SNX9 – a prelude to vesicle release

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Summary

The sorting nexin SNX9 has, in the past few years, been singled out as an important protein that participates in fundamental cellular activities. SNX9 binds strongly to dynamin and is partly responsible for the recruitment of this GTPase to sites of endocytosis. SNX9 also has a high capacity for modulation of the membrane and might therefore participate in the formation of the narrow neck of endocytic vesicles before scission occurs. Once assembled on the membrane, SNX9 stimulates the GTPase activity of dynamin to facilitate the scission reaction. It has also become clear that SNX9 has the ability to activate the actin regulator N-WASP in a membrane-dependent manner to coordinate actin polymerization with vesicle release. In this Commentary, we summarize several aspects of SNX9 structure and function in the context of membrane remodeling, discuss its interplay with various interaction partners and present a model of how SNX9 might work in endocytosis.

Key words: SNX9, Dynamin, N-WASP, Clathrin, Endocytosis, Membrane remodeling

Introduction

The interior of a eukaryotic cell contains a multitude of membrane structures that are constantly changing in shape and composition. The concept of a lipid-bilayer membrane design, which yields elegantly simple physical barriers, is employed by cells in a number of ways to facilitate the separation of chemical reactions into various compartments. For the creation, maintenance and dynamics of the shape of membrane-enclosed structures, an intricate interplay between proteins and constituent membrane lipids is required. Recent developments in membrane biophysics and the identification of proteins with membrane-modulating properties have begun to shed some light on the complex processes that are involved in intracellular membrane reshaping [for recent reviews on membrane remodeling, see the following articles (Campelo et al., 2008; McMahon and Gallop, 2005; Zimmerberg and Kozlov, 2006)]. One of the central membrane-active proteins is sorting nexin 9 (SNX9), which is involved in the progression of endocytosis.

Endocytosis is the collective designation of various processes that mediate the uptake of fluid, molecules and particles from the exterior of the cell. Common to these processes is the formation of a curved membrane, which yields a lipid-bilayer envelope around the engulfed matter. The geometry and composition of such envelopes are carefully controlled to allow for their proper transport to the cell interior. If the enveloped structures are sufficiently small (with a diameter of less than 200 nm or so), they are called vesicles, and these can be spherical or can have elongated tubular structures (Fig. 1). Once inside the cell, the vesicles meet and fuse with an elaborate system of membranous structures that are known as endosomes, where the endocytosed cargo is sorted and delivered to the appropriate destination.

One of the quantitatively dominant and best-studied schemes for the uptake of macromolecules is characterized by the presence of the coat protein clathrin, and is termed clathrin-mediated endocytosis (CME) (for reviews, see Benmerah and Lamaze, 2007; Edeling et al., 2006; Mousavi et al., 2004; Rappoport, 2008; Schmid and McMahon, 2007; Ungewickell and Hinrichsen, 2007). In CME, clathrin oligomerizes on the cytoplasmic side of the plasma membrane, which gradually becomes invaginated through the action of several proteins that give directionality to the process. A central molecule in this respect is adaptor protein 2 (AP-2), which binds to cargo proteins to be taken up and enriches them in clathrin-coated areas; at the same time, AP-2 recruits accessory proteins that are essential for the formation of the vesicular coat and the determination of its shape. At later stages, when the coated membrane has developed into a highly invaginated bud, only a narrow membrane neck remains that connects the vesicle to the plasma membrane. The separation of the vesicle from the originating membrane is accomplished by the action of the large GTPase dynamin, which – in a process that is still not fully understood – overcomes the energy barrier for bilayer fusion and membrane separation. Dynamin, however, is not alone in mediating this process, and several recent studies have indicated that SNX9 is an important partner protein in vesicle scission.

The complex interplay between the plasma membrane and the actin cytoskeleton requires numerous regulatory proteins and is indispensable in many cellular processes (Doherty and McMahon, 2008). Although the precise role of the actin cytoskeleton in CME in mammalian cells is not understood, it is evident that invagination of the plasma membrane has to influence and involve the actin cytoskeleton. It has been shown that actin polymerization occurs in a temporally regulated manner at cortical sites at which clathrin-coated pits are formed (Merrifield et al., 2002). The timing of actin bursts is indicative of a role in the inward movement and scission of vesicles. A driving force from actin polymerization that is coordinated with the molecular scission machinery might be necessary for endocytic release of vesicles [for reviews on the role of actin in endocytosis, see the following articles (Engqvist-Goldstein and Drubin, 2003; Perrais and Merrifield, 2005; Upadhyaya and van Oudenaarden, 2003)]. Several factors have the potential to function as links between endocytic-vesicle formation and activation of the actin-polymerizing system, and recently SNX9 has been added to this list of multi-functional proteins as one of the prime candidates for communication between dynamin-dependent membrane scission and actin dynamics.

