The contribution of proteins and lipids to COPI vesicle formation and consumption

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Cover picture: HeLa cells stained with an antibody against a resident Golgi protein (GalT, red), an antibody against a member of the p24 family of proteins (p27, green), and a nuclear stain (blue).

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Abstract

The contribution of proteins and lipids to COPI vesicle formation and consumption

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Abstract:

In the secretory pathway, movement of proteins between compartments occurs through small (50-100 nm) vesicles. The coat of COPI vesicles is composed the small GTPase ARF1 and the large coatomer complex. In the secretory pathway, these vesicles mediate Golgi-to-ER and intra-Golgi transport. During vesicle formation, recruitment of cytosolic coatomer to the membrane by ARF1 represents the key step. This generates a bud, which subsequently separates from the donor membrane as a vesicle. During consumption, the vesicle tethers to the target membrane, followed by docking and fusion, resulting in the merging of the two bilayers. This thesis has been devoted to COPI vesicle formation and consumption.

We examined the role of the lipid diacylglycerol (DAG) in bud formation. We demonstrate that efficient inhibition of DAG synthesis by the addition of the inhibitor Propranolol causes rapid dissociation of the ARFGAP1 protein from the membrane. Upon electron microscopy examinations of treated cells, we find that this results in smooth Golgi membranes devoid of budding profiles. Washout of Propranolol resulted in a marked increase of buds and associated vesicles. Cells expressing low amounts of ARFGAP1 were treated similarly. In such cells, removal of the inhibitor caused an increase in membrane buds but not vesicles. This suggests that DAG is needed at an early stage of bud formation whereas ARFGAP1 is required at a later step.

We investigated the function of two new ARFGAPs in COPI vesicle formation in living cells. We demonstrate that stimulation of vesicle budding by addition of aluminum fluoride causes accumulation of ARFGAP2, ARFGAP3, and coatomer on the Golgi, but not of ARFGAP1. Fluorescence recovery after photobleaching (FRAP) analysis of the association with the Golgi demonstrates that this accumulation also reflects irreversible binding of ARFGAP2 and ARFGAP3 with the membrane. The degree of immobilization was close to that of coatomer, suggesting a closer role than of ARFGAP1. The ability to generate the COPI coat lattice in cells lacking different combinations of ARFGAP1-3 was investigated. Absence of the ARFGAP2 and ARFGAP3 pair but not ARFGAP1 prevented coat lattice formation. This suggests that these two ARFGAPs play an overlapping role in COPI vesicle formation in the Golgi.

We looked into the factors that influence the docking and fusion of COPI vesicles with Golgi cisternae using an *in vitro* assay for the reconstitution of intra-Golgi transport. We find that vesicle fusion is regulated by the presence of $PI(4,5)P_2$ on vesicles. The pre-treatment of vesicles with a kinase stimulated fusion and treatment with a phosphatase inhibited fusion. The ability of ARF1 to generate $PI(4,5)P_2$ on the Golgi membrane may therefore prime vesicles for the following fusion event.

We analyzed a property of the cytosol, molecular crowding, and its consequences for diffusion by fluorescence correlation spectroscopy. We find that fluorescent dextrans diffuse normally in water but become subdiffusive upon microinjection into cells or in artificially crowded solutions. This phenomenon can have important consequences for the function of proteins, such as coatomer, that depend on diffusion for the association with the membrane.

Keywords: ARF1, COPI, ARFGAP, vesicle, fusion, diffusion

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List of papers

The thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I: Lennart Asp, **Fredrik Kartberg**, Julia Fernández-Rodríguez, Maria Smedh, Markus Elsner, Frédéric Laporte, Montserrat Bárcena, Karen A Jansen, Jack A Valentijn, Abraham J Koster, John J.M. Bergeron and Tommy Nilsson.
Early stages of Golgi vesicle and tubule formation require diacylglycerol
In press, Molecular Biology of the Cell

II: **Fredrik Kartberg**, Lennart Asp, Maria Smedh, Julia Fernández-Rodríguez and Tommy Nilsson.
ARFGAP2 and ARFGAP3 are essential for COPI coat assembly on the Golgi membrane of living cells
Submitted

III: Frédéric Laporte, **Fredrik Kartberg**, Johan Hiding, Francois Lepine, Markus Grabenbauer, Anirban Siddhanta, Dennis Shields, Joel Lanoix, Joachim Ostermann, John J.M. Bergeron and Tommy Nilsson.
$PI(4,5)P_2$ promotes fusion of COPI-derived vesicles with Golgi cisternae, *in vitro*
In manuscript

IV: Matthias Weiss, Markus Elsner, **Fredrik Kartberg** and Tommy Nilsson.
Anomalous subdiffusion is a measure for cytoplasmic crowding in living cells
Biophysical Journal vol 87 November 2004 3518-3524

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Abbreviations

ALPS	ARFGAP1 lipid packing sensor
AlF	aluminum fluoride
AP	adaptor protein
ARF	ADP-ribosylation factor
BAR	Bin, amphiphysin, Rvs
BARS	Brefeldin A ADP-ribosylated substrate
BFA	Brefeldin A
BIG	BFA-inhibited GEF
CAPS	Ca^{2+}-dependent activator protein for secretion
CGN	*cis*-Golgi network
COG	conserved oligomeric Golgi
COP	coat protein
CtBP	C terminal binding protein
DAG	diacylglycerol
EM	electron microscopy
ER	endoplasmic reticulum
ERES	endoplasmic reticulum exit sites
ERGIC	ER-Golgi intermediate compartment
FCS	fluorescence correlation spectroscopy
FRAP	fluorescence recovery after photobleaching
GalNacT2	N-acetylgalactoseamine transferase 2
GAP	GTPase activating protein
GBF	Golgi BFA-resistance factor
GEF	guanine exchange factor
GGA	Golgi-localized γ-ear containing ADP-ribosylation factor binding
GFP	green fluorescent protein
GTP	guanine triphosphate
MSD	mean square displacement
NSF	N-ethylmaleimide sensitive factor
PA	phosphatidic acid
PAP	phosphatidate phosphohydrolase
PC	phosphatidylcholine
PH	pleckstrin homology
PI	phosphatidylinositol
PK	proteinase K
PLD	phospholipase D
ProPr	Propranolol
SNAP	soluble NSF attachment protein
SNARE	soluble NSF attachment protein receptors
tER	transitional endoplasmic reticulum
TGN	*trans*-Golgi network
TLC	thin layer chromatography
VSV	vesicular stomatitis virus
VTCs	vesicular tubular clusters
VTP	vesicular tubular profiles

Introduction

Cells are in constant communication with their environment, secreting proteins such as signaling molecules and digestive enzymes into the surrounding tissues to perform a wide variety of functions. Through the efforts of Palade and others it was demonstrated that secretory proteins make their way through several of the organelles of the eukaryotic cell, including the endoplasmic reticulum (ER) and the Golgi apparatus, before being secreted [1]. This secretory pathway is shared by the newly synthesized proteins that reside in most of the compartments of the endomembrane system as well as the proteins of the plasma membrane, making it the transport route of at least a third of all proteins synthesized in the cell. The movement of proteins through this pathway requires mechanisms for the selective transfer of proteins and lipids between different compartments. This is achieved by formation and consumption of transport vesicles. This thesis has been devoted to the investigation of the contribution of proteins and lipids to the formation and consumption of COPI vesicles in the early secretory pathway.

1. The early secretory pathway

The ER of mammalian cells comprises a continuous, tubular membrane network that extends throughout the cytoplasm and is a major site of protein and lipid synthesis [2]. This organelle represents the point of entry into the secretory pathway. Proteins that enter this route contain a hydrophobic signal sequence in their N-terminus that is exposed to the cytosol upon translation on cytosolic ribosomes. This attracts the signal recognition particle which binds to the signal sequence, arrests elongation and directs the ribosome to a receptor in the rough ER membrane [3]. At the ER membrane, the ribosome associates with the translocon that generates an aqueous pore through the membrane [4, 5]. As protein synthesis then proceeds, soluble proteins are co-translationally transferred through the translocon pore into the ER lumen and the transmembrane sequences of integral membrane proteins are embedded into the membrane. In the ER, proteins are subjected to a number of modifications including initiation of N-linked glycosylation and disulphide bond formation. The main task of the ER is to ensure that proteins reach their native conformation which is assisted by an abundance of chaperones and folding enzymes [6]. These factors monitor non-native structures of proteins, such as exposure of hydrophobic patches or improperly folded glycosylated proteins. They transiently associate with them and catalyze folding and prevent formation of protein aggregates. The ability of the ER to retain improperly folded proteins or target them for degradation is a crucial part of a quality control machinery. This ensures that only proteins in their native conformation proceed along the secretory pathway [7, 8].

Proteins exported from the ER are delivered to the Golgi apparatus (the Golgi), the central organelle of the secretory pathway. The Golgi apparatus exhibits a striking morphology [9-12]. In most mammalian cells, the Golgi is a single-copy organelle, located close to the nucleus (juxtanuclear) next to the centrosome (Figure 1A). The basic functional units of the Golgi apparatus are flattened membrane discs (cisternae), which assemble into stacks with usually 3-8 cisternae in each stack (see Figure 1B and Paper I). The cisternae are typically 2-3 μm wide with a narrow lumen of 10-20 nm and are in very close proximity to each other. In the cell, the Golgi apparatus is made up of several parallel stacks that are connected to one another by tubular connections, forming a continuous membrane structure referred to as the Golgi ribbon. All cisternae are fenestrated and holes of adjacent cisternae align to form openings (wells) into the Golgi stack. In spite of this structural complexity, the Golgi has been demonstrated to have a functional polarity. Proteins enter the Golgi stack on one side (the *cis*-side) and exit the Golgi on the other side (the *trans*-side), with the intermediate cisternae referred to as the medial Golgi. The *cis*-Golgi often appears as a tubular network in electron microscopy (EM) micrographs and is termed the *cis*-Golgi network (CGN). Correspondingly, at the *trans*-most part of the Golgi, a network of branching tubules from the cisternae is referred to as the *trans*-Golgi network (TGN). The Golgi apparatus performs a number of post-translational modifications on the proteins that pass through this organelle, including glycosylation (both N-linked and O-linked), tyrosine sulfation, phosphorylation and limited proteolytic cleavage of some secretory proteins.

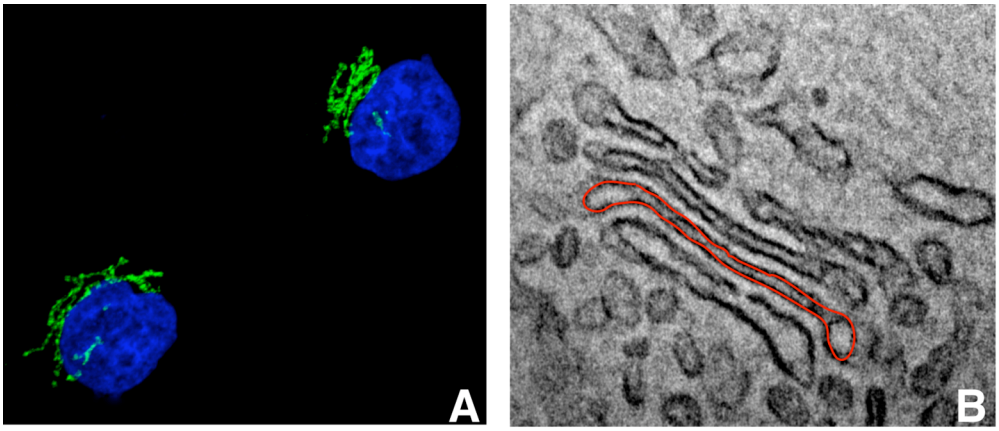

Figure 1. The Golgi apparatus. (A) The juxtanuclear position of the Golgi apparatus in the cell. HeLa cell stained with an antibody against a resident Golgi protein (green) stained with a nuclear stain (blue). (B) Golgi cisternae are assembled in stacks as demonstrated on an EM micrograph. One cisterna is highlighted in red.

The glycosylation of proteins, by the sequential removal and addition of sugar residues to the N-linked oligosaccharide added in the ER, is the major modification that occurs in the Golgi [13, 14]. Briefly, the oligosaccharide that is attached to the newly synthesized protein in the ER is trimmed by the removal of glucose and mannose residues in the ER and the Golgi. In the Golgi cisternae, sugar moieties (such as mannose, N-acetylglucosamine, galactose and sialic acid) are added to elongate it to the final oligosaccharide, a process that continues throughout the Golgi stack. The sequential addition of sugars in the Golgi is carried out by the glycosyltransferases, the most prominent family of Golgi-resident proteins with some 100-200 members in the human genome [15]. Glycosyltransferases are type II transmembrane proteins with a small N-terminal cytoplasmic tail, a single transmembrane domain and the large globular catalytical domain exposed to the lumen of the Golgi [16, 17]. The glycosyltransferases show gradient-like distributions over the Golgi stack with typical *cis*, medial or *trans* localizations over several cisternae [18]. This compartmentalized localization corresponds to the order in which they act on the substrate [19]. The mechanism behind the targeting of Golgi glycosyltransferases to certain parts of the Golgi stack is not fully known but is thought to be mediated by complex formation between enzymes (kin recognition) and/or by the sensing of membrane bilayer thickness by the transmembrane domain of the glycosyltransferases [20, 21].

