Sorting Nexin 9
in Clathrin-mediated Endocytosis

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Umeå 2004
Tillägnad min älskade familj
Samuel, Elias och Ida
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# ABBREVIATIONS

<table>
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<tr>
<td>Φ</td>
<td>Bulky hydrophobic residue</td>
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<tr>
<td>AAK</td>
<td>Adaptor associated kinase</td>
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<td>AP</td>
<td>Adaptor protein</td>
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<td>AP-180</td>
<td>Adaptor protein 180</td>
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<td>BAR</td>
<td>Bin/Amphiphysin/Rvs</td>
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<td>CALM</td>
<td>Clathrin assembly lymphoid myeloid leukaemia</td>
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<td>CCV</td>
<td>Clathrin-coated vesicles</td>
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<td>CME</td>
<td>Clathrin-mediated endocytosis</td>
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<tr>
<td>DsCAM</td>
<td>Down’s syndrome cell adhesion molecule</td>
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<td>Dyn2</td>
<td>Dynamin-2</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>EH</td>
<td>Eps15 homology</td>
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<td>ENTH</td>
<td>Epsin NH2-terminal homology</td>
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<td>Eps15</td>
<td>Epidermal growth factor protein substrate 15</td>
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<td>GTPase effector domain</td>
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<td>G-protein coupled</td>
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<td>PI</td>
<td>Phosphatidylinositol</td>
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<td>Phox homology</td>
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<tr>
<td>RNAi</td>
<td>RNA interference</td>
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<td>SH3</td>
<td>Src homology 3</td>
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<td>SNX</td>
<td>Sorting nexin</td>
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<td>SNX9</td>
<td>Sorting nexin 9</td>
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<tr>
<td>TGN</td>
<td>trans-Golgi network</td>
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<td>WASP</td>
<td>Wiscott Aldrich syndrome protein</td>
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ABSTRACT

Clathrin-mediated endocytosis is a process by which cells can internalise diverse molecules such as nutrients, antigens and signalling-surface receptors. The creation of clathrin-coated vesicles demands interplay between the plasma membrane lipids, cargo molecules and the proteins that build up the coat. The major coat proteins involved in clathrin-mediated endocytosis are clathrin and adaptor-protein (AP) complex 2, but a symphony of additional proteins are required for appropriate coat formation. This thesis deals with the identification and characterisation of sorting nexin 9 (SNX9) as a new component of the endocytic machinery.

To study mechanisms of vesicle creation we wanted to identify and compare additional proteins working together with AP complexes. In eukaryotic cells, four different AP complexes are known as major players in the regulation of coated vesicle transport in the late secretory and endocytic pathways. Structural analysis of purified individual β-appendage domains from these AP complexes revealed both similarities and differences. Protein binding assays showed overlapping and distinct interactions of the specific subunits. One of the major specific-binding partners of AP-2, identified by sequencing and mass-spectrometry, was sorting nexin 9 (SNX9). This protein belongs to a large family of proteins based on the presence of a phox-homology (PX) domain. In addition, SNX9 harbours an N-terminal src-homology (SH3) domain followed by a region with predicted low-complexity and a C-terminal Bin/amphiphysin/Rvs (BAR) homology domain.

Interaction studies with the different domains in SNX9 demonstrated that SNX9 interacted with the endocytic core components clathrin and AP-2 through dual overlapping motifs. The major binding partner of the SH3 domain in SNX9 was dynamin-2, a GTPase known to be crucial for vesicle scission. The C-terminal region, containing the PX and BAR domains bound to phosphatidylinositols and targeted SNX9 to artificial liposomes and cellular membranes.

Consistent with a role in endocytosis, a large portion of SNX9 co-localised with AP-2 and dynamin-2 but not with markers for early endosomes, Golgi or lysosomes. Over-expression of truncated variants of SNX9 in K562 and HeLa cells interfered with the uptake of transferrin.

We found that in cytosol, SNX9 formed a resting complex together with dynamin-2 and the metabolic enzyme aldolase. This complex was inactive for membrane binding. Activation involved ATP hydrolysis and correlated with phosphorylation of SNX9 and the release of aldolase. Aldolase bound to a tryptophan-containing acidic region near the clathrin and AP-2 motifs and blocked lipid binding of purified SNX9 derivatives.

SNX9 was required for membrane targeting of dynamin2 in vitro and knockdown of SNX9 in HeLa cells by RNAi resulted in less punctuate and more diffuse dynamin-2 staining, suggesting impaired membrane localisation. Together these results argue strongly for a role of SNX9 in recruiting and linking of dynamin-2 to sites of vesicle creation.
This thesis is based on the following papers, which will be referred to by their roman numerals (I-III)


OVERVIEW

1. INTRODUCTION

The eukaryotic cell is built up of different compartments (organelles), separated by individual membrane systems. The protein and lipid constitution of each organelle is critical for proper function and maintained through active and specific transport. In the endomembrane system, commonly divided into the secretory and endocytic pathways, transport is mediated by membrane vesicles. The secretory pathway delivers newly synthesised proteins from the endoplasmatic reticulum to the Golgi and via the trans-Golgi network (TGN), dispersing them to the plasma membrane or endosomes. The endocytic pathway governs the uptake from the cell surface and further trafficking to endosomes and lysosomes (see fig.1).

To ensure correct trafficking of proteins in these systems, sorting is powered by complex protein machineries that generate membrane vesicles containing molecules to be sorted (cargo) (reviewed in [1-3]). Vesicular trafficking requires coat proteins, specific proteins at the cytoplasmic face of membranes that have the potential to recognise and collect cargo molecules. These coat proteins are integrated into complex protein networks that deform the membrane into a budding vesicle. Finally, the mature vesicle is released from the donor membrane. Through active or passive transport, it can dock and fuse with a receptor organelle and deliver the cargo molecules.

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The aim of this study was to identify and characterise additional proteins involved in the various mechanisms of vesicular transport, with a view to understand more about how this process is regulated.

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Fig. 1. Schematic representation of the secretory and endocytic pathways of the eukaryotic cell.
2. ADAPTOR PROTEIN COMPLEXES

Adaptor protein (AP) complexes are the major coat proteins in the late secretory and endocytic pathways. These complexes participate both in the recognition of cargo and the formation of a carrier vesicle. There are four homologous AP complexes expressed in higher eukaryotes (AP-1, AP-2, AP-3 and AP-4) (reviewed in [4]). AP-1 carries out transport between the TGN and endosomes, while AP-2 mediates rapid uptake from the plasma membrane – so-called clathrin-mediated endocytosis (CME). AP-3 is involved in trafficking from TGN and endosomes to melanosomes, late endosomes and lysosomes. AP-4 has been suggested to be important for transport from the TGN and basolateral sorting in fibroblasts (reviewed in[5;6])

Each tetrameric AP complex is composed of two large subunits \((\gamma, \alpha, \delta, \varepsilon \text{ and } \beta 1-4)\) in the different AP complexes, respectively), one medium subunit \((\mu 1-4)\) and one small subunit \((\sigma 1-4)\) (see fig. 2). The specific subunits from each complex are homologous to one another. The general image of the AP complexes, visualised by electron microscopy and crystallography, resembles a compact trunk with two protruding appendages that are connected via flexible hinge domains [7;8]. The trunk domain is built up of the N-terminal parts of the two large subunits and the medium and small subunits. The hinge and appendage domains are composed of the C-terminal parts of the large subunits.

The medium subunits of the AP complexes are involved in sorting signal recognition. The small subunit is thought to stabilise the trunk structurally, while the two appendage domains are responsible for recruitment of accessory proteins. AP-1 and AP-2 recruit the membrane deforming protein clathrin for help at sites of vesicle creation. The flexible hinge domain harbours an LLNLND sequence, termed the clathrin box motif, which binds to the \(\beta\)-propeller domain in clathrin (see next section). AP-3 contains a clathrin box that can interact directly with clathrin and assemble clathrin on liposomes in vitro. [9;10]. However, there are no in vivo data to support these findings. AP-4 has no clathrin box and was observed at clathrin-free regions on membranes, suggesting that AP-4 is part of a non-clathrin coat [11].

