Golgi-to-phagosome transport of acid sphingomyelinasmand prosaposin is mediated by sortilin

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Summary
Sortilin, also known as neurotensin receptor 3 (NTR3), is a transmembrane protein with a dual function. It acts as a receptor for neuromediators and growth factors at the plasma membrane, but it has also been implicated in binding and transport of some lysosomal proteins. However, the role of sortilin during phagosome maturation has not been investigated before. Here, we show that in macrophages, sortilin is mainly localized in the Golgi and transported to latex-bead phagosomes (LBPs). Using live-cell imaging and electron microscopy, we found that sortilin is delivered to LBPs in a manner that depends on its cytoplasmic tail. We also show that sortilin participates in the direct delivery of acid sphingomyelinasne (ASM) and prosaposin (PS) to the phagosome, bypassing fusion with lysosomal compartments. Further analysis confirmed that ASM and PS are targeted to the phagosome by sortilin in a Brefeldin-A-sensitive pathway. Analysis of primary macrophages isolated from Sort1−/− mice indicated that the delivery of ASM and PS, but not pro-cathepsin D, to LBPs was severely impaired. We propose a pathway mediated by sortilin by which selected lysosomal proteins are transported to the phagosome along a Golgi-dependent route during the maturation of phagosomes.

Key words: Golgi, Phagosome, Sortilin

Introduction
Sortilin is a 95 kDa glycoprotein transmembrane receptor that binds neurotensin (NT) with high affinity. In contrast to neurotensin receptors NTR1 and NTR2, sortilin has only a single transmembrane domain and is not a G-protein-coupled receptor (Mazella, 2001; Mazella et al., 1998). Sortilin was originally identified by its ability to bind associated protein (RAP), a member of the low-density lipoprotein (LDL) receptor family (Petersen et al., 1997). Whereas NTR1 and NTR2 are predominantly localized to the cell surface, sortilin is predominantly intracellular and only a small proportion (≈10%) is associated with the plasma membrane (Morinville et al., 2004).

Sortilin belongs to the family of mammalian Vps10p domain (Vps10p-D) receptors and it contains two copies of a similar domain in the luminal part. The Vps10 domain is able to bind and transport carboxypeptidase Y (CPY) from the trans-Golgi network (TGN) to the lysosomal vacuole in yeast (Marcusson et al., 1994). In spite of recent advances in the last few years, the functional and intracellular properties of sortilin are far from being fully elucidated (Westergaard et al., 2004). Based on its properties as a surface receptor for neurotensins and nerve growth factor (NGF), neuronal functions for sortilin have been shown (Jansen et al., 2007; Nykjaer et al., 2004; Willnow et al., 2008). It has been proposed that sortilin mediates the intracellular sorting of some selected lysosomal enzymes, in agreement with the evidence that the major pool of sortilin is localized in the Golgi complex (Morinville et al., 2004). The short cytoplasmic tail of sortilin is similar to the cytoplasmic segment of the cation-independent mannose-6-phosphate receptor (CI-MPR). This cytoplasmic tail of sortilin binds to the Golgi-localized gamma-adaptin ear-containing ADP-ribozylation factor-binding proteins (GGAs) (Nielsen et al., 2001), an interaction that is important for the trafficking of sortilin from the Golgi to the late endosomal or lysosomal compartment (Ni and Morales, 2006; Nielsen et al., 2001).

It has been shown that sortilin provides an alternative route to mannose-6-phosphate receptors (MPRs) for directing soluble lysosomal proteins from the TGN to the lysosomes. These proteins include acid sphingomyelinasne (ASM), the lipid-binding proteins prosaposin (PS) and GM2-activator protein (GM2AP) (Lefrancois et al., 2003; Ni and Morales, 2006). The emerging hypothesis is that one set of lysosomal proteins is targeted by the two MPRs towards the lysosomes whereas a separate pool of lysosomal proteins is targeted via sortilin; however, this hypothesis remains to be formally proven.

