# *In vitro* reconstitution of *trans*-Golgi exit

# and

# the effect of GAG attachment on protein sorting

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Dekan

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# Summary

**The *trans*-Golgi network is one of the main sorting stations for proteins and lipids in the eukaryotic endomembrane system. Yet, the mechanisms for sorting and transport out of the *trans*-Golgi network remain poorly understood. In this work, an *in vitro* procedure to reconstitute carrier formation at the *trans*-Golgi was established. Using this assay, it could be shown that formation of carriers containing the asialoglycoprotein receptor H1 and the cation dependent mannose 6-phosphate receptor is independent of the clathrin machinery, but dependent on GTP hydrolysis. This is in contrast to the common notion that the mannose 6-phosphate receptor exits the Golgi in clathrin coated vesicles, but is consistent with recent evidence for tubular export carriers or maturation.**

**The assay is based on sulfation, which can only take place in the *trans*-Golgi and thus defines the starting compartment. To improve the radioactive sulfation signal in the assay, a novel tag was developed that contains an attachment site for glycosaminoglycans (long, linear and heavily sulfated carbohydrate polymers). To investigate the influence of glycosaminoglycan attachment on protein sorting, biosynthetic exocytosis, endocytosis, endocytic recycling, and overall stability of H1 with or without glycan was examined. While stability and recycling were unaffected, exocytosis was accelerated and endocytosis was slowed down by carbohydrate attachment. Endocytosis was impaired due to interactions of glycosaminoglycan with the extracellular matrix, but the alteration of exocytosis rate requires further investigation, as it suggests that the glycan diverts the protein to a different sorting route.**

# Introduction

The eukaryotic cell is subdivided into several compartments, also called organelles. They are delineated by membranes, which can be exchanged between organelles through closed carriers in a process called membrane traffic. The compartmentalization is prerequisite for efficient regulation of the complex interaction network between lipids, proteins and carbohydrates. Organization within a cell is itself the basis for regulation of interactions between different cells that give rise to tissue and ultimately a multicellular organism. Subcellular distribution of functions (e.g. protein degradation in lysosomes, or metabolite uptake at the plasma membrane (PM)) is the foundation of cellular activity. To achieve this, molecules involved in these functions have to be sorted to specific places inside the cell. Some proteins are sorted to an organelle of permanent residence, for example lysosomal hydrolases that degrade proteins to lysosomes. For other proteins, transport between compartments is part of their function, for example endocytic receptors that shuttle nutrients from the plasma membrane to the endosomes and then recycle to the PM for another round of ligand binding.

It is therefore very rewarding for a scientist to understand and help elucidate the trafficking events inside the cell. Sorting at the *trans*-Golgi network (TGN), one of the most important organelles involved in intracellular distribution of newly synthesized proteins, is the main subject of this thesis.

# Sorting of proteins in the eukaryotic endomembrane system

Proteins are synthesized on ribosomes that are either free in the cytosol, for cytosolic, nuclear, peroxisomal and mitochondrial proteins (also chloroplast proteins in plants), or at the membrane of the endoplasmic reticulum (ER). At the ER, proteins destined to the secretory pathway (i.e. the Golgi apparatus, the ER-Golgi intermediate compartment (ERGIC) and ER), the endosomal-lysosomal system, the plasma membrane (PM) or for secretion are translated through the translocation pore into the lumen. In this chapter, I will briefly describe the mechanism of sorting and trafficking itineraries of these proteins (Fig. 1).

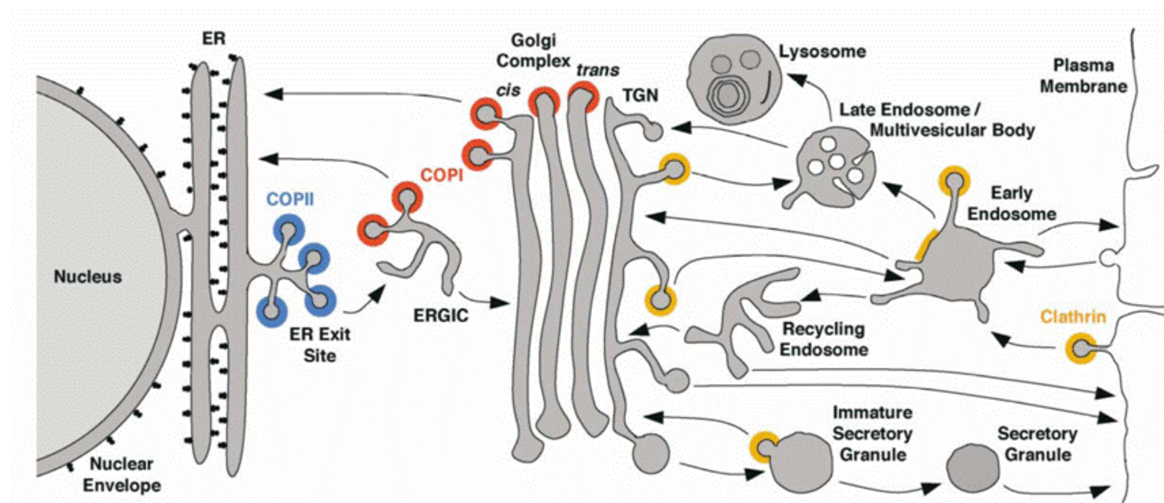


***Fig. 1: Schematic overview of the secretory pathway.*** *All common sorting pathways between major secretory pathway compartments are indicated with arrows. COPI coats (red) form at the ERGIC and Golgi membranes, COPII coats (blue) form at the ER, and clathrin coats (yellow) form on the TGN, the endosomes, the immature secretory granules, and the PM. Adapted from (Bonifacino and Glick, 2004).*

## Mechanisms for vesicular membrane traffic

Each organelle in the eukaryotic endomembrane system is defined by a specific set of lipids and proteins. Some lipids, such as phosphoinositides, can be produced and consumed at the membrane of a specific compartment (Di Paolo and De Camilli, 2006), while others, such as cholesterol need to be transported (Maxfield and Tabas, 2005). Similarly, cytosolic proteins can be transiently recruited to an organelle, while transmembrane and lumenal proteins need to be transported there from the site of

their synthesis. The sorting information of such a protein is contained within the protein itself. It can reside in a short amino acid stretch, a whole domain, or a posttranslational modification. The post-ER sorting of proteins relies on membrane enclosed carriers that bud from the donor compartment and fuse with the membrane of the acceptor compartment.

The best studied mechanism for formation of membrane carriers is mediated by cytoplasmic coat proteins that bend the membrane to form a bud. In order to ensure specific targeting, SNARE proteins that are responsible for fusion are also incorporated. The last step in the formation of a vesicle is its scission from the donor membrane, for example performed by dynamin. The released vesicle is then at least partially uncoated and moves, possibly on cytoskeleton tracks to the acceptor membrane. Specific factors tether the vesicle to the target compartment and fusion is mediated by SNARE proteins. The vesicle SNARE (v-SNARE) binds a SNARE on the target membrane (t-SNARE) and vesicle membrane becomes incorporated into the membrane of the acceptor compartment (Fig. 2; Bonifacino and Glick, 2004).

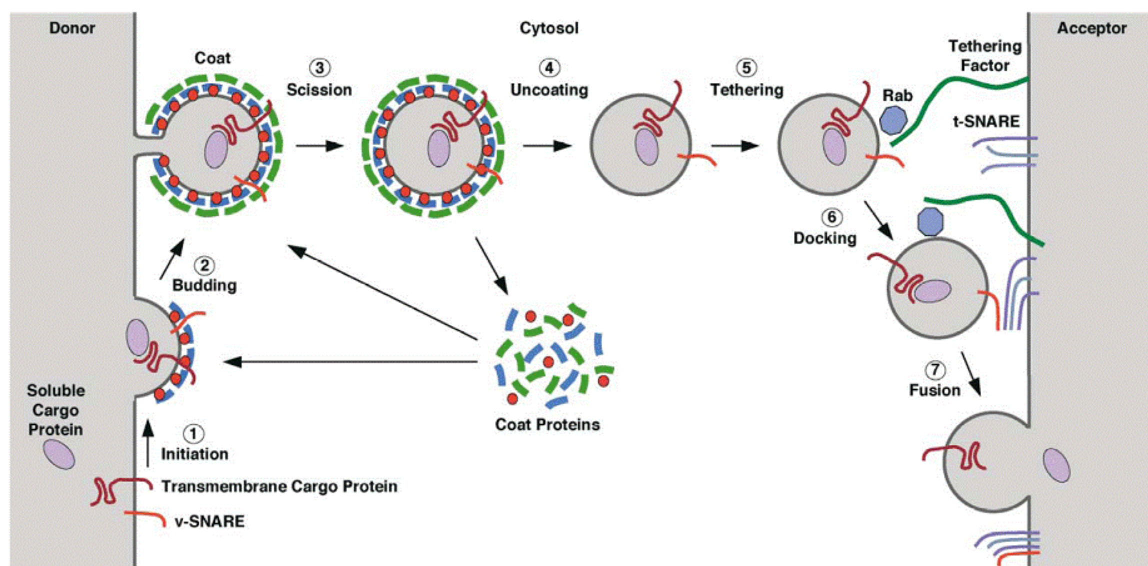


***Fig. 2: Mechanism of vesicle budding and fusion.*** *Cytosolic coat proteins are recruited to cargo in donor membranes, where they form a vesicle. The vesicle moves to the acceptor membrane to which it is tethered and subsequently fused (see details in text). Adapted from (Bonifacino and Glick, 2004).*

Coat proteins bind sorting signals in the cytosolic portion of transmembrane proteins. Soluble proteins rely on a transmembrane cargo receptor. Coat polymerization causes concentration of cargo and is coincident with generation of vesicles.