In this Commentary, we discuss the structure of SNX9 and the functions of its constituent domains, and describe how SNX9 is thought to function in membrane remodeling during endocytosis. We describe the proposed interplay between SNX9 and dynamin

at the neck of endocytic vesicles, as well as the role of SNX9 in coupling vesicle formation and actin remodeling. Finally, we discuss potential mechanisms of SNX9 regulation and outline a model of its function in CME.

The SNX9-family proteins

SNX9 was first described in 1999 as a Src homology 3 (SH3)-domain and phox homology (PX)-domain protein that interacted with the metalloproteinases ADAM9 and ADAM15, and was proposed to regulate the turnover of these enzymes (Howard et al., 1999). The sorting nexins are a rather weakly related group of proteins that all contain the phosphoinositide-binding PX domain [for reviews on sorting nexins, see the following articles (Carlton et al., 2005; Cullen, 2008; Seet and Hong, 2006; Worby and Dixon, 2002)]. Outside this domain, the proteins are quite different and they clearly have different roles in the cell. In terms of function, it is more relevant to group together those sorting nexins that possess a Bin-amphiphysin-Rvs (BAR) domain in addition to a PX domain. This group of proteins includes SNX1 and SNX2, which target highly curved membranes and are required for endosomal trafficking by the retromer complex. The PX-BAR structural unit yields an effective device to modulate and stabilize membrane shape (see below). A few of the PX-BAR-containing sorting nexins also have an SH3 domain. This subgroup of sorting nexins, which we prefer to call 'the SNX9 family', comprises SNX9, SNX18 and SNX33 (previously annotated as SNX30) (Håberg et al., 2008).

The three proteins of the SNX9 family have the same domain organization, which consists of an SH3 domain at the N-terminus and a PX-BAR unit at the C-terminus (Fig. 2). The region that differs the most between the three proteins lies between the SH3 domain and the PX domain. This sequence, which is predicted to be mostly without ordered secondary structure, is termed the low-complexity domain (LC domain), and has been found to harbor several sequence motifs for interprotein interactions (Lundmark and Carlsson, 2003; Lundmark and Carlsson, 2004; Shin et al., 2008). SNX9, SNX18 and SNX33 localize differently in cells and appear to function in different trafficking pathways (Håberg et al., 2008). It is therefore believed that the LC domain confers functional specificity on the three proteins. Whereas SNX9 localizes to plasma-membrane endocytic sites, SNX18 is found on peripheral endosomal structures. The LC domain of SNX18 contains a binding motif for AP-1, which is another adaptor protein that is known to associate with clathrin. Our findings indicate, however, that SNX18 functions in an endosomal trafficking pathway that does not rely on clathrin, but that is dependent on AP-1 and the retrograde-trafficking protein PACS1 (Håberg et al., 2008). Very little is known about the cellular function of the third member, SNX33.

In Fig. 2 and its legend, we have summarized the identified protein partners for SNX9 according to the literature. Not all of these interactions have been confirmed by experiments in the biological context, and several of the indicated proteins should be considered as potential interacting partners. The SH3 domain of SNX9 (and also that of SNX18 and SNX33) binds most strongly to so-called class I polyproline sequences (Alto et al., 2007; Håberg et al., 2008), which are found in dynamin 1 and 2, the bacterial exotoxin EspF, and the actin regulators WASP and N-WASP, among others. From its 'interactome', it appears that SNX9 is centrally positioned at the interface between endocytosis and cytoskeletal organization. It is noteworthy that yeast and protozoa do not express any orthologs to the SNX9-family proteins. A single variant appears in invertebrates, and in vertebrates all three proteins are present. In
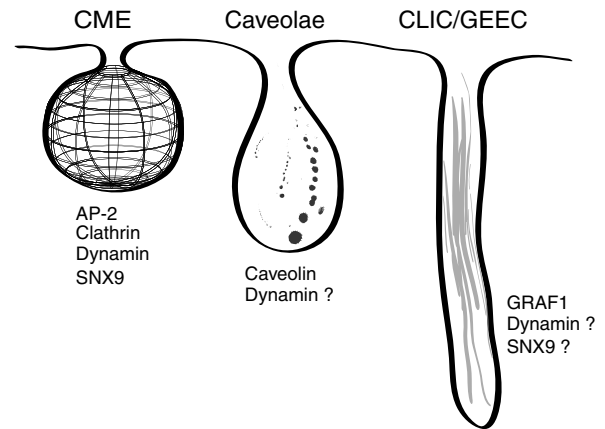

Fig. 1. Vesicular and tubular endocytic pathways in the cell. The main uptake pathways involve clathrin-mediated endocytosis (CME), caveolae and clathrin-independent carriers/GPI-enriched endocytic compartments (CLIC/GEEC). Proteins that are involved in carrier formation and/or release are indicated below each pathway. Question marks indicate uncertainties or yet-to-be-established findings. GRAF1 is a recently described BAR-domain protein that marks, and is indispensable for, the CLIC/GEEC pathway (Lundmark et al., 2008).

at least some mammalian cells, SNX9, SNX18 and SNX33 are co-expressed (Håberg et al., 2008), which hints that the proteins have acquired separate roles in evolution as the cells have become more complex.