![Fig. 2. Schematic depiction of the four adaptor protein (AP) complexes.](image)

3. CLATHRIN

Clathrin adopts a basket-like protein lattice upon assembly at the plasma membrane or intracellular membranes, ultimately generating so-called clathrin-coated vesicles (CCV).
CCVs are dense vesicles covered by a clathrin cage, and are known to be responsible for receptor-mediated endocytosis and regulated trafficking from the TGN (reviewed in [12-14]). The clathrin cage-like structures are built up of basic units, the clathrin triskelions, which consist of three heavy chains and three light chains to form a three-legged structure. The heavy chain has a globular domain in its N-terminus, called the β-propeller, which contains binding sites for endocytic proteins. The C-terminal region mediates trimerisation and the middle, curved, domain results in the characteristic kinked structure – thus creating a proximal and distal leg. The light chain binds to the proximal leg but its exact function is uncertain.

Purified clathrin triskelion self-assemble into polyhedral cages in mildly acidic, high-Ca²⁺, low-ionic strength buffers. This intrinsic ability to assemble into facet-like pentamere and hexamere structures originate from multiple interaction sites along the proximal and distal legs (see fig. 3.). In the assembled clathrin, each side of a polygonal cell is formed by two anti-parallel proximal legs and two anti-parallel distal legs with the β-propeller domains projecting towards the membrane. In the cell, clathrin assembly is tightly regulated and involves numerous weak interactions that coordinate and specify the cage-structure precisely [15]. Numerous endocytic proteins bind clathrin either through derivatives of the traditional clathrin box now defined as LΦpΦ (-) (where Φ denotes a bulky hydrophobic residue, p denotes a polar residue and (-) is a negatively charged residue) or through other divergent motifs (reviewed in [16]). The data that are available suggest that this network of interactions forces clathrin to adopt the correct conformation for stabilising the coat.

Fig. 3. The structure of clathrin. Clathrin triskelions are depicted schematically and assembly of triskelions into cages is depicted both schematically and as a cryo electron micrograph (modified from Alberts et. al; The Cell, fourth edition)

4. ENDOCYTOSIS

Eukaryotic cells are shielded from the extracellular environment by the plasma membrane. Integral membrane pumps and channels facilitate entry of small essential molecules such as amino acids, ions and sugars. To take up macromolecules, the cell needs to engulf them in vesicles derived from the plasma membrane, in a process called endocytosis. Endocytosis can be divided into two major phenomena: phagocytosis and pinocytosis. Phagocytosis or “cell eating” is the way in which specialised cells take up and destroy bacteria, yeast or large cell debris. Pinocytosis or “cell drinking” occurs in all cells and facilitates the uptake of
macromolecules. There are different mechanisms for pinocytosis which include macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis and clathrin- and caveolae-independent endocytosis – of which clathrin-mediated endocytosis is the major pathway [17].

5. CLATHRIN-MEDIATED ENDOCYTOSIS

The process of clathrin-mediated endocytosis (CME) is used by virtually all eukaryotic cells to internalise extracellular molecules, either constitutively or in response to stimuli (reviewed in [17;18]). This process is used for active uptake of trans-membrane channels, transporters, receptors and extracellular ligands such as nutrients, antigens, hormones and growth factors. Endocytosed trans-membrane receptors are taken to the early endosomes, from where they can be recycled to the plasma membrane or shuttled to the lysosomes for degradation [19]. CME is crucial for intercellular communication during development and modulates signal transduction by regulating the levels of signalling receptors at the cell surface. At nerve terminals, CME is used for recycling of synaptic vesicles and controlling the strength of synaptic transmission (reviewed in [20;21]).

The uptake of cargo is concentrated to hot spots on the plasma membrane – clathrin coated pits – where lipids, coat proteins and clathrin are integrated to form a mechanical invagination of the membrane. It is believed that lipid composition instigates these hot spots, recruiting coat proteins which collect cargo and collectively assemble clathrin. The invagination process is assisted by a number of modular proteins – the so-called accessory proteins – which are integrated into a complex network. The continuous assembly of accessory proteins and clathrin causes increased curvature of the membrane, which invaginates into a budding vesicle. Eventually, this will lead to the formation of a mature vesicle which is released from the plasma membrane by a mechanism driven by the large GTPase dynamin (reviewed in [13;22;23]).

Fig. 4. Schematic overview of the sequential steps and key components involved in the creation of clathrin coated vesicles
5.1 Structure and function of AP-2

AP-2, the main adaptor of clathrin-coated vesicles (CCV), has been studied extensively over the years (reviewed in [13]). The first function assigned to AP-2 was a role in assembly of the coat, since addition of adaptors to an assembly reaction containing clathrin in vitro generated coats of uniform size. It was later shown that AP-2 targets clathrin to the membrane and that membrane receptors could be concentrated at clathrin-coated pits by AP-2.

The crystal structures of the individual AP-2 subunits nicely depict how the complex can collect cargo, assemble clathrin and at the same time recruit endocytic accessory proteins [8;24-27]. The trunk domain is composed of the structurally very similar N-terminal domains of α and β2 linked together by the σ2 subunit. A phosphoinositide-binding site at the N-terminus of α mediates the membrane recruitment of AP-2 [28]. The μ2 subunit, also located in the trunk, is able to recognise the peptide motif YxxΦ in cargo molecules and to interact with phosphoinositides following activation ([24;29] reviewed in [30]). The trunk domain in the inactive complex hides these binding sites [8]. Upon phosphorylation of μ2, a conformational change exposes these sites for cargo and lipid binding, locking AP-2 to the membrane. The adaptor-associated kinase AAK and GAK/Auxilin, members of the Ark/Prk family of kinases can phosphorylate μ2 in vitro and have been suggested to be responsible for μ2 activation ([31-33] reviewed in [34]). Clathrin was found to promote this activation by stimulation of kinase activity [35;36].

The C-termini of the α and β2 subunits, referred to as the ears or appendages, have similar structures – built up from an inner and outer domain. The outer domain resembles a platform and has been shown to contain a binding site for the motif DPF/W [25-27]. In addition, the α-subunit platform has been shown to recognise Fx DxF and WxXF motifs, and to harbour an extra site for interaction with DPF/W motifs [37;38]. Binding motifs for AP-2 appendages are being found in a growing number of proteins with accessory roles in CME. Since each platform contains at least two binding sites for such motifs, multiple motifs are often found in proteins associated with AP-2 (reviewed in [20]). The ears are linked to the trunk by a long hinge region with no secondary structure, allowing the ears to reach out and collect accessory proteins. The β2-subunit contains two binding sites for clathrin. The strong clathrin box is found in the hinge region [39] and there is a weaker binding site in the appendage domain [27].

5.2 Plasma membrane recruitment

Targeting of endocytic proteins to the plasma membrane is crucial for establishment of the correct connections for vesicle creation. Biological membranes are asymmetrical fluid mosaics of different lipids and proteins. The major lipid constituents are glycerophospholipids, sphingolipids and cholesterol. The term glycerophospholipid comprise lipids made up of two fatty acid chains and a polar head group linked to glycerol and includes subgroups like phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylethanolamin (PE), with different properties and shape. PI contains five exposed hydroxyl groups, three of which are known to be targets of phosphorylation – thus
generating several options for single, double or triple phosphorylated PI (see fig 5). The phosphorylated derivatives of PI, known as phosphoinositides, have been shown to function as second messengers to recruit and/or activate proteins in a number of systems (reviewed in [40]). The levels of phosphoinositides are tightly regulated by kinases and phosphatases, which can yield highly-localised concentrations of a particular phosphoinositide.

