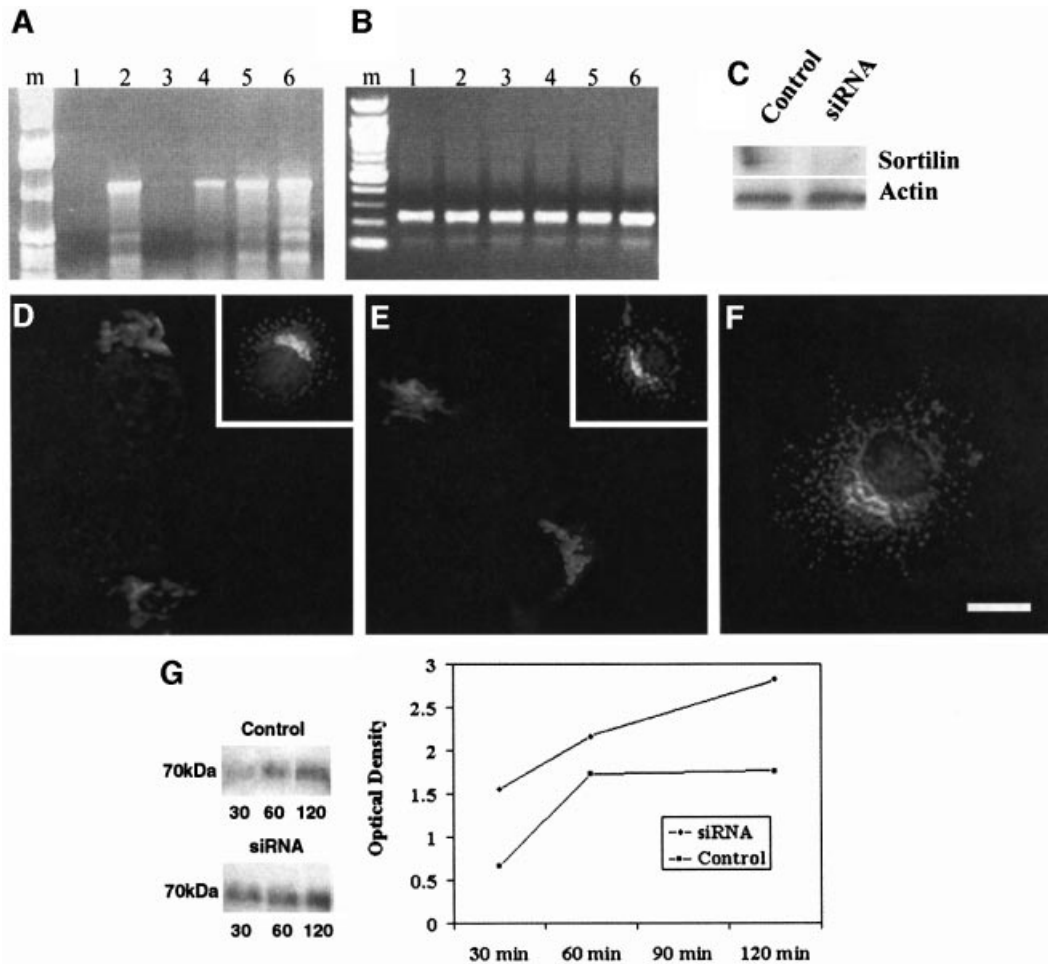
Fig. 5. Use of siRNAs to knock-down sortilin. (**A**) Six siRNAs were tested for their ability to knock-down sortilin mRNA in COS-7 cells. siRNA 1 and 3 (lanes 1 and 3) were able to reduce sortilin mRNA, while the others could not (lanes 2, 4, 5 and 6). (**B**) β-Actin is present in all samples, showing that the drop in sortilin mRNA is specific and not dependent on a total decrease in mRNA. (**C**) Western blot of cell lysate from COS-7 cells transfected or not with siRNA 1. (**D**) COS-7 cells transfected with sortilin siRNA and stained for anti-Golgin and anti-prosaposin. An untransfected cell is shown in the inset. (**E**) COS-7 cells transfected with sortilin siRNA and stained for anti-Golgin and anti-G_{M2}AP. The inset shows a non-transfected cell. (**F**) A COS-7 cell transfected with sortilin siRNA and stained for anti-Golgin and anti-cathepsin B. Scale bar =10 μm. (**G**) A representative pulse–chase analysis of COS-7 cells transfected with the sortilin siRNA 1 (bottom) shows that inactivation of sortilin mRNA increases the secretion of prosaposin into the culture medium. The top panel shows basal secretion of prosaposin in control cells. The quantitative values in optical density support this observation. Scale bar = 10 μm.