### *Small GTPases regulate membrane traffic*

Coat assembly and tethering of the vesicle to the acceptor membrane is regulated by small GTPases like Arf family and Rab family proteins (Munro, 2004). These proteins act as molecular switches, important in regulation of, amongst other cellular events, trafficking. They are activated by guanine nucleotide exchange factors (GEFs), which exchange GDP for GTP, and remain active until GTP hydrolysis. GTPase activating proteins (GAPs) stimulate GTPases to hydrolyse GTP and are in some cases necessary for this.

Rab proteins, which are GTPases required for tethering, are important regulators of membrane traffic. They can incorporate into the cytosolic leaflet of the vesicle membrane and by interaction with their effectors, also called tethering factors, direct vesicles to the correct acceptor membranes. Rabs are also able to form domains on organelles thereby defining organellar identity. For example Rab5, which is involved in endosomal tethering, is able to induce a positive feedback loop by recruiting its own GEF Rabex-5 to endosomal membranes, which in turn recruits more Rab5. This GTPase also recruits the phosphatidyl inositol 3-phosphate (PI3P) kinase (PI(3)K), which generates lipids typical for endosomes (i.e. PI3P). The combination of lipid and Rab recruits various effectors, which cluster in this Rab domain (Zerial and McBride, 2001).

### *Vesicle coats and sorting signals they decode*

The three best characterized coats in the mammalian endomembrane system are COPI, COPII and clathrin coats (Fig. 1; Kirchhausen, 2000). COPI coats act on Golgi and ERGIC membranes. They consist of a polymer of heptameric coatomer complexes. These are recruited to the membrane by the small GTPase Arf1 and bind e.g. the KKXX motif at the cytosolic C-terminus of transmembrane proteins (Lee et al., 2004). In absence of cargo, coatomer as well as ArfGAP1 cause rapid GTP

hydrolysis, and hence release of coat proteins to the cytosol. If cargo is present however, Arf1 remains active thus allowing coat formation (Nie et al., 2003).

COPII coats generate vesicles on ER membranes. Coat assembly is regulated by the small GTPase Sar1, which recruits the inner layer of COPII coats, the Sec23/24 dimer. The sec23 subunit binds the GTPase and acts as a GAP, while sec24 binds cargo. The outer layer that is responsible for membrane bending consists of sec13/31. A plethora of sorting signals mediate binding to sec23/24, but the best characterized are the di-acidic ([D/E]X[D/E]) and di-hydrophobic (FF, LL, YY, FY near or at the C-terminus) motifs in the cytoplasmic portion of transmembrane cargo (Sato and Nakano, 2007).

The best studied sorting signals in the secretory pathway recruit the clathrin transport machinery. These include tyrosine signals, with the consensus sequence YXXΦ (Φ being a bulky, hydrophobic residue) or di-leucine signals (LL or LI preceded by a short, acidic amino acid stretch; Bonifacino and Traub, 2003). These motifs act as signals for internalization at the PM, endosomal/lysosomal targeting, recycling from endosomes to the PM and sorting to the basolateral PM in polarized cells. Another tyrosine signal, with the NPXY motif mediates internalization only. Sorting signals are not recognized by clathrin, but by an adaptor protein layer that itself recruits the clathrin scaffold. Coat assembly is regulated by Arf proteins (D'Souza-Schorey and Chavrier, 2006). Clathrin is responsible for curving the membrane and the GTPase dynamin for vesicle scission (Sweitzer and Hinshaw, 1998). Adaptors connect clathrin to cargo and membrane lipids, and provide trafficking specificity, since clathrin coats mediate transport to and from various different organelles (Owen et al., 2004), i.e. the Golgi apparatus, the endosomes and the PM.

The first clathrin adaptors to be discovered were AP complexes (assembly polypeptides, later renamed to adaptor proteins; Zaremba and Keen, 1983). There are 4 different AP complexes (AP-1 through AP-4), each with specific trafficking itineraries. They are heterotetramers, and consist of two ~100 kDa adaptins (β1 through β4, and α, γ, δ, ε in AP-1 through AP-4, respectively), one medium subunit of ~50 kDa (μ1 through μ4) and one small subunit of ~20 kDa (σ1 through σ4). APs

bind tyrosine sorting signals with their μ subunit (Ohno et al., 1995). The crystal structure for this interaction has been solved for the μ2 subunit of AP-2 (Owen and Evans, 1998). The combinations of γ/σ1 in AP-1 and δ/σ3 in AP-3 bound di-leucine signals in a yeast three-hybrid assay (Janvier et al., 2003). Even though all APs mediate trafficking events (Robinson, 2004), AP-3 does not need, and AP-4 is not able to bind clathrin. These two adaptors together with AP-1 localize to the Golgi and endosomes based on immuno-fluorescence microscopy (with AP-4 predominantly on the Golgi and AP-3 predominantly on endosomes). AP-2 is the endocytosis adaptor and thus localizes to the PM (Owen et al., 2004).

The different AP isoforms provide organelle specificity. For example, AP-1 requires activated Arf1 to be recruited to membranes (Zhu et al., 1999). Since Arf1 localizes to Golgi and endosomal membranes, AP-1 can only be recruited to these organelles. Arf1 binding is not sufficient for recruitment; the adaptor exhibits a preference for PI4P, a Golgi specific lipid (Heldwein et al., 2004; Wang et al., 2003). Once recruited, AP-1 can bind cytosolic tails of transmembrane proteins and polymerize, thereby concentrating cargo independently of clathrin polymerization (Meyer et al., 2005).

Another clathrin adaptor type acting on Golgi and on endosomes is the GGA protein family (Golgi-localized, γ ear-containing, Arf-binding proteins; GGA1 through 3) (Ghosh and Kornfeld, 2004). They are monomeric and have 4 domains: the VHS domain binds di-leucine signals, GAT domain binds to Arf1 and ubiquitin (Scott et al., 2004; Shiba et al., 2004), the hinge domain binds clathrin, and the γ-ear domain binds accessory proteins (Bonifacino, 2004); they are thus *bona fide* clathrin adaptors. The sorting signal recognized by GGAs differs from the one recognized by AP-1, as the former binds the DXXLL motif and the latter the [D/E]XXXL[L/I] motif. GGAs also bind mono-ubiquitin. Attachment of a single ubiquitin, a small 8.5 kDa protein, to a lysine residue in the cytoplasmic domain of transmembrane cargo is a signal for endocytosis and lysosomal targeting (Traub and Lukacs, 2007). In contrast, polyubiquitination (generation of ubiquitin chains) has previously been shown to mediate proteasomal degradation.

Many other proteins contribute to selectivity and formation of CCVs. Epsins and Hrs (Vps27p in yeast) for example also bind ubiquitin, while Dab2 and ARH are specific for NPXY motif mediated endocytosis of low density lipoprotein (LDL) receptors. Other accessory proteins, such as AP180/CALM or the amphiphysins bind lipids. Amphiphysins are also responsible for recruitment of dynamin and later synaptojanin, which aids in vesicle uncoating (reviewed in (Owen et al., 2004)).

## Itineraries of membrane traffic

Proteins in the secretory pathway follow specific routes. For example, it is unusual for a protein to directly travel from the ER to the PM or from the PM to the ER. In this chapter, trafficking routes of ER-synthesized proteins will be described alongside the organelles to which these proteins can be sorted (Fig. 1).

### *Targeting to ER membranes*

The ER is a membranous meshwork contiguous with the nuclear membrane. Proteins synthesized on the rough ER bear a signal sequence (Blobel and Dobberstein, 1975a, b) that, as soon as it emerges from the ribosome, is bound by the signal recognition particle (SRP). SRP then binds to the SRP receptor that is integrated in the ER membrane and the ribosome docks to the translocon. This protein forms a proteinaceous channel through the membrane, through which the emerging peptide is usually co-translationally translocated in higher eukaryotes; posttranslational translocation is also possible, but common only in yeast and bacteria.

Transmembrane proteins can laterally exit the translocon and become integrated into the membrane. In a process called topogenesis, the preferred orientation of a transmembrane domain is established. It can insert so that its N-terminus is cytoplasmic and its C-terminus lumenal or the opposite way (Goder and Spiess, 2001). Another form of membrane attachment is accomplished by the glycosylphosphatidylinositol (GPI) glycolipid on the lumenal side.

### *Translocation, posttranslational modifications and quality control in the ER*

In the ER proteins fold and misfolded proteins need to be efficiently removed, for example since they have the tendency to form cytotoxic aggregates. Upon emergence from the translocon on the lumenal side most proteins are modified with N-linked glycans. The oligosaccharide is transferred from a dolichol precursor to an asparagine residue where it is trimmed and modified. A cycle of glucose trimming and readdition to the glycan regulates binding to calnexin and calreticulin, which act as so called chaperones. In general, chaperones sense the folding status (e.g. BiP) and help proteins to fold by, for example, formation (or cleavage and reformation) of di-sulfide bonds carried out by protein disulfide isomerase (PDI) or isomerization of proline peptide bonds by prolyl-peptidyl isomerase (PPI). Calnexin and calreticulin retain unfolded proteins and only upon proper folding the proteins are released and allowed to exit from the ER (Ellgaard and Helenius, 2003). Proteins that do not pass this ER quality control because they are misfolded, are exported back through the translocon (in a process called ER associated degradation (ERAD; Yoshida, 2007)) for degradation by proteasomes.