A number of protein domains have evolved which interact specifically with different phosphoinositides and can be used by proteins involved in diverse cellular processes for attachment to membranes (reviewed in [41;42]). At the plasma membrane, PI(4,5)P$_2$ is known to be important for the recruitment proteins involved in CME (reviewed in [43]). AP-2 is probably localised to the membrane by the trunk domain of the $\alpha$-subunit, since this region has been shown to interact with PI(4,5)P$_2$ and PI(3,4,5)P$_3$ in vitro [44]. The conserved ENTH (epsin NH2-terminal homology) domain of epsin and the similar ANTH (AP-180 NH2-terminal homology) domain found in adaptor protein 180 (AP-180) and the non-neuronal homologue CALM (clathrin assembly lymphoid myeloid leukaemia) was shown to interact preferably with PI(4,5)P$_2$ [45;46]. Despite the similarity in structure, the binding sites for PI(4,5)P$_2$ are different and in addition the ENTH domain can generate curvature in the membrane ([47] reviewed in [48]).

A subclass of the double zinc-finger domains, the FYVE (Fab 1p, YQTB, Vac1p, EEA1) domain is found in proteins such as EEA1 (early endosome autoantigen). This domain specifically interacts with PI(3)P since the shallow binding pocket is incompatible with other PIs (reviewed in [49]). The BAR (Bin/Amphiphysin/Rvs) domain found in an increasing number of proteins like the amphiphysins functions as a dimer and is known to interact with phosphoinositides and curved membranes. In some proteins, BAR domains are used in combination with adjacent lipid binding modules [50]. Lipid-binding modules belonging to the same class are not necessarily tied to particular lipid specificity. Even subtle modifications can alter the specificity, enabling their use in various systems [51]. Different variants of the PX (phox homology) domain (discussed in 6.2) found in numerous proteins can recognise divergent phosphoinositides (reviewed in [42;52]). The well-characterised PH
(pleckstrin homology) domain, present in a multitude of proteins, can be divided into different classes based on recognition of different phosphoinositides (reviewed in [53]). PH domains in class 1 bind strongly to PI(3,4,5)P₃, while members of class 2 prefer PI(4,5)P₂. Class 3 includes proteins that are able to bind both PI(3,4)P₂ and PI(3,4,5)P₃, and class 4 – represented by dynamin – have low affinity for phosphoinositides. The binding affinities of most of these protein domains are quite low, suggesting that in vivo these proteins function in oligomeric complexes.

5.3 Accessory protein network

For additional recruitment of coat and accessory proteins and fine-tuning of the network, protein-protein interactions are used. Proteins involved in molecular networks are often built up of binding modules and specific sequence motifs that help to unite these molecules. Characteristic protein binding modules such as the src-homology (SH3) domain, Eps15 homology (EH) domain, proline-rich domain (PRD), NPF and DPW/F motifs facilitate a highly dynamic regulation of assembly. Motifs for binding to the platform of AP-2 are found in nearly all accessory proteins and have almost become the criteria for involvement in CME. Proteins that function as dimers are often linked by more stable coiled-coil interactions.

The exact functions of all the different accessory proteins are not completely understood, but it is clear that they can have several functions and act at several stages (see Fig. 6.) (reviewed in [18;20]). Proteins such as AP-180/CALM and epsin are thought to function at the initiation of coated pit formation. They bind membranes through ENTH/ANTH domains and can through protein binding motifs interact with cargo and AP-2, and promote clathrin assembly (reviewed in [48]). Their distinct roles are based on findings that epsin cause membrane curvature [47], while AP-180 was shown to determine vesicle size in vitro and in vivo [54-56].

Other accessory proteins such as intersectin and Eps15 (epidermal growth factor substrate 15) seem to specialise in linking and coordination. Intersectin has multiple binding domains which interact with dynamin, WASP (Wiscott Aldrich syndrome protein), Sos, synaptojanin, and epsin [57-60], suggesting a role in coordination of signalling, endocytosis and actin dynamics. Eps15 lacks a membrane targeting domain but harbours multiple sites for protein-protein interactions including a EH domain. Eps15 is thought to strengthen interactions between AP-2 and proteins containing NPF and W/FW motifs, such as epsin and AP180 (reviewed in [61]).

At later stages, the need for deeper invagination and scission calls for recruitment of specialised proteins. Proteins such as syndapin, endophilin, intersectin and amphiphysin have been suggested to interact sequentially with dynamin to mediate constriction and fission of vesicles [62]. Some accessory proteins, in addition to protein and lipid binding modules, harbour enzymatic activity. Synaptojanin, a regulator of coat assembly and disassembly, contains a lipid-phosphatase domain and has PI(4,5)P₂ as its main physiological substrate, which is degraded to PI(4)P. Endophilin exhibits lipid transferase activity that converts lysophosphatidic acid to phosphatidic acid ([63] reviewed in [64]).
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<td>ANTH</td>
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<td>Binds PI(4,5)P₂ through ANTH domain</td>
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<td>Involved in: coated pit initiation, control of vesicle size</td>
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<td>ENTH UIM NPF</td>
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<td>Interacts with: AP-2, epsin, intersectin, synaptojanin, and AP-180. Forms dimers</td>
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<td>Involved in: dynamin recruitment, scission, and linkage of the endocytic and actin machineries</td>
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<tr>
<td>Intersectin</td>
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<td>EH EH coiled-coil SH3 SH3 SH3 SH3</td>
<td>Interacts with: Eps15, epsin, dynamin, Sos, synaptojanin. Forms dimers</td>
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<td>Involved in: coordination of the sequential steps in vesicle creation and links the endocytic and actin machineries</td>
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<tr>
<td>Synaptojanin</td>
<td></td>
<td>Sac1 5´phosphatase PRO NPF</td>
<td>Interacts with: endophilin, syndapin, amphipysin, AP-2 Phosphoinositide phosphatase</td>
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<td>Involved in: PI metabolism, invagination, scission and uncoating</td>
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<tr>
<td>SNX9</td>
<td></td>
<td>SH3 PX BAR</td>
<td>Interacts with: AP-2, clathrin, dynamin, Nck and WASP</td>
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<td>Binds membranes through PX and BAR domains</td>
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<td>Involved in: dynamin recruitment, axonal guidance, actin rearrangements, and cargo sorting</td>
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Fig. 6. Summary of the domain organisation and proposed functions of endocytic accessory proteins. References are found in the text and in references therein.
5.4 Cargo selection

The dynamic plasma membrane is filled with transmembrane receptors that mediate uptake of nutrients and sense the surroundings, to transmit signals to the inside of the cell. Each cell type has its own uniquely composed set of membrane receptors which must be constantly updated, and receptors are recycled between the plasma membrane and endosomes or ultimately degraded in the lysosomes.

For rapid endocytosis, transmembrane proteins require an internalisation signal (reviewed in [30;65;66]). Several membrane receptors use short linear sequence motifs as signals for uptake. The most common motif is the tyrosine-based motif with the consensus sequence YxxΦ. This motif binds directly to the µ2 subunit of AP-2 and is found in proteins such as the epidermal growth factor (EGF) receptor, mannose 6-phosphate receptor and transferrin receptor. The target specificity of the other major endocytic signal, the [DE]xxx[LI] motif, is poorly understood. This motif interacts with AP-2, although it is has not yet been established whether binding concerns the µ2 or the β2 subunit.

The common view that AP-2 is responsible for the recognition and sorting of cargo molecules to sites of vesicle budding has been challenged by a number of observations (reviewed in [67;68]). The motif FxNPxY found in, for example, the LDL receptor does not compete with the YxxΦ motif in the transferrin receptor for CME [69;70], and was found to be structurally incompatible with the µ2 binding site [8]. Instead, this motif has been shown to interact with the phosphotyrosine binding (PTB) domain-containing proteins Disabled, Numb and ARH and there is evidence to support their role in trafficking (reviewed in [71]). Recently, elegant work using RNA interference showed that knockdown of the expression of AP-2 abolishes the uptake of transferrin receptor but leaves endocytosis of the EGF receptor and LDL receptor unchanged [72;73]. Inactivation of AP-2 by over-expression of AAK gave the same distinguished result in receptor uptake (Conner and Schmid 2003). Despite silencing of AP-2, clathrin-coated pits can be observed – although in fewer numbers – suggesting that other proteins may be able to recruit clathrin, select cargo and promote vesicle creation.