able to pull-down full-length (Figure 6A, lane 3) and truncated sortilin (Figure 6A, lane 4), demonstrating that sortilin interacts with both SAPs. On the other hand, cathepsin B did not pull-down either truncated or full-length sortilin (Figure 6A, lanes 5 and 6). *In vivo* Co-IP confirmed the *in vitro* data as prosaposin and G_{M2}AP were able to pull-down sortilin from COS-7 cell lysates (Figure 6B, lanes 1 and 2). On the other hand, cathepsin B could not pull-down sortilin (Figure 6B, lane 3).

Dominant-negative GGA abolishes the transport of SAPs

To substantiate our experimental evidence showing that prosaposin and G_{M2}AP use sortilin to traffic to the lysosomes, COS-7 cells transfected with the dominant-negative GGA3 construct were immunostained with anti-prosaposin (Figure 7A), anti-G_{M2}AP (Figure 7B), anti-cathepsin B (Figure 7C), anti-LAMP-2 antibodies (Figure 7D) or anti-myc (sortilin) and stained with a

secondary antibody conjugated to Alexa 568. The GGA3 construct lacking the 'hinge and ear' domains was linked to the green fluorescent protein (GFP). Thus, transfected cells were recognized from non-transfected cells by their green fluorescence. While transfected cells did not exhibit immunostaining for anti-cathepsin B, anti-prosaposin or anti-G_{M2}AP antibodies, the anti-LAMP-2 antibody produced a strong granular and perinuclear immunostaining (Figure 7A–D). Non-transfected COS-7 cells were immunostained by the four antibodies and showed punctate lysosomal staining. Sortilin staining was also abolished from punctate structures and remained in the perinuclear region of cells transfected with GFP–GGA3 (Figure 7E).

Discussion

Sortilin belongs to a growing family of multiligand type-1 receptors with homology to the yeast receptor Vps10p
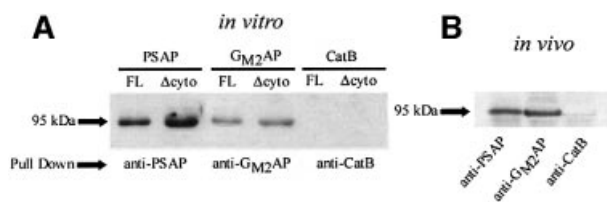
A *in vitro* **B** *in vivo*

Fig. 6. Co-immunoprecipitation showing the association of sortilin with SAPs. (**A**) *In vitro* translated full-length and truncated sortilin were incubated with SAPs or cathepsin B. Lanes 1 and 2 demonstrate that prosaposin pulls-down both full-length and truncated sortilin. Lanes 3 and 4 show that $G_{M2}AP$ pulls-down full-length and truncated sortilin, while lanes 5 and 6 demonstrate that cathepsin B is unable to bind sortilin. (**B**) *In vivo* co-immunoprecipitation using COS-7 cell lysates. Lanes 1 and 2 demonstrate the ability of prosaposin and $G_{M2}AP$ to pull-down sortilin, while cathepsin B was not able to pull-down sortilin (lane 3).

(Nielsen *et al.*, 2001). Based on structural features and experimental evidence, sortilin was proposed to traffic lysosomal proteins from the TGN to the lysosomes (Nielsen *et al.*, 2001). Chimeric sortilin containing the dileucine motifs linked to the luminal domain of the M6P-R restored lysosomal trafficking of soluble hydrolases in M6P-R-deficient cells (Nielsen *et al.*, 2001). However, no lysosomal proteins are known to interact with sortilin. In this investigation, we tested the hypothesis that sortilin is involved in the sorting of SAPs (prosaposin and $G_{M2}AP$) and that the lysosomal trafficking of these proteins requires monomeric adaptor protein GGA.