SNX9 structure and the mechanism of membrane remodeling

A common approach to the dissection of different properties and functions in multi-domain proteins is to study the domains separately. In our laboratory, several attempts were made to produce the individual PX and BAR domains of SNX9; however, all such manipulations inevitably led to folding problems. The explanation for this phenomenon, which has also been noted by others (Yarar et al., 2008), came when we obtained the structure of the whole PX-BAR unit (Pylypenko et al., 2007). The two domains are in close contact with each other and, furthermore, are interconnected by an additional folding unit that we termed the 'yoke domain' (Fig. 3A). The yoke domain is formed from two separate regions that are located on either side of the PX domain in the primary structure, and unites the membrane-binding PX and BAR domains to form a single 'superdomain' for membrane sculpting. The contact area between the BAR domain and the yoke and PX domains is large, which ensures that the unit is held together once it is formed. The expression in bacteria or eukaryotic cells of SNX9 constructs in which either the PX or the BAR domain is deleted is expected to have global effects on SNX9 folding, and might lead to aggregation of the protein. Folding of the SH3 domain, however, seems not to be affected in the constructs, so the phenotypes that are observed in studies with such proteins are likely to be mediated by this domain and/or by the unstructured LC domain.

BAR domains form dimers [for reviews on BAR-domain proteins, see the following articles (Dawson et al., 2006; Gallop and McMahon, 2005; Habermann, 2004; Ren et al., 2006)]. The dimerized BAR domain in SNX9 creates a folding unit with a bent structure (Fig. 3A). The PX domains in the dimer align with the bent structure to give a protein with a rather shallow curvature for
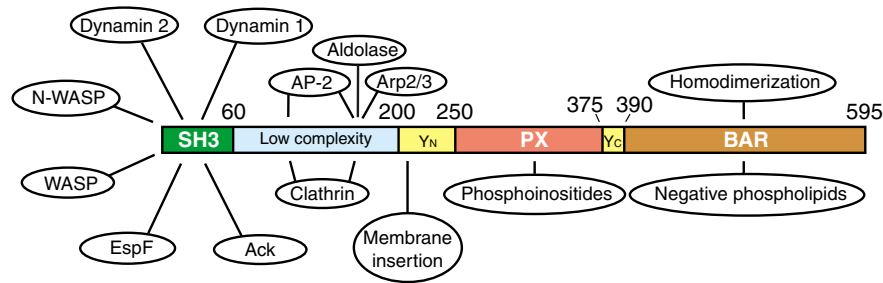
Fig. 2. Domain structure and interaction partners of SNX9. The domains are shown as colored rectangles, and numbers refer to the last amino acid in each domain in human SNX9. The sequences that form the yoke domain, which connects the PX and BAR domains in the tertiary structure, are from two regions that are denoted as Y_N and Y_C. The protein and phospholipid partners of SNX9 that are discussed in the text are shown. Further protein interaction partners that have been described in the literature, but are not shown in the figure, are as follows: ADAM9 and ADAM15 (Howard et al., 1999), Sos1 and Sos2 (Schulze and Mann, 2004), synaptojanin (Miele et al., 2004), phosphoinositide 3-kinase p85 (Badour et al., 2007) and PtdIns(4)P 5-kinase (Shin et al., 2008). *Drosophila* SNX9 has been reported to bind to WASP, AP-2, Dscam and Nck (Worby et al., 2001; Worby et al., 2002). Also indicated in the figure are two properties of the membrane-binding yoke-PX-BAR structural unit (membrane insertion and homodimerization).

membrane interaction. The BAR dimer is held together by extensive hydrophobic and polar interactions over an unusually large contact area (3191 Å^2) (Pylypenko et al., 2007), which probably precludes the existence of monomer-dimer equilibrium in the cell. Consequently, SNX9 is always isolated as a dimer (Lundmark and Carlsson, 2003; Lundmark and Carlsson, 2005; Yarar et al., 2008) or as multimeric aggregates at low ionic strength (Lundmark and Carlsson, 2002), and we have never been able to detect monomeric SNX9 in gel-filtration chromatography or in sedimentation assays. In line with this, analysis in the same cell extract of SNX9, SNX18 and SNX33, which show significant sequence homology in their BAR domains, revealed that they form separate homodimeric proteins; heterodimers between the paralogs were not detected (Håberg et al., 2008). It is therefore concluded that the homodimer is the smallest functional unit in SNX9-family proteins.