Several such connectors have been suggested. HIP1 (Huntingtin interacting protein 1) links the AMPA receptor to clathrin, and endocytosis is impaired in mice lacking HIP1 [74]. It is accepted that phosphorylation of the serine-rich region in signalling G protein-coupled (GPC) receptors promotes binding to the β-arrestin family of proteins which mediates their uptake [75;76]. Ligand-stimulated monoubiquitinylation of signalling receptors such as the receptor tyrosine kinases and GPC receptors serves as signals for internalisation and degradation. Proteins such as epsin which contain a ubiquitin recognition motif (UIM) bind to monoubiquitinated receptors and mediate uptake of the receptors [77;78].

A picture is emerging whereby constitutively recycling receptors bearing traditional signal motifs are internalised by interactions with AP-2, whereas signalling receptors might use specific coat proteins and post-translational modifications for their uptake. Conceivably, these processes are linked so that different coat proteins are integrated to form common vesicles.
5.5 Membrane curvature

How the dramatic changes in membrane curvature associated with vesicle budding are accomplished has been a key question for our understanding of vesicular trafficking. Different lipid species have distinct shapes which support the properties of the membrane.

Two main themes for control of membrane curvature have evolved: enzyme-dependent changes in the lipid composition and mechanical protein-induced deformation. Lipid modifying enzymes can alter the shape of lipids and by converting inverted cone-shaped lipids to cone-shaped lipids, proteins such as the endophilins are suggested to favour bending of the membrane. (reviewed in [64])

Through interactions with coat proteins, high concentrations of a particular lipid can be achieved. In addition, several coat proteins have been shown to mechanically bend membranes. The assembly of clathrin pentameres and hexameres was originally identified as the cause of membrane deformation. Over the years, additional coat proteins with membrane-deforming capacity have been characterised. Proteins like dynamin, amphiphysin and endophilin have been shown to tubulate membranes on their own in vitro (reviewed in [79]). The presence of BAR domains explain the tubulating activity of amphiphysin and endophilin [50], clarifying why endophilin lacking enzymatic activity still can tubulate membranes [80]. These proteins are thought to function in the later stages were extreme positive and negative curvature is needed. At the initial bud formation proteins like epsin might generate bending of the membrane. The ENTH domain of epsin was shown to tubulate membranes reviewed in [81;82].

The crystal structure solved in the presence of inositol 1,4,5-triphosphate showed that an additional helix forms in epsin during membrane binding [47]. Mutations in this region severely affect the tubulation ability of epsin. The hydrophobic nature suggests that this helix is inserted into the membrane, where it pushes the head groups apart, thereby bending the membrane. Several of these membrane-bending proteins are known to interact with clathrin and AP-2. The general impression is that a flexible protein network is responsible for generating curvature, whereas clathrin functions merely as a stabilising coat.

5.6 Dynamin and vesicle fission

A critical step in vesicular trafficking is the release of mature vesicles from the donor membrane. The GTPase dynamin has proven to be necessary for this process since mutants abolish endocytosis and have been seen to cause paralysis, due to inhibition of synaptic vesicle generation in Drosophila.(reviewed in [79]) Mammals express three isoforms of dynamin with additional splice variants. Dynamin 2 is ubiquitously expressed while Dynamin1 is neuron-specific and Dynamin 3 is restricted to testis, brain and lung (reviewed in [83]).

Dynamin is a multi domain protein made up of an N-terminal GTPase domain and an adjacent middle domain with the potential for self-assembly followed by a PH domain that
allows binding to PI(4,5)P₂ in the oligomerised state. The C-terminus contains an assembly-dependent GTPase effector domain (GED) known to stimulate GTPase activity and self-assembly, and a proline-rich domain (PRD) (reviewed in [84]). Dynamin is known to self-assemble around the vesicle neck in its GTP-bound form [85]. *In vitro*, purified dynamin forms rings and spirals at low ionic strength and oligomerises on lipid membranes [86]. GTP hydrolysis accompanied with conformational changes in the assembled dynamin has been proposed to generate a force aiding vesicle fission [87-90]. The fact that over-expression of dynamin GED mutants increased the rate of endocytosis [91] has led to conflicting ideas about the function of dynamin.

There are two main models for how dynamin exerts its role at the vesicular neck (reviewed in [84;92-94]). In one model, the hydrolysis of GTP will force dynamin into a conformational change that mechanochemically releases the vesicle from the membrane. In the other model, the oligomerisation of dynamin leads to recruitment of effector molecules that are responsible for the fission reaction. In the latter model, dynamin acts as a GTPase – in the sense that it functions as a molecular switch. In both models proteins interacting with dynamin such as endophilin and actin may aid the fission process.

Truncated variants of dynamin which lack the PRD fail to accumulate at clathrin-coated pits and disruption of interactions between SH3 domains and the PRD interfered with endocytosis[95;96], showing that this region is responsible for targeting of dynamin. The SH3 domains of several proteins including amphiphysins, endophilin and syndapin interact with the PRD domain of dynamin and their over-expression inhibits endocytosis [62;97]. The SH3 domains of amphiphysin have been shown to sterically inhibit self-assembly of dynamin [98], while this was not seen using the full-length protein [99]. This multitude of interactions highlights the importance of sequential SH3 domain-mediated interactions in the later stages of endocytosis.

In addition to its role in fission, dynamin has been shown to be important for earlier stages such as vesicle constriction [89]. It is likely that dynamin is recruited to the membrane at an early stage and subsequent guided to its sites of action in endocytosis through sequential interactions with multiple SH3-containing proteins.

### 5.7 Role of actin in endocytosis

Monomeric actin is a building block of the cell, with the potential to form filamentous structures important for numerous biological functions. Actin filaments regulate cell structure and mobility and are responsible for cellular outgrowths such as lamellipodia and filopodia (reviewed in [100]). A role for actin in vesicular trafficking in both the secretory and endocytic pathways has been suggested, since treatment of cells with drugs against actin affects vesicular transport (reviewed in [101-104]). Although quite elusive, actin may play different roles in endocytosis – mediated through interactions with different regulatory proteins. Local clearance of the rigid cortical actin cytoskeleton is seen in the immediate vicinity to coated pits [105]. Spatial organisation through specific anchoring of actin filaments could generate hot spots for endocytosis by generating a shield to stop endocytic
proteins from diffusion. The force generated by actin fibres could aid invagination, fission and transport of vesicles.

The Rho-GTPases are known to regulate actin polymerisation. At the plasma membrane, the Rho-GTPase cdc42 together with PI(4,5)P₂ and SH3 domain-containing proteins such as Grb2 activate the autoinhibited WASP family of proteins (reviewed in [106]). These proteins are known to initiate polymerisation and branching of actin via the Arp2/3 complex. Several proteins, such as Hip1, syndapin and intersectin link dynamin and other clathrin-associated proteins to this actin polymerisation machinery (reviewed in [103]). Endocytic vesicles have been shown to be transported at the tips of actin tails [107] and an intensification of dynamin staining at vesicular scission is followed by an increased actin signal, suggesting that dynamin and actin have sequential roles at the late stages of endocytosis [108].

5.8 Vesicle uncoating

For a vesicle to be able to fuse with the target membrane, it has to be uncoated. This process is directed by the heat shock protein Hsc70 [109] and its cofactor GAK/auxilin which targets Hsc70 to AP-2 and clathrin and promotes its ATPase activity [31;110]. Over-expression of ATPase-deficient Hsc70 mutants inhibits uncoating of CCVs in vivo [111]. It is believed that the complex between Hsc70 and GAK/auxilin acts by dissociating individual clathrin triskelions. Another protein implicated in uncoating is the lipid phosphatase synaptojanin which can dephosphorylate PI(4,5)P₂ into PI(4)P. The change in lipid composition will function in reverse of the recruitment process and lead to de-attachment of coat proteins. The role of synaptojanin in uncoating was emphasised by its knockout in mice, leading to the accumulation of CCVs [112].

