Functional characterization of the secretory pathway and the role of COPI vesicles

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ABSTRACT

In the secretory pathway, proteins synthesized in the endoplasmatic reticulum move through the Golgi apparatus in a cis-to-trans direction and then sorts into different types of vesicles at the trans-Golgi network. How intracellular transport takes place has been the focus of cell biology research for a long time. In order to understand how the organelles within the pathway functions it would be very helpful to know all the proteins from which it is composed. However there is no complete and accurate map of the proteins within the organelles of the secretory pathway.

In order to achieve such a cell map, isolated subcellular organelles from rat liver were analyzed by tandem mass spectrometry. Redundant peptide counting was used to determine the relative abundance of proteins in the highly enriched organelle preparations. Peptide counting is based on the idea that when normalized for protein size, abundant proteins will generate more peptides than relatively rare proteins. The method was validated by comparing the results from quantitative western blotting and standard enzyme assays. The study resulted in a quantitative proteomic map of more than 1400 proteins with spatial information and relative presence of each protein of the rough ER, smooth ER and Golgi apparatus. Of the 345 proteins of unknown function discovered the subcellular locations for 230 of these were predicted. Extending the study to biochemical subfractions and organelle subcompartments resulted in the observation that COPI vesicles were enriched with Golgi-resident proteins while largely excluding secretory cargo. This would support a function for COPI vesicles in retrograde transport from trans to cis within the Golgi stack, which is in line with the cisternal maturation model of biosynthetic protein transport.

Coatomer is recruited to Golgi membranes in an ARF1-dependent manner that couples protein sorting to vesicle formation. Using a modified intra-Golgi transport assay with highly purified COPI vesicles, demonstrated that fusion of retrograde-directed vesicles with Golgi membranes requires phosphatidylinositol-(4,5)-bisphosphate (PI(4,5)P2). The dependency on PI(4,5)P_2 appears to be COPI vesicle associated since pre-treatment of vesicles with either a specific 5-phosphatase or a specific 5-kinase, resulted in inhibition or gain of fusion, respectively. As ARF1 recruits the kinase responsible for PI(4,5)P_2 synthesis in a GTP-dependant manner to Golgi membranes, ARF1 can effectively prime COPI vesicles for fusion during vesicle formation.

The rate whereby functional COPI vesicles bind to Golgi membranes can be measured independently of the fusion reaction, *in vitro*. Proteinase K was used to investigate the effect on vesicle binding and fusion when proteins are removed by proteolysis. While binding was accelerated by protease-accessible factors such as SNARE proteins, these are not required for fusion. That proteins on COPI vesicles where removed was confirmed by Western blotting as well as by quantitatively determining NSF and SNAP requirements for vesicle fusion before and after proteolysis. As shown for vacuolar fusion, we propose that the principal role of SNARE proteins is to ensure the recruitment of cytoplasmic fusion proteins to the docking site rather than to mediate the actual fusion event of COPI vesicles to Golgi membranes.

LIST OF PUBLICATIONS

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

I Quantitative Proteomics Analysis of the Secretory Pathway

Annalyn Gilchrist*, Catherine E. Au*, Johan Hiding*, Alexander W. Bell, Julia Fernandez-Rodriguez, Souad Lesimple, Hisao Nagaya, Line Roy, Sara J.C. Gosline, Michael Hallett, Jacques Paiement, Robert E. Kearney, Tommy Nilsson, and John J.M. Bergeron

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* These authors contributed equally o this work

II $PI(4,5)P_2$ is Required for the Fusion of COPI-Derived Vesicles with Golgi Cisternae

Frédéric Laporte, Johan Hiding, Fredrik Kartberg, François Lépin, Markus Grabenbauer, Anirban Siddhanta, Dennis Shields, Joachim Ostermann, John J.M. Bergeron and Tommy Nilsson

Manuscript

III Membrane Binding and Fusion Ability of COPI Vesicles upon Protease Treatment

Frédéric Laporte*, Johan Hiding*, Joachim Ostermann*, Fredrik Kartberg, Joel Lanoix, John J.M. Bergeron and Tommy Nilsson

Manuscript

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ABBREVIATIONS

ALPS	ARFGAP1 lipid packaging sensor
ARF1	ADP ribosylation factor 1
BFA	brefaldin A
BLAST	basic local alignment search tool
CGN	cis-Golgi network
CHO	Chinese hamster ovary
COG	oligomeric Golgi complex
COPI	coat protein complex I
COPII	coat protein complex I
ER	endoplasmic reticulum
ERGIC	ER-Golgi intermediate compartment
GalT	beta1,4 galactosyltransferase
GEFs	guanidine exchange factors
GlcNacT-1	N-Acetylglucosaminyltransferase-I
(L)DCV	(large) dense core vesicles
MannII	alpha1,3-1,6 mannosidase II
MS	mass spectrometry
NSF	N-ethylmaleimide sensitive factor
PAGE	polyacrylamide gel electrophoresis
PDI	protein disulphide isomeras
PI(4)P	phosphatidyl-4-phosphate
PI(4,5)P_2	Phosphatidylinositol -4,5-bisphosphate
PK	proteinase K
PMSF	phenylmethylsulphonyl fluoride
RM	rough microsomes
SM	smooth microsomes
SNAP	soluble NSF attachment protein
SNARE	soluble NSF attachment receptor
TGN	trans-Golgi network
TRAPP	transport protein particle
VSV	vesicular stomatitis virus
VSV-G	vesicular stomatitis virus membrane glycoprotein
VTC	vesicular tubular cluster

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BACKGROUND

The secretory pathway – an overview

Eukaryotic cells contain a complex set of different organelles, each with a specific function. For a cell to be organized and functional, the vast amount of proteins produced have to be sorted, transported and localized to the correct cellular membrane or organelle. To achieve this, organelle-specific mechanisms exist that recruit and package the correct set of proteins and lipids into vesicles for transport to an acceptor membrane [1]. In the secretory pathway, vesicles with a distinct coat that enable them to detach from a donor membrane in a process called budding primarily mediate the movement of cargo between different compartments. When the vesicle is completely formed the coat is lost and the vesicle can dock and fuse with a target membrane [2]. In this way, a cell can transfer secretory proteins to the extra cellular space or insert transmembrane proteins into different membranes. Additionally, membrane lipids can move from one compartment to another as a part of a vesicle membrane. The endocytic and the secretory pathway are the two major transport machineries in the cell. The endocytic pathway transport proteins from the plasma membrane to the endosomes and further to lysosomes or back to the plasma membrane. The secretory pathway on the other hand transports newly synthesized proteins from the endoplasmic reticulum (ER) through the Golgi complex to the cell surface or the endosomes and lysosomes. Synthesized proteins can also be recycled to the endoplasmatic reticulum from Golgi [3].

The secretory pathway consists of several functionally and structurally different membrane compartments. They include the ER, ER-Golgi intermediate compartment (ERGIC) or vesicular tubular cluster (VTC), *cis*-Golgi network (CGN), the Golgi stacks and *trans*-Golgi network (TGN) [4]. Proteins destined for the secretory pathway contain an N-terminal sequence that is recognized by the signal recognition particle (SRP). This sequence direct the nascent chain complex to the rough ER and the proteins enter the ER co-translationally through the Sec-translocon [5]. Protein disulphide isomeras (PDI) catalyses the rearrangement of disulphide bonds and thereby accelerates the folding of newly synthesized proteins. Only properly folded and assembled proteins are transported from the ER to the Golgi complex. Proteins destined for export from the ER are selectively incorporated into COPII (COat Protein)-coated vesicles and transported to the Golgi complex [6, 7]. Membrane cargo is sorted into the vesicles by a direct interaction between the cytosolic domain of the cargo protein and the coat components whereas soluble cargo requires the involvement of a transmembrane receptor to mediate the interaction [8]. The COPII coated vesicles uncoat and fuse with each other to form vesicular tubular clusters or ERGIC [9, 10]. This intermediate compartment is transported by dynein along microtubular tracks towards the CGN [11] on the *cis* side of the Golgi complex. Misfolded or unassembled subunits are transported back through the translocon into the cytosol where they will be degraded by the ubiquitin-mediated proteolytic pathway [12]. Resident ER proteins wrongly transported

and vesicle components needed for another round of transport are returned to the ER by retrograde transport [13]. Membrane proteins contain a dilysine motif (K(X)KXX) [14-16] and soluble proteins a tetrapeptide sequence KDEL [17] that will be recognized and sorted into COPI coated vesicles for retrograde transport [18-20].