2. Protein transport in the early secretory pathway

Membrane traffic between the organelles of the early secretory pathway (defined here as the ER and the Golgi apparatus and associated membrane structures) is in part mediated by transport vesicles, small round membrane profiles of 50-100 nm diameter (see Paper I). These vesicles pinch off a donor membrane, carrying protein and lipid cargo, to be delivered to a target membrane. Vesicles are generated and consumed by protein machineries that are conserved from yeast to human, indicating the universality of this transport mechanism in eukaryotic cells [22]. There are two key steps in the life cycle of a vesicle, the deformation of the donor membrane that produces the vesicle (budding) and the fusion of the vesicle with the target membrane leading to vesicle consumption [23]. For an overview of the life cycle of a vesicle see Figure 2. Vesicle budding is driven by the recruitment of coat proteins (COPs) to a donor membrane. This recruitment is under the control of small molecular weight guanine triphosphatases (GTPases). These act like binary switches promoting coat assembly in their GTP-binding state and coat disassembly in their GDP-binding state. The initiation of vesicle formation occurs by the exchange of GDP-for-GTP on the GTPase catalyzed by guanine-exchange factors (GEFs).

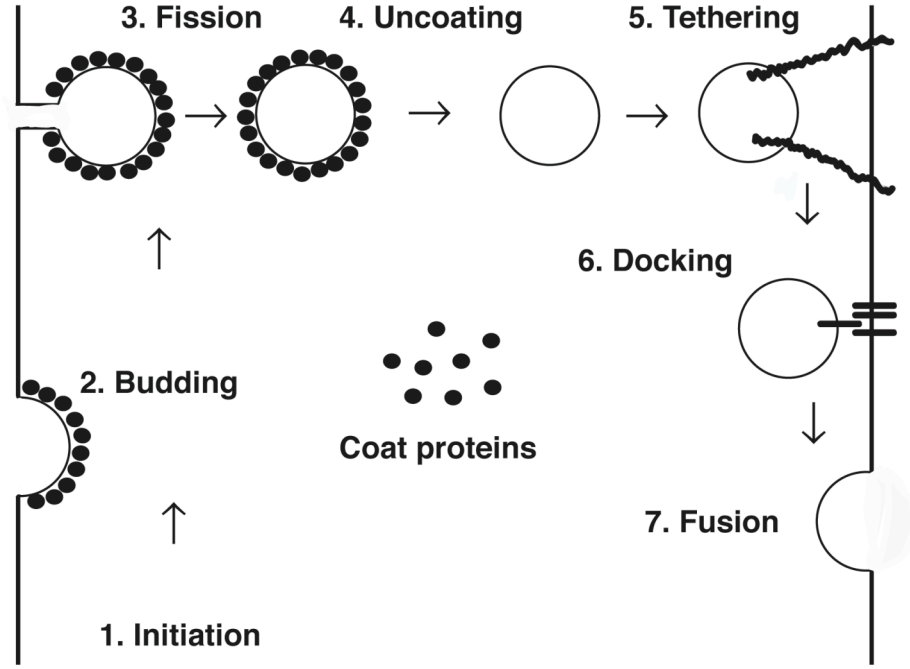

Figure 2. The life cycle of a vesicle. A schematic view of the key steps in the formation and consumption of a COPI vesicle (see text for more details). (1) Initiation of vesicle formation occurs by nucleotide exchange on ARF1, which recruits coatomer to the membrane in its GTP form. (2) On the membrane, coatomer selects cargo and induces membrane deformation generating a bud. (3) Fission. The neck of the vesicle is constricted, separating the vesicle from the donor membrane. (4) Uncoating. The GTP hydrolysis by ARF1 causes the coat to dissociate from the vesicle, exposing factors necessary for vesicle consumption (not shown). (5) The initial interaction of the vesicle with the membrane is thought to be mediated by tethering factors. (6) Docking occurs by the assembly of the SNARE complex. (7) Fusion occurs, which mixes the two bilayers and delivers vesicle content to the acceptor membrane.

A second critical function of the coat proteins is to promote the incorporation of correct protein cargo into the vesicles (cargo sorting). This is primarily mediated by direct interaction between cytoplasmic domains of cargo proteins with the coat proteins [23]. Soluble proteins of the lumen of the donor organelle may associate with cargo receptors that in turn project through the membrane for interactions with coat proteins. In addition, several other events must occur for efficient transfer of a vesicle form one compartment to the other (see Figure 2). The coat that generated the vesicle should be removed to expose protein factors necessary for fusion (uncoating) and the vesicle must attach to the correct target membrane (tethering/docking) before fusion. The life of a transport vesicle can therefore be divided the following stages: initiation, budding, fission (also termed scission), uncoating, tethering, docking and ultimately fusion [23].

In the early secretory pathway, three main coats have been described that use the vesicular transport paradigm described here. Coat protein complex II (COPII) vesicles mediate export of proteins from the ER, coat protein complex I (COPI) vesicles operate between the Golgi and the ER and in the Golgi, and clathrin-derived vesicles mediate transport from the TGN. Although these are likely to be the major coats that operate in the secretory pathway, the discovery of new molecular compositions of the coats and subpopulations of vesicles demonstrates that we currently do not have a complete account of the vesicles-dependent pathways that operate in the cell.

2.1 ER-to-Golgi transport

In mammalian cells, protein export occurs at distinct sites of specialized ER, the so-called transitional ER (tER), which is devoid of ribosomes and enriched in budding profiles. A typical mammalian cell has around 100-200 of these ER exit sites (ERES) that are scattered over the ER network across the cytoplasm but tend to be concentrated toward the Golgi region. The ERES are characterized by the presence of the COPII coat components that associate with the membrane for the generation of transport vesicles [24]. The core protein machinery consists of five proteins: the small molecular weight GTPase Sar1 and two coat complexes Sec23/Sec24 and Sec13/Sec31. In the absence of other proteins these proteins are able to generate COPII vesicles of 60 nm diameter from chemically defined

liposomes, *in vitro* [25]. Sec12 is an ER-resident transmembrane GEF that initiates COPII-coated vesicle formation by catalyzing the GDP-for-GTP exchange on Sar1 at the ERES. This induces a conformational change in Sar1 that exposes an N-terminal amphipatic helix that is in turn inserted into the outer leaflet of the ER membrane to anchor it to the membrane [26]. Sar1 then recruits the heterodimeric Sec23/Sec24 to the membrane [27]. Cargo selection into COPII vesicles is mediated mainly by the Sec24 subunit of the coat which interacts with cytoplasmic export signals of transmembrane proteins [28]. A dibasic motif (usually a DXE stretch) or a motif consisting of two bulky hydrophobic residues (usually FF) has been shown to interact with one of three independent cargo-binding sites on the Sec24 subunit. To complete COPII vesicle budding, a heterotetramer of Sec13/Sec31 associates with the inner layer of the pre-budding complex Sar1-Sec23/Sec24. The assembly of this outer layer into a scaffold drives both further membrane deformation as well as the collection of Sar1-Sec23/Sec24-cargo complexes into the nascent bud. Following the fission event, the uncoating of COPII vesicles is driven by the GTP hydrolysis by Sar1, causing retraction of its amphipatic helix. The GTPase activity of Sar1 is intrinsically low and dependent on the interaction with the Sec23 subunit of the coat that is a GAP for Sar1. The addition of the outer shell of Sec13/31 further promotes GTP hydrolysis by Sar1 and ensures uncoating of the vesicle [29]. Free COPII vesicles can be observed in close proximity to the ERES *in vivo* [30]. These vesicles undergo fusion with each other (homotypic fusion) to generate a new compartment for further transport of proteins along the secretory pathway [31].

The ER-Golgi intermediate compartment (ERGIC) is a distinct compartment located in close proximity to the ERES. At the ultrastructural level it appears as a collection of vesicular-tubular clusters (VTCs) or large pleiomorphic bodies (with a diameter of 0.4 to 1 μm) with numerous buds [32, 33]. The ERGIC lacks the components of the COPII coat and is instead distinguished by the presence of the transmembrane protein ERGIC-53 and components of the COPI coat. The ERGIC is an important site of concentration of secretory cargo in the secretory pathway [34]. This is thought to occur by the selective retrieval of ER-resident proteins from the ERGIC to the ER by vesicles formed by the COPI coat [35]. From the ERGIC, the secretory protein is then transported along microtubule tracks towards the Golgi apparatus [36]. This was initially thought to be mediated by transport of the ERGICs themselves but evidence now suggests that this may require the formation of specialized carriers as the ERGICs are largely immobile in the cell [37].

2.2 Intra-Golgi transport

The first model for intra-Golgi transport was based on EM examinations of the Golgi apparatus. In this model, cisternae were seen as transitory structures, formed at the *cis*-side and consumed on the *trans*-side by the generation of into secretory vesicles [38]. The analysis of the secretion of algal scales (glycosylated molecules too large to fit in the vesicles surrounding the stack) implied that these molecules traveled the Golgi in "progressing" cisternae (see Figure 3). This "cisternal progression" model was subsequently replaced by a more static view of the Golgi apparatus following the observation that the organelle remained intact in the absence of protein synthesis. Following the mapping of glycosyltransferases to certain parts of the stack (*cis*, medial, *trans*) and the identification of the Golgi-associated COPI vesicles, once thought to mediate intra-Golgi transport of secretory protein, a new model replaced the old one. The "vesicular transport-stable compartment" model for Golgi transport was proposed in which secretory cargo is ferried between the cisternae of the stack by several rounds of budding and fusion of COPI vesicles for sequential glycosylation.

This model became difficult to reconcile with several different observations that revealed the dynamic nature of the Golgi apparatus and how proteins are transported through the Golgi [39]. The Golgi apparatus was shown to undergo dramatic reorganizations upon the addition of pharmacological agents and during mitosis. In addition, the localization of glycosylation enzymes was found not to be restricted to a single cisternae but rather over several cisternae contradicting the notion that each cisternae is a distinct compartment [18]. Transport of large and small secretory cargo was demonstrated to occur within the cisternae of the Golgi from *cis* to *trans* in mammalian cells [40, 41]. It was shown that the coat of COPI vesicles interacts with the cytoplasmic tails of ER-resident to mediate retrograde transport to the ER (see section 3.2). More recently, the concept of maturation, that cisternae change their protein makeup over time, was directly visualized in yeast [42, 43].

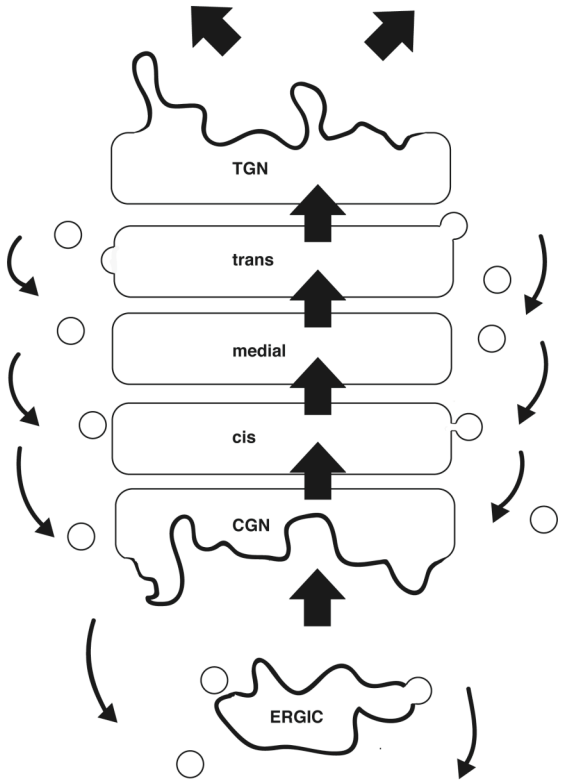

Figure 3. The cisternal progression/maturation model. A schematic view of the cisternal progression/maturation model for intra-Golgi transport (see text for details). Secretory cargo is transported in the forward (anterograde) direction, in the cisternae (thick arrows). At the levels of the TGN, proteins are further transported through different carriers to their final destination. In the early secretory pathway, proteins are recycled in the retrograde direction (thin arrows) through COPI vesicles. Figure is not to scale.

Such studies are in support of a cisternal progression/maturation model scheme transport, which is currently the most widely accepted model for intra-Golgi transport [39, 44]. In this view, new Golgi cisternae are formed by the continuous delivery of carriers derived from the ERGIC along the microtubule tracks. At the Golgi, these carriers either fuse with each other (or with a pre-existing cisternae) to generate a new *cis*-most Golgi cisternae at the level of the CGN. This continuous addition of new material at the *cis*-side drives the progression of the rest of the Golgi cisternae in the *trans*-direction (see Figure 3). During their passage through the stack, the cisternae mature by changing their content of resident glycosylation enzymes. At the level of the TGN, the cisternae are consumed by the formation of vesicles and other transport carriers for further transport. At all levels of the pathway proteins are recycled by COPI vesicles. This generates not only the steady-state distributions of the proteins resident to the pathway but also maintains membrane homeostasis in the pathway.