During phagosome maturation, the phagosome sequentially acquires lysosomal enzymes (Claus et al., 1998; Haas, 2007; Rogers...
This key process, together with acidification, allows the degradation of ingested pathogens and also antigen processing (Beron et al., 1995; Jutras and Desjardins, 2005; Vieira et al., 2002). There is some evidence that the acquisition of specific pools of lysosomal proteins, including proteins that are transported by sortilin such as ASM and PS, is a regulated process that has an impact in crucial events during the development of the immune response (Utermohlen et al., 2008; Winau et al., 2004; Yuan et al., 2007). In fact, ASM and PS have been implicated in processes that regulate bacterial killing, lipid antigen loading and phagosome maturation (Kolter et al., 2005; Schramm et al., 2008).

It is known that not all the hydrolytic enzymes and phagosomal proteins are acquired simultaneously by phagosomes during their maturation, suggesting differential delivery from distinct organelles (Claus et al., 1998; Rogers and Foster, 2007). However, the mechanisms and pathways controlling these steps of delivery are poorly understood.

In a previous study, we identified sortilin as a protein that is upregulated in response to the infection of macrophages with mycobacteria. During the infection, mycobacteria are efficiently killed by the macrophages within phago-lysosomes (Gutierrez et al., 2008) suggesting a role for sortilin during phagosome maturation.

In the present study, we investigated the function of sortilin during the phagocytic pathway using the latex-bead model (Desjardins and Griffiths, 2003). We found that in macrophages, sortilin is associated with the Golgi, but not with late endosomes or lysosomes. Using live-cell imaging and electron microscopy, we found that sortilin is delivered to phagosomes in a manner that depends on its cytoplasmic tail. This route was independent of fusion of phagosomes with late endosomes or lysosomes and was sensitive to Brefeldin A (BFA), a drug that blocks exit out of the ER or Golgi. We also show that sortilin is important for the efficient delivery of ASM and PS to the phagosome. Finally, the delivery of ASM and PS to the phagosome was drastically reduced in macrophages from sortilin-knockout mice, whereas the delivery of pro-cathepsin D (pro-CtsD) remained unaffected. Altogether, the data presented here support the hypothesis that ASM and PS are delivered to the phagosome in a sortilin-dependent way.

Results
Endogenous sortilin is associated with the Golgi in RAW264.7 macrophages and delivered to latex-bead phagosomes

Previous observations provided evidence that sortilin can deliver some lysosomal proteins to lysosomes and late endosomes (Lefrancois et al., 2003; Ni and Morales, 2006). Since gradual acquisition of lysosomal enzymes is a characteristic feature of phagosome maturation, we studied the behavior of sortilin during this process. For that, we first internalized 3 μm IgG-coated latex beads for 1 hour in RAW264.7 macrophages and endogenous sortilin was detected by indirect immunofluorescence. We found that sortilin was strongly associated with latex-bead phagosomes (LBPs) (Fig. 1A-C). Similar results were obtained with 1 μm and 3 μm avidin-coated latex beads (data not shown). Quantitative analysis of the number of phagosomes that were positive for sortilin was performed.

Fig. 1. Sortilin is associated with LBPs.
(A) RAW264.7 macrophages were incubated with 3 μm IgG Alexa Fluor 546-coated latex beads for 1 hour and subsequently fixed and stained with anti-sortilin antibody followed by Alexa Fluor 488-coupled anti-rabbit IgG. Scale bar: 10 μm. (B) Profile of fluorescence intensity along the phagosome (indicated as a white bar in inset in A). (C) 3D-colocalization plot obtained from inset of A. (D) Quantitative analysis of the presence of endogenous sortilin on LBPs. Cells were processed as indicated above and for the indicated time, the colocalization of sortilin on phagosomes was analyzed. The total number of LBPs counted in each experiment was 100. The data indicate means ± s.e.m. of three independent experiments. (E) Western blot analysis of RAW246.7 cell lysates (RAWtotal) and purified LBPs with anti-sortilin antibody showing a specific band at ~90 kDa. Tubulin was detected as a loading control in the same membrane. (F) Macrophages fixed and processed for indirect immunofluorescence for GM130 or TGN38 and sortilin. (G) LAMP-2 was detected as indicated above and for lysotracker (lyso) staining; cells were incubated with lysotracker at 1:20,000 for 2 hours and subsequently washed, fixed and stained for sortilin. Scale bars: 15 μm.
at different time points revealed that the association of sortilin with LBPs occurred prominently by 15 minutes after uptake and decreased with time until it reached 29% at 24 hours (Fig. 1D). We detected sortilin at the expected molecular mass of 95 kDa by western blotting of the total cell lysates; supporting the notion that sortilin is expressed in this cell line (Fig. 1E). Sortilin was also present in purified LBPs, confirming our microscopic observations (Fig. 1E). Next, we studied the intracellular localization of sortilin in macrophages. In agreement with previous observations in other cell types, endogenous sortilin significantly colocalized with Golgi markers including GM130, TGN38 (Fig. 1F) and syntaxin-6 (Stx6, data not shown). Surprisingly, only a low level of colocalization was observed between sortilin and LAMP-2-positive compartments, which correspond to late endosomes and lysosomes. Sortilin was also excluded from acidic compartments as measured by lysotracker staining (Fig. 1G). Taken together, our data indicate that sortilin is acquired by phagosomes soon after their internalization and in macrophages, little colocalization of sortilin with late endocytic or lysosomal compartments was observed.