The lysosomal targeting mechanism for sphingolipid activator proteins (prosaposin and $G_{M2}AP$) has not been identified conclusively. Earlier reports from several laboratories have demonstrated that both SAPs can reach the lysosomes in an M6P-R-independent manner (Rijnboutt *et al.*, 1991; Rigat *et al.*, 1997). Immunocytochemical analysis of tunicamycin-treated cells demonstrated that non-glycosylated prosaposin can be targeted more efficiently to lysosomes, and biochemical analysis revealed that prosaposin is associated with Golgi membrane fractions and that this association is not disrupted by free mannose 6-phosphate (Igdoura *et al.*, 1996). A carbohydrate-independent pathway of native $G_{M2}AP$ has been characterized recently (Rigat *et al.*, 1997). Taken together, all these reports indicate the existence of an alternative mechanism of sorting and transport of lysosomal proteins. Although it has been suggested that the alternative mechanism of transport of these lysosomal proteins is that of secretion and internalization by other receptors (Hiesberger *et al.*, 1998), our data do not support this model as a general mechanism. First, we only find the 65 kDa form of prosaposin in the lysosomal compartment as opposed to the 70 kDa form. If the mechanism of transport to the lysosome consisted primarily of the secreted form, the 70 kDa form should be more abundant, not the 65 kDa form. Also, unpublished data from our laboratory of cells that had a non-functional LRP receptor showed that they contained prosaposin in their lysosomes. These results indicate the existence of a novel intracellular route.

Three approaches were used to test our first hypothesis (i.e. that sortilin is involved in the sorting of SAPs): a dominant-negative competition experiment; siRNA; and

Co-IP assays. The dominant-negative competition experiment was performed using a truncated sortilin over-expressed in COS-7 cells. The truncated sortilin lacked the cytoplasmic domain necessary to bind GGAs and exit from the Golgi apparatus (Nielsen *et al.*, 2001). This protein could, however, compete and bind its specific ligands. Overexpression of truncated sortilin abolished the punctate cytoplasmic immunostaining of anti-prosaposin and anti-$G_{M2}AP$ antibody but not the Golgi-like peri-nuclear staining. This result indicates that truncated sortilin competed for and probably retained a fraction of prosaposin and $G_{M2}AP$ in the Golgi apparatus. However, pulse–chase analysis demonstrated that the bulk of prosaposin is secreted to the culture medium (data not shown). This result suggests that after saturation of sortilin receptors, prosaposin enters a default secretory pathway. As expected, overexpression of truncated sortilin had no effect on cathepsin B immunostaining since this hydrolase is trafficked to the lysosomal compartment via the M6P-R (Mort and Buttle, 1997). To confirm further the require-ment for sortilin in the sorting of prosaposin, RNAi technology was used to knock-down sortilin mRNA in COS-7 cells. Sortilin-deficient cells were not able to route prosaposin to the lysosomal compartment. Interestingly, pulse–chase analysis showed that ablation of sortilin increased the secretion of the mature form of prosaposin into the culture medium, demonstrating the requirement for sortilin in the trafficking of prosaposin to the lysosome.

To confirm whether prosaposin or $G_{M2}AP$ were inter-active partners of sortilin, an *in vitro* Co-IP assay was conducted. Truncated and wild type-sortilin as well as prosaposin, $G_{M2}AP$ or cathepsin B were translated and labeled in a reticulocyte lysate system. Each lysosomal protein was incubated with full-length or truncated sortilin, and immunoprecipitated with anti-prosaposin, anti-$G_{M2}AP$ or anti-cathepsin B antibody. The immuno-precipitated complex was pulled-down with protein G-coated Sepharose beads, electrophoresed, transferred to nitrocellulose paper and visualized with a non-radioactive detection assay. The results demonstrated that both full-length and truncated sortilin co-immunoprecipitated with both prosaposin and $G_{M2}AP$ but not with cathepsin B. Finally, *in vivo* Co-IP demonstrated that unlike cathepsin B, prosaposin and $G_{M2}AP$ were able to pull-down sortilin.