Disruption of the interaction between endophilin and synaptojanin by a peptide or an antibody inhibits uncoating [113]. Recently, some elegant work has confirmed that endophilin localises and stabilises synaptojanin to sites of synaptic vesicle recycling, and links the enzymatic activities of these proteins [114;115]. This complex probably acts at several stages, such as fission, uncoating and actin disassembly, where their lipid-modifying properties are needed.

5.9 Regulation of coat formation

Regulation of CME can be envisioned on several planes, where cargo abundance, lipid composition, actin arrangement and assembly of endocytic proteins could all influence the rate of vesicle formation. Post-translational modifications such as reversible phosphorylation seem to serve as regulatory mechanisms (reviewed in [116]).

Work in nerve cells identified dynamin 1, AP180, synaptojanin, amphiphysin I/II, epsin, Eps15 and AP180 as dephosphins that were phosphorylated in their resting state and dephosphorylated at the initiation of synaptic vesicle endocytosis [117;118]. The current model is that phosphorylation inhibits interactions between endocytic proteins, thereby
preventing their assembly – as seen in the case of the dephosphins. Several kinases such as PKC and cdk5 have been implicated in this process [119-121]. The most abundant kinase in CCV, casein kinase 2, has been shown to be responsible for the majority of phosphorylation events in CCVs in vitro [122].

In the case of AP-2, phosphorylation has been shown to modulate both membrane binding, cargo recognition and coat assembly. GAK/auxilin and AAK1 can phosphorylate the µ2 subunit in vitro, which is linked to cargo recognition (reviewed in [34]). Phosphorylation of β2 has been suggested to promote binding to the di-leucine signal in the EGF receptor [123]. Dephosphorylation of the appendages appears to regulate clathrin assembly [124].

6. SORTING NEXIN 9

Sorting nexin 9 (SNX9) is a ubiquitously expressed protein [125] found in the cytosol and associated with membranes in cells (PAPER II). The highest level of transcription was found in heart and placenta, whereas relatively low expression was detected in thymus and leucocytes [125]. Homologues have been found in C. elegans, Drosophila, and higher eukaryotic cells, but not in yeast. The protein has been classified as a member of the sorting nexin (SNX) family of proteins on the basis of the fact that it contains a variant of the PX domain, which is characteristic of this family of proteins. SNXs are thought to function in protein sorting events in different compartments of the cell. Sequence analysis of SNX9 has revealed that it is built up from an N-terminal SH3 domain followed by a 200 amino acid-long stretch of (predicted) low complexity. The PX-domain is located in the middle of the primary structure and is followed by a linker and the C-terminal α-helical rich region which is most likely a BAR domain (see fig 6). This particular domain organisation is only shared by the sequentially similar SNX18 which can be considered as an isoform. In addition, these proteins are the only sorting nexins to have an SH3 domain.

6.1 SH3 domains

SH3 domains are well-characterised protein-interaction modules that function in numerous biological processes of the cell. The SH3 domains are used frequently in processes that require signalling cascades and brief interactions (reviewed in [126] [127]). In endocytosis, proteins such as the amphiphysins, endophilin, synaptojanin and intersectin all find their target using SH3 domains.

SH3 domains were identified as sequentially homologous protein-binding regions in proteins such as the Src family of tyrosine kinases. The targets of SH3 domains were recognised as proline-rich regions with the consensus motif PxxP. The globular structure of SH3 domains consists of two anti-parallel β-sheets and variable loops termed the RT loop, the n-Src loop, and the distal loop. The hydrophobic binding surface of an SH3 domain consists of three shallow pockets composed of conserved aromatic residues located between the RT loop and the n-Src loop in the tertiary structure. The proline motif adopts a left-handed helical conformation resembling a triangle with the base facing the SH3 domain.
In addition to the traditional PxxP-variant, divergent proline motifs such as the Eps8 SH3 consensus PxxDY \[128\] and yet another variant in the protein kinase PAK have been identified \[129\]. An SH3 domain can interact with two proteins simultaneously, using the conventional PxxP motif binding site and another binding site for a non-PxxP motif \[130;131\]. In addition, the affinity for the target protein can be increased through dual interactions \[132\]. The target specificity of SH3 domains can be altered by post-translational modifications such as phosphorylation. The phosphorylated SH3 domain of SNX9 in *Drosophila* has been shown to prefer binding to Nck over binding to WASP \[133\]. The affinity and specificity of SH3 domains are quite low, but high local concentration and multiple interaction sites enables their use in biological processes.

The SH3 domain of SNX9 resembles that of the signal adaptor protein Grb2. Two PxxP motifs are present in SNX9, one in the PX domain and one in the linker preceding the BAR domain. These motifs are potential sites for the regulation of SNX9.

### 6.2 PX domains

The phox-homology (PX) domain has been shown to be one of the increasing numbers of membrane binding domains. PX domains function in a variety of distinct biological processes such as membrane trafficking and enzyme localisation (reviewed in \[42;52;134\]). Work on the p47phox and p40phox protein components of the NADPH oxidase complex identified this domain as a phosphoinositide binding region which targets p47 to the membrane.

The PX domains are about 100-130 amino acids long. Structural studies of several proteins have revealed that the compact structure of PX domains is composed of three β-strands, forming an anti-parallel β-sheet, and three α-helices. Conserved basic residues in the β3-α1 region and α2 region are thought to form a basic binding pocket for negatively charged phosphate groups of the PI. Membrane binding is further supported by a hydrophobic loop between the α1 and α2 helices which conducts non-specific hydrophobic interactions with the membrane. A conserved proline motif (see previous section on SH3 domains) is located in the loop between the α1 and α2 helices. This motif is a potential mediator of SH3 interactions and PX domain regulation. The membrane binding of p47phox has been suggested to be regulated by an intra-molecular interaction between one of the SH3 domains and the PX domain.

### 6.3 BAR domains

The BAR (Bin/Amphiphysin/Rvs) domain comprise a structurally conserved region of 200 amino acids approximately, based on long α-helices. Although the sequence similarity is relatively weak, BAR domains have now been identified in many proteins such as the amphiphysins, endophilins and Tuba \[20;50;135\]. These proteins have been shown to dimerise and tubulate membranes *in vitro*, a phenomena thought to reflect the presence of a BAR domain \[99;135;136\].
As determined by X-ray crystallography, the BAR domain is a functional dimer with an elongated banana-shaped structure [50]. The monomer is a coiled-coil of three extended kinked α-helices that intersect to form a six-helix bundle around the dimer interface. Since the dimer is the functional domain, the monomer should be referred to as a half-BAR domain. The banana shape is generated by a kink in helices 2 and 3 and by the way in which monomers are joined by hydrophobic interactions. Conserved positively-charged patches at the concave surface have been suggested to mediate interactions with phospholipids. It can be envisioned how BAR-containing proteins force membranes to tubulate by oligomerisation. Point mutations in the BAR domain of amphiphysin have revealed that this region is responsible for tubulation. In addition, this domain structure could be used for sensing curvature, as suggested by the preference of some BAR domains for binding to liposomes of a particular size. Using sequence alignment, several proteins have been proposed to form BAR domains – including sorting nexins [50]. We aligned SNX9 to this consensus sequence and found that SNX9 indeed contains a half-BAR domain in the C-terminal part (designated CT in PAPER II and PAPER III) (see Fig. 7). Interestingly, dimerisation of the C-terminal region of Drosophila SNX9 has been shown using the yeast two-hybrid system [137], suggesting that SNX9 can form a functional BAR domain.