### *From the ER to the Golgi apparatus*

The mechanism of carrier generation at the ER is the COPII coat formation (Sato and Nakano, 2007). Efficient export from the ER is mediated by direct interaction with COPII (Hammond and Glick, 2000) or indirectly, by interaction with cargo receptors, such as ERGIC-53, that bind the coat (Appenzeller et al., 1999). Export competent transmembrane proteins are gathered at ER exit sites (ERES) and packaged into ~60-70 nm vesicles. Also cargo that exceeds the size of a vesicle, like procollagen-I, is dependent on COP II proteins (Fromme and Schekman, 2005); recent elucidation of the structure of the scaffolding component of COP II, Sec13/31, suggests that the cage formed by these proteins can adapt various shapes and sizes, in addition to small, spherical vesicles (Stagg et al., 2006).

After ER exit, proteins pass the ER-Golgi intermediate compartment (ERGIC) on the way to the Golgi. This compartment is an important sorting platform in protein traffic between Golgi and ER in vertebrate cells (Appenzeller-Herzog and Hauri, 2006). The

anterograde (to Golgi) and retrograde (to ER) transport, as well as transport between two ERGIC entities, is dependent on microtubules (Ben-Tekaya et al., 2005).

ER-resident proteins that escape the ER are retrieved from Golgi and ERGIC to the ER in a process mediated by COPI coats (reviewed by Lee et al., 2004). These bind e.g. the KKXX ER retrieval motif at the cytosolic C-terminus of transmembrane proteins. A protein may possess such a signal and mask it upon folding or oligomerization, thus preventing its retrieval after exiting the ER. Retrograde transport of soluble proteins is mediated by the KDEL-receptor. It binds this sequence and has itself a cytosolic KKXX signal.

### *The Golgi apparatus*

As seen by electron microscopy, the Golgi apparatus is composed of stacked, flat, disc-shaped cisternae. On both sides of the stack and on the edges, tubular/vesicular structures emanate from the cisternae. The Golgi can be divided into 3 regions: *cis*, *medial* and *trans*. The entry site of proteins, the *cis*-Golgi, consists of a tubular network, the *cis*-Golgi network, and the *cis*-Golgi cisternae. The secretory pathway continues through a cisternal stack of the *medial*-Golgi, to the *trans*-Golgi cisternae. The *trans*-most side is composed of a complex tubular/vesicular network, the *trans*-Golgi network (TGN), which serves as a sorting station for proteins (Polishchuk and Mironov, 2004). From here, proteins can traffic towards the endosomes and the plasma membrane (Rodriguez-Boulan and Musch, 2005). In specialized cells, such as neurons and endocrine cells, secretion to the PM can take place in a regulated manner; secretory proteins are enriched and densely packed into secretory granules that are transported to the cell periphery where they undergo fusion with the PM triggered by an external stimulus.

The best studied, although clearly not the only, machinery for generating TGN carriers is the Arf1 dependent formation of AP-1/clathrin vesicles. Also AP-3 and AP-4 adaptors act on the TGN. An alternative mechanism is the release of tubular elements from the TGN presumably also mediated by dynamin (Rodriguez-Boulan and Musch, 2005). The traffic through and out of the Golgi apparatus and the mechanisms involved will be discussed later in detail, as they are the main subject of this thesis.

The Golgi, next to its sorting function, is important for a variety of posttranslational modifications. Here, O-linked glycosylation and processing takes place. Also, trimming and modification of the N-linked glycan precursor is performed sequentially, as the protein passes from the *cis* to the *trans* side. Another set of carbohydrates, the glycosaminoglycans (GAGs), are synthesized in the Golgi. These are heavily sulfated on the *trans* side. Also proteins can be sulfated in the *trans*-Golgi network, although not many natural proteins carry a sulfation motif (Huttner, 1988).

### *Endocytosis at the plasma membrane*

The plasma membrane is the border of the cell and thus responsible for interaction with the cell exterior. In terms of membrane traffic this is either exocytosis or endocytosis. On one hand exocytic carriers, such as secretory vesicles, fuse with the PM and release their content (e.g. hormones) to the cell exterior. Endocytosis on the other hand is the uptake of extracellular macromolecules or particles (Fig 3).

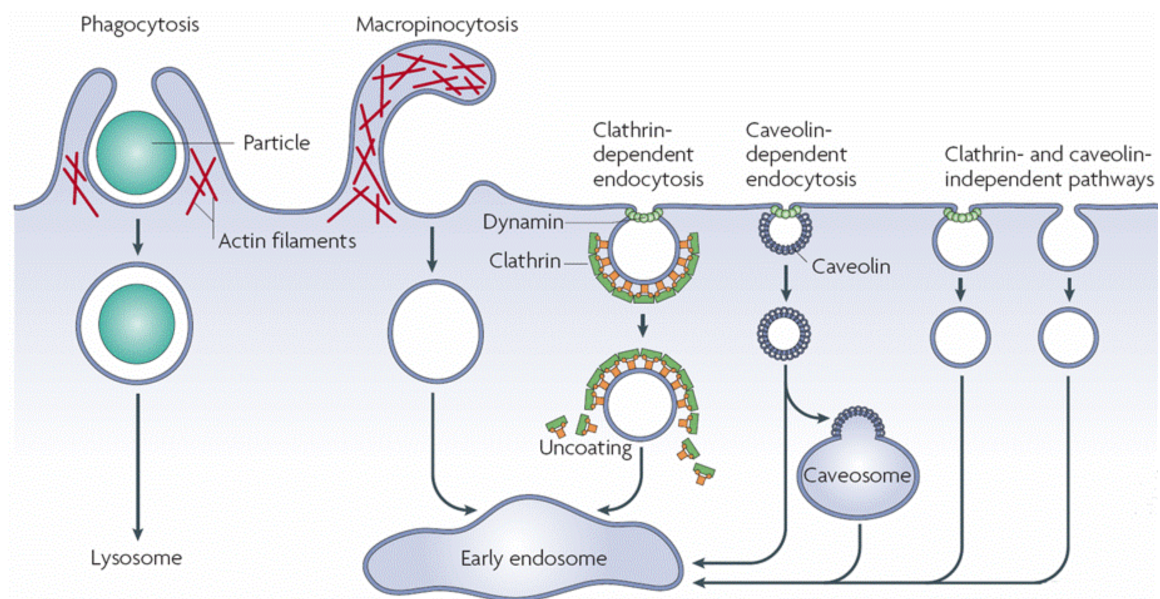


***Fig. 3: Overview of all known mechanisms for endocytosis.*** *From left to right: phagocytosis (uptake of larger particles) and pinocytosis (fluid phase uptake) depend on actin; dynamin dependent endocytosis can be mediated by cytosolic clathrin coat components, membrane integrated caveolin, or independently of these two mechanisms; dynamin independent endocytosis is common but poorly understood. Adapted from (Mayor and Pagano, 2007).*

Historically, phagocytosis (intake of large particles including pathogenic bacteria) and macropinocytosis (fluid phase uptake) were the first endocytic events observed. Both are actin dependent and mediated by remodeling of the PM at a large scale. Uptake of

soluble cargo molecules is typically mediated specific receptors. AP-2/clathrin dependent machinery, which is regulated by Arf6, is the best studied mechanism for receptor mediated endocytosis (Edeling et al., 2006). Other mechanisms that rely on dynamin scission but are coat independent, such as caveolin endocytosis, are also common. Dynamin-independent endocytosis is possible and common as well. This is referred to as constitutive endocytosis and its mechanism is poorly understood, but often depends on small GTPases, such as CDC42 or Arf6 (reviewed by Mayor and Pagano, 2007)).

### *The endosomal-lysosomal system*

Endosomes receive cargo arriving either from the plasma membrane (internalized via endocytosis) or from the Golgi. From endosomes, the proteins can be targeted to the plasma membrane, to lysosomes or to the TGN. The best studied carriers in the endosomal-lysosomal system are clathrin coated vesicles. Endosomes can be divided into early and late species. Early endosomes are implicated in sorting of internalized material to the lysosomes. After receptor mediated endocytosis, the cargo/receptor complex is sorted to the endosomes. Here, in the acidic environment of the endosomal lumen (~pH 6), cargo dissociates, and is sorted to lysosomes for degradation, while the receptor is recycled to the PM; e.g. asialoglycoprotein receptor subunit (ASGPR) H1, recycling of which depends on AP-1/clathrin machinery and Rab4 (Pagano et al., 2004). Alternatively, the receptor can be degraded in lysosomes with its ligand as a means of downregulation of PM expression (e.g. epidermal growth factor receptor; EGFR). Retrograde transport from endosomes to the TGN can also be mediated by AP-1, as is the case with mannose-6-phosphate receptor (M6PR; Meyer et al., 2000; Wan et al., 1998).

Early endosomes differ in their appearance and function, but can be divided into 2 classes: sorting endosomes, which are peripheral, and recycling endosomes, which are perinuclear. Either Rab5 alone or both Rab5 and Rab4 are markers for the former; Rab4 and Rab11 are markers for the latter. Sorting endosomes are the first transport stage for a protein after endocytosis. Endocytic vesicles can fuse with each other and/or with sorting endosomes in a Rab5 dependent manner. As these organelles are highly dynamic, they can fuse with each other as well. This process is also mediated

by Rab5. From here, rapid recycling is possible, or transport to recycling endosomes (Zerial and McBride, 2001).