While there is little doubt about the role of COPI in Golgi-to-ER transport, their function within the Golgi stack is subject to intensive debate. Especially, the content and directionality of the COPI vesicles associated with the Golgi is at the center of the controversy between different models for intra-Golgi transport [45]. One key prediction of the cisternal maturation model is that Golgi-resident proteins are recycled by COPI vesicles [46]. The enrichment of glycosylation enzymes into COPI vesicles, seems to confirm this prediction [47-50]. By electron microscopy, glycosylation enzymes have been shown to gain access to, and become concentrated in, vesicles associated with the Golgi [51, 52]. These findings none-withstanding, the incorporation of glycosylation enzymes into peri-Golgi COPI vesicles is still questioned [53-55]. Furthermore, secretory cargo has been identified in COPI vesicles, suggesting that there could be a role for these vesicles in anterograde transport in the Golgi [49, 56].

In the cisternal progression/maturation model, there are two pathways mediated by COPI vesicles, the Golgi-to-ER pathway and retrograde, intra-Golgi transport. In scale-producing algae and plants, two COPI vesicle populations can be identified based on morphological criteria [57]. One of these is strictly found between the *cis*-Golgi and the ER and the other adjacent to medial and *trans*-cisternae, with no

overlap in the localization of the two. In mammalian cells, two populations have been identified biochemically of which one is enriched in glycosylation enzymes [49, 58].

A complement to the cisternal maturation model for intra-Golgi transport proposes that the Golgi stack is a continuous membrane system with tubular connections between cisternae [59]. Tubular connections between cisternae extending in the *cis*-to-*trans* direction have been observed in cells [60, 61]. In contrast to transport vesicles, however, such intermediates are poorly characterized and appear primarily in cells that are highly active in transport. Nonetheless, the notion that the Golgi stack is a continuous membrane system may help to explain how secretory cargo is exported from this organelle [62]. In this view, protein cargo is delivered to the Golgi and immediately distributes across the stack for partitioning into processing domains (containing glycosylation enzymes) or export domains. This scheme is not easily reconciled with the cisternal maturation/progression model in its purest form [62].

2.3 Post-Golgi transport

The TGN represents a major site of sorting in the cell, where proteins are directed to a wide array of post-Golgi compartments. Briefly, soluble secretory proteins and integral membrane proteins can be delivered to the plasma membrane by constitutive secretion or regulated secretion. In polarized cells, an additional level of sorting ensures the correct targeting of proteins to the basolateral or apical plasma membrane [63]. The transport from the TGN involves a number of different types of carriers. Constitutive secretion is achieved by transport of large tubular structures that are formed from the cisternae of the TGN and subsequently move along microtubules towards the plasma membrane [64-66]. In specialized secretory cells, dense-core granules are formed from the TGN and serve as long-term storage for secretory proteins that are released to the extracellular milieu by appropriate stimulus [67].

The role for classical (50-100 nm) transport vesicles at the TGN is primarily to export proteins to the organelles of the endocytic pathway. Clathrin was the first coat to be described and has a well-established role in receptor-mediated endocytosis at the plasma membrane but is also mediates vesicle formation at the TGN [68, 69]. Clathrin-coated buds are restricted to the very last cisternae of the TGN, in contrast to other coats that are found throughout the Golgi stack [10]. The basic units of the clathrin coat are the triskelia formed by the association of three heavy-chains and three light chains of clathrin [70, 71]. At the TGN, adaptor proteins (APs) are recruited to the Golgi membrane by the small GTPase ADP-ribosylation factor 1 (ARF1) and are able to interact with sorting signals in the cytoplasmic domains of transmembrane proteins [72]. Two clear roles for different APs at the TGN have been identified. AP1 functions to concentrate the mannose-6-phosphate receptor into clathrin-coated vesicles for transport of lysosomal enzymes to endosomes [73] and AP-3 associates with other transmembrane cargo to mediate a separate transport pathway from the TGN to endosomes [74]. Another class of adaptors, the monomeric Golgi-localized γ-ear containing ADP-ribosylation factor binding (GGA) proteins are recruited to the Golgi by ARF1 and may work together with AP-1 for cargo selection into clathrin-coated vesicles [75]. In this way, ARF1 is bound to transmembrane cargo together with APs and form an inner layer that selects vesicle cargo in clathrin vesicle formation [76].

3. COPI vesicle formation

Even though the precise roles of COPI vesicles are still debated, the molecular machinery that generates these vesicles has been described in some detail. First, I will review the proteins that control initiation of vesicle formation, coat assembly, cargo recognition and the role of GTP hydrolysis for these events (see also Figure 2).

3.1 Initiation and coat assembly

The COPI coat consists of two components, ARF1 and the heptameric protein complex coatomer. The ARF family of proteins constitute one of five major protein subfamilies of the Ras superfamily of GTPases [77]. Mammalian cells have six members in the ARF family (ARF1-6) that are divided into three classes based on sequence identity: class I (ARF1-3), class II (ARF4-5), and class III (ARF6)

[78]. In humans, ARF1 and ARF3 are >96% identical and are the most abundantly expressed members of this family. The class II ARFs are less abundant but may have important roles in maintaining of Golgi structure and membrane traffic in the early secretory pathway [79]. In contrast to these ARFs, ARF6, resides at the plasma membrane where it regulates endocytosis and actin and plasma membrane remodeling [80]. In the Golgi apparatus (and pre-Golgi membranes) ARF1 regulates the association of a number of coat complexes (including coatomer and clathrin associated adaptors) as well as lipid-modifying enzymes [81].

The role for ARF1 in regulating COPI vesicle formation was established by the observations that inhibition of GTP hydrolysis caused a block in intra-Golgi transport and that ARF1 was responsible for transport in the early secretory pathway [82-84]. By blocking GTP hydrolysis by the non-hydrolyzable GTP analogue GTPγS it was possible to accumulate (non-clathrin) coated vesicles, and analysis of the coat constituents revealed that ARF1 was an major component [85]. Subsequently, ARF1 bound to GTP was shown to be necessary for the recruitment of soluble coatomer to isolated Golgi membranes, *in vitro* [86, 87]. In intact cells, the overexpression of mutated ARF1 that is deficient in GTP hydrolysis (and therefore locked in its GTP-state) promotes the association of coatomer with the membrane [88]. As the overexpression of this mutant (as well as the overexpression of the GDP-restricted form of ARF1) blocks transport in the secretory pathway it demonstrated the importance of regulated nucleotide exchange on ARF1 for proper COPI function [89].

In its GDP form, ARF1 is primarily a cytosolic protein that associates weakly with the membrane. The posttranslational modification in the form of the addition of a myristate fatty acid to the N-terminus (myristoylation) of the protein is essential for this weak association [90]. Interestingly, it has been demonstrated that ARF1-GDP can interact with membrane proteins of the ER-Golgi interface, such as membrin and p23 [91, 92]. These studies suggest that ARF1-GDP binding to a "receptor" in the correct membrane is an important step in the earliest stages of vesicle formation [93]. Once it has associated with the membrane ARF1 undergoes GTP-for-GDP exchange and this results in a conformational change that ejects and extends the N-terminal 17-amino acid amphipatic helix into the membrane. The insertion of several hydrophobic residues of this helix (previously hidden in the protein core) into the membrane ensures the tighter ARF1-GTP membrane association [94]. This nucleotide exchange that initiates membrane binding and coat recruitment is catalyzed by dedicated ARFGEFs [95-97]. The catalytical domain of the ARFGEFs consists of a 200 amino acid stretch, the Sec7 domain, which is sufficient for nucleotide exchange. Using this domain as a classification one can identify 15 human genes that encode peripheral membrane proteins that are divided into several subfamilies. Only large, multi-domain, ARFGEFs found in all eukaryotes. They can be divided into two families: the Golgi Brefeldin-A-resistance factor (GBF) family and the Brefeldin-A-inhibited GEF (BIG) family. The roles of the domains outside of the Sec7 region are poorly understood but have been suggested to be important for protein interactions and localization [98]. In mammalian cells, the three members of these two families (GBF1, BIG1, BIG2, respectively) localize to membranes of the early secretory pathway.

The fungal metabolite Brefeldin A (BFA) is useful for studying ARFGEF function [99]. This small molecule intercalates the Sec7 domain of some ARFGEFs and ARF1-GDP to form an abortive ternary complex that prevents the activation of ARF1 [100]. In turn, this inhibits the recruitment of coatomer to the Golgi membrane, which rapidly dissociates into the cytosol together with ARF1 [101]. This dissociation of coatomer from the membrane inhibits transport in the secretory pathway and causes the relocalization of Golgi glycosylation enzymes to the ER [102]. As all three large ARFGEFs are sensitive to BFA *in vivo* [95].

Whereas GBF1 mainly localizes to pre-Golgi membranes (the ERGIC) and the *cis*-Golgi, BIG1 and BIG2 localize to the *trans*-part of the Golgi [103-105]. The overexpression of BIG2 alters the association of AP1 at the TGN without any effect on COPI function [106, 107]. GBF1 is thought to mediate the association of COPI with the pre-Golgi and Golgi membranes for the regulation of COPI vesicle formation. This is supported by the observation that the overexpression of GBF1 protects against the BFA-induced dissociation of coatomer from Golgi membranes [104, 108]. Expressing a catalytically inactive mutant of GBF1 induces coatomer dissociation and impairment of Golgi structure [109]. Exposing cells to a 15°C temperature block (which inhibits ER-to-Golgi transport) accumulates GBF1 on peripheral structures consistent with a role in this transport step [103, 104]. Finally, microinjection of antibodies against GBF1 causes the dissociation of coatomer from membranes *in vivo* [105]. Several studies have reported the nature of the membrane association-dissociation cycle of GBF1 with the Golgi membrane using fluorescence recovery after photobleaching (FRAP) [105, 110].

From these studies it is clear that GBF1 exists in a large soluble cytosolic pool that dynamically associates with the ERGIC and Golgi membranes by a cycle of binding and release events.

Activated ARF1 is necessary for recruiting coatomer, the major component of the COPI coat [111-113]. Coatomer is a large protein complex that consists of the subunits α- (160 kDa), β- (107 kDa), β'- (102 kDa), γ- (100 kDa), δ- (60 kDa) ε- (35 kDa) and ζ-COP (20 kDa) and is stabilized by a number of interactions between the subunits [114]. The sequential assembly of the individual subunits *in vivo* results in a very stable cytosolic complex that is recruited as one unit 'en bloc' to the Golgi membrane by ARF1-GTP [115, 116]. Interestingly, there exists two paralogue isoforms of the γ- and ζ-subunits in higher eukaryotes (termed γ1/2 and ζ1/2 respectively) that are assembled into different coatomer complexes with separate distributions within the Golgi stack, but the relevance for this is not known [117, 118]. The recruitment of coatomer is accomplished by several GTP-specific interactions between ARF1 and subunits of coatomer [119, 120]. An intriguing aspect of ARF1-GTP function is its ability to, independent of coatomer, induce positive membrane curvature that may contribute to vesicle formation [121-123]. However, although ARF1-GTP may induce membrane deformation (see section 5) making it conducive for further budding, the requirement for both ARF1 and coatomer for vesicle formation has been established in a number of *in vitro* budding systems [124-128]. Most strikingly, it is possible to generate COPI-coated vesicles from protein-free liposomes *in vitro*, using only purifed ARF1 and coatomer, if GTP hydrolysis is inhibited, demonstrating that basic budding and fission functions are supplied by these two factors [126].

3.2 Cargo recognition

The canonical sorting motif for COPI vesicles, the dilysine motif, was discovered as an ER-targeting motif in the cytoplasmic tail of the adenoviral transmembrane protein E3/19K [129]. This motif consists of two positively charged amino acids (lysine) in the –3 and –4 position (relative to the C-terminus) and is found in many of the transmembrane proteins of the ER [130]. The alternative positioning of the lysine residues in the –3 and –5 positions was also shown to be efficient for ER retrieval and this motif is denoted K(X)KXX to indicate this. A important discovery demonstrated that the K(X)KXX sorting motif interacts with coatomer for the retrieval of proteins to the ER [131, 132]. At least two binding sites for proteins carrying the K(X)KXX sorting signal have been demonstrated on the α and β' subunits of the coatomer complex [132, 133]. Another sorting motif consists of two positively charged arginine residues (usually in an RXR sequence). This motif can be found in cytoplasmic loops or tails of individual subunits of large oligomeric proteins (such as ion channels) that function in the plasma membrane [134]. The exposure of this motif targets the subunits to the ER and prevents export until the oligomeric complex is properly assembled. Coatomer therefore mediates their retention in the secretory pathway [135]. Upon assembly of individual subunits, the sorting signals that interact with coatomer are masked, which allows for forward transport of the oligomeric complex to the plasma membrane through the secretory pathway [136].