The Golgi complex

Named after its discoverer Camillo Golgi [21], the Golgi consists of polarized stacks of flattened cisternae. Proteins from the ER enter the Golgi complex at the *cis*-side and exit at the *trans*-side. A Golgi matrix built up by a family of coiled-coil proteins termed golgins and other peripheral membrane components is thought to maintain this structure [22, 23]. The primary function of the Golgi complex is to modify, process, and sort newly produced proteins that arrive from the ER. Each membrane compartment of the stack contains different sets of glycosyltransferases and other resident proteins. The glycosylation enzymes can both add and remove N- and O-linked oligosaccharides to the proteins transported through the Golgi complex [24]. The distribution of several of the enzymes has been mapped. For example the α1,3-1,6 mannosidase II (Man II) is present in the *medial* and *trans* cisternae whereas β1,4 galactosyltransferase (GalT) is present in the *trans* cisternae and the TGN [25, 26]. The enzymes have a gradient like distribution over the stacks [26] and their localization in the Golgi complex is retained by a signal sequence in the membrane-spanning domain [27].

Intra-Golgi transport

Vesicular transport within the Golgi apparatus is mediated by COPI vesicles. They were first identified when Golgi membranes were incubated together with cytosol and GTPγS [28]. The role of COPI in intra-Golgi transport is still controversial. Two major models have been suggested to explain the movement of newly synthesized proteins through the Golgi stack, the cisternal maturation/progression model [29] and the vesicular transport/stable compartment model [30]. The distinction between the two models concerns the content and directionality of the COPI coated vesicles. In the vesicular transport/stable compartment model, anterograde COPI vesicles transport secretory cargo proteins excluding resident Golgi proteins. In the cisternal maturation/progression model, the entire cisternae function as the transporting entity and retrograde COPI vesicles recycle resident Golgi proteins to a younger cisternae excluding secretory proteins.

The cisternal maturation/progression model originate from morphological studies using electron microscopy revealing membranes at the *cis* and *trans* side to be less ordered and suggested that cisternal membranes form at the *cis* side and disassembly at the *trans* side. In this model, new cisternae mature and push the former *cis* cisternae forward and newly synthezised proteins would move forward to the *trans* side within the cisternae where they would be released for further transport by vesicles. This dynamic model requires a constant input of new proteins. This was

proven to be wrong when it was shown that when protein synthesis was turned of, the Golgi stacks remained the same [31]. Thus, the Golgi seamed to be stable, and combined with the observation of vesicles close to the cisternae the vesicular transport/stable compartment model was adopted [32]. This was also supported by observations involving the explanation of N-linked oligosaccharides processing [33], characterization of yeast mutants effecting Golgi function [34] and the use of a in vitro assay to identify components involved in vesicular transport [35]. The processing of N-linked oligosaccharides agreed with a view of a stable Golgi with a unique set of enzymes in each cisterna. In addition, an in vitro assay suggested that vesicles transported vesicular stomatitis virus membrane glycoprotein protein (VSV-G) from N-Acetylglucosaminyltransferase-I (GlcNAc-T1) deficient cisternae to cisternae that could complement the deficiency. Together with yeast genetics and morphology, this confirmed the vesicle transport/stable compartment model where COPI vesicles play a central role in transporting cargo forward from cisterna to cisterna in a cis to trans or anterograde direction. However, this model could not explain the transport of large complexes such as collagen precursors [36] or lipoproteins [37] to large to fit into vesicles. New observations indicated that the Golgi is not as stable as one first thought. Brefeldin A (BFA), a fungal metabolite was shown to strongly inhibit the protein secretion by disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum [38]. When BFA was washed out, the Golgi reformed indicating a dynamic system and the possibility of Golgi to self-assembly [39, 40]. Furthermore, it is now known that glycosylation enzymes are not restricted to a single cisternae but exist in a gradient–like distribution over the stacks [26, 41]. A dynamic Golgi was not the only observation to challenge the vesicular transport/stable compartment model. The previously mentioned in vitro assay was found to register the transport of GlcNAc-T1 rather than the VSV-G protein [42] and the vesicles were found to include glycosylation enzymes. It was also shown that proteins containing the ER recycling motif K(X)KXX interacts with the coat of the COPI vesicles [18] and that the COPI vesicles are responsible for retrograde transport from Golgi to ER [19]. This data supports a role for COPI in recycling resident enzymes and thereby maintaining a steady state of the Golgi in agreement with a cisternal maturation/progression model. Even though most observations now supports a cisternal maturation/progression model there are observations of little or no concentration of either resident enzymes or secretory cargo in COPI vesicles [43, 44]**.** In an attempt to merge the two models the existence of two types of COPI vesicles involved in bidirectional traffic at the Golgi level has been suggested [45]. This is also in line with the observation of sub-populations of COPI vesicles [46, 47].

Formation of COPI vesicles

The cytosolic multi-protein complex coatomer consists of the seven subunits α-(~160kDa), β-(107 kDa), β′-(102 kDa), γ-(~100 kDa), δ-(~60 kDa), ε-(36 kDa) and ζ-(20 kDa) COP; [48]). The coatomer can be split into two sub-complexes, one containing β-, γ-, δ- and ζ-COP and one containing α-, β′- and ε-COP[49]. The basic model for coat assembly and formation of COPI-vesicles starts with the activation of the GTPase ADP ribosylation factor 1 (ARF1) initially in complex with GDP. Membrane associated guanidine exchange factors (GEFs) defined by the presence of a Sec7 domain catalyze the exchange of GDP to GTP on ARFs [50]. This nucleotide exchange leads to a conformational change exposing the myristoylated N-terminus that together with hydrophobic and basic residues on the N-terminal alpha helix interacts with the Golgi membrane[51, 52]. Once bound to the membrane ARF1 will recruit the complete coatomer complex by binding to the β- and γ-COP subunits [53]. WD40 motifs of α- and β′-COP can also bind to cytoplasmic tails of transmembrane proteins containing a KKXX sequence [54]. Coat polymerization then leads to membrane budding and fission of coated vesicles. In order to fuse with its target membrane, the COPI vesicle needs to be uncoated. This is facilitated by the hydrolysis of ARF1-bound GTP [55], a process catalyzed by an ARF1 GTPase-activating protein (GAP) [56].

Incorporation of cargo

First, it was thought that ARFGAP1 solely facilitates the uncoating of vesicles by acting as an inactivator of Arf leading to dissociation of the COPI coat. The formation of coated vesicles could be inhibited and already formed vesicles could be uncoated by addition of a truncated ARFGAP1 mutant that contained the active GAP domain but not the C-terminal targeting domains [57]. However, when studying the packaging of cargo into COPI vesicles it was shown that the efficiency of the uptake was reduced in the presence of a non-hydrolysable GTP analog (GTPγS) as compared to GTP [58]. The target GTPase involved in cargo uptake was identified as Arf1 both *in vitro* [59] *in vivo* [60]. Indeed, studies using rat liver Golgi membranes and cytosol showed that GTP hydrolysis by ARF1 is important for cargo incorporation as addition of a GTPγS or a GTP-restricted ARF1 (Q71L) mutant resulted in decreased amount of Golgi resident cargo incorporated into vesicles [61]. An interaction between ARFGAP1, coatomer and ARF1 suggests a tripartite complex that can interact with resident cargo in different ways [62]. Kinetic studies of GAP-stimulated GTP hydrolysis using a membrane-independent mutant form of ARF1 (Δ17-ARF1) and a catalytic fragment of GAP1, showed that ARFGAP1 activity is strongly stimulated by coatomer in solution [63]. This effect could be blocked by the cytosolic tails of the Golgi cargo protein p24 [62]. When using full length ARFGAP1 and wild type, myristoylated ARF1 in the presence of Golgi membranes the effect of coatomer was much lower [64]. Nevertheless, peptides corresponding to p24 and p23 tails showed inhibition ability in an assay using full-length Arf1 and ArfGAP1 on both liposomes and Golgi membranes [46]. This led to

the proposition of a model for sorting of cargo based on kinetic control of ARF1-mediated GTP hydrolysis. In this model, coatomer bound to ARF1 without sorting signals on cargo that inhibit GAP activity would lead to fast GTP hydrolysis and dissociation of ARF1 and coatomer from membranes. In contrast, coatomer bound to both ARF1 and a sorting signal from for example p24, would be more stably associated with the membrane due to the inhibited GAP activity. In this way, only coatomer bound to cargo would stay long enough on the membrane to be polymerized. Support for this mechanism to mediate sorting was confirmed by computer simulation [65]. Other studies examining the regulation of GAP1 has shown that increased membrane curvature stimulates GAP1 activity [66]. This would ensure efficient uncoating of the budded vesicle. With the use of liposomes with defined size, it was shown that stimulation of ARF-mediated GTP hydrolysis increased as the liposome radius decreased. This effect is due to an ARFGAP1 lipid packaging sensor (ALPS) motif in the non-catalytic domain of AFRGAP1 [67]. Association with this motif with the bilayer stimulates GAP activity. On a flat membrane with lipids packed tight together ARFGAP1 could therefore be inactive. Whereas on highly curved membranes this motif can insert into the less tightly packed lipids which would increase the GTP hydrolysis rate and lead to uncoating of the vesicle.