Traffic from endosomes to lysosomes is still a matter of debate. Clearly, transport of some proteins, e.g. melanin to melanosomes (lysosome related organelles), depends on AP-3 (Daugherty et al., 2001; Dell'Angelica et al., 1999; Rous et al., 2002). However, maturation of endosomes to lysosomes has also been proposed. Endosomes adopt different shapes, from tubular elements to multivesicular bodies (Maxfield and McGraw, 2004) (Fig. 1). The former are parts of early endosomes, the latter are also referred to as multivesicular carrier vesicles; these form from globular domains of early endosomes. Their membrane invaginates towards the lumen to create intralumenal vesicles. This is mediated by the ESCRT protein family. Multivesicular bodies can fuse witch each other or mature to form late endosomes (van der Goot and Gruenberg, 2006). Early to late endosome/lysosome transport is mediated by Hrs proteins that bind mono-ubiquitin and polymerize clathrin into flat lattices. Also GGAs bind this sorting signal, which indicates that they are involved in lysosomal transport. Indeed, silencing of the GGA3 gene resulted in endosomal accumulation of M6PR and EGFR (Puertollano and Bonifacino, 2004).

Lysosomes are also diverse in their morphology. They are characterized by acidic pH of ~5. This is prerequisite for the action of acid hydrolases, enzymes that are involved in degradation, the main function of lysosomes. These organelles can degrade proteins, larger particles like LDL (consisting of proteins and lipids), phagocytosed material or even entire organelles in a process called autophagy.

### *Trafficking in epithelial cells*

Polarized cells possess different plasma membrane domains. Borders between tissue and lumen (e.g. gut) or the exterior (skin) of an organism are maintained by polarized cells i.e. epithelial cells. The epithelial cells that are best characterized on molecular level are the Madin Darby canine kidney cell lines (reviewed by Rodriguez-Boulan et al., 2005). They serve as a model system, and many polarized trafficking events representative for other polarized cells were discovered or confirmed in them. These cells, when grown in culture, form a tight monolayer with an apical (top) and basal (bottom) side. In the upper region, tight junctions form, which seal the space between

single cells. The tight junctions also divide the plasma membrane into an apical and a basolateral domain with different compositions, thus requiring differential protein and lipid sorting.

Polarized sorting occurs at the TGN and endosomes. At the former, soluble proteins are packaged into basolateral or apical secretory vesicles; the latter sort transmembrane receptors (e.g. LDLR) in the recycling pathway. But also in the biosynthetic pathway, i.e. coming from TGN, sorting of e.g. the ASGPR (Leitinger et al., 1995) or the transferrin receptor (TfnR; Futter et al., 1995) to the basolateral PM occurs in endosomes. In some cells, such as hepatocytes, basolateral transmembrane cargo is first sorted to the basolateral PM, and only then transported to the apical PM in a process called transcytosis.

Basolateral sorting of transmembrane cargo relies on the clathrin sorting machinery. Recently, an epithelial specific clathrin adaptor isoform, AP-1B has been discovered. It is identical to the original AP-1 complex (now renamed to AP-1A), with the exception of the μ subunit. The μ1B subunit is 80% identical to the μ1A subunit. Tyrosine motifs are the best studied basolateral sorting signals; they bind μ1B (Folsch et al., 1999). Interestingly, basolateral localization of LDLR and TfnR is dependent on AP-1B, but they bind the adaptor independently of their tyrosine motifs (Sugimoto et al., 2002). Also AP-4, the adaptor that does not bind clathrin was implicated in basolateral sorting (Simmen et al., 2002).

Apical sorting has been studied extensively and many non-cytoplasmic signals have been identified that mediate sorting to the apical PM, yet no sorting machinery has been characterized that binds these signals. N-glycosylation (Scheiffele et al., 1995; Urban et al., 1987) and O-glycosylation (Yeaman et al., 1997) mediate apical localization; recently, another set of glycans, the glycosaminoglycans, have been shown to contain sorting information (Prydz and Dalen, 2000). A lectin involved in trafficking of any of these sorting determinants has yet to be identified. In recent studies a cytoplasmic apical sorting determinant was identified (reviewed by Altschuler et al., 2003), but similar to lumenal signals, the receptor remains to be discovered.

In many cases apical sorting appears to be mediated by lipid rafts (Hanzal-Bayer and Hancock, 2007). Lipid rafts are microdomains of the membrane that have different lipid compositions from the surrounding membrane. They are enriched in cholesterol and glycolipids and are more resistant to mild detergents (i.e. 1% Triton X-100) than other membranes. The first apical sorting determinant to be discovered was the GPI-anchor (Lisanti et al., 1989), which is integrated into lipid rafts. Also, the transmembrane domain of influenza hemagglutinin (HA) was shown to integrate into rafts (Scheiffele et al., 1997). It is clear that rafts are sorted to the apical PM, but the mechanism for this remains a mystery. However, a minus end kinesin, KIF3, was implicated in raft sorting, linking apical transport to microtubules (Noda et al., 2001). In contrast, basolateral transport relies on the actin cytoskeleton (since interfering with the function of cdc42, an actin regulator, disrupts exit of basolateral cargo from TGN; Kroschewski et al., 1999; Musch et al., 2001). Also, there is evidence that apical cargo can directly recruit a motor protein; i.e. rhodopsin, which binds dynein (Tai et al., 1999; Tai et al., 2001) The involvement of the cytoskeleton in polarized trafficking is reviewed in (Rodriguez-Boulan et al., 2005).

## *Protein sorting in the Golgi apparatus*

The mechanism of anterograde transport (from the *cis* side to the *trans* side) within the Golgi is still a matter of debate; even more so, the exit from this organelle at the TGN, one of the most important sorting stations in the cell. The current understanding of Golgi traffic will be described in this chapter.

### Vesicular intra-Golgi transport versus cisternal maturation

Vesicular transport within a cell, as proposed by Palade (Palade, 1975), has been a paradigm for many years. Concerning anterograde traffic between Golgi cisternae, evidence has been provided for this mechanism (Balch et al., 1984). Particularly, COPI vesicles were found by electron microscopy to contain the vesicular stomatitis virus glycoprotein (VSVG) on its way to the plasma membrane (Orci et al., 1986). Later this was contradicted by experiments indicating that this very protein is excluded from peri-Golgi vesicles (Martinez-Menarguez et al., 2001). Moreover, they contained mannosidase II, a Golgi resident protein, albeit not concentrated, but in concentrations similar to that in the cisternae. This was explained with a cisternal

maturation model as opposed to vesicular anterograde transport between individual cisternae. The model predicts that the *cis*-most cisterna forms upon fusion of pre-Golgi carriers, and matures to the *trans*-cisterna without the need for the cargo to ever leave the lumen. Oddly enough, this was already proposed in the 1960s, based on EM studies of plants and algae. One of the most important arguments in favor of this hypothesis is the fact that procollagen-I (PC I), a secretory protein that is too large for COPI vesicles, does not leave the cisternae (Bonfanti et al., 1998). The authors show the same for VSVG, further contributing to the debate. It has later been shown that large aggregates of a chimeric reporter protein, but not PC I, can traverse the Golgi in so called megavesicles (Volchuk et al., 2000).

Also for post-Golgi, endosomal traffic, maturation has become a valuable option. The key finding to support this was recently provided by immuno-fluorescence video microscopy (Rink et al., 2005). The authors track single organelles and show that Rab5 positive organelles (early endosomes) successively lose Rab5 and acquire Rab7, a late endosomal marker. Another maturation based protein transport event is the formation of secretory granules. During and after formation of large immature secretory granules, clathrin coats are recruited, which remove non-cargo proteins and membranes, resulting in concentration of *bona fide* cargo and granule maturation (Borgonovo et al., 2006).

Recently, Golgi maturation has been visualized by 3-D video microscopy in *Saccharomyces cervisiae* (Losev et al., 2006; Matsuura-Tokita et al., 2006), where cisternae are not stacked but distributed throughout the cell, thus allowing resolution by light microscopy. The authors fluorescently labeled an early Golgi and a late Golgi resident protein with different spectral versions of GFP and tracked single cisternae as they changed colors. Additionally, they compared the maturation kinetics with transport kinetics of cargo, measured by pulse-chase experiments, and found that they correlate. According to the maturation hypothesis, COPI vesicles would mediate retrograde transport between cisternae, as they clearly do between Golgi and ER (Letourneur et al., 1994). Surprisingly, Matsuura et al. found that in COPI deficient yeast cells, maturation still occurs, although three times more slowly. This raises the question, what mechanism maintains the localization of Golgi-resident proteins.

Yet another model could explain this as proposed by Mironov et al. (reviewed in Mironov et al., 2005). It relies on the fact that secretory traffic can trigger formation of temperature sensitive continuities between Golgi cisternae (Trucco et al., 2004). This group has previously found that these connections are not important for cargo (VSVG and PC I) transport (Mironov et al., 2001), but contain Golgi enzymes (Kweon et al., 2004). Since membrane continuities between Golgi cisternae were observed by several groups before (Mironov et al., 2005) the stack is also referred to as Golgi ribbon (or Golgi stack ribbon; Mogelsvang et al., 2004).