**Delivery of sortilin to LBPs depends on its cytoplasmic tail**

The signal for sortilin localization and exit from the Golgi is localized in the cytoplasmic tail of the protein (Nielsen et al., 2001), where a GGA-binding domain has been suggested to direct sortilin to the lysosomal compartment (Nielsen et al., 2001). We therefore decided to investigate the role of the cytoplasmic tail in the targeting of sortilin to the phagosome. For this, we tested the distribution of sortilin fused to GFP in the (cytoplasmic) C-terminus (sort-GFP) and a truncated form of sortilin (sortT-GFP) that lacks the cytoplasmic tail (residues 779-831). When sort-GFP was expressed in macrophages, the perinuclear signal was mainly associated with the Golgi complex (identified by GM130), but almost no colocalization with the late endosome or lysosome marker LAMP-2 was observed (supplementary material Fig. S1A). Importantly, sort-GFP expressed in macrophages colocalized precisely with the endogenous sortilin, as seen by fluorescence microscopy, thus ruling out any significant mis-targeting of the expressed protein (data not shown). As expected, when the truncated form was expressed, the protein was exclusively localized to the Golgi in the majority of the cells (supplementary material Fig. S1B). By contrast, in cells with high levels of expression, the truncated protein was also strongly associated with the plasma membrane (identified by CD14), in addition to the Golgi (supplementary material Fig. S1B). Based on these observations, we selected only cells with low levels of sortilin expression for all further analysis. As shown in Fig. 2A, sort-GFP was also targeted to phagosomes and the presence of expressed sortilin in phagosomes dramatically decreased in cells expressing the truncated form (61.1% vs 19.6%, respectively) (Fig. 2B,C). To confirm these observations at the ultrastructural level, we performed immunoelectron microscopy analysis using thawed cryo-sections. The truncated form labeled LBPs at a lower efficiency compared with full-length sortilin (Fig. 2D,E, quantified in F). Taken together, this set of data indicated that sort-GFP is also targeted to LBPs and that the trafficking of sort-GFP to the phagosome is dependent on its cytoplasmic domain.

**Sortilin is delivered to LBPs independently of late-endosomal-lysosomal fusion**

Although we observed low levels of localization of sortilin in LAMP-2- and lysotracker-positive compartments, it is possible that sortilin is acquired by phagosomes by fusion with late-endocytic organelles. To address this possibility, we internalized latex beads and labeled both endogenous sortilin and markers of endosomal-lysosomal fusion

![Image](https://example.com/image.png)