Tethering

After a vesicle loses its coat, it must be targeted to the correct acceptor compartment. The initial interaction between a vesicle and its target membrane is termed tethering. Together with Rabs, small GTPases of the Ras superfamily [68], proteins called tethers or tethering factors have an important role in determining the specificity of vesicle targeting [69]. Tethers mainly belong either to a group of long coiled-coil proteins or to multi-subunit tethering complexes. The long coiled-coil proteins called Golgins consist of at least two a-helices that wrap around each other into a super-helical coil. One of the most studied tethers is the tether p115, a peripheral-Golgi membrane protein that was purified on the basis of its ability to stimulate transport between Golgi cisternae [70]. It has been shown that p115 binds the N-terminus of the Golgi protein GM130 [71] as well as Giantin, a typ II protein on COPI vesicles and could thereby form a link between COPI vesicles and Golgi membranes [72]. However, this bridging model for GM130-p115-giantin tethering of COPI vesicles has been questioned by studies showing that GM130 and giantin compete for the same binding site on p115 [73]. Golgin tethers has also revealed sub-populations of COPI vesicles containing glycosylation enzymes defined by the Golgin-84-CASP tethers or p24 proteins defined by the Giantin-p115-GM130 tethers [47]. The multi-subunit tethering complexes include both quatrefoil tethers such as the oligomeric Golgi complex (COG) and non-quatrefoil tethering complexes, including the transport protein particle (TRAPP). It has been suggested that COG function as a tether between *cis*-Golgi and COPI as COG has been shown to associate with COPI subunits both in mammalian and yeast [74, 75]. TRAPP II, localized in the *cis*-Golgi has been shown to be functionally linked to ARF1 and coatomer and is believed to be involved in intra-Golgi traffic [76, 77].

Fusion

Soluble N-ethylmaleimide sensitive factor (NSF) attachment protein (SNAP) receptors (SNAREs) mediate the final docking of vesicles with the target membrane and catalyze the fusion reaction [78]. They are short membrane associated proteins with a characteristic SNARE motif of around 60 amino acids [79]. SNAREs located on the vesicles are called v-SNAREs, while those on the target membrane are called t-SNAREs. When the tethering factors have brought the opposing membranes close together, one v-SNARE and three t-SNAREs can form a four-helix bundle called trans-SNARE or SNAREpins, which catalyzes the fusion of the vesicle with the target membrane [80]. In the middle of the bundle, the helices are connected by layers of hydrophobic amino acids except for a hydrophilic central layer. This layer is formed by three glutamines (Q) from the t-SNARE and one arginine (R) from the v-SNARE, leading to the alternative nomenclature Q- and R-SNAREs, respectively [81]. The mechanism is unclear but one popular model suggest that the trans-SNARE complex brings the membranes close to each other and thereby ‘zipping’ them together, overcoming the energy barrier required for fusion of the lipid bilayers [82, 83]. Membrane fusion results in a conversion from a trans-SNARE to a cis-SNARE. This complex then recruits α-SNAP and NSF to disassemble the complex and recycle the SNARE proteins for further rounds of fusion [84].

Organellar proteomics – experimental strategy

Mass spectrometry (MS) based proteomics has gained recognition as an effective experimental strategy to determine the identity and function of organelles. The experimental strategy is composed of sample preparation, mass-spectrometric analysis and data evaluation. For organellar proteomics, highly enriched and purified organelle fractions are characterized by a combination of biochemical assays, Western blots and electron microscopy. The protein components of the organelle are then separated by one-dimensional (1D) polyacrylamide gel electrophoresis (PAGE) followed by in-gel enzymatic degradation with trypsin after reduction and alkylation. The resulting peptide mixtures are separated by liquid chromatography and subsequently analyzed by tandem MS. The resulting raw-data are processed to generate a peaklist of all tandem MS. The matching of the tandem MS to tryptic peptides in a protein database involves the theoretical digestion of all proteins and fragmentation of tryptic peptides. Experimental and calculated peptide masses are matched followed by scoring of the matching of the experimental and theoretical fragment ions. After matching the tandem MS to peptides, the corresponding proteins are listed and the minimum number of proteins required to account for all peptides is generated. Finally, further grouping by BLAST analysis to gather isoforms of proteins into a single group followed by literature analysis to assign names and eliminate database errors will generate the fully annotated protein list.

PRESENT INVESTIGATION

AIMS OF THIS WORK

Intracellular transport has been the focus of cell biology research for a long time. However, there is no complete and accurate map of all the proteins within the secretory pathway. In order to achieve such a cell map the subcellular organelles rough microsomes, smooth microsomes and Golgi membranes were isolated and analyzed by quantitative tandem MS. Further, an *in vitro* budding assay to purify Golgi-derived COPI vesicles was used in order to learn more about their role in intra-Golgi transport. The COPI vesicles was both analyzed by tandem MS and used in an *in vitro* transport assay to study the requirements for vesicle fusion.

RESULTS AND DISCUSSION

QUANTITATIVE PROTEOMICS ANALYSIS OF THE SECRETORY PATHWAY (PAPER I)

Historically, the studies of the Secretory pathway have often focused on morphology, biochemistry and genetics. Here, a proteomics approach was taken to investigate the protein content of the rough microsomes (RM), smooth microsomes (SM), Golgi cisternae, COPI-vesicles and vesicles formed in presence of the non-hydrolysable analogue GTPγS to monitor the effects of impaired sorting. Proteomics has previously mapped the protein contents of targets such as the nuclear pore complex in yeast [85] and clathrin-coated vesicles in rat brain and liver [86, 87]. Efforts have also been made to compile ER and Golgi proteins. However, a complete and accurate protein map of the secretory pathway has not been established. To achieve this isolated subcellular organelles and tandem MS to map proteins to their intracellular locations were used. Highly enriched organelles were solubilized and their protein content separated by SDS-PAGE. The protein lanes were cut into slices and trypsinized before subjected to tandem MS.

Quantification by redundant peptide counting

The resulting data sets were analyzed using redundant peptide counting as a quantitative representation of what the tandem mass spectrometer observed. Peptide counting is based on the idea that when normalized for protein size, an abundant protein within a sample will generate more peptides than a relatively rare on. This method has previously been used to show that varying amounts of proteins in a yeast lysate have a linear correlation between redundant peptide counts and protein abundance over 2 orders of magnitude with respect to percent of the total protein content (Liu 2004). To further test the validity of the method this was compared to quantitative western blotting using ^{125}I-labeled antibodies to albumin, which showed a similar distribution (fig. 1B, paper I). In addition, the total number of peptides detected for six different proteins in increasing amounts of SM fractions on a SDS-PAGE gel (fig. 1SB) further support the method as an index of protein abundance. The redundant peptide counts were used in order to present the tandem MS data by similar expression profiles across organelles using hierarchical clustering commonly used for microarray data grouping proteins by different levels of protein similarity [88, 89]. In this way, proteomics clustering profiles can be analyzed to ascertain the biological significance of the data.

The ER proteome

All proteins and associated mass spectra were assigned into one of 23 functional categories (fig.1C, paper I). For RM, the highest number of peptides was for proteins involved in protein synthesis and folding. For SM, this category as well as proteins involved in detoxification showed the highest peptide abundance. The most abundant protein was serum albumin with 570 redundant peptides in nine experiments. Only two orders of magnitude were thus available for the detection of less abundant proteins. To detect these proteins, membranes were biochemically subfractionated by salt wash to concentrate cytosolic membrane peripheral proteins and detergent extraction with Triton X-114 to separate the soluble and luminal proteins from the membrane proteins based on their partition in the aqueous or the detergent phase, respectively [90].

