

Recruitment of AP-1 Clathrin Adaptors to Liposomal Membranes

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SUMMARY

Protein and membrane traffic between organelles within the endocytic and exocytic pathway is mediated most prominently by coated vesicles. These vesicles are formed by the assembly of cytosolic coat proteins onto the donor membrane, which deform it into a bud so that vesicles can pinch off. Clathrin with its associated adaptors, COPI and COPII are the three major coats. Various *in vitro* studies allowed insight into the mechanism of coat formation. COPI and COPII vesicle budding from chemically defined liposomes has been reconstituted *in vitro*, using pure coat compounds. Further, it has been demonstrated that cargo is sorted into these vesicles. The mechanism of clathrin-coated vesicle formation appears to be more complicated.

The AP-1 clathrin adaptor is involved in vesicle formation at the trans-Golgi network and endosomes. This work presents an *in vitro* assay where AP-1 is recruited to peptidoliposomes, presenting covalently linked peptides corresponding to sorting signals. In this system, AP-1 recruitment depends on myristoylated ADP-ribosylation factor 1 (ARF1), GTP or GMP-PNP, tyrosine signals and a small amount of phosphoinositides, most prominently phosphatidyl inositol 4,5-bisphosphate. In such a minimal system AP-1 is recruited as a high-molecular weight complex indicating the formation of a precoat in the absence of clathrin. GTP hydrolysis, induced by ARF GTPase-activating protein 1 (ARFGAP1), disassembled this complex. Further, AP-1 is able to enhance the GAP activity of ARFGAP1 on myristoylated ARF1, suggesting a regulatory function of GTP hydrolysis in early steps of coat recruitment.

This work provides insights into the mechanism of AP-1 clathrin coat formation which might also be used to investigate the recruitment of other coats.

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Abbreviations

AAK1	adaptor-associated kinase 1
AC-LL	acidic cluster dileucine
AP-1, -2, -3, -4	adaptor protein 1, 2, 3, 4
ARF	ADP-ribosylation factor
ARFGAP1	ADP-ribosylation factor GTPase-activating protein 1
BFA	Brefeldin A
CALM	clathrin assembly lymphoid myeloid leukaemia protein
CD	cation-dependent
CHC	clathrin heavy chain
CI	cation-independent
CK2	casein kinase II
COPI / II	coat protein I / II
CSV	constitutive secretory vesicle
EEA1	early endosome antigen 1
EH	Eps15 homology
EM	electron microscopy
ENTH	epsin N-terminal homology
EPS15	Epidermal growth factor protein substrate 15
epsin1	EPS15 interacting protein
ER	endoplasmatic reticulum
ERGIC	ER-Golgi intermediate compartment
EST	expressed-sequence tags
GAK	cycling G-associated kinase
GAP	GTPase-activating protein
GAT	GGA and Tom
GDI	GDP dissociation inhibitor
GEF	guanine nucleotide exchange factor
GGA	golgi-localized, γ ear-containing, ADP ribosylation factor binding protein
GMP-PNP	guanylyl imidodiphosphate
GTP γ S	guanosine 5'-O-(3-thiotriphosphate)
ISG	immature secretory granule
Lamp1	lysosome-associated membrane protein-1
LAP	like AP-180
MPR	mannose 6-phosphate receptor
MSG	mature secretory granule
NSF	N-ethylmaleimide-sensitive-factor
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PIP	phosphoinositide
PM	plasma membrane
PP2A	protein phosphatase 2A
PS	phosphatidylserine
PTEN	phosphatase and tensin homology
RER	rough ER
SER	smooth ER
SNAP	soluble NSF attachment protein
SNARE	Soluble N-ethylmaleimide-sensitive-factor attachment protein receptor
TfR	transferrin receptor
TGN	trans-Golgi network
VHS	Vps, Hrs, and STAM
VSV-G	vesicular stomatitis virus G protein

1 INTRODUCTION

1.1 Intracellular membrane transport

A cell consists of different organelles which have to fulfill various functions. They are delimited by membranes and contain specific proteins. All proteins are synthesized in the cytosol. Except for mitochondria and chloroplasts, all organelles receive their proteins directly or indirectly from the endoplasmic reticulum (ER), the major entry point for organellar proteins. The major means of transport of membrane proteins between organelles is the budding of vesicles from the starting organelle and their fusion with the target membrane.

Cargo that leaves the ER may therefore be captured and packed several times into vesicles before reaching its destination organelle. Similarly, a cell is able to take up macromolecular material from the extracellular space by vesicles. Receptors at the plasma membrane capture cargo and deliver it into the cell to endosomes where they are further transported to lysosomes for degradation.

There are several major sorting stations in a cell, most prominently the trans-Golgi network (TGN) and the endosomes. At the TGN proteins are sorted towards endosomes or to the plasma membrane. In endosomes they can be sent to lysosomes for degradation or recycle back to the plasma membrane or the TGN. These sorting organelles have to ensure that resident proteins are not packed into vesicles, and if this happens there has to be a mechanism in place to send them back. Therefore anterograde and retrograde transport have to be tightly regulated. This is maintained by a combination of sorting signals within the cargo and a set of accessory proteins that recognize these signals and deliver the cargo to the proper destination.

1.1.1 The exocytic / biosynthetic pathway

The endoplasmic reticulum (ER), the largest intracellular compartment, is made of an array of interconnecting membrane tubules and cisternae that extend throughout the cell including the nuclear envelope. It performs many

functions, including protein synthesis and folding, lipid metabolism, detoxification and regulation of calcium ion gradients. The ER consists of smooth (SER) and rough (RER) regions. Protein synthesis is performed by ribosomes, either free in the cytosol or associated with the RER. The SER is believed to function in lipid synthesis, detoxification and calcium regulation (Lippincott-Schwartz et al., 2000).

The ER is a major site of protein synthesis. Associated ribosomes synthesize the nascent chain into the ER lumen. N-linked oligosaccharides are attached to many of these proteins. The ER is the entry point for secretory and membrane proteins into the exocytic pathway. Newly synthesized proteins interact with luminal chaperones like BiP, calnexin, calreticulin, and protein disulfide isomerase. Their function is to facilitate folding reactions necessary for protein maturation and oligomerization. In addition they are part of the ER quality control system. Quality control mechanisms distinguish correctly from incorrectly folded proteins (Ellgaard and Helenius, 2003). Incorrectly folded proteins remain associated with chaperones and are retained. Eventually these proteins will be retrotranslocated to the cytosol and degraded by proteasomes. Secretory cargo, which is correctly folded, is actively sorted into ER exit sites and exported via COPII coated vesicles (see section 1.2.3). It travels via the **ER-Golgi intermediate compartment (ERGIC)** to the Golgi apparatus. The ERGIC is a dynamic membrane system composed of tubulovesicular clusters that connects the ER with the Golgi. It plays an important role in sorting of protein traffic; anterograde to the Golgi and retrograde to the ER.

The Golgi apparatus was one of the first organelles to be described. It consists of four to six cisternae, arranged somewhat like a stack of pancakes. Cargo arrives from the ERGIC at the cis-Golgi network. Secreted proteins pass through the different cisternae, may be modified throughout, and leave the Golgi at the trans-Golgi network (TGN). From the cis-Golgi on, N-glycosylated proteins are modified, O-linked glycosylation is initiated and eventually modified before reaching the TGN, and finally sulfation is achieved in the TGN. Two models, how proteins move through the Golgi are still under

discussion. In the vesicular transport model the cisternae build a static structure and all the cargo moves via vesicles. Proteins that pass through are transported in vesicles moving forward, recycling receptors and missorted ER resident proteins are sent back by retrograde vesicles. In contrast to this model is the cisternal maturation model. The Golgi functions as a dynamic structure in which the cisternae themselves move. The vesicular structures that arrive from the ERGIC fuse to become the cis-Golgi network, which slowly matures to the medial Golgi and the TGN where it dissolves into budding vesicles. In this model everything moves forward by bulk flow, whereas resident Golgi enzymes are sent back by a steady flow of retrograde vesicles. Evidence suggests that transport occurs by a combination of the two models. Some cargo might move fast via vesicular transport whereas slower structures move slowly corresponding to the rate of cisternal maturation (Pelham and Rothman, 2000).

The main sorting station for biosynthetic / exocytic transport is the TGN. Except for Golgi resident proteins all cargo that passes the Golgi is sorted in the TGN. There are several routes that a protein can take. Proteins can be brought to the **plasma membrane** (PM) either via a constitutive pathway (in polarized cells pathways to the apical and the basolateral surface) or via a regulated pathway in secretory granules. The regulated secretion pathway is normally found in specialized secreting cells, mediated by specialized secretory granules. In this manner hormones and neuropeptides are secreted. It is thought that aggregation of secretory proteins in the TGN leads to membrane deformation, that buds as an immature secretory granule. These granules still contain proteins, which are not destined for regulated secretion. As the granules mature, these proteins are captured by clathrin-coated vesicles (CCVs) and travel back to the TGN (Dittie et al., 1996; Klumperman et al., 1998). The release of the cargo into the extracellular space is triggered by an external stimulus.

In a further pathway cargo is sent from the TGN to **endosomes** where it enters the endocytotic membrane system and is further sorted to the PM or to lysosomes (Leitinger et al., 1995; Lippincott-Schwartz et al., 2000).

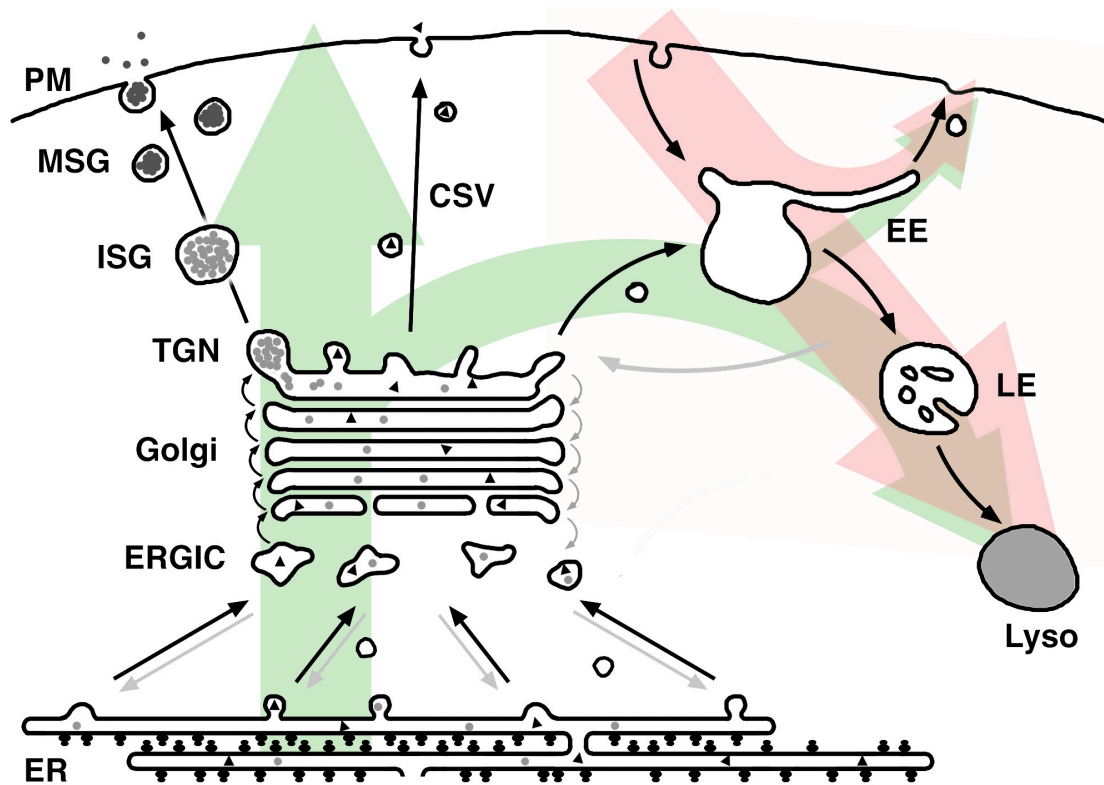


Figure 1

The major membrane traffic pathways. In the exocytic / biosynthetic pathway (in green), newly synthesized molecules are transported from the endoplasmic reticulum (ER) through the ER-Golgi intermediate compartment (ERGIC) and Golgi to the plasma membrane (PM) or via endosomes to lysosomes and plasma membrane (ISG=immature secretory granule, MSG=mature secretory granule, CSV=constitutive secretory vesicle). In the endocytic pathway (in red) molecules are internalized at the plasma membrane and transported via early endosomes to lysosomes or recycle back to the plasma membrane. Anterograde transport is indicated with black, retrograde transport with gray arrows (adapted with courtesy of M.Spiess).

1.1.2 The endocytic pathway

The bilayer of the **PM** is the barrier separating the inside of a cell from the outside. It is the entry point of all extracellular material that a cell has to import. Small molecules can pass the bilayer directly (e.g. lipid hormones), or in a controlled way through channels or transporters. The transport of larger molecules is performed by endocytosis. There are at least three types of endocytosis: receptor-mediated endocytosis, fluid-phase endocytosis, and caveoli formation.

In receptor-mediated endocytosis, receptors interact with cargo at the PM, invaginate and bud in vesicles into the cell. Endocytic transport receptors

(receptors for LDL, ASGP) travel to the early **endosomes** close to the PM. Endosomes form a set of heterogeneous membrane-enclosed tubes that extend from the PM to the perinuclear region, where they are often close to the Golgi. There are two main classes of endosomes, the early and the late endosomes. The early endosome can further be subdivided into at least two populations, the recycling and the sorting endosomes (Pillay et al., 2002). The acidic environment of the early endosome leads to a conformational change in the receptor such that the ligand is released and usually sent via late endosomes to lysosomes where it is degraded. Some receptors can be recycled back to the PM via a recycling endosome, whereas others are downregulated via late endosomes and lysosomes (e.g. EGF-, insulin receptor).

Lysosomes are organelles of about 0.5 μ m diameter. They are usually regarded as the terminal compartment of the endocytic pathway where proteins are degraded (Kornfeld and Mellman, 1989). Lysosomes form heterogeneous membrane-enclosed compartments that are filled with acidic hydrolases, which are optimally active at the low pH of lysosomes. The pH gradually drops on the way from the early endosome to the lysosome. Lysosomes contain various types of enzymes including proteases, nucleases, glycosidases, lipases, phospholipases, phosphatases, and sulfatases. These enzymes are usually highly glycosylated, which protects them from being digested themselves. Some are mannose-6-phosphate tagged. The mannose-6-phosphate receptors (MPR) recognize these enzymes in the TGN from where they are delivered to lysosomes via endosomes. The receptors recycle back to the TGN before they reach the lysosome. Lysosomes are probably generated by fusion of late endosomes with existing lysosomes, forming a hybrid organelle that matures into a lysosome (Luzio et al., 2000).

1.2 Coated vesicles

Typically, transport vesicles are formed by the recruitment of cytosolic proteins forming a coat that induces membrane curvature and vesicle budding. Three major coat systems how transport vesicles are formed and

cargo is sorted into vesicles are known. Depending on the origin of a vesicle it is packed into a COPI, a COPII, or a clathrin coated vesicle. COPII coated vesicles mediate traffic between the ER and the Golgi, COPI vesicles traffic from the Golgi back to the ER and mediate intra Golgi transport. The clathrin pathway has several routes. It transports vesicles from the Golgi to endosomes, from endosomes to lysosomes or the PM, and from the PM to the early endosome. The most important feature of coated vesicles is an identifiable coat. The basic principle of coat formation is the same for all three coats. Cargo proteins that have to be packed into vesicles present short sorting signals in the cytoplasm. These are recognized directly by the coat proteins or via an adaptor protein. The function of the coat is to concentrate the cargo and to physically bend the membrane to form a coated vesicle (Kirchhausen, 2000b; Robinson and Bonifacino, 2001; Schekman and Orci, 1996).

1.2.1 The clathrin coat

Clathrin-coated vesicles (CCVs) are the most prominent carrier between the TGN and endosomes, endosomes and lysosomes, and between the plasma membrane and endosomes. The coat has a basket like shape and consists of clathrin and clathrin adaptors (Kirchhausen, 2000b; Robinson and Bonifacino, 2001). Since CCVs are quite abundant this was the first coat to be discovered (Pearse, 1976; Roth and Porter, 1964). This, together with the relative ease to purify CCVs has made it to be the most studied coat.

Clathrin

Clathrin is the scaffold protein of the coat. The assembly unit is the “triskelion” (Ungewickell and Branton, 1981)(Fig. 2). A triskelion is composed of three heavy chains (CHC / 192 kDa each) each associated with a light chain (CLCa or CLCb / ~25 kDa each). Each heavy chain forms a 45-50nm long leg of the triskelion. The carboxy terminal third of the CHC is known as the hub. This region binds to the CLC and includes sequences that mediate trimerization of clathrin. The N-terminal third of the leg comprises a globular domain, which is able to interact with various proteins (ter Haar et al., 1998). Clathrin does not

directly interact with the membrane. It needs an adaptor protein (AP) to be recruited (Vigers et al., 1986). APs interact with various players on the membrane (see below) and are able to bind clathrin. Therefore the specificity of coat formation determined by the recruitment of the adaptor. APs contain several clathrin-binding motifs. The clathrin box (L ϕ X ϕ [D,E]), the PWDLW sequence, the LLDLL sequence and short DLL repeats. These motifs bind to the amino-terminal domain of the CHC (Brodsky et al., 2001; Kirchhausen, 2000a; Ungewickell, 1999).

The CLC binds to the hub region of the CHC. Two subforms with a sequence identity of about 60% are known in vertebrates. It is believed that clathrin initially forms a flat network of hexagons. Some of them are able to convert into pentagons and thereby drive the curvature of the membrane (Heuser, 1980). The exact mechanism of this conversion is unknown.

In vitro it has been shown that CLC-free clathrin shows an enhanced affinity for self-assembly at physiological pH. With CLC the polymerization can only occur at a pH below 6.5. Clathrin adaptors reverse this inhibition so that polymerization occurs again at physiological pH (Ybe et al., 1998). Therefore the light chain might have a regulatory function that ensures that the formation of a CCV can only occur in the presence of adaptors.

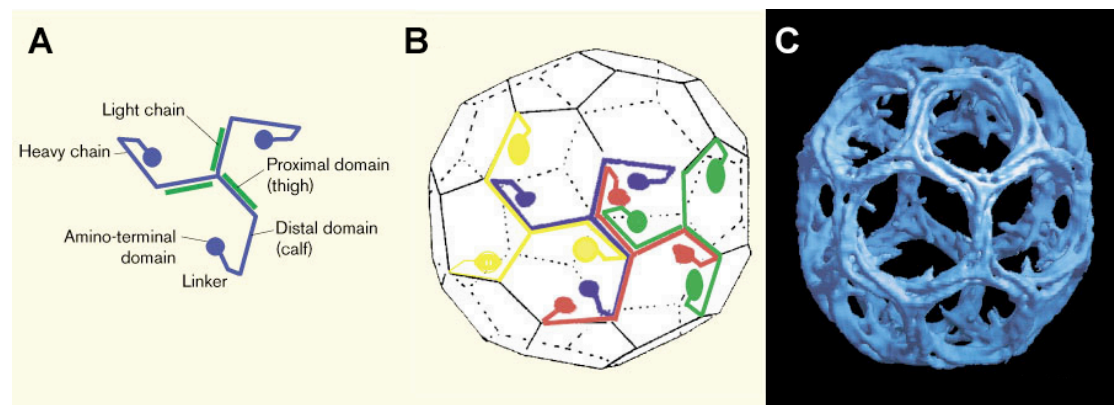


Figure 2

The domain structure of a clathrin triskelion and how it is arranged in the clathrin coat. (A) Domain organization of a clathrin triskelion. (B) Schematic representation of how clathrin is packed into a coat. For reasons of clarity the light chains have been removed. The linker- and the amino-terminal domains point into the center of the sphere. (C) Map of a clathrin coat (adapted from Ungewickell, 1999).

Sorting signals for clathrin coats

Sorting of cargo occurs through coated areas of membranes. Prior to budding the cargo is concentrated in this area. This process has to be regulated to capture only the proteins that have to be packed into the vesicle. The sorting is mediated by signals that are present in the cytosolic domains of transmembrane proteins. Clathrin adaptors interact with these sorting signals, connecting the cargo with the coat. Most of the signals consist of a short linear sequence of four to seven residues. Two major types of sorting signals are known for the clathrin coat, tyrosine- and dileucine-based signals (Bonifacino and Dell'Angelica, 1999; Bonifacino and Traub, 2003; Heilker et al., 1999; Kirchhausen et al., 1997)

The tyrosine-based signals can be grouped into NPXY and YXX Φ (Φ =bulky hydrophobic) signals. NPXY signals mediate rapid internalization of some type I proteins like the LDL receptor, integrin β , and the β -amyloid precursor protein families. A phenylalanine or a tyrosine at position minus two is also important for proper sorting. Most of the signals therefore contain a hexapeptide of the form [F,Y]XNPXY (reviewed by Bonifacino and Traub, 2003). These signals are normally part of a medium length cytosolic tail of 40 to 200 amino acids. They are never exactly at the carboxy terminus of the proteins and the

distance from the membrane is more than ten amino acids (Bonifacino and Traub, 2003).

The YXX Φ signal is more widespread than the NPXY motif. This motif is found in endocytic receptors like the transferrin and the asialoglycoprotein receptor, intracellular sorting receptors like the CI (cation independent) and the CD (cation dependent) MPRs, lysosomal protein sorting such as Lamp1 and Lamp2, and in TGN proteins like TGN 38 (Bonifacino and Traub, 2003). The tyrosine is essential and the X in the tetrapeptide and surrounding residues participate in the fine-tuning of the sorting signal (Gough et al., 1999; Rous et al., 2002). The position of the motif within the tail also plays an important role. Endocytic motifs are often situated at position 10-40 from the transmembrane domains but not at the carboxy termini of the protein. Lysosomal proteins on the other hand contain short cytosolic parts with the sorting signal at the carboxy termini at position 6-9 from the transmembrane domain. It has been shown that changing the distance of the sorting signal of Lamp1 from the membrane impairs lysosomal sorting (Rohrer et al., 1996).

Dileucine-based signals can be divided into two subgroups. The [D, E]XXXL[L, I] and the DXXLL, respectively. The [D, E]XXXL[L, I] motif has been discovered in the CD3- γ receptor where the signal is DKQTLL. The receptor is present at the PM and is internalized upon down-regulation. An alanine scan mutagenesis of the sorting signal revealed the importance of the two leucines and an acidic cluster (Letourneur and Klausner, 1992). The CD3- γ internalization signal is regulated by phosphorylation of a serine next to the aspartic acid. A regulatable signal has also been reported in other proteins, e.g. the CD4 receptor (Pitcher et al., 1999). Transmembrane proteins with constitutive active signals of this type are mainly localized to late endosomes and lysosomes (e.g. LIMP2). The position relative to the transmembrane domain is similar to the YXX Φ signal. They have short cytoplasmic tails with the signal near their carboxy or the amino termini. A distance of 6-7 amino acids from the transmembrane domain has been demonstrated to be optimal for the downregulation of CD3- γ (Geisler et al., 1998).

The second group of dileucine signals is found in transmembrane proteins

and receptors that cycle between TGN and endosomes, such as the CI- and the CD-MPRs. They are incorporated into CCVs that bud from the TGN and travel to endosomes (Johnson and Kornfeld, 1992). These signals often have an upstream serine residue that can be phosphorylated. Most of the DXXLL signals are close to the carboxy terminus of the protein. The distance from the transmembrane domain is longer and more variable.