An important family of transmembrane proteins that are included into COPI vesicles are the p24 family of proteins. These localize to the membranes in the ER-Golgi interface by constant cycling between the compartments [137]. Members of this family were identified as major transmembrane proteins of COPI vesicles that were purifed from *in vitro* budding assays [138, 139]. The p24 family of proteins (consisting of at least six members in mammals) are type I transmembrane proteins with large (20 kDa) luminal domains, a single transmembrane domain, and small (12-18 amino acid) cytoplasmic domains [137]. The cytoplasmic domains of the p24 proteins contain two sorting signals, a di-phenylalanine motif close to the transmembrane region (all members) and a K(X)KXX-like motif at the cytoplasmic terminus (some members). These cytoplasmic domains mediate both binding to the COPII and COPI coats and would explain how they cycle between the ER and Golgi [138, 140, 141]. The p24 proteins exist as monomers and oligomers, which is thought to regulate their localization and function, such as their incorporation into COPI vesicles [142-144]. The cytoplasmic domains of the p24 protein may preferentially interact with two binding sites within the γ-subunit of coatomer for the inclusion into COPI vesicles [145]. The large luminal domains of the p24 proteins have been proposed to function as cargo receptors for transport of soluble proteins in the ER-Golgi shuttle, but no clear binding partners have yet been characterized [146]. Other functions have been identified that could contribute to COPI vesicle formation. The presence of the p23 cytoplasmic domains on liposomes facilitates COPI vesicle formation by purifed coatomer and ARF1 [128]. Binding by coatomer to p23 has been suggested to

promote coatomer polymerization [147]. The role of p23 in recruiting ARF1-GDP to the membrane (see section 3.1) and of p24 in regulating GTP hydrolysis (see section 3.3) also suggest a central role for these proteins in COPI vesicle formation.

A well-known example of a protein that cycles between the ER and the Golgi is the KDEL-receptor (KDEL-R). Certain abundant luminal proteins of the ER are characterized by a conserved KDEL sequence at their C-terminus that is sufficient to mediate the retrieval from later compartments of the secretory pathway [148]. This sequence is recognized by the KDEL-R, a 26 kDa, seven integral membrane protein that localizes mainly to the Golgi complex and the ERGIC [149]. Upon binding to a KDEL-containing protein, the KDEL-R is thought to oligomerize and associate with components of the COPI coat through a K(X)KXX-like motif [150-152]. The interaction between the KDEL-R and its ligand is thought be triggered by the decrease in pH that extends over the secretory pathway [153]. This could induce the association of ligands with the KDEL-receptor in the Golgi and the release of free ligands upon retrograde transport to the ER.

The molecular events described above propose a straightforward model for COPI vesicle formation (the classical model). In this model, the activation of ARF1 results in the recruitment of cytosolic coatomer to the membrane. The interaction of coatomer with cytoplasmic domains of proteins in the membrane selects these proteins for incorporation into the vesicle. The polymerization of coatomer subunits results in membrane deformation that generates a vesicle, which dissociates from the donor membrane (see Figure 2).

3.3 Role of GTP hydrolysis

In the classical model for vesicle formation, the coat is lost from the vesicle (termed uncoating) by the GTP hydrolysis of ARF1 present in the coat lattice [154]. The conversion of ARF1 into its GDP-binding conformation would destabilize the association of coatomer with the vesicle membrane in the same way as it does with the donor membrane (see section 3.1). The significance of proper uncoating is highlighted by the accumulation of transport-incompetent vesicles upon blocking GTP hydrolysis [82, 111]. As ARF1 was isolated and characterized, it was shown that it has non-detectable GTPase activity. This postulated the existence of an ARF1 GTPase-activating protein (ARF1GAP) [155].

By fractionating the ARFGAP activity from rat liver cytosol it was possible to isolate a protein that stimulated GTP hydrolysis by ARF1 [156]. This protein, ARFGAP1, is a peripheral 45 kDa protein that localizes to the Golgi apparatus in a BFA-sensitive manner, thus implying a role in ARF-GTP dependent vesicle budding [157]. The catalytical activity was localized to the 130 amino acids of the N-terminus (the ARFGAP domain) of the protein that includes a characteristic $CX_2CX_{16}CX_2CX_4R$ sequence where an invariant arginine is essential for GAP activity [158, 159]. The four cysteine residues coordinate a zinc ion resulting in a ‘zinc finger’ motif of structural importance [160]. The proper localization and function of ARFGAP1 on the Golgi membrane requires regions of primarily its C-terminus where multiple hydrophobic residues mediate the interaction with the membrane [161-164]. A number of observations support a role for ARFGAP1 in regulating COPI association with the Golgi membrane. Overexpression of ARFGAP1 induces the dissociation of coatomer from the membrane and redistribution of Golgi resident enzymes to the ER (that is, a BFA-phenotype) consistent with the notion that it drives the membrane pool of ARF1 into its cytosolic GDP-form [151, 161, 165]. The catalytic activity of recombinant ARFGAP1 on Golgi membranes is stimulated approximately two-fold by the addition of coatomer [166]. This could be mediated by a role of coatomer in facilitating the interaction between ARFGAP1 and ARF1-GTP [158, 166]. *In vitro*, a catalytical fragment of ARFGAP1 is able to efficiently uncoat COPI-coated vesicles generated from liposomes demonstrating that the uncoating reaction can be mediated by ARFGAP1 [127].

ARFGAP1 is the founding member of a large family of ARFGAPs of which there are 31 predicted members that can be divided into ten families based on domain similarities outside of the ARFGAP domain [97, 167, 168]. Many of these members are larger multidomain proteins that regulate the roles of ARF1-6 in endosomal trafficking and actin remodeling at the plasma membrane. Some of these ARFGAPs contribute to vesicle formation by binding to coat components and as part of vesicle coats. The notion that ARFGAP1 is included in the COPI coat as a structural component is consistent with a number of observations.

The C-terminus of ARFGAP1 has been shown to interact with the KDEL-R and to be an important event in the recruitment of ARGFAP1 to the Golgi membrane [151, 169, 170]. Furthermore, ARFGAP1 has been demonstrated to bind to the p24 protein of the p24 family [58]. More recently ARFGAP1 was shown to directly enhance the binding of coatomer to the cytosplasmic tail of a cargo protein *in vitro*, consistent with a role as a part of the COPI coat [171]. A role of ARFGAP1 to stimulate vesicle formation is suggested by studies that employ a two-step assay for the generation of COPI vesicles with high amounts of ARFGAP1 from liposomes or Golgi membranes [152, 171]. This ability of ARFGAP1 to stimulate COPI vesicle formation is apparently dependent on the catalytical activity. Finally, interactions between ARFGAP1 and coatomer have been demonstrated both *in vitro* and *in vivo* [165, 171]. Based on these findings it is likely that ARFGAP1 contributes to COPI vesicle formation in at least two ways: as a GTPase-activator of ARF1 (for uncoating) and as a coat component (by interacting with coatomer and cargo).

The role for GTP hydrolysis in vesicle formation has also been extended to the process of cargo sorting into the nascent vesicle. The addition of non-hydrolyzable analogs of GTP (such as GTPγS) to *in vitro* budding assays markedly decrease the amount of cargo that is incorporated into COPI vesicles generated from purified Golgi membranes [47, 172]. The addition of GTP-restricted ARF1 to these assays produced similar negative effects on cargo sorting, demonstrating that the small GTPase is the major target of GTPγS for this effect [47, 173]. *In vivo*, the microinjection of GTPγS or expression of GTP-restricted ARF1 causes accumulation of COPI-coated vesicles containing less cargo [174]. These findings suggest that there is active ARF1 GTP-hydrolysis occurring early in vesicle formation and that it is not strictly linked to coat dissociation. Two separate mechanisms have been proposed to contribute to the regulation of ARFGAP1 to achieve this [175, 176].

Sorting by inhibition

The first such mechanism occurs through ‘sorting by inhibition’ and centered on cargo-induced inhibition of ARFGAP activity. It was demonstrated that the cytoplasmic tail of the p24 protein could inhibit coatomer- and ARFGAP1-dependent GTP hydrolysis by truncated ARF1 in solution [177]. By examining the effect of the p24 cytoplasmic peptide on ARFGAP1 activity on both liposomes and Golgi membranes it has been shown to inhibit GTP hydrolysis mediated by full-length ARFGAP1 [58, 158]. Furthermore, it was shown to inhibit cargo incorporation into COPI vesicles in a manner similar to GTPγS and GTP-restricted ARF1 when introduced into the *in vitro* vesicle budding assay [58].

The classical model for COPI vesicle formation can then be modified to incorporate a role for ARFGAP1-catalyzed GTP hydrolysis for cargo concentration. Briefly, the activation of ARF-GTP recruits coatomer and subsequently ARFGAP1 to the Golgi membrane where this complex can probe the membrane and interact with proteins carrying the K(X)KXX-sorting motif. If no cargo is present, the activity of ARFGAP1 is high and mediates the ARF-1 dependent release of coatomer into the cytosol again for further rounds of GTP hydrolysis. This continuous cycling of coatomer on the membrane could mediate the formation of membrane patches enriched with cargo by continuously recruiting such proteins. Another way of creating such membrane patches could be by formation of large complexes of cargo (e.g., glycosylation enzymes or p24 proteins) due to the triggering by the luminal milieu of the Golgi [178]. These domains containing large numbers of cargo proteins (including the cytoplasmic domain of p24) then down-regulate the activity of ARFGAP1 and cause coatomer to reside on the membrane longer, allowing it to polymerize and drive bud formation and subsequent fission of a cargo-laden vesicle [58, 175, 179].

Control by curvature

The second proposed mechanism for regulation of ARFGAP1 the ‘control by curvature’ model, focuses on the role of the membrane. In an *in vitro* assay it was demonstrated that ARFGAP1 bound preferentially to liposomes with diacylglycerol (DAG) carrying monounsaturated acyl chains resulting in increased catalytical activity [180]. This is not to dependent on the chemical composition of DAG but rather its geometrical shape (see section 5 and Figure 6). This cone-shaped lipid, reduces the packing of the lipid bilayer and was proposed to facilitate the interaction of ARFGAP1 with the membrane [180]. Furthermore, lipid packing can influence the catalytic activity of ARFGAP1 [181]. Here, smaller liposomes (50 nm in diameter) supported ARFGAP1-catalyzed ARF1-GTP hydrolysis and COPI-coat disassembly much more efficiently than larger liposomes (150 nm) [181]. The region of

ARFGAP1 that senses membrane curvature was narrowed down to a stretch of 40 amino acids, termed the ARFGAP1 lipid packing sensor motif (ALPS) [182]. This motif is located to the central region of ARFGAP1 (see Figure 4) and consists of a number of hydrophobic residues, thought to be unstructured in solution but form an amphipatic helix upon binding to small liposomes [182]. Subsequently, a second ALPS motif with similar properties was discovered in ARFGAP1 that further contributes to this effect [183]. Residues of these two motifs (ALPS1 and ALPS2) also contribute to Golgi localization in combination with other regions of the protein [163].

In this way the ALPS motifs are able to 'sense' the mismatching of the shape of the lipids and the membrane curvature to insert hydrophobic residues of the ALPS motifs between the loosely packed lipids for efficient binding. The nature of the ALPS motifs suggests an elegant mechanism for regulation of coat dissociation during vesicle formation [175, 184]. In this model, the tight lipid packing that exists in a flat membrane or in the base of the bud keeps ARFGAP1 in low activity within the COPI coat and thus protects it from premature dissociation. The membrane is deformed into a bud and subsequently into a vesicle by ARF1 and coatomer (see section 5 and Figure 6). The highly curved membrane of the vesicle activates ARFGAP1 (by way of the ALPS switch). This would ensure efficient removal of the coat and subsequently complete uncoating upon vesicle fission.

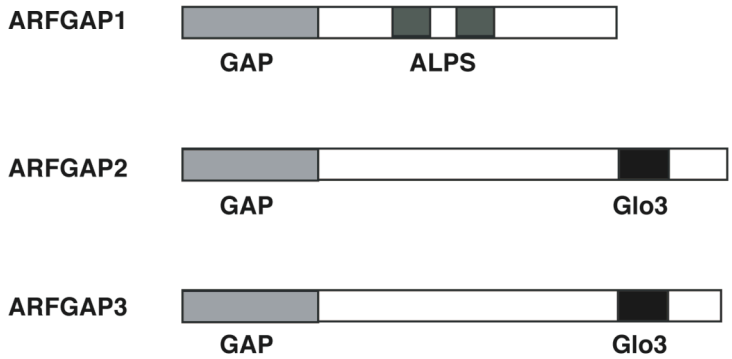

Figure 4. The ARFGAPs investigated in this thesis. In addition to the ARFGAP domain, ARFGAP1 carries two ALPS stretches in the central region. The defining motif of ARFGAP2 and ARFGAP3 is the Glo3 motif of still unknown function. GAP = ARFGAP domain, ALPS = ARFGAP1 lipid-packing sensor, Glo3 = Glo3 motif.