After applying hierarchical clustering analysis of the suborganellar ER fractions (fig. 3A, paper I), it turned out that 77% of all the assigned peptides (tab. S3B) were present in six clusters, each representing a distinct functional category (fig. 3B). For the BiP cluster, most proteins were molecular chaperones and protein-folding enzymes known to be soluble luminal ER proteins. The actin cluster consisted mostly of insoluble cytoskeletal proteins including molecular motors and was largely localized to the SM. Insoluble proteins in the RM included ribonucleoproteins, RNA splicing factors together with DnaJ paralogs that co-clustered with the poly(A)-binding protein. The arginase cluster consisted of metabolic enzymes and proteins involved in translation or folding. The cytochrome P450 cluster reflects a major function of ER in drug detoxification and the ribophorin cluster defines integral membrane proteins of functional significance in the ER for protein modification including N-glycosylation. In total, this supports the conclusion that biochemical subfractionation of the SM and RM led to the concentration of proteins with specific suborganellar locations and significantly extended the ER proteome.

The Golgi proteome

Golgi membranes were treated in a similar way as the SM and RM. This did not extend the Golgi proteome significantly over previous studies (Bell 2001). Therefore the study was extended to include Golgi derived COPI vesicles. The vesicles were formed as described by Kartberg *et al* [91] with Golgi membranes, cytosol, an ATP-regenerating system and either GTP or the nonhydrolysable analog GTPγS. As vesicles can fuse once formed, the reaction was supplemented with a dominant mutant of α-SNAP to inhibit NSF-specific fusion. Electron micrographs confirmed that a homogenous population of COPI vesicles with a size of around 45-60 nm could be isolated (fig. 4, paper I). Vesicles formed with GTPγS were found to have a broader diameter distribution possibly due to a higher degree of coated vesicles.

Quantitative representation of protein abundance (fig. S6A, paper I) show for the Golgi fraction a prevalence of soluble secretory cargo proteins, while COPI vesicles contain proteins involved in posttranslational modification such as glycosylation enzymes. The vesicles produced with GTPγS show a broader distribution over the 23 functional categories with a notable amount of

coat proteins. Using hierarchical clustering of the data, focuse was put on nine of the clusters, representing 71% of the peptides (fig. S7B, paper I). The Rab6 and p115 clusters contain proteins classified as biosynthetic cargo including serum albumin and apolipoprotein B with a high abundance in the Golgi fraction. These proteins are on the other hand present in a two fold decreased amount in the vesicles. This result is consistent with earlier studies based on immunogold-labeling of thin frozen sections, revealing a reduced amount of soluble synthetic cargo in peri-Golgi vesicles including both albumin and apolipoprotein E [37].

Most of the COPI coat components were found in the COPI cluster with the two isoforms γ-COP1 and γ-COP2, readily identified. This indicates that there are more than one class of COPI vesicles in the COPI fraction [92]. All the coat components increase in the GTPγS fraction indicating more coated structures, consistent with the broader size distribution found for this fraction. Taken together, the COPI coatomer cluster emphasize the COPI nature of both vesicle fractions.

The ERV29 cluster shows proteins only enriched in the fraction with vesicles formed using GTPγS. Since this cluster contains mostly ER proteins involved in detoxification, it suggests that this fraction does not represent a faithful extension of the Golgi proteome. Therefore, these fractions were only used to analyze proteins also found in the Golgi and COPI fractions such as the coat proteins. The rest of the clusters in figure S7B highlight proteins that are enriched in the COPI vesicles as compared to the Golgi fraction. For example, the Rab1 cluster contains Rab1 and Rab2 together with the SNARE proteins rBET1 and Gos-28. The membrin cluster includes the coatomer component β' and the SNARE proteins sec22b and membrin. The Golgin 84 cluster also contain CASP previously used to highlight different populations of COPI vesicles [47]. That both are enriched in the COPI fraction further highlights the COPI nature of the fraction. Sixteen SNAREs were observed in the study, the most outstanding being the R-SNARE sec22 and the Q-SNARE Gos-28. These were found in the COPI vesicles along with the less abundant Q-SNAREs membrin, Vti-1A, Bet1 and GS15. Syntaxin 5 on the other hand was diminished in in COPI vesicles. These predicted distributions would enable more direct testing to learn more about the mechanism of SNARE-mediated fusion in Golgi traffic. In fact, studies of the fusion mechanism between COPI vesicles and target Golgi membranes will be discussed in paper II and III.

The role of COPI vesicles

A predicition of the cisternal maturation model is that secretory cargo would remain in the cisternae while Golgi-resident proteins are sorted into COPI vesicles [93]. To test this hypothesis the abundance of nine different secretory cargo proteins in three biological replicates of RM, SM, Golgi and COPI vesicle fractions generated with GTP or GTPγS were analyzed (fig 5, paper I). A consistent pattern of enrichment and concentration of these proteins in the Golgi cisternae and an exclusion of these proteins in COPI vesicles was found (fig. 5A, paper I). The opposite

result was seen with resident Golgi proteins where a maximal enrichment in concentration was observed in COPI vesicles. The incorporation of resident proteins into COPI vesicles required GTP hydrolysis highlighting an active sorting mechanism. The significant enrichment in resident proteins over biosynthetic cargo suggests a main role for COPI-vesicles in the recycling of resident proteins within the Golgi as predicted by the cisternal maturation/progression model. However, also transmembrane biosynthetic cargo was found in the vesicles. This would support a bidirectional model of COPI vesicle transport were membrane protein is distinguished from soluble cargo. Previous studies using p24γ3 to immunoisolate vesicles suggest that there are subpopulations of vesicles [46]. This is recently supported by the finding that the tethering factors CASP and p115 reside in different vesicle populations [47]. Whether COPI vesicles also have role in transporting anterograde cargo remains to be resolved.

Crosscontamination

A concern in studies including subcellular fractionation is that organelles are potentially contaminated with proteins from other compartments of the cell. To test the degree of cross-contamination, a set of established organellar markers for the ER and Golgi apparatus was selected (fig. 1A-D, paper I). Peptides assigned to the translocon constituent sec61a are highly enriched in isolated RM fractions. Lower amount is observed in the SM and only a few peptides in the Golgi fraction (fig. 1A, paper I). Proteins expected to be evenly distributed between RM and SM like calreticulin and calnexin, indeed showed such a distribution with only low amounts in observed in the Golgi fractions (fig 1B, paper I). On the other hand, Golgi resident proteins were largely enriched in the Golgi fractions, with only a few peptides detected in the RM and SM fractions (fig. 1D, paper I). Overall, this shows the ER character of the RM and SM fractions as well as the Golgi character of the Golgo fractions. Nevertheless, proteins from other cells together with mitochondrial, perixosomal, lysosomal and nuclear proteins were readily detected. Approximately 20% of proteins came from other organelles in the different fractions.

Proteins of unknown function

Totally 345 proteins were characterized as proteins of unknown function in all the analyzed fractions. The locations of 234 proteins were indicated by their co-distribution with marker proteins of 14 selected clusters. The protein distributions predicted by the proteomics to were tested by chimeric YFP protein expression for three of these proteins. The redundant peptide counting predicts a location in the ER for all three as indicated in figure 7C, paper I. Monomeric YFP expressed as fusion proteins in HeLa cells also revealed an ER localization for these proteins (fig. 7B, paper I), validating the proteomic predictions. Interestingly, during our study another group uncovered and described one of the proteins as cytosolic [94]. Therefore, a peptide-specific antibody against this protein was made. Immunolocalization then confirmed the YFP chimera prediction that the protein is ER localized (fig. S9A, paper I). Taken together, this

proteomic study resulted in a quantitative proteomic map of more than 1400 proteins with spatial information and relative presence of each protein of the rough ER, smooth ER and Golgi apparatus. In addition, by extending the study to biochemical subfractions and organelle subcompartments more evidence could be provided that COPI vesicles concentrate Golgi-resident proteins while largely excluding secretory cargo. This supports a role for COPI vesicles in transporting Golgi resident proteins in a retrograde direction from trans to cis within the Golgi stack.