Clathrin Adaptors

Adaptor proteins (APs) link the cargo to clathrin. They recognize the sorting signals, they are able to bind and polymerize clathrin, and they recruit accessory proteins that regulate coat formation. AP-1 and AP-2 are the founding members of this protein family. Since then two more APs, AP-3 and AP-4, have been discovered. All four are heterotetramers containing two large (~100kDa), a medium (~50kDa), and a small (~20kDa) subunit. Electron microscopic analysis of APs revealed a characteristic morphology resembling a head with two ears (Heuser and Keen, 1988). AP-1, -2, and -3 interact with clathrin (Kirchhausen, 2000b; Robinson and Bonifacino, 2001). More recently the GGAs, a new group of monomeric clathrin adaptors, were discovered (Dell'Angelica et al., 2000; Hirst et al., 2000).

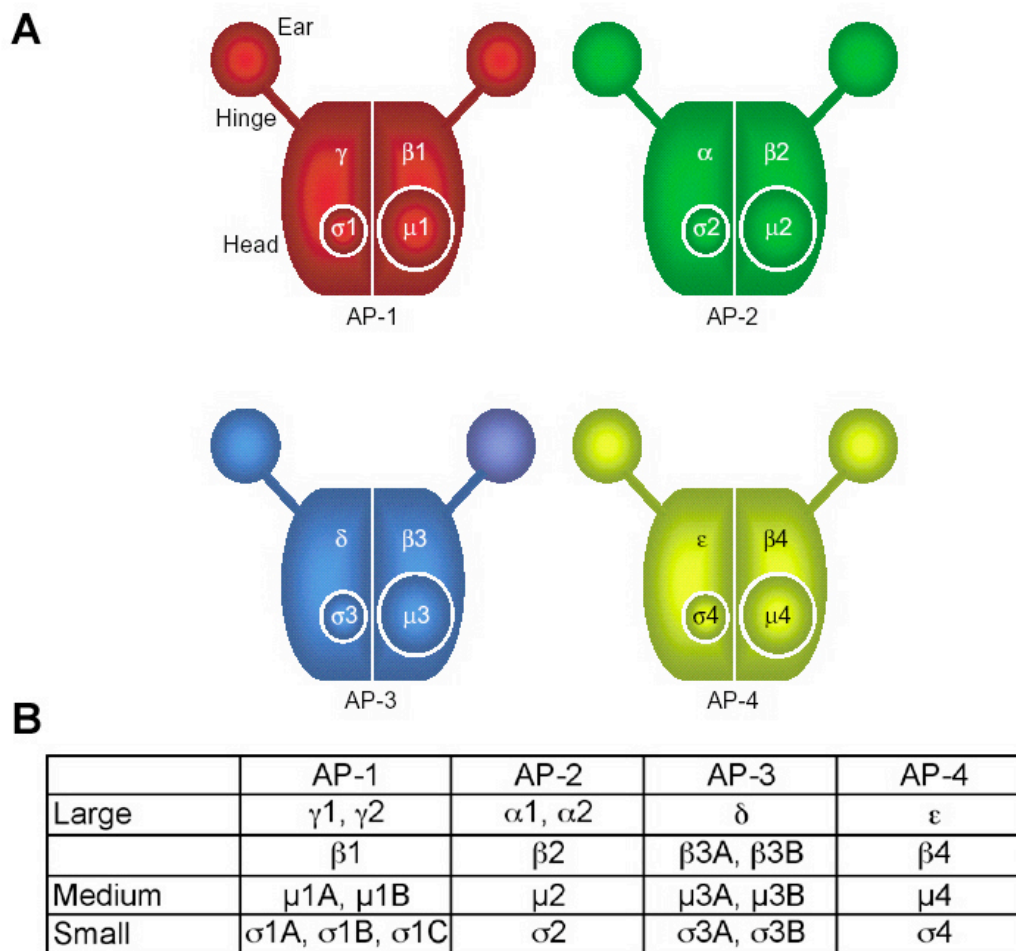


Figure 3

Clathrin adaptors (A) Schematic diagrams of the four heterotetrameric clathrin adaptor complexes. Each consists of two large a medium and a small subunit (Robinson and Bonifacino, 2001). (B) Nomenclature of the subunits with their identified mammalian isoforms (adapted from Boehm and Bonifacino, 2001)

AP-1

AP-1 plays an essential role in packaging membrane proteins into CCVs at the TGN and endosomes. The two large AP-1 subunits are $\beta 1$ and γ , the medium is $\mu 1A$ (of AP1-A) or $\mu 1B$ (AP-1B), and the small is $\sigma 1$. The two isoforms of AP-1 (AP-1A and AP-1B) have distinct functions. AP-1A is ubiquitously expressed; AP-1B expression is restricted to polarized epithelial cells (Ohno et al., 1999). The γ and σ subunit are found as different isoforms, however the functional difference between the γ and the σ isoforms is not known (Boehm and Bonifacino, 2001).

AP-1 recognizes tyrosine and dileucine based sorting signals. Yeast-two

hybrid studies have identified the μ 1 subunit as the one to interact with the YXX Φ motif (Bremnes et al., 1998; Ohno et al., 1995). Proteins with this type of sorting signals have been identified to interact with AP-1, such as the CD- and the CI-MPR, Lamp1, TGN38 and furin (Bonifacino and Traub, 2003). The μ 1 or the β 1 subunit have been proposed to interact with the [D, E]XXXL[L, I] type dileucine signals (Bremnes et al., 1998; Rapoport et al., 1998). However, a recent yeast three-hybrid assay demonstrated interaction with a combination of the γ and the σ 1 subunits (Janvier et al., 2003). Binding of AP-1 to an artificially introduced dileucine motif (Heilker et al., 1996), the CD3- γ receptor (Dietrich et al., 1997) and LIMPII (Fujita et al., 1999) have been demonstrated in vitro.

Originally AP-1A was thought to mediate transport from the TGN to endosomes (Ahle et al., 1988). More recent findings demonstrated that it might also regulate transport from endosomes to the TGN. In fibroblasts that were deficient in μ 1A the steady-state distribution of CD- and CI-MPRs were shifted to early endosomes (Meyer et al., 2000). Recruitment of AP-1 is initiated by nucleotide exchange of ADP-ribosylation factor 1 (ARF1)-GDP for ARF1-GTP and its binding to the place of recruitment. The μ 1 subunit interacts with the sorting signal of cargo proteins (Ohno et al., 1996; Ohno et al., 1995) and the trunk region of γ adaptin and β 1 adaptin interact with membrane bound ARF (Stamnes and Rothman, 1993; Traub et al., 1995; Traub et al., 1993) Clathrin is recruited through its interaction with the clathrin box motif in the hinge domain of β 1 adaptin (Shih et al., 1995) or γ adaptin (Doray and Kornfeld, 2001).

The formation of an AP-1 CCV is highly regulated. A set of accessory proteins has recently been identified that binds the ear domain of γ adaptin (see below). The β 1 adaptin and the μ 1 subunit can be phosphorylated. Gosh and coworkers demonstrated that phosphorylation of μ 1 strongly enhances binding to some sorting signals whereas dephosphorylation by protein phosphatase 2A (PP2A) is involved in uncoating. Phosphorylation of β 1 is differentially regulated. This subunit becomes dephosphorylated upon recruitment (Ghosh and Kornfeld, 2003a).

AP-1B, expressed in epithelial cells differs from AP-1A only in the μ 1 subunit. The two subunits share about 80% amino acid sequence identity (Ohno et al., 1999). The trafficking route of the AP-1B subform differs from the one of AP-1A. The polarized epithelial cell line LLC-PK1 does not express μ 1B. Basolateral proteins, such as the LDL receptor and the transferrin receptor, are miss-sorted to the apical surface. Transfection with μ 1B restores basolateral sorting, indicating a function of AP-1B in basolateral sorting (Folsch et al., 1999). The site of AP-1B recruitment is still not clear. It probably controls polarized sorting at the TGN and endosomes (Gan et al., 2002). Recent findings implicate that the two subforms of AP-1 are localized on distinct membrane domains, which leads to the formation of different types of vesicles (Folsch et al., 2003).

AP-2

AP-2 was the first clathrin adaptor to be discovered. It is probably still the most studied and therefore the best understood. It is composed of the two large subunits α 1 or α 2, and β 2, the medium subunit μ 2, and the small subunit σ 2. Mediating endocytosis of transmembrane proteins, it is responsible for the major entry pathway into the cell. Three subunits participate in clathrin coat recruitment. The α and μ 2 subunits bind to PI(4,5)P₂ located on the PM. This interaction positions the adaptor on the membrane (Collins et al., 2002; Gaidarov et al., 1999; Rohde et al., 2002). The carboxy-terminal α appendage interacts with a large number of accessory proteins involved in the regulation of coat recruitment (Slepnev and De Camilli, 2000). A clathrin-binding sequence (clathrin box) is present in the β 2 subunit. It binds to the CHC promoting lattice assembly (Brodsky et al., 2001; Owen et al., 2000). Some regulatory proteins are also able to bind to the β subunit. The sorting signal of the cargo is recognized by the μ 2 subunit (Ohno et al., 1995). It interacts with the FXNPXY or the YXX ϕ motif of cargo such as the transferrin receptor (TfR), the LDL receptor, or TGN38 (Boll et al., 2002; Ohno et al., 1995). In addition the μ 2 or the β 2 subunit may recognize [D, E]XXXL[L, I] motifs (Bremnes et al., 1998). The tyrosine- and the dileucine-based signals are recognized by

two different binding pockets (Marks et al., 1996). $\sigma 2$, the fourth subunit, has probably structural functions (Collins et al., 2002).

Phosphorylation regulates the recruitment of AP-2. Cargo binding is enhanced when the tyrosine 156 of the $\mu 2$ subunit is phosphorylated by the adaptor-associated kinase 1 (AAK1) (Conner and Schmid, 2002; Ricotta et al., 2002). Many accessory proteins have been identified that regulate the formation of a vesicle or are involved in coat release (see below).

AP-3

For a long time AP-1 and AP-2 were the only known clathrin adaptors. The hypothesis that there must be more than just these initiated the search for new adaptors. Modern technologies enabled homology screens in cDNA libraries and databases that revealed a set of new heterotetrameric and monomeric clathrin adaptors. The first to be discovered was AP-3 (Pevsner et al., 1994; Simpson et al., 1996). It consists of the large subunits δ , $\beta 3A$ or $\beta 3B$, the medium subunits $\mu 3A$ or $\mu 3B$ and the small subunit $\sigma 3A$ or $\sigma 3B$. $\beta 3A$ and $\mu 3B$ are specific for neuron and endocrine cells. All other subunit isoforms are ubiquitously expressed (Robinson and Bonifacino, 2001). Loss-of-function mutation of AP-3 are not lethal in mammals and *Drosophila melanogaster*. Therefore it was possible to study the function of AP-3 in vivo. The *garnet* gene of *Drosophila melanogaster* encodes the ortholog of the AP-3 δ subunit (Ooi et al., 1997; Simpson et al., 1997). This gene is one of the classical eye color genes. Since pigment granules in the eyes show similarities to lysosomes and loss-of-function mutations of the *garnet* gene show defective pigment granules, AP-3 is thought to be involved in lysosomal trafficking (Ooi et al., 1997). Naturally occurring mutations in human and mouse have served to establish the role of AP-3 in lysosomal trafficking. Studies in fibroblasts with a drastically reduced level of AP-3 revealed that in these cells lysosomal membrane proteins travel via the PM (Dell'Angelica et al., 1999b; Le Borgne et al., 1998).

AP-3 interacts with YXX Φ motifs of lysosomal proteins such as CD63 and LAMP1 (Le Borgne et al., 1998; Rous et al., 2002). Lysosomal proteins with

[D, E]XXXL[L, I] signals are also targeted via AP-3, such as LIMP II and tyrosinase (Honing et al., 1998; Le Borgne et al., 1998).

It is still controversial whether AP-3 is associated with clathrin. It is not enriched in purified CCVs (Simpson et al., 1996). However, both $\beta 3$ isoforms contain a clathrin-binding motif that can interact with clathrin in vitro (Dell'Angelica et al., 1998). Immunofluorescence and electron microscopy studies yielded no co-localization in some studies (Simpson et al., 1996; Simpson et al., 1997) and co-localization in others (Dell'Angelica et al., 1998). The recruitment of AP-3 to membranes is ARF1 dependent (Ooi et al., 1998).

AP-4

The fourth adaptor has been identified via a database search for proteins with homology to the AP-1, AP-2, and AP-3 complexes (Dell'Angelica et al., 1999a; Hirst et al., 1999). AP-4 is composed of the subunits ϵ , $\beta 4$, $\mu 4$ and $\sigma 4$. Immunofluorescence- and immunogold staining has localized AP-4 to the cytoplasmic face of non-clathrin coated vesicles in the region of the TGN (Hirst et al., 1999). Treating cells with Brefeldin A (BFA) disrupted this pattern. This indicates that the recruitment of AP-4 is also regulated via ARF (Dell'Angelica et al., 1999a; Hirst et al., 1999). A later study by Boehm and coworkers demonstrated that the GTP bound form of ARF1 interacts with the ϵ subunit. Further interaction has been found between $\mu 4$ and ARF1 independently of the activation state of ARF1. Redistribution into the cytosol of AP-4 has been detected upon overexpression of a dominant negative mutant of ARF1 and ARF3 (Boehm et al., 2001). The $\mu 4$ subunit recognizes the tyrosine based sorting signals of TGN 38, Lamp1, Lamp2, TfR and CD63 in vitro (Aguilar et al., 2001; Simmen et al., 2002). In cells with depleted $\mu 4$ subunit basolateral proteins get missorted to the apical membrane indicating its participation in basolateral sorting (Simmen et al., 2002). However, further investigations are needed for detailed understanding of the regulation of AP-4 recruitment and to determine its exact trafficking route.

GGAs

By searching the EST database for proteins with similarity to the γ adaptin subunit of AP-1 at least three proteins were found named Golgi-localized, γ ear-containing, ADP ribosylation factor binding protein (GGA) 1-3 (Dell'Angelica et al., 2000; Hirst et al., 2000). They are monomeric adaptor proteins that contain three domains, an amino-terminal VHS (Vps, Hrs, and STAM) domain, followed by a GAT (coiled-coiled GGA and Tom) domain, a variable hinge region and a COOH-terminal appendage that is homologous to the ear of γ adaptin. The same group of proteins was found in an independent approach searching for interacting partners of ARF3 (Boman et al., 2000). All three GGAs localize predominantly to the trans-Golgi region (Boman et al., 2000; Dell'Angelica et al., 2000; Hirst et al., 2000) and are involved in trafficking to the early and late endosomes in mammalian cells and to the vacuole in yeast (Boman et al., 2000; Costaguta et al., 2001). They are monomeric in the cytosol and polymerize with each other on Golgi membranes (Ghosh et al., 2003).

Recruitment of GGA to the TGN is mediated by interaction of the GAT domain with ARF1 (Collins et al., 2003). The VHS domain interacts with cargo. It binds to an acidic cluster/dileucine (AC-LL) sorting motif on the cytoplasmic tails of the two MPRs (Puertollano et al., 2001; Takatsu et al., 2001; Zhu et al., 2001). Clathrin interacts with GGA through a clathrin box in the hinge domain. The latter domain is also able to interact with AP-1 (Doray et al., 2002b; Puertollano et al., 2001; Zhu et al., 2001). Other accessory proteins can bind to the ear domain (reviewed by Boman, 2001). GGA1 and 2 colocalize with AP-1 in buds and CCVs at the TGN (Doray et al., 2002b; Puertollano et al., 2003).

The recruitment of GGA1 and 3 is regulated via phosphorylation. The hinge domain contains an AC-LL sequence. Phosphorylation of a serine that is located three residues upstream of this motif induces a conformational change in the GGA. This causes the binding of the VHS domain to the internal AC-LL motif, which in turn inhibits the ability to bind to cargo. Phosphorylation is driven by a Casein kinase II (CK2) activity associated with AP-1 (Doray et al.,

2002a; Ghosh and Kornfeld, 2003b).

A model of how the GGAs act at the TGN has emerged. Monomeric GGAs are recruited from the cytosol onto the TGN in an ARF1 dependent manner. There they form a complex that stabilizes the TGN structure and is able to interact with cargo and AP-1. Phosphorylation by CK2 releases the cargo from the GGAs and hands it over to AP-1 that will then pack it into CCVs (Doray et al., 2002b; Ghosh et al., 2003; Ghosh and Kornfeld, 2003b).

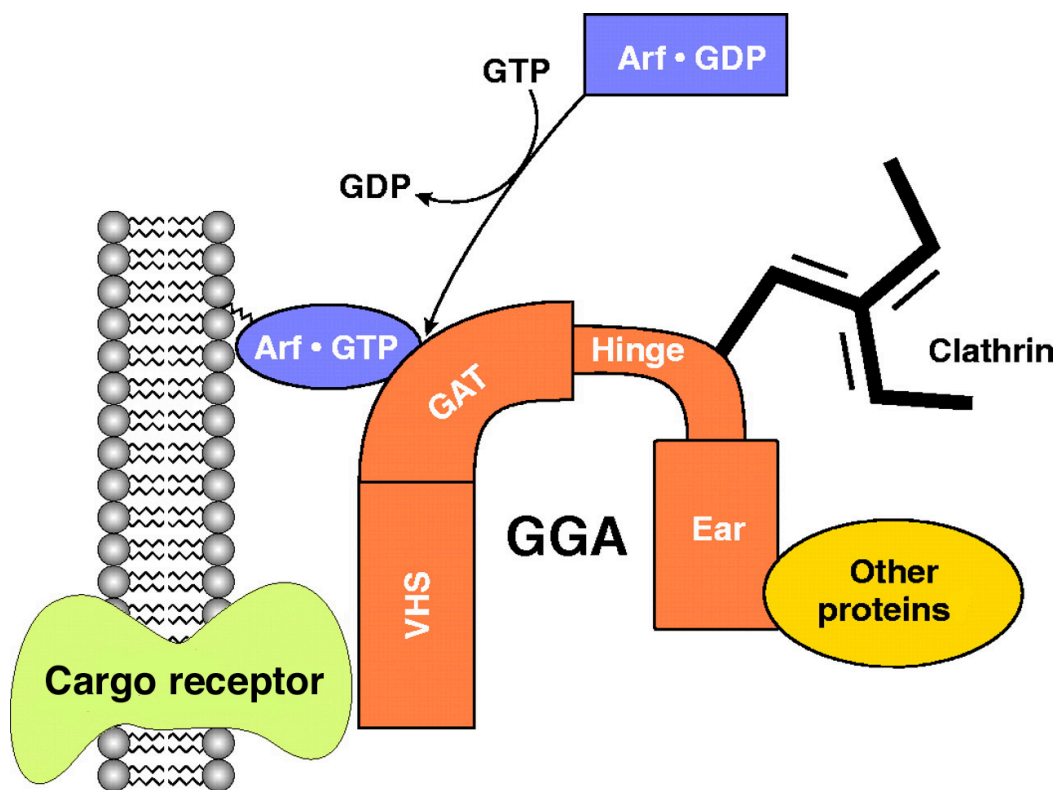


Figure 4

Model of GGA interactions with other proteins (adapted from Boman, 2001)

Accessory proteins

CCV formation is a highly regulated process. A number of accessory proteins have been identified that participate in cargo selection, coat formation, membrane deformation, vesicle scission, or coat release. Initially many AP-2 interacting proteins were discovered. Some bind directly to a “binding platform” within the α and $\beta 2$ ear domains, others interact with AP-2 via other accessory proteins. Together they form a network with AP-2 as a central compound (Mousavi et al., 2004; Slepnev and De Camilli, 2000). Recent

studies demonstrated that AP-2 depleted cells internalize some endocytic receptors as efficiently as wild type cells. This suggests that AP-2 is not essential for CCV formation and that some accessory proteins may function as alternative adaptors (Motley et al., 2003). Many accessory proteins contain an ENTH (epsin N-terminal homology) or an EH (Eps15 homology) domain. The ENTH domain binds PI(4,5)P₂ and probably other PIPs. It is able to interact with the bilayer in a way that it becomes buried in its cytoplasmic region and therefore helps to induce curvature (Ford et al., 2002). The EH domain is able to interact with a NPF motif found in proteins implicated in CCV formation at the PM.

Until recently not many AP-1 accessory proteins have been known. Database searches and yeast two-hybrid screens identified new candidate accessory proteins at the TGN. They interact with the γ ear of AP-1. The search for AP-1 interactors just started. In the near future more such proteins will probably be discovered, leading to a clearer picture of the regulation of coat recruitment.

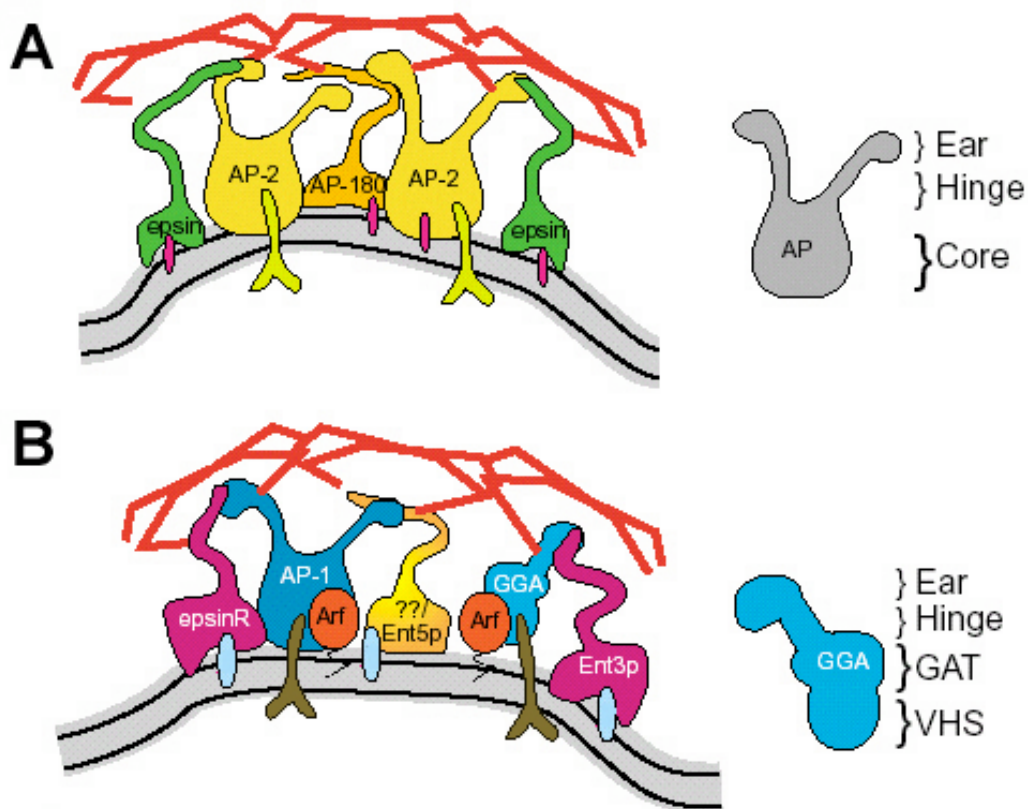


Figure 5

Schematic view of CCV formation and interactions with some accessory proteins. (A) CCV formation at the plasma membrane. AP-2 recognizes sorting signals of cargo receptors (in yellow). Epsin1, AP180 and AP-2 interact with each other, with clathrin and PI(4,5)P₂ (in purple). (B) CCV formation at the TGN / endosomes. AP-1 interacts with cargo (in brown). Accessory proteins that interact with AP-1. EpsinR, Ent3p and Ent5p interact with PI(4)P (in blue). Question mark indicates a possible mammalian homolog of Ent5p (adapted from Duncan and Payne, 2003).

Accessory proteins at the TGN

γ -Synergin

In a yeast two-hybrid assay γ -synergin has been identified to interact with the γ ear of AP-1 (Page et al., 1999). It is ubiquitously expressed and associated with AP-1 in the cytosol as well as on CCVs. γ -Synergin is able to interact with other proteins through its EH domain. Since interaction with AP-1 is localized to a different part of γ -synergin, it might provide a protein interaction platform that allows other proteins to interact with AP-1 through γ -synergin (Page et al., 1999). AP-2 where the α ear is replaced by the γ ear still localizes to the PM. This chimera can miss-localize γ -synergin to the PM. Therefore it is thought

that γ -synergin follows AP-1 to the membrane (Page et al., 1999).