**Fig. 2. Role of the sortilin cytoplasmic tail in the association with LBPs.** Macrophages were transfected with full-length sort-GFP (A) and truncated sortT-GFP (B). Transfected macrophages were incubated for 1 hour with 3 μm IgG Alexa Fluor 546-coated latex beads. Cells were fixed and analyzed by confocal microscopy. Scale bars: 10 μm. (C) Quantitative analysis of the number of phagosomes positive for sort-GFP or sortT-GFP. The data indicate means ± s.e.m. of three independent experiments **P<0.001. (D) Macrophages expressing sort-GFP incubated with avidin-coated 3 μm latex beads for 1 hour (uptake) plus 1 hour chase and then processed for cryosectioning. Sortilin was detected using a rabbit anti-sortilin antibody followed by protein-A-gold (15 nm) labeling. (E) Macrophages expressing sortT-GFP processed as indicated above. Arrows indicate the presence of sortilin in the phagosomal membrane. (F) Quantitative analysis of the number of gold particles in phagosomes in cells expressing sort-GFP or sortT-GFP.
internalisation, approximately 40-60% of phagosomes were positive for endogenous sortilin, but clearly negative for bis(monoacylglycero)phosphate (BMP) commonly known as LBPA (the acronym used hereafter) and LAMP-2, markers of late endosomal or lysosomal compartments (Fig. 3A-D). In addition, sort-GFP was also clearly present in LBPs that were negative for LAMP-2 (supplementary material Fig. S2). A quantitative analysis clearly showed that a subpopulation of phagosomes positive for sortilin, but negative for LBPA and LAMP-2, was present during the first 2 hours of internalization (Fig. 3B,D). Altogether, these findings argue that sortilin is acquired by phagosomes independently of late-endosomal–lysosomal fusion.

Sortilin is delivered from the Golgi to phagosomes

Lysosomal enzymes are transported between the TGN and the late endosomal compartment by receptors such as MPRs and sortilin (Braulke and Bonifacino, 2009; Rouille et al., 2000). Although sortilin was present on the phagosomal membrane, we observed little localization of sortilin to late-endocytic and lysosomal compartments. This suggests that sortilin could be directly transported from the biosynthetic pathway to the phagosome, as suggested before (Fratti et al., 2002; Fratti et al., 2003; Ullrich et al., 1999). To further analyze this possibility, we performed live-cell imaging in macrophages expressing sort-GFP. We observed that small vesicles and tubules positive for sortilin were present in the Golgi region, and they were directly targeted to and fused with LBPs (Fig. 4A-D). These vesicles containing sort-GFP were positive for Stx6 and perhaps equivalent to the Stx6-positive vesicles targeted to the LBP, as described previously (Fratti et al., 2003) (supplementary material Fig. S1A). A quantitative analysis of the fluorescence associated with the phagosome confirmed that the association of sortilin with the LBP occurs early (Fig. 4B and supplementary material Movie 1). Additionally, tubules emerging from the Golgi were also observed interacting via direct contacts with LBPs (Fig. 4D and supplementary material Movie 2). As expected, live-cell imaging of macrophages expressing sortT-GFP revealed that the GFP signal remained associated with the Golgi without any obvious evidence of tubule formation (supplementary material Movie 3).
Sortilin delivery to LBPs is sensitive to Brefeldin A

To functionally extend the above observations, we carried out experiments using Brefeldin A (BFA). BFA targets ARF-exchange proteins, inhibits the exit of most cargo proteins from the Golgi and fuses the Golgi with the ER or intermediate compartment (Lippincott-Schwartz et al., 1991). After treatment with BFA, the distribution of sortilin changed from a Golgi to a Golgi-ER distribution (Fig. 4E) and endogenous sortilin was no longer associated with the phagosome (Fig. 4F). Next, macrophages were incubated with or without BFA for 30 minutes and cells were subsequently incubated for 1 hour with latex beads in the continued presence or absence of BFA. LBPs were purified and the associated proteins detected by western blotting. In agreement with the fluorescence data, acquisition of sortilin by LBPs was significantly reduced in BFA-treated macrophages, which indicates that sortilin acquisition by phagosomes depends on a functional Golgi (Fig. 4G). This reduction was also accompanied with a dramatic decrease of both ASM and PS in LBPs, but the levels of neither pro-CtsD nor LAMP-2 were different (Fig. 4G; quantified in Fig. 4H). These data provide strong evidence for a sorting pathway between the TGN and LBPs mediated by sortilin. Our observations also argue that this pathway is the main mechanism for delivering ASM and PS to the LBPs, but not for the delivery of membrane (LAMP-2) or luminal (pro-CtsD)
lysosomal proteins, which remain unaffected by treatment with BFA.