Another important line of investigation has been *in vivo* studies measuring the dynamic association of GFP-tagged COPI coat components with the Golgi membrane [185]. A main conclusion of these studies has been that the core COPI components (ARF1, coatomer and ARFGAP1) associate with the membrane partially independently of each other. In support of this, ARF1 and coatomer was found to associate with the Golgi with different residence times as measured by FRAP [186, 187]. A model was proposed that suggested that coatomer is recruited to the Golgi by ARF1-GTP but remains on the membrane after GTP hydrolysis (and ARF1 dissociation) in a meta-stable coat lattice that is able to generate a vesicle. ARF1-independent association was suggested by a study of ARFGAP1-YFP dynamics on the membrane (by localizing to the Golgi in the presence of BFA) [165]. ARFGAP1 would therefore become associated with ARF1-GTP and coatomer after their association with cargo for inclusion into the COPI coat lattice. A caveat of these observations is the fact that ARF1 has multiple roles on the Golgi membrane and that strict coupling between the Golgi-localization of ARF1 and coatomer may still exist in a subset of ARF1 molecules. Moreover, the slow cytosolic diffusion of coatomer (see section 6 and Paper IV) will cause diffusion-limited binding kinetics to the Golgi membrane and will be observed as a two-fold slower binding rate when analyzed by FRAP which could explain the different residence times for ARF1 and coatomer [187]. In addition, the observation that ARFGAP1-YFP is able to localize to the Golgi membrane in the presence of BFA is contrast to the original observation that endogenous ARFGAP1 is sensitive to this treatment [157]. Therefore, the exact nature of uncoupled kinetics as well as its consequences for COPI vesicle formation is still a matter of some debate (see Paper II).

The Glo3 ARFGAPs

Another subfamily of ARFGAPs has been linked to COPI vesicle formation. This group, the ARFGAP2 subfamily, consists of two members, ARFGAP2 and ARFGAP3 [167]. In similarity to ARFGAP1 they carry their ARFGAP domain at their N-terminus but lack apparent ALPS motifs (see Figure 4). ARFGAP2 was identified as a zinc finger protein of 521 amino acids with unknown function in mouse mammary epithelial cells [188]. ARFGAP3 was identified as a protein of 516 amino acids with a predicted ARFGAP domain and sequence similarity to rat ARFGAP1 [189]. Subsequently, ARFGAP3 was demonstrated to localize to the juxtanuclear region and catalyze GTP hydrolysis on ARF1 *in vitro* [190]. The identification of a motif consisting of two repeats of 15 amino acids separated by some 20 amino acids, termed the Glo3-motif, enabled the classification of ARFGAP2 and ARFGAP3 into a separate subfamily of ARFGAPs together with those of other species [191, 192]. In mammalian cells, ARFGAP2 interacts strongly with γ-COP for the localization to the Golgi membrane and both ARFGAP2 and ARFGAP3 co-localize with coatomer on the Golgi apparatus as well as pre-Golgi structures [191, 193]. Vesicles generated from purified Golgi membranes *in vitro* contained higher amounts of ARFGAP2 and 3 than of ARFGAP1, suggesting that they could be a more prominent component of the coat if GTP hydrolysis is blocked [191]. In the living cell however, all three ARFGAPs are likely to be required as only triple knockdown (and not single or double) is lethal [191].

A similar situation of overlapping ARFGAP function exists in yeast where the two ARFGAPs Gcs1p (the yeast orthologue of mammalian ARFGAP1) and Glo3p provide essential and overlapping functions for retrograde transport [194, 195]. Glo3p has been shown to give a stronger and more direct contribution to COPI vesicle formation. The single deletion of Glo3p in yeast leads to severe structural phenotypes on the secretory pathway and impairment in the retrieval of K(X)KXX-tagged proteins to the ER [194, 196]. Furthermore, Glo3 binds to coatomer both *in vitro* and *in vivo* in contrast to Gcs1p [197, 198]. The binding site between Glo3p and Sec21 (yeast γ-COP) appears to be conserved from yeast to human [193]. Most significantly, it appears that Glo3p is essential for generation of COPI vesicles from yeast Golgi membranes. This function depends on the recruitment of Glo3p to the membrane by a member of the p24 family [197, 199]. Whether or not all of these characteristics extend to ARFGAP2 and ARFGAP3 in mammalian cells remains to be determined.

4. COPI vesicle consumption

Following the fission and uncoating events the vesicle is poised to deliver its cargo to the target membrane (see Figure 2). Consumption requires the tethering, docking and fusion of uncoated vesicles.

4.1 Tethering

Tethering describes the initial interaction between a vesicle and a target membrane that precedes the merging of the two membranes (fusion). The concept of vesicle tethering in the early secretory pathway was suggested by the observations of apparent protein strings (tethers) between the cisternae of the Golgi stack and vesicles on EM examinations [200-202].

Tethering is mediated in part by the activity of the Rab proteins, a large subfamily of the Ras-like GTPases with some 60 members in mammals [77]. Like the members of the ARF-family, the Rabs cycle between cytosolic- and membrane-bound forms. Rabs associate strongly with membrane through their prenylated C-termini that contain the information necessary for Rab targeting [203]. Following the activation by specific RabGEFs, Rab-GTP interacts with proteins (termed Rab effectors) to mediate various downstream events which are terminated by the catalytical actions of RabGAPs [204]. Rabs play important roles in many aspects of vesicle formation and consumption and several members are localized to the membranes of the early secretory pathway where at least one (Rab1) is essential for ER-to-Golgi transport. Rab1 regulates the association of several different tethering factors.

An important group of Rab effectors are the Golgins, which are a diverse group of peripheral and integral Golgi membrane proteins with extensive coiled-coil domains [205, 206]. These proteins contribute to vesicle tethering events and to regulation of the stacking of Golgi cisternae for

maintaining normal Golgi structure. One of the most well-characterized members of this family is the peripheral membrane protein p115, identified as a factor essential for intra-Golgi transport and later determined to localize to both the ERGIC and the *cis*-Golgi [207]. At the *cis*-Golgi, p115 has been proposed to tether COPI vesicle together in concert with other members of the Golgin family, GM130 and giantin [208]. These latter two Golgins are both Rab1 effectors that localize to *cis*-Golgi membranes and are also capable of interacting with p115. In contrast to GM130, which is a peripheral membrane protein mostly associated with the membrane, Giantin is an integral membrane protein identified in COPI vesicles [206, 209]. It was proposed that COPI vesicles would become tethered by a bridging mechanism where the vesicle-associated giantin would bind to p115 that in turn would bind to the Golgi membrane by interacting with GM130 [208]. More recently, this model has been questioned as it has been demonstrated that GM130 and giantin bind to the same site on p115 [210]. These two interactions with p115 may therefore represent two different tethering events. Another Golgin-mediated vesicle tethering event relevant for COPI vesicle consumption was recently described [49]. Golgin-84, a transmembrane coiled-coil protein localized to the *cis*-Golgi, is a Rab1 effector and crucial for maintaining Golgi structure [211]. It localizes primarily to COPI vesicles and suggested to mediate the tethering of these retrograde intra-Golgi COPI vesicles (enriched in glycosylation enzymes) to the CGN by binding to the membrane-localized Golgin CASP [49].

The incorporation of appropriate tethering factors may also be important during vesicle formation. Interestingly, Rab1 modulates the association of coatomer with the Golgi membrane [212]. This is mediated by a direct interaction between Rab1 and GBF1, suggesting that it may serve to stabilize ARF1 on the membrane for COPI vesicle formation [213]. Furthermore, GBF1 is able to interact with p115 directly on the Golgi membrane [214]. Rab1 may therefore be an essential component in coordinating COPI vesicle formation (by recruiting GBF1) and downstream tethering and fusion events (by incorporating tethering factors).

The second large group of tethering factors consists of large oligomeric protein complexes linked to different trafficking steps [206, 215, 216]. Several of these complexes have been implicated in tethering of vesicles in the early secretory pathway. The Dsl1 complex is located to the ER membrane where it may regulate the tethering of COPI vesicles derived from the Golgi as suggested by studies in yeast. The TRAPP complex comes in two forms which both associate with the Golgi complex where they mediate tethering of COPII vesicles and possibly COPI vesicles [217]. The conserved oligomeric Golgi (COG) complex is thought to be especially important for retrograde transport within the Golgi complex [218]. Certain integral membrane proteins that recycle within the Golgi stack are localized through the combined function of the COG and COPI complexes [219]. In this view, the COG complex acts to tether COPI vesicles to Golgi cisternae during retrograde transport [220]. Strikingly, knocking down COG subunits causes accumulation of non-tethered vesicles around the Golgi apparatus [221].

4.2 Docking and fusion

Once a vesicle is tethered with the correct target membrane, a tighter and more stable interaction (docking) occurs before the two bilayers merge (fusion) to deliver the vesicle cargo to the donor membrane (see Figure 2). The key proteins responsible for vesicle docking and fusion are the ‘soluble NSF attachment protein receptors’ (SNAREs), a family of small (100-300 amino acids) proteins with some 36 members in mammals [222]. The conserved SNARE motif, a stretch of 60-70 amino acids, is the hallmark of these proteins. For most SNAREs, a single transmembrane domain on the C-terminus and more variable N-terminal domains flank the SNARE motif. For docking and fusion, closer membrane contact promotes the formation of a *trans*-SNARE complex consisting of four SNARE motifs contributed by SNAREs in the vesicle and the target membrane. Therefore, SNAREs can be classified functionally based on their presence in the vesicle (v-SNARE usually consisting of one polypeptide) or in the presence in the target membrane (t-SNARE consisting of two or three polypeptides). Following fusion, v-SNAREs and t-SNAREs reside together in the acceptor membrane as an inactive *cis*-SNARE complex [222].

A more unambiguous classification of SNAREs is based on the presence of an arginine or glutamine residue (R or Q, respectively) at a key position within the SNARE motif. The Q-SNAREs are further subdivided into Qa-, Qb- and Qc-SNAREs, respectively. In our current understanding of the assembly of the *trans*-SNARE complex, one member of each subfamily (Qa, Qb, Qc and R) contributes a single

SNARE motif. These motifs are unstructured in solution but assemble into a stable parallel bundle of four intertwined, parallel helixes. This core complex is an elongated coiled-coil that is stabilized by 15 hydrophobic layers of interacting side chains of the SNARE motifs. According to the 'zippering' hypothesis, the assembly of the *trans*-SNARE complex starts at the N-termini of the SNAREs and continues towards the C-terminal transmembrane region and that this provides the mechanical force that overcomes the energy barrier for fusion. The inactive *cis*-SNARE complex is subsequently disassembled by the combined efforts of two essential SNARE-regulators, the N-ethylmaleimide sensitive factor (NSF) and the soluble NSF attachment protein (α-SNAP). To achieve this, three molecules of α-SNAP bind to the *cis*-SNARE complex. In turn NSF binds and provides the metabolic energy necessary for disassembly by hydrolyzing ATP. The free SNAREs are then available for recycling by retrograde transport (for the v-SNARE) or for the generation of a new t-SNARE in the same membrane [222-225].

The original SNARE hypothesis stated that the specific pairing of certain 'cognate' SNAREs provided the specificity in vesicular transport. Since then this has been modified to account for observations demonstrating that SNAREs can functionally replace each other *in vivo* (by combining different Qa-, Qb-, Qc- and R-SNAREs) and that a single SNARE can be involved in several transport steps [223]. Nonetheless, specific SNARE complexes have been invoked in COPI vesicle fusion. The *trans*-SNARE complex that mediates fusion of retrograde COPI vesicles with ER has been proposed to consist of the Syntaxin18 (Qa), Sec20 (Qb), Slt1 (Qc) and Sec22b (R) SNAREs. Analysis in both yeast and mammalian cells suggest that Sec22b is the relevant v-SNARE in this retrograde pathway [226, 227]. In intra-Golgi transport, a complex consisting of the Syntaxin5 (Qa), GS28 (Qb), GS15 (Qc) and Ykt6 (R) SNAREs may mediate the fusion of COPI vesicles with Golgi cisternae [228, 229]. Studies in yeast suggest that GS15 may perform the v-SNARE function in this complex [229]. A second complex consisting of Syntaxin5 (Qa), GS27 (Qb), Bet1 (Qc) and Sec22b (R) may also be involved in transport within the Golgi stack [230].

The v-SNAREs are incorporated into vesicles as cargo (for recycling) or as functional molecules (for fusion) althpugh they lack classical sorting motifs. SNAREs have been demonstrated to interact with coatomer subunits and ARF1, and in this respect could function as membrane receptors for the spatial regulation of vesicle formation [91, 171, 228]. In yeast, the ARFGAPs Gcs1p and Glo3p have been proposed to modify the conformation of v-SNAREs for recruitment of ARF1 onto membranes [231]. This was recently extended to t-SNAREs suggesting a novel role for ARFGAPs in regulating SNARE-complex formation [232]. In addition, tethering factors, such as the COG complex, have been shown to interact with several intra-Golgi SNAREs and could represent another level of SNARE regulation [225]. A striking example of this is p115 which has been demonstrated to catalyze the specific assembly of the trans-SNARE complexes that are necessary for COPI vesicle docking and fusion [233].

5. The role of the lipid bilayer in COPI function

During the formation of a transport vesicle, different degrees of positive and negative curvature are generated from a donor membrane (Figure 5). The early stages of budding produce a membrane bud with a dome-shape of almost entirely positive curvature. The continued invagination of the bud generates a vesicle that is ready to bud off and this has a more complex membrane topology. The body of the vesicle is still dome-shaped and positively curved but the region between the bud neck and the dome has a concave shape (negative curvature). The neck itself has regions of both positive as well as zero curvature (flat membrane). As the neck of the bud is constricted and eventually severed (the actual fission event), a vesicle of entirely positive membrane curvature and separated from the donor membrane is generated [234].