PX domains are known to be phosphoinositide-binding units [for reviews on PX domains and phosphoinositides, see the following articles (Di Paolo and De Camilli, 2006; Lemmon, 2008; Seet and Hong, 2006)]. Our early studies on the phosphoinositide specificity of the SNX9 PX domain indicated that it is rather promiscuous in its phosphoinositide binding compared with other PX domains (Lundmark and Carlsson, 2003), and this result was subsequently confirmed by others (Shin et al., 2008; Yarar et al., 2008). The structure of SNX9 PX-BAR gives a logical explanation for this lack of strict specificity, as it was found that the canonical phosphoinositide-binding pocket is wider than usual and can therefore be anticipated to accommodate various phosphoinositide species (Pylypenko et al., 2007). Under stringent assay conditions, however, a slight preference for phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2] is observed. This fits with the proposed role of SNX9 at PtdIns(4,5)P_2-enriched sites at the plasma membrane (Lundmark and Carlsson, 2003), but it is possible that SNX9 can also take part in processes that are linked to the production of other phosphoinositides (Badour et al., 2007). Unlike other PX-domain structures, the PX domain of SNX9 has a unique extra α-helix that is located at the edge of the yoke-PX-BAR unit (PX α-helix 4 in Fig. 3A). This α-helix, which is highly conserved among species and is also present in SNX18 and SNX33, was found to create a second basic crevice on the membrane-interacting surface of SNX9, into which negatively charged lipids could bind. Mutations of positive amino acids in this α-helix gave a striking decrease in membrane-binding affinity (Pylypenko et al., 2007). Interestingly,

mutations of amino acids in the canonical phosphoinositide-binding site abolished the binding to phosphatidylinositol 3-phosphate [PtdIns(3)P] but only weakened the binding to PtdIns(4,5)P_2, a result that opens up the possibility that the second site has specificity for PtdIns(4,5)P_2. Further structural studies are required to fully decipher the phosphoinositide-binding properties of SNX9.

In pure protein-liposome assays, the yoke-PX-BAR structural unit has a high capacity to modulate spherical liposomes into long, uniform lipid tubules with a diameter of 20 nm, as detected by negative stain in electron microscopy (Pylypenko et al., 2007). We found that a sequence just upstream of the determined structure was necessary for this activity (Pylypenko et al., 2007). This sequence, which is likely to be flexible and therefore invisible in the crystal structure, was suggested to form an amphipathic helix when contacting the membrane. Partial membrane insertion by this helix might be needed to displace membrane lipids for the generation of highly curved membrane. In Fig. 2, we have extended the yoke domain to include the sequence of the proposed amphipathic helix, as it clearly belongs to the functional membrane-sculpting unit.