Enthoprotein / clint / epsinR

A novel protein with an ENTH domain that localizes to the TGN was identified by three independent groups using different methods (mass spectrometry of brain CCVs, mass spectrometry of AP-1 γ -ear binding partners and database searches for ENTH-domain proteins). Consequently the protein has three different names, enthoprotein, clint and epsinR (Kalthoff et al., 2002; Mills et al., 2003; Wasiak et al., 2002). In this report it will be referred to as epsinR. EpsinR interacts with the γ -ear of AP-1 and with clathrin and it is enriched in CCVs (Kalthoff et al., 2002; Mills et al., 2003; Wasiak et al., 2002).

It interacts with PI(4)P in a nitrocellulose overlay and liposome binding assay (Mills et al., 2003). Lipid interaction with its ENTH domain seems to be important for the proper localization of epsinR (Mills et al., 2003). Overexpression of epsinR leads to impaired sorting of cathepsin D, which indicates a possible role in vesicle budding between the TGN and endosomes (Mills et al., 2003).

Ent3p / Ent5p

Two ENTH domain containing yeast proteins named Ent3p and Ent5p have been identified (Duncan et al., 2003). Both proteins interact with Gga2p and clathrin, in addition Ent5p interacts with AP-1 (Duncan et al., 2003; Friant et al., 2003). The two proteins are very similar to one another (Duncan et al., 2003). Ent3p and Ent5p co-localize with clathrin. Single deletion mutants showed no effect, whereas cells lacking both proteins displayed defects in clathrin localization and cargo that is sorted via CCVs at the TGN showed a severe delay in maturation (Duncan et al., 2003).

Accessory proteins at the PM

AP180

AP180 is a brain specific protein. It is concentrated in nerve terminals and it

co-purifies with CCVs (Ahle and Ungewickell, 1986). The ubiquitously expressed functional homologue of AP180 is called CALM (clathrin assembly lymphoid myeloid leukaemia protein) (Tebar et al., 1999). AP180 binds to the ear domains of the two adaptins of AP-2 (Owen et al., 1999; Owen et al., 2000). It has been demonstrated that the complex of AP-2 and AP180 has a much stronger ability to assemble clathrin than each protein separately (Hao et al., 1999). Since these vesicles are smaller and more homogenous in the presence of AP180 its function has been proposed to regulate the vesicle size (Ye and Lafer, 1995). These findings have been confirmed in vivo. The number and size of vesicles in the nerve terminal was reduced in *Drosophila melanogaster* where the LAP ("like AP180") gene has been disrupted .

AP180 is also able to interact with phosphoinositides (Hao et al., 1997). The crystal structure revealed an ANTH domain, which is similar to the ENTH domain and binds to PI(4,5)P₂ (Ford et al., 2001). AP180 might serve to tether clathrin to the membrane. Monolayers incubated with clathrin and AP180 form a flat lattice whereas adding AP-2 induces coated pit formation (Ford et al., 2001).

Eps15

Eps15 (Epidermal growth factor protein substrate 15) was found to interact with the ear domains of AP-2. This interaction is mediated via a carboxy-terminal DPF motif (Benmerah et al., 1996). The N-terminus contains three EH domains, which bind several endocytic proteins including epsin. EPS15 is localized to the edges of clathrin-coated pits. This points to a possible function in molecular dynamics at the periphery of the coat where it might function in recruiting dynamin (Tebar et al., 1996). Overexpression of the EPS15 carboxy-terminus inhibited endocytosis of transferrin, probably due to binding competition with the endogenous EPS15 for AP-2 binding. This shows that interaction of AP-2 with EPS15 is required for efficient receptor mediated endocytosis (Benmerah et al., 1998).

Epsin1

Epsin1 (EPS15 interacting protein) is the main binding partner of EPS15. It interacts via its carboxy-terminal region. The central region binds clathrin and AP-2. The most prominent part of the protein is its amino-terminus. It contains a highly conserved region, the ENTH domain. This domain derives its name from epsin1. Adding epsin1 or just its ENTH domain to liposomes converts them into tubules (Ford et al., 2002). In vitro studies with PI(4,5)P₂containing monolayers demonstrated that epsin 1 is able to recruit clathrin to the monolayer and to induce curvature (Ford et al., 2002). Since the expression of fragments or microinjection of antibodies inhibits clathrin-mediated endocytosis, epsin1 seems to play an important role in this process (Chen et al., 1999; Rosenthal et al., 1999).

Amphiphysin1

Amphiphysin1 was initially identified as a brain specific protein that is localized to synaptic vesicles (Lichte et al., 1992). It contains a SH3 domain, which specifically interacts with dynamin (David et al., 1996). Microinjection of this domain into synapses functions as a dominant negative mutant. This led to a massive block in endocytosis, pointing to a function in this pathway (Shupliakov et al., 1997). Amphiphysin1 binds clathrin, AP-2, and the lipid modifying proteins endophilin, synaptojanin, and phospholipase 1 & 2 (Slepnev and De Camilli, 2000). This network of interactions and the severe effect of the dominant negative mutants leads to the conclusion that Amphiphysin1 might function as an adaptor that recruits coat proteins to the membrane and targets dynamin and synaptojanin to the coat (Wigge and McMahon, 1998).

Auxilin

Auxilin can be found in two forms, a neuronal specific and a ubiquitously expressed form. The latter is also known as cycling G-associated kinase (GAK) (Brodsky et al., 2001). It contains an amino-terminal phosphatase and tensin homology (PTEN) domain, followed by a clathrin-binding domain and a

J domain (Umeda et al., 2000; Ungewickell et al., 1995). The PTEN domain could bind to actin and might play a function in phosphorylation of APs. The J domain is essential for stimulating the ATPase activity of hsc70, an important factor for uncoating (Kelley, 1998).

Auxilin can recruit hsc70 to the CCV through the interaction with clathrin and APs and stimulate uncoating activity (Brodsky et al., 2001). Clathrin association to membranes is disrupted when auxilin is overexpressed. This points to a function of auxilin as a cofactor in CCV uncoating (Umeda et al., 2000).

Dynamin 1 & 2

Dynamin is a GTPase that forms tetrameres that can stack to form rings and tubules (Hinshaw, 2000). Dynamin 1 is found at the PM, whereas dynamin 2 is associated with the TGN. Dynamin plays a role in scission of CCVs. The exact mechanism is still discussed. Two models were proposed. In the first model dynamin provides the mechanical force. It oligomerizes as a ring around the bud neck. This leads to constriction of the membrane, which will drive the vesicle to pinch off. The second model suggests that dynamin plays a role in attracting other proteins that mediate vesicle scission. The self-assembly would stimulate the GTPase activity, which would act as a sensor for vesicle closure (Marks et al., 2001; McNiven et al., 2000; Sever et al., 2000).

1.2.2 The COPI coat

Initially, clathrin was thought to be the only carrier for all vesicular transport. However, in yeast a viable mutant missing the clathrin heavy chain was shown to grow slowly but to secrete proteins at a normal rate (Payne and Schekman, 1985). Furthermore, a cell free reaction reproducing vesicular transport within the Golgi complex was shown not to depend on clathrin. This observation was underlined by the morphological observation that not all Golgi associated vesicles were covered by clathrin (Orci et al., 1986). This non-clathrin coat is called COPI. COPI coated vesicles form in the Golgi. They

consist of coatomer, a complex of seven subunits ($\alpha, \beta, \beta', \gamma, \delta, \epsilon$ and ζ) (Malhotra et al., 1989; Waters et al., 1991), and ARF1 (Orci et al., 1993). Coatomer can be reversibly dissociated into two subcomplexes, the F-COPI (β, γ, δ and ζ) and the B-COPI (α, β and ϵ) complex (Fiedler et al., 1996). Each of the subunits of the F-COPI complex contains significant sequence homology to subunits of AP-2. β and γ COP share similarity with the α and β 2 subunit, δ - and ζ COP show sequence similarity with μ 2 and σ 2 respectively. The structure of γ -COP has recently been solved. It shows a similar overall structure as the α and β appendage of AP-2 (Hoffman et al., 2003; Watson et al., 2004). Since these appendages of AP-2 interact with other proteins it is not surprising that also γ COP is able to interact with other proteins e.g. ARFGAP2 (Watson et al., 2004).

The COPI coatomer captures cargo containing a dilysine signal (KKXX or KXKXX) (Cosson and Letourneur, 1994) pointing to the function of retrieving cargo back to the ER. The KDEL receptor, which binds and retrieves luminal proteins containing a KDEL carboxy terminal sequence, is also transported via COPI vesicles (Cosson and Letourneur, 1997; Kirchhausen, 2000b).

1.2.3 The COPII coat

The COPII coat, was first discovered in yeast (Barlowe et al., 1994) in which most of the subsequent studies have been done. COPII coated vesicles form at the ER. They segregate exocytic cargo from ER resident proteins. On the surface of the ER the activated small GTPase Sar1p binds to the membrane followed by the recruitment of the Sec23p-Sec24p (Sec23/24p) complex, building a pre-budding complex (Springer et al., 1999). The structure of Sec23/24 has been analyzed by high-resolution electron microscopy (EM). It forms a bone like structure, indicating interaction of Sec 24p with the cargo and Sec 23p with Sar1p (Lederkremer et al., 2001; Matsuoka et al., 2001). The binding of Sec13p-Sec31p (Sec13/31p) drives polymerization of the coat and induces curvature (reviewed by Barlowe, 2002).

Different types of cargo are transported in COPII coated vesicles. In some transmembrane proteins, for example the vesicular stomatitis virus G protein

(VSV-G) and potassium channel proteins, a di-acidic motif (EXD) at the carboxy terminus, is important for ER export. In the VSV-G tail an additional tyrosine based motif was found to be important for optimal ER export (Ma et al., 2001; Nishimura and Balch, 1997). Other proteins integrate into COPII coated vesicles by interaction with cargo receptors, such as ERGIG53 and the p24 proteins that cycle between the ER and the Golgi. Their export depends on a pair of hydrophobic residues (e.g. FF or LL) contained in their cytoplasmic tail sequence (Dominguez et al., 1998; Kappeler et al., 1997). Another recently identified protein, Erv29p, serves as a receptor for soluble cargo (Belden and Barlowe, 2001).

1.2.4 ARF GTPases in protein trafficking

The hydrolysis of GTP to GDP and inorganic phosphate (Pi) plays a key role in numerous vital processes such as cell growth, protein synthesis, protein targeting, or vesicular transport. GTP binding proteins catalyze this process. Membrane traffic and organelle structure are regulated via the Ras-like ADP-ribosylation factors (ARFs). The GDP bound form is generally soluble and the GTP bound form binds to the membrane via its myristoyl tail (Goldberg, 1998). On a membrane, ARFs interact with their effectors and regulators, the guanine nucleotide exchange factors (GEFs), and the GTPase-activating proteins (GAPs). ARF effectors include lipid-modifying proteins and vesicle coat proteins. Mammals have six ARFs named ARF1-6. They are categorized into three classes based on sequence similarity. Class I comprise ARF1, 2 and 3, class II ARF4 and 5, and class III ARF6. Most extensive studies have been done on the class I ARFs, especially ARF1. These ARFs regulate the assembly of coat protein complexes onto vesicle-budding sites including COPI, GGA 1-3 and the AP-1, -3 and -4 clathrin adaptor complexes. ARF6, the only Class III member, functions in the endosome-plasma membrane recycling system and in the remodeling of actin cytoskeleton. Little is known about the Class II ARFs. (reviewed by Donaldson et al., 1992; Jackson and Casanova, 2000; Nie et al., 2003b; Scheffzek et al., 1998).

GEFs

Small Ras like monomeric GTPases undergo structural changes in response to binding of GTP or GDP. Different partner proteins recognize the different nucleotide state of the GTPase proteins. The exchange of GDP for GTP is not spontaneous, but is catalyzed by a guanine nucleotide exchange factor (GEF). First the GEF forms a low affinity complex with the GDP bound GTPase. GDP dissociates from this complex, which becomes a high affinity GEF-GTPase complex. Upon GTP binding, this intermediate rapidly dissociates, which leaves the GTPase in its active, GTP bound, state (Cherfilis and Chardin, 1999).

ARF GEFs build a large and diverse family of proteins. They all share a Sec7 domain that consists of about 200 amino acids. This domain alone is sufficient to catalyze nucleotide exchange (Chardin et al., 1996). The variable sequences outside the Sec7 domain are probably necessary for substrate specificity (Jackson and Casanova, 2000). The GEFs can be subdivided into two major families. The high molecular weight GEFs (>100 kDa) include the yeast GEF Sec7p, Gea1p and Gea2p and the mammalian GBF1, BIG1 and BIG2. They have been reported to be localized in the Golgi region and to be involved in membrane trafficking (Claude et al., 1999; Morinaga et al., 1997; Yamaji et al., 2000). The other family of low molecular weight GEFs (45-50kDa) include mammalian ARNO, cytohesin-1, GRP1/ARNO3, cytohesin-4, and EFA6. They are involved in endosomal recycling and cytoskeletal reorganization through activating ARF6 primarily (Cherfilis and Chardin, 1999).

BFA, a fungal metabolite, inhibits most of the ARF GEFs of the high molecular weight family. It blocks the activation of ARF, leading to the disassembly of the Golgi (Mansour et al., 1999). If the targeting of a protein to a membrane is inhibited by BFA it might indicate the involvement of an ARF. Therefore BFA is widely used as a tool to detect ARF dependent processes (Robinson and Kreis, 1992).

GAPs

GTP hydrolysis by ARF1 induces COPI uncoating (Tanigawa et al., 1993).

Since ARF1 alone has low intrinsic GTPase activity, it needs a GAP to activate it (Kahn and Gilman, 1986). ARFGAP1, the first identified GAP for ARF1 (Cukierman et al., 1995), is present in a cytosolic and a Golgi localized pool. The catalytic N-terminal GAP domain consists of ~140 residues (Cukierman et al., 1995) and is conserved in all known ARFGAPs. The C-terminal targeting domain is important for proper localization of the GAP (Huber et al., 1998).

The activity of ARFGAP1 can be modulated with a set of co-GAPs. This has been studied extensively in the COPI coat. Coatamer enhances the GAP activity of ARFGAP1, suggesting a mechanism that induces uncoating once the coat is made (Goldberg, 1999; Szafer et al., 2001). Golgi resident cargo, for example the p24 family of proteins which are packed into COPI coated vesicles, reduce the GAP activity of ARFGAP1. Adding peptides with the signal sequence of p24a to the catalytic domain of ARFGAP1 also inhibits its activity. In this case the GTPase acts as a timer giving cargo more time to be packed into vesicles (Goldberg, 2000; Lanoix et al., 2001). Another factor that influences the GAP activity is the lipid environment. It was found that in vitro the binding of ARFGAP1 and its activity on membrane bound ARF-GTP is increased by diacylglycerols with monounsaturated acyl chains (Antonny et al., 1997b). Since these are secondary products from the hydrolysis of phosphatidylcholine by ARF-activated phospholipase D this might provide a feedback mechanism for the regulation of uncoating (Brown et al., 1993). New findings demonstrate that the membrane bilayer curvature influences the GAP activity, proposing a model in which the activity is low as long as the bilayer is flat. The formation of a vesicle bends the bilayer which enables the GAP to access ARF1 (Bigay et al., 2003).

The regulation of GTP hydrolysis is similar in the COPII coat. Here, the GAP is Sec23p, which is already part of the coat. It induces GTP hydrolysis on the GTPase Sar1p. Once the coat is completed Sec13/31 acts as a co-activator for the GAP activity of Sec 23/24p, which leads to rapid uncoating (Antonny et al., 2001).

1.2.5 Vesicle targeting

The specificity of membrane tethering and fusion is critical for the proper flow of cargo within a cell. Two major classes of proteins play a critical role in this process. First the Rabs mediate the correct tethering of the incoming vesicle. Following this the specific pairing of SNAREs (Soluble N-ethylmaleimide-sensitive-factor attachment protein receptor) between the vesicle and the acceptor membrane ensures specific fusion at the correct place.

The Rabs form the largest family within the Ras family of small GTPases. More than 60 mammalian Rabs have been identified. They can be found as regulators of almost all steps of membrane traffic. The regulatory function of the Rabs lies in their ability to switch between the active GTP-bound and the inactive GDP-bound form (Rybin et al., 1996). Activated Rabs are membrane bound; they are prenylated, which helps them to anchor to the membrane. They recruit soluble effectors to the membrane that transduce the signal of the Rab GTPase into the transport mechanism. They can recruit membrane tethering and docking factors as well as motor proteins for vesicle transport. Different compartments contain distinct Rabs. For example Rab 1 can be found associated with the ER, Rab6 on the Golgi, Rab5 on early endosomes and Rab7 on late endosomes (Zerial and McBride, 2001).

The cycle of Rab5, an organizer of the early endosome, is well established. Rab5 is activated by the GEF Rabex-5 (Horiuchi et al., 1997), which initiates its association with the membrane. PI(3)-kinase, the enzyme that produces PI(3)P, interacts with Rab5, producing PI(3)P in the place where Rab5 is recruited (Christoforidis et al., 1999). The combination of these two signals creates two binding sites for early endosome antigen 1 (EEA1). In addition, Rab5 recruits the effectors rabaptin5 and rabenosin-5 to the same area of the membrane. SNAREs that are involved in the fusion of the membrane with the target membrane interact with these effectors. Normally the Rab stays active until fusion is completed. Afterwards it returns to the compartment of origin. This is mediated by the Rab GDP dissociation inhibitor (GDI) (Pfeffer, 2001). This protein has a high affinity to Rab-GDP. GTP hydrolysis takes place after fusion and converts to a substrate for GDI capture (Pfeffer, 2001; Zerial and

McBride, 2001).

Fusion of a vesicle with a membrane requires that SNARE proteins associated with the vesicle (v-SNARE) bind to SNARE proteins associated with the target membrane (t-SNARE)(Sollner et al., 1993). Since this naming scheme is confusing for homotypic fusion events, a structurally based scheme was introduced. A single key residue is either arginine (R-SNARE) or glutamine (Q-SNARE)(Fasshauer et al., 1998). Fusion of two membranes generally requires four SNAREs, where at least one of the membranes contributes multiple SNAREs. Most of the fusion reactions require one R-SNARE, usually contributed by the vesicle, and three Q-SNAREs, usually contributed by the target membrane (Bock et al., 2001; McNew et al., 2000). Before fusion the two membranes are bridged by this trans-SNARE complex, which then converts into a very stable cis-SNARE complex with all the SNAREs associated with the same membrane. This stable complex has to be disassembled, freeing the SNARE for productive trans-SNARE assembly and recycling those that have already mediated membrane fusion. The chaperones NSF (N-ethylmaleimide-sensitive-factor) and SNAP (soluble NSF attachment protein) mediate this process by consuming energy of ATP hydrolysis (Reviewed by Ungar and Hughson, 2003).

1.3 The role of inositol lipids as regulators of membrane traffic

Initially phosphoinositides (PIPs) have been identified as sources of second messengers diacylglycerol, inositol(1,4,5) trisphosphate, and phosphatidylinositol(3,4,5)trisphosphate (PI(3,4,5)P₃). Later experiments revealed that inositol lipids are able to function as a reversible recruiting device for proteins to transiently bind to membranes as regulators of other proteins. The inositol portion can be reversibly phosphorylated at positions 3', 4', or 5', leading to seven different forms which can interact with different proteins. The parent lipid of all these variations is phosphatidylinositol (PI). About 5-8% of all the lipids in mammalian cells consists of PI, which is synthesized in the ER (Whatmore et al., 1999). PI is present on the

cytoplasmic side of most membranes. It is delivered by a phosphatidylinositol transfer protein (PITP), which binds a single PI molecule and can exchange PI for PC depending on the PI content of the membrane (Hsuan and Cockcroft, 2001).

A set of various kinases and phosphatases modify PI. The distribution of these enzymes governs the specific distribution of the PIPs. ARF1, which is important for the initial step of AP-1 clathrin and COPI recruitment at the Golgi, interacts with different lipid kinases. It regulates the synthesis of PI(4,5)P₂ by recruiting PI5K β and PI5K α from the cytosol (Godi et al., 1999; Jones et al., 2000). Phospholipase D1 (PLD), which catalyse the hydrolysis of PC to PA and choline, is also recruited by ARF1. In vitro studies demonstrated that the activity of PI5K is increased in the presence of PA (Arneson et al., 1999). This might be an additional regulator for PI(4,5)P₂ synthesis. Since the GEF for the activation of ARF1 at the Golgi is able to bind to PIPs the recruitment of ARF1 is also regulated by the presence of PIPs (Jackson and Casanova, 2000).

In endocytosis PI(4,5)P₂ plays an important role in coat recruitment and vesicle release. AP-2 and many accessory proteins have been identified to contain binding sites for PI(4,5)P₂ (see section 1.2.1). Overexpression of a PH domain shows a severe inhibitory effect on endocytosis (Lee et al., 1999).

The use of PI3K inhibitors demonstrated the importance of PI(3)P at the stage of endosomal fusion. EEA1 was identified as the direct binding partner for PI(3)P through its FYVE domain (Stenmark and Aasland, 1999). In addition it interacts with the GTP bound form of Rab5, which serves as a dual recognition system and acts as a tethering device between two Rab5 positive membranes. This elegant principle of regulation might also apply for other lipids that are able to interact with proteins and therefore regulate important steps in membrane trafficking.

The finding that PIPs play an important role in trafficking was accomplished by the identification of various PIP binding domains. Binding partners to all the possible headgroups, except for PI(5)P have been identified in vivo. Those PIPs with a phosphate group at the 3 position are the least abundant. They

have to be recognized with high affinity and specificity. The PH domain fulfills these requirements and it is the only identified domain to bind to PI(3,4,5)P₃ and PI(3,4)P₂. PI(3)P has a large number of known specific binding partners. They all contain a FYVE or PX domain. PI(4,5)P₂ is the most abundant PIP. Therefore its binding domain has to be less specific. Proteins containing an ENTH or the homolog ANTH domain as well as some PH domain containing proteins have been identified to interact with PI(4,5)P₂ (Lemmon, 2003).

1.4 Coat recruitment and vesicle formation in chemically defined systems

To assess the mechanism of coat recruitment, various *in vitro* studies have been performed. In such a system it is possible to study the influence and the timing of the different players by manipulating their concentration or activity. COPII was the first coat where budding has been reconstituted using purified coat proteins and chemically defined liposomes. Matsuoka and coworkers were able to stepwise recruit Sar1p and Sec23/24p followed by Sec13/31p to liposomes. These coat compounds were sufficient to pinch off vesicles from chemically defined protein-free liposomes, which contained a small amount of PI(4)P or PI(4,5)P₂ (Matsuoka et al., 1998b). Later, GST bound sorting signals were coupled to the liposomes. In this system COPII proteins were sufficient to selectively sort cargo proteins into the vesicles (Matsuoka et al., 1998a). Coating of a liposome and the formation of small vesicles lead to changes in light scattering. This has been used to monitor the dynamics of COPII vesicle formation and disassembly (Antonny et al., 2001). Sec 23/24p was added to liposomes preloaded with activated Sar1p. Upon addition of Sec13/31p, vesicle formation could be observed. These vesicles were uncoated within seconds when GTP was used to activate Sar1p. When nonhydrolyzable GMP-PNP was used, the coat stayed on the liposome (Antonny et al., 2001).

