1 INTRODUCTION

1.1 Overview of membrane traffic in eukaryotic cells

Eukaryotic cells contain a variety of specialized organelles surrounded by single or double membrane bilayers to fulfill different functions inside the cell. Unlike prokaryotes in which cellular functions are mostly coordinated by diffusion in the cytosol, eukaryotes have to employ other strategies of intracellular communication, i.e. by regulated membrane trafficking.

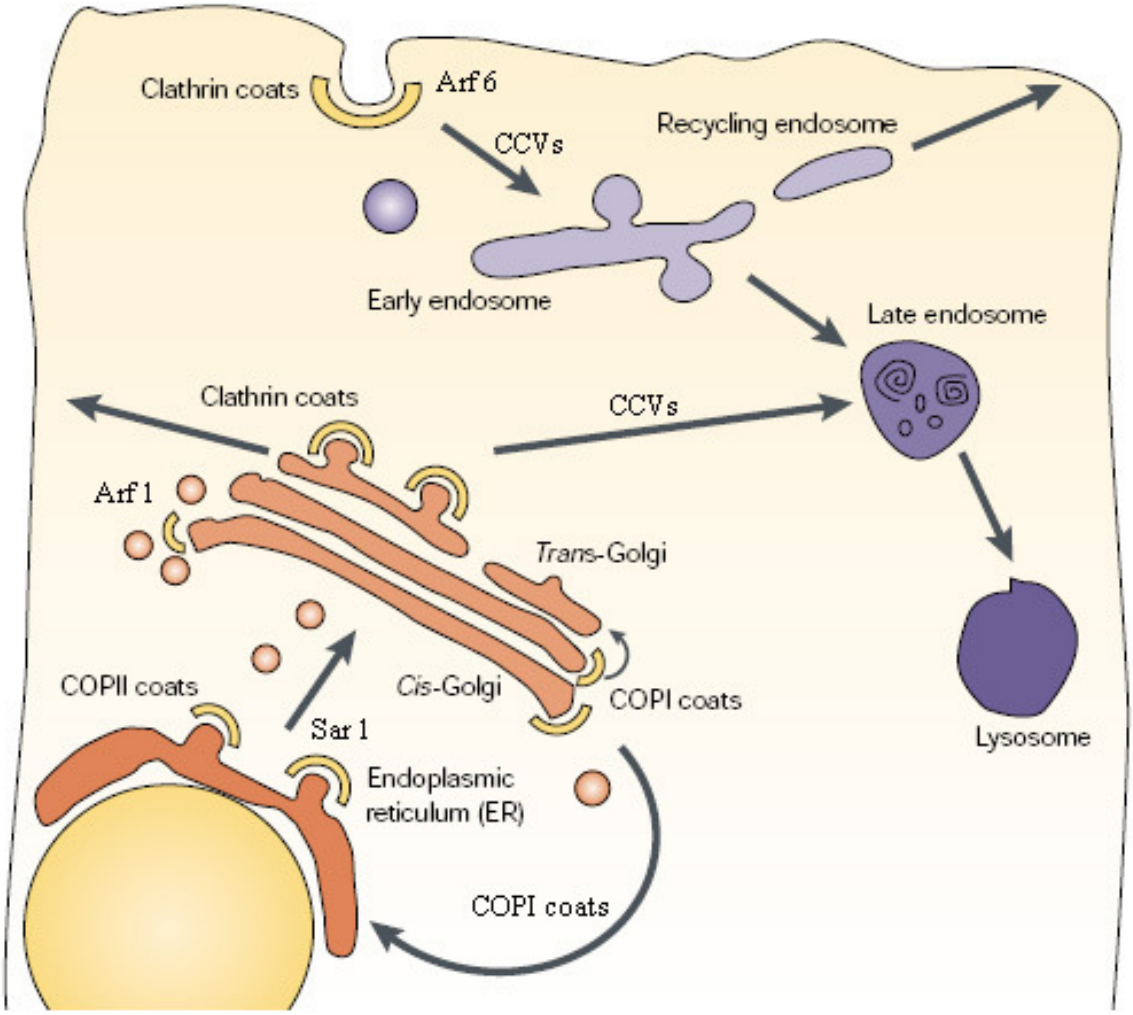

Figure 1-1 Membrane trafficking pathways of vesicle carriers coated with clathrin, COPII and COPI in eukaryotic cells

In the biosynthetic pathway, newly synthesized molecules are transported from the endoplasmic reticulum to the Golgi and from one cisterna of the Golgi to the next until they reach the *trans*-Golgi network. There, sorting occurs, directing traffic to the plasma membrane or to endosomes. In the endocytic pathway, macromolecules are internalized at the plasma membrane and forwarded to early endosomes, from where they are either recycled to the plasma membrane through recycling endosomes or forwarded toward degradation in late endosomes and lysosomes. Two regulatory proteins indispensable for coated-vesicle formation, the small GTPases Arf and Sar1, at certain donor organelle membranes are shown. CCVs, clathrin-coated vesicles (Modified from Kirchhausen T 2000).

Membrane trafficking includes multiple ways of transport facilitated by the cooperation of proteins, lipids and sometimes also carbohydrates. Pathways of membrane trafficking include endocytosis at the plasma membrane (PM), the secretory pathway from the endoplasmic reticulum (ER) via the Golgi apparatus to the plasma membrane, and the degradation route from endosomes to lysosomes. Most of these transport intermediates have to be encapsulated with membrane which can be formed at specialized domains of the donor organelle.

Frequently, membrane exchange along the secretory and endocytic pathways is mediated by coated-vesicle carriers with a diameter of 40-100 nm (**Figure 1-1**). Vesicle formation is accompanied by the recruitment of cytosolic or transmembrane cargo proteins at donor membranes which following budding are delivered to acceptor compartments. In this scenario, two basic questions arise. Firs, how do cargo proteins get selected and enriched in the coated-vesicle pits? Second, how is membrane curvature generated and maintained? To understand these questions, I will briefly summarize the current view on the molecular mechanism of coated-vesicle mediated membrane trafficking.

1.2 Scaffolding, adaptor, regulatory proteins and cargo in coated-vesicle mediated membrane trafficking

Trafficking vesicles have been identified by their different coat proteins decorating the cytoplasmic side of the vesicle membrane. Three of the best-understood coat proteins identified are: COPII, COPI (COP stands for coat protein complex) and clathrin (Kirchhausen 2000). COPII-coated vesicles mediate anterograde transport from the ER to the early Golgi, whereas COPI-mediated transport contributes to both inter-Golgi cisternal and retrograde traffic from the Golgi to the ER. Clathrin is used at different locations including the *trans*-Golgi network (TGN), endosomes and the PM in combination with specific adaptor proteins and thus, may contribute to both the secretory and endocytic pathways.

1.2.1 Vesicle coat scaffolding proteins

Naked small lipid vesicles with diameters of 40-00 nm are energetically unfavorable due to the high membrane tension and curvature. Although coated vesicles are supposed to undergo an uncoating process either immediately after vesicle fission from the donor membrane or just before docking and priming on the acceptor membrane, biochemically isolated trafficking vesicles were shown to be protein-coated. Thus, vesicle coats represent scaffolding proteins needed for the stability of vesicles at least during budding. One general

property of all coat proteins is their intrinsic curvature which should fit the highly bent vesicle membrane.

1.2.1.1 Clathrin

The basic assembly unit of clathrin is a trimer called triskelion from the Greek for three legged. The triskelion is composed of three heavy chains (~190 kDa) and three affiliated light chains (~25k Da) (**Figure 1-2 A**). The intrinsic curvature upon triskelia polymerization is supposed to bend the membrane and form a coated vesicle. A recent high resolution subnanometer cryo-EM analysis of *in vitro* reconstructed clathrin assemblies revealed how triskelia form the clathrin lattice (**Figure 1-2 B**) (Fotin, Cheng et al. 2004). Clathrin lattices and coated buds can be seen on the inner leaflet of the PM under electron microscopy (**Figure 1-2 C**).

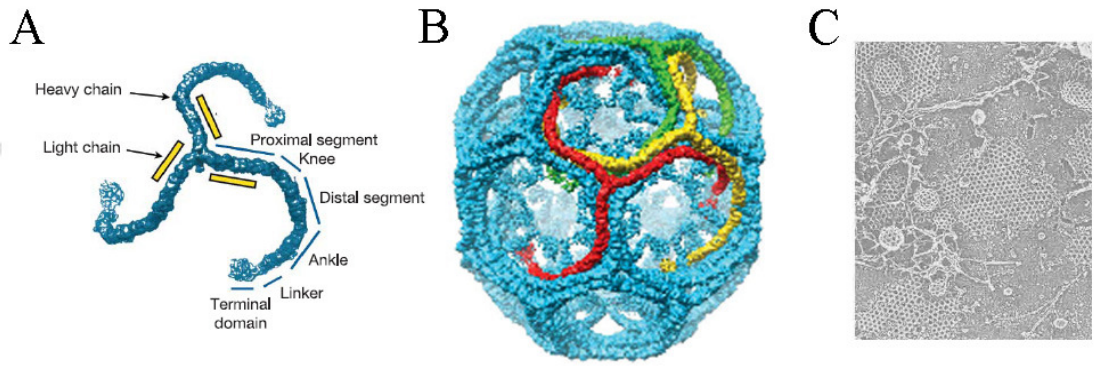

Figure 1-2 Clathrin structure and clathrin-coated vesicles

(A) Schematic representation of a clathrin triskelion. The N terminal end of the heavy chain is at the terminal domain, the C terminal at the vertex. Positions of the light chains are shown schematically. (B) Reconstruction of a clathrin coated vesicle based on Cryo-EM data. There are 36 clathrin triskelia in the structure, which has a D6 symmetry. Thus, there are three symmetry-independent triskelia (or nine symmetry-independent legs). The coloured triskelia show one choice of the three independent triskelions. Noisy central density, from spatially disordered and substoichiometric AP-2 complexes, has been flattened. (Taken from Fotin A *et al.*, 2004). (C) Free fracture EM picture of CCV from inner plasma membrane (Courtesy of John Heuser, Washington University).