## TGN exit

The *trans* side of the Golgi apparatus is the main sorting station for proteins in the secretory pathway. Therefore it is a very dynamic structure that is hence difficult to visualize. As the Golgi cisternae and thus the TGN were assumed for a long time to be stable compartments, the vesicular mechanism of TGN exit dominates the text books. It is undeniable that this happens, as there is a plethora of publications showing coated pits on and coated vesicles adjacent to the TGN by EM (e.g. Geuze et al., 1985; Fig. 4). However, while analysis of intra-Golgi cisternal maturation progressed, the question about the fate of the terminal cisterna was not fully answered. Detailed EM studies revealed that the 3 *trans*-most cisternae contribute to the TGN (Ladinsky et al., 1994), as opposed to one tubular network. Only the *trans*-most cisterna contained clathrin coats (Mogelsvang et al., 2004), while the others seemed to peel off the stack. These might represent post-Golgi carriers. Indeed, such carriers were observed *in vivo* to be involved in traffic to the plasma membrane (Polishchuk et al., 2000), to endosomes (Ang et al., 2004), or even retrograde, from endosomes to the TGN (Huang et al., 2001).

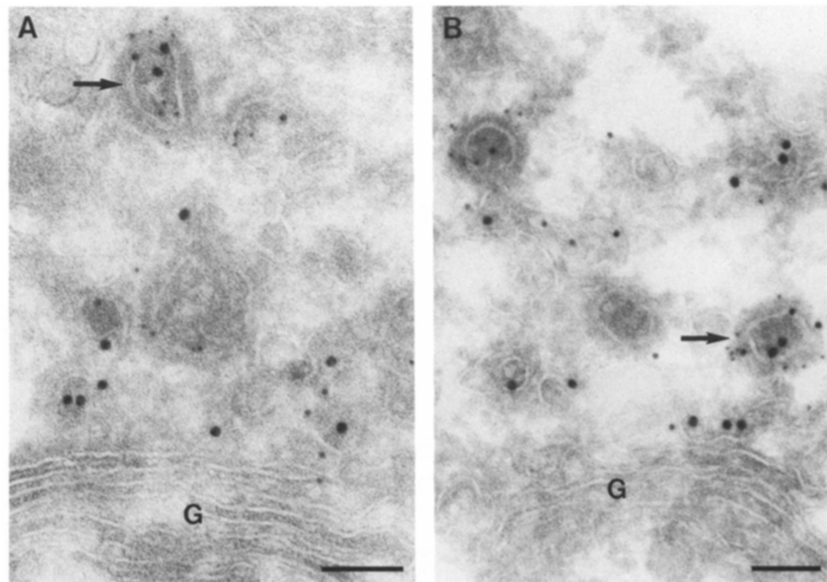


***Fig. 4: MPR and AP-1 colocalize to coated structures adjacent to the TGN.*** *(A) and (B): CD-M6PR (10 nm gold) and CI-M6PR (15 nm gold) colocalize with the γ-adaptin of AP-1 (5 nm gold) in clathrin coated vesicles (arrows) adjacent to the Golgi complex (G). Bars: 0.1 μm. Adapted from (Klumperman et al., 1993).*

### Clathrin coated vesicles

By electron microscopy, AP-1 was shown to form clathrin coated vesicles on the TGN for both, the di-leucine signal (Klumperman et al., 1993; Fig. 4), and for tyrosine signal containing proteins (Honing et al., 1996). With the discovery of GGAs, a second clathrin adaptor was provided, which can cooperate with AP-1 at the TGN. The sorting signal binding site on GGAs can be obstructed (autoinhibition) upon phosphorylation in a casein kinase 2 site (Doray et al., 2002a), thus providing a regulatory mechanism. GGAs 1 and 3 colocalize with mannose 6 phosphate receptors (M6PR) and AP-1 in clathrin coated pits at the TGN (Doray et al., 2002b). Since mutation of the GGA binding site for M6PR prevents it from entering AP-1 positive structures, the authors speculate that the GGAs hand over the cargo to AP-1. They went further to propose a model, in which the AP-1 associated casein kinase phosphorylates the GGAs thus releasing them to the cytosol (Ghosh and Kornfeld, 2003). Interestingly, in a recent *in vitro* study GGA1 without AP-1 polymerized clathrin into tubules (Zhang et al., 2007) instead of vesicles.

### *Non-vesicular carriers*

The advent of video microscopy and GFP has made it possible to visualize traffic in live cells. One of the first Golgi video microscopic studies showed TGN exit of fluorescently tagged VSVG in tubular structures (Hirschberg et al., 1998; Fig 5). Later, using correlative light electron microscopy (CLEM), these tubular carriers were shown to be up to 1.7 μm in length. These carriers did not arise from fusion of small vesicles, as PC-I was sorted within them (Polishchuk et al., 2003). Further, they aligned with microtubules and stained with anti kinesin anitbodies. The authors concluded that kinesin pulls out tubules from the TGN, which are then cleaved by an unknown mechanism. The itineraries of tubular carriers also remain fairly elusive. For example, VSVG that was used as a reporter in most of these studies, was found to bypass endosomes on its route to the basolateral PM in PtK2 cells (Keller et al., 2001), but to involve them in MDCK cells (Ang et al., 2004). More recent studies however, by using LDLR as a basolateral marker and p75NTR as an apical marker, could clearly distinguish between basolateral and apical pathways, confirming microtubule dependence of the apical (Kreitzer et al., 2003).

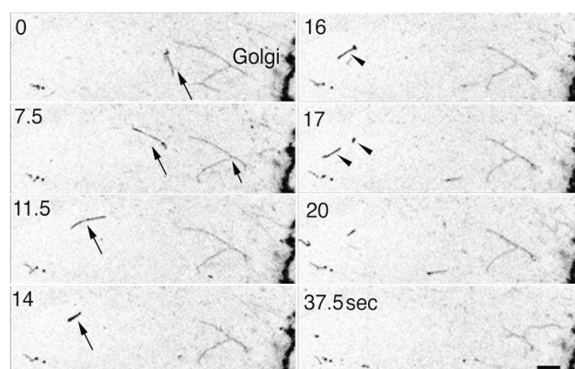


***Fig. 5: Dynamic nature of tubular structures containing VSVG after TGN exit.*** *After the release from the 20°C TGN block VSVG leaves the TGN within seconds. Short arrows indicate tubule bifurcating, long arrows indicate post-Golgi carriers extending and retracting during translocation, and arrowheads indicate carriers breaking in half. Bar: 2 μm. Adapted from (Hirschberg et al., 1998).*

Dynamin is a candidate for scission of these carriers. *In vitro* studies showed that dynamin can cleave lipid tubules generated from liposomes by kinesin (Roux et al., 2006). Another candidate is CtBP3/BARS, which was shown to mediate scission of tubular carriers containing VSVG, but not p75NTR (an apical protein), at the TGN in MDCK and COS-7 cells (Bonazzi et al., 2005). Interestingly, TGN exit of both

markers depended on dynamin, but not CtBP3/BARS in BHK cells, indicating that these scission mechanisms are somewhat redundant. The authors later showed that for COPI vesicle formation, these two scission machineries are interchangable and dependent on ArfGAP1 (a GAP for Arf1; Yang et al., 2006). Yet another Golgi exit related process regulated by Arf1 is recruitment of cortactin. This protein regulates the assembly of actin (on the PM and the TGN) and thereby aids the fission event (Cao et al., 2005).

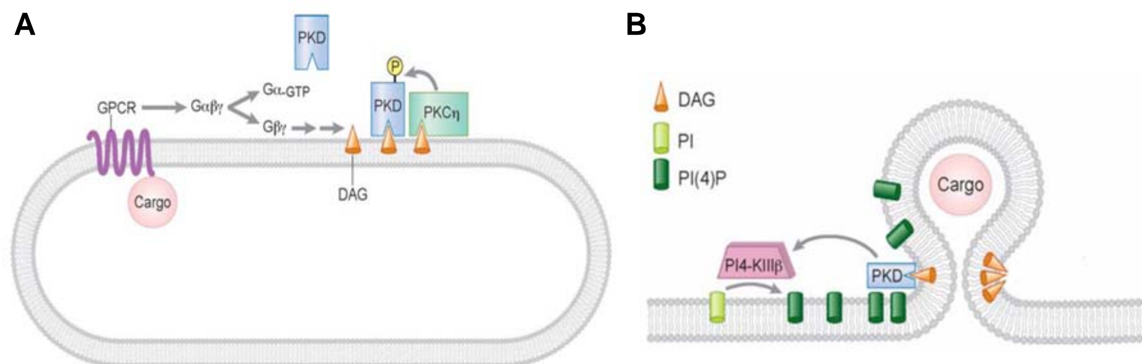


***Fig. 6: A model of PKD mediated carrier scission at the TGN.*** *(A) Cargo stimulates an unknown G-protein coupled receptor (GPCR), which activates a trimeric G-protein, leading to production of DAG. DAG recruits PKD and PKCη and subsequently the latter activates the former by phosphorylation. (B) PKD activates PI-4KIIIβ leading to localized production of PI4P. This lipid is either used as a precursor for DAG or it forms a domain that reinforces the DAG domain by mutual exclusion. DAG is conical and induces the formation of a bud-neck that facilitates scission. Adapted from (Bard and Malhotra, 2006).*

Malhotra et al. proposed a compelling model for regulation of generation of tubular carriers (Bard and Malhotra, 2006). Protein kinase D (PKD) is a regulator of scission of Golgi derived carriers (Liljedahl et al., 2001) that are destined to the basolateral PM in polarized cells (Yeaman et al., 2004). A trimeric G protein activates phospholipase C β3 (PLCβ3) at the TGN (Diaz Anel, 2007). This generates diacylglycerol (DAG), which recruits PKD (Maeda et al., 2001) as well as protein kinase Cη (PKCη; Diaz Anel and Malhotra, 2005). PKCη phosphorylates and thereby activates PKD (Fig. 6). One substrate of PKD is phosphatidylinositol 4-kinase IIIβ (PI4-KIIIβ) that generates PI4P at the TGN membrane (Hausser et al., 2005). This might induce a feedback loop, in which PI4P is converted to PI(4,5)P2 and cleaved by PLCβ3 to generate more DAG, and recruit more PKD and PKCη. DAG itself is a conical lipid that induces negative membrane curvature (bud necks). Malhotra et al.

suggest that this alone could be sufficient for scission, but other PKD substrates, along with accessory proteins that bind PI4P, could also contribute; for example, a recently characterized protein family, the FAPPs (four-phosphate-adaptor proteins), that are regulated by Arf1 (Godi et al., 2004). In particular FAPP2 was shown to be necessary in transport of HA, an apical marker, from the TGN to the plasma membrane (Vieira et al., 2005). In a very recent report the importance of FAPP2 was dramatically elevated: it was shown to link synthesis of glycosphingolipids to their Arf1 and PI4P regulated export from the TGN (D'Angelo et al., 2007).