$PI(4,5)P_2$ IS REQUIRED FOR THE FUSION OF COPI-DERIVED VESICLES WITH GOLGI CISTERNAE (PAPER II)

Although much has been learned about the processes involved in vesicle transport, exactly how a carrier vesicle finds and fuses with its target remains an unanswered question. The formation of $PI(4,5)P_2$ is required for fusion or the regulation of fusion in several membrane systems. This has been shown in yeast where $PI(4,5)P_2$ is required for SEC18p(NSF)-dependent priming of SNAREs [95] and in PC12 cells where $PI(4,5)P_2$ is required for the recruitment of cytosolic factors needed for fusion of dense core vesicles with the plasmamembrane [96]. $PI(4,5)P_2$ may have a similar role in the Golgi apparatus. Inhibition of $PI(4,5)P_2$ formation results in the fragmentation and vesicularisation of the Golgi apparatus [97, 98]. This suggests a possible requirement of $PI(4,5)P_2$ in the maintenance of the Golgi apparatus via membrane fusion. In this study, the role of $PI(4,5)P_2$ in COPI vesicle fusion was studied *in vitro*, using a modified version of a well-characterized intra Golgi transport assay which monitors the fusion of Golgi-derived COPI vesicles with the early Golgi membranes [61, 99].

The *in vitro* transport assay

Cells of the GlcNacT-1-deficient Chinese hamster ovary (CHO) cell line, Lec1, were first infected with vesicular stomatitis virus (VSV) to express the N-linked glycosylated and temperature-sensitive protein G. Upon synthesis, VSV-G protein is accumulated and retained in the *cis*-Golgi network by transferring cells to 15 °C [100]. Cells were homogenised and Golgi membranes containing the VSV-G protein purified and mixed with cytosol and isolated COPI vesicles without the VSV-G protein but high GlcNacT-1 enzyme activity. When the COPI vesicles dock and fuse with the Golgi membranes it will result in modification of N-liked oligosaccharides attached to VSV-G. The assay then registers the incorporation of ^{3}H-GlcNac onto the N-linked oligosaccharides of VSV-G [35].

Four parameters have been identified that describes the kinetics of this transport assay (fig. 1A, paper II) [101]. The first parameter, termed apparent vesicle concentration (C_v^{app}) describes the concentration of vesicles available for fusion. The second parameter describes a time-dependent decrease of C_v^{app}. The third parameter describes the binding and fusion of COPI vesicles with the Lec1 Golgi membranes and the fourth parameter describes the transfer of GlcNac onto VSV-G. The overall titration curve seen in figure 1C is defined by the equation $a+c(1-e(-bx))$, where “a” corresponds to the minimal signal or background in the assay, “b” is the slope of the curve or concentration of vesicles that can generate a signal and “c” is the maximum signal or the amount of VSV-G present in the assay.

A role for phosphoinositides in fusion

When vesicles are pre-incubated at 37°C without cytosol, fusion activity of vesicles decreases over time. To investigate if this might correspond to changes in phosphoinositides, neomycin or Wortmannin were added to the fusion assay, both known PI(4,5)P_2 affectors. Both showed inhibitory effects supporting a role for phosphoinositides in fusion. To test more specifically, a monoclonal antibody specific for PI(4,5)P_2 was added. Increasing amounts of antibody revealed an antibody-specific inhibition (fig. 2b, paper II) suggesting that phosphoinositides such as PI(4,5)P_2 are required for the fusion process of COPI vesicles. A mouse PI4P5-kinase was added directly into the assay but the kinase had only a low stimulatory effect (fig. 3B, paper II). However if the kinase was introduced an hour before the addition of Lec1 target membranes the time-dependant inactivation was inhibited, supporting a role for PI(4,5)P_2 in fusion of COPI vesicles. If PI(4,5)P_2 is needed for fusion, then treatment of COPI vesicles with PI(4,5)P_2 phosphatase should block fusion. Indeed, pre-incubation of vesicles with phosphatase before addition to the Lec1 membranes resulted in an inhibitory effect (fig. 4B, paper II). As pre-incubation of vesicles alone or in the presence of either phosphatase or kinase effects the concentration of vesicles available for fusion, it can be concluded that the presence of PI(4,5)P_2 is required on the COPI vesicles for fusion with target Golgi membranes *in vitro*.

MEMBRANE BINDING AND FUSION ABILITY OF COPI VESICLES UPON PROTEASE TREATMENT (PAPER III)

SNARE proteins are together with tethering factors and other accessory molecules thought to mediate the specificity for fusion so that correct membranes fuse with each other. In this study, the relative sensitivity of SNARE proteins to protease treatment was investigated, comparing COPI vesicles and acceptor Golgi membranes in the transport assay previously described.

Target membranes are inactivated by proteolysis

Could SNARE proteins in COPI vesicles be degraded by proteolysis? The vesicles were incubated with increasing amount of proteinase K (PK) for 30 minutes on ice in order to digest peripherally exposed SNAREs and other cytoplasmically exposed proteins. The SNARE GS28 has been suggested for taking part in intra-Golgi vesicular transport and it is mostly cytoplasmically oriented [102]. Here the proteolytic removal of GS28 from COPI vesicles formed *in vitro* (fig. 1, paper III) was monitored abd it was estimated that at least 90% were degraded. When Golgi membranes were treated with PK, it was seen that GS28 was equally sensitive as it was in the vesicles. The amount of PK necessary to inactivate the Lec1 target Golgi membranes with which the COPI vesicles fuse was tested. Target membranes were incubated with PK and then treated with phenylmethylsulphonyl fluoride (PMSF) to inactivate PK before the membranes was added to the transport reaction. For all PK concentration tested, the fusion of COPI vesicles with target membranes was efficiently inhibited (fig. 2, paper III) showing that the ability of target membranes to fuse with transported COPI vesicles is highly sensitive to degradation of proteins on their surface. However, proteolysis did not seem to inactivate vesicles in a similar manner since even after treatment with PK vesicles readily continued to fuse with Golgi membranes.

Vesicle activity is partially resistant to proteolysis

Compared to target membranes, determining the fraction of vesicles that inactivates is a bit more complicated. The variable that is monitored in the transport assay is the rate at which the maximum signal is obtained. The relationship between the amounts of vesicles added describes an inverse exponential dos-response curve and the initial increase or slope of this curve is proportional to the amount of functional vesicles in the assay [101]. At saturating amounts of vesicles, the obtained assay signal is proportional to the concentration of target membranes capable of fusion with the added vesicles. To study the kinetic parameters of the fusion event more closely increasing amounts of vesicles to a fixed amount of Golgi membranes in the assay was added (fig. 3A, paper III). The vesicles were either non-treated or incubated with PK before used in the assay. The maximum signal obtained and the slope of the dose-response curve was determined by curve fitting of the appropriate mathematical expression to the data [101]. When

vesicles were incubated with PK the slope decreased to around two thirds of the control incubation without PK. Even at very high PK concentrations, the vesicles remained mostly active (fig. 3B, paper III). However, even though there was no further reduction in the slope the maximum signal was reduced up to 50%. This was most likely due some of the PK had escaped the inactivation by PMSF. Even small amounts of PK would reduce the assay signal due to the highly sensitive Golgi membranes.

The partial inactivation of vesicles could be due to a decreased ability for the vesicles to dock and bind to the target membranes since the PK treatment would remove cytoplasmically oriented proteins involved in this process. The inactivation-to-binding ratio can be measured by determining the effect of diluting the reaction. Dilution slows the binding step as it increases the distance between vesicles and target membranes and thus reducing the number of docking events. If vesicles would not inactivate the dilution would have no effect on the number of vesicles that have fused at the end of the reaction. If inactivation were much faster than binding, almost all vesicles would inactivate leading to an assay signal produced by the small number of vesicles that would bind before inactivation. Therefore, the fraction of vesicles that bind and fuse would be reduced by half if the reaction were diluted by half. When testing a two-fold dilution with and without proteolysis (fig. 4A-B, paper III) a decrease of the dose-response curve corresponding to an inactivation of around 35% for untreated and almost 60% for protease treated vesicles was observed. The reduction in the binding to inactivation ratio can be explained as a reduction in binding kinetics to about one third of the control value. The reduction of vesicles that bind and fuse rather than inactivate is in good agreement with the observed reduction of the amount of vesicles available for fusion. This suggests that the reduction of the binding kinetics is the principal cause for the observed partial inactivation of a vesicle, not an inhibition of fusion.