To examine our second hypothesis (i.e. that the lysosomal trafficking of SAPs requires monomeric adaptor protein GGA), we tested the effect of mutant adaptor proteins on the transport of prosaposin and $G_{M2}AP$. Two classes of adaptor proteins have been implicated in the transport of lysosomal proteins from the Golgi apparatus to the lysosomes, namely the GGAs and AP-3. GGAs have been shown to bind sortilin (Nielsen *et al.*, 2001). To investigate the involvement of GGAs in the transport of prosaposin from the Golgi to the lysosomes, a truncated form of GGA lacking the hinge and ear domains was transfected and overexpressed in COS-7 cells. Overexpression of this mutant created a dominant-nega-tive competition with the endogenous GGA. Since the transfected construct lacked the clathrin-binding subunits, all lysosomal proteins whose transport is dependent on sorting receptors that interact with GGAs were misrouted away from lysosomes. Our results demonstrated that prosaposin and $G_{M2}AP$ immunostaining were absent in the
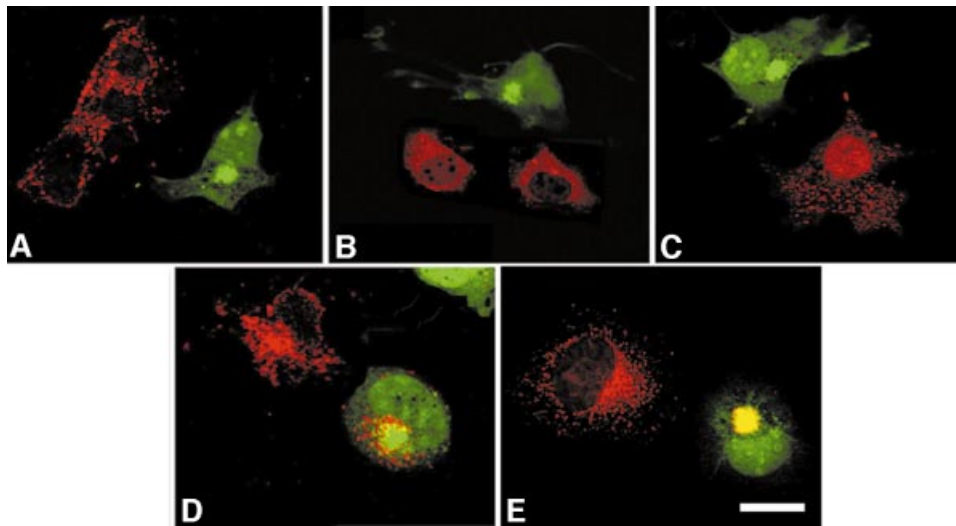
Fig. 7. Effect of a dominant-negative GGA3–GFP construct in COS-7 cells immunostained with anti-prosaposin (**A**), anti-$G_{M2}AP$ (**B**), anti-cathepsin B (**C**), anti-LAMP-2 antibody (**D**) or anti-sortilin (**E**). GGA3–GFP-transfected cells are recognized by their green fluorescence. While anti-prosaposin, anti-$G_{M2}AP$ and anti-cathepsin B antibodies yielded an intense punctate staining, transfected cells (green fluorescence) are not stained. LAMP-2 staining is not affected, while that of sortilin is reduced to the perinuclear area. Scale bar = 15 μm.

lysosomes of transfected cells, indicating that their transport is dependent on the presence of GGAs. The absence of immunostaining in the lysosomes with anti-cathepsin B antibody was expected because GGAs are required to traffic M6P-R-bound proteins including cathepsin B (positive control).

In a recent study, the selective deletion of prosaposin functional domains demonstrated that the C-terminus was required for its targeting to lysosomes. When the D domain and the C-terminus of prosaposin were added to albumin, this chimeric construct was rerouted to lysosomes (Zhao and Morales, 2000). Based on these results, it was hypothesized that prosaposin interacts with membrane lipids via its D domain and with a sorting receptor, other than the M6P-R, via its C-terminus (Lefrancois *et al*., 2002).

The C-terminus of prosaposin contains a saposin-like motif that forms a short α-helix stabilized by two conserved disulfide bonds (Zhao *et al*., 1998). This region is significantly similar (66%) to the N-terminus of surfactant-B protein (SP-B) (Zaltash and Johansson, 1998). SP-B requires the presence of the N-terminal region for its transient routing to multivesicular and lamellar bodies in type II neumocytes (Lin *et al*., 1996; Stahlman *et al*., 2000).