## *Elucidation of sorting mechanisms at the TGN*

Most early studies concerning Golgi traffic were performed using purified Golgi membranes from genetically modified yeast (Balch et al., 1984). While very important, this technique is not easily transferable to mammalian systems, because genetic manipulation is more difficult and more importantly, the mammalian Golgi apparatus is far more complex than that of *S. cervisiae* and thus nigh impossible to purify in native form.

Modern investigations of TGN exit in mammalian cells almost exclusively rely on time-lapse microscopy of fluorescently labeled trafficking markers (see above), which is the most direct method, because one can observe the formation of carriers and movement of proteins. But, as every other technique, it has limitations. The limitation lies in the resolution of light microscopy, which is $\lambda/2$, i.e. 250 nm for green or even 300 nm for red light (the two most used colors). This is a low resolution to investigate vesicles, which are <100 nm, or even tubular carriers, which can be up to 1.7 µm long but only <100 nm thick. By electron microscopy the resolution barrier can be overcome, in contrast to light microscopy however, only snapshots of trafficking events can be visualized.

A limitation of *in vivo* studies in general is that only long-term effects can be observed, because elimination or overexpression of a protein takes hours to days. This may cause indirect effects or adaptation, as exemplified by the use of a VSVG mutant, VSVG ts045, which can be accumulated in the ER at 40°C. The cells are incubated for long times (e.g. over night; Ang et al., 2004) in order to generate a bulk of proteins

to enhance the signal and possibly to deplete the cells of peripheral GFP signal. The bulk of the reporter protein is then released and reaccumulated in the TGN at 20°C. Under these conditions not only is TGN exit stopped, but also the morphology of this organelle is changed (Ladinsky et al., 2002), possibly rendering the system non-physiological. Moreover, the marker could saturate its sorting machinery and "spill over" to another.

Thus, we have chosen to complement these studies with an *in vitro* TGN carrier formation assay in semi-intact cells based on sulfation, where cells are minimally manipulated until perforation of the PM. Sulfation of proteins is a TGN specific event (Huttner, 1988), and is thus a very suitable marker of this organelle. Few proteins are naturally sulfated; hence, a sulfation tag needs to be fused to the reporter protein. This tag does not affect basic trafficking properties of a protein (Leitinger et al., 1994) and was used in earlier trafficking studies (Leitinger et al., 1995). The TGN carrier formation assay allows the testing of candidate proteins for their importance in TGN exit of a reporter protein. Theoretically, this assay is expandable to any reporter that passes the TGN, no matter what trafficking routes it takes and the selection of the reporter determines which exit carrier formation is monitored. We initially concentrated our efforts on requirements for TGN-to-endosome carrier formation. We chose M6PR as a reporter, since its TGN-to-endosome traffic is studied in detail and is yet not fully elucidated, but widely accepted to be dependent on AP-1/GGA/clathrin carriers (Ghosh et al., 2003). We also investigated the ASGPR subunit H1; this protein is a typical recycling receptor that travels from the TGN to endosomes on its biosynthetic pathway (Leitinger et al., 1995), but the mechanism of this trafficking step is unknown.

## Mannose 6-phosphate receptor

There are two M6PRs, a cation independent (CI-M6PR, ~300 kDa) and the cation dependent (CD-M6PR, ~46 kDa). Both are type I transmembrane proteins and their function is to bind lysosomal hydrolases that expose an N-glycan with a mannose 6-phosphate in the TGN. The receptor then shuttles these hydrolases to late endosomes, where because of acidic pH the lysosomal protein is released and the M6PR is transported back to the TGN. The CI-M6PR has additional functions that affect its

trafficking, thus, the CD-M6PR, which is smaller and more amenable to manipulation, was more appropriate for this work and will be described in this chapter (henceforth referred to as MPR).

MPR was shown to be packaged into clathrin coated vesicles at the TGN by EM (Fig. 4), and clathrin coated vesicles containing MPR-acid hydrolase complexes were purified from tissue (Campbell and Rome, 1983) and metabolically labeled cell cultures (Schulze-Lohoff et al., 1985). This suggests that CCVs represent a trafficking intermediate between TGN and late endosomes (either direct or indirect). Lysosomal enzyme delivery, but not binding to AP-1, depended on the di-leucine signal in the cytosolic portion of MPR (Johnson and Kornfeld, 1992), and it was later shown that this motif is bound by GGAs. Consequently, GGAs, AP-1 and MPR colocalize in clathrin coated buds and vesicles at the TGN (Doray et al., 2002b). GGAs bind MPR prior to AP-1, as mutant GGAs defective in binding AP-1 trap MPRs in the TGN (Puertollano et al., 2001). Based on these studies, it appeared plausible that MPR is a positive control for AP-1/clathrin dependent TGN exit.

## Asialoglycoprotein receptor subunit H1

The ASGPR is a hepatic lectin recognizing tri- and tetra-antennary desialylated oligosaccharides, exposing a galactose or N-acetyl galactosamine (GalNAc). ASGPR is directed to the basolateral PM, internalizes its cargo via clathrin mediated endocytosis and brings it to endosomes of hepatocytes, where ligand dissociates from the receptor upon lowered pH; the receptor recycles back to the PM and the ligand is degraded after transport to lysosomes (Fuhrer and Spiess, 1996). The ASGPR is a heterotetramer composed of 2 H1 and 2 H2 subunits; when H1 is expressed alone, it builds homotrimers. While only the heterooligomeric complex can bind ligand, H1 contains the main determinant for rapid endocytosis and basolateral targeting in a tyrosine dependent manner (Fuhrer et al., 1994; Fuhrer et al., 1991; Geffen et al., 1993).

H1 is a type II transmembrane protein. After biosynthesis and transport to the Golgi, it is sorted to endosomes in the TGN (Leitinger et al., 1995). The mechanism of this transport step is unknown and is therefore one of the topics of this work. Interestingly,

while TGN exit is independent of the tyrosine sorting signal, deletion of the entire cytoplasmic domain caused retention of the protein in the TGN (Wahlberg et al., 1995).

## TGN carrier formation

*In vitro* biochemical approaches allow the monitoring of single transport events in a complex trafficking route. An early example of such a reconstitution is the revolutionary assay developed by Balch et al. (Balch et al., 1984): Golgi membranes from a wild type and an N-acetyl glucosamine (GlcNAc) transferase deficient, but VSVG transfected yeast strain were purified; transport between these two Golgi species was demonstrated by incorporation of [3H]GlcNAc into the N-glycan of VSVG (Fig 7). Later, on the basis of this intra-organellar assay, inter-organellar approaches for investigation of trafficking in yeast were developed; these approaches include ER-to-Golgi transport (Baker and Schekman, 1989), retrograde Golgi-to-ER transport (Spang and Schekman, 1998), endosome-to-vacuole transport (Vida and Gerhardt, 1999) and, most importantly for this work, Golgi-to-late endosome (Blanchette et al., 2004). While very powerful in terms of throughput, this assay did not distinguish between early endosomal and late Golgi membranes (Brickner et al., 2001).