To test if protease treated vesicles differ in other kinetic parameters from untreated controls, the overall reaction kinetics and the effect of protease treatment was looked at. Vesicles were pre-incubated with or without PK before added to target membranes and at indicated times, fusion was blocked by the calcium-specific chelator BAPTA. Only small differences could be observed (fig. 5, paper III). Since the vesicle-binding rate is reduced after proteolysis, the overall vesicle consumption rate must also be affected. As the vesicle consumption rate also includes the inactivation rate, the relative change in the consumption rate is smaller than the change in binding rate. Therefore, the change in the binding rate alone is enough to explain the kinetic changes before and after proteolysis of vesicles. No additional effects such as changes in membrane fusion could be observed after proteolysis of vesicles. In summary, target membranes are highly sensitive to protease treatment in contrast to COPI vesicles that are only partially inactivated by protease treatment and do not effect their ability to fuse. This suggests a heterotypic fusion event between COPI vesicles and Golgi cisternae supporting the role of COPI vesicles as transport carries that are both biochemically and functionally distinct from Golgi cisternae.

SUMMARY AND CLOSING REMARKS

The proteome is constantly changing through interactions with the genome and the environment. An organism will have different protein expression in different cell types, in different stages of its life cycle and in different environmental conditions. Proteomics have expanded the size of biological studies from simple biochemical analysis of single proteins to measurements of complex protein mixtures. This study represents a quantitative proteomic map of the rough ER, smooth ER, and Golgi membranes isolated from rat liver. The use of highly enriched organelles and the extension of the study to biochemical subfractions and organelle subcompartments resulted in the identification of more than 1400 different proteins assigned to the secretory pathway. This proteome of hepatic ER and Golgi may be nearly complete as predicted by bioinformatic tools [103]. Additionally, quantitative proteomics is not just a method for generating a list of proteins but can be used as a predictive tool. This is highlighted by prediction of the subcellular location for 230 of the 345 previously unidentified proteins that was assigned to the ER-Golgi proteome. Together with bioinformatics, this is an important tool in increasing our understanding of the phenotypes of both normal and diseased cells.

An effort was also made to increase the understanding of the role of COPI vesicles. Much care was taken to produce high quality, functionally intact and homogenous vesicles (fig. 4, paper I). The function of these vesicles is controversial and two roles have been suggested. They could either mediate forward transport of cargo proteins or carry resident Golgi proteins in a retrograde direction. Analysis of the data set shows that cargo proteins were largely excluded from vesicles whereas Golgi-resident proteins were enriched (fig. 5, paper I). Though a role for COPI vesicles in transporting resident cargo in a retrograde direction according to cisternal the maturation model is the most likely concept, some biosynthetic cargo was found in the vesicle fraction.

The COPI vesicles was further used in an *in vitro* transport assay demonstrating that fusion of retrograde-directed COPI vesicles with target Golgi membranes requires $PI4,5P_2$. The role for $PI(4,5)P_2$ in membrane fusion was previously shown for the fusion of (large) dense core vesicles ((L)DCVs) with the plasma membrane [96, 104]. Now this may be extended to include COPI-mediated transport. Although Golgi membranes contain very low levels of $PI(4,5)P_2$ at steady state [105, 106], the results suggest that ARF1 could bind the kinase required for $PI(4,5)P_2$ synthesis in a GTP specific manner [107, 108]. In this way, ARF1 could prime budding COPI vesicles for fusion. Support for such a COPI-dependent coupling between budding and fusion has been reported previously [109]. When protease K was used to determine the effect on vesicle binding and fusion it was shown that protease treatment decreases the rate of binding but had little effect on the fusion event. In contrast, when target membranes were protease treated it efficiently blocked both binding and fusion. This would suggest heterotypic fusion between COPI vesicles and Golgi membranes and strengthen the view of COPI vesicles as biochemically and functionally distinct from Golgi cisternae.

REFERENCES

1. Schekman, R. and L. Orci, *Coat proteins and vesicle budding*. Science, 1996. **271**(5255): p. 1526-33.
2. Rothman, J.E., *Mechanisms of intracellular protein transport*. Nature, 1994. **372**(6501): p. 55-63.
3. Dean, N. and H.R. Pelham, *Recycling of proteins from the Golgi compartment to the ER in yeast*. J Cell Biol, 1990. **111**(2): p. 369-77.
4. Saraste, J. and E. Kuismanen, *Pathways of protein sorting and membrane traffic between the rough endoplasmic reticulum and the Golgi complex*. Semin Cell Biol, 1992. **3**(5): p. 343-55.
5. Blobel, G. and B. Dobberstein, *Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma*. J Cell Biol, 1975. **67**(3): p. 835-51.
6. Rexach, M.F., M. Latterich, and R.W. Schekman, *Characteristics of endoplasmic reticulum-derived transport vesicles*. J Cell Biol, 1994. **126**(5): p. 1133-48.
7. Barlowe, C., et al., *COPII: a membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum*. Cell, 1994. **77**(6): p. 895-907.
8. Wieland, F. and C. Harter, *Mechanisms of vesicle formation: insights from the COP system*. Curr Opin Cell Biol, 1999. **11**(4): p. 440-6.
9. Aridor, M., et al., *Sequential coupling between COPII and COPI vesicle coats in endoplasmic reticulum to Golgi transport*. J Cell Biol, 1995. **131**(4): p. 875-93.
10. Bannykh, S.I. and W.E. Balch, *Membrane dynamics at the endoplasmic reticulum-Golgi interface*. J Cell Biol, 1997. **138**(1): p. 1-4.
11. Presley, J.F., et al., *ER-to-Golgi transport visualized in living cells*. Nature, 1997. **389**(6646): p. 81-5.
12. Sommer, T. and D.H. Wolf, *Endoplasmic reticulum degradation: reverse protein flow of no return*. Faseb J, 1997. **11**(14): p. 1227-33.
13. Pelham, H.R., *Evidence that luminal ER proteins are sorted from secreted proteins in a post-ER compartment*. Embo J, 1988. **7**(4): p. 913-8.
14. Nilsson, T., M. Jackson, and P.A. Peterson, *Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the endoplasmic reticulum*. Cell, 1989. **58**(4): p. 707-18.
15. Jackson, M.R., T. Nilsson, and P.A. Peterson, *Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum*. Embo J, 1990. **9**(10): p. 3153-62.
16. Jackson, M.R., T. Nilsson, and P.A. Peterson, *Retrieval of transmembrane proteins to the endoplasmic reticulum*. J Cell Biol, 1993. **121**(2): p. 317-33.
17. Munro, S. and H.R. Pelham, *A C-terminal signal prevents secretion of luminal ER proteins*. Cell, 1987. **48**(5): p. 899-907.
18. Cosson, P. and F. Letourneur, *Coatomer interaction with di-lysine endoplasmic reticulum retention motifs*. Science, 1994. **263**(5153): p. 1629-31.
19. Letourneur, F., et al., *Coatomer is essential for retrieval of dilysine-tagged proteins to the endoplasmic reticulum*. Cell, 1994. **79**(7): p. 1199-207.
20. Townsley, F.M., D.W. Wilson, and H.R. Pelham, *Mutational analysis of the human KDEL receptor: distinct structural requirements for Golgi retention, ligand binding and retrograde transport*. Embo J, 1993. **12**(7): p. 2821-9.
21. Droscher, A., *Camillo Golgi and the discovery of the Golgi apparatus*. Histochem Cell Biol, 1998. **109**(5-6): p. 425-30.
22. Warren, G. and V. Malhotra, *The organisation of the Golgi apparatus*. Curr Opin Cell Biol, 1998. **10**(4): p. 493-8.
23. Seemann, J., et al., *Matrix proteins can generate the higher order architecture of the Golgi apparatus*. Nature, 2000. **407**(6807): p. 1022-6.
24. Kornfeld, R. and S. Kornfeld, *Assembly of asparagine-linked oligosaccharides*. Annu Rev Biochem, 1985. **54**: p. 631-64.
25. Rabouille, C. and T. Nilsson, *Redrawing compartmental boundaries in the exocytic pathway*. FEBS Lett, 1995. **369**(1): p. 97-100.
26. Rabouille, C., et al., *Mapping the distribution of Golgi enzymes involved in the construction of complex oligosaccharides*. J Cell Sci, 1995. **108 (Pt 4)**: p. 1617-27.
27. Nilsson, T. and G. Warren, *Retention and retrieval in the endoplasmic reticulum and the Golgi apparatus*. Curr Opin Cell Biol, 1994. **6**(4): p. 517-21.
28. Orci, L., B.S. Glick, and J.E. Rothman, *A new type of coated vesicular carrier that appears not to contain clathrin: its possible role in protein transport within the Golgi stack*. Cell, 1986. **46**(2): p. 171-84.
29. Grasse, P.P., *[Ultrastructure, polarity and reproduction of Golgi apparatus.]*. C R Hebd Seances Acad Sci, 1957. **245**(16): p. 1278-81.