The bud-neck severing step (fission) requires bending the membrane and close positioning (a few nanometers) of the luminal leaflets. The lipid bilayer is stabilized by the hydrophobic effect which glues the leaflets of the bilayer together and makes stretching of the membrane difficult [235]. Membrane bending for the generation of a spherical transport vesicle therefore requires the input of energy, which is supplied primarily by proteins. Peripheral membrane proteins can contribute to membrane bending by acting as scaffolding proteins that assemble on the membrane. Such proteins must expose a curved interaction surface toward the membrane and must possess sufficient rigidity to

counteract the tendency of the membrane to resume its original shape [236]. It must also be able to interact with the polar head groups of the membrane lipids to accommodate the bilayer in its binding groove. Several families of proteins are thought to have these characteristics including members of the dynamin family, coat proteins, caveolin and proteins with the so called "Bin, amphiphysin, Rvs" (BAR) domain. A second mechanism for introducing membrane curvature by proteins is by active helix insertion into the membrane. By inserting sidechains into the bilayer, this 'local spontaneous curvature' mechanism generates curvature by displacing the lipid headgroups and reorienting the lipid acyl chains to favor higher curvature.

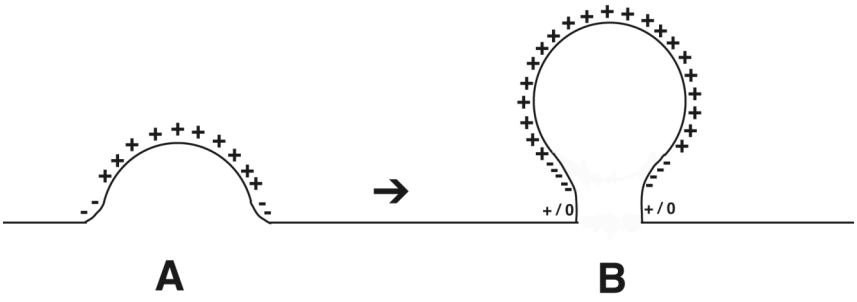

Figure 5. Membrane curvature during vesicle formation. Different degrees of membrane curvature are generated during vesicle formation. (A) The curvature of the initial bud has mostly positive curvature. (B) A later-stage budding vesicle has a more complex membrane topology with positive curvature around the body of the vesicle. Between the neck and the vesicle there is negative curvature and the neck-region has both positive and zero curvature.

Lipids can also make contributions to membrane curvature through a number of mechanisms [234, 237, 238]. Primarily, as specific lipids serve as attachment sites for peripheral membrane proteins, modifications of the lipid composition can facilitate recruitment of proteins involved in membrane bending (e.g., ARF1 and coatomer). Second, the generation of an area difference (asymmetry) between the two monolayers, by energy-driven translocation of lipids, will result in immediate membrane bending (the bilayer-couple mechanism). A third mechanism occurs by transbilayer curvature asymmetry and depends on the presence of different lipid species in the two monolayers, Each monolayer will have a characteristic spontaneous curvature depending on the type of lipid that is included [235]. The ability of certain lipids to affect the spontaneous curvature of a monolayer depends on its geometrical shape. Type I lipids (such as the lysophospholipids), have an inverted cone shape whereas the type II lipids (such as DAG) are cone-shaped (see Figure 6). In contrast, lipids like phosphatidylcholine (PC) have a cylindrical shape [237]. By manipulation the relative abundance of cone-shaped or inverted cone-shaped lipids between the two monolayers, negative or positive curvature may be induced and thus facilitate membrane bending. Based on energetic considerations, however, lipid mechanisms alone would not be sufficient for generating a transport vesicle [236]. In a more likely scenario, therefore, the role of lipids may be to produce a permissive environment for membrane curvature by recruiting the proteins that deform the membrane and to reduce the energy needed for membrane bending.

The membrane bending that is generated by the combined actions of ARF1 (by helix insertion) and coatomer (by a scaffolding mechanism) is sufficient to generate COPI-coated vesicle from synthetic liposomes *in vitro* [126]. Additional factors may assist in the fission process *in vivo*, however. The addition of a long-chain acyl, palmitoyl-CoA, to Golgi membranes pre-incubated with ARF1 and coatomer stimulates fission of COPI-coated vesicles *in vitro* [239]. A similar approach suggests that ARFGAP1 may also serve a crucial role in driving fission [152, 171]. More recently, a member of the C terminal-binding protein (CtBP) family has been suggested to contribute to the constriction of the membrane neck-region during fission in several trafficking steps and for mitotic Golgi partitioning [240]. The Brefeldin-A ADP-ribosylated substrate (BARS) protein was required for COPI vesicle formation from stringently washed Golgi membranes [241]. Interestingly, BARS may regulate vesicle fission through interactions with both palmitoyl-CoA and ARFGAP1, although the mechanism by which this occurs is not clear.

Figure 6. Geometrical shape of lipids. Lipids can be classified based on the geometrical shapes. The cylindrical lipids (e.g. phosphatidylcholine) are also referred to as bilayer-preffering lipids. Type I lipids (e.g. lysophospholipids) have an inverted cone-shape whereas the type II lipids (e.g. diacylglycerol) are cone-shaped. The relative abundance of the inner and outer monolayer of such lipids may influence the curvature of the lipid bilayer.

In addition to regulating the association of several coat proteins with the Golgi, ARF1 also controls a number of lipid-modifying enzymes. Phospholipase D (PLD) enzymes are activated by the members of the ARF family and catalyze the hydrolysis of phosphatidylcholine (PC) to generate choline and phosphatidic acid (PA) [242, 243]. The two isoforms of PLD (PLD1 and PLD2) both localize to the Golgi membrane where PLD2 has been demonstrated to be enriched at the cisternal rims [244]. The formation of COPI vesicles from Golgi membranes is stimulated by PLD *in vitro* by facilitating coatomer recruitment to the membrane [245]. Both ARF1 and coatomer can bind to PA individually and the generation of PA at the budding site could stimulate the interaction with the membrane to facilitate vesicle formation [246]. Recently, PA generated via PLD2 was suggested to interact with BARS and could in this way stimulate vesicle fission [247].

Phosphatidic acid also serves a role as an important precursor for the synthesis of DAG, which is generated by the removal of the negatively charged phosphate group by phosphatidic acid phosphatases (PAPs) [248]. As a consequence of its small and electrically neutral head group and its strong type II (cone-shaped) nature it is predicted to contribute significantly in facilitating membrane bending [249]. The role of DAG in the secretory pathway has been mainly studied at the level of the TGN where it has been shown to be essential to the recruitment of DAG-binding proteins and for formation of transport intermediates. A characteristic relevant for COPI vesicle formation is the ability of DAG to facilitate the binding of ARFGAP1 to the membrane [180]. More recently, the inhibition of DAG formation was shown to decrease the association of ARFGAP1 with the membrane and inhibit COPI vesicle formation at the level of fission in the Golgi-to-ER retrograde pathway [250].

In addition to activating PLD, ARF1 also recruits kinases for the generation of different species of phosphatidylinositol (PI) on the Golgi membrane [251, 252]. The reversible phosphorylation of the inositol ring of PI at the 3', 4' or 5' positions can generate several different lipids of negative charge. Throughout the cell, the PIs regulate different processes by recruiting proteins through the interaction with modular binding domains such as the pleckstrin homology (PH) domain. The generation of $PI(4,5)P_2$ occurs primarily through sequential phosphorylation of the 4' and the 5' of the inositol ring of PI. In the cell $PI(4,5)_2$ may constitute up to 1% of all phospholipids and is a versatile lipid characterized mainly for its many roles at the plasma membrane in exocytosis, endocytosis and actin polymerization . In the Golgi, PI(4)P is generated from PI by the actions of three PI4 kinases that have been localized to this organelle termed PI4KIIα, PI4KIIIα and PI4KIIIβ. The PI(4)P pool in the Golgi is large and is the result of a extensive PI4 kinase activity in the organelle [253, 254]. Interfering with the activity of PI4KIIIβ perturbs the structure of the Golgi apparatus [252]. The PI(4)P pool regulates the association of several proteins with the TGN, including AP-1, for generation of clathrin-coated vesicles. In addition to these specific roles, PI(4)P is also used as a precursor for the synthesis of $PI(4,5)P_2$. There are three members of the type I PIP5 kinase family (α, β and γ, respectively) that have been identified and localized mainly in the plasma membrane, endosomes, and the nucleus. In the Golgi, ARF1 stimulates the formation of $PI(4,5)P_2$ both directly and indirectly. First, by recruiting the PI4IIIβ kinase and a type I PIP4 5' kinase to the Golgi, ARF1 delivers the relevant enzymes [251, 252]. Second, by activating PLD, ARF1 generates PA which stimulates the same kinases [255]. Third, PLD itself is stimulated by $PI(4,5)P_2$. This generates a positive feedback loop which have been shown to generate large amounts of $PI(4,5)P_2$ on the Golgi [252]. At steady-state, Golgi membranes contain modest levels of $PI(4,5)P_2$ [256]. This can be explained in part by the demonstration that Golgi membranes intrinsically possess a high level of 5' phosphatase activity, resulting in the rapid removal of $PI(4,5)P_2$ [257, 258]. One of the two phosphatases that have been identified in the Golgi, INPP5B, may have a role in ERGIC-to-ER retrograde transport [259]. Nonetheless, this lipid has a critical role in maintaining the stability of the Golgi structure as inhibition of $PI(4,5)P_2$ formation causes the fragmentation and vesicularization of the Golgi [257, 258].

6. Protein diffusion

The recruitment of cytosolic coatomer to membrane-bound ARF1-GTP represents a key step in COPI vesicle formation. In the cytosol, proteins move randomly due to the thermal noise of surrounding molecules, termed Brownian motion or 'diffusion'. To quantify these random particle motions, the mean square distance traveled can be measured [260, 261]. For a collection of diffusing molecules, the square of the average distance traveled is expressed as the mean square displacement (MSD) which grows linearly over time according to the equation

$r(t)^2 = 2*D*t$ (Equation 1)

where D is termed the diffusion coefficient. The diffusion coefficient for a spherical free particle in solution can also be described by the Stokes-Einstein formula

$D = (k*T) / (6*\pi*\eta*R)$ (Equation 2)

where D is the diffusion coefficient, T is the absolute temperature, η is the viscosity of the solution, k is the Boltzmann constant and R is the hydrodynamic radius of the particle. The most critical factor of this equation is the hydrodynamic radius (R), which is determined mostly by protein radius and shape.

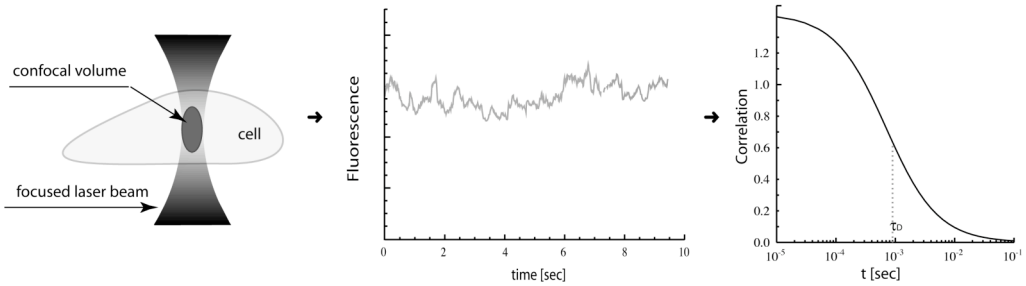

Figure 7. The concept of fluorescence correlation spectroscopy (FCS). (A) A laser beam is focused in a cell that contains fluorescently labeled molecules and the emitted photons are collected by the detectors. (B) Over time, the fluorescence fluctuates around an average value. These fluctuations depend on the diffusion in and out of the confocal volume by labeled molecules. (C) The autocorrelation curve is calculated from the fluctuations and the half-time of the decay is related to the mobility of the particle, making it possible to derive a diffusion coefficient.

The diffusion coefficient of proteins tagged with a fluorescent protein, such as the green fluorescent protein (GFP) can be determined using fluorescence correlation spectroscopy (FCS) carried out with a confocal microscope [262-264]. For FCS, a laser beam is focused in a cell and the pinhole of the confocal microscope is used to remove out-of-focus light. This creates a very small confocal detection volume of less than $1\mu m^3$ from which photons are collected (see Figure 7A). As fluorescent proteins diffuse in and out of the volume this results in a fluorescent signal that fluctuates around an average value (see Figure 7B). These fluctuations reflect the number of molecules in the confocal volume and the average time of diffusion for each molecule across the confocal volume, which makes it possible to derive both molecular concentrations and diffusion coefficients. In order to extract the information from the fluorescence data, the so-called autocorrelation function is calculated. This mathematical procedure calculates the self-similarity of the fluorescence curve and is defined as

$G(\tau) = \langle \delta F(t)* \delta F(t+\tau) \rangle / \langle F(t) \rangle^2$ (Equation 3)

where δF is the deviation of fluorescence from the mean [262-264]. The resulting autocorrelation curve (see Figure 7C and Paper IV for examples) describes the decreasing probability that a particle remains inside the confocal volume after a certain time point. This experimental data is subsequently fitted with a theoretically derived formula for the appropriate type of diffusion studied (e.g., free 3D diffusion in the cytoplasm). By doing this, one obtains the time point τ_D, when the autocorrelation curve has dropped to half its value. This value is in turn inversely proportional to the diffusion coefficient D according to the following equation:

$\tau_D = r^2/4D$ (Equation 4)

where r is the radius of the confocal volume. In addition, the mean number of particles in the confocal volume can be calculated from the amplitude $G(\tau=0)$. The exact functional form of the autocorrelation function is determined by the dynamic process studied. For example, free 3D diffusion has an autocorrelation function of the form:

$$G(\tau) = G(0)\frac{1}{(1+\tau/\tau_D)(1+a^{-2}(\tau/\tau_D)^2} \quad \text{(Equation 5)}$$

where a is the elongation of the confocal volume along the optical axis. Active transport along one direction gives:

$$G(\tau) = G(0)e^{-\left(\frac{\tau \cdot v}{r}\right)^2} \quad \text{(Equation 6)}$$

where v is the transport speed, and r the diameter of the confocal volume.