![Fig. 7. The BAR domain of SNX9. A. The C-terminal sequences of SNX9 from several species were manually aligned to the BAR consensus sequence described in [50]. Amino acids that matched the consensus are highlighted in gray. Prefixes in designations of SNX9: h, human; m, mouse; z zebrafish; d, drosophila melanogaster; a, anopheles; e C. elegans. B. Model of the tertiary structure of a SNX9 dimer.](image-url)
6.4 Role of SNX9

SNX9 was first characterised as a protein that bound to metalloproteinase disintegrins MDC9 and MDC15 via the SH3 domain, suggesting a role for SNX9 in trafficking of these molecules [125]. In *Drosophila*, SNX9 has been implicated in axonal guidance [137;133]. SNX9 was found to interact directly with the *Drosophila* orthologs of human Nck, Down’s syndrome adhesion molecule (DsCAM), Ack and WASP. This suggested that it links the DsCAM receptor to the actin skeleton together with Nck and WASP, and mediates trafficking and localisation of DsCAM. SNX9 was tyrosine-phosphorylated by Ack at position Y56 following EGF stimulation of cells. This phosphorylation regulated the SH3 domain to favour binding of Nck instead of WASP. Interestingly, knock-down of the expression of SNX9 in fly embryos resulted in pleiotropic effects disrupting the formation of head structures. This suggests a more general role in trafficking than merely regulating the DsCAM receptor [133;137]. Further support for EGF-stimulated phosphorylation of SNX9 by Ack came from a study showing that Ack2 also complexes with SNX9 and clathrin [138]. In addition, the cellular localisation of SNX9 have been reported to respond to insulin stimulation [139]. These results suggest that SNX9 is involved in trafficking of various membrane proteins. The identification and characterisation of SNX9 as a component of the endocytic machinery will be presented in the Results section of this thesis.

6.5 The sorting nexin (SNX) family

The SNX family includes proteins identified in organisms ranging from yeast to mammals and which are involved in trafficking between endocytic compartments. The current databases assign 28 proteins to this protein family, based on the presence of a subclass of the PX-domain. The divergent members of this group of proteins in addition contain various previously characterised interaction modules such as the RGS (regulator of protein-G signalling), BAR, SH3, and RA (RasGTP effector) domains [140]. The common theme seems to be that SNXs function in the endocytic pathway where they can associate in a reversible fashion with membranes. Most SNXs localise to endosomal compartments and are thought to form functional homo- or heterodimers.

Several sorting nexins have been suggested to interact with membrane receptors. The most well-studied family member, SNX1, has been shown to bind to the cytoplasmic tail of the EGF receptor and, by association with endosomes, mediate sorting and degradation of this receptor [141-144]. Like SNX1, SNX2 and SNX4 also recognise receptors such as the EGF receptor, PDGF receptor [145] and Insulin receptor, and oligomerise to form homo- and heteromeric complexes [146;147]. SNX1 and SNX2 are homologous and have essential redundant functions, shown by knockout studies in mice [148].

Other SNXs such as SNX6 and SNX17 have also been identified in regulation of membrane receptor trafficking [149;150].Some SNXs, such as SNX3, are small, comprising little sequence other than the PX domain. SNX3 is thought to be important for maintaining the tubular structure that is characteristic of early endosomes [151].
RESULTS AND DISCUSSION

The β-appendages of the four adaptor-protein (AP) complexes: structure and binding properties, and identification of sorting nexin 9 as an accessory protein to AP-2. [PAPER I]

The crystal structure of the α- and β-appendages of AP-2 revealed that, despite low sequence similarities, the tertiary structures of these subunits were highly homologous [25-27]. The appendages are made up of an inner and an outer domain, where the latter was shown to harbour binding pockets for accessory endocytic proteins containing DPW/F motifs.

We were interested in comparing the appendage domains of all AP complexes in terms of structure and protein interactions. We focused on the β-appendages and by using secondary structure predictions in combination with manual sequence analysis, we were able to align not only the β1 and β2 subunits that are homologous, but also the β3 and β4 subunits. Importantly, after alignment of the sequences the predicted secondary elements also aligned, albeit in some cases where helices were predicted instead of β-strands or vice versa. Interestingly, we found that the short β4 appendage most likely comprised only the outer subdomain. A similar analysis of the α, γ, δ, and ε appendages showed that all aligned with both subdomains except for the γ-chain, which aligned only with the inner domain (unpublished results). This result was later confirmed by the tertiary structure determination of the γ-subunit [152].

The C-terminal regions equivalent to the inner and outer domains of the β-appendages were cloned and expressed as GST fusion proteins in bacteria. To examine the secondary structure experimentally, the GST tag was cleaved off and the appendages were analysed by CD spectroscopy. As expected, the spectra of β1 and β2 were very similar and consistent with the crystal structure of β2 in terms of helix/β-sheet content. Both β3 and β4 showed a higher proportion of α-helices, which in the case of β4 is in agreement with our proposal that it only comprises the α-helical-containing outer subdomain. In addition the secondary structures revealed by CD spectroscopy analysis indicated that the recombinant proteins were properly folded.

In a comparison of the protein binding specificity between the β-subunits using pull-down assay, β1 and β2 showed similar but distinct binding patterns, while β3 displayed a unique pattern. β4 did not pull down any specific proteins – again suggesting that this is truncated and may have other properties. We used this technique as a broad nonbiased scan for interacting proteins from heamatopoetic cell cytosol and interesting candidates were analysed by amino acid sequencing of CNBr-cleavage fragments. Since little was known about the function of AP-3, we tried hard to identify a specific protein that bound to β3 (designated band-8 in PAPER I), but sequencing failed to reveal the identity of this protein.

It is intriguing that so many accessory proteins have been identified for AP-2 while the mechanisms for AP-3 and AP-4 function remain mysterious. We have also used the δ and ε subunits in similar pull-down experiments but failed to identify any specific interactors and we believe that the generally low expression of AP-3 and AP-4 in cells limit the usefulness of
this method. The γ-appendage of AP-1 has been shown to recruit several accessory proteins and a potential consensus motif for interacting proteins was recently described [153]. It is likely that the γ-appendage defines the specificity for interactions with AP-1 while the β1-subunit stabilise these interactions.

To further analyse interacting proteins we used an overlay assay that allowed the identification of several proteins that in their denatured state bound to β2, arguing for the use of sequence motifs. Using antibodies, we could confirm binding of the β2 appendage to Eps15, epsin and clathrin in haematopoetic cells and show for the first time that in addition β2 interacted with CALM, the non-neuronal homologue of AP-180. Interestingly, β1 also showed affinity for these proteins, although much weaker, suggesting overlap in specificity. Maybe these interactions reflect an ability of β-1 to bind a broad range of molecules as suggested above.

One of the major β2-interacting proteins from haematopoetic cytosol was identified by amino acid sequencing as SNX9. At the time of our discovery, this protein was essentially unknown, and only one paper had been previously published on SNX9 [125]. Using antibodies, we determined that SNX9 bound specifically to both appendages of AP-2 but not to the β-appendages of any other AP complex. SNX9 has been suggested to interact with the γ subunit of AP-1 [154], showing the same overlap in binding specificity between AP-1 and AP-2 appendages as we observed for epsin and Eps15. We found that SNX9 co-precipitated with AP-2 but not with AP-1 arguing for a specific interaction in vivo.

When proteins in the cytosol were separated by gel filtration, we found that SNX9 eluted in fractions near the void volume – as did AP-2, clathrin, and AP-1, suggesting integration into multimer complexes. Determination of the cellular localisation by analysis of separated cytosolic and membrane fractions concluded that the major part of SNX9 was found in the cytosol. These results at first appears contradictory to results presented in PAPER II and PAPER III but originates from the divergent properties that SNX9 display under different buffer conditions, and emphasise the importance of choosing the appropriate conditions for in vitro experiments. We now know that membrane association of SNX9 is highly dependent on the salt concentration during extraction of cytosol from cells. At low salt conditions membrane bound SNX9 together with clathrin, AP-1 and AP-2 are released as part of large complexes into the cytosol. In addition SNX9 has a propensity to form aggregates at high ionic-strength and these effects are emphasised in the experiments. Notably, the association of SNX9 with AP-2 was detected in cell-lysates obtained from cytosol and membranes, but not when only cytosol was used, suggesting an association at the membrane. Concordant with PAPER II and III we believe that SNX9 indeed interacts with AP-2 in large assemblies in vivo, but that these assemblies are restricted to the plasma membrane.