The cytoplasmic tail of sortilin is required for the delivery of ASM and PS to phagosomes

The experiments performed using BFA suggested that sortilin-dependent trafficking is the main pathway for delivery of ASM and PS from the TGN to the phagosome. We decided to further evaluate the functional role of sortilin in the delivery of ASM and PS to phagosomes. For this, we first tested whether both proteins are indeed present in LBPs. ASM and PS were both detected in phagosomes, as observed by fluorescence microscopy, western blot and electron microscopy (supplementary material Fig. S4A). The acquisition of these proteins was an early event during LBP maturation (data not shown). We then tested whether the cytoplasmic tail of sortilin has a role in the acquisition of ASM and PS by LBPs. Macrophages expressing GFP, sort-GFP or sortT-GFP were incubated with latex beads, fixed after 1 hour and PS and ASM were detected by immunofluorescence microscopy. As shown in Fig. 5, both PS and ASM were acquired by the majority of the phagosomes that were positive for sort-GFP. By contrast, when the truncated form of sortilin was expressed, the fraction of phagosomes containing ASM or PS was significantly reduced. In addition, we also observed a dramatic decrease in the intracellular levels of both PS and ASM, suggesting that the expression of the truncated form could induce the secretion of these proteins into the culture medium. Only an intracellular pool of both enzymes associated with the Golgi was observed. This is consistent with previous observations indicating that the expression of the truncated form acted as a dominant-negative mutant (Ni and Morales, 2006).

Delivery of ASM and PS to the phagosome is impaired in Sort1–/– primary macrophages

In the above experiments with expressed sortilin, the endogenous protein was still present and could influence the interpretation of the data. We therefore performed experiments in bone marrow macrophages (BMMs) isolated from wild type and sortilin-knockout (Sort1–/–) mice (Jansen et al., 2007). BMMs from wild-type mice showed the typical distribution of sortilin associated with the Golgi complex (supplementary material Fig. S4A). As expected, BMMs from Sort1–/– mice completely lacked sortilin, as determined by immunofluorescence and western blot analysis (supplementary material Fig. S4A). We then internalized IgG-coated 3 µm latex beads for 1 hour in wild-type and Sort1–/– BMMs and analyzed the association of sortilin, ASM and PS to LBPs by confocal microscopy. Immunofluorescence studies showed that sortilin was present in LBPs from wild-type BMMs but not from Sort1–/– BMMs (supplementary material Fig. S4B). The distribution of ASM and PS in Sort1–/– BMMs was not significantly different from that in wild-type BMMs (data not shown). However, the number of phagosomes that accumulated ASM (Fig. 6A) and PS (Fig. 6B) was significantly reduced in phagosomes from Sort1–/– BMMs, relative to LBPs isolated from wild-type macrophages. To extend these observations, the recruitment of sortilin, ASM and PS to purified phagosomes was determined by western blotting. As shown in Fig. 6C-D, ASM and PS were present in phagosomes from wild-type BMMs. However, the capacity of the early phagosomes to accumulate ASM and PS in Sort1–/– BMMs was severely impaired. Interestingly, levels of pro-CtsD were not affected, indicating that delivery of CtsD follows a sortilin-independent pathway to the phagosomes. From these observations, we conclude that sortilin constitutes the major pathway that conveys ASM and PS into phagosomes.