The mechanism for membrane remodeling by SNX9 has not yet been fully elucidated. As discussed above, it is clear from mutagenesis studies that certain structural features in the yoke-PX-BAR unit are important for tubulation activity (Pylypenko et al., 2007). Tight binding to the membrane, which is mediated by large patches of positively charged amino acids on the concave surface (including two or more phosphoinositide-binding sites), probably triggers the insertion of the amphipathic helix and the formation of higher-order structures of the protein on the membrane surface. It has been demonstrated that membrane binding stimulates the formation of oligomers of SNX9 (Yarar et al., 2008). Interestingly, we have observed that membrane-induced SNX9 oligomers require an intact PX α-helix 4, which thereby would have a dual role in SNX9 (our unpublished results). We speculate that SNX9 oligomerizes on the membrane surface through lateral contacts, which are at least partially mediated by PX α-helix 4, to form spirals that will drive tube formation. A spiral arrangement would explain why 20-nm tubes can be stabilized by the concave surface of SNX9 despite the fact that its curvature is shallower (Pylypenko et al., 2007). The proposed mechanism is similar to the recently described arrangement of F-BAR domains required to propagate membrane tubulation (Frost et al., 2008).
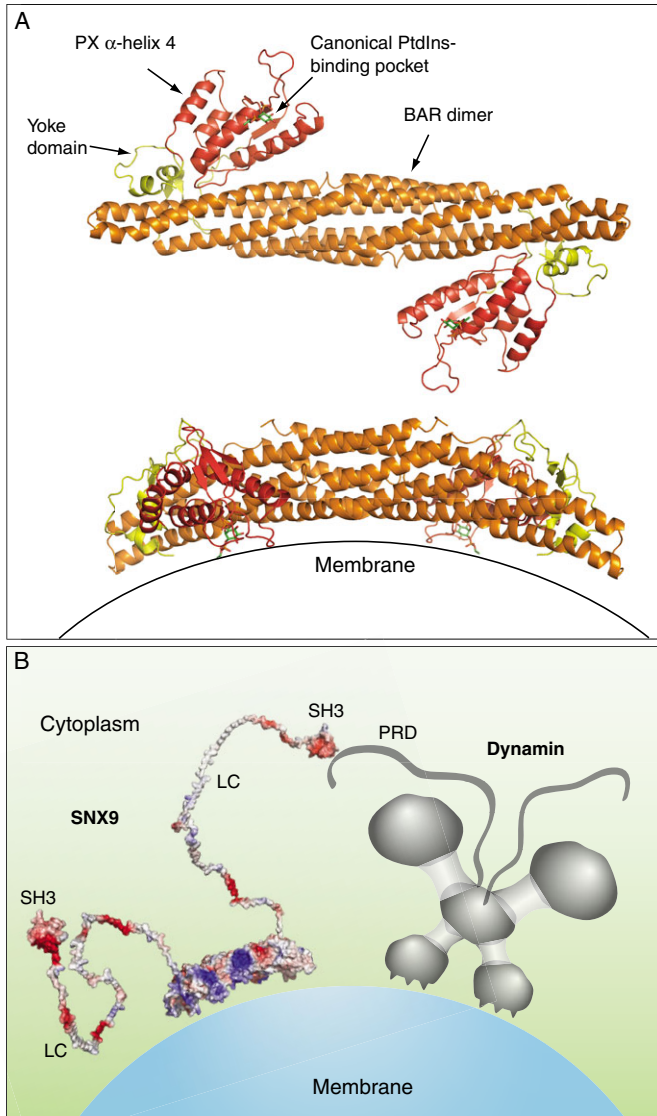
Fig. 3. Structure of SNX9. (A) Ribbon diagram of the crystal structure of the membrane-remodeling yoke-PX-BAR dimer [Protein Data Bank (PDB) entry 2RAK], color-coded as in Fig. 2. In the upper structure, the membrane-binding surface is towards the viewer; the lower structure is a side view. Phosphoinositides (green) are bound to the canonical phosphoinositide-binding pockets. (B) Size comparison of SNX9 and dynamin. To the left is the full-length dimer of SNX9 with the electrostatic surface potential shown in red (negative charge) and blue (positive charge). The SH3-domain structure is from the PDB entry 2ENM and the LC domain is randomly depicted to denote its flexibility. To the right is a schematic model of dimeric dynamin that is drawn roughly to scale with SNX9. The model is based on cryoelectron micrographs (Mears et al., 2007). The GTPase domains of dynamin are the large balls at the top of the structure and the membrane-binding PH domains are at the bottom. The proline-rich domains (PRDs) are depicted as unstructured ribbons that emanate from the central region, which comprises the middle domains and the GTPase effector domains. The figure shows that the SNX9 SH3 domain and the dynamin PRD can interact with each other above the bulk of either protein and that SNX9 can reach out above dynamin to enable additional interactions, such as with N-WASP, while it is assembled on the membrane surface.

Recently, the structure of the SNX9 PX-BAR domain was confirmed in an independent study (Wang et al., 2008) (the crystallized protein did not, however, include the complete yoke domain). An alternative mechanism of membrane tubulation was proposed from structural models based on small-angle X-ray-scattering data (Wang et al., 2008). According to these models, the dimerized BAR domains can adopt two different conformations that yield different domain curvatures that would drive membrane re-shaping. Such a phenomenon has not been previously found for any BAR-containing protein and, given the large contact area between the BAR-domains, it is not clear how a transition between the conformations can be accomplished.

The interplay between SNX9 and dynamin

Ubiquitously expressed dynamin 2 (Lundmark and Carlsson, 2003) and brain-specific dynamin 1 (Soulet et al., 2005) both bind effectively to SNX9, as detected by in vitro and in vivo assays, and dynamin is considered to be a major binding partner of the SNX9 SH3 domain. SNX9 and dynamin colocalize at the plasma membrane (Fig. 3B) (Lundmark and Carlsson, 2003) and, by using total internal reflection fluorescence (TIRF) microscopy on living cells, it was seen that SNX9 is recruited to clathrin-enriched spots simultaneously with dynamin just before scission of the clathrin-coated vesicle (Soulet et al., 2005). Although there is evidence for recruitment of dynamin at earlier stages of clathrin-coated-vesicle formation as well (Damke et al., 2001) (which might be independent of SNX9), the major burst of dynamin localization that coincides with vesicle scission is probably the result of new recruitment of dynamin that is triggered by the architecture at the vesicle neck. We have found that a proportion of dynamin 2 exists as a complex together with SNX9 in the cytosol and, by using cell-free assays and RNA interference in cells, we showed that dynamin 2 requires SNX9 for efficient membrane recruitment (Lundmark and Carlsson, 2003; Lundmark and Carlsson, 2004). In view of recent advances in our understanding of SNX9 function in membrane binding and remodeling, we speculate that SNX9 brings dynamin to the highly invaginated clathrin-coated pit. The specificity in localization comes both from the affinity of SNX9 for AP-2 and clathrin (Lundmark and Carlsson, 2003), and its preference for PtdIns(4,5)P_2 (which is built up in clathrin-enriched areas by the action of lipid kinases), as well as from the preference of SNX9 for binding to curved membranes (Pylypenko et al., 2007). At the vesicle neck, SNX9 might further modulate the membrane into a short, narrow tube that allows the compilation of dynamin into a fission-competent assembly.