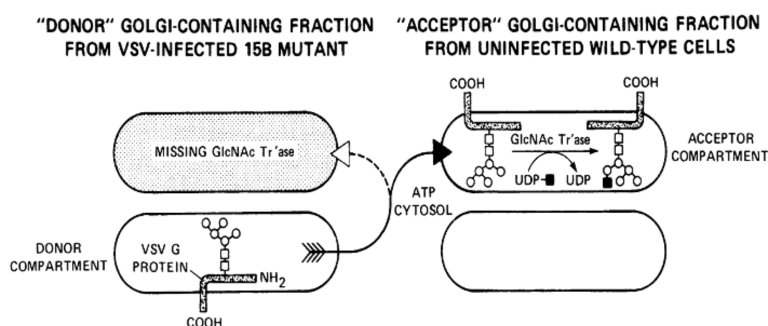


***Fig. 7: In vitro intra Golgi transport assay****. Purified Golgi membranes from a mutant yeast cell line lacking GlcNAc transferase but expressing VSVG are incubated with purified Golgi membranes from wild type yeast cell line that does not express VSVG but does express GlcNAc transferase. Only if transport between these two membrane species takes place, radioactive GlcNAc can be incorporated into the N-glycan of VSVG. This transport is dependent on ATP and cytosol. Adapted from (Balch et al., 1984).*

In mammalian cells, intra-endosomal or early-to-late endosome transport could be reconstituted from purified early endosomes (Aniento et al., 1993), but in general, organelles (specifically the Golgi apparatus) are more difficult to purify in this system and thus such studies are very challenging. Again, it was Balch and colleagues who found a solution (Beckers et al., 1987) and who developed an assay to reconstitute ER-to-Golgi transport *in vitro*. Instead of purifying the organelle, they used specific markers for the compartment of interest; for ER, this was [$^{35}$S]methionine. Cells were radio-labeled and permeabilized with a hypo-osmolar buffer, thus leaving the cells semi-intact. Cytosol and peripheral membrane proteins could be washed away using a high-salt buffer and transport reconstituted in defined conditions, i.e. with cytosol and an energy mix. This technique was later adapted for late endosome-to-TGN transport (Goda and Pfeffer, 1988); instead of labeling the reporter in the donor compartment, the authors took advantage of a mutant cell line that lacked sialyl transferase. These cells were used as donor membranes, while acceptor membranes were from wild type cells that could modify the reporter with [$^{3}$H]sialic acid. In another elegant approach, endosome-to-plasma membrane vesicle formation was reconstituted (Pagano et al., 2004). Reporter proteins were biotinylated at the cell surface, then internalized and surface biotin was removed, resulting in endosomes loaded with the biotinylated reporter.

Inspired by these studies we designed an *in vitro* TGN carrier formation assay in semi-intact cells. Different exit pathways out of the TGN could be examined with a pathway specific marker; e.g. $\alpha$1 anti-trypsin (A1Pi) for direct TGN-to-PM traffic of secretory proteins, or MPR and H1 for TGN-to-endosome traffic. Identification of the donor organelle was based on sulfation, as this only occurs in the *trans*-Golgi. Sulfation can take place on the protein itself, specifically on the tyrosine sulfation tag fused to lumenal portion of the reporter, yielding one sulfate molecule per sulfation motif (Leitinger et al., 1994). An alternative would be to fuse a glycosaminoglycan attachment site to the reporter; a GAG chain can incorporate up to hundreds of sulfate molecules thus dramatically enhancing the signal (Prydz and Dalen, 2000).

Glycosaminoglycans are linear carbohydrate polymers. There are four classes of GAGs: chondroitin sulfate (CS)/dermatan sulfate (DS), heparan sulfate (HS)/heparin, keratan sulfate (KS) and hyaluronic acid. The first 3 are attached to proteins and

synthesized in the Golgi apparatus, while hyaluronic acid is synthesized outside the cells. KS is initiated on N- and O-linked glycans, while HS/heparin and CS/DS GAGs are synthesized on proteins with a xylosylation site. This site is transplantable and was in our case 14 amino acids long. In order to use this GAG-tag in our budding assay, we needed to characterize its effects on the trafficking properties of one of our reporters, i.e. H1. Attachment of GAG resulted in accelerated exocytosis, which implied that it redirected the reporter to another TGN exit route. Thus, the GAG tagged protein did not represent wild type behavior and was omitted from TGN carrier formation experiments. Results from the characterization of GAG attachment to a reporter protein are presented in part I of the results chapter (in form of a manuscript), while the TGN carrier formation is described in part II.

# Results-Part I

## The effect of GAG attachment on protein sorting

## *Introduction*

The information for the intracellular sorting of proteins resides either in the amino acid sequence of the polypeptide itself (Bonifacino and Traub, 2003) or in its posttranslational modifications (e.g. N- and O-linked glycans; Potter et al., 2006). The best studied signals for sorting in the late secretory pathway are short cytosolic amino acid motifs that directly interact with the sorting machinery, cytosolic proteins that form a coat at the membrane and produce transport vesicles. Examples are the tyrosine motif with the consensus YXXΦ (Φ being a bulky, hydrophobic residue) and the di-leucine motif (LL or LI preceded by a short, acidic amino acid stretch), both of which are recognized by clathrin-associated adaptor proteins (APs) mediating endocytosis, basolateral sorting in polarized cells, and lysosomal transport (Bonifacino and Traub, 2003; Robinson, 2004).

In contrast, carbohydrate modifications have been shown to be involved in apical sorting, for which no general amino acid motifs have been identified yet. Treatment of MDCK cells with tunicamycin, which abrogates N-glycosylation, resulted in nonpolarized secretion of a normally apically secreted protein (Urban et al., 1987). Conversely, insertion of N-glycosylation sites into growth hormone that is normally secreted in a nonpolarized fashion resulted in apical secretion (Scheiffele et al., 1995). When a domain containing O-glycosylation sites was removed from the neurotrophin receptor p75NTR, the protein lost its apical localization (Yeaman et al., 1997). Furthermore, attachment of a glycosylphosphatidylinsoitol anchor to a polypeptide was shown to mediate apical sorting (Lisanti et al., 1989).

Glycosaminoglycans (GAGs), the third major class of glycan modifications, has also been shown to contain sorting information (Prydz and Dalen, 2000). Heparan sulfate (HS)/heparin and chondroitin sulfate (CS)/dermatan sulfate (DS) GAGs are synthesized on proteins with a xylosylation site, a serine in a Ser-Gly sequence with a flanking acidic amino acid cluster (Esko and Zhang, 1996; Zhang and Esko, 1994). A xylose, two galactose residues and a glucuronic acid (GlcUA) are sequentially attached to form a linker tetra-saccharide, before alternating addition of N-acetyl galactosamine and GlcUA, or N-acetyl glucosamine and GlcUA produces long, linear chains of CS/DS or HS/heparin, respectively. In HS, GlcUA can later be isomerized

to iduronic acid. It is not fully elucidated what mechanisms decide which GAGs are synthesized. Insertion of a tryptophan after the Ser-Gly di-peptide (Zhang and Esko, 1994) or the presence of a large, globular domain (Chen and Lander, 2001) were shown to promote HS assembly, whereas sulfation of the galactose residues in the linker has been speculated to drive CS production (Sugahara and Kitagawa, 2000; Ueno et al., 2001). Both CS/DS and HS/heparin GAGs are synthesized and heavily sulfated in the late Golgi (reviewed by Silbert and Sugumaran, 2002; Sugahara and Kitagawa, 2002).

In Madin-Darby canine kidney (MDCK) cells, HS proteoglycans (HSPGs) are predominantly secreted from the basolateral side (Caplan et al., 1987). CS, on the other hand, is preferentially sorted to the apical domain in the same cell line, whether it is attached to a protein, or to hexyl β-D-thioxyloside added as a competing acceptor molecule (Kolset et al., 1999). Glypican, a HS proteoglycan, is sorted preferentially basolaterally in Caco-2 cells and in a non-polarized manner in MDCK cells despite its GPI-anchor (Mertens et al., 1996). Upon removal of GAG chains, glypican is almost exclusively delivered to the apical membrane. Not only polarized sorting, but also secretion in general, may depend on GAGs. In CHO cells, secretion of decorin, an extracellular matrix proteoglycan, was dependent on either its N-glycan or its CS chain, by an unknown mechanism (Seo et al., 2005).

GAGs contribute to the extracellular matrix, where they regulate signal transduction and development of multicellular organisms (reviewed by Bishop et al., 2007; Gorsi and Stringer, 2007; Sugahara et al., 2003), but they also contribute to sorting of proteins on the subcellular level (reviewed by Kolset et al., 2004). For example, knock-out of NDST-2, an enzyme involved in maturation of heparin, causes reduced delivery of granule proteins to secretory granules in mast cells (Forsberg et al., 1999; Humphries et al., 1999). Proteoglycans can also serve as endocytosis receptors for cationic polymers (Payne et al., 2007).

To investigate the role of GAGs in intracellular sorting, we introduced a GAG-attachment sequence into the asialoglycoprotein (ASGP) receptor subunit H1 that normally lacks this modification. The ASGP receptor is a typical recycling receptor that mediates the uptake of galactose-terminal glycoproteins into hepatocytes. The

cytoplasmic domain of H1 contains a typical tyrosine motif necessary for clathrin-mediated endocytosis (Fuhrer et al., 1991) and basolateral sorting in polarized cells (Geffen et al., 1993). Basolateral transport was shown to depend on the epithelial-specific adaptor isoform AP-1B (Sugimoto et al., 2002), while non-polarized recycling depended on AP-1A (Pagano et al., 2004). Upon biosynthesis, H1 is transported from the TGN to the plasma membrane (PM) via endosomes (Leitinger et al., 1995).

We determined the stability, the steady-state distribution, and the kinetics of exocytosis, endocytosis, and recycling of two forms of H1, with and without an attached GAG chain. We observed a clear inhibition of endocytosis and acceleration of exocytosis upon GAG-attachment. GAG chains thus significantly affect receptor trafficking in a dominant manner.

## *Results*

### Modifying H1 with a CS glycosaminoglycanation site

In order to analyze the effect of GAG-attachment on protein sorting, GAG-attachment sequences were introduced into the sequence of the ASGP receptor H1. H1 is a type II single-spanning membrane protein that can be modified at its exoplasmic C-terminus by short sequences, such as a myc-epitope tag (Pagano et al., 2004) or a tyrosine sulfation sequence derived from pro-cholecystokinin (Fig. 8; Leitinger et al., 1994) without disturbing protein folding or transport. Similarly, we now fused short sequences including the well characterized GAG-attachment sites of amyloid precursor-like protein 2 (APLP2) and decorin to the C-terminus of H1. APLP2 and decorin were shown to be modified by chondroitin sulfate at specific serine residues (Fig. 8; Esko and Zhang, 1996). As a control for a receptor protein without GAG-attachment, H1 with the tyrosine sulfation motif (H1-TS) was used. Tyrosine sulfation, like GAG sulfation, is restricted to the TGN (Huttner, 1988). In [$^{35}$S]sulfate-labeling experiments, H1-TS and H1-GAG are thus directly comparable with respect to post-Golgi protein transport.