Discussion

There is evidence that sortilin is the only neurotensin receptor expressed in both human and mouse microglial macrophages (Martin et al., 2005; Martin et al., 2003). This is a strong argument that sortilin has an important role in this type of professional
We report here that sortilin is recruited to phagosomes and has an essential role in the acquisition of ASM and PS in macrophages. Moreover, there is compelling evidence showing that NTRs have an important function in the phagocytic and immune function of mouse macrophages (De la Fuente et al., 1993; Kim et al., 2006). Sortilin is able to signal from the plasma membrane to regulate the migration of microglial cells in response to neurotensins (Martin et al., 2005). Based on our data, we suggest that sortilin in macrophages could mirror the dual function played by CI-M6PR: one intracellular function delivers lysosomal proteins to the phagosome and the other at the plasma membrane regulates ligand-receptor signaling. Receptors of lysosomal enzymes are normally thought to be recycled back from endocytic compartments, where the majority of these proteins are located, once the cargo has been released (Bonifacino and Rojas, 2006). In the case of sortilin, the recycling back to the Golgi from endocytic compartments is mediated by sortin nexin 1 (SNX1) (Mari et al., 2008). In light of our results, it would be interesting to investigate the presence of SNX1 in phagosomes, because it has been recently shown that early phagosomes containing Salmonella recruit this protein (Bujny et al., 2008).

Although the content of PS and ASM was severely reduced in phagosomes isolated from Sort1<sup>−/−</sup> macrophages, we observed that low amounts of those proteins are still able to reach the phagosome. This suggests an alternative pathway for the trafficking of these proteins to the phagosome. Although lysosomal enzymes and CI-MPRs are found in early endosomes, they are only transient components of the early endosomes (Ludwig et al., 1991). Therefore, it is unlikely that the acquisition of the bulk of ASM and PS observed in phagosomes occurs by fusion of phagosomes with early endosomes.

Neither endogenous, nor expressed sortilin were found to be associated with late-endosomal or lysosomal compartments, as observed by staining for LAMP-2, lysotracker or LBPA. These observations confirm and extend previous data in HepG2 cells, where the CD63- and LAMP-1-positive compartments were devoid of sortilin (Mari et al., 2008). The delivery of sortilin and its cargo to the phagosome is inhibited by BFA, and sortilin is acquired

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**Fig. 6. Phagosomes from Sort1<sup>−/−</sup> macrophages contain significantly less ASM and PS.** (A,B) BMMs isolated from BALB/c WT and Sort1<sup>−/−</sup> mice exposed to IgG Alexa Fluor 546-coated 3 μm latex beads. After 1 hour, macrophages were immunostained for ASM (A) or PS (B). Insets show representative phagosomes with the corresponding fluorescence-intensity profile. Images are representative of at least three separate experiments of each type. Scale bar: 10 μm. (C) BMMs isolated from WT and Sort1<sup>−/−</sup> mice (ko) loaded with latex beads for 1 hour, homogenized and LBPs purified and processed for western blot (50 μg protein per lane) and developed with antibodies against sortilin, PS, ASM or CtsD. Tubulin was detected as a loading control. (D) Densitometry quantification of three representative western blots of sortilin, PS and ASM from wt and Sort1<sup>−/−</sup> (ko) phagosomes. The data indicate means ± s.e.m. of three independent experiments; **P<0.001 between phagosomes from wt and Sort1<sup>−/−</sup> macrophages for the indicated proteins.
within 15 minutes of phagosome formation, without any evidence for a direct fusion with late-endosomal or lysosomal compartments. Therefore, our results suggest that the route sortilin uses for delivery of a lysosomal protein subset to the phagosome could be related to the previously described direct Golgi (TGN)-to-phagosome pathway (Fratti et al., 2002; Fratti et al., 2003; Ulrich et al., 1999). One possibility is that several post-TGN vesicles that are distinct from the classical secretory pathway selectively target different cargo for fusion with the phagosomes. However, these putative vesicles need to be further identified and characterized. The cytoplasmic tail of sortilin contains a GGA-binding domain, which is likely to be required for delivery to the phagosome. There is also the possibility that other motifs present in this cytoplasmic tail could be required for the exit of sortilin – a possibility that needs further investigation.

Transient interactions of phagosomes containing Salmonella with the Golgi complex have been previously reported (Salcedo and Holden, 2003). Chlamydia inclusions also interact with vesicles derived from the Golgi, but not with the classical endosomal-lysosomal pathway (Hackbarth et al., 1996; Hackstadt et al., 1995). Interestingly, we observed sortilin-containing tubules emerging from the Golgi region and contacting the phagosome, in agreement with previous observations in Dictyostelium discoideum, which showed tubules labeled with a GFP-Golgi marker contacting phagosomes (Gersch et al., 2004). This suggests that the interaction between Golgi tubules and phagosomes represents a conserved, evolutionarily ancient mechanism.