The interplay between SNX9 and dynamin does not end with their recruitment to clathrin-coated pits. It is well known that the GTPase activity of dynamin is important for the scission reaction [for reviews on various aspects on dynamin action, see the following articles (Conner and Schmid, 2003; Hinshaw, 2000; Kruchten and McNiven, 2006; Orth and McNiven, 2003; Praefcke and McMahon, 2004)]. It has been shown that SNX9 has a profound effect on the GTPase activity of dynamin, especially in the presence of PtdIns(4,5)P_2-containing liposomes (Soulet et al., 2005). This result was interpreted to mean that SNX9 potentiates the assembly-stimulated activity of dynamin on the membrane surface. Full-length SNX9 was required for this effect, which indicates that membrane remodeling mediated by the yoke-PX-BAR unit of SNX9 is involved in activation of dynamin. These results were corroborated by the finding that mutations in either the PX domain or the BAR domain of SNX9 affected the GTPase-stimulatory activity (Yarar et al., 2008). Recent studies using spectroscopy of fluorescently labeled dynamin showed that the protein acts by partial insertion into the bilayer of highly curved, PtdIns(4,5)P_2-dense membranes (Ramachandran and Schmid, 2008). The hydrolysis of GTP elicits

a conformational change that favors dynamin dissociation and destabilization of the membrane. Addition of SNX9 was found to stabilize the association of dynamin with the membrane during stimulated GTP hydrolysis, which would further destabilize the membrane structure. Interestingly, amphiphysin (a brain-enriched protein that shares many properties with SNX9) was found to have the opposite effect (Ramachandran and Schmid, 2008) and was previously shown to inhibit dynamin assembly (Owen et al., 1998). This might mean that SNX9 and amphiphysin operate differently in uptake processes.

SNX9 and actin remodeling

It can be envisioned that dynamin and SNX9 act together to narrow down the membrane-tubule diameter at the neck of a clathrin-coated vesicle to a minimum; this might be followed by destabilization of the phospholipid bilayer to the point at which a sudden pushing force is able to separate the vesicle from the membrane in conjunction with rearrangements of the lipids in the bilayer. Such a force might be mediated by the locally controlled polymerization of actin. In this scenario, the SH3 domains of assembled SNX9, which are clustered on the membrane tubule, can be used for binding and activation of N-WASP to trigger actin polymerization via the actin-nucleating Arp2/3 complex. In this way, the SH3 domains of SNX9 might shift sequentially in function, from recruitment and assembly of dynamin to binding and activation of N-WASP. Strikingly, when SNX9 that lacks the SH3 domain is overexpressed in cells, it can no longer mediate the interactions with dynamin and N-WASP. This leads to the production of membrane tubules that can sometimes be several micrometers long (Håberg et al., 2008; Pylypenko et al., 2007; Shin et al., 2008) (Fig. 4); this is probably because the scission machinery is not recruited and activated. N-WASP is an important regulator of the Arp2/3 complex and actin nucleation, and is auto-inhibited in the resting state. Binding of PtdIns(4,5)P_2, Cdc42 or SH3 domains from several proteins relieves the auto-inhibition and exposes the binding site for Arp2/3 [for reviews on N-WASP and Arp2/3 function, see the following articles (Goley and Welch, 2006; Takenawa and Suetsugu, 2007)]. Although SNX9 is unable to interact directly with actin, it has been found to bind to the Arp2/3 complex via the LC domain (Shin et al., 2008). Indeed, there is a motif in this region that matches the acidic region that is found in activators of the Arp2/3 complex. The SNX9-Arp2/3 interaction might aid the precise localization and stabilization of actin nucleation, or might temporally regulate the action of Arp2/3 at the vesicular neck.

The functional importance of the interaction between SNX9 and N-WASP has been studied both in vivo and in vitro (Badour et al., 2007; Shin et al., 2007; Shin et al., 2008; Yarar et al., 2007; Yarar et al., 2008). Using a fluorometric pyrene-actin polymerization assay, Yarar et al. showed that, when SNX9 was pre-incubated with liposomes, it worked synergistically with phospholipids to activate N-WASP (Yarar et al., 2007; Yarar et al., 2008). This effect was seen with PtdIns(3,4)P_2, PtdIns(3,5)P_2, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3, which again indicates that SNX9 is promiscuous in its interaction with lipids. Mutations in either the PX domain or the BAR domain reduced this synergistic effect. Although the SH3 domain is sufficient for binding to N-WASP and necessary for its activation, full-length SNX9 is required for full activation of N-WASP (Yarar et al., 2007; Yarar et al., 2008). The requirement for pre-incubation indicates that assembly of SNX9 into structurally ordered oligomers on membranes and remodeling of membrane
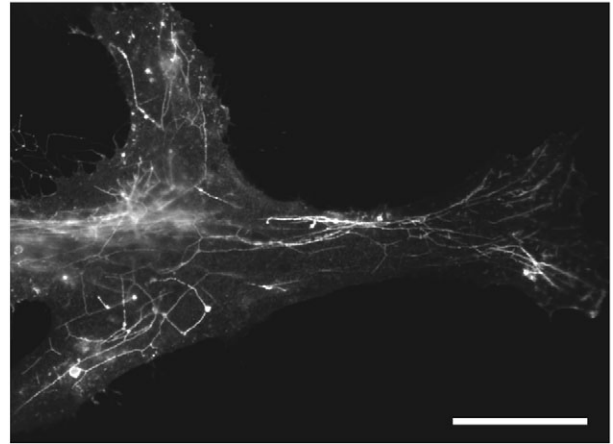