Fitting the experimental curve to those theoretically derived functions can therefore not only provide information about the speed of the diffusion of a given protein (and therefore about its effective size) but also about the nature of its mode of movement. If the same molecule participates in several dynamic processes, the resulting autocorrelation curve will be a superposition of the respective functions. A two or more component fitting procedure can reveal the presence of those processes and allow for the determination of the proportion of the molecules undergoing the respective mode of transport.

A complicating factor in assessing protein diffusion in cells is that the cytoplasm is not a simple water-like buffer solution but rather a highly complex mixture of dissolved macromolecules (protein, RNA and DNA), higher order structures (components of the cytoskeleton) and organelles [265]. In the environment of the cell, diffusion is therefore far from the ideal situation expressed in equation 1 and 2. One example of this is that as proteins diffuse in the cytosol, the presence of macromolecules will impair their normal random motions by restricting the space that is available. This phenomenon is termed molecular crowding. This will cause the MSD to grow more slowly over time than during normal diffusion as the MSD becomes proportional to t^{α}:

$r(t)^2 = 2*D*t^{\alpha}$ (Equation 7)

where $\alpha < 1$. This is referred to as 'anomalous subdiffusion' or subdiffusion [260]. Such deviations from the normal diffusion pattern have been demonstrated in both the membranes and cytoplasm in different model systems. When diffusion is analyzed through FCS, subdiffusion will influence the slope of the autocorrelation curve. Specifically, a lower α-value will yield an autocorrelation function exhibiting a more shallow decay as a function of time compared to free diffusion (see paper IV for examples) [260].

Aims

This thesis has been devoted to investigating the contribution of proteins and lipids to COPI vesicle formation and consumption. Specifically, we have addressed the following issues:

1. The role of local lipid conversion in the early steps of vesicle formation (bud formation and fission).

2. The function of the recently described ARFGAP2 and ARFGAP3 in the generation of the COPI coat lattice in cells.

3. The mechanism by which COPI vesicles dock and fuse with Golgi cisternae for intra-Golgi retrograde transport.

4. The effect of the highly crowded nature of the cytoplasm on diffusion.

Results and discussion

Paper I

Early stages of Golgi vesicle and tubule formation require diacylglycerol

The recruitment of cytosolic coatomer by ARF1 is the key step for both cargo incorporation as well as deformation of the membrane. The function of ARF1 in regulating lipid metabolism in the Golgi and its consequences for vesicle formation is less clear but may contribute (see section 5). In this paper, our aim was to investigate the role for DAG in peri-Golgi bud formation and vesicle fission, *in vivo*.

To perturb the levels of DAG in the Golgi membrane we added the pharmaceutical agent Propranolol (proPr), which inhibits the conversion of PA to DAG by inhibiting the activity of a phosphatidate phosphohydrolase (PAP) enzyme. By analyzing the binding of ARFGAP1 to Golgi membranes upon addition of cytosol, we demonstrate that the relevant PAP activity inhibited by proPr resides in the cytosol (Fig. 1A). The addition of 300 µM proPr to cells for 3 minutes caused a rapid and reversible dissociation of the entire pool of over-expressed ARFGAP1-GFP as well as the endogenous protein from the Golgi (Fig. 1B-F and Supplemental Fig. 2). Significantly, coatomer remains associated with the Golgi membrane demonstrating a partial impairment of the COPI machinery. We found that the addition of lower concentrations of proPr (60 µM) was less effective in dissociating ARFGAP1-EGFP from the Golgi membrane with some 25% remaining even after longer treatments (Fig. 1B). To be able to elucidate the effects of proPr-induced (and complete) dissociation of ARFGAP1 we therefore employed the higher concentration in all subsequent experiments.

To examine the effect of proPr treatment on the formation of peri-Golgi vesicles, we examined the appearance of the Golgi apparatus on the ultra structural level by EM and quantified the levels of buds and vesicular-tubular-profiles (VTPs) in the Golgi area (Fig. 2). The addition of proPr to cells resulted in smooth cisternal membranes lacking both membrane buds as well as associated VTPs consistent with a block in bud and subsequent vesicle formation (Fig. 2B and E). Strikingly, the removal of proPr resulted in a dramatic increase in membrane buds as well as VTPs, which also caused consumption of the cisternae (Fig. 2C and 2F). We examined thick plastic sections of treated cells followed by tomography (Fig. 3). The reconstruction of representative areas of Golgi treated with proPr (Fig. 3A, middle) and samples examined 2 minutes after the removal of proPr (Fig. 3A, right) showed similar differences in the levels of buds, vesicles and tubules to those obtained by quantification of thin slices. This confirms that the VTPs that are generated through the washout of proPr are genuine vesicle profiles.

This suggested that there is a requirement for PA-derived DAG in peri-Golgi bud formation leading to the generation of vesicles and tubules. However, based on these results it was still possible that this corresponded to the concurrent loss of ARFGAP1 from the membrane rather than through a specific effect on DAG levels. To characterize the role of ARFGAP1 function in bud and VTP formation in more detail we made use of the dramatic increase of Golgi-associated buds and VTPs occurring upon the removal of proPr from treated cells (Fig. 2C and G). We tested for the role of ARFGAP1 by transfecting cells with an siRNA against ARFGAP1 resulting in efficient knockdown of ARFGAP1 but unperturbed levels of coatomer (Supplementary Fig. 5). Interestingly, the cells transfected with siRNA against ARFGAP1 showed a marked increase in associated buds (Fig. 5A and quantification in 5D) with a simultaneous decrease in VTPs compared to that of mock-transfected cells. This is significant as it suggested an impairment of vesicle fission as a result of lowering the endogenous ARFGAP1 level. An even more pronounced effect on vesicle fission was seen upon proPr addition followed by washout to knocked-down cells. As in the case for non-transfected cells the addition of proPr resulted in a clear decrease in buds and VTP associated with the Golgi (Fig. 5B and D). Strikingly, after removal of proPr for 2 minutes the cells lacking ARFGAP1 demonstrated a drastic increase in membrane buds over that of VTPs compared to that of mock-transfected cells (for quantification see Fig. 5D). Cells lacking ARFGAP1 also exhibited fewer associated VTPs suggesting that the fission was unable to complete under these condition.

A previous study has used a similar approach in identifying a role for DAG in peri-Golgi vesicle formation for transport in the Golgi-to-ER retrograde pathway. A key difference between that study

and the one presented here is that we use a higher concentration of proPr (300 μM vs 60 μM). By utilizing this more stringent treatment, we are able to demonstrate that there are two distinct steps of COPI vesicle formation with different lipid and protein requirements.

The first step, bud formation, requires conversion of PA into DAG but not the ARFGAP1 protein as buds are readily formed in cells lacking ARFGAP1. What would be the role for DAG in bud formation? The geometrical shape of DAG is that of a cone-shaped lipid which strongly favors negative curvature upon addition into a monolayer. The introduction of DAG into a bilayer would therefore facilitate the formation of negative curvature in the vesicle bud-neck region and decrease the energy barrier for membrane deformation. Theoretical modeling suggests that DAG, rather than the precursor PA would be more efficient in this aspect [249]. The ability of DAG to readily flip-flop across the membrane bilayer could localize it to the luminal leaflet of the vesicle as well. It remains to be demonstrated where this pool of DAG is located during vesicle formation, however.

The Golgi-localized pool of ARFGAP1 is extremely sensitive to perturbations of DAG synthesis as demonstrated in this paper. Therefore a crucial role for DAG would be to enable ARFGAP1 binding for its role(s) within the coat lattice. In this paper, we find that one of these roles is at a late stage of fission. Interestingly, such a role for ARFGAP1 was suggested by employing an *in vitro* assay that monitored the release of COPI-coated vesicles upon the addition of ARFGAP1 to Golgi membranes pre-incubated with ARF1 and coatomer [152]. The exact mechanism by which ARFGAP1 would promote fission is still unclear and remains to be determined. The BARS-50 protein is involved in constriction of the bud neck for COPI vesicle fission and it interacts with ARFGAP1. However, whereas BARS-50 seems to be restricted to the bud-neck region, ARFGAP1 is present over the entire bud [241]. An alternative hypothesis is that ARFGAP1-catalyzed coat dissociation on the bud promotes fission. The presence of ARFGAP1 at the tip of the bud, where the catalytical activity is high (see section 3.3) may point in this direction. A catalytically inactive mutant of ARFGAP1 was incapable of stimulating vesicle fission in the two-step *in vitro* assay, which is consistent with such a role [171].

Paper II

ARFGAP2 and ARFGAP3 are necessary for the formation of the COPI coat lattice on the Golgi membrane of living cells

In order to fully understand the mechanisms that lead to the formation of a COPI vesicle, it is necessary to have a complete account of the factors that are involved in this process. The role of ARFGAP1 has been extensively investigated but remains controversial. Therefore, the identification of a new class of ARFGAPs with a link to COPI function (see section 3.3) represents an important development. In this study, our aim was to investigate the function of the human ARFGAP2 and ARFGAP3 proteins in cells and to compare that to the function of the better characterized ARFGAP1.

We generated fluorescent protein constructs as well as affinity purified antibodies in order to be able to monitor the characteristics and dynamic behaviour of these proteins in cells. We find that endogenous ARFGAP2 and ARFGAP3 localize primarily to the *cis*-Golgi through its extensive co-localization with coatomer, a result similar to that demonstrated by another group [191]. At the Golgi membrane, the association of ARFGAP2 and ARFGAP3 was found to be dependent on ARF-GTP as the addition of BFA resulted in rapid (<1 minute) redistribution to the cytosol (Fig. 1). A recent study demonstrated that ARFGAP1-YFP associates with the Golgi independently of ARF1-GTP [165]. In light of this, the clear effect of BFA presented here is especially important as it demonstrates that ARFGAP2 and ARFGAP3 do not attach to the membrane in anticipation of, but rather as a response to ARF1 activation. In addition, we could not verify the persistence of ARFGAP1 on the Golgi in the presence of BFA (discussed below).

Next, we examined the effect of promoting ARF function, which we accomplished through the use of the compound aluminum fluoride (AlF), trapping ARF1 in an active state. To establish the effect of AlF in cells, we added the compound and analyzed the morphology of the Golgi by EM (Fig. 2). In treated cells, we found a massive accumulation of vesicles surrounding the cisternae of the Golgi stack in responses to AlF. We then examined the effect of AlF on the association of the three ARFGAPs with the Golgi membrane and compared it to coatomer by quantifying the Golgi levels. To our surprise, we found that both ARFGAP2 and ARFGAP3, together with coatomer, accumulated (an estimated 40-50%) upon the addition of AlF but the levels of ARFGAP1 did not change significantly (Fig. 3). The effect on coatomer association by AlF had been noted before but only quantified *in vitro* and not in cells [266, 267]. This suggests that upon AlF-induced vesicle budding ARFGAP2 and ARFGAP3 are recruited to the coat of vesicles whereas ARFGAP1 was excluded. This results are in agreement with those published by Duden and co-workers who demonstrated that vesicles generated under conditions of blocked GTP hydrolysis *in vitro* contain ARFGAP2 and ARFGAP3 but very little ARFGAP1 [191].

As AlF could mediate its effect through heterotrimeric G proteins, we tested if the expression of GTP-restricted ARF1-Q71L would cause the permanent recruitment of ARGFAP2 and ARFGAP3 to the Golgi membranes. We show that expression of GTP-restricted ARF1, but not wild-type ARF1, prevented the effect of BFA on the Golgi localization of ARFGAP2 and ARFGAP3 in a significant portion of cells. This suggests that the ARFGAPs were incorporated into a BFA-insensitive complex with ARF1-Q71L (Fig. 5).

In previous studies, the addition of AlF to cells was combined with FRAP analysis and this was shown to immobilize the entire pool of coatomer on the Golgi [186, 187]. The effect of AlF on ARFGAP2 and ARFGAP3 suggested that they could become recruited to a complex with coatomer on the membrane. To test this, we expressed the GFP-tagged versions in cells and analyzed their binding properties to the Golgi membrane in untreated and perturbed cells as has been done for other components of the COPI coat. Strikingly, ARFGAP2 and ARFGAP3 became immobilized on the Golgi, almost to the same extent as coatomer after the addition of AlF (Fig. 6). This is different to the effect on ARFGAP1, which is affected much less. Although we do see an immobilization of a smaller component of ARFGAP1 (20%), the effect was clearly more pronounced for ARFGAP2 and ARFGAP3 (50-60%) and coatomer (more than 60%).