**TS (Tyr sulfation)**
proCCK: ...SEADYEYPS•

**GAG**
APLP2: ...ALPENEGSGVGEQD•
Decorin (L): ...FMLEDEASGIGPEVPDD•
Decorin (S): ...EDEASGIGPE•

***Fig. 8: Sulfation tags****. The tyrosine sulfation (TS) tag was derived from procholecystokinin (proCCK), GAG-attachment sites from amyloid precursor-like protein 2 (APLP2) and decorin in a short (S) and a long version (L). Sulfatable tyrosines and serines to which a GAG can be attached are underlined. The C-terminus of the sequence is indicated by •.*

Transiently transfected COS-1 cells were [$^{35}$S]sulfate labeled for 1 h. After immunoprecipitation of H1, SDS-gel electrophoresis and autoradiography, a very strong signal in a broad band of ~50–90 kDa could be observed (Fig. 9, lane 1 for APLP2 tag), which is indicative of GAG-attachment. Based on sulfation of the fusion proteins in transiently transfected HeLa cells, the efficiency of GAG-attachment was similar for all three sequences (not shown). The subsequent experiments were all performed with H1-GAG containing the GAG-attachment sequence derived from APLP2.

Upon [$^{35}$S]methionine labeling, the high mannose glycosylated ER form of ~40 kDa was detected (Fig. 9, lane 2) and after a chase in medium with unlabeled sulfate, this was partially converted to the Golgi modified form (Fig. 9, lane 3). While the heterogeneous GAG-modified products were barely visible, a 45 kDa species was produced, corresponding to the complex glycosylated protein free of GAGs. This indicates that GAG-attachment was quite inefficient in transiently transfected COS-1 cells.

To avoid overexpression and potential saturation of GAG-attachment/xylosylation, stable HeLa cell lines expressing H1-GAG or H1-TS were generated. GAG-attachment and sulfation were found to be very efficient in this system, since hardly any complex glycosylated forms free of GAG-attachment could be detected upon [$^{35}$S]methionine labeling and chase (Fig. 9, lane 6).

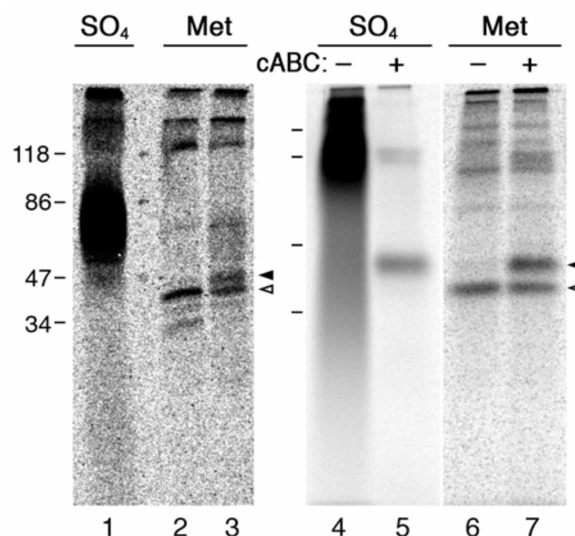


***Fig. 9: Expression and modification of H1-GAG with chondroitin sulfate.*** *Transiently transfected COS-1 cells expressing H1-GAG were labeled for 1 h with [$^{35}$S]sulfate (SO4; lane 1), or for 2 h with [$^{35}$S]methionine (Met; lane 2) and chased for 5 h (lane 3) before immuno-precipitation, SDS-gel electrophoresis, and autoradiography. Stable HeLa cell lines expressing H1-GAG were labeled for 2 h with [$^{35}$S]sulfate (lanes 4 and 5) or overnight with [$^{35}$S]methionine (lanes 6 and 7). To distinguish between CS and HS, immuno-precipitated H1-GAG was incubated with (+) or without (-) chondroitinase ABC (cABC) before gel electrophoresis and autoradiography. Filled and open arrowheads mark the positions of complex glycosylated and the high-mannose forms of H1 without GAGs, respectively. The positions of molecular weight markers (in kDa) are indicated.*

Because the type of GAG chains (HS or CS) produced on the same xylosylation site may vary depending on the cell type (as for example shown for serglycine; Kolset et al., 2004), we tested the GAGs attached to the APLP2 xylosylation site in H1-GAG in HeLa cells for sensitivity to chondroitinase ABC (cABC). This enzyme is specific for CS/DS and hyaluronan, but it does not hydrolyze HS/heparin. Upon digestion of CS/DS chains, at least the first five carbohydrate residues on the protein (the linker tetra-saccharide plus the first N-acetyl galactosamine) remain resistant to hydrolysis. Therefore, even after cABC digestion, sulfates on this minimal glycan structure may produce a detectable radioactive signal. Indeed, upon treatment of [$^{35}$S]sulfate-labeled H1-GAG with cABC, the heterogeneous products collapsed to a distinct band with the size of mature wild-type H1 (Fig. 9, lanes 4 and 5; the more slowly migrating band of ~90 kDa represents a post-lysis dimer of H1). When the cells were labeled with [$^{35}$S]methionine overnight, a barely visible smear could be observed in the size range of the [$^{35}$S]sulfate signal which was also collected in the 45 kDa band upon cABC treatment (Fig. 9, lanes 6 and 7).

## Stability and steady state distribution of H1-GAG

To test whether GAG affects stability of the receptor, cells expressing H1-TS or H1-GAG were labeled with [$^{35}$S]sulfate and the receptor was analyzed over a period of 20 h by immuno-precipitation, SDS-gel electrophoresis and autoradiography (Fig. 10). H1-GAG and H1-TS showed similar half-lives of ~7 h and ~9 h respectively.

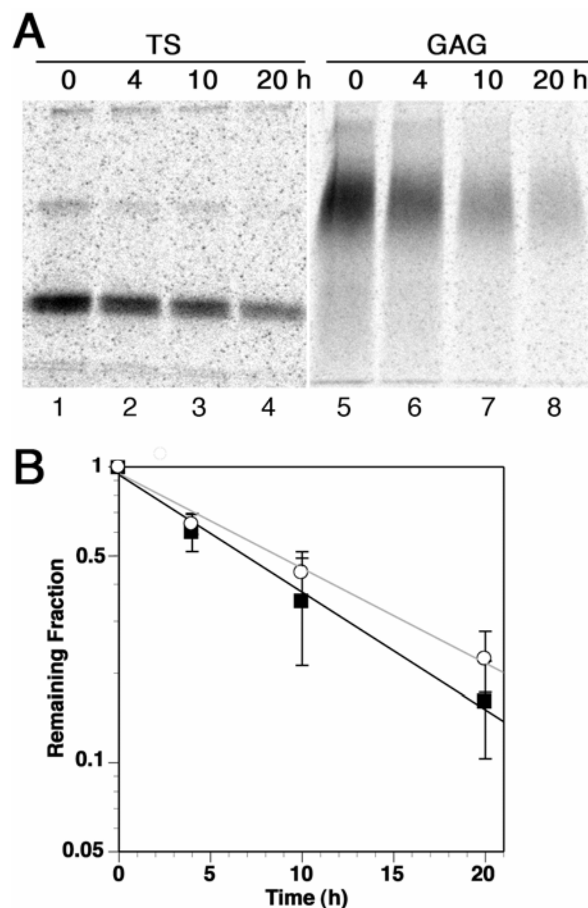


***Fig. 10: GAG-attachment does not significantly affect protein stability**. (A) HeLa cells stably expressing H1-TS (lanes 1-4) or H1-GAG (lanes 5-8) were labeled for 2 h with [$^{35}$S]sulfate and chased for 0, 4, 10, and 20 h before analysis by immuno-precipitation, gel electrophoresis and autoradiography. (B) Autoradiographs were quantified and the values were plotted on a logarithmic scale against time (average with standard deviation of triplicate determinations). H1-GAG, filled squares; H1-TS, open circles.*

As a simple assay for receptor traffic, we determined the steady-state distribution of H1-GAG between the cell surface and intracellular (endosomal) compartments. HeLa cells stably expressing H1-GAG or H1-TS were pulse-labeled for 7 min with [$^{35}$S]sulfate. This time had previously been shown to be too short to allow TGN exit of significant amounts of labeled protein (Leitinger et al., 1994, and references

therein). Indeed, upon proteinase K treatment of pulse-labeled intact cells to digest surface proteins, the labeled receptor was protected (Fig. 11, lanes 2 and 6). The signals obtained by autoradiography were even somewhat increased after proteinase K digestion compared to untreated samples, suggesting that the immuno-precipitation efficiency was nonspecifically increased by the treatment. After a chase, approximately half of H1-TS was protected intracellularly (Fig. 11, lane 4), which is in agreement with previous measurements in COS cells (Leitinger et al., 1994). In contrast, only ~15% of H1-GAG was resistant (Fig. 11, lane 8). The clear difference in steady-state distribution suggests that the GAG modification affects trafficking of the receptor in the endocytic cycle.