Fig. 4. Overexpression of the yoke-PX-BAR unit of SNX9 in cells produces numerous long membrane tubules. HeLa cells were transfected with a plasmid encoding Myc-tagged yoke-PX-BAR, stained with anti-Myc antibodies and fluorescently labeled secondary antibodies, and visualized by epifluorescence microscopy. Scale bar: 10 µm.

shape might induce N-WASP activation. In addition to its presence in endocytic pits, SNX9 has been observed to localize together with N-WASP at actin-enriched peripheral ruffles and dorsal rings (Yarar et al., 2007). This localization appears to be independent of the membrane-binding ability of SNX9, as mutations in the PX or BAR domains were without effect. This suggests that the SH3 domain of SNX9 might be sufficient for localization because N-WASP is enriched at these sites. What role SNX9 might have in this situation is not known.

Microbes are known to secrete toxins that affect elementary processes in target cells. Notably, SNX9 was found to bind to the *Escherichia coli* exotoxin EspF and to colocalize with this protein in enteropathogenic *E. coli* (EPEC)-infected cells (Marches et al., 2006). As described above, this interaction was shown to require the SH3 domain of SNX9 and high-affinity proline-rich motifs in EspF (Alto et al., 2007; Marches et al., 2006). In addition, EspF targets N-WASP through additional binding sites, near the proline-rich motifs, that promote N-WASP-mediated actin polymerization. Overexpression of EspF together with SNX9 in cells resulted in numerous short tubular structures that co-stained with actin (Alto et al., 2007). It seems, therefore, that the bacterium uses EspF to couple the actin-polymerization ability of N-WASP to the efficient membrane-remodeling activity of SNX9. As the SNX9 SH3 domain is occupied by EspF and SNX9 therefore cannot bind to dynamin, regulated fission is inhibited and instead longer pleiomorphic structures are generated, as was shown by electron microscopy (Alto et al., 2007). Although the benefit and precise use of this bacterial cellular strategy is not known, it is striking that EspF so specifically targets SNX9. These data further reinforce the role of SNX9 in actin dynamics.

The regulation of SNX9 in the cellular context

It is expected that a protein such as SNX9, which works at the interface between different molecular processes, will be tightly regulated in its activity. So far, very little is known about the cellular regulation of SNX9, and it is anticipated that future experiments will give insights into this matter. Several investigators have noted that SNX9 binds to and is tyrosine

Vesicular release →

Fig. 5. Integration of SNX9 and dynamin function with actin remodeling during vesicular release. The figure shows a model that is outlined in the text. SNX9 recruits dynamin to the neck of a highly invaginated clathrin-coated pit. Ordered assembly of the proteins on the membrane surface leads to the formation of a narrow tubule that, through SNX9-assisted GTP hydrolysis by dynamin, becomes destabilized. Recruitment and activation of N-WASP by SNX9 triggers a burst of actin polymerization, which might aid in the final release of the vesicle.

Legend:
- SNX9
- Dynamin
- N-WASP
- Actin polymer
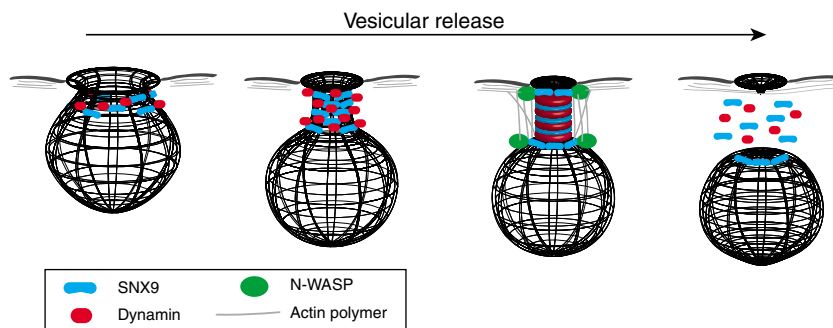
phosphorylated by Ack (Ack1 and Ack2 in human, DACK in *Drosophila*) (Childress et al., 2006; Lin et al., 2002; Worby et al., 2002; Yeow-Fong et al., 2005), which is a clathrin-associated kinase. Ack phosphorylates the SH3 domain of SNX9, after which the domain can no longer bind to proline-rich sequences. In *Drosophila*, phosphorylation by Ack switched the binding preference of the SNX9 SH3 domain from Wasp to the adaptor protein Nck (also known as Dock) (Worby et al., 2002). It is possible that SNX9 can take part in signaling pathways, and even act as a cargo receptor, through its SH3 domain. SNX9 was shown to be necessary for the CME-mediated uptake of the T-cell antigen CD28 through a WASP-dependent process (Badour et al., 2007), and the turnover of other receptors and enzymes has also been proposed to be regulated by SNX9 (Howard et al., 1999; Lin et al., 2002; MaCaulay et al., 2003).