1.2.1.2 COPII

COPII coats are comprised of the five different subunits Sar1, Sec23/24 and Sec13/31. Sar1 is a small GTPase similar to Arfs (ADP-ribosylation factor), but lacks myristoyl modification. Its membrane recruitment is mediated by Sar1 specific GEFs (Guanine nucleotide-exchange factors) at ribosome-free ER membranes (Barlowe, d'Enfert et al. 1993; Yoshihisa, Barlowe et al. 1993). Membrane anchored Sar1 then recruits the Sec23/24 heterodimer (Yeung, Yoshihisa et al. 1995) to the membrane where Sec23 works as a GAP (GTP-activating protein) for Sar1 and Sec24 engages IN cargo binding (Aridor, Weissman et al. 1998; Miller, Beilharz et al. 2003; Aridor, Fish et al. 2001). The outer most layer of COPII coats is formed

by the Sec13/31 complex recruited by the Sec23/24 (Lederkremer, Cheng et al. 2001) to assist the final step of vesicle formation. Sec23/24 and Sec13/31 complexes display curved (Miller, Beilharz et al. 2003) and cage-like structures (Stagg, Gurkan et al. 2006), respectively.

1.2.1.3 COPI

The COPI consists of seven proteins including α, β, β', γ, δ, ε, ζ which display a limited structural similarity to clathrin and heterotetrameric AP complexes (see below). The AP-2 (Adaptor protein 2, see below "Adaptor protein") α appendage domain is conserved in β- and γ-COP (Hoffman, Rahl et al. 2003; Watson, Frigerio et al. 2004), δ-COP is similar to μ_2 as involved in cargo binding (Andag and Schmitt 2003). To initiate COPI coatomer recruitment to Golgi membranes; Arf1 in its active form is needed as in the case of COPII coat recruitment by Sar1. The GTP hydrolysis rate of Arf1 depends on associated GAPs and determines the time-point of vesicle uncoating. Therefore, both Sar1 and Arf1 work as a timer for COPII- and COPI-coated vesicle maturation, respectively.

1.2.2 Adaptor proteins

Two slightly different mechanisms are used in coated vesicle formation. The simpler one is that coat proteins directly attach to the membrane themselves as exemplified by COPII and COPI. In the case of COPII, two of the five proteins are responsible for direct membrane recruitment. Sar1 binds to a cognate GEF at the ER which triggers a GDP-GTP exchange to release its amino-terminal amphipathic helix for membrane insertion and stabilization (Lee, Orci et al. 2005); the other stabilization factor is Sec24 in the pre-budding complex which can bind to sorting motifs in the cytosolic tail of membrane cargo proteins. For COPI, both the small GTPase Arf1 and δ-COP are required for membrane anchoring (Bethune, Wieland et al. 2006). The more complicated process is CCV formation where clathrin itself does not bind directly to membrane and cargo (Dell'Angelica 2001). This missing link is supplied by adaptor proteins that can simultaneously bind membrane and cargo on one side and clathrin on the other side and also provide "cross-linking" capacity.

Based on this criterion, many adaptor proteins have been identified at various membranes where they are used for clathrin-mediated endocytosis (CME) or clathrin coated vesicle formation at internal membranes. These are collectively named clathrin-associated sorting proteins (CLASPs) which include ARH (autosomal recessive hypercholesterolemia) (He, Gupta et al. 2002), Disabled-2 (Morris and Cooper 2001), numb (Santolini, Puri et al. 2000),

β-arrestin (Zhang, Ferguson et al. 1996; Goodman, Krupnick et al. 1996) and AP-2 etc (Traub 2003). Schematic representation of monomeric adaptor proteins is shown in **Figure 1-3**.

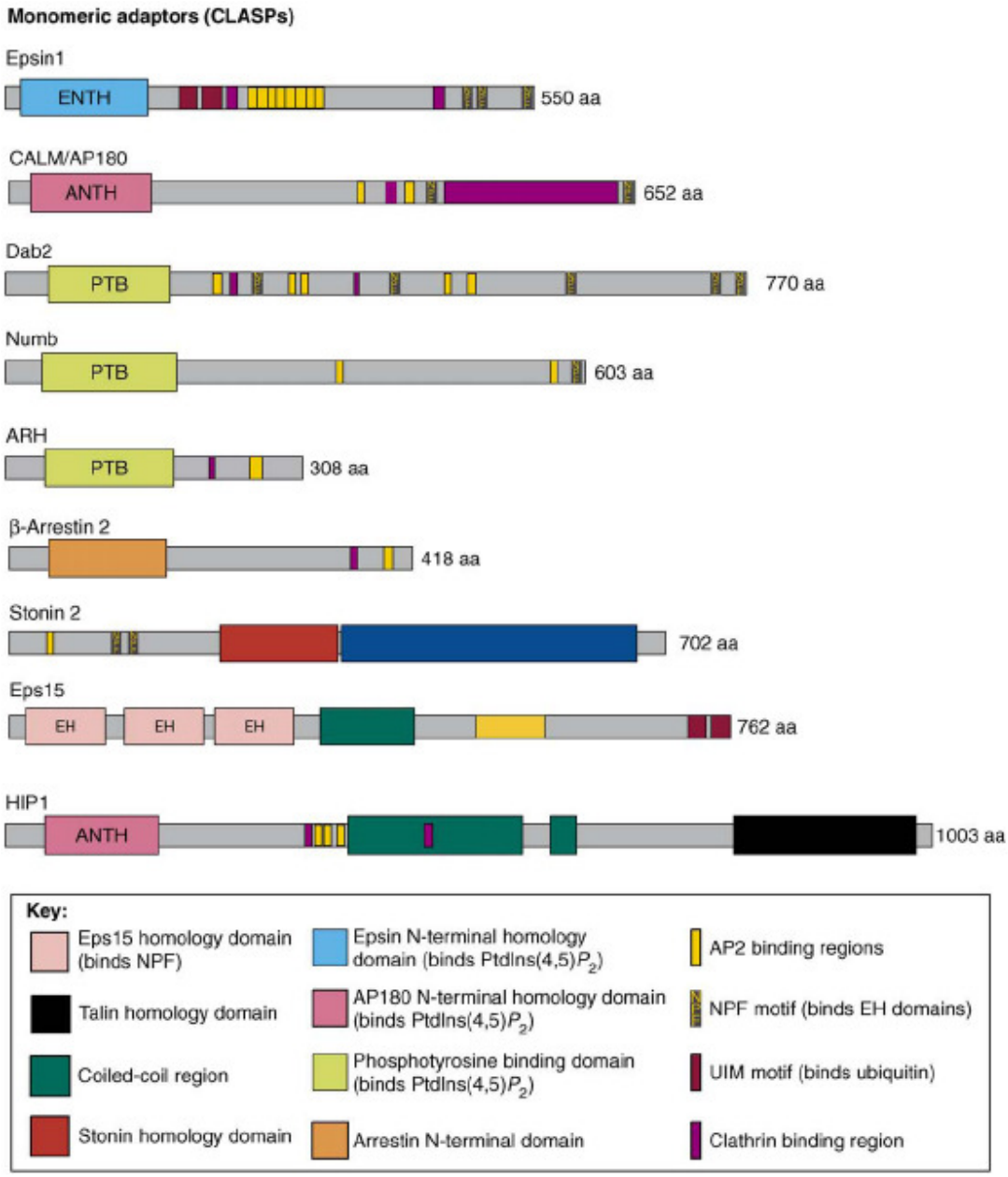

Figure 1-3 Monomeric clathrin endocytic adaptors

A schematic representation of the overall domain/motif organization of selected human endocytic adaptor proteins is shown. Thick boxes indicate folded domains and thin boxes indicate unstructured regions (Modified from Maldonado-Baez and Wendland 2006).