Similar to COPII, COPI coated vesicles could be produced by incubating protein-free liposomes with coatomer, ARF1 and GTP γ S (Spang et al., 1998). Vesicle formation was most efficient when a lipid composition was used that is similar to the one of ER derived microsomes. However, for vesicle formation

from liposomes with a Golgi-like lipid composition, sorting signals were needed. These could be linked to liposomes by the use of lipopeptides. When these peptidoliposomes were incubated with ARF1 and coatamer, vesicle formation could be observed (Bremser et al., 1999). Therefore the minimal machinery to generate a COPI coated membrane vesicle consists of activated ARF1, coatamer and sorting signals. Recently the reconstitution of a full round of COPI recruitment followed by uncoating has been reported. Vesicles produced as described above became uncoated when they were incubated with ARFGAP1 (Reinhard et al., 2003).

The reconstitution of clathrin budding with chemically defined components however, seems more complicated. Interaction of the different APs with sorting signals has been studied *in vitro* using surface plasmon resonance (Heilker et al., 1996; Honing et al., 1998; Simmen et al., 2002). This method detects interaction of purified adaptors with immobilized peptides containing cytoplasmic sorting signals. It allows to define sorting signals and to calculate their binding affinities of the APs. However, the influence of the lipid bilayer and other factors cannot be assessed by this method. Studies of AP-1 recruitment to Golgi membranes provide a more physiological insight into early steps of coat formation. ARF1 binding generates transient high-affinity docking sites where AP-1 and clathrin can be recruited (Zhu et al., 1998). Golgi membranes prepared from cells devoid of MPRs lead to similar AP-1 recruitment. This suggests a MPR independent recruitment, possibly pointing to independence of any signal of this docking site (Zhu et al., 1999b). If ARF1 binds independent of sorting signals it should be possible to recruit AP-1 and clathrin to protein free liposomes that have been primed with ARF1-GTP. The Kornfeld Lab developed an assay where they incubated liposomes with ARF1, GTP γ S and cytosol and analyzed the bound material by pelleting the liposomes (Zhu et al., 1999a). In this assay AP-1 recruitment was possible to pure lipid membranes but depended on the presence of an unknown cytosolic factor. Using this assay the assembly of AP-3 containing CCVs was studied. ARF1 (or ARF5), GTP γ S, AP-3 and clathrin were sufficient to generate coats and to form CCVs (Drake et al., 2000).

Since AP-2/clathrin budding probably involves more accessory factors, it was not yet possible to reconstitute this process *in vitro*. However, when a lipid monolayer containing 10% PI(4,5)P₂ was incubated with AP-2 and AP180, flat clathrin coated pits, with a few invaginations could be observed (Ford et al., 2001). Incubating such a monolayer with AP-2 and epsin1 the formation of curved coated pits could be observed (Ford et al., 2002). These experiments point to the involvement of additional factors needed for a minimal system for AP-2 clathrin budding *in vitro*.

1.5 Aim of this thesis

As a transport vesicle is generated, several processes and functions occur simultaneously or in rapid succession (site selection, stepwise recruitment of coat components, cargo selection, induction of curvature, vesicle scission, and uncoating). These can be dissected in an *in vitro* system where pure components can be added in a defined order and contents can be manipulated. It was possible to reconstruct COPI and COPII recruitment and budding *in vitro* using only defined liposomes and purified components.

In vitro binding studies with clathrin adaptors revealed the role of sorting signals. Recruitment to liposomes was used to address the influence of lipids in coat recruitment. However, this was done in the absence of sorting signals and the addition of cytosol was essential. Until now, reconstituted coat recruitment of clathrin adaptors in a chemically defined minimal system was not reported.

Our first goal was to establish an *in vitro* system where the role of sorting signals and lipids in coat recruitment could be studied. We tried to define the minimal system required for AP-1 recruitment, it contains known interacting proteins, such as ARF1 and it was possible to find the lipid composition that is needed to recruit AP-1. The results are presented in Part I of this thesis.

The aim of part II is to understand the molecular mechanism of coat recruitment in more detail. First we explored the stability and the oligomeric state of recruited AP-1. Further a GAP was introduced into the system to understand the function of GTP hydrolysis in coat recruitment and uncoating.

Using AP-1 or other clathrin adaptor coats, we hope to eventually be able to reconstitute the whole cycle of coat recruitment, vesicle budding and uncoating in vitro. We should be able to monitor and control all the steps involved in this budding event, which will lead to a better understanding of how cargo travels through a cell and how these routes are regulated in vivo.

2 RESULTS

2.1 ARF1·GTP, Tyrosine-based Signals, and Phosphatidylinositol 4,5-Bisphosphate Constitute a Minimal Machinery to Recruit the AP-1 Clathrin Adaptor to Membranes

(Mol. Biol. Cell 13, 3672–3682)

Abstract:

At the trans-Golgi network, clathrin coats containing AP-1 adaptor complexes are formed in an ARF1-dependent manner, generating vesicles transporting cargo proteins to endosomes. The mechanism of site-specific targeting of AP-1 and the role of cargo are poorly understood. We have developed an in vitro assay to study the recruitment of purified AP-1 adaptors to chemically defined liposomes presenting peptides corresponding to tyrosine-based sorting motifs. AP-1 recruitment was found to be dependent on myristoylated ARF1, GTP or nonhydrolyzable GTP-analogs, tyrosine signals, and small amounts of phosphoinositides, most prominently phosphatidylinositol 4,5-bisphosphate, in the absence of any additional cytosolic or membrane bound proteins. AP-1 from cytosol could be recruited to a tyrosine signal independently of the lipid composition, but the rate of recruitment was increased by phosphatidylinositol 4,5-bisphosphate. The results thus indicate that cargo proteins are involved in coat recruitment and that the local lipid composition contributes to specifying the site of vesicle formation.

My contribution:

Lipid dependence study, purify and analyze pure AP-1, cytosolic adaptor recruitment

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ARF1-GTP, Tyrosine-based Signals, and Phosphatidylinositol 4,5-Bisphosphate Constitute a Minimal Machinery to Recruit the AP-1 Clathrin Adaptor to Membranes

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At the *trans*-Golgi network, clathrin coats containing AP-1 adaptor complexes are formed in an ARF1-dependent manner, generating vesicles transporting cargo proteins to endosomes. The mechanism of site-specific targeting of AP-1 and the role of cargo are poorly understood. We have developed an *in vitro* assay to study the recruitment of purified AP-1 adaptors to chemically defined liposomes presenting peptides corresponding to tyrosine-based sorting motifs. AP-1 recruitment was found to be dependent on myristoylated ARF1, GTP or nonhydrolyzable GTP-analogs, tyrosine signals, and small amounts of phosphoinositides, most prominently phosphatidylinositol 4,5-bisphosphate, in the absence of any additional cytosolic or membrane bound proteins. AP-1 from cytosol could be recruited to a tyrosine signal independently of the lipid composition, but the rate of recruitment was increased by phosphatidylinositol 4,5-bisphosphate. The results thus indicate that cargo proteins are involved in coat recruitment and that the local lipid composition contributes to specifying the site of vesicle formation.

INTRODUCTION

Sorting of membrane proteins is generally mediated by cytosolic coats which serve the dual role of creating a scaffold to form coated buds and vesicles and of selectively concentrating cargo proteins by interacting with cytosolic signals. The best studied systems are COPI in intra-Golgi and Golgi-to-endoplasmic reticulum (ER) transport, COPII in ER-to-Golgi transport, and clathrin with associated adaptor proteins in the formation of vesicles at the plasma membrane, the *trans*-Golgi network (TGN) and endosomes. There are different types of clathrin-associated adaptor proteins (APs), heterotetrameric complexes composed of two ~100-kDa adaptors, a ~50-kDa medium (μ), and a ~20-kDa small (σ)

chain (Robinson and Bonifacino, 2001). The adaptor complexes form the inner layer of the coat that specifies the site of coat formation and interacts with cargo molecules. AP-1 adaptors are primarily functional at the TGN generating vesicles destined for endosomes but have also been found on sorting endosomes and implicated in (basolateral) recycling to the plasma membrane (Futter *et al.*, 1998). AP-2 adaptors are found at the plasma membrane to form coated vesicles for endocytosis. AP-3 adaptors are involved in lysosomal transport from the TGN or endosomes. The different adaptor complexes recognize similar tyrosine and dileucine signals in cargo molecules, and in many cases the same signals are recognized by several adaptor types (Bonifacino and Dell'Angelica, 1999; Heilker *et al.*, 1999).

Recruitment of the different coats to their specific membranes appears to involve common basic mechanisms. With the exception of AP-2/clathrin coats, all the coats mentioned above require small GTPases that are activated from their soluble GDP-bound to their membrane-associated GTP-bound form by a guanine nucleotide exchange factor (GEF) at the correct membrane. For COPII coats in yeast, the GTPase Sar1p is activated by the GEF Sec12p in the ER membrane. In an assay with chemically defined liposomes containing acidic lipids like phosphatidic acid (PA), phosphatidylserine (PS), or phosphoinositides, these components were sufficient to recruit the subunits of COPII, first

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Abbreviations used: AP, adaptor protein; ARF1, ADP-ribosylation factor 1; ER, endoplasmic reticulum; GEF, guanine nucleotide exchange factor; GMP-PNP, guanylyl imidodiphosphate; GTP γ S, guanosine 5'-O-(3-thiotriphosphate); Lamp-1, lysosome-associated membrane protein-1; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIP, phosphoinositide; PS, phosphatidylserine; TGN, *trans*-Golgi network.

2.1.1 Introduction

Sorting of membrane proteins is generally mediated by cytosolic coats which serve the dual role of creating a scaffold to form coated buds and vesicles and of selectively concentrating cargo proteins by interacting with cytosolic signals. The best studied systems are COPI in intra-Golgi and Golgi-to-endoplasmic reticulum (ER) transport, COPII in ER-to-Golgi transport, and clathrin with associated adaptor proteins in the formation of vesicles at the plasma membrane, the trans-Golgi network (TGN) and endosomes. There are different types of clathrin-associated adaptor proteins (APs), heterotetrameric complexes composed of two ~100-kDa adaptins, a ~50-kDa medium (μ), and a ~20-kDa small (σ) chain (Robinson and Bonifacino, 2001). The adaptor complexes form the inner layer of the coat that specifies the site of coat formation and interacts with cargo molecules. AP-1 adaptors are primarily functional at the TGN generating vesicles destined for endosomes but have also been found on sorting endosomes and implicated in (basolateral) recycling to the plasma membrane (Futter et al., 1998). AP-2 adaptors are found at the plasma membrane to form coated vesicles for endocytosis. AP-3 adaptors are involved in lysosomal transport from the TGN or endosomes. The different adaptor complexes recognize similar tyrosine and dileucine signals in cargo molecules, and in many cases the same signals are recognized by several adaptor types (Bonifacino and Dell'Angelica, 1999; Heilker et al., 1999).

Recruitment of the different coats to their specific membranes appears to involve common basic mechanisms. With the exception of AP-2/clathrin coats, all the coats mentioned above require small GTPases that are activated from their soluble GDP-bound to their membrane-associated GTP-bound form by a guanine nucleotide exchange factor (GEF) at the correct membrane. For COPII coats in yeast, the GTPase Sar1p is activated by the GEF Sec12p in the ER membrane. In an assay with chemically defined liposomes containing acidic lipids like phosphatidic acid (PA), phosphatidylserine (PS), or phosphoinositides, these components were sufficient to recruit the subunits of

COPII, first Sec23p/24p and then Sec13p/31p, to form coated buds and vesicles (Matsuoka et al., 1998b). In the presence of cargo membrane proteins (the v-SNAREs Sec22p or Bos1p), these were selectively incorporated (Matsuoka et al., 1998a).

For COPI coats, the GTPase ARF1 (ADP-ribosylation factor 1) is activated by a Golgi-associated GEF. On liposomes made of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) with unsaturated fatty acids or containing acidic phospholipids, ARF1·GTP γ S and COPI complexes were sufficient to form coats and vesicles (Spang et al., 1998; Bremser et al., 1999). However, with saturated lipids of different compositions, COPI recruitment was only achieved in the presence of liposome-associated cargo sequences (Bremser et al., 1999).

Recruitment of the clathrin adaptors AP-1 and AP-3 also involves ARF1, together with specific GEFs (e.g., BIG2; Shinotsuka et al., 2002). ARF·GTP γ S, AP-3, and clathrin were sufficient to generate coats on liposomes made from soybean lipids (containing 20% PC and various other lipids) and to bud coated vesicles (Drake et al., 2000). Based on various studies (Dittié et al., 1996; Mallet and Brodsky, 1996; Seaman et al., 1996; Zhu et al., 1998, 1999a), the following model for AP-1/clathrin coat formation has been proposed (Zhu et al., 1998). After nucleotide exchange in ARF1 by a GEF at the site of coat initiation, ARF1·GTP will interact rapidly with putative docking protein(s) to generate high-affinity binding sites for AP-1. In turn, clathrin trimers will bind to immobilized AP-1 and laterally associate to form the characteristic lattice. Cargo molecules will associate with AP-1 despite the low affinity of interaction, because AP-1 is highly concentrated in the coat. GTP hydrolysis induced by an ARF GTPase-activating protein will eventually inactivate the docking protein. As the growing coat soon interacts with multiple cargo proteins, it will stay membrane bound even as docking proteins and ARF1·GDP dissociate.

It has been proposed that the mannose-6-phosphate receptors form the major docking sites for AP-1 at the TGN (Le Borgne and Hoflack, 1997), a concept that has been challenged by studies with Golgi membranes devoid of mannose-6-phosphate receptors (Zhu et al., 1999b). In addition, the finding

that AP-1 could be recruited in an ARF1-dependent manner to protein-free soybean liposomes, which can be easily pelleted, in the presence of cytosol indicated that integral membrane proteins are not necessary (Zhu et al., 1999a). Yet, the cytosol dependence of the process suggested the involvement of a soluble cytosolic factor(s) that peripherally attaches to the liposomes and functions as the AP-1 docking site. Peripheral membrane proteins have also been shown to bind to AP-1 on affinity chromatography (Mallet and Brodsky, 1996), and a Tris-strippable factor was shown to be required for AP-1 binding to immature secretory granules (Dittié et al., 1996). AP-1 binding to liposomes was dependent on the lipid composition, which thus might play a role in the binding of a cytosolic factor to the membrane. A soybean lipid mixture containing 20% PC and acidic lipids was optimal, whereby PS, but to some extent also phosphatidylinositol (PI) or PA seemed to contribute (Zhu et al., 1999a).

In the present study, we have analyzed the minimal requirements for the recruitment of AP-1 adaptor complexes to a membrane in vitro using chemically defined liposomes in a floatation assay that does not require the liposomes to be pelletable. In particular, the contributions of cargo-sorting signals and lipids were tested. Stable AP-1 recruitment was found to require in addition to myristoylated ARF1·GTP also the presence of membrane-anchored tyrosine signals and specific phosphoinositides but no further cytosolic factors.

2.1.2 Materials and methods

Reagents

Guanylyl imidodiphosphate (GMP-PNP), guanosine 5'-O-(3-thiotriphosphate; GTP γ S), and GTP were from Roche Diagnostics. Superose-6 (Prep grade) and ECL reagent were from Amersham Pharmacia Biotech (Piscataway, NJ). N-((4-maleimidylmethyl)cyclohexane-1-carbonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (MMCC-DHPE) was from Molecular Probes (Eugene, OR). Egg PC, liver PI, liver PE, and brain PS were from Avanti Polar Lipids (Alabaster, AL), phosphatidylinositol 3-phosphate (PI3P), PI5P, and PI(3,4)P₂ from Echelon Research Laboratories Inc. (Salt Lake City, UT), PI(3,5)P₂ from Calbiochem (La Jolla, CA), and PI(3,4,5)P₃ from Matreya Inc. (Pleasant Gap, PA). mAb 100/3 (anti γ -adaplin), horseradish peroxidase-coupled anti-mouse IgG antibody, PI4P, PI(4,5)P₂, soybean PC (azolectin, P-5638), mixed phosphoinositides (P-6023), GDP, and dipalmitoyl-PA were purchased from Sigma (Buchs, Switzerland). Peptides were synthesized on a Pioneer synthesizer (PerSeptive Biosystems, Framingham, MA) using Fmoc (fluorenylmethoxycarbonyl) protected amino acids with TBTU (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate) as coupling reagent. Cleaved and deprotected peptides were first purified via reverse phase HPLC (RP C18, Vydac, Hesperia, CA) and then verified by MALDI-TOF mass spectrometry (TOFSPEC-2E, Micromass, Manchester, UK). mAb 1D9 against ARF1 was a kind gift by Richard Kahn (Emory University, Atlanta, GA).

Purification of AP-1 and ARF1

Clathrin-coated vesicles were purified from calf brains, freshly obtained at the local slaughterhouse as described (Campbell et al., 1984). All the procedures were performed at 4°C. The coats were released by homogenizing vesicles with one volume of 1.5 M Tris-HCl (pH 7.0), 6 mM EDTA, 0.6 mM DTT, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), and 10 μ g/ml benzamidine and 2 μ g/ml pepstatin A, leupeptin, antipain, and chymostatin. After overnight

incubation at 4°C membranes were spun for 30 min at 100,000 x g, and the supernatant was loaded in 2-ml portions on a 50 x 1.6 cm Superose-6 column equilibrated with 0.5 M Tris-HCl (pH 7.0), 2 mM EDTA, 0.2 mM DTT and run at 0.5 ml/min. Mixed adaptors were collected between 55 and 64 ml of elution. To eliminate the remaining clathrin, mixed adaptors were dialyzed into 0.1 M MES, 1 mM EGTA, 0.5 mM MgCl₂, 0.2 mM DTT (pH 6.6) to form clathrin cages and centrifuged for 1 h at 400,000 x g. Although clathrin was only found in the pellet with most of AP-2 and AP180, AP-1 largely stayed in solution in accordance with its lower cage-promoting activity (Keen, 1989; Lindner and Ungewickell, 1992). The supernatant was dialyzed into 20 mM ethanolamine, pH 8.9, 2 mM EDTA, 1 mM DTT (MonoQ buffer; Ahle et al., 1988) and loaded on a 2-ml CHT-II hydroxyapatite column (Bio-Rad, Cambridge, MA) that was equilibrated and washed with 0.5 M Tris-HCl, 2 mM K/PO₄, pH 7.0, followed by 10 mM phosphate in the same buffer. AP-1 was eluted stepwise with 50 mM and 100 mM phosphate. Purified AP-1 was dialyzed against MonoQ buffer containing 0.5 mM PMSF and stored at 4°C with protease inhibitors. The 70-kDa protein was identified after Coomassie staining and in-gel digestion with trypsin (Perrot et al., 1999) by analysis on a Reflex III MALDI-TOF instrument (Bruker, Bremen, Germany) using -cyano-hydroxy-cinnamic acid as matrix. Protein identification was done using the Mascot software (Matrix Science Ltd., London, UK).

Plasmids encoding bovine ARF1 with residues 3-7 from yeast Arf2p (Liang et al., 1997) and yeast N-myristoyltransferase (pBB131; Duronio et al., 1990) were generous gifts by Stuart Kornfeld and Jeffrey Gordon, respectively (both at Washington University, St. Louis, MO). After cotransformation of both plasmids into *Escherichia coli* BL21(DE3), myristoylated ARF1 was purified as described (Liang and Kornfeld, 1997). This ARF1 preparation bound to Golgi membranes (Martín et al., 2000), indicating its efficient myristoylation. Nonmyristoylated ARF1 was also prepared and purified and showed the expected mobility shift on SDS gel electrophoresis (Franco et al., 1995; Liang and Kornfeld, 1997). Proteins were quantified using the bicinchoninic acid assay (BCA; Pierce, Rockford, IL) or the Bradford assay (Bio-Rad; for

samples containing Tris), using bovine serum albumin as standard. Silver staining of polyacrylamide gels was performed as described (Morrissey, 1981).

Preparation of Peptidoliposomes

Five micromoles of egg PC (3.8 mg) were combined with 125 nmoles MMCC-DHPE (2.5 mol %). When indicated, other lipids were used to replace some of the PC. The organic solvent was evaporated under a stream of nitrogen. Dichloromethane was added and evaporated twice. Dried lipids were resuspended into 1 ml 10 mM HEPES (pH 6.5), 0.1 M NaCl, 0.5 mM EDTA and freeze-thawed five times in liquid nitrogen and then extruded 11 times through a 400-nm Nucleopore polycarbonate membrane (Corning, Corning, NY) using a homemade hand-driven extruder. The liposomes (0.3 ml) were immediately incubated with 120 μ g of peptide (i.e., about a fourfold excess over the coupling lipid, assuming half of it is exposed) for 1 h at room temperature, and then stored at 4°C with 0.02% (wt/vol) NaN₃ for up to 2 weeks. The coupling efficiency varied from ~30 to 50% as judged by measuring the amount of peptide associated with the liposomes the bicinchoninic acid assay after extensive dialysis of the liposomes against phosphate-buffered saline. We found it unnecessary to remove free peptides from the liposomes before the AP-1 recruitment assay (negligible inhibition of adaptor binding to immobilized peptides had also been observed in surface plasmon resonance assays; Heilker et al., 1996).

Liposome Recruitment Assay

Peptidoliposomes (200 μ l; 1 μ mol lipid) were first incubated for 30 min at 37°C with 5 μ g of ARF1 and either 0.2 mM GMP-PNP (or GTP γ S), or 2 mM GTP or GDP. When GTP or GDP were used, 3 mM phosphate was also added to inhibit hydrolysis by a spurious phosphatase (Franco et al., 1995). Samples were returned to ice and 10 mM MgCl₂ was added to stabilize the loaded ARF1 (Franco et al., 1995) as well as 10 μ g of mixed adaptors or 0.5 μ g of AP-1. After 15 min on ice, samples of 250 μ l were mixed with 0.5 ml of 60% (wt/vol) sucrose in assay buffer (10 mM HEPES, pH 7.0, 150 mM NaCl, 10

mM KCl, 3 mM potassium phosphate, 2 mM MgCl₂, 0.2 mM dithiothreitol; Höning et al., 1997), overlaid with 3.07 ml of 20% sucrose in assay buffer and with 0.18 ml of buffer in a 4-ml centrifuge tube, and centrifuged in a TST60 rotor (Kontron, Zurich, Switzerland) at 55,000 rpm (300,000 × g_{av}) for 1 h at 4°C. Four 1-ml fractions were collected from the top and precipitated with 8% (wt/vol) trichloroacetic acid. Acetone-washed pellets were analyzed by 7.5-15% PAGE and immunoblotting using antibodies to γ -adaptin (100/3) or ARF1 (1D9), a peroxidase-coupled secondary antibody, and ECL reaction. Quantitation was performed using a Multimage Light Cabinet from Alpha Innotech Corporation (San Leandro, CA).

Cytosol was obtained from calf brain or bovine adrenals (gift of Kitaru Suda, Biozentrum, Basel, Switzerland) as the high-speed supernatant after homogenization (Campbell et al., 1984), supplemented with protease inhibitors, and clarified by centrifugation before use. Peptidoliposomes (0.5 μ mol lipid) were incubated for 30 min at 37°C with 0.5 mg of cytosol, 5 μ g of ARF1, and 0.2 mM GMP-PNP in 200 μ l of assay buffer. Samples were returned to ice and mixed with 0.4 ml of 60% (wt/vol) sucrose in assay buffer, and liposomes were floated as described above.

Nucleotide Exchange Assay

Nucleotide exchange was measured using [³⁵S] GTP γ S and the filtration assay according to Franco et al. (1995) under the experimental conditions used for the recruitment assay.