Through the previously demonstrated interactions with coatomer subunits, these ARFGAPs could be incorporated into the COPI coat lattice. A higher concentration of these ARFGAPs in this lattice

predicts a more crucial role in its generation than compared with ARFGAP1. To test such a role, we used a knockdown approach that efficiently reduced the expression of the ARFGAPs (Fig. 7). In cells treated with siRNA for the knockdown of one or two ARFGAPs we assayed the ability of AlF to generate a BFA-resistant pool of coatomer [266]. We found that control knockdown or single knockdown of any of the ARFGAPs did not affect this ability. Interestingly, only the double knockdown of ARFGAP2 and ARFGAP3 caused a significant portion of coatomer to become cytosolic upon AlF-BFA treatment (Fig. 8). No combinations with ARFGAP1 showed this effect. This suggests that the ARFGAP2 and ARFGAP3 pair performs an overlapping function that is distinct from that of ARFGAP1 and is essential for vesicle formation.

Based on current evidence presented here and by others we propose that these two ARFGAPs have an essential role in participating in COPI coat lattice formation on the Golgi membrane. Furthermore, we show that this role in cells is overlapping between ARFGAP2 and ARFGAP3 but that it cannot be substituted for by ARFGAP1. It represents a division of labor between these ARFGAPs and could help resolve the puzzling nature of the functional redundancy between ARFGAPs that has been observed both in yeast and mammalian cells [191, 194].

These results raise important concerns regarding the role and function of ARFGAP1 on the Golgi membrane and in vesicle formation. A recently proposed model for the function of ARFGAP1 suggests that it associates with the Golgi membrane in advance of coat lattice formation [165]. This model is challenged by the findings presented here where we observe a clear ARF-GTP dependency in Golgi association for all three ARFGAPs, including ARFGAP1 which was shown previously [157]. An alternative explanation for the partial effect of AlF on the association of ARFGAP1 with the Golgi membrane could be that it is involved in clathrin-coated vesicle formation at the TGN. The effect of AlF may therefore be more specific to the components of the COPI coat.

Paper III

$PI(4,5)P_2$ is necessary for the fusion of COPI vesicle with Golgi cisternae, *in vitro*

The consumption of a transport vesicle entails tethering to the target membrane, followed by the docking and fusion steps (see section 4). By recruiting kinases to the Golgi membrane, ARF1 is able to promote the synthesis of $PI(4,5)P_2$ and this is critical for maintaining Golgi structure (see section 5). As $PI(4,5)P_2$ has been shown to be involved in several different fusion steps in the cell, we aimed to investigate the possible function of $PI(4,5)P_2$ in regulating the fusion of COPI vesicles with Golgi cisternae, *in vitro*.

To study COPI vesicle fusion, we employ an *in vitro* assay for the study of intra-Golgi transport [268]. Golgi membranes from the CHO cell line Lec1, deficient in the N-acetylglucosaminyltransferase-I glycosyltransferase, are isolated from cells infected with the vesicular stomatitis virus (VSV). To complement this glycosylation deficiency, purified Lec1 Golgi membranes are incubated with those derived from wild-type CHO cells, together with cytosol and radiolabeled UDP-N-acetylglucosamine. This assay was demonstrated to register the transfer of GlcNAc-T1 from wild-type membranes to the Lec1 membranes, mediated by COPI vesicles (i.e., an intra-Golgi retrograde transport event) [47, 48]. In order to be able to evaluate this assay more quantitatively, highly purified COPI vesicle generated in an *in vitro* budding assay were titrated to a constant amount of acceptor membrane and the resulting fusion was quantified [269].

A typical response curve, together with its mathematical description $a + c(1-e^{(-bx)})$ is shown in Fig. 1C. In this equation, "a" corresponds to the background signal generated in the absence of vesicles, "b" describes the slope of the curve and "c" is the maximal signal that can be detected. The rate of the fusion reaction depends on the amount of vesicles that is added as well as their ability to fuse, and this influences the slope of the response curve. Therefore, since the slope depends on the number of vesicles that can be detected, or that are apparent, the factor "b" is termed C_v^{app}, for the concentration of vesicles that is apparent. This variable is able to quantify the ability of the vesicles to fuse. A concern about the assay is that the transfer of GlcNAc-T1 to target cisternae was not specific to the COPI-derived vesicles but could also be mediated by other vesicle types derived from the membranes, such as clathrin or COPII vesicles. To address this, we depleted coatomer from the cytosol used for the generation of vesicles and used the resulting vesicle fraction in the fusion assay. Strikingly, this reduced the C_v^{app} more than 10-fold (Fig. 2B) as compared with the control. We conclude that our fusion assay is specific for the fusion of COPI-derived vesicles.

Our next investigation revealed that COPI vesicles gradually lost their ability to fuse over time as determined by a decrease in C_v^{app}, upon incubation at 37°C, suggesting that they are inactivated by an intrinsic mechanism (Fig. 2C). We speculated that this could be due to a decrease in levels of $PI(4,5)P_2$ and tested this possibility. The addition of several inhibitors, including a monoclonal antibody against $PI(4,5)P_2$, affected fusion negatively. To be able to manipulate the levels of $PI(4,5)P_2$ more specifically we purified a PI4P 5-kinase which generated this lipid efficiently on both Golgi membranes and isolated vesicles as judged by mass spectrometry analysis and thin layer chromatography (TLC) (Fig. 3A). The addition of the kinase to the fusion assay directly only produced a mild stimulatory effect (Fig. 3B). However, when allowed to compete with the time-dependent inactivation of vesicles the kinase was found to restore fully the ability of the vesicles to fuse, and this was reflected in the corresponding C_v^{app} values (Fig. 3B and 3C). A prediction of this result is that the addition of a 5' phosphatase would exacerbate the time-dependent inactivation of fusion. To test this we purified the catalytical domain of a 5' phosphatase and found that this was efficient in consuming $PI(4,5)P_2$ on COPI vesicles as judged by TLC experiments (Fig. 4A). Pre-incubation of the vesicles with the phosphatase to caused a concentration-dependent inhibition of C_v^{app} (Fig. 4B). To determine if these two effects could complement each other, vesicles were subjected to a two-step incubation procedure. As predicted, the phosphatase was found to abolish the effect of adding the kinase to vesicles, demonstrating further the influence of $PI(4,5)P_2$ on fusion (Fig. 5).

We observed no effect on fusion upon pre-treatment of target Lec1 membrane with either kinase of phosphatase which suggested to us that the role of $PI(4,5)P_2$ resides only on the surface of COPI vesicles (Fig. 6). There are three possible molecular mechanisms that could mediate $PI(4,5)P_2$-

dependent fusion of COPI vesicles. The role of $PI(4,5)P_2$ could be to: (1) facilitate the SNARE-mediated fusion by regulating their function or (2) through the recruitment of a protein from the cytosol or (3) the target membrane. To test this, a proteinase K (PK) treatment of vesicles was performed, effectively removing most of the cytoplasmic oriented domains of proteins on COPI vesicles including, several SNAREs and tethering factors (Fig. 7). When employed in the fusion assay these vesicles still fused although albeit less efficiently (Fig. 8A), revealing a partial disturbance in fusion. We expected that the removal of so many proteins important for tethering and fusion would produce a more significant effect on fusion and sought to specify this step. Critically, PK-treated vesicles still demonstrated a time-dependent decrease in C_v^{app}, which demonstrated that the $PI(4,5)P_2$ pool still influenced the fusion process (Fig. 9).

During the incubation, vesicles may bind and fuse to Golgi membranes or inactivate (Fig. 1A). To determine if the removal of peripheral membrane proteins by PK affected the binding or fusion step, we performed a dilution experiment of the reaction. By diluting the reaction two-fold the C^v_{app} decreased to 2/3 of the untreated vesicles due to an increased likelihood that the vesicles inactivate before reaching a cisternae [269]. If the PK treatment would affect the binding step then the effect of the dilution reaction should be higher which was confirmed experimentally (Fig. 8B). As the PK treated vesicles still fused in an NSF/α-SNAP dependent manner, their ability to fuse does seem unperturbed, suggesting a block in vesicle binding rather than fusion. This is consistent with the result of the dilution experiment (Fig. 8C and D).

There are a number of fusion events that have already been demonstrated to be influenced by $PI(4,5)P_2$. In yeast, the SNARE-dependent fusion of vacuoles involves $PI(4,5)P_2$ [270]. This is thought to occur by binding an oligomeric tethering factor to $PI(4,5)P_2$ in the membrane which stimulates the formation of the relevant trans-SNARE complex. In mammalian cells, the fusion of large dense core vesicles as well as synaptic vesicles at the plasma membrane is mediated by $PI(4,5)P_2$ which recruits the cytosolic Ca^{2+}-dependent activator protein for secretion (CAPS) proteins for promotion of Ca^{2+}-triggered fusion of docked secretory vesicles [271]. Through what mechanism $PI(4,5)P_2$ regulates the fusion of COPI vesicles is still unclear. Using this experimental system, however, it should be possible to screen for relevant proteins (possibly with a similar role to CAPS). Furthermore, these results point to a role of ARF1 in priming vesicles for fusion by incorporation of factors that are necessary for downstream events.

Paper IV

Anomalous subdiffusion is a measure for cytoplasmic crowding in living cells

In an earlier study, we had observed that the diffusion of coatomer was much slower than expected from its size [187]. This slow diffusion could be due to subdiffusion as a result of either interactions with other components in the cytosol or molecular crowding (see section 6). The subdiffusion makes a protein much slower and therefore more localized which might have important consequences for biological events. To examine this phenomenon, we employed fluorescent dextran particles of different sizes and determined their diffusion in solution by FCS. We compared this diffusion with the naturally crowded solution of the cell and an artificially crowded solution to analyze the effect of such an environment.

In aqueous solution, the dextrans exhibited normal diffusion (i.e. α was approximately 1), as determined by fitting the experimental data with the analytical function that describes subdiffusion (see Fig. 2 for representative curves). Upon the microinjection of the dextrans into cells however, they all exhibited anomalous diffusion to various extents (see Fig. 3 and Table 1). This was not the result of obstruction by higher order structures in the cells. To test this, we perturbed cellular structures (microtubules, actin filaments and the ER) by adding appropriate drugs to the cells. This did not affect the degree of anomalous subdiffusion of the dextrans (see Table 2). This suggested to us that the observed subdiffusion resulted from other mechanisms, such as molecular crowding (see section 6). To reproduce the crowded nature of the cytosol, we analyzed the diffusion of labeled dextrans in a concentrated solution of unlabelled dextrans and determined the degree of subdiffusion (Fig. 5). We observed a similar correlation between the size of the dextrans and the degree of anomalous diffusion as in living cells (Fig. 5). Strikingly, the degree of subdiffusion increased as the level of molecular crowding increased (higher concentration of unlabelled molecules) (Fig. 5). To confirm that molecular crowding and not other inter-molecular forces are causative for the observed anomalous diffusion, we constructed a computer model of a crowded solution. The simulations confirmed our interpretation of the experimental data.

A striking observation both *in vitro* and *in vivo* was that the degree of anomalous subdiffusion was not linear with respect to the size (Table 1 and Fig. 5). The 40 kDa dextran was determined to be more subdiffusive than the 500 kDa molecule. A likely explanation for this is the polymeric nature of dextrans, which can intrinsically contribute to subdiffusion, through a process called reptation. In contrast, larger dextranes are more globular and are more likely to be influenced by molecular crowding alone which is reflected in the higher α-values as a function of molecular crowding (Table 1 and Fig. 5).

The results presented in this paper demonstrate that molecules diffuse in a qualitatively slower rate in the cytosol as compared to in solution. This anomalous subdiffusion can be used as a measure for the cytoplasmic crowding of living cells. How could anomalous subdiffusion contribute to an event like COPI-vesicle formation? Once coatomer has been delivered to the Golgi membrane by long-range diffusion it would stay closer to the membrane as a result of anomalous subdiffusion after being released from the membrane due to hydrolysis by ARF1, which could facilitate its recruitment for vesicle formation making the process more effective.

Concluding remarks

In this thesis, aspects of COPI vesicle formation and consumption are investigated. These aspects are all centered on different roles of ARF1 function. The effects are a result of the activities of ARF1 (on lipid-modifying enzymes that generate DAG or $PI(4,5)P_2$) or by regulating the function of ARF1 (for the ARFGAP proteins). We conclude that:

Paper I

The formation of vesicles from Golgi membranes can be divided into two steps where the first requires the formation of DAG (bud formation) and later stages is dependent on ARFGAP1 (vesicle fission).

Paper II

The recently described ARFGAP2 and ARFGAP3 are more closely linked to coatomer function than ARFGAP1 and are required for the generation of the COPI coat lattice on the Golgi membrane of living cells.

Paper III

The fusion of COPI vesicles with Golgi cisternae is promoted by the presence of $PI(4,5)P_2$ on the vesicle surface. The removal of cytoplasmic domains of vesicle-associated proteins primarily affects tethering/docking.

Paper IV

The macromolecular crowding of the cytosol causes subdiffusion in living cells.

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