1.2.2.1 Heterotetrameric assembly (adaptor) protein (APs)

The first clathrin-binding adaptor identified was the heterotetrameric AP-2 complex. Early EM studies of CCVs by Pearse *et al.* (Vigers, Crowther et al. 1986) showed that a protein moiety was positioned between clathrin and the enclosed membrane. The AP-2 complex then turned out to be the first identified member of a heterotetrameric adaptor family which now

includes AP-1, aforementioned AP-2, AP-3, AP-4 and β, γ, δ and ζ subunits of COPI (Owen 2004). AP complexes comprise a proteolysis-resistant trunk or core domain and two flexible appendages or ear domains (**Figure 1-4**). The AP-2 200 kDa trunk core domain consists of μ_2, σ_2 and the amino-terminal parts of α and β_2 (Collins, McCoy et al. 2002), The Carboxy-terminal regions of α and β_2 fold into the ear domains (Owen, Vallis et al. 1999; Owen, Vallis et al. 2000) linked with the trunk domain by proteolytically sensitive linker regions. The structure of AP-2 is consistent with its role as a central protein-protein interaction hub in CME. AP-2 is believed to exist in two conformational statuses involve the association of amino-terminus of its α subunit with the phosphatidylinositide (4,5)- bisphosphate ($PI(4,5)P_2$)-enriched hot spots on the plasmalemma. In this conformation the binding site for tyrosine-based endocytic motifs (see below "cargo selection") in μ_2 is buried within the core domain and can not engage in cargo recognition. A presumed conformational change induced by phosphorelation of Thr156 μ_2 by AAK1 (adaptor associated kinase 1) or GAK (cyclin G-associated kinase) exposes the tyrosine motif binding pocket and the second $PI(4,5)P_2$ binding site within the Carboxy-terminal domain of μ_2 (Honing, Ricotta et al. 2005). Acidic dileucine sorting motif in CME is discussed in the section of "cargo selection". Additional interactions between the β-hinge region and the clathrin heavy chain aid in clathrin lattice formation at the cargo-rich sites (Edeling, Mishra et al. 2006). In addition to the essential components for coat formation, CME needs variety of accessory proteins functioning in curvature generation, fission, uncoating, and actin rearrangements.

The α appendage domain of AP-2 consists of two subdomains, a platform and a sandwich domain (Traub, Downs et al. 1999; Owen, Vallis et al. 2000). The platform domains can recruit accessory proteins including epsin (Drake, Downs et al. 2000), amphiphysin (Slepnev, Ochoa et al. 2000), Eps15 (Owen, Vallis et al. 1999) through DX[F/W] (K_d=120μM) and FXDXF (K_d=30-50μM) motifs whereas the sandwich domain interacts with W××F motifs (K_d<10μM) present in other accessory proteins, for example stonin2 (Jha, Agostinelli et al. 2004; Walther, Diril et al. 2004), synaptojanin (Jha, Agostinelli et al. 2004) and NECAPs (adaptin-ear-binding coat-associated protein) (Ritter, Denisov et al. 2004). Compared with the low affinity binding of accessory proteins to the α appendage domain, the binding site for motifs found in ARH (autosomal recessive hypercholesterolemia) and β-arrestin (Mishra, Keyel et al. 2005) within the β_2 appendage is of comparatively high affinity (K_d=1-2μM). Clathrin can be recruited by a canonical clathrin box, which is a linear five amino acid

peptide of LΦxΦ [DE] (where Φ is a bulky hydrophobic residue) within the linker region of β_2 subunit (Owen, Collins et al. 2004).

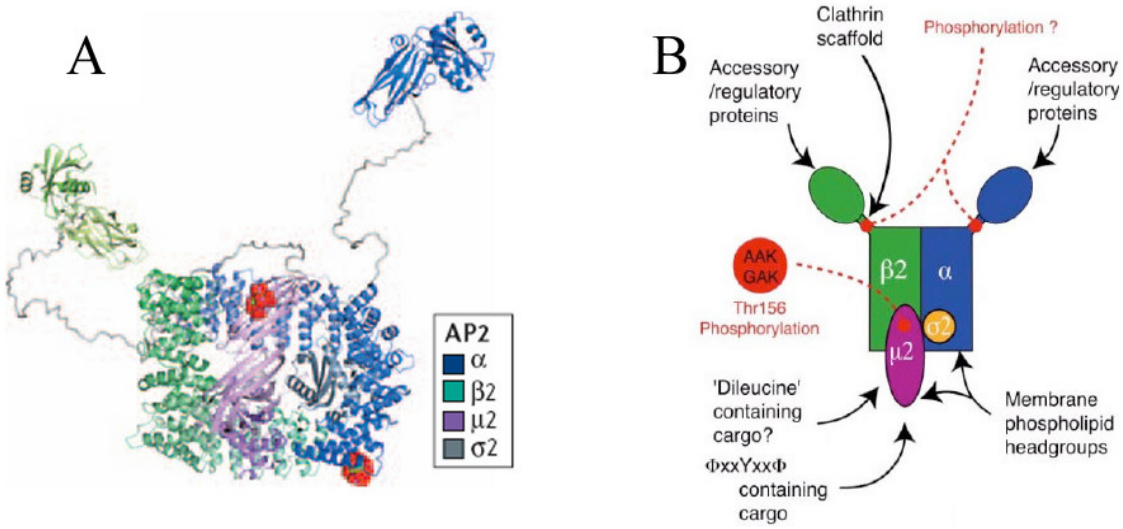

Figure 1-4 Structural model of the AP-2 adaptor complex

(A) Model of AP-2 adaptor was made using structures that were determined by x-crystallography. This structure is in an inactive, closed state, because its μ-subunit YXXΦ (X represents any amino acid and Φ represents a large hydrophobic residue) is blocked by its β-subunit (Edeling, Smith *et al*. 2006). (B) Profile of protein and lipid binding to AP-2 complex (Owen and Evans, 2006).

1.2.2.2 GGAs

Compared with the rich arsenal of adaptor proteins available at the PM for CME, only three APs are known to work within the TGN/endosome system. AP-3 and AP-4 work in a partial overlapping way to AP-1 and share structure similarity with endocytic adaptor AP-2. Since α and γ appendage domains of AP-2 and AP-1 serve as a hub for accessory protein recruitment, searching for new proteins based on potential γ ear similarity characterized the frist time the monomeric adaptor protein at TGN GGAs (Golgi-localized, γ-ear containing, Arf-binding family of proteins) by several groups (Boman, Zhang et al. 2000; Dell'Angelica, Puertollano et al. 2000; Hirst, Lui et al. 2000; Poussu, Lohi et al. 2000). They are monomeric proteins with a Carboxy-terminal domain which is similar to the Carboxy-terminal γ_1 and γ_2-ear domain of AP-2 adaptin isoforms. They are ubiquitously expressed and localized in TGN and endosomes. There are three human GGAs with GGA1 illustrated in **Figure 1-5 A**.

The amino-terminal sequence encodes a ~140-residue VHS (Vps27, Hrs, Stam) domain that is also found in other proteins like yeast Vps27 (vacuolar protein sorting 27), Hse1 (Hbp, Stam, EAST) and their mammalian orthologues. Like heterotetrameric APs the VHS domain of GGAs can bind specifically to DXXLL (where X is any amino acid) modules which can be

found in the cation-dependent (CD-MPRs) and cation-independent MPRs (CI-MPRs) (Puertollano, Aguilar et al. 2001); (Zhu, Doray et al. 2001; Takatsu, Katoh et al. 2001). X-ray crystalographic structure analysis showed that the important residues in mammalian GGAs for DXXLL sorting motif binding were not found in other VHS domain containing proteins like TOM1 (target of myb1), Hrs (hepatocyte-growth-factor-receptor substrate), Stam (signal-transducing adaptor molecule).

A short linker region of ~20 residues connects VHS to the next ~150 residue GAT (GGA and TOM) domain. The first hint of its function came from two studies where the authors showed that the GAT domain can bind to GTP-Arf1 and Arf3 but not GDP-bound Arfs (Dell'Angelica, Puertollano et al. 2000; Boman, Zhang et al. 2000). Another protein shown to interact with the GAT domain is Rabaptin 5 which is a Rab4/Rab5 effector localized in endosomal compartments.