2.1.3 Results

An Assay for AP-1 Recruitment to Model Membranes

To assess the interaction of AP-1 adaptors to sorting signals in the context of a chemically defined membrane, we coupled synthetic peptides via an N-terminal cysteine to a maleimide derivative of PE, thus creating lipid-anchored peptides. The reactive lipid was mixed with PC or various lipid mixtures at 2.5 mol %, and large unilamellar liposomes were produced by extrusion through a 400-nm pore-size filter. Peptides were then coupled via an N-terminal cysteine to the reactive lipid (Figure 1A). The peptides used (Lamp1Y and TGN38Y) corresponded to the C-terminal cytoplasmic domain of Lamp-1 (lysosome-associated membrane protein-1) and a portion of the cytoplasmic domain of TGN38 (trans-Golgi network protein of 38 kDa), two proteins with well characterized tyrosine-containing sorting signals (Figure 1B). The same peptides with the tyrosines mutated to alanine (Lamp1A and TGN38A) were used as negative controls. Lamp-1 is sorted from the TGN via endosomes to lysosomes (Hunziker and Geuze, 1996) and has been demonstrated by immunogold electron microscopy in AP-1-positive clathrin-coated buds and vesicles at the TGN (Höning et al., 1996). TGN38 cycles between the TGN and the plasma membrane. An interaction with AP-1 is less clearly established (Ohno et al., 1995; Boll et al., 1996; Stephens et al., 1997).

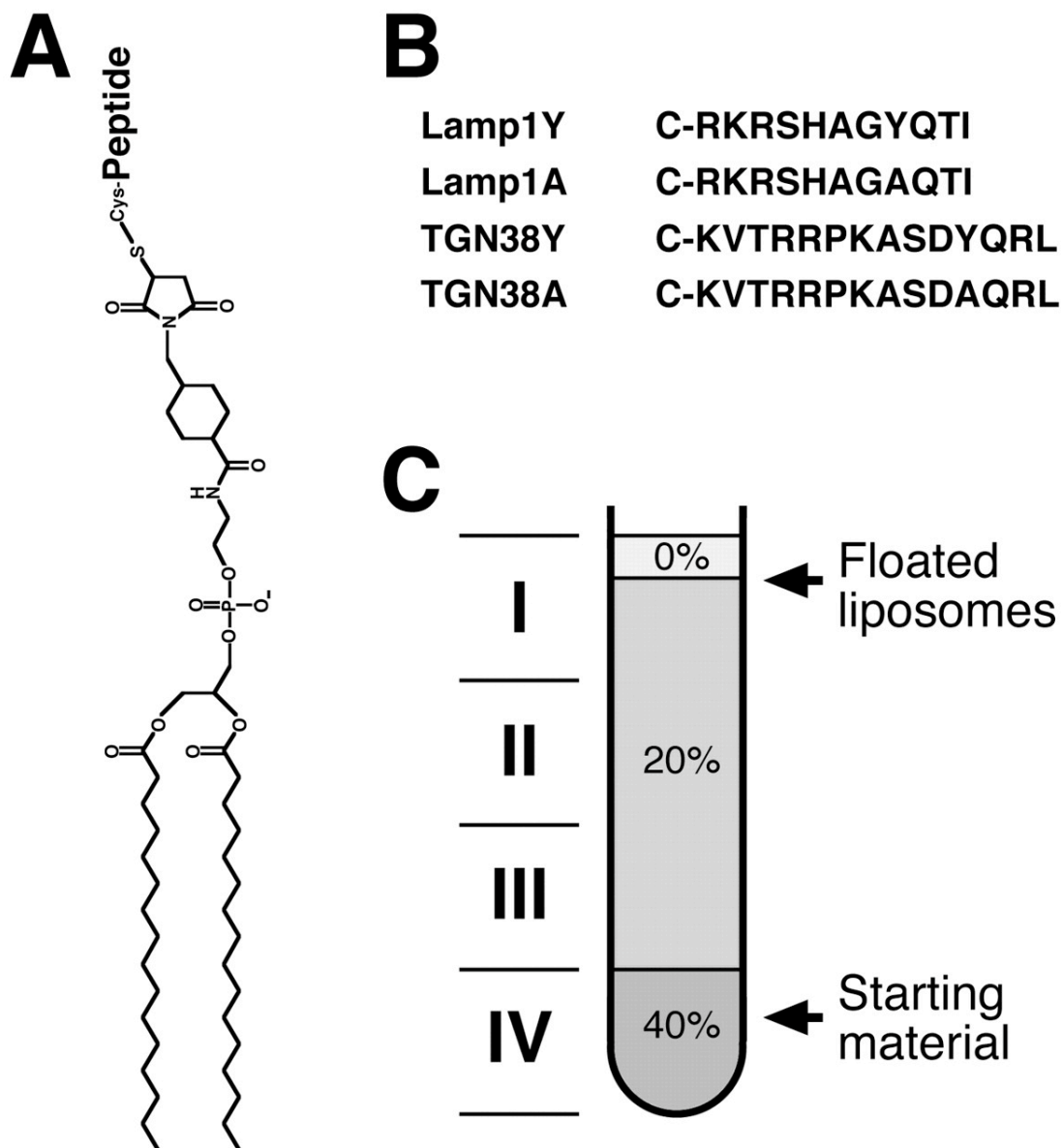


Figure 1

Peptidoliposomes to assay AP-1 recruitment in vitro. The maleimide derivative of PE MMCC-DHPE was used to couple synthetic peptides via an N-terminal cysteine to a lipid (A). The peptides used correspond to the cytoplasmic domain of Lamp1 (B, Lamp1Y) or the segment of TGN38 that has previously been shown to contain the functional tyrosine motif (Boll et al., 1996). Lamp1A and TGN38A are the control peptides with the critical tyrosine mutated to alanine. After incubation of peptidoliposomes with AP-1 and with or without ARF1, they were floated from the bottom of a sucrose step gradient (C). Four fractions were collected as indicated, with fraction I containing the floated liposomes with bound proteins and fraction IV including the loading zone with unbound proteins.

Adaptor complexes were isolated from calf brain coated vesicles by releasing the coat with 1 M Tris followed by gel filtration to remove the bulk of clathrin. This mixed adaptor preparation (containing both AP-1 and AP-2) was incubated with the peptidoliposomes. The mixture, supplemented with sucrose to a concentration of 40%, was then loaded below a 20% sucrose cushion and a small amount of sucrose-free buffer (Figure 1C) and centrifuged for 1 h at 300,000 x g to separate the liposomes and bound proteins from free adaptors. The gradient was collected from the top in four fractions (I-IV), with fraction I containing the floated liposomes with recruited proteins and fraction IV containing unbound material. Aliquots of the four fractions were analyzed by SDS-gel electrophoresis and probed by immunoblot analysis.

Because *in vivo* recruitment of AP-1 to the TGN requires the GTPase ARF1 in its active GTP-bound form (Stamnes and Rothman, 1993; Traub et al., 1993), the potential requirement of ARF1 in our assay was tested by incubating purified ARF1 with the peptidoliposomes together with GTP or a nonhydrolyzable GTP analog (GMP-PNP or GTP γ S) at 37°C for 30 min before addition of adaptors. It has previously been shown that liposomes induce guanine nucleotide exchange on ARF1 and thus activate it (Antonny et al., 1997), a function performed *in vivo* by specific GEFs at the TGN.

Recruitment of AP-1 Adaptors to Liposomes Requires a Tyrosine-based Signal, ARF1, and Specific Lipids

In previous *in vitro* assays, AP-1 was shown to bind to the cytoplasmic sequence of Lamp-1 immobilized on beads or on the sensor surface in surface plasmon resonance experiments (Höning et al., 1996). In our assay, however, no recruitment of AP-1 could be observed to Lamp1Y presented on liposomes made of PC or of a 1:1 mixture of PC and soybean lipids (azolectin; Figure 2A, lanes 1-4). -Adaptin, a 100-kDa subunit of AP-1 complexes, was detected exclusively in fraction IV of the step gradients, which represents the loading zone. This result is consistent with the apparent dissociation rates of adaptors from immobilized tyrosine motifs in surface plasmon resonance experiments (Heilker et al., 1996; Höning et al., 1996), which would not allow

interacting adaptors to stay bound to the peptidoliposomes during a 1-h floatation.

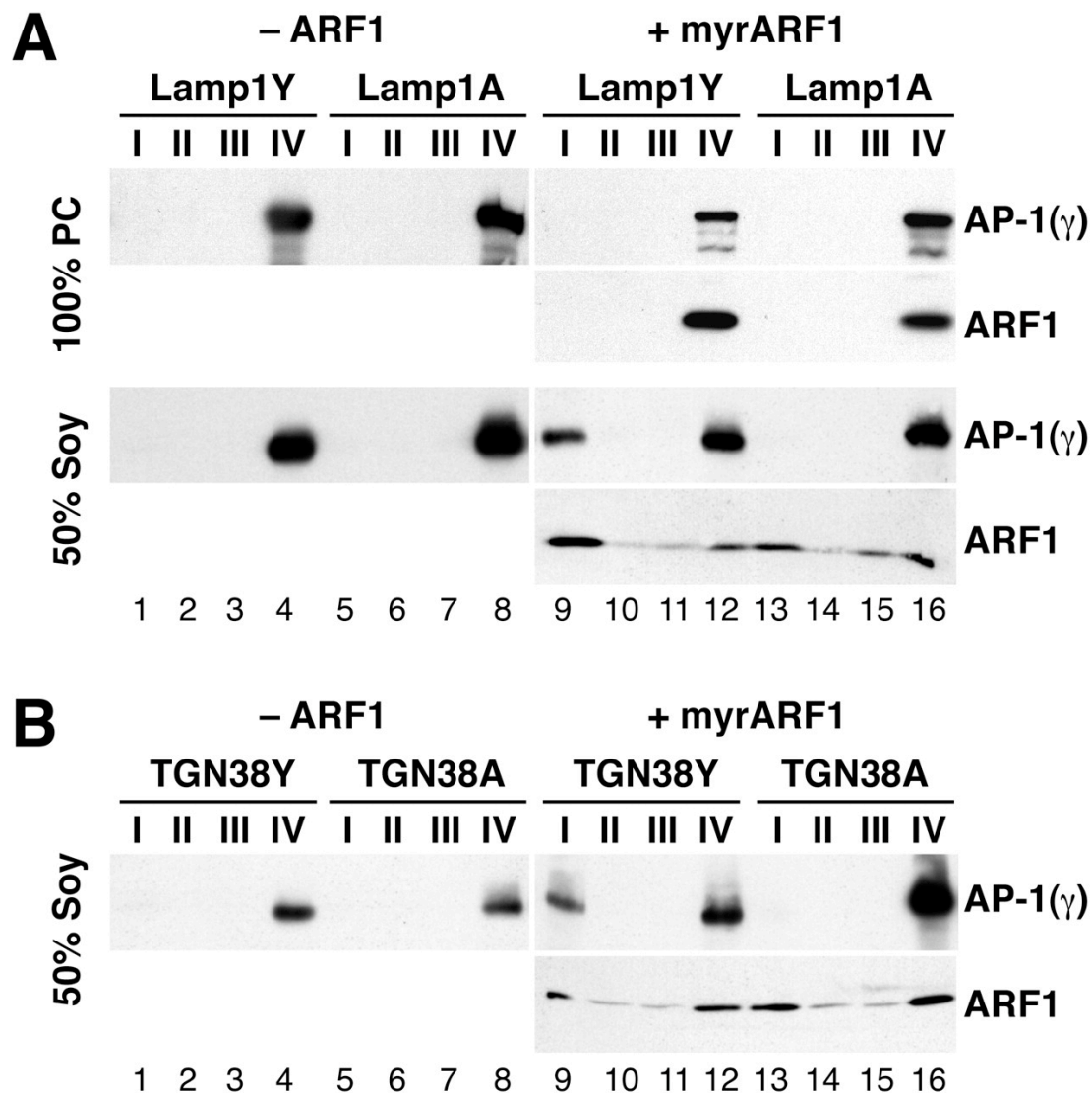


Figure 2

AP-1 recruitment to peptidoliposomes is signal-, ARF1- and lipid-dependent. (A) Peptidoliposomes made of 100% PC or 50% PC/50% soybean lipids and presenting Lamp1Y or Lamp1A peptides were incubated with a mixed adaptor preparation and with or without myristoylated ARF1 and GMP-PNP. After flotation on a sucrose step gradient, four fractions (I-IV, as shown in Figure 1C) were collected from the top and analyzed by immunoblotting for -adaplin or ARF1. (B) The same experiments were performed using peptidoliposomes made of 50% PC/50% soybean lipids and presenting TGN38Y or TGN38A peptides.

However, if purified myristoylated ARF1 with GMP-PNP was added to the Lamp1Y peptidoliposomes and incubated at 37°C before addition of adaptors, a significant fraction of AP-1 was floated to the top of the gradient (fraction I) together with liposomes containing 50% soybean lipids (Figure 2A, lanes 9-12). AP-1 was not recruited to liposomes presenting Lamp1A peptides or to

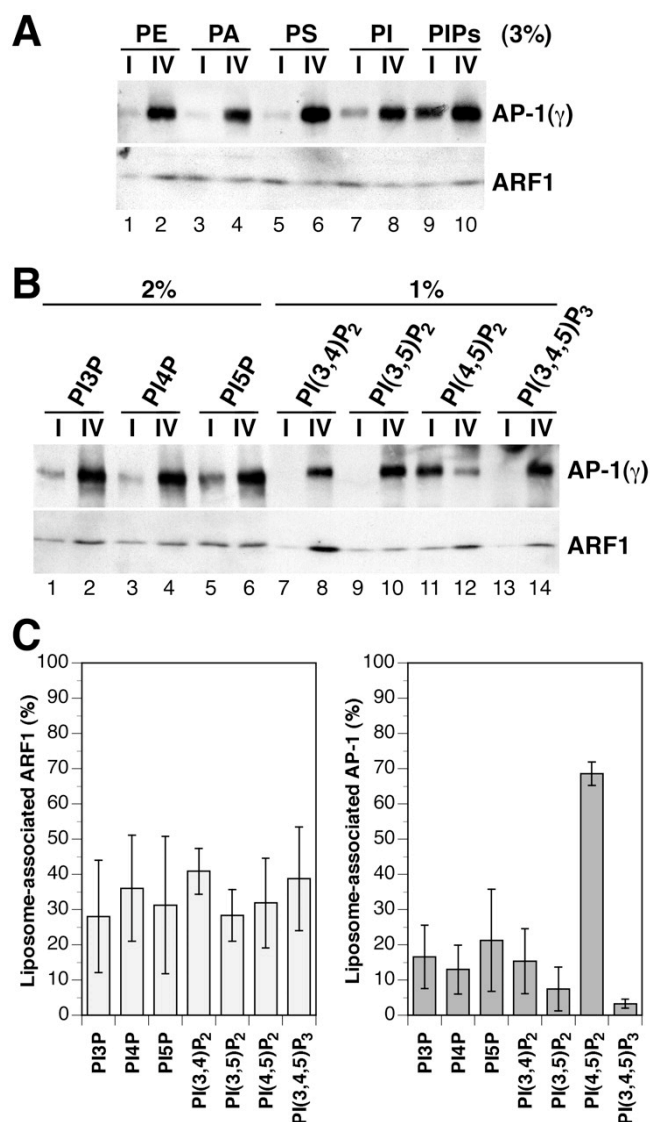
liposomes composed entirely of PC (lanes 9-16) even in the presence of ARF1·GMP-PNP.

AP-1 recruitment to the membrane was rather stable, because the middle fractions II and III of the gradient were entirely devoid of γ adaptin, indicating that bound adaptors did not significantly dissociate during the floatation. This is in contrast to the interaction of the bulk of ARF1 with liposomes. On nucleotide exchange, the active ARF1 exposes its myristoyl tail, which allows it to interact with lipid bilayers (Antonny et al., 1997). The equilibrium between lipid-associated and soluble ARF1 is shifted by the addition of soy lipids in favor of the lipid-associated form: although ARF1 is not dragged out of the loading zone (fraction IV) by pure PC liposomes (in agreement with Helms et al., 1993), approximately half of ARF1 was floated to fraction I in the presence of 50% soybean lipid, with considerable trailing into fractions II and III. The residual clathrin in the adaptor preparation was not corecruited with AP-1.

Like Lamp1Y, the tyrosine motif peptide TGN38Y was similarly able to recruit AP-1 only in the presence of ARF1·GMP-PNP and with liposomes containing 50% soybean lipids (Figure 2B). Again, recruitment depended on the tyrosine signal, because TGN38A was not functional. ARF1, in contrast, was associated with liposomes irrespective of the peptides coupled to them. The results show that recruitment of AP-1 to liposomes requires activated ARF1, functional tyrosine motifs, and a particular lipid composition.

Phosphoinositides Are Required to Recruit AP-1

The soybean lipids used in Figure 2 contain 20% PC and an ill-defined mixture of other lipids. To identify which components are responsible for AP-1 recruitment, 3% of PE, PA, PS, PI, or a mixture of phosphoinositides (PIPs) were added to PC to produce peptidoliposomes presenting Lamp1Y in our assay (Figure 3A). AP-1 was not significantly recruited to the liposomes containing PE, PA, or PS and only slightly to those containing 3% PI. Most efficient recruitment was reproducibly observed to liposomes containing phosphoinositides.

**Figure 3**

Lipid requirement for AP-1 recruitment to peptidoliposomes. (A) Three percent of the indicated lipid was incorporated into PC peptidoliposomes exposing Lamp1Y. After incubation with a mixed adaptor preparation and with myristoylated ARF1-GMP-PNP, fractions I and IV of a flotation gradient were analyzed by immunoblotting. PIPs indicates a commercial mixture of phosphoinositides. (B) Two percent of PI-monophosphates and 1% of PI-bis- and trisphosphates were incorporated into PC peptidoliposomes exposing Lamp1Y and analyzed as in A. (C) The recruitment of AP-1 and ARF1 to liposomes containing different phosphoinositides (2% of PI-monophosphates and 1% of PI-bis- and trisphosphates) were densitometrically quantified. The amount recovered in fraction I is expressed in percent of the total in fractions I plus IV. The average and SDs of at least three experiments, including those shown in B, are presented.

To determine which phosphoinositides are capable of stimulating AP-1 recruitment, we compared Lamp1Y/PC peptidoliposomes containing 2% of the monophosphorylated phosphoinositides PI3P, PI4P, or PI5P, or 1% of the phosphatidylinositol bisphosphates PI(3,4)P₂, PI(3,5)P₂, or PI(4,5)P₂, or phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P₃). At these concentrations

the phosphoinositides with one and two phosphates on the inositol ring introduce approximately the same negative charge to the membranes.

Among the monophosphorylated phosphoinositides, PI5P was the most effective in recruiting AP-1 (Figure 3, B and C), whereas PI3P and PI4P were only marginally functional. However, the most efficient AP-1 recruitment of all was obtained with PI(4,5)P₂, even though it was used at only half the concentration of the monophosphorylated phosphoinositides. The other bis- or trisphosphorylated molecules were unable to sustain AP-1 recruitment. In contrast to the pronounced lipid dependence of AP-1 recruitment, the amount of ARF1 recovered in fraction I did not show significant differences for different lipids used.

AP-1 Recruitment Depends on Myristoylated ARF1 in Its Active Conformation

In the above experiments, GMP-PNP, a nonhydrolyzable analogue of GTP was used, indicating that GTP hydrolysis is not required for AP-1 recruitment to peptidoliposomes. In Figure 4, we further analyzed the nucleotide requirement using myristoylated ARF1 and liposomes with 10% mixed phosphoinositides and Lamp1Y peptides. No AP-1 recruitment and no ARF1 association with liposomes was detected when only GDP was added to the ARF1/peptidoliposome incubation (lanes 9 and 10), demonstrating that AP-1 binding required active ARF1. No significant differences in the efficiency of AP-1 recruitment were observed when GTP, GTP γ S, or GMP-PNP were used as the nucleotide. In contrast, ARF1 association with liposomes reproducibly depended on the type of GTP analog used. ARF1-GTP γ S floated more efficiently with liposomes than ARF1-GMP-PNP, whereas ARF1-GTP did so the least (lanes 3-8). This is possibly due to slight differences in conformation and/or to some hydrolysis of GTP. Both AP-1 recruitment and ARF1 association with peptidoliposomes depended on incubation of ARF1 with liposomes at 37°C because they were almost completely abolished at 4°C (Figure 4, lanes 1-4). This reflects the fact that nucleotide exchange is temperature dependent. As expected, unmyristoylated ARF1 was not

functional in the assay (lanes 11 and 12).

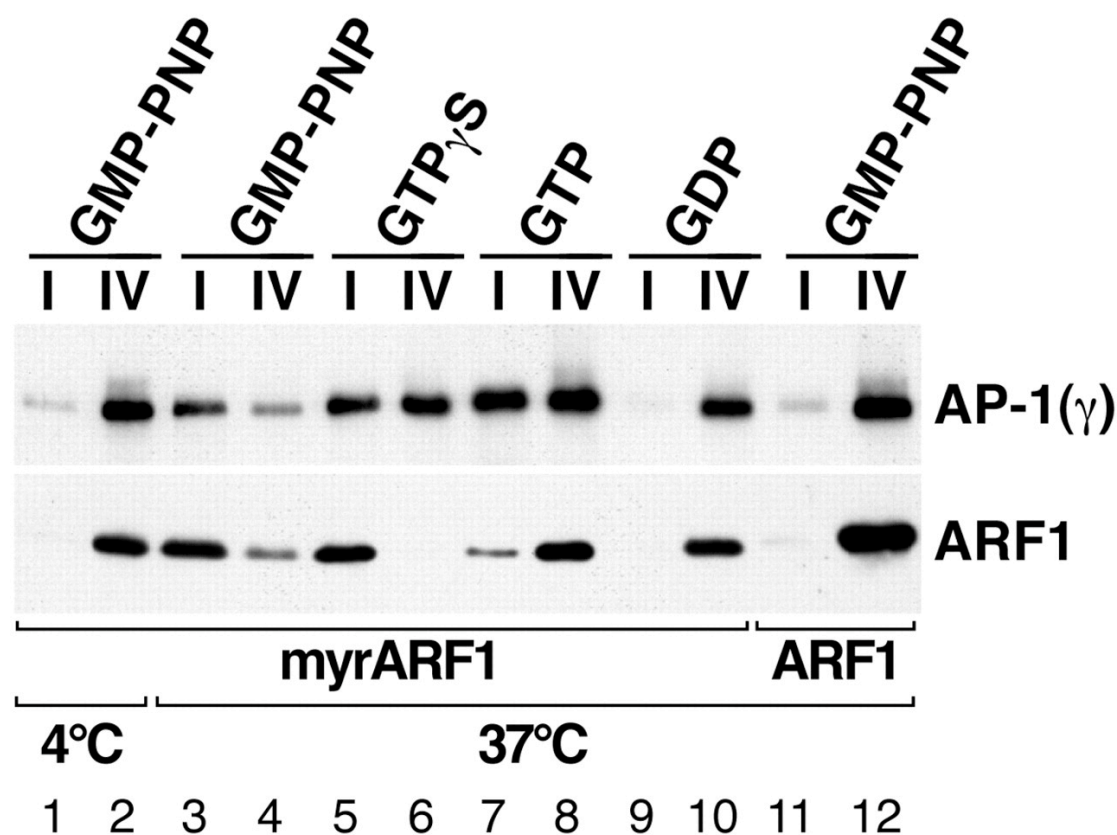


Figure 4
Nucleotide dependence of AP-1 recruitment to peptidoliposomes. The indicated nucleotide was incubated with myristoylated or nonmyristoylated ARF1 and peptidoliposomes containing 3% of mixed inositides and exposing Lamp1Y. The analysis was performed as in Figure 3.