Members of the superfamily of aspartic proteinases also contain a common saposin-like domain (Kervinen *et al*., 1999). Other proteins containing saposin-like domains are acyloxyacyl hydrolase and acid sphingomyelinase (Muniz and Riezman, 2000). Evidence from several laboratories suggests that saposin-like motifs in aspartic proteinases promote membrane–lipid binding and vacuolar targeting (Ponting and Russell, 1995).

To our surprise, the overexpression of truncated sortilin abolished the transport of the $G_{M2}AP$ to the lysosomes. Although the $G_{M2}AP$ does not contain a saposin-like motif, a striking feature of its structure is the presence of a short α-helix stabilized by a disulfide bridge between C112 and C138. Patients with a C138 substitution are associated with the AB variant form of G_{M2} gangliosidosis

characterized by the absence of $G_{M2}AP$ in lysosomes. Taken together, all these results indicate the existence of an alternative mechanism of sorting and transport of lysosomal proteins in addition to the M6P-R pathway (Lefrancois *et al*., 2002).

In conclusion, our data disclosed a novel mechanism of lysosomal sorting and trafficking mediated by sortilin. Although sortilin is involved in the sorting and trafficking of both prosaposin and $G_{M2}AP$, this receptor may be responsible for the targeting of other soluble lysosomal proteins.

Materials and methods

Cell culture and transfection

COS-7 cells were cultured in DMEM (Sigma, St Louis, MO) supplemented with 10% fetal bovine serum (FBS) and antibiotics. The COS-7 cells were harvested then plated on coverslips (5×10^5/10 ml) in NuSerum supplemented medium overnight in preparation for a transient transfection. The cells were transfected using a DEAE–dextran/ chloroquine phosphate protocol (Zhao and Morales, 2000) and then fed with DMEM supplemented with 10% FBS and antibiotics overnight.

Immunofluorescent staining

The cells were washed three times in phosphate-buffered saline (PBS) and, in the case of some cells, also incubated in 60 nM LysoTracker (Molecular Probes Inc., Eugene, OR) for 30 min then fixed with 3.8% paraformaldehyde (Sigma) for 30 min at room temperature followed by two washes in PBS. The cells were treated with 0.5% Triton X-100 (Boehringer Mannheim, Indianapolis, IN) for 30 min at room temperature and then blocked with 100 μl of 10% goat serum for 1 h, followed by 100 μl of primary antibody diluted 1:200 in PBS overnight at 4°C. The cells were then washed in 0.05% Tween-20 (Sigma) three times for 5 min each. The appropriate Alexa-conjugated secondary antibody (Molecular Probes) was diluted to 1:200 and 100 μl of the antibody solution was placed on each coverslip for 1 h at room temperature. The cells were then washed with 0.05% Tween-20 three times for 5 min each, followed by a rinse with distilled water. The coverslips were mounted face down on microscope slides with VectaShield (Vector Labs, Burlingame, CA) and sealed with nail polish to be viewed on a Zeiss 410 confocal microscope (Carl Zeiss, Germany). The slides were stored in a light-proof black box.

Plasmids and antibodies

The mutant GGA3–GFP construct is from Dr Juan Bonifacino's lab (CBMB, NIH, Bethesda, MD). The full-length sortilin construct was from

Dr Claus Petersen (University of Aarhus, Denmark). The truncated form of sortilin was generated in our lab. It included the luminal and transmembrane regions without the cytosolic portion subcloned into the pcDNA3.1B expression vector. The anti-cathepsin B antibody was a generous gift from Dr John S.Mort (Shriner's Hospital, Montreal, Quebec). The anti-G$_{M2}$AP antibody and plasmid were a generous gift from Dr Don Mahuran (Hospital for Sick Children, Toronto, Ontario). The anti-prosaposin antibody was generated in our laboratory against the N-terminus and the functional domains A–B of prosaposin. The characterization and specificity of the prosaposin antibody were discussed in a previous report (Zhao and Morales, 2000). The anti-myc antibody was purchased from Invitrogen (Mississauga, Ontario). The anti-sortilin antibody was purchased from BD Biosciences (Toronto, Ontario).