30. Balch, W.E., B.S. Glick, and J.E. Rothman, *Sequential intermediates in the pathway of intercompartmental transport in a cell-free system*. Cell, 1984. **39**(3 Pt 2): p. 525-36.
31. Jamieson, J.D. and G.E. Palade, *Intracellular transport of secretory proteins in the pancreatic exocrine cell. 3. Dissociation of intracellular transport from protein synthesis*. J Cell Biol, 1968. **39**(3): p. 580-8.
32. Jamieson, J.D. and G.E. Palade, *Intracellular transport of secretory proteins in the pancreatic exocrine cell. I. Role of the peripheral elements of the Golgi complex*. J Cell Biol, 1967. **34**(2): p. 577-96.
33. Baenziger, J. and S. Kornfeld, *Structure of the carbohydrate units of IgA1 immunoglobulin. I. Composition, glycopeptide isolation, and structure of the asparagine-linked oligosaccharide units*. J Biol Chem, 1974. **249**(22): p. 7260-9.
34. Novick, P., C. Field, and R. Schekman, *Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway*. Cell, 1980. **21**(1): p. 205-15.
35. Balch, W.E., et al., *Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of N-acetylglucosamine*. Cell, 1984. **39**(2 Pt 1): p. 405-16.
36. Marchi, F. and C.P. Leblond, *Radioautographic characterization of successive compartments along the rough endoplasmic reticulum-Golgi pathway of collagen precursors in foot pad fibroblasts of [3H]proline-injected rats*. J Cell Biol, 1984. **98**(5): p. 1705-9.
37. Dahan, S., et al., *Concentration of intracellular hepatic apolipoprotein E in Golgi apparatus saccular distensions and endosomes*. J Cell Biol, 1994. **127**(6 Pt 2): p. 1859-69.
38. Fujiwara, T., et al., *Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum*. J Biol Chem, 1988. **263**(34): p. 18545-52.
39. Misumi, Y., et al., *Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes*. J Biol Chem, 1986. **261**(24): p. 11398-403.
40. Lippincott-Schwartz, J., et al., *Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER*. Cell, 1989. **56**(5): p. 801-13.
41. Nilsson, T., et al., *Overlapping distribution of two glycosyltransferases in the Golgi apparatus of HeLa cells*. J Cell Biol, 1993. **120**(1): p. 5-13.
42. Love, H.D., et al., *Isolation of functional Golgi-derived vesicles with a possible role in retrograde transport*. J Cell Biol, 1998. **140**(3): p. 541-51.
43. Orci, L., et al., *Anterograde flow of cargo across the golgi stack potentially mediated via bidirectional "percolating" COPI vesicles*. Proc Natl Acad Sci U S A, 2000. **97**(19): p. 10400-5.
44. Orci, L., et al., *Exclusion of golgi residents from transport vesicles budding from Golgi cisternae in intact cells*. J Cell Biol, 2000. **150**(6): p. 1263-70.
45. Pelham, H.R. and J.E. Rothman, *The debate about transport in the Golgi--two sides of the same coin?* Cell, 2000. **102**(6): p. 713-9.
46. Lanoix, J., et al., *Sorting of Golgi resident proteins into different subpopulations of COPI vesicles: a role for ArfGAP1*. J Cell Biol, 2001. **155**(7): p. 1199-212.
47. Malsam, J., et al., *Golgin tethers define subpopulations of COPI vesicles*. Science, 2005. **307**(5712): p. 1095-8.
48. Waters, M.G., T. Serafini, and J.E. Rothman, *'Coatomer': a cytosolic protein complex containing subunits of non-clathrin-coated Golgi transport vesicles*. Nature, 1991. **349**(6306): p. 248-51.
49. Lowe, M. and T.E. Kreis, *In vitro assembly and disassembly of coatomer*. J Biol Chem, 1995. **270**(52): p. 31364-71.
50. Chardin, P., et al., *A human exchange factor for ARF contains Sec7- and pleckstrin-homology domains*. Nature, 1996. **384**(6608): p. 481-4.
51. Franco, M., et al., *Myristoylation-facilitated binding of the G protein ARF1GDP to membrane phospholipids is required for its activation by a soluble nucleotide exchange factor*. J Biol Chem, 1996. **271**(3): p. 1573-8.
52. Antonny, B., et al., *N-terminal hydrophobic residues of the G-protein ADP-ribosylation factor-1 insert into membrane phospholipids upon GDP to GTP exchange*. Biochemistry, 1997. **36**(15): p. 4675-84.
53. Zhao, L., et al., *GTP-dependent binding of ADP-ribosylation factor to coatomer in close proximity to the binding site for dilysine retrieval motifs and p23*. J Biol Chem, 1999. **274**(20): p. 14198-203.
54. Eugster, A., et al., *The alpha- and beta'-COP WD40 domains mediate cargo-selective interactions with distinct di-lysine motifs*. Mol Biol Cell, 2004. **15**(3): p. 1011-23.
55. Tanigawa, G., et al., *Hydrolysis of bound GTP by ARF protein triggers uncoating of Golgi-derived COP-coated vesicles*. J Cell Biol, 1993. **123**(6 Pt 1): p. 1365-71.
56. Cukierman, E., et al., *The ARF1 GTPase-activating protein: zinc finger motif and Golgi complex localization*. Science, 1995. **270**(5244): p. 1999-2002.
57. Reinhard, C., et al., *Functional reconstitution of COPI coat assembly and disassembly using chemically defined components*. Proc Natl Acad Sci U S A, 2003. **100**(14): p. 8253-7.
58. Nickel, W., et al., *Uptake by COPI-coated vesicles of both anterograde and retrograde cargo is inhibited by GTPgammaS in vitro*. J Cell Sci, 1998. **111 (Pt 20)**: p. 3081-90.
59. Malsam, J., et al., *A role for ADP ribosylation factor in the control of cargo uptake during COPI-coated vesicle biogenesis*. FEBS Lett, 1999. **462**(3): p. 267-72.