Purified clathrin-coated vesicles showed no enrichment of SNX9 as compared to cell lysates depleted of nuclei, suggesting that SNX9 is not incorporated into the released vesicles, as has been shown for several other accessory factors. This result is in contrast with another study showing that SNX9 is indeed enriched in clathrin-coated vesicles [154]. The discrepancy is probably due to differences in purification procedures and the buffer-sensitivity of SNX9 described above. In addition, the cellular fraction to which enrichment in purified CCVs was
compared varied in the two experiments and might have influenced the interpretations of the results. The results presented in PAPER II, is consistent with the presence of SNX9 in CCVs. In conclusion, SNX9 was identified in vitro and in vivo as a specific interactor of AP-2 in non-neuronal cells, suggesting an accessory function in endocytosis.

Sorting nexin 9 participates in clathrin-mediated endocytosis through interactions with the core components. [Paper II]

To establish the inferred role of SNX9 together with AP-2 in endocytosis, we cloned and expressed full-length SNX9 and individual regions of SNX9 as GST fusion proteins. Using these proteins in pull-down experiments from cytosol, several interacting proteins could be detected. Two of the major proteins pulled down by the amino-terminal one-third of SNX9 were identified by mass-spectrometry as clathrin heavy chain and dynamin-2 (Dyn2). Interestingly, we also identified the lipid phosphatase synaptojanin as a potential interaction partner (unpublished results). The latter finding has not been further evaluated.

The amino terminal region in SNX9 contains an SH3 domain and a region of (predicted) low complexity. Further interaction studies showed that purified proline-rich domain of Dyn2 bound directly to the SH3 domain of SNX9, while clathrin and the $\alpha$-appendage of AP-2 interacted with the low-complexity region (LC). Mapping of the binding sites for AP-2 and clathrin using point mutations of specific residues revealed that dual overlapping motifs are used. As expected, the DPW motif in SNX9 interacted with the $\alpha$ appendage but in addition the motif WDEDW was determined to mediate binding. Mutations of the tryptophans to serines in these motifs abolished binding of both the $\alpha$-appendage and purified clathrin. The overlap in binding sites could imply that SNX9 is unable not interact with clathrin and AP-2 at the same time. These sites may be used sequentially to coordinate assembly, or maybe SNX9 is capable to assemble clathrin in the absence of AP-2. Interestingly, binding of full-length SNX9 to the $\alpha$-appendage and clathrin was hampered compared to constructs of the LC region, suggesting an intramolecular inhibition. A possible interaction between the SH3 domain and the PxxP motif found in the PX-domain could be one explanation for obstructing the LC region, but LC in it self might in the full-length molecule fold in a way prohibiting binding.

SNX9 forms a discrete complex in cytosol together with Dyn2, and a protein of 41 kDa (see PAPER III), as determined by co-immunoprecipitation experiments. Sedimentation velocity analysis of cytosol showed that most of SNX9, part of Dyn2 and p41 are components of this approximately 400-kDa complex. The complex was found to be sensitive to phosphate containing buffers (unpublished observation) and easily dissociates upon dilution at physiological ionic strength, arguing for a dynamic arrangement of the complex.

We found a large portion of SNX9 together with AP-2, Dyn2 and clathrin in large detergent-resistant assemblies at membranes. These complexes were sensitive to low-ionic strength and high concentrations of Tris, and revealed to us that the results obtained on the cellular localisation in PAPER I should be reinterpreted as discussed above.
The N-terminal binding sites for Dyn2, AP-2 and clathrin could potentially target SNX9 to clathrin coats at the membrane. To investigate whether SNX9 has a lipid-binding activity of its own, we analysed the binding of recombinant SNX9, and SNX9 derivatives, to artificially prepared liposomes. SNX9 was found to interact specifically with phosphoinositides, but with no specific preference. This binding was dependent on the C-terminal part of SNX9, harbouring the PX- and BAR-domains. PX domains of SNXs are generally thought to target PI(3)P for endosomal localisation. As we see no preference in specificity, the binding unit could be a combination of a PX and a BAR domain. BAR domains have recently been shown to bind curved membranes in the dimerised form. The presence of GST in the recombinant SNX9 mediate the formation of dimers, and maybe the BAR domain binds to liposomes of the appropriate curvature as shown for oligophrenin and centaurin [50]. It is conceivable that the lipid binding modules function in combination so that the PX domain targets SNX9 to membranes while the BAR recognise curvature thereby providing precision in the localisation of SNX9.

In confocal microscopy, endogenous SNX9 displayed numerous small dots at, or close to, the plasma membrane of HeLa cells, again showing that SNX9 is reversibly localised to membranes. In agreement with a role of SNX9 in endocytosis and the presence in coated pit assemblies, extensive co-localisation was seen with AP-2 and Dyn2, but not with AP-1 or markers for early endosomes or lysosomes. We demonstrated that SNX9 is involved in the endocytic process by the fact that over-expression of truncated versions interfered with the uptake of transferrin. In both HeLa and K562 cells, expression of C-terminally truncated variants containing the binding sites for dyn2, AP-2 and clathrin reduced endocytosis of transferrin as determined by immunofluorescence and biochemical analysis. Over-expression of proteins is commonly used to emphasise their role in a particular process. We believe that the effect we see on transferrin uptake is caused by sequestering of either AP-2 or dynamin. Endocytosis seem unaffected in cells expressing full-length SNX9, suggesting that this molecule is functional and incorporated into the machinery.

Regulated membrane recruitment of dynamin-2 is mediated by sorting nexin 9. [PAPER III]

Since SNX9 and Dyn2 formed a complex in the cytosol and also co-localised on membranes, we were curious to know what triggered the membrane binding of these molecules. We tested the membrane-binding activity of SNX9 and Dyn2 from cytosol by incubation in the presence of permeabilised K562 cells from which the cytosol had been depleted. We could show that proper membrane recruitment of SNX9 from the cytosol required incubation at 37°C in the presence of ATP. In addition, the non-hydrolysable GTP analogue GTPγS and ortovanadate, an inhibitor of tyrosine phosphatases, were needed for full activity. Under optimal conditions essentially all SNX9 associated with membranes. Dyn2 followed the binding pattern of SNX9 in terms of requirement, except for an absolute requirement for GTPγS. As expected, purified recombinant SNX9 bound to membranes in this assay without incubation at 37°C or any additional factors, suggesting that complex-bound SNX9 from cytosol is obstructed from membrane binding. It makes sense that proteins involved in assemblies and oligomerisation at membranes should somehow be regulated since such events would be detrimental to the cell if they occurred at the wrong location.
By separate immuno-depletion of cytosol, we demonstrated that SNX9 was essential for Dyn2 recruitment, whereas the reverse what not the case. Reconstitution of SNX9-depleted cytosol with recombinant full-length SNX9, but not with truncated variants of SNX9, restored membrane binding of Dyn2. This suggests that SNX9 is responsible for a regulated recruitment of Dyn2 and that the membrane-binding activity resides in the C-terminal region of SNX9 that has been shown to bind phosphoinositides (PAPER II). We therefore tested lipid binding of the cytosolic SNX9/Dyn2 complex in a liposome-based assay. Indeed, the requirements for activation were the same as in the previous assay, given that the liposomes contained phosphoinositides.