The LC domain of SNX9 is clearly involved in the regulation of the activities that have been discussed here. This long region (see Fig. 3B) is a motif domain and possesses at least two binding sites each for clathrin and the α- and β2-appendages of AP-2 (Lundmark and Carlsson, 2002; Lundmark and Carlsson, 2003). We believe that this region is the primary determinant for targeting of the SNX9-dynamin complex to the growing clathrin-coated pit. It has been shown that the LC domain is subjected to phosphorylation (Lundmark and Carlsson, 2004), which is likely to fine-tune the interactions of SNX9 with the clathrin coat. We previously presented evidence that the glycolytic enzyme aldolase binds strongly to a specific sequence in the LC domain, and aldolase can even be found in a complex together with SNX9 and dynamin in the cytosol (Lundmark and Carlsson, 2004). At present, the significance of aldolase binding is not clear, but it is interesting to note that aldolase also binds to N-WASP with high affinity (Buscaglia et al., 2006; St-Jean et al., 2007). As the tetrameric aldolase also has affinity for F-actin, one possibility is that SNX9 and N-WASP use the interaction with aldolase to achieve high local concentrations near the plasma membrane through the association of aldolase with cortical actin. It is conceivable that this arrangement functions as a 'depot' for SNX9 and N-WASP, and that further regulation (such as by phosphorylation) can free these molecules to interact with the clathrin machinery.

Conclusions and perspectives

Studies over the past several years have established a clear role for SNX9 in CME. The evidence supports a mechanism whereby SNX9 is recruited together with dynamin to clathrin-coated pits through interactions with AP-2, clathrin and a highly curved membrane surface enriched in PtdIns(4,5)P_2. SNX9 might further remodel the vesicular neck into a narrow tubule onto which dynamin can self-assemble. In addition, SNX9 stabilizes the assembled dynamin oligomer that, through several rounds of GTP hydrolysis, destabilizes the membrane bilayer. Finally, SNX9 localizes and activates N-WASP, and subsequent actin polymerization might lead to release of the clathrin-coated vesicle (Fig. 5). It has been shown that SNX9 is enriched in purified preparations of clathrin-coated vesicles (Hirst et al., 2003), so a fraction of SNX9 might remain attached to the leaving vesicle and take active part in a putative actin-dependent process of vesicle movement.

The uptake of transferrin (which occurs through CME) can be blocked by overexpression of the SNX9 SH3 domain, whereas expression of the full-length protein or a protein that lacks the SH3 domain does not affect this process. Depletion of SNX9 in cells results in a reduction in membrane-targeted dynamin 2 (Lundmark and Carlsson, 2004) and endocytosed transferrin (Soulet et al., 2005) (and our unpublished results). However, similar to other proteins that are involved in the formation of clathrin-coated vesicles (such as epsin and AP-180), depletion of SNX9 from cells results in a relatively modest reduction in transferrin uptake (Huang et al., 2004; Soulet et al., 2005). This is probably because of redundancy in the molecular mechanism, and highlights the importance of the CME process.

Our knowledge of the functional importance of SNX9 would greatly benefit from in vivo experiments in animals, but so far no data have been presented in a vertebrate model organism in which SNX9 has been depleted. As SNX9 is ubiquitously expressed, it is certainly required for basic processes that are fundamental to different cell types, but it might also have specialized functions. It has been suggested that SNX9, together with dynamin 1 and N-WASP, has a role in synaptic-vesicle endocytosis (Shin et al., 2007). How this uptake relates to the different mechanisms of synaptic vesicular retrieval, and to the role of amphiphysin (see above), remains to be seen. It is also not known whether SNX9 is important for the CME of any particular cargo, or whether its activity is a general prerequisite for vesicular release in different processes, as has been suggested for dynamin (Praefcke and McMahon, 2004). Indeed, it has been proposed that SNX9 is not limited to CME but also takes part in fluid-phase uptake, together with dynamin and N-WASP (Yarar et al., 2007). Altogether, the importance of SNX9 is clearly manifested by its role in the fundamental process of endocytosis, and future studies are likely to underscore its significance.

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