60. Pepperkok, R., et al., *COPI vesicles accumulating in the presence of a GTP restricted arf1 mutant are depleted of anterograde and retrograde cargo*. J Cell Sci, 2000. **113 (Pt 1)**: p. 135-44.
61. Lanoix, J., et al., *GTP hydrolysis by arf-1 mediates sorting and concentration of Golgi resident enzymes into functional COP I vesicles*. Embo J, 1999. **18**(18): p. 4935-48.
62. Goldberg, J., *Decoding of sorting signals by coatomer through a GTPase switch in the COPI coat complex*. Cell, 2000. **100**(6): p. 671-9.
63. Goldberg, J., *Structural and functional analysis of the ARF1-ARFGAP complex reveals a role for coatomer in GTP hydrolysis*. Cell, 1999. **96**(6): p. 893-902.
64. Szafer, E., M. Rotman, and D. Cassel, *Regulation of GTP hydrolysis on ADP-ribosylation factor-1 at the Golgi membrane*. J Biol Chem, 2001. **276**(51): p. 47834-9.
65. Weiss, M. and T. Nilsson, *A kinetic proof-reading mechanism for protein sorting*. Traffic, 2003. **4**(2): p. 65-73.
66. Bigay, J., et al., *Lipid packing sensed by ArfGAP1 couples COPI coat disassembly to membrane bilayer curvature*. Nature, 2003. **426**(6966): p. 563-6.
67. Bigay, J., et al., *ArfGAP1 responds to membrane curvature through the folding of a lipid packing sensor motif*. Embo J, 2005. **24**(13): p. 2244-53.
68. Stenmark, H. and V.M. Olkkonen, *The Rab GTPase family*. Genome Biol, 2001. **2**(5): p. REVIEWS3007.
69. Cai, H., K. Reinisch, and S. Ferro-Novick, *Coats, tethers, Rabs, and SNAREs work together to mediate the intracellular destination of a transport vesicle*. Dev Cell, 2007. **12**(5): p. 671-82.
70. Waters, M.G., D.O. Clary, and J.E. Rothman, *A novel 115-kD peripheral membrane protein is required for intercisternal transport in the Golgi stack*. J Cell Biol, 1992. **118**(5): p. 1015-26.
71. Nakamura, N., et al., *The vesicle docking protein p115 binds GM130, a cis-Golgi matrix protein, in a mitotically regulated manner*. Cell, 1997. **89**(3): p. 445-55.
72. Sonnichsen, B., et al., *A role for giantin in docking COPI vesicles to Golgi membranes*. J Cell Biol, 1998. **140**(5): p. 1013-21.
73. Linstedt, A.D., et al., *Binding relationships of membrane tethering components. The giantin N terminus and the GM130 N terminus compete for binding to the p115 C terminus*. J Biol Chem, 2000. **275**(14): p. 10196-201.
74. Suvorova, E.S., R. Duden, and V.V. Lupashin, *The Sec34/Sec35p complex, a Ypt1p effector required for retrograde intra-Golgi trafficking, interacts with Golgi SNAREs and COPI vesicle coat proteins*. J Cell Biol, 2002. **157**(4): p. 631-43.
75. Zolov, S.N. and V.V. Lupashin, *Cog3p depletion blocks vesicle-mediated Golgi retrograde trafficking in HeLa cells*. J Cell Biol, 2005. **168**(5): p. 747-59.
76. Sacher, M., et al., *TRAPP, a highly conserved novel complex on the cis-Golgi that mediates vesicle docking and fusion*. Embo J, 1998. **17**(9): p. 2494-503.
77. Sacher, M., et al., *TRAPP I implicated in the specificity of tethering in ER-to-Golgi transport*. Mol Cell, 2001. **7**(2): p. 433-42.
78. Sollner, T., et al., *SNAP receptors implicated in vesicle targeting and fusion*. Nature, 1993. **362**(6418): p. 318-24.
79. Weimbs, T., et al., *A conserved domain is present in different families of vesicular fusion proteins: a new superfamily*. Proc Natl Acad Sci U S A, 1997. **94**(7): p. 3046-51.
80. Weber, T., et al., *SNAREpins: minimal machinery for membrane fusion*. Cell, 1998. **92**(6): p. 759-72.
81. Fasshauer, D., et al., *Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs*. Proc Natl Acad Sci U S A, 1998. **95**(26): p. 15781-6.
82. Monck, J.R. and J.M. Fernandez, *The exocytotic fusion pore and neurotransmitter release*. Neuron, 1994. **12**(4): p. 707-16.
83. Hanson, P.I., et al., *Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep-etch electron microscopy*. Cell, 1997. **90**(3): p. 523-35.
84. Sollner, T., et al., *A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion*. Cell, 1993. **75**(3): p. 409-18.
85. Rout, M.P., et al., *The yeast nuclear pore complex: composition, architecture, and transport mechanism*. J Cell Biol, 2000. **148**(4): p. 635-51.
86. Blondeau, F., et al., *Tandem MS analysis of brain clathrin-coated vesicles reveals their critical involvement in synaptic vesicle recycling*. Proc Natl Acad Sci U S A, 2004. **101**(11): p. 3833-8.
87. Girard, M., et al., *Non-stoichiometric relationship between clathrin heavy and light chains revealed by quantitative comparative proteomics of clathrin-coated vesicles from brain and liver*. Mol Cell Proteomics, 2005. **4**(8): p. 1145-54.
88. Cox, B., T. Kislinger, and A. Emili, *Integrating gene and protein expression data: pattern analysis and profile mining*. Methods, 2005. **35**(3): p. 303-14.
89. Kislinger, T., et al., *Global survey of organ and organelle protein expression in mouse: combined proteomic and transcriptomic profiling*. Cell, 2006. **125**(1): p. 173-86.
90. Bordier, C., *Phase separation of integral membrane proteins in Triton X-114 solution*. J Biol Chem, 1981. **256**(4): p. 1604-7.

91. Kartberg, F., Hiding, J., and Nilsson, T., *Purification of COPI vesicles.*, in *Cell Biology: A Laboratory Handbook*, N.C. J. Celis, K. Simmons, V. Small, T. Hunter, and D.M. Shotton, Editor. 2005.
92. Wegmann, D., et al., *Novel isotypic gamma/zeta subunits reveal three coatomer complexes in mammals*. Mol Cell Biol, 2004. **24**(3): p. 1070-80.
93. Malhotra, V. and S. Mayor, *Cell biology: the Golgi grows up*. Nature, 2006. **441**(7096): p. 939-40.
94. Bornhauser, B.C., P.A. Olsson, and D. Lindholm, *MSAP is a novel MIR-interacting protein that enhances neurite outgrowth and increases myosin regulatory light chain*. J Biol Chem, 2003. **278**(37): p. 35412-20.
95. Mayer, A., et al., *Phosphatidylinositol 4,5-bisphosphate regulates two steps of homotypic vacuole fusion*. Mol Biol Cell, 2000. **11**(3): p. 807-17.
96. Grishanin, R.N., et al., *CAPS acts at a prefusion step in dense-core vesicle exocytosis as a PIP2 binding protein*. Neuron, 2004. **43**(4): p. 551-62.
97. Sweeney, D.A., A. Siddhanta, and D. Shields, *Fragmentation and re-assembly of the Golgi apparatus in vitro. A requirement for phosphatidic acid and phosphatidylinositol 4,5-bisphosphate synthesis*. J Biol Chem, 2002. **277**(4): p. 3030-9.
98. Siddhanta, A., et al., *Fragmentation of the Golgi apparatus. A role for beta III spectrin and synthesis of phosphatidylinositol 4,5-bisphosphate*. J Biol Chem, 2003. **278**(3): p. 1957-65.
99. Lin, C.C., et al., *ER/Golgi intermediates acquire Golgi enzymes by brefeldin A-sensitive retrograde transport in vitro*. J Cell Biol, 1999. **147**(7): p. 1457-72.
100. Saraste, J. and E. Kuismanen, *Pre- and post-Golgi vacuoles operate in the transport of Semliki Forest virus membrane glycoproteins to the cell surface*. Cell, 1984. **38**(2): p. 535-49.
101. Ostermann, J., *Stoichiometry and kinetics of transport vesicle fusion with Golgi membranes*. EMBO Rep, 2001. **2**(4): p. 324-9.
102. Nagahama, M., et al., *A v-SNARE implicated in intra-Golgi transport*. J Cell Biol, 1996. **133**(3): p. 507-16.
103. Scott, M.S., D.Y. Thomas, and M.T. Hallett, *Predicting subcellular localization via protein motif co-occurrence*. Genome Res, 2004. **14**(10A): p. 1957-66.
104. Tucker, W.C., et al., *Identification of synaptotagmin effectors via acute inhibition of secretion from cracked PC12 cells*. J Cell Biol, 2003. **162**(2): p. 199-209.
105. Watt, S.A., et al., *Subcellular localization of phosphatidylinositol 4,5-bisphosphate using the pleckstrin homology domain of phospholipase C delta1*. Biochem J, 2002. **363**(Pt 3): p. 657-66.
106. Wenk, M.R., et al., *Phosphoinositide profiling in complex lipid mixtures using electrospray ionization mass spectrometry*. Nat Biotechnol, 2003. **21**(7): p. 813-7.
107. Godi, A., et al., *ARF mediates recruitment of PtdIns-4-OH kinase-beta and stimulates synthesis of PtdIns(4,5)P2 on the Golgi complex*. Nat Cell Biol, 1999. **1**(5): p. 280-7.
108. Jones, D.H., et al., *Type I phosphatidylinositol 4-phosphate 5-kinase directly interacts with ADP-ribosylation factor 1 and is responsible for phosphatidylinositol 4,5-bisphosphate synthesis in the golgi compartment*. J Biol Chem, 2000. **275**(18): p. 13962-6.
109. Elazar, Z., et al., *ADP-ribosylation factor and coatomer couple fusion to vesicle budding*. J Cell Biol, 1994. **124**(4): p. 415-24.