The Effect of Phosphoinositides Is Not via the Nucleotide Exchange Activity of Liposomes

The efficiency of AP-1 binding to peptidoliposomes with different lipid compositions did not correlate with the relative or absolute amounts of ARF1 that floated with the liposomes to the top fraction of the gradient (Figure 3). It appears that all acidic lipids increased ARF1 association to the liposomes compared with pure PC, whereas AP-1 recruitment was much more specific. Nevertheless, it was conceivable that the effect of the functional phosphoinositides on AP-1 recruitment was indirect by increasing the rate or extent of nucleotide exchange in ARF1, which in our assay is performed in an unphysiological manner by the liposome surface. To test this possibility, a nucleotide exchange assay was performed using liposomes made of PC only

or of PC with 10% mixed phosphoinositides. ARF1 was incubated with these liposomes and [^{35}S]GTP γ S for different times, after which the samples were filtered and the amount of radioactivity bound to ARF1 was determined. As is shown in Figure 5, the rate of nucleotide exchange in the presence of liposomes is more than 10 times higher than in the absence of membranes. Yet, there is no significant difference in the kinetics or the final extent of GTP γ S loading of ARF1 in the presence or absence of phosphoinositides that could explain the dramatic difference in AP-1 recruitment observed with these lipid compositions (compare Figure 4, lanes 3 and 6, with Figure 2A, lanes 9-12, top panel). Thus, the phosphoinositides must affect other aspects of ARF1 function or must act on the AP-1 adaptors.

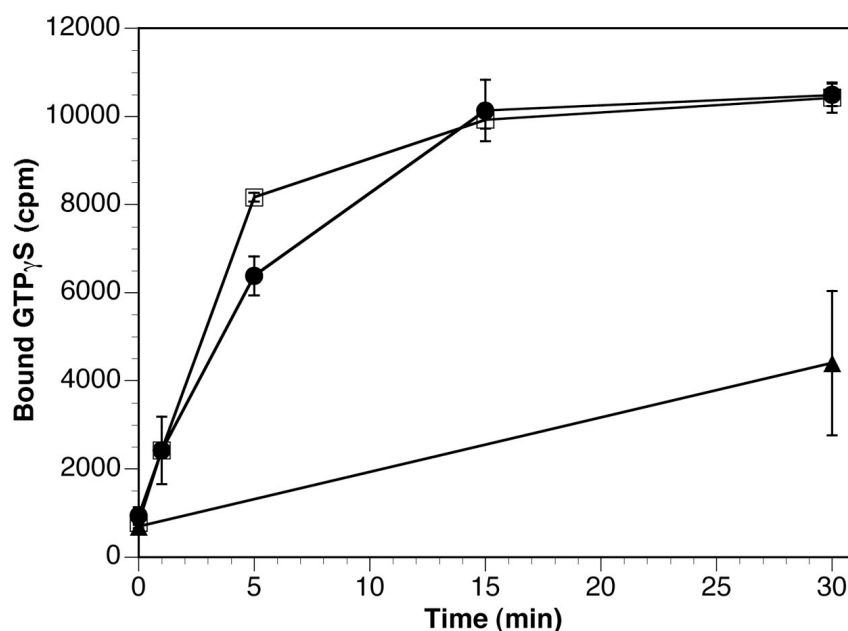
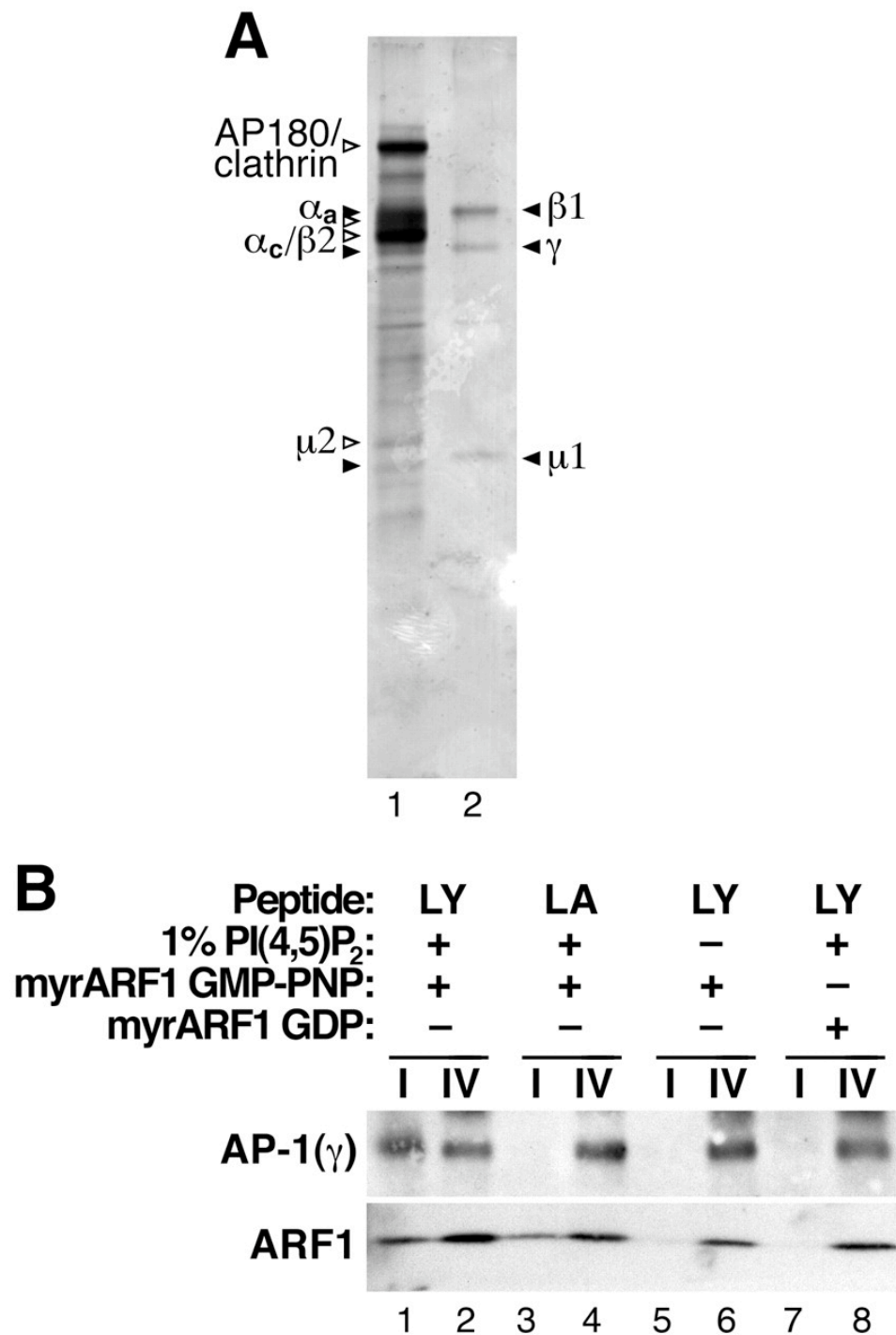


Figure 5

Nucleotide exchange on ARF1. Myristoylated ARF1 was incubated at 37°C with [^{35}S]GTP γ S and either buffer only (▲), PC liposomes (●), or PC with 10% mixed phosphoinositides (□). At the indicated times, samples were quickly filtered through a nitrocellulose filter. After washing, the radioactivity on the filter, corresponding to GTP γ S bound to ARF1, was counted.

A Minimal Machinery for AP-1 Recruitment

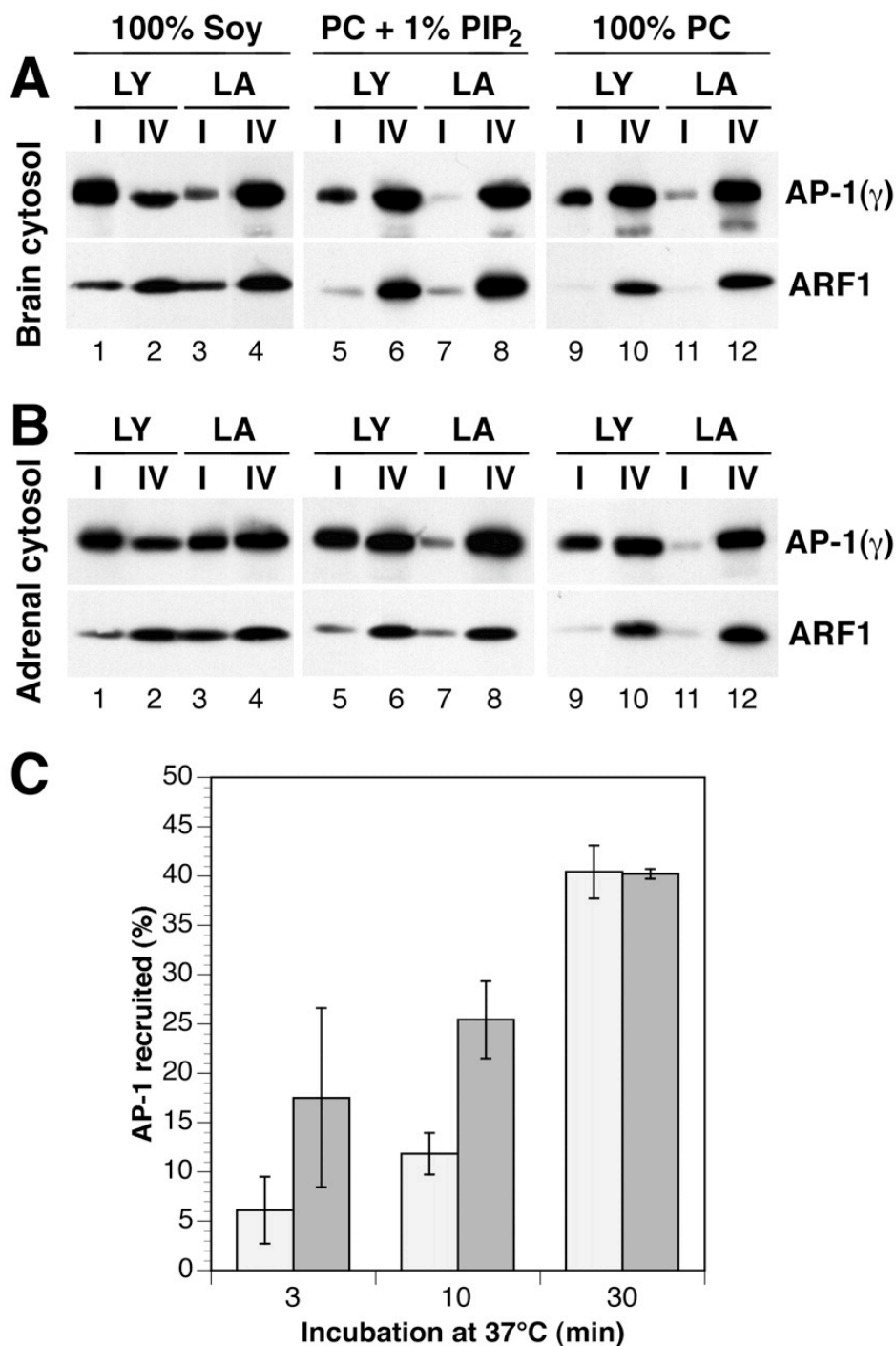
The mixed adaptor preparation used in the experiments described so far contains in addition to AP-1 also AP-2 adaptors, AP-180, and a number of unknown contaminating bands, which might directly or indirectly contribute to AP-1 recruitment. To identify the minimal set of proteins required, we purified AP-1 adaptors to near homogeneity. Figure 6A shows aliquots of the mixed adaptor preparation (lane 1) and of the purified AP-1 preparation (lane 2) containing the same amount of AP-1 (as judged by immunoblot analysis) on an SDS-gel stained with silver. All contaminating proteins except for one of ~70 kDa were removed below detection in the purified sample. By mass spectrometry, this copurifying contaminant was identified to be hsc70, the uncoating ATPase of clathrin-coated vesicles (Schlossman et al., 1984; DeLuca-Flaherty and McKay, 1990), which is highly unlikely to contribute to coat recruitment and could not be detected in the floated fraction. Using this AP-1 preparation, again robust recruitment of AP-1 complexes was achieved to liposomes containing 1% PI(4,5)P₂ presenting the Lamp1Y peptides and in the presence of myristoylated ARF1 loaded with GMP-PNP (Figure 6B, lanes 1 and 2). Using Lamp1A lacking the tyrosine, liposomes lacking the phosphoinositides, or GDP-loaded ARF, each individually abolished AP-1 association with the liposomes. This result thus defines the minimal machinery to recruit AP-1 to a membrane to consist of a peptide with a functional tyrosine motif and anchored to a lipid membrane containing a small amount of PI(4,5)P₂, and myristoylated ARF1 loaded with GTP or a nonhydrolyzable GTP analog.

**Figure 6**

Recruitment of pure AP-1 to peptidoliposomes. (A) Aliquots of the mixed adaptor preparation (lane 1) and of hydroxyapatite-purified AP-1 (lane 2) containing the same amount of AP-1 (as judged by immunoblot analysis) were separated by SDS-gel electrophoresis and stained with silver. AP-1 subunits β_1 , γ and μ_1 are indicated by filled arrowheads, whereas AP180 and AP-2 subunits, α_a , α_c , β_2 and μ_2 are indicated by open arrowheads. (B) AP-1 recruitment assays were performed using liposomes made of PC with or without 1% PI(4,5)P₂ and exposing Lamp1Y (LY) or Lamp1A (LA) peptides in the presence of myristoylated ARF1 loaded with GMP-PNP or GDP. The analysis was performed as in Figure 3.

Signal and Lipid Dependence of AP-1 Recruitment from Cytosol

Zhu et al. (1999a, 1999b) observed signal-independent AP-1 recruitment from cytosol to soybean liposomes in a pelleting assay. Therefore, using our floatation assay, we also investigated AP-1 recruitment from cytosol. Peptidoliposomes were mixed with cytosol supplemented with purified ARF1 and incubated for 30 min at 37°C before floatation of the liposomes as before. Consistent with the results by Zhu et al. (1999a), significant recruitment of AP-1 from brain cytosol to soybean liposomes presenting Lamp1A was observed (Figure 7A, lanes 3 and 4). This tyrosine-independent binding was even stronger using adrenal cytosol (which was used by Zhu et al. 1999a; Figure 7B, lanes 3 and 4). With both types of cytosol, however, AP-1 recruitment was clearly enhanced when functional Lamp1Y peptides were presented (Figure 7, A and B, lanes 1 and 2). If liposomes made of PC with 1% PI(4,5)P₂ or of pure PC were used, recruitment to Lamp1A was detectable, but very low (lanes 7 and 8, and 11 and 12, respectively), whereas recruitment to Lamp1Y-presenting liposomes was robust with ~40% (lanes 5 and 6, and 9 and 10).

**Figure 7**

Recruitment of AP-1 from cytosol. AP-1 recruitment assays were performed using brain cytosol (A) or adrenal gland cytosol (B), and peptidoliposomes made of soybean lipids (lanes 1-4), PC with 1% PI(4,5)P₂ (lanes 5-8), or pure PC (lanes 9-12), exposing Lamp1Y (LY) or Lamp1A (LA) peptides. Cytosol supplemented with purified ARF1 and GMP-PNP was incubated with the peptidoliposomes for 30 min at 37°C before separation by gradient centrifugation. (C) To determine the kinetics, AP-1 recruitment assays were performed using brain cytosol and liposomes exposing Lamp1Y peptides prepared of either PC alone (white bars) or PC containing 1% PI(4,5)P₂ (dark bars) at different incubation times (average and SD of 3 determinations).

The finding that AP-1 could be recruited from cytosol to pure PC liposomes

with Lamp1Y peptides (lanes 5 and 6) is in contrast to our observation with purified AP-1 derived from clathrin coats, which was not recruited to pure PC membranes (Figure 2A). However, analysis of the time-course of AP-1 recruitment from cytosol to PC liposomes with or without 1% PI(4,5)P₂ revealed that the kinetics were significantly faster to peptidoliposomes containing 1% PI(4,5)P₂ than to those made of PC alone (Figure 7C).

2.1.4 Discussion

Vesicular transport requires the recruitment of coat components to the specific donor membrane in the cell and the selection and incorporation of cargo proteins as well as of proteins necessary for vesicle targeting and fusion (e.g., the appropriate v-SNAREs). Two models for how this is accomplished have been proposed for different transport steps. Coat components may be targeted to the donor compartment by binding to a specific, high-affinity docking protein. Cargo molecules will diffuse into the coated area and be trapped by specific, but rather low-affinity interactions with coat molecules. Alternatively, it is the cargo itself that induces coat formation in combination with a site-specific feature like a particular lipid composition or a GEF for an accessory GTPase.

This second concept is attractive, because cargo selection and coat recruitment are coupled. This provides a mechanism to adjust vesicle formation to the amount of cargo to be transported, as has, for example, been observed experimentally for AP-2/clathrin coats in dependence of transferrin receptor overexpression (Iacopetta et al., 1988; Miller et al., 1991). However, the two models are not mutually exclusive. A docking protein is implicated in the nucleation of AP-2/clathrin coats, and there is evidence that synaptotagmin plays this role (Zhang et al., 1994). Binding of AP-2 to synaptotagmin is stimulated by tyrosine-based endocytosis motifs, i.e., by cargo (Haucke and De Camilli, 1999). Because in addition both AP-2 and synaptotagmin bind to phosphoinositides, particularly PI(4,5)P₂ (Beck and Keen, 1991; Südhof and Rizo, 1996), it was proposed that the lipid composition might be an additional level of regulating AP-2 recruitment (Takei and Haucke, 2001).

Our results using liposomes show that a docking protein is not necessary for AP-1 recruitment. The minimal machinery in our assay consists of myristoylated ARF1-GTP (or GMP-PNP or GTP γ S), membrane-anchored tyrosine-containing sorting motifs of cargo proteins and a small amount of specific phosphoinositides. In the absence of any other membrane-associated

proteins, ARF1 thus must interact directly with AP-1 to stimulate its recruitment. Such an interaction has recently been shown between ARF1 and the β 1 and γ -adaptins of AP-1 bound to immature secretory granules by cross-linking experiments (Austin et al., 2000). Similarly, a direct interaction has been shown between ARF1 and COPI complexes (Zhao et al., 1997). ARF1·GTP may dramatically increase AP-1 affinity for tyrosine signals or alternatively induce AP-1 to oligomerize, forming a surface patch with multiple cargo interactions already before addition of clathrin. AP-1 may thus behave similarly to COPI coatomer, which is induced to polymerize by a peptide corresponding to the cytoplasmic sequence of the COPI cargo protein p23 (Reinhard et al., 1999).

The third component required for AP-1 recruitment besides ARF1 and cargo signals was a lipid composition containing phosphoinositides, particularly PI(4,5)P₂ and to a lesser extent PI(5)P, at physiologically low concentrations in the range of a few mole-percent. The phosphoinositide contribution is clearly specific and does not simply correlate with charge, because different isomers showed vastly different effectiveness and other acidic phospholipids at higher concentrations were inactive.

The lipid composition also affected the equilibrium distribution of activated ARF1 between the membrane-associated and the free form, as was apparent from the amount of ARF1 that was associated with the floated liposomes. However, all acidic lipids increased membrane association of ARF1, and there was no correlation between the recruitment of AP-1 and the fraction of floated ARF1. Phosphoinositides, which stimulated AP-1 recruitment, also did not affect the rate or extent of nucleotide exchange in ARF1 (in agreement with Antonny et al., 1997). Furthermore, recruitment of AP-3 or COPI, which are also ARF1 dependent, to liposomes was largely independent of the lipid composition (Bremser et al., 1999; Drake et al., 2000). The major effect of the lipid composition on AP-1 recruitment is thus unlikely to be exerted via ARF1, but rather via AP-1.

Phosphoinositides have indeed been shown to modulate tyrosine signal recognition of both AP-1 and AP-2 using a cross-linking assay with

lipid/detergent micelles in the absence of ARF1. The interactions between the TGN38 motif and AP-2 (Rapoport et al., 1997) as well as between the Lamp-1 motif and AP-1 (Rapoport et al., 1998) were found to be enhanced by PI(3,4)P₂. This phenomenon thus does not explain the lipid dependence of AP-1 recruitment in our system. However, the most efficient lipid for AP-1 recruitment, PI(4,5)P₂, and the appropriate kinases for their synthesis have in fact been localized to the Golgi apparatus (Cockcroft and De Matteis, 2001). There, ARF1 was shown to regulate the synthesis of PI(4,5)P₂ by recruiting, and thus activating, PI 4-kinase and PI(4)P 5-kinase from the cytosol (Godi et al., 1999; Jones et al., 2000). Activation of ARF1 at the TGN may therefore contribute to preparing the ground with respect to the optimal lipid environment for AP-1 recruitment.

When a tyrosine signal was present, recruitment of AP-1 from cytosol was found not to be absolutely dependent on the lipid composition. This either reflects a difference between cytosolic and coat-derived AP-1 adaptors or contributions by unknown cytosolic factors. Yet, even in this system, the presence of PI(4,5)P₂ significantly enhanced the kinetics of the process. Generation of this phosphoinositide is thus a likely mechanism of regulating coat formation.

AP-1 recruitment in our assay is strongly dependent on tyrosine motifs presented on the membrane surface. The tyrosine motif of Lamp-1 has been shown to bind to both AP-1 and AP-2 in vitro (Höning et al., 1996; Ohno et al., 1996). The tyrosine motif of TGN38, also interacted with AP-2 adaptors in vitro (Ohno et al., 1995) but only weakly with AP-1 (Boll et al., 1996); yeast two-hybrid assays with μ 1 yielded variable results (Ohno et al., 1995, 1996; Rapoport et al., 1997; Stephens et al., 1997; Stephens and Banting, 1998). There is evidence that at least some membrane proteins are transported from the TGN to the basolateral surface via endosomes rather than in a direct vesicular transport route to the plasma membrane (Futter et al., 1995; Leitinger et al., 1995; Laird and Spiess, 2000; Orzech et al., 2000). Together with the recent discovery of a μ 1 isoform (μ 1B) involved in basolateral sorting (Fölsch et al., 1999; Ohno et al., 1999), AP-1 adaptors are thus potentially

involved in surface transport of basolateral proteins, including TGN38. AP-1 recruitment by the TGN38Y sequence in our assay might be related to this function.

In summary, our results define minimal requirements for AP-1 recruitment to a membrane and suggest the following modified model of the molecular events. Whereas in our assay ARF1 was activated by spontaneous nucleotide exchange on the lipid bilayer, ARF1 activation in the cell is a controlled and catalyzed process. Already ARF1.GDP may be concentrated at the membrane as indicated by its interaction with a putative PKA-activated receptor at the Golgi (Martín et al., 2000). It is activated to ARF1.GTP by a specific brefeldin A-sensitive GEF like BIG2 (Shinotsuka et al., 2002). The second factor specifying the site of AP-1 recruitment is likely to be the lipid composition in the TGN, i.e., the local production of PI(4,5)P₂, which is further stimulated by ARF1.GTP activating appropriate lipid kinases. Productive AP-1 recruitment will only take place, when a sufficient concentration of cargo proteins with AP-1 recognition sequences is present. Interaction with ARF1, PI(4,5)P₂ and tyrosine signal may induce a conformational change in AP-1 inducing AP-1 oligomerization. The resulting structures will be stably attached to the membrane by multiple low-affinity interactions with cargo molecules and lipids. In our assay, this is reflected in the fact that, unlike ARF1, AP-1 attachment to the liposomes survived a 1-h floatation through a sucrose gradient without "bleeding" into the middle fractions. Subsequent binding of clathrin will then induce coat and membrane curvature. Because ARF1 is scarce in isolated clathrin-coated vesicles (Zhu et al., 1998), it must dissociate at some point, most likely upon GTP hydrolysis. Interaction of ARF1.GTP with AP-1 might activate its GTPase activity. If AP-1 has not associated with other AP-1 complexes when GTP is hydrolyzed, it will be released from the membrane. Thus, ARF1 might function as a timer regulating coat assembly. It remains to be tested whether AP-1 acts as a GTPase-activating protein for ARF1, like the COPII components Sec23p/24p for Sar1 (Antonny et al., 2001).