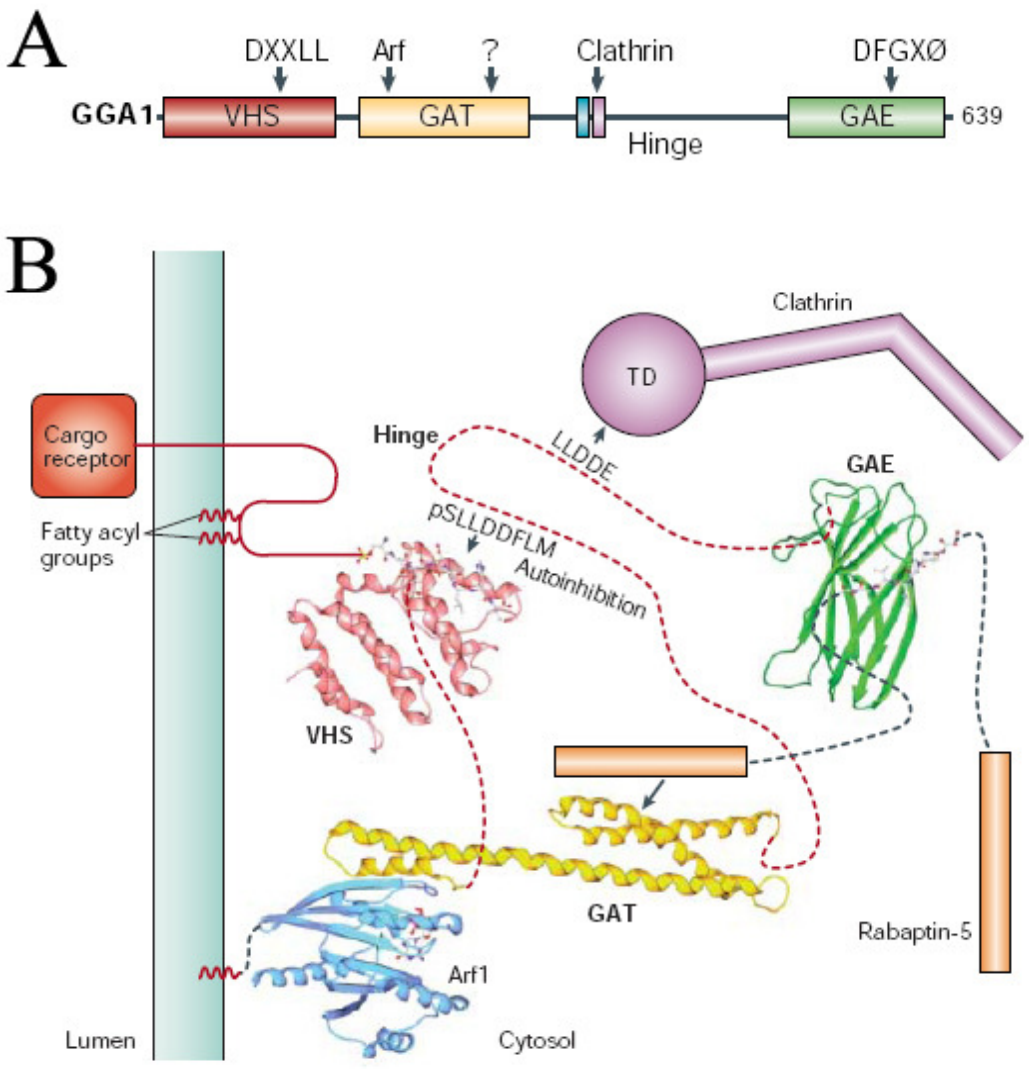

Figure 1-5 Schematic domain structure of GGA1 and assembly of GGA on membranes

(A) Schematic representation of GGA1 domain organization. The sequences or proteins that bind to each domain are indicated by arrows. The VHS (Vps27, Hrs, Stam) domain binds DXXLL-type sequences (where X is any amino acid) similar to an autoinhibitory DXXLL sequence in the hinge segment of the GGAs. The GAT (GGA and TOM (target of myb)) domain has two binding sites, one for Arf and the other for an unidentified protein that is indicated by a question mark. The hinge segment of GGA1 contains variants of the clathrin-box motif (LLDDE in GGA). The GAE (γ-adaptin ear) domains bind DFGXØ-type sequences (where Ø is a bulky hydrophobic residue). (B) Cartoon representation of the assembly of a GGA-containing carrier. Membrane-tethered Arf (ADP-ribosylation factor)–GTP (blue) binds to the GAT (GGA and TOM (target of myb); yellow) domain, which results in the recruitment of the GGA (Golgi-localized, γ-ear-containing, Arf-binding protein) to the membrane. The VHS (Vps27, Hrs, Stam; pink) domain binds DXXLL-type signals (where X is any amino acid) in the tails of mannose-6-phosphate receptors and other transmembrane cargo. An autoinhibitory, internal DXXLL sequence (that is, pSLLDDFLM in GGA1, where pS indicates phosphoserine) in the hinge segment regulates signal recognition. The hinge segment binds through clathrin-box-like sequences (for example, LLDDE in GGA1) to the terminal domain (TD) of clathrin, and the GAE domain (γ-adaptin ear; green) binds through DFGXØ-like sequences (where Ø is a bulky hydrophobic residue) to accessory proteins. The order of these different steps has not been established. Other proteins such as adaptor protein (AP) 1 and enthoprotin/Ent5 might intercalate into these coats and might also participate in the recruitment of clathrin, cargo and accessory proteins. The red dashed lines represent the unstructured sequences in the GGA, and the black dashed lines represent the unstructured sequences in Rabaptin5 (Taken and modified from Bonifacino JS 2004).

The largely unstructured hinge segment following the GAT domain contains several clathrin binding peptides which were shown to interact with clathrin *in vitro* (Costaguta, Stefan et al. 2001; Mullins and Bonifacino 2001; Zhu, Doray et al. 2001). GGA1 and GGA3 hinge regions also contain an internal DXXLL motif that can bind to the VHS domain to cause autoinhibition, which can be released by phosphorylation on the flanking residues (Doray, Bruns et al. 2002; Ghosh and Kornfeld 2003).

The Carboxy-terminal GAE (γ-adaptin ear) domain has been found to interact with a cohort of accessory proteins including γ-synergin (Page, Sowerby et al. 1999), p56 (Lui, Collins et al. 2003), Rabaptin5 (Shiba, Takatsu et al. 2002), enthoprotin/EpsinR/CLINT (clathrin interacting protein localized in the *trans*-Golgi region) (Wasiak, Legendre-Guillemin et al. 2002; Mills, Praefcke et al. 2003; Kalthoff, Groos et al. 2002). These proteins contain canonical DFGXØ motifs (where Ø is a bulky hydrophobic residue). The GAE domain can also interact with clathrin (Puertollano, Randazzo et al. 2001), but the underlying mechanism is not known.

A cartoon scheme of how GGA1 coordinate cargo, Arf1 and clathrin binding at the TGN is illustrated in **Figure 1-5 B**.

1.2.3 Arf GTPases and vesicle budding

Small GTPase such as either Arfs or Sar1 directly or indirectly regulate coat protein recruitment to membrane. As mentioned above, Sar1 is a component of the COPII coat complex and Arf1 is indispensable for the recruitment of COPI in the Golgi apparatus. In

clathrin mediated transport, Arfs seem to function as regulators to recruit adaptor proteins like APs or GGAs to membranes depending on the location of their cognate GEF proteins and presence of membrane phosphoinositides.

Arfs belong to the Ras superfamily of small GTP binding proteins. Although Arfs were originally identified as cofactors for cholera-toxin-catalyzed ADP-ribosylation of the α-subunit of heterotrimeric G proteins in a cell-free *in vitro* system (Kahn and Gilman 1986; Kahn and Gilman 1984), they were later shown to work as regulators of different cellular trafficking pathways (Roth 1999; Donaldson 2003).

Like all small GTP-binding proteins, Arfs undergo cycles of GTP- and GDP-induced conformational changes. GTP hydrolysis is GAP-dependent and GDP-GTP replacement is activated by GEFs. All Arfs are post-translationally modified by amino-myristoylation, which is critical for their tethering to membranes *in vivo*.

There are 6 members of mammalian Arfs that can be categorized into 3 classes. Class I proteins Arf1, Arf2 (lost in human) and Arf3 share 96% identity and regulate different secretory and trafficking pathways (Bonifacino and Glick 2004). The function of class II (Arf4, Arf5) Arfs still remains largely unknown but they are suggested to be involved in TGN/endosomal transport with an overlapping and redundant role with class I Arfs (Volpicelli-Daley, Li et al. 2005; Claude, Zhao et al. 1999; Takatsu, Yoshino et al. 2002; Liang and Kornfeld 1997), they are 90% identical to each other and 81% to Arf1. The only member of the class III family is Arf6 that is the most divergent one with 66-70% identity to other Arfs and believed to work at the PM and in recycling endosomal trafficking (D'Souza-Schorey and Chavrier 2006). Other structurally related proteins include Arf-like proteins (ARL) (Burd, Strochlic et al. 2004), Arf-related protein ARFRP (Mueller, Joost et al. 2002) and Sar1 (secretion-associated and Ras-related protein) (Nakano and Muramatsu 1989). The essential differences between Arfs and other Arf-related proteins are the former (1) serve as cofactors for cholera toxin, (2) rescue the lethal *arf1*$^{-}$*arf2*$^{-}$ deletion in *Saccharomyces cerevisiae*, and (3) directly activate phospholipase D (PLD).

The crystal structures of non-myristoylated Arfs have been solved in both GDP- and GTP-bound conformations. The two β strands (also called interswitch) that connect the nucleotide-sensitive switch 1 and switch 2 regions adopt a distinct conformation in the GDP- versus the GTP-bound forms. (Amor, Harrison et al. 1994; Goldberg 1998; Menetrey, Macia et al. 2000;

Pasqualato, Menetrey et al. 2001). When GDP is bound, the amino-terminal amphipathic helix and myrsitoyl group lock and cap the interswitch region which is retracted to the core structure of Arfs. A GTP-induced conformational change then releases the amphipathic-helix and myristoyl group followed by a two amino acid residue out-shift of the interswitch which will engage in the interaction with other proteins (**Figure 1-6**).