There could be several explanations for the regulated inhibition of membrane binding. Somehow the C-terminal region of SNX9 must be obstructed in the complex prior to activation. Since there was an additional component of the complex, this protein could function as an inhibitor. Surprisingly, the third component of the complex, p41, was identified by mass spectrometry as the glycolytic enzyme fructose 1,6-bisphosphate aldolase. We confirmed that aldolase was part of the complex using immunochemical detection. Mapping of the binding site in SNX9 showed that aldolase bound to an acidic region close to the binding sites of AP-2 and clathrin. Mutation of two tryptophans in this segment completely abolished the binding of aldolase. These results suggest that aldolase competes with AP-2 and clathrin for binding of SNX9, and since aldolase is a large molecule, the lipid binding activity of SNX9 might be sterically hindered by aldolase. As a direct test, we assayed liposome binding of recombinant SNX9 constructs in the presence of pure aldolase. Clearly, a minimal construct containing the aldolase- and phosphoinositide-binding sites was specifically inhibited in its membrane binding after the addition of aldolase. That common proteins like aldolase could be used as passive inhibitors of processes far from their main function is not unusual. It is conceivable that evolution has provided SNX9 with a docking site for aldolase that is used to prohibit membrane recruitment and assembly of two endocytic proteins.

How is then this complex activated? There should be a signal that coordinates membrane targeting with coated pit formation. Since activation of the complex involves ATP hydrolysis, a possible mechanism might involve phosphorylation. We could show that SNX9 does indeed become phosphorylated when activated for lipid binding. Concomitantly with phosphorylation of SNX9, aldolase is released from the complex. In vitro-phosphorylation assays showed that the region in SNX9 that was significantly phosphorylated was the low-complexity region containing the binding site for aldolase. This can be argued to be circumstantial rather than proof, but substantial evidence points to a role of phosphorylation in the activation step. The influence of ortovanadate on activation suggests a direct or indirect role of a tyrosine kinase. Tyrosine kinases mediate several signalling processes in the cell and tyrosine phosphorylation is known to activate other types of kinases like serine/threonine kinases. The regulatory mechanism we propose is in agreement with the emerging evidence that reversible phosphorylation function as a regulatory mechanism in clathrin mediated endocytosis (see 5.9)
To study the role of SNX9 in recruitment of Dyn2 \textit{in vivo}, we knocked down the expression of SNX9 using RNA interference. Immunofluorescence analyses of cells with reduced SNX9 expression showed that Dyn2 displayed a less punctuate and more diffuse staining in agreement with a role for SNX9 in recruiting Dyn2. As one of the controls, cells were depleted of AP-2 by siRNA. Interestingly these cells did not display an altered distribution of SNX9 (data not shown). Although SNX9 depletion reduced the number and intensity of Dyn2 assemblies at the membrane, part of Dyn2 could still be detected as characteristic puncta. We have however not been able to determine if this Dyn2 staining corresponds to coated pits at the plasma membrane. It is possible that SNX9 is responsible for specific targeting of Dyn2 to sites of vesicle creation. Since Dyn2 is used in a number of systems, it is plausible that in addition to SNX9, other proteins might mediate recruitment of Dyn2. This could occur by a different mechanism, as we would anticipate from our \textit{in vitro} data, were SNX9 was essential for Dyn2 recruitment. We could not detect an alteration of the cellular distribution of AP-2 or clathrin in SNX9 depleted cells, arguing for a specific role of SNX9 in Dyn2 membrane recruitment.

**GENERAL PERSPECTIVES**

During the last years a symphony of accessory endocytic proteins has been identified. The interaction mechanisms and characteristics of different accessory proteins are surprisingly similar which suggests that the endocytic machinery is based on an intricate network. Probably, accessory proteins are needed at several stages and sequential association will promote specific functions. One obvious question is if all these accessory proteins are needed for the general creation of a vesicle or if they are used in different tissues or in specific transport processes?

The work we have done on SNX9 led us to purpose a model of the function of SNX9 in vesicle creation in PAPER III. This model involves an appealing regulatory mechanism for recruitment of Dyn2 and implies additional roles of SNX9 at the coated pit. I believe that in the further perspective SNX9 will provide us with additional clues to the mechanisms behind vesicle creation. Studies dealing with the influence of SNX9 on the biochemical properties of dynamin, like oligomerisation and GTPase activity, will shed light on the regulation of vesicle constriction events. An interesting question in this era of new accessory proteins is at what stages of vesicle creation each protein is needed. The possible ability of SNX9 to sense membrane curvature generates obvious projects in characterising this capacity and its applications. I favour a view were different membrane binding molecules like SNX9 can sense the curvature of the maturating vesicle and recruit additional factors.

The discovery that AP-2 is not essential for all aspects of clathrin-mediated endocytosis and the fact that accessory proteins have been shown to recognise cargo molecules allow for a more general role of several of the accessory proteins. Sorting nexins are known to bind cargo molecules and direct their transport in the endosomes. Taken together that SNX9 has been shown to interact with transmembrane proteins like the metalloproteinases, DsCAM receptor, and Insulin receptor and the link to the core components of endocytosis suggests a
specific role for SNX9 in sorting. It has to be clarified if SNX9 functions in trafficking of individual molecules or if the role is more general for vesicle creation. The emerging links between cell signalling, endocytosis and actin rearrangements are appealing. We have found that SNX9 can interact with WASP, and cells depleted of SNX9 were small and showed less lamellipodia formation, indicating a role in actin rearrangement. Further studies will reveal the different roles of SNX9 and how these processes are coordinated.
CONCLUSIONS

• The β appendages of the four AP complexes have a similar structural organisation consisting of one or two subdomains but exhibit distinct interactions with accessory proteins.

• SNX9 can be considered as an accessory protein in endocytosis due to specific interactions of SNX9 with the appendage domains of AP-2 and with clathrin via tryptophane-based binding motifs.

• SNX9 recycles between the cytosol and the plasma membrane were it co-localises with Dyn2, AP-2 and clathrin, consistent with a role in coated pit formation.

• A direct SH3-PRD interaction generates a native cytosolic complex between SNX9 and Dyn2. A third component in the complex, identified as aldolase, obstructs the membrane binding activity of SNX9. Activation of the complex releases aldolase, leading to recruitment of SNX9 and Dyn2 to the membrane.

• Affecting the normal function of SNX9 in vivo results in impaired endocytosis and interferes with membrane targeting of Dyn2
ACKNOWLEDGEMENT

I would like to take the opportunity to thank the nice people at the department of Medical Biochemistry and Biophysics for creating a warm and friendly environment. Despite the quite small group that I have worked in I have never felt alone. You have always been supportive and easy to ask for help.

In addition, I would especially like to thank:

My supervisor Sven Carlsson: For your trust in me and the patience you have showed. Your nice personality and our common interests have made working together with you a real pleasure. I admire your knowledge and have especially enjoyed our long discussions from which I have learned a lot.

Ingrid: for your positive attitude and desire to help. Lola: for non-scientific chats and providing means to work. Urban: for supporting the teaching.

People in Eriks group: for sharing lab space and letting me know what it feels like to have lab mates. Per: for introducing me to over-expression. Martin: for help with CD analysis. Per Ingvar: for introducing me to Hörmän and the identification analysis
The gang from undergraduate studies: for your friendship and for understanding that anyone can do science

The PhD students: for understanding the importance of kindness and sympathy at work. The coffee break people: for an oasis at work. Artur: for exercising my vocabulary. Nasim: for your merry and daring personality. Pelle: for your rewarding friendship, positive attitude and curiosity. Magnus: for being a god thoughtful friend. You have shared my ups and downs during undergraduate studies, the PhD period and fishing trips.

Innebandy and football players: for keeping me in physical shape and putting up with my raging temper.

Linda och Jonas: for being great and loyal friends.

Mother and farther in law: for all your help and support with the kids.

My parents, Simon and Malin: for your care and the security you give me.

Samuel and Elias: for your unconditioned love and for reminding me what really matters. You’ve accepted that dad couldn’t play sometimes because he had to mix, and feed the cells.

Above all, my dear Ida whom I can’t thank enough. Our love and care keeps me going.
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