RNAi

A silencer siRNA construction kit was purchased from Ambion (Austin, TX) and used to produce six siRNAs to human sortilin with 3′-overhanging uridine dimers. The siRNAs were chosen to correspond to sequences located in the 5′, 3′ or medial regions of sortilin mRNA. The siRNAs were transfected into COS-7 cells using the DEAE–dextran/chloroquine phosphate protocol. Total RNA isolation and analysis was performed using Qiagen's Rneasy kit (Mississauga, Ontario) after a 72 h incubation with the siRNA. The mRNA levels were quantified by spectrophotometry. The expression level of sortilin RNA in both target and control was determined by RT–PCR using primers designed in our lab. First-strand cDNA was synthesized from total RNA with the Omniscript Reverse Transcriptase Kit (Qiagen) using oligo(dT) primer. Then, 2 µg of total RNA sample was added in a 20 µl reaction. For PCR, 2 µl of cDNA was used as a template in a volume of 25 µl of reaction mixture. The PCR mixture contained cDNA, 2.5 µl of 10× PCR buffer (20 mM MgCl$_2$), 1 µl of a dNTP mixture, 0.5 U of *Taq* (Qiagen) and 1 µl of each primer. Amplification was performed under the following conditions: 30 cycles at 94°C during 1 min to denature, 55°C during 1 min to anneal, 72°C for 2 min for extension, and a final extension for another 10 min at 72°C. The PCR products were separated by electrophoresis in a 1% (w/v) agarose gel, and 0.4% ethidium bromide was added during the gel preparation.

Pulse–chase labeling

COS-7 cells were plated to 50% confluency in 100 mm dishes 1 day before transfection and maintained in DMEM containing 10% FBS. After 24 h transfection with a sortilin siRNA, the cells were washed with PBS twice and incubated in methionine/cysteine-free DMEM containing 5% dialyzed FBS for 40 min at 37°C. Then, the cells were pulse-labeled with 0.8 mCi of [^{35}S]methionine/cysteine (Promix AGQ0080, Amersham, Boston, MA) in methionine/cysteine-free DMEM for 15 min at 37°C. Subsequently, the cells were washed with warm PBS twice and chased in 3 ml of chase medium (DMEM containing 5 mM cold methionine/cysteine) for 0, 30, 60 and 120 min. Untransfected COS-7 cells were used as control. Finally, the cells were harvested, lysed and spun down to remove cellular debris. The supernatant was collected and pre-cleared with 50 µl of protein A–beads during 1 h (50% slurry). Then the supernatant was incubated with anti-prosaposin antibody at 4°C overnight followed by a 1 h incubation in 50 µl of a 50% slurry of protein A–beads at 4°C. After spinning down at 10 000 r.p.m. for 1 min, the supernatant was discarded and the pellet washed with IP buffer (150 mM NaCl, 1.0% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris pH 8.0) three times, and then with 50 mM Tris pH 8.0. The immunoprecipitates were resolved by SDS–PAGE and visualized by autoradiography.

Co-immunoprecipitation assays

Proteins were *in vitro* translated using Promega's TnT Coupled Reticulocyte Lysate System (Promega, Madison, WI). Briefly, prosaposin, G$_{M2}$AP, sortilin and truncated sortilin plasmids were incubated with the rabbit reticulocyte lysate from the kit. Proteins were labeled using the Transcend Non-Radioactive Translation Detection System (Promega) for visualization. The proteins were then incubated together with the appropriate antibody. The complex was pulled-down using protein A-coated Sepharose beads and washed using MatchMaker Co-IP Kit solutions (Clontech, Palo Alto, CA). The proteins were then suspended in reducing sample buffer and boiled at 100°C for 5 min. The samples were subsequently run on a 12.5% acrylamide gel, transferred to nitrocellulose paper, and visualized with the Transcend Non-Radioactive Translation Detection System (Promega). Conversely, cell lysates were prepared from COS-7 cells using TNE (50 mM Tris, 2 mM EDTA, 150m M NaCl, 1% Triton X-100). After incubating the cells for 10 min in the above buffer on ice, the cell debris was pelleted at 14 000 r.p.m. for 30 min. The

cell lysates were immunoprecipitated with prosaposin, G$_{M2}$AP or cathepsin B antibody for 2 h. A 50 µl aliquot of protein G-coated Sepharose beads was added and incubated for 2 h, and spun down at 8000 r.p.m. for 5 min. The immunoprecipitated fractions were run on a 12% polyacrylamide gel and transferred to nitrocellulose paper. The blot was then probed for sortilin using an anti-sortilin antibody.

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