Our results do not exclude that docking proteins able to recruit AP-1 exist. In fact, we have reproduced the previous finding that AP-1 can be targeted to

certain lipid compositions in a signal-independent, but cytosol-dependent manner. This might provide a mechanism for generating a basal level of cargo-independent vesicle budding as might be required to guarantee transport of lipids or recycling of v-SNARES for endosome-to-Golgi transport when cargo proteins are few. Interestingly, the v-SNARE VAMP4 has been recently shown to bind AP-1 via a di-leucine motif (Peden et al., 2001). Various membrane proteins thus may be able to nucleate AP-1/clathrin coats, as has also been proposed by Springer and Schekman (1998).

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2.2 The AP-1 clathrin adaptor forms a high-molecular weight precoat which can be disassembled by ARFGAP1

(Manuscript in preparation)

Abstract

Recruitment of AP-1 clathrin adaptors can be reconstituted *in vitro*. Myristoylated ARF1, GTP or non-hydrolysable analogues, a small amount of specific lipids, and sorting signals are sufficient to recruit AP-1 to membranes. In current models AP-1 recognizes sorting signals; coat polymerization and membrane curvature is induced by clathrin. Here we present evidence that AP-1 is organized in high-molecular weight complexes in the absence of clathrin. This “precoat” contains ARF1 GTP, since it is sensitive to hydrolysis induced by the GTPase activating protein ARFGAP1. Furthermore we could show that, similar to the COPI coat, AP-1 enhances the activity of ARFGAP1. These results suggest a novel model of coat recruitment, where AP-1 might play an additional role in coat polymerization, whereas the main function of clathrin might be to induce curvature.

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2.2.1 Introduction

Intracellular transport between membrane compartments is initiated by the recruitment of cytosolic coat components onto membranes. These proteins select and concentrate cargo proteins, and polymerize into a coat that deforms the target membranes into buds and vesicles. The three major known coats are: coat protein I (COPI), COPII, and clathrin in combination with associated adaptors (Kirchhausen, 2000b). The COPI coat mediates intra Golgi and Golgi-to-endoplasmic reticulum (ER) transport. It consists of the small Ras-like GTPase ADP-ribosylation factor 1 (ARF1) and coatomer, a complex of seven proteins. COPII, which consists of Sar1-GTP, Sec23/24 and Sec13/31, is involved in the transport from the ER to the Golgi. Clathrin, with associated adaptor proteins (APs), forms coats at the plasma membrane, the trans-Golgi network (TGN) and endosomes.

APs concentrate cargo by interacting with sorting signals, build the scaffold to form coated pits and vesicles, and are able to recruit accessory proteins. Several clathrin-associated APs are known. They are all heterotetrameric complexes that consist of two large (ca. 100 kDa), a medium (μ ca. 50 kDa) and a small subunit (σ ca. 20kDa) (Robinson and Bonifacino, 2001). The adaptors form the inner layer, which connects the clathrin with cargo molecules. AP-1 adaptors are involved in post Golgi sorting and mainly found in coated vesicles associated with the TGN and endosomes. AP-2 adaptors mediate the formation of endocytotic vesicles at the plasma membrane. AP-3 forms transport vesicles at the TGN or endosomes for lysosomal transport. Least is known about the AP-4 adaptor complex, which was reported to be involved in basolateral transport (Simmen et al., 2002).

Small GTPases are required for the initial recruitment step of all coats except AP-2 / clathrin. These associate with a membrane upon nucleotide exchange by a guanine nucleotide exchange factor (GEF). In the COPII coat, the GTPase is Sar1p in the remaining coats (COPI, AP-1, 3) it is activated ARF1.

The formation of the three coats has been studied in vitro. Chemically defined liposomes, purified coat components and non-hydrolysable nucleotides are

sufficient for in vitro COPII- and AP-3/clathrin coated vesicle formation (Drake et al., 2000; Matsuoka et al., 1998a; Matsuoka et al., 1998b). However, additional cargo signals are required to form COPI coated buds and vesicles from liposomes with a Golgi-like lipid composition (Bremser et al., 1999). In contrast, AP-1 recruitment to liposomes and clathrin coated vesicle (CCV) formation required cytosol (Zhu et al., 1999a). When covalently linked sorting signals were used, cytosol independent AP-1 recruitment in a minimal system of liposomes containing a small amount of specific lipids and of activated ARF1 was reconstituted (Crottet et al., 2002).

GTP hydrolysis, catalyzed by a GTPase activating protein (GAP), has been associated with uncoating in COPI and the COPII. The GAP activity has to be regulated to obtain an appropriately timed inactivation of Sar1p or ARF1 (reviewed by Nie et al., 2003b). Sec23p, the GAP that activates the activity of Sar1p, is part of the COPII coat. Once the coat is completed, Sec13/31p acts as an activator for the GAP activity of Sec 23/24p (Antonny et al., 2001), leading to disassembly of the coat. In COPI, hydrolysis is also used to regulate sorting. The hydrolysis is catalyzed by the ARF1 GTPase activating protein 1 (ARFGAP1) (Cukierman et al., 1995). GAP activity is accomplished on one hand by coatomer, which stimulates ARF1 mediated GTP hydrolysis in a GAP dependent manner (Goldberg, 1999; Szafer et al., 2001). Cargo, on the other hand is able to inhibit the GTPase activity and thereby allows the coatomer to polymerize (Goldberg, 2000; Lanoix et al., 2001; Weiss and Nilsson, 2003).

The role of hydrolysis in the AP-1/clathrin coat is not clear. It appears that it is not involved in uncoating, since ARF1 is not found in CCVs (Zhu et al., 1998). Two GAPs have been associated with clathrin traffic. The targeting domain of ARFGAP1 has been found to interact with the ear domain of γ adaptin (Hirst et al., 2003). AGAP1 has been found to localize to endosomes where it co-localizes with AP-1 (Nie et al., 2002) and AP-3 (Nie et al., 2003a). It interacts with AP-3, and overexpression changes the distribution of AP-3 but does not affect the distribution of other coat proteins.

To study the function of hydrolysis we recruited AP-1 to liposomes in the

absence of clathrin. We found that these adaptors polymerize into a high-molecular weight complex which is sensitive to hydrolysis, induced by ARFGAP1. This suggests, in contrast to current models, that the main function of clathrin in coat formation is to induce curvature, rather than coat polymerization.

2.2.2 Materials and Methods

Reagents

GMP-PNP, GTP γ S, and GTP were from Roche Diagnostics. Superose-6 (Prep grade) was from Amersham Pharmacia Biotech, ECL reagent was from Pierce. N-((4-maleimidymethyl)cyclohexane-1-carbonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (MMCC-DHPE) was from Molecular Probes (Eugene, OR). Monoclonal antibody 100/3 (anti- γ -adaptin), horse-radish peroxidase-coupled anti-mouse IgG antibody, soybean PC (azolectin, P-5638), charcoal, SDS, and MOPS were purchased from Sigma (Buchs, Switzerland). [γ ³²P] GTP 6000 Ci/mmol was from Perkin Elmer, 0.45 μ m HA-type membrane filters were from Millipore. Peptides were synthesized on a Pioneer synthesizer (PerSeptive Biosystems) as described earlier (Crottet et al., 2002). Peptide sequences were CRKRSHAGYQTI-COOH (Lamp-1Y) and CRKRSHAGAQTI-COOH (Lamp-1A). Polyclonal rabbit antibody against ARF1 was from Santa Cruz Biotechnology. Polyclonal rabbit antibody against ARFGAP1 was prepared as described (Cukierman et al., 1995).

Plasmids: bovine ARF1 with residues 3-7 from yeast Arf2p (Liang et al., 1997) and N-myristoyltransferase (pBB131(Duronio et al., 1990)) were generous gifts by Stuart Kornfeld and Jeffrey Gordon, respectively (both at Washington University, St. Louis, MO).

Purifications

Clathrin coated vesicles were isolated from calf brains (local slaughterhouse) as described (Campbell et al., 1984). The coat was released and mixed APs were purified as described earlier (Crottet et al., 2002). To obtain pure AP-1, mixed APs were dialyzed into MonoQ buffer A (20mM ethanolamine, pH 8.9, 2mM EDTA, 1mM DTT), loaded on a MonoQ HR 5/5 (Amersham Pharmacia Biotech) and eluted by a 5 ml linear gradient 0-150 mM NaCl followed by a 50ml linear gradient 150-450 mM NaCl in starting buffer (adapted from Ahle et al., 1988). AP-1 containing fractions were pooled after excluding the

presence of ARFGAP1 by immunoblotting. These fractions were further purified on a hydroxyapatite column as described (Ahle and Ungewickell, 1986; Crottet et al., 2002).

Myristoylated ARF1 was purified as described in (Liang and Kornfeld, 1997).

His-tagged ARFGAP1 was expressed in and purified from Sf9 cells, ARFGAP1 (6-136) was expressed in and purified from E.coli BL21 (DE3) cells as described elsewhere (Huber et al., 2001).

Liposome Recruitment assay

Peptidoliposomes were produced and the first step of adaptor recruitment was performed as described earlier (Crottet et al., 2002), except that the adaptors were added at the beginning and co-incubated at 37°C, 100% soybean PC was used. Fraction 1 was split in two, 10µg of full length ARFGAP1 was added as mentioned and incubated at 37°C or left on ice for 30 min as required. Samples of 0.5 ml were mixed with 0.5 ml of 60% sucrose in assay buffer (10mM HEPES, pH 7.0, 150 mM NaCl, 10mM KCl, 3mM potassium phosphate, 2mM MgCl₂, 0.2 mM dithiothreitol (Honing et al., 1996) overlaid with 2.82 ml 20% sucrose in assay buffer and with 0.18 ml of assay buffer and centrifuged as in the first step of the recruitment. Four fractions were collected from the top and precipitated with 8% (wt / vol) trichloroacetic acid. Acetone washed pellets were analyzed by 7.5-15% PAGE and immunoblotting using antibodies against γ adaptin or ARFGAP1, a peroxidase-coupled secondary antibody, and ECL reaction. Sedimentation experiments

340 µl of floated liposomes were collected from the top. 340 µl of assay buffer and 0.2 % Triton X-100 (final conc.) were added and the sample was loaded on top of a 4.32 ml 10-25% sucrose gradient in assay buffer in a 5ml centrifuge tube and centrifuged at 90,000g for 5h at 4°C. 10 0.5 ml fractions were collected from the top and analyzed as described in the text.

ARFGAP1 activity assay

Myristoylated ARF1 was loaded with [γ ³²P]GTP in the presence of soybean liposomes. Loading mixture contained 4 µM myristoylated Arf1, 2.5 mM

MOPS, pH 7.4, 100 mM KCl, 1mM MgCl₂, 2 mM EDTA, 25 μM GTP (supplemented with GTP [³²P]), and 1 mg/ml liposomes. Loading proceeded for 15 min at 30°C and was terminated by transfer to ice. Loading efficiency with respect to [³²P]-GTP was typically 40-50%. GAP assay contained 40 nM [³²P]GTP-loaded ARF1, 5 mM MgCl₂, 25 mM MOPS pH 7.4, 40 mM KCl, 1 mM dithiothreitol. ARFGAP1 (6-136) was used at 0.1μM, coatomer and AP-1 at 0.25μM. Assay volume was 25 μl. Reactions were preincubated for 5 min at room temperature and initiated by the addition of ARFGAP1 (6-136). The reactions were incubated at 30°C for 1-15 min and terminated by addition of 20 μl of 0.5% SDS. Then, 0.5 ml of cold charcoal suspension (5% charcoal in 50 mM NaH₂PO₄) was added. Following centrifugation, the amount of ³²Pi in the supernatant was determined by scintillation counting.

2.2.3 Results

AP-1 is stably recruited to peptidoliposomes in the absence of clathrin

In a previous study we have shown that AP-1 can be recruited in an in vitro system containing only ARF1-GTP and tyrosine-based signals coupled to liposomes (Crottet et al., 2002). Peptides with the wild type signal sequence of lysosome-associated membrane protein-1 (Lamp1Y) were coupled to liposomes made of soybean lipids and incubated at 37°C with purified myristoylated ARF1, GTP or GMP-PNP, and clathrin adaptors purified from calf brain. These liposomes were supplemented with sucrose to a concentration of 40%, and loaded below a 20% sucrose cushion. After centrifugation for 1 h at 300,000 x g four fractions were collected from the top. Fraction 1 contained floated liposomes and bound proteins, whereas fraction 4 contained unbound material. The four fractions were analyzed by SDS-gel electrophoresis and probed by immunoblot analysis. The recruitment was similar whether ARF1-GTP or ARF1-GMP-PNP was used (Fig. 1, top). To assess whether AP-1 is stably bound, the floated material of fraction 1 was collected and reincubated at 37°C for 30 min. As a control half the sample was kept on ice. The samples were then loaded at the bottom of a new gradient and the liposomes were floated again as before (Fig. 1, bottom). AP-1 was quantitatively recovered from the liposome fraction. No protein was detected in the starting zone at any conditions. This result indicates that in the absence of clathrin AP-1 recruited to the peptidoliposomes is stably associated with the membrane.

Recruited AP-1 forms high molecular weight complexes

The intrinsic affinity of AP-1 to sorting signals is relatively low (Heilker et al., 1996) Stable binding maybe achieved by the additional interaction with ARF1 and lipids. Alternatively, formation of an oligomer with multiple low-affinity interactions to signal peptides, lipids and ARF1 might be responsible for the stable recruitment observed. To test the oligomeric state of recruited AP-1,

fraction 1 of a floatation experiment using soybean peptidoliposomes with Lamp1Y signals, was supplemented with Triton X-100 to solubilize the lipid membrane and loaded on top of a linear 10 – 25% sucrose gradient. After 5h of centrifugation at 90,000 x g, fractions were collected from the top and analyzed by Western blotting. Starting APs and fraction 4, containing non-floated APs, were analyzed in parallel gradients. These adaptors could be detected mainly in fraction 2 and 3 of the gradient (Fig 2A). In contrast, recruited AP-1 moved deeply into the gradient and in part even to the bottom fraction. Thus AP-1 was present as high-molecular weight complexes of at least 2-5 monomers, resistant to detergent solubilization of the underlying membrane. This could be observed whether GTP or GMP-PNP was used for the activation of ARF1 (Fig 2B). Using pure AP-1, further purified from the AP preparation by MonoQ and hydroxyapatite chromatography (purified to homogeneity as judged by silver staining), showed the same behavior (Fig. 2A).

GTP hydrolysis causes disassembly of the AP-1 oligomers

Since free activated ARF1 is also associated with the membranes, it is difficult to determine directly whether ARF1 is part of the AP-1 oligomers. Free ARF1 “contaminates” the floated liposomes and obscures ARF1 potentially associated with AP-1 oligomers. Indirectly, the presence of ARF1 can be tested by determining the effect of GTP hydrolysis induced by an added GAP. ARFGAP1 has been shown to interact with the ear domain of γ -adaptin (Hirst et al., 2003) and is therefore a likely physiological partner of ARF1 in AP-1/clathrin coat formation. To analyze the effect of GTP hydrolysis, we added ARFGAP1 to the recruited, membrane-bound AP-1. After a recruitment experiment, fraction 1 was collected and split in two portions. Half the sample was incubated for 30 min at 37°C with ARFGAP1 whereas the other half was incubated without. The liposomes were refloated and four fractions were analyzed. When GTP was used to recruit AP-1 and hydrolysis was induced, the adaptors were partially released. Using GMP-PNP, where hydrolysis is prevented AP-1 stayed associated with the liposomes (Fig 3A). To distinguish whether AP-1 disassembled into individual adaptors or whether it released en

bloc, Triton X-100 was added to fraction 1 after incubation with or without ARFGAP1 and loaded on top of a sedimentation gradient. As expected no significant effect could be observed on the size distribution in the presence of GMP-PNP (compare to Fig. 2B). However, sedimentation of AP-1 recruited with GTP and incubated with ARFGAP1 displayed a shift towards lower molecular weight complexes when compared to incubation without ARFGAP1. This indicates disassembly into smaller complexes or individual APs (Fig. 3B) and suggests that ARFGAP1 downregulates ARF1 as part of the complex. Mechanistically, hydrolysis might regulate the lifespan of these complexes and therefore be involved in sorting, similar to the proposed function in COPI vesicles (Lanoix et al., 1999; Lanoix et al., 2001).

AP-1 stimulates the GAP activity of ARFGAP1

Coatomer and the corresponding sorting signals have previously been proposed to modulate the GAP activity of ARFGAP1 (Goldberg, 1999; Szafer et al., 2001). We investigated whether AP-1 also alters the activity of ARFGAP1. Two different types of liposomes were used, containing either the Lamp1Y signal or a peptide where the essential tyrosine was replaced by an alanine (Lamp1A). ARF1 was activated on these liposomes in the presence of radioactively labeled [γ ³²P] GTP. GAP activity was determined by adding the catalytic domain of ARFGAP1 and effectors and measuring GTP hydrolysis as detected by radioactive free phosphate after various incubation times at 30°C. Using low concentration of ARFGAP1 (0.1µM) did not result in detectable GTP hydrolysis within 15 min (Fig. 4A circles). As expected, coatomer that was added stimulated the GAP activity; independently of the liposomes used (Fig. 4A, triangles). Enhanced GAP activity was observed, however, with pure AP-1 and ARFGAP1 added on Lamp1Y liposomes. Using Lamp1A liposomes the activity was similar to background level (Fig4B, rectangles). To exclude an intrinsic GAP activity, pure AP-1 was added to LampY liposomes in the absence of ARFGAP1 (Fig. 4B, diamonds).

2.2.4 Discussion

In some respects, AP-1/clathrin and COPII have similar principles of coat formation. Both coats consist of a two-layer system. A first component (AP-1 or Sec23/24p) interacts with GTPases (ARF1 or Sar1) and cargo and offers a platform to recruit a second layer (clathrin or Sec13/31) which crosslinks the coat and induces curvature. Current models imply that AP-1 is recruited as individual adaptors which are crosslinked upon interaction with clathrin. Our results demonstrate that without clathrin, AP-1 assembles into a stable high-molecular weight complex, a “precoat”. This does not exclude that in the presence of clathrin the formation of the coat may happen almost simultaneously. The first layer of the COPII coat can be recruited separately in so-called pre-budding complexes (Springer et al., 1999). Whether they form polymers as we have found for AP-1 is not known. Sedimentation experiments, similar to the ones presented here, could be used to find out. Our findings suggest an additional role in polymerization for the first layer. The main function of the second layer would therefore be to induce curvature, and to form and release vesicles.

Small GTPases are involved in the initial steps of coat formation. GTP hydrolysis has been linked to quality control and vesicle uncoating in COPI and COPII. COPI uncoating has been demonstrated by incubating *in vitro* formed vesicles with ARFGAP1 (Reinhard et al., 2003). Sec23p, the GAP for COPII, is already part of the coat. When GTP is used, hydrolysis causes the COPII coat to dissociate as soon as a vesicle is formed (Antonny et al., 2001). With respect to uncoating, clathrin coats clearly differ from the two COP coats. Evidently uncoating is not linked to GTP hydrolysis, which makes clathrin coats relatively long-lived and facilitates the purification of CCVs. Uncoating of AP-1/clathrin involves hsc70, cyclin G-associated kinase (GAK/auxilin2) and dephosphorylation by the cytosolic phosphatase PP2A (Ghosh and Kornfeld, 2003a; Hannan et al., 1998; Umeda et al., 2000). Our results show that AP-1 recruitment to membranes in the absence of clathrin is sensitive to GTP hydrolysis. GTP is necessary to stabilize the precoat but is dispensable for the

integrity of the final AP-1/clathrin coat. If at all, only little ARF1 is present in CCVs (Zhu et al., 1998) suggesting that hydrolysis of GTP has taken place without release of AP-1. Apparently, recruitment of clathrin stabilizes the precoat. In the completed coat, as an additional mechanism to phosphorylation / dephosphorylation, GTP hydrolysis may prepare the AP-1 layer for dissociation after removal of clathrin by hsc70 and GAK/auxilin.

In the COPI system GTP hydrolysis acts as a timer, which is involved in quality control of vesicle formation. Coat components regulate the GAP activity of ARFGAP1. In agreement with Goldberg, (1999) and Szafer et al., (2001) an increased GAP activity was observed in the presence of coatomer. This was independent of the tyrosine-sorting signal presented on the liposome. It has been proposed that cargo plays a role in sorting into COPI vesicles. Cargo that has to be incorporated can reduce the GAP activity of ARFGAP1 (Goldberg, 2000; Lanoix et al., 2001), allowing more time for the coat components to assemble. When we analyzed the influence of AP-1 on the GAP activity, we observed a stimulatory effect in the presence of the Lamp1Y sorting signal. It seems that, as for the COPI system, AP-1 is able to stimulate the GAP activity of ARFGAP1. However, stimulation was not observed in the control experiment using the Lamp1A peptide. This might be explained by the fact that Lamp1Y recruits AP-1, which brings all three players into physical proximity, so that AP-1 can act on ARFGAP1. Alternatively the Lamp1Y sorting signal could induce a conformational change in AP-1, which is able to enhance the GAP activity of ARFGAP-1. This possibility could be addressed by preincubating AP-1 with the soluble Lamp1Y peptide and measuring the influence on the GAP activity on peptide-free liposomes.

Together these findings lead to a novel model of cargo selection and coat recruitment of AP-1/clathrin coats (Fig. 5). Initially ARF1 is activated on a membrane with the help of a membrane associated GEF. With the correct sorting signals and lipids, this creates binding sites that allow stable AP-1 recruitment. On the membrane, AP-1 polymerizes into a stable precoat. If an individually recruited AP-1 does not interact with other APs, it may dissociate back into the cytosol. ARF1 is still active and important for the stability of the

precoat. At this stage and in the absence of clathrin, hydrolysis will trigger disassembly. As clathrin interacts with the precoat and adds a second layer, AP-1 becomes resistant to GTP hydrolysis. Clathrin induces curvature that a vesicle can be formed. Curvature, as a signal of productive coat formation, might trigger a further increase in ARFGAP1 activity as observed in the COPI coat (Bigay et al., 2003). Only when clathrin is released, regulated by Hsc70, and auxilin/GAK, the adaptors disassemble as well.

Further experiments have to be done to demonstrate the protecting function of clathrin, to confirm this model. It is also important to assess the influence of clathrin on the GAP activity of ARFGAP1. Furthermore it remains to be elucidated whether AP-1 is the only adaptor to form a precoat or whether this is a common mechanism that is involved in the coat formation of the other clathrin adaptors.

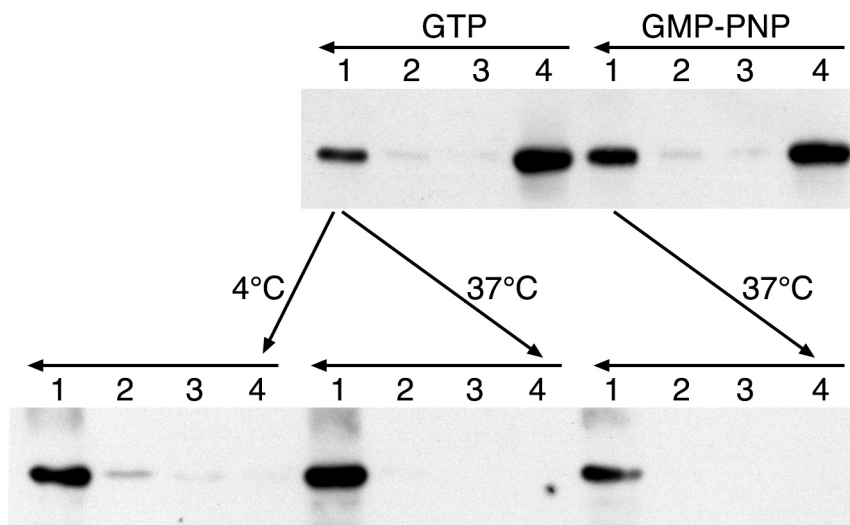


Figure 1

AP-1 stably recruits to peptidiliposomes. Top row: peptidiliposomes made of soybean lipids and presenting Lamp1Y peptides were incubated with a mixed adaptor preparation, myristoylated ARF1 and GTP or GMP-PNP. After floatation on a sucrose step gradient, four fractions (1-4) were collected from the top and analyzed by immunoblotting for γ -adaplin. Bottom row: fraction 1 was further incubated at 4°C or 37°C and refloatated on a similar sucrose step gradient. Horizontal arrows represent liposomal movement from loading to floating fraction.