Recently, it has been shown that maturation of phagosomes does not proceed as discrete steps of fusion, but is highly dynamic (Rogers and Foster, 2007) and includes both total fusion and ‘kiss-and-run’ processes of fusion (Desjardins et al., 1994). Our data support the hypothesis that different subsets of lysosomal enzymes are sorted with different kinetics into the phagosome by different receptors. We have earlier shown that at least four different late-endocytic compartments need to fuse with phagosomes to kill mycobacteria (Añes et al., 2006). Interestingly one of those compartments was negative for LAMP-1 and positive for vacuolar ATPase, which is consistent with the notion that late-endocytic organelles are functionally heterogeneous.

Although it has been reported that sortilin also mediates the lysosomal targeting of CtsD (Canaud et al., 2008), we observed normal CtsD trafficking to the phagosomes in macrophages from Sort1−/− mice. This could be due to differences in cell types, since in this report we used macrophages that could behave differently to COS-7 cells or fibroblasts. A key question is why in macrophages do ASM and PS, and perhaps additional proteins, need a separate, MPRs-independent mechanism? An alternative pathway to the phagosome mediated by sortilin could represent another level of regulation during the development of the immune response. For example, some lipid antigens are loaded into CD1c molecules that bypass the lysosomal compartment (Van Rijn et al., 2009).

Although both the conventional (MPR-sorted) pool of lysosomal enzymes and the sortilin pool are involved in anti-microbial defense, including bacterial lysis, lipid-antigen loading and phagosome maturation (Kolter et al., 2005; Schramm et al., 2008), there are significant differences between the two classes. PS and ASM belong to the family of saposin-like proteins (SAPLIPs) that have membrane-perturbing properties and three-dimensional structures similar to the pore-forming peptide of Entamoeba histolytica (Leippe et al., 2005). In addition, the presence of these enzymes in the vesicle changes the lipid composition of the vesicles that contain it, presumably altering the fate of the phagosome. Additional studies are required to investigate the impact of phagosomes with low levels of ASM and PS such as those seen in Sort1−/− mice on phagosome maturation, antigen presentation and bacterial killing.

Our study highlights the role of the sortilin pathway in the context of phagosomes containing intracellular pathogens. We have previously reported that sortilin is upregulated during the process of mycobacterial killing in macrophages in an NFκB-dependent manner, arguing that sortilin is part of the response to pathogens (Gutierrez et al., 2008). However the precise role(s) of this protein during mycobacterial killing is not known. It is tempting to hypothesize that sortilin delivers key proteins and enzymes needed for bacterial killing. In summary, our findings identify a novel and important contribution of sortilin to the delivery of a sub-class of lysosomal proteins to phagosomes. Based on our data, it would be interesting to investigate the role of this sortilin-mediated pathway during the killing of intra-phagosomal pathogens.

Materials and Methods

Reagents

The following antibodies were used: rabbit anti-ASM, rabbit anti-CtsD (Santa Cruz Biotech); rat anti-CD14, mouse anti-GM130 (BD Biosciences); anti-saposin-C as described before (Vielhaber et al., 1996); rat anti-LAMP-2 (HybriDoma Bank); mouse anti-TGN38 (Novus Biologicals); anti-LBPA was a gift from Jean Graevenstein, University of Geneva, Geneva, Switzerland. Two antibodies against sortilin were used: a rabbit polyclonal antibody anti-sortilin (kindly provided by Claus Petersen, University of Aarhus, Aarhus, Denmark) for immunostaining and a mouse anti-sortilin (BD Biosciences) for western blots. pEGFP-sortilin (sort-GFP) and pEGFP-sortilin-truncated (sortT-GFP) plasmids were kindly provided by Carlos Morales (McGill University, Montreal, Canada).