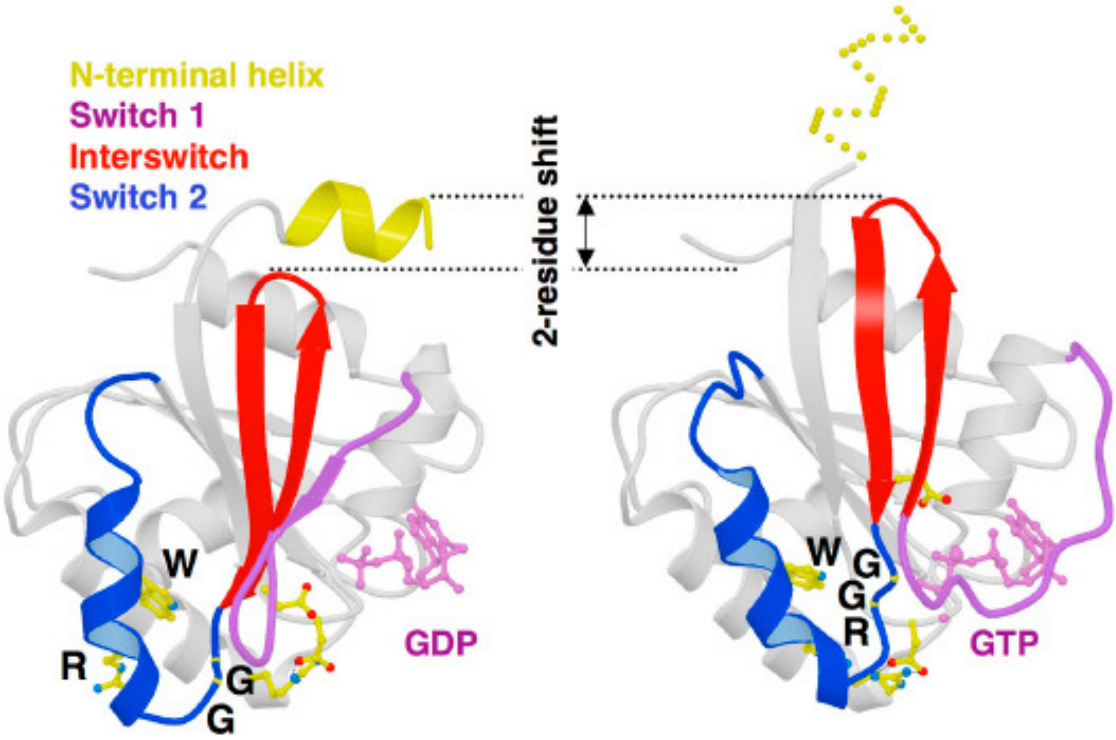

Figure 1-6 Interswitch toggles of Arfs

The retracted interswitch region (in red) in the GDP-bound conformation is locked by the NH_2-terminal helix; upon GTP replacement of GDP, a two-residue distance movement was initiated by the release of the amino-terminal helix and strengthened by the W/GG/R signature (GDP- and GTP-bound Arf6 are shown here) (Kahn, Cherfils *et a*l. 2006).

Arfs only function in their active states on membranes where GEF proteins initiate the nucleotide exchange from GDP to GTP. It is thus reasonable to speculate that the localization of GEFs for different Arfs contributes to their versatile cellular functions. Brefeldin A (BFA), a fungal metabolite, prevents completion of the nucleotide exchange reaction of Sec7-domain-containing GEFs through a non-competitive mechanism in which the drug traps the Sec7 domain together with GDP-bound Arfs in an abortive complex (Magner and Papagiannes 1988; Morinaga, Tsai et al. 1996). Many Sec7-domain containing GEFs for Arf1 have been identified to function at different intracellularlocations. These proteins include GBF1 (Golgi-associtated BFA-resistant) at the TGN, BIG1 and BIG2 at the *cis*-Golgi and Golgi/endosomal compartments, respectively (Yamaji, Adamik et al. 2000; Zhao, Lasell et al. 2002; Garcia-Mata, Szul et al. 2003; Niu, Pfeifer et al. 2005; Szul, Garcia-Mata et al. 2005; Zhao, Claude et al. 2006). Identified GEFs for Arf6 include the EFA6 family proteins and

Arf-GEP100/BRAG2 (Dunphy, Moravec et al. 2006; Sakagami, Suzuki et al. 2006). They are insensitive to BFA treatment.

The two best-understood Arfs are Arf1 and Arf6. Arf1 at the TGN can recruit the adaptor proteins AP-1 and GGA to facilitate subsequent membrane targeting of clathrin. Arf1 is essential for the COPI coat assembly at the Golgi apparatus. Arf6 either directly (Paleotti, Macia et al. 2005) or indirectly via stimulation of phosphatidylinositol-4-phosphate kinase I gamma (PIPKIγ) and local generation of $PI(4,5)P_2$ (Krauss, Kinuta et al. 2003) contributes to the recruitment of the clathrin adaptor AP-2.

1.2.4 Cargo selection

Coated vesicles are utilized to selectively transport cargo from one organelle to another compartment according to the cellular requirements. As coated vesicles form at the donor membrane, soluble or transmembrane proteins are selected as cargos to pre-existing coated pits. This process is facilitated by the recognition of linear peptide motifs within the cytosolic tails of transmembrane cargo proteins themselves or receptors for soluble cargos by either coat (COPII and COPI) or adaptor proteins (clathrin coated vesicle).

1.2.4.1 Sorting signals in CME

The best-understood mechanism of cargo selection is the direct interaction of the YXXΦ (where Φ is a bulky hydrophobic residue and X is any residue) motif by the μ_2 subunit of AP-2. According to crystallographic data a YXXΦ-containing peptide in an extended conformation is sitting in a hydrophobic pocket of AP-2 μ2 (Owen and Evans 1998). A distinct class of tyrosine based motifs, FXNPXY, was first found in the cytosolic tail of low density lipoprotein (LDL) receptor which can bind to the phosphotyrosine-binding (PTB) domain of Disabled-2 (Dab2) and to autosomal recessive hypercholesterolemia (ARH) through its non-phosphorylated NPXY motif. Another endocytic motif is the acidic cluster dileucine motif [D/E]XXXL[L/I] (where residues at position four and, less frequently, five from the first leucine are typically acidic, X is any amino acid and the second leucine can be replaced by isoleucine) which is present in a plethora of transmembrane proteins and is recognized by the trunk domain of the β2 subunit of AP-2 as detected using photoactivatable cross-linking (Rapoport, Chen et al. 1998) or by μ2 subunit as suggested by *in vitro* affinity binding assay (Rodionov and Bakke 1998). Monoubiquitylation, the convalent attachment of a single ubiquitin molecule to a protein can also serve as sorting signals for CME (Polo, Sigismund et al. 2002). Endocytic proteins like epsin and Eps15 contain UBD (ubiquitin-

binding domains) that can recognize ubiquitylated cargos at the PM and undergo ubiquitylation modification themselves at distinct sites from UBD domains. Mechanisms of cargo sorting in CME are illustrated in **Figure 1-7**.

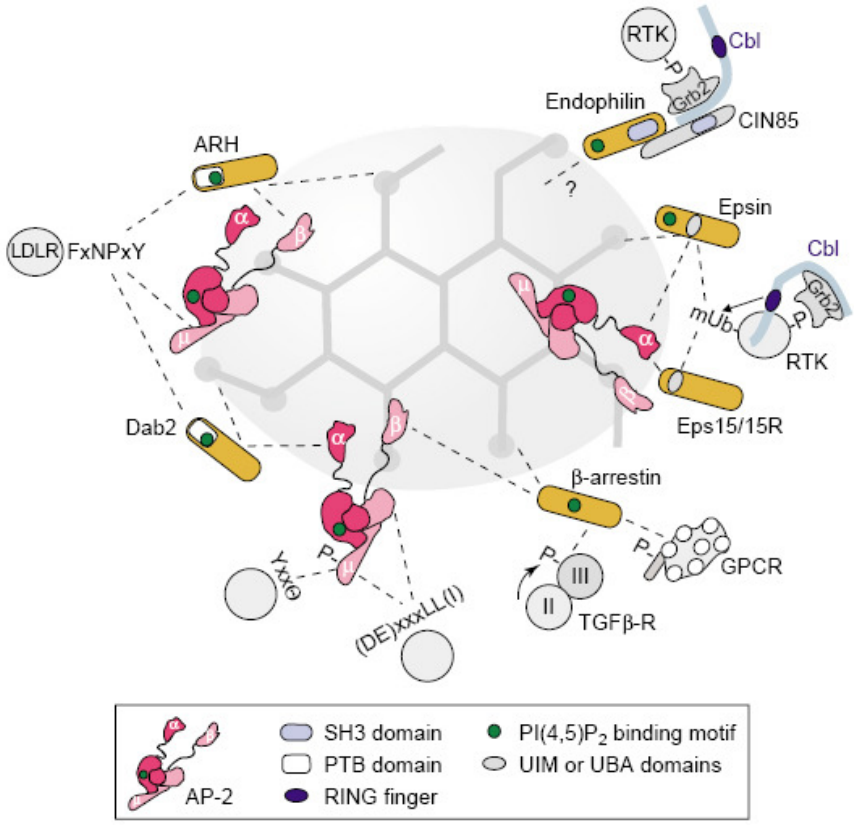

Figure 1-7 Mechanisms of cargo recruitment into clathrin coated pits

Possible interactions of adaptor proteins with cargos, clathrin are shown by dashed lines. Arrows show enzymatic reactions of protein ubiquitinylation and phosphorylation. The question mark indicates that the mechanism of the recruitment of the endophillin-CIN85 complex into coated pits is unclear. P, phosphorylated tyrosine, serine or threonine residues; Ub, monoubiquitin; x, variable residue; Θ, bulky hydrophobic residue (Sorkin A 2004).

One specialized form of CME is used at central nervous system synapses where synaptic vesicles are retrieved following exocytosis (Maycox, Link et al. 1992). Synaptotagmin 1, a type I synaptic vesicle transmembrane protein, had long been believed to regulate Ca(2+)-dependent fast synchronous neurotransmitter release. It has also been shown to recruit AP-2 through its C2 domain and thus as nucleation site for CME of synaptic vesicles (Zhang, Davletov et al. 1994). One interesting observation is that two putative tyrosine based endocytic motifs present in the synaptic vesicle transmembrane protein SV2 (synaptic vesicle protein 2) can stimulate binding of synaptotagmin 1 to AP-2 (Haucke and De Camilli 1999). Since synaptic vesicles contain many exclusive proteins that do not contain any obvious endoytic motif, one open question is how these proteins are sorted together with synaptotagmin 1 and SV2 to maintain synaptic vesicle composition and sustain the vesicle cycle. More details of the SV cycling will be discussed in section 1.4.

1.3 Membrane bending and fission

1.3.1 Membrane deformation

Vesicle-mediated membrane traffic is intimately linked to membrane deformation. Compared with cellular organelles and dynamic membrane re-shaping like pseudopodia and lamellipodia, the multi-step coated-vesicle budding process requires a much more exquisite regulation by cytosolic proteins due to its small size (40-100nm). Possible mechanisms for membrane deformation are summarized in **Figure 1-8**. These include lipid composition changes, membrane oligomerization, cytoskeleton rearrangement, scaffolding protein bending and helix insertion.

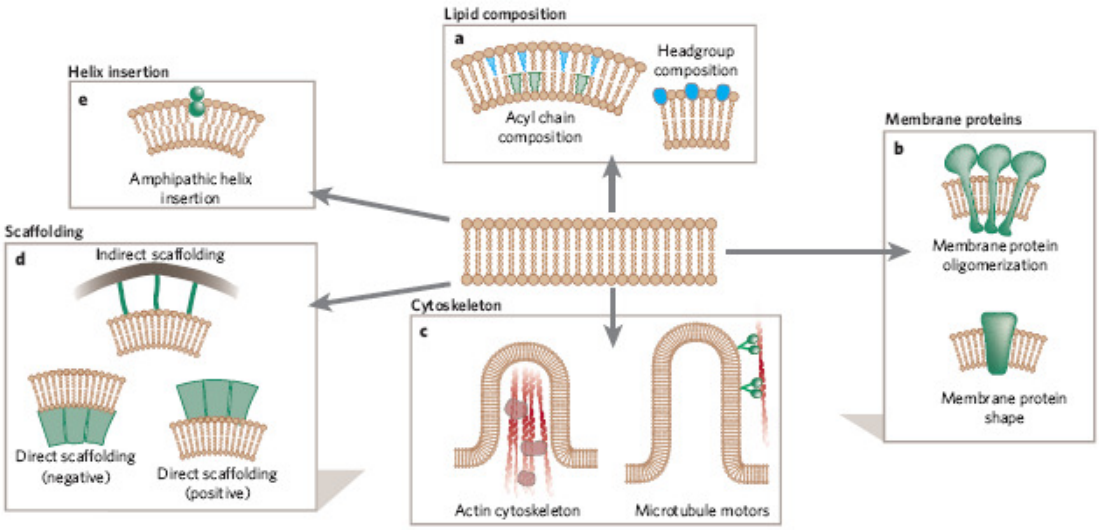

Figure 1-8 Mechanisms of membrane deformation

Five different mechanisms (a-e) to deform membranes are summarized (Taken from Gallop and McMahon, 2005). a, asymmetric membrane lipid distribution, for example, Bis(monoacylglycero)phosphate (LBPA) at the multivesicular body (Matsuo, Chevallier et al. 2004), or lipid shape changes due to modification of fatty acyl chains or headgrourp; b, oligomerization of membrane proteins (Bauer and Pelkmans 2006; Voeltz, Prinz et al. 2006); c, cytoskeleton polymerization; d, direct or indirect curvature induction by scaffolding proteins (Habermann 2004); e, amphipathic helix insertion into one leaflet of the membrane bilayer (Ford, Mills et al. 2002; Lee, Orci *et al*. 2005).

For cargo selection and enrichment at coated-pits, local positive curvature (a curvature pointing toward the cytoplasm) is needed to initiate vesicle budding from the relatively flat donor membrane. One of the first hints of how coated-vesicle curvatures are achieved came from a pioneering study by Takei *et al.* (Takei, Haucke et al. 1998) when an *in vitro* system was established to produce coated intermediates of CME on protein-free liposomes. By incubating cytosol, clathrin coat proteins or purified dynamin, respectively with liposomes of defined composition, vesicle buds and tubules similar to the synaptic membrane budding

process in CME were observed. This finding indicated that cytosolic coat proteins play a fundamental role in deforming lipid bilayers in clathrin-mediated budding events.

Using the *in vitro* assay developed by Takei and colleagues, a varity of proteins has been found to be involved in different stages of coated-vesicle budding on donor lipid membrane or membrane tubulation (Takei, Slepnev et al. 1999; Farsad, Ringstad et al. 2001; Ford, Mills et al. 2002; Lee, Marcucci et al. 2002; Peter, Kent et al. 2004; Itoh, Erdmann et al. 2005; Lee, Orci et al. 2005; McMahon and Gallop 2005; Roux, Uyhazi et al. 2006; Tsujita, Suetsugu et al. 2006; Voeltz, Prinz et al. 2006).

1.3.2 Coated vesicle budding

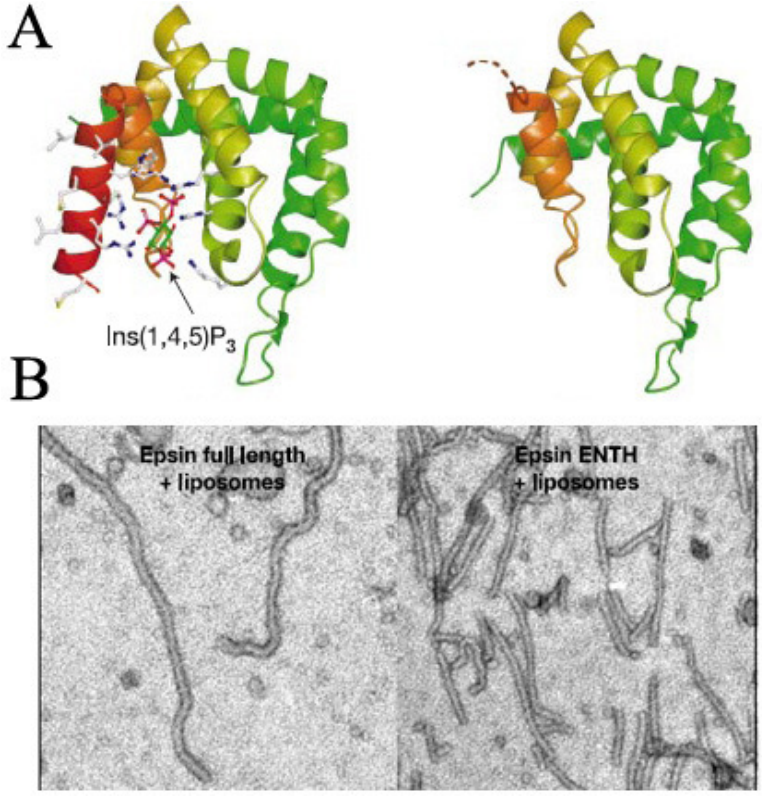

Figure 1-9 Epsin forms an amino-terminal amphipathic helix upon PI(4,5)P_2 binding and tubulates liposomes *in vitro*

(A) Ribbon diagrams of epsin ENTH domain bound to Ins(1,4,5)P_3 (Protein DataBank (PDB) accession number 1H0A) on the left compared with epsin ENTH structure solved in the absence of Ins(1,4,5)P_3 (PDB 1EDU). The structures are colored red to blue from N- to Carboxy-terminal. (B) Electron microscopy of tubulated liposomes in the presence of the indicated protein or domains (Ford *et al* 2002).

Since the first clathrin coated bud was shown by John Heuser (Heuser 1980) about two decades ago, more than 20 molecules have been shown to work coordinately in the precise regulation of clathrin-coated vesicle generation. These proteins participate in different steps

of coated vesicle transport, i.e. membrane bending, coated vesicle budding and fission, uncoating and fusion with target membranes.

It has been observed that several accessory proteins in CME including endophilin (Farsad, Ringstad et al. 2001), amphiphysin (Takei, Slepnev et al. 1999) and dynamin can produce tubular structures when incubated with liposomes *in vitro*. The molecular mechanism of how these factors generate and sense curvature were just recently uncovered by crystallographic studies (Weissenhorn 2005; Masuda, Takeda et al. 2006; Gallop, Jao et al. 2006; Peter, Kent et al. 2004; Habermann 2004; Gallop and McMahon 2005). An amino-terminal NBAR domain, consisting of an amphipathic helix and a BAR (Bin/amphiphysin/Rvs) domain in endophilin and amphiphysin form a crescent-shaped dimer that binds preferentially to highly curved negatively charged membranes. BAR domains are sufficient for the liposome tubulation *in vitro*. Another mechanism to tubulate membranes is the insertion of an amphipathic helix. Ford *et al.* (Ford, Mills et al. 2002) showed that epsin, an early-stage adaptor protein in CME, can form an amphipathic helix upon $PI(4,5)P_2$ binding and this induced amphipathic helix is enough to initiate liposome tubulation *in vitro* (**Figure 1-9**).

Membrane curvature generation is not only needed for CCV budding, but also for COPII- and COPI-mediated vesicle trafficking. Lee *et al.* demonstrated that the small GTPase Sar1 can generate curvature itself by inserting an amino-terminal amphipathic helix into the membrane, in a simillar way to epsin (Lee, Orci et al. 2005). Sar1 contributes to both the initiation, fission and coat disassembly in COPII-coated vesicle traffick (Bi, Corpina et al. 2002).

An important unresolved question is whether clathrin- and COPI-coated vesicle formation at the Golgi apparatus employs a similar molecular mechanism to bend membranes? If so, what is the molecular identity? It has been known that Arf proteins contain an amino-terminal amphipathic helix that is likely to be exposed to the membrane upon GDP to GTP exchange. Therefore it is reasonable to speculate that Arf family proteins may assist and initiate membrane curvature in a manner similar to epsin and Sar1.

1.3.3 Fission

The final step of coated-vesicle budding is the detachment of vesicles from the donor membrane, a process called fission. COPII-, COPI- and clathrin-coated vesicles use different mechanisms for this step. CCV budding at the PM involves dynamin (**Figure 1-10**), a large GTPase. Dynamin was first identified by using drosophila temperature-sensitive mutant

shibire. At 19°C, synaptic vesicle cycling is normal, in contrast at 29°C endocytosis is completely blocked at presynapses (Koenig and Ikeda 1989). In early EM studies it was shown that GTPγS (a non-hydrolysable GTP analogue) treated nerve terminals contained tubular invaginations decorated with dynamin at the neck region (Takei, McPherson et al. 1995), suggesting that they were arrested at the fission stages. Additionally, the first experimental data from many groups demonstrated that dynamin is indeed an indispensible player in the last step of vesicle fission (Hinshaw and Schmid 1995; Sweitzer and Hinshaw 1998; Marks, Stowell et al. 2001; Zhang and Hinshaw 2001). Its exact mechanism of action though is still under debate. A recent study by Roux *et al.* (Roux, Uyhazi et al. 2006) suggested that dynamin acts as a mechanoenzyme in which GTP-induced constriction of oligomeric dynamin rings cooperate with the generation of longitudinal tension to catalyze membrane fission. Dynamin may also assist fission of clathrin-coated vesicles at the TGN (Jones, Howell et al. 1998).

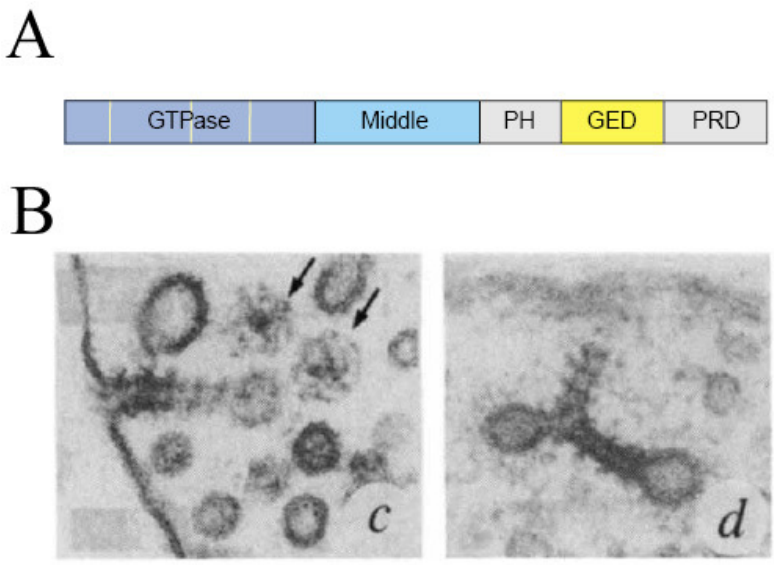

Figure 1-10 Dynamin works at the last step of CCV budding

(A) Domain structure of dynamin. Amino-terminal GTPase domain; PH: pleckstrin homology domain; GED: GTPase enhancing domain; PRD; proline rich domain (Sever 2002). (B) Electron micrographs showing the tubular membrane invagination coated by regularly spaced striations in nerve terminals incubated with GTPγS (Takei, McPherson *et al.* 1995).

COPII-vesicles apparently undergo fission without assistance from additional dynamin factors. Sar1 GTPase activity is stimulated by the GAP function of Sec23 following assembly of the Sec13/31 complex on the ER. The GTP-GDP exchange of Sar1 will promote the fission process in the neck region. Fission of COPI-vesicles needs Arf1 GTP hydrolysis, but instead of stimulation by a coat protein like Sec23 for Sar1 an extra GAP protein called ArfGAP-1 is necessary. ArfGAP-1 also has an amphipathic helix like Arfs, Sar1 and epsin

but is only used to sense the mature COPI-vesicle curvature ready for fission instead of membrane deformation.

1.4 Synaptic vesicle membrane traffic at chemical synapses

1.4.1 Synaptic vesicle cycle

Synaptic vesicles are specialized organelles (**Figure 1-11 A**) with a diameter of about 40 nm and enriched within presynapse of central nervous system neurons. Calcium influx through voltage-gated channels upon action potential arrival initiates SNARE-facilitated exocytosis of SVs at the synaptic active zone. The released neurotransmitters then diffuse across the synaptic cleft and bind to post-synaptic receptors. To sustain the physiological function of the synapse, synaptic vesicles have to be recycled back at the peri-active zone to maintain the recycling vesicle pool for the next round of exocytosis (**Figure 1-11 B**) (Galli and Haucke 2004; Fernandez-Alfonso and Ryan 2006).

Two routes of synaptic vesicle recycling have been proposed (**Figure 1-12**). According to the "kiss-and run", pathway synaptic vesicles do not fuse completely with the PM, but open transiently to allow neurotransmitter release through narrow fusion pore (Valtorta, Meldolesi et al. 2001). Pore closing will allow re-sealed vesicles to return to the active zone and be reloaded with neurotransmitter for the next round of exocytosis. Most of the data supporting this model come from electrophysiological, amperometric and opto-biophysical dye studies (Ales, Tabares et al. 1999; Harata, Choi et al. 2006; He, Wu et al. 2006). The second pathway represents "classical" or "slow" CME that occurs outside of the active zone and includes a full collapse of the synaptic vesicle membrane into the PM (Jarousse and Kelly 2001).

Compared with a clear role of CME for synaptic vesicle retrieval which is supported by an overwhelming set of genetic (Zhang 2003; Di Paolo, Moskowitz et al. 2004) and biological data (Shupliakov, Low et al. 1997), the existence of a "kiss-and-run" mechanism is still controversial and hotly debated question (LoGiudice and Matthews 2006). Genetic evidence for the existence of a "kiss-and-run" model came from *Drosophila* mutants of *endophilin (endo)* and *synaptojanin (synj)* (Verstreken, Kjaerulff et al. 2002), but a recent study by Dickman *et al.* has questioned these findings (Dickman, Horne et al. 2005). Granseth *et al.*

used an improved fluorescent reporter comprising pHluorin fused to synaptophysin and found that only the slow mode of CME operates when vesicle fusion is triggered by single nerve impulses or short bursts (Granseth, Odermatt et al. 2006). It thus seems that clathrin-mediated endocytosis is the predominant, if not the only pathway, of synaptic vesicle retrieval in small synapses of CNS neurons.

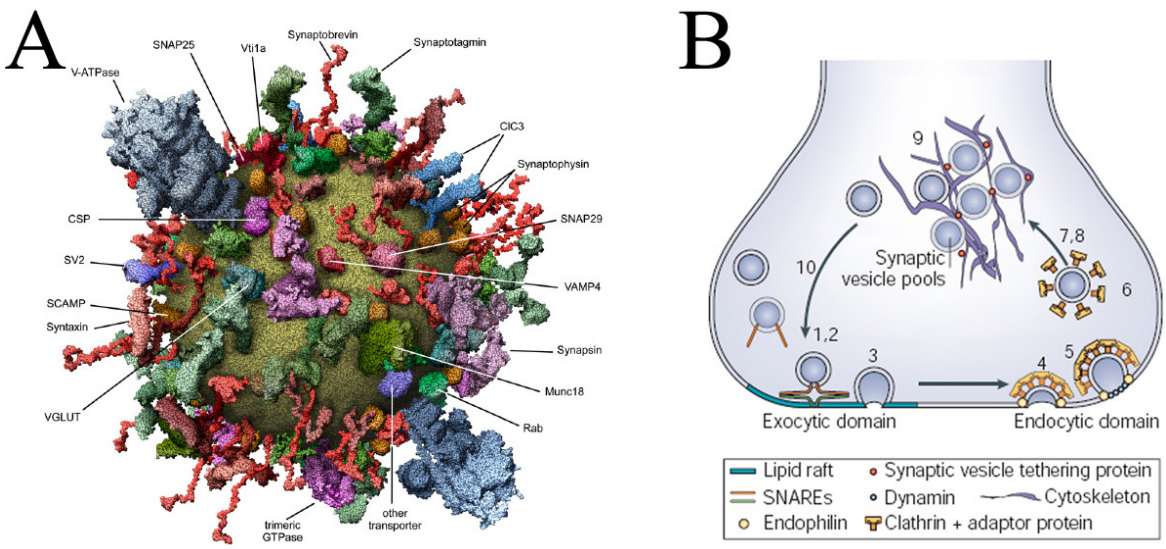

Figure 1-11 Synaptic vesicle cycling

(A) Outside view of a vesicle with several predominant proteins indicated by different colors. Taken from Shigeo Takamori and Matthew Holt 2006. (B) Diagram of a presynaptic terminal, depicting the main stages of the synaptic vesicle, characterized by complete fusion and CME. Ten stages can be defined: (1) synaptic vesicle docking to the plasma membrane, (2) vesicle priming for fusion, (3) Ca^{2+}-triggered vesicle fusion, (4) clathrin-mediated budding and synaptic vesicle formation, (5) fission of a new vesicle, (6) clathrin uncoating, (7) neurotransmitter loading, (8) vesicle trafficking, (9) tethering in a reserve pool and (10) mobilization and targeting to the plasma membrane release site. The plasma membrane region that is highlighted turquoise shows where exocytosis occurs in lipid-raft domains. Endocytosis occurs in adjacent specialized regions, which might have distinct raft-like features. Endophilin regulates membrane shaping and faciliatates vesicle budding. Cytoskeletal and tethering proteins interact with visicle lipids to traffic and sequester synaptic vesicles (Taken from Rohrbough and Broadie).

As mentioned above, if CME is the predominant form of synaptic vesicle retrieval, one important question how the majority of transmembrane proteins are selectively internalized by CME at the peri-active zone together with synaptotagmin 1, the main AP-2-coated pit nucleating protein?

Upon exocytosis, synaptic vesicle lipids and proteins could either mix up completely with PM lipids and proteins or be patched together awaits their immediate internalization. Although it remains possible that synaptic vesicle proteins are sorted individually, mixing of synaptic vesicle lipids and proteins with those of the PM following fusion may unlikely to be favored. Alternatively, synaptic vesicle proteins may somehow be clustered together with the aid of membrane lipid, for example cholesterol, and internalized as a package by clathrin-

mediated endocytosis (Rohrbough and Broadie 2005). This hypothesis has proven to be difficult to validate due to kinetics process, small size of synaptic vesicles (~40 nm), and low spatial resolution of fluorescent microscopes. Using high resolution stimulated emission-depletion (STED) microscopy imaging of actively cycling vesicles, Willig *et al* demonstrated that synaptotagmin 1 remains clustered at the PM both under mild and strong stimulation conditions (Willig, Rizzoli et al. 2006). This reveals that at least some constituents of synaptic vesicles could be patched together after fusion within PM. Evidence from neuroendocrine PC12 cells (Huttner and Schmidt 2000; Thiele, Hannah et al. 2000) and primary neurons (Nagler, Mauch et al. 2001) suggests that membrane cholesterol content is critical for synaptic vesicle protein clustering and endocytic cycling. These functional data are paralleled by the observations that synaptophysin and synaptotagmin 1 directly bind to cholesterol *in situ* (Thiele, Hannah et al. 2000) and that synaptophysin forms a cholesterol-dependent protein complex with synaptobrevin 2 within the synaptic vesicle membrane (Galli, McPherson et al. 1996; Mitter, Reisinger et al. 2003; Pennuto, Dunlap et al. 2002). Exocytic synaptic vesicle fusion sites are also dependent on membrane cholesterol, which facilitates clustering of both SNARE proteins (Chamberlain, Burgoyne et al. 2001; Lang, Bruns et al. 2001) and PI(4,5)P_2 (Pike and Miller 1998), an essential factor in coordinating the exo-and endocytic limbs of the vesicle cycle (Cremona and De Camilli 2001; Di Paolo, Moskowitz et al. 2004). Additionally, cholesterol may play a role in the organization of the pre- and postsynaptic actin cytoskeleton (Murthy and De Camilli 2003).

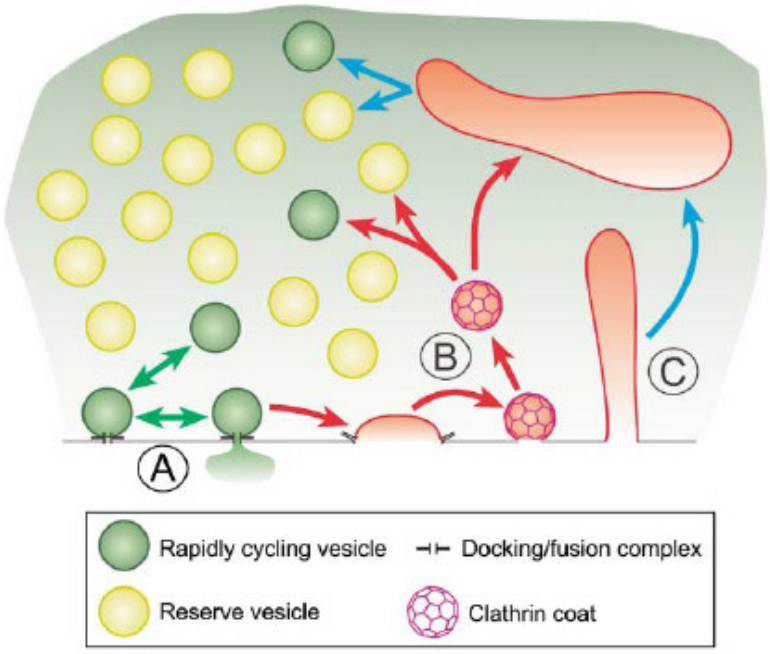

Figure 1-12 Postulated pathways of synaptic vesicle recycling

In pathway A, neurotransmitter is released via reversible opening of a fusion pore (kiss-and-run). In pathway B, the vesicle membrane merges with the plasma membrane and is later retrieved by clathrin-dependent endocytosis. The resulting coated vesicles may directly give rise to releasable vesicles (green) or reserve vesicles (yellow) or they may merge to form intermediate endosomes (cisternae). A third pathway (C) retrieves vesicle membrane by forming membrane invaginations, which pinch off to form endosomes. Whether formed via pathway B or C, the endosomes later give rise to recycled synaptic vesicles (Taken from (Matthews 2004).

Since the PM is considered to be heterogeneous and may contain entities termned "membrane microdomains" that are enriched in cholesterol, we speculate that membrane microdomains could serve as spatial coordinators for cargo selection in the process of synaptic vesicle cycling.

1.4.2 Membrane Microdomains

Membrane microdomains were first observed when van Meer and Simons studied how sphingolipids are transported to the apical side of polarized epithelial (Madin-Darby canine kidney) cells. They found that sphingolipids can aggregate together into distinct domains in the Golgi complex (van Meer, Stelzer et al. 1987). Later, sphingolipids and GPI-anchored proteins were shown to be resistant to extraction by nonionic detergents applied in the cold and float to the top of density gradients (Brown and Rose 1992). These membrane microdomains defined as sphingolipid- and cholesterol-enriched microdomains resistant to cold nonionic detergent extraction were thus called detergent-resistant membranes (DRMs) (van Meer and Sprong 2004). Since the debut of the "raft microdomain" hypothesis, a varity of putative functions of membrane microdomains have been put forward including signal transduction and protein sorting (Helms and Zurzolo 2004).

There are generally two main methods to study membrane microdomains: one is biochemical isolation of DRMs using cold nonionic detergent extraction of biological samples; the other method is based on immunofluorescence or fluorescence-based live cell imaging (Kusumi and Suzuki 2005). One concern of biochemical isolation procedures involving detergents is the fact that results may be dependent on the nature of the detergent and thus perhaps represent *in vitro* artefacts (Heerklotz 2002; Staneva, Seigneuret et al. 2005), and may not reflect *in vivo* situations. Fluorescence-based methods also suffer from potential drawbacks. For example, crosslinking could cause "patching" by fixatives or multivalent probes for immunofluroscence.

Although lipid-lipid interaction has long been believed to be the main driving force for local DRM formation, protein-protein interactions can also drive membrane microdomain formation. In the process of immunological synapse formation upon T cell receptor

stimulation, proteins like LAT seems to play a prominent role in generating protein-protein network microdomains at the PM (Douglass and Vale 2005; Nichols 2005).