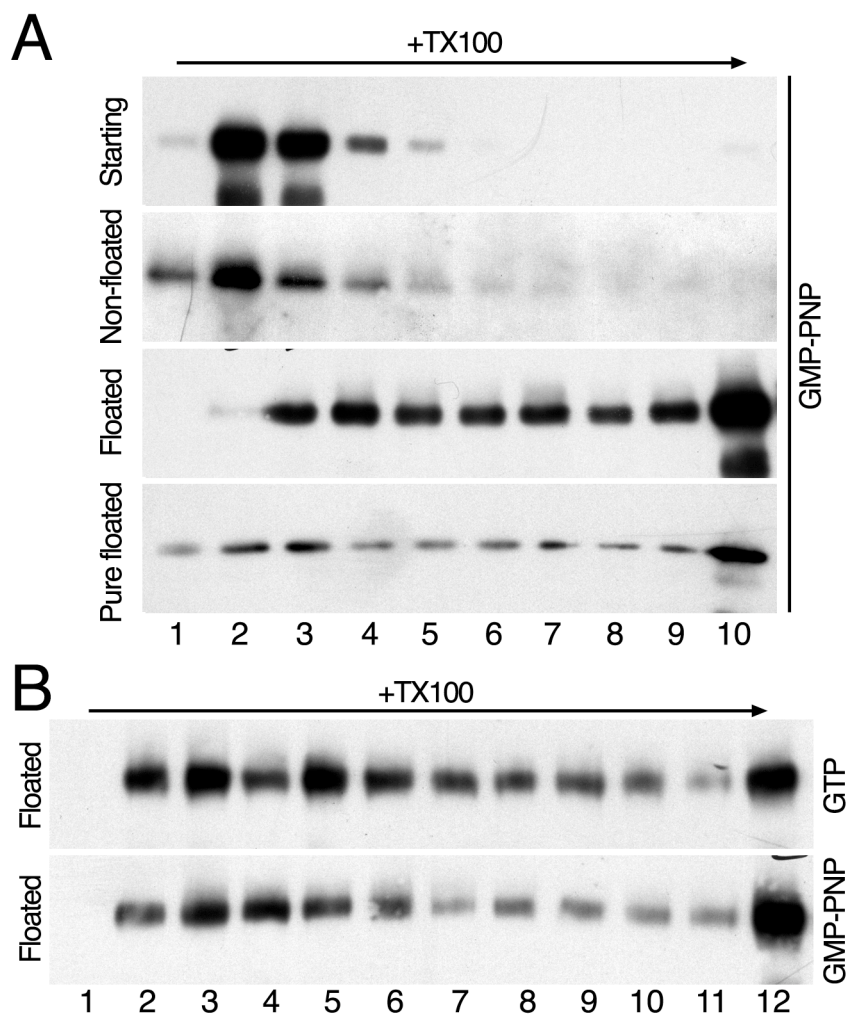


Figure 2

Recruited AP-1 forms a high-molecular weight complex. (A) Mixed adaptors were incubated with liposomes presenting the Lamp1Y peptide and ARF1 GMP-PNP. Starting material, non floated and floated fraction were supplemented with Triton X-100 and loaded on top of a 10-25% sucrose gradient and spun for 5h at 90,000 x g. Ten fractions were unloaded from the top and analyzed by immunoblotting for γ -adaptin. The floated fraction of a recruitment experiment with pure AP-1 was loaded on top of a similar sucrose gradient and analyzed for AP-1. (B) Sedimentation is independent of nucleotide. Mixed adaptors were incubated with liposomes as in A with ARF1 GTP or ARF1 GMP-PNP. Top fraction, supplemented with Triton X-100 was loaded on top of a 10-25% sucrose gradient, 12 fractions were unloaded from the top and immunoblotting was performed as in (A).

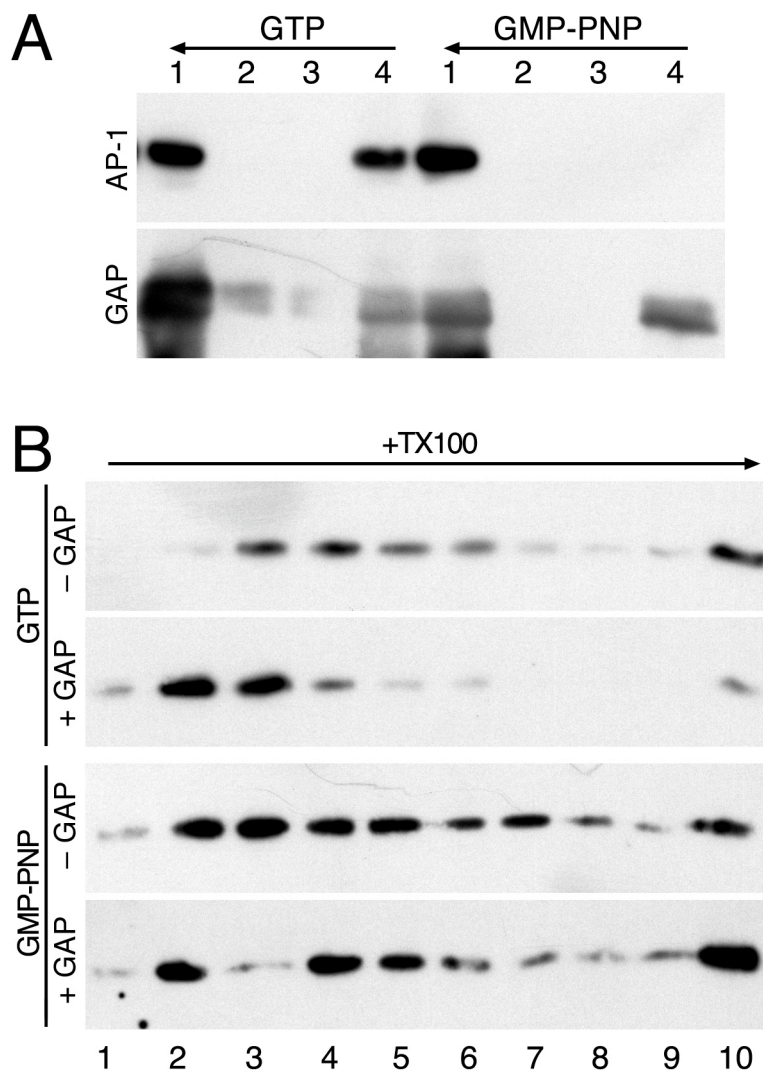
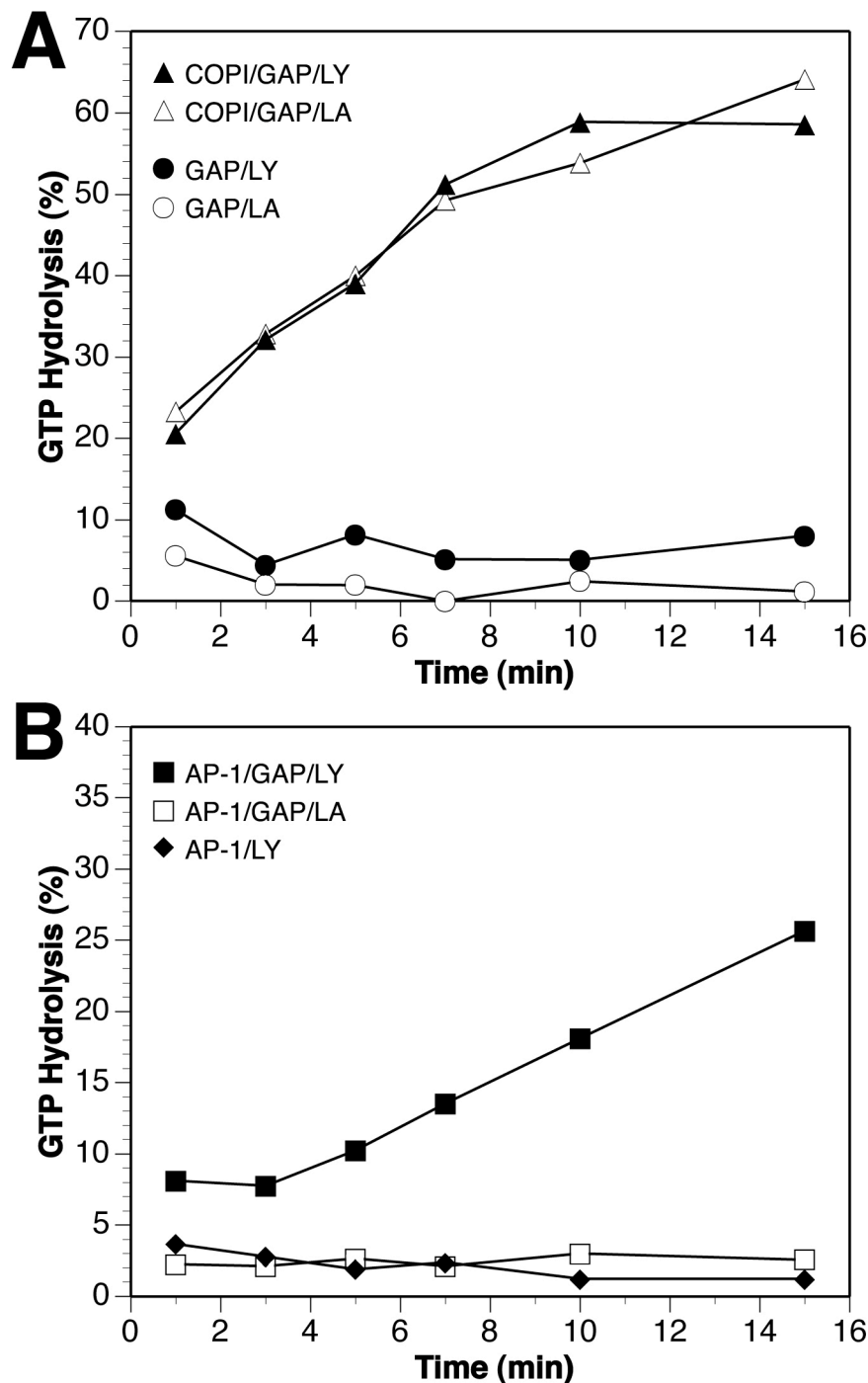


Figure 3

GTP hydrolysis induces dissociation of the AP-1 oligomers. Mixed APs were incubated with soybean Lamp1Y peptidoliposomes and ARF1 GTP or GMP-PNP and the floated fraction was used for further analysis. (A) The floated fraction was incubated with ARFGAP1, loaded on a new gradient and analyzed by immunoblotting for γ -adaptin or ARFGAP1. Partial uncoating can be observed when the initial recruitment step was performed in the presence of GTP. (B) The floated fraction was incubated with ARFGAP1 or buffer, supplemented with Triton X-100 and loaded on top of a 10-25% sucrose gradient and sedimented for 5h at 90000 x g. 10 fractions were unloaded from the top and analyzed by immunoblotting for γ -adaptin.

**Figure 4**

AP-1 stimulates the GAP activity of ARFGAP1. ARF1 was activated in the presence of [γ - 32 P] GTP on liposomes containing the LampY (LY, filled markers) or the LampA (LA, empty markers) peptide. Effectors were added and free phosphate was measured at the indicated time points. (A) The catalytic domain of ARFGAP1 has no detectable GAP activity when added at $1\mu\text{M}$ (circles). Coatomer enhances the GAP activity independently of the tyrosine sorting signal used (triangles). (B) AP-1 is able to enhance the activity of ARFGAP1 when incubated with the LY liposomes (filled squares). No stimulation was detected with LA liposomes (empty squares). AP-1 has no detectable intrinsic GAP activity (diamonds). All the experiment have been performed at least three times, figure shows the result of a representative experiment.

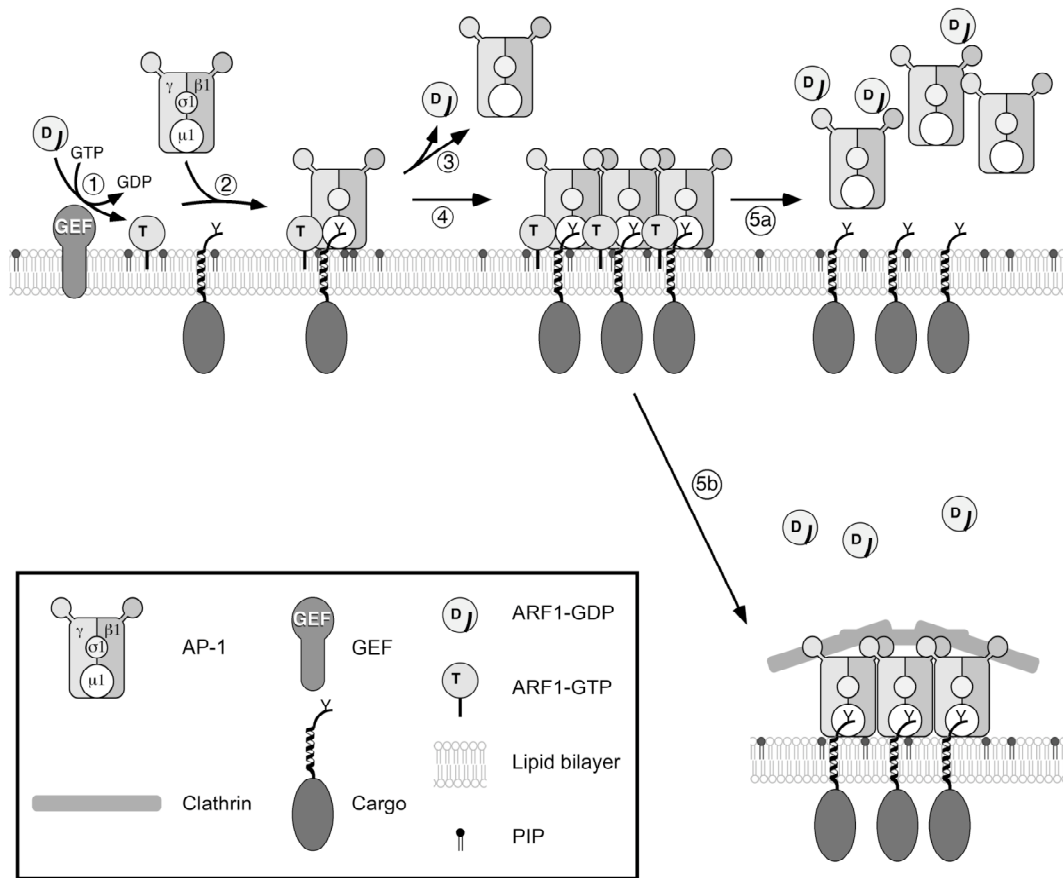


Figure 5

Model for AP-1 clathrin coat recruitment. (1) ARF1 is activated by a GEF at the membrane and creates binding sites together with cargo and lipids. (2) AP-1 is recruited and (4) polymerizes into a precoat or (3) dissociates from the membrane if it does not interact with other APs. (5a) Without interaction with clathrin the precoat disassembles, triggered by GTP hydrolysis (5b) Clathrin polymerization makes the AP-1 layer insensitive to hydrolysis.

3 DISCUSSION

3.1 Coat formation – similar mechanisms, different players?

This work, taken together with other studies, reveals similarities of basic mechanisms of coat formation. Clathrin, COPI and COPII coats form a polymer by the ordered assembly of cytosolic proteins, which concentrate cargo and shape the membrane to produce transport vesicles. This process can be broken down into different stages: initiation, cargo recognition and concentration, polymerization, membrane deformation to form a vesicle, scission, uncoating, and fusion with the target membrane.

Most of the coats need a small-activated GTPase to initiate the site of vesicle formation, creating a high-affinity docking site. In the COPII coat it is Sar1 that is able to interact directly with the membrane upon GTP exchange. For COPI, AP-1, -3, -4 and the GGAs it is activated ARF1. Only AP-2/clathrin coats seem to form independently of small GTPases.

However, the activated GTPase is generally not sufficient to specifically recruit the coats. Lipids, docking and/or sorting signals are additional factors. It is still discussed whether sorting signals help recruiting coat proteins. In one model, the coat is recruited to the membrane first and cargo then diffuses laterally into the coated area where it is trapped and concentrated. In another model coat recruitment and signal binding are simultaneous processes. As a result, the density of cargo proteins enhances coat formation as has been observed *in vivo* (Iacopetta et al., 1988; Miller et al., 1991). It has been shown *in vitro* that COPI and COPII vesicle budding from liposomes can occur in the absence of sorting signals (Matsuoka et al., 1998b; Spang et al., 1998), demonstrating the intrinsic ability of the coat to deform a membrane. However, COPI and COPII cargo might facilitate vesicle budding, since peptides containing sorting signals are properly sorted into vesicles (Bremser et al., 1999; Matsuoka et al., 1998a). For the clathrin coat it has been discussed whether a cytosolic docking component is needed to initiate vesicle formation

or whether the cargo on its own acts as a docking place. Several studies from the Kornfeld lab indicated that sorting signals are not necessary to recruit clathrin adaptors to liposomes in the presence of cytosol (probably providing a “docking partner”) (Doray and Kornfeld, 2001; Zhu et al., 1999a). However, our results presented here indicate that sorting signals are sufficient for stable AP-1 recruitment, in the absence of any other potential docking proteins. We conclude that signals can at least contribute to coat recruitment. The existence of docking proteins cannot be excluded, however.

Coat components need to be polymerized at one point during coat formation. Since the COPI coat consists only of ARF1 and coatamer, it has to be coatamer that performs coat polymerization. Recent structural data lead to a model in which the F-COPI subcomplex (β , γ , δ and ζ) is involved in cargo selection and the B-COPI subcomplex (α , β and ϵ) induces polymerization and curvature (Hoffman et al., 2003).

COPII and clathrin recruitment seem to be two-step processes. Sec 23/24 interacts with cargo and forms a pre-budding complex together with Sar1. This complex is thought to diffuse on the membrane and collect cargo. Sec 13/31 is assumed to crosslink the Sec23/24/Sar1 complexes. Since no budding can be observed in the absence of Sec13/31, this has to be responsible for inducing curvature.

So far it was believed that clathrin plays the dual role of coat polymerization and curvature induction. Here we present evidence that AP-1 alone is already able to form an oligomeric complex, leaving clathrin with the function of curvature induction. It remains to be tested whether the other AP complexes work similarly.

When a small GTPase is involved in initiation of coat recruitment one would expect an effect of GTP hydrolysis. GAPs, activators of the GTPase, have been identified in most coat systems. Probably the best-studied system is COPI, where ARFGAP1 was identified (Cukierman et al., 1995). Initially its activity was linked to uncoating. Later studies revealed a surprising function of GTP hydrolysis in cargo sorting (Goldberg, 2000; Lanoix et al., 2001). The

correct cargo is able to slow down GTP hydrolysis, which allows more time to be sorted into a vesicle. In COPII, the GAP Sec23 is part of the coat. This makes it more difficult to investigate its function isolated from coat formation. Upon coat recruitment and vesicle formation, GTP hydrolysis is stimulated and uncoating occurs. The GAP activity can be enhanced by the GAP activator Sec13/31, which causes rapid uncoating once the vesicle is budded off. Recent findings suggest a new model, where GTP hydrolysis is as well linked to cargo sorting. The Sec23/24-cargo pre-budding complex polymerizes rapidly with Sec13/31 into COPII vesicles before GTP hydrolysis occurs. In contrast, when incorrect cargo interacts with the pre-budding complex, the polymerization into COPII coated vesicles is slow. Here, GTP hydrolysis induced coat disassembly occurs before the vesicle can form (Sato and Nakano, 2004). Only recently the involvement of GAPs in the clathrin coats have been reported. AGAP1 was shown to regulate the formation of AP-3 coats (Nie et al., 2002). For AP-1 there was so far no GAP identified. Here we demonstrated that ARFGAP1 is able to disassemble the AP-1 precoat. In addition we could show that AP-1 is able to work as a GAP activator, similar to coatomer. We also found a stimulation difference between different sorting signals. However, it remains to be elucidated whether GTP hydrolysis is involved in cargo sorting. Together, these findings suggest that in all three coat systems GTP hydrolysis has been linked to important regulatory functions.

Structural data and sequence alignment revealed a surprising similarity between subunits of clathrin adaptors and the coatomer. Some evolutionary studies indicate that these coats might have evolved from a single coat (Boehm and Bonifacino, 2001; Schledzewski et al., 1999). This explains the similar basic principles and participants involved in coat formation. COPII subunits on the other hand seem to be unrelated, nevertheless significant mechanistic parallels can be identified between all coat systems.

Table I shows a summary of key players and interactions of the different coats. Many proteins play a role in more than one coat. This demonstrates that in a cell there are additional regulators that make sure a coat is only

formed at the time and place where it is required.

Coat	AP-1	AP-2	AP-3	AP-4	COPI	COPII
Major coat compounds on the vesicle	AP-1 clathrin	AP-2 clathrin	AP-3 clathrin (?)	AP-4	Coatomer ARF1	Sec 13/31 Sec 23/24 Sar1
GTPase / initiation factors	ARF1, 3	PI(4,5)P ₂	ARF1, 3, 5	ARF1, 3	ARF1, 3	Sar1
GAP	ARFGAP1	-	AGAP1	?	ARFGAP1	Sec23
recognized sorting signals	YXXΦ ¹⁾ [DE]XXXL[L] ²⁾	YXXΦ ¹⁾ [DE]XXXL[L] ²⁾ FXNPXY ³⁾	YXXΦ ¹⁾ [DE]XXXL[L] ²⁾	YXXΦ	KKXX KXKXX FFXRRXX	DXE
Signal recognition	¹⁾ μ1 ²⁾ μ1, β1 or γ & σ1	¹⁾ μ2 ²⁾ μ2or β2 ³⁾ μ2	¹⁾ μ3 ²⁾ σ3 & δ	μ4	coatomer (F-COPI)	Sec24
Polymerization inducer	AP-1	AP-2 / clathrin?	AP-3?	AP-4?	coatomer (B-COPI)	Sec13/31
Curvature inducer	clathrin	clathrin, epsin1, other proteins?	clathrin (?)	AP-4 ?	coatomer	Sec13/31
GAP function	precoat formation?	-	uncoating	?	uncoating, sorting	uncoating, sorting

Table I

Key players and their function in coat formation

3.2 Outlook

Further studies have to be done to describe the regulatory function of GTP hydrolysis in more detail and to analyze a possible role of ARFGAP1 in cargo sorting. The effect of clathrin on coat stability and GAP activity also has to be addressed. Finally we would like study the formation of an AP-1 precoat by means of electron microscopy. We found specific lipids to be involved in AP-1 recruitment. However, it is not clear in what way they influence coat recruitment, or which part of the coat interacts with lipids.

The *in vitro* system presented here offers possibilities to elucidate the role of accessory factors in AP-1 coat recruitment. In addition it can be used to study the requirements of other clathrin and non-clathrin coats. A liposome-based assay could further be used to investigate general interactions between proteins and lipids.

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2000 / 01 / 02 Biochemistry block course
2001 “introduction to biology” (tutorial)

Publications:

Crottet, P., Meyer, D. M., Rohrer, J., and Spiess, M. (2002). ARF1.GTP, tyrosine-based signals, and phosphatidylinositol 4,5- bisphosphate constitute a minimal machinery to recruit the AP-1 clathrin adaptor to membranes. *Mol Biol Cell* 13, 3672-3682.

Meyer, D. M., Crottet, P., Degytar, E., Cassel, D., Spiess, M. The AP-1 clathrin adaptor forms a high molecular weight precoat which can be disassembled by ARFGAP1. (Manuscript in preparation)