Cells

RAW 264.7 macrophages were maintained in Dulbecco’s modified Eagle medium (D-MEM, Invitrogen) supplemented with 4.5 g/l glucose, L-Glutamine (Invitrogen), penicillin-streptomycin (Invitrogen) and 10% heat-inactivated fetal calf serum (FCS, PAA) at 37°C in a 5% CO2 atmosphere. BMMs were obtained from 6-week-old Balb/c female wild-type mice from Jackson Laboratory and Sort1−/− mice (Jansen et al., 2007). Mice were killed by cervical dislocation, and the femur and tibia bones were removed. The bones were trimmed at both ends, and the marrow was flushed out with D-MEM containing 10% FCS. The cell suspension was centrifuged and the pellet was homogenized in D-MEM supplemented with 10% FCS, 20% L929 supernatant and 5% horse serum and plate into bacterial Petri dishes. After 2 days, the monolayer was washed with PBS and incubated for more 5 days. At this point more than 95% of the cells were positive for CD14 as tested by flow cytometry.

Macrophage transfection

Transfection of RAW 264.7 macrophages was performed using Lipofectamine 2000 (Invitrogen). Briefly, cells were washed twice with PBS and then incubated with the transfection mix for 6 hours at normal cell culture conditions. After incubation, 500 µl D-MEM culture medium containing serum was added. Cells were subsequently used for uptake of latex beads, immunofluorescence and live-cell imaging.

Coating of latex beads

400 µl latex beads (1 µm or 3 µm in diameter, Biosciences) supplied as a 2.5% aqueous solution were coupled to either 50 µg IgG-Alexa Fluor 488 (Invitrogen), 50 µg IgG-Alexa Fluor 546 (Invitrogen) or 500 µg Avidin (Invitrogen). Coupling was performed in presence of MES buffer, pH 6.7, containing EDAC crosslinker (Sigma) and incubation for 1 hour at room temperature on a rotating wheel. The reaction was stopped by washing the beads with 1% Triton X-100 (Sigma) in 10 mM Tris buffer (Sigma), pH 9.4. After washing in PBS, the beads were stored in 0.02% fish-skin gelatine (Sigma) and 0.02% NaN3 (Merck) in PBS at 4°C.

Internalization of latex beads

For internalization, the IgG-coated beads were diluted 1:1000 in complete medium, and applied to macrophages seeded in a 24-well plates with a final concentration of ~10 beads per cell. After the indicated times of uptake and chase, cells were washed with PBS and fixed for 15 minutes with 3% PFA in 10 mM HEPES.

Indirect immunofluorescence

Fixed cells on coverslips were quenched for 15 minutes in 50 mM glycine in PBS followed by a 30 minute incubation with 1% bovine serum albumin (BSA, Sigma) and 0.01% saponin (Sigma) in PBS. The primary and secondary antibodies were
diluted in PBS and incubated for 1 hour. Nuclear staining was performed using 4',6-
diamidino-2-phenylindole (DAPI, Invitrogen). After staining, the cells were mounted on slides using aqueous mounting medium (Dako Cytomation). Samples were analyzed by confocal fluorescence microscopy using a Zeiss LSM 510 FCS or Leica SP2 microscope.

Live-cell Imaging

For live-cell imaging, macrophages were seeded on 35 mm glass-bottom dishes (MatTek Corporation) in DMEM, 10% FCS. Transfections were performed as described in the dishes on the day before the imaging. Then, RAW 264.7 cells were washed with PBS and cells were subsequently maintained in CO2-independent imaging medium (DMEM without Phenol Red, 10% FCS, 25 mM HEPES, pH 7.4, 2 mM L-Glutamine). A solution of latex beads was prepared in imaging medium and added directly to the dishes. After 30 minutes of incubation, imaging was started with a Leica SP5 confocal microscope with AOBS and AOTF equipped with objective, argon laser (488 nm) and DPSS laser (561 nm) with scanner frequency of 630 mm/s. Images were acquired using a Leica III CCD Camera and AnalySIS 3.2 acquisition software.

Statistical analysis

Data are presented as means ± s.e.m. of at least three independent experiments; P-values (ANOVA, two ways) are relative to the control.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/4/2502/DC1

References


Sortilin in phagosome maturation


A. Sortilin-GFP

B. SortilinT-GFP

LOW expression

HIGH expression

GM130

LAMP-2

Sort-GFP

Stx6

Sort-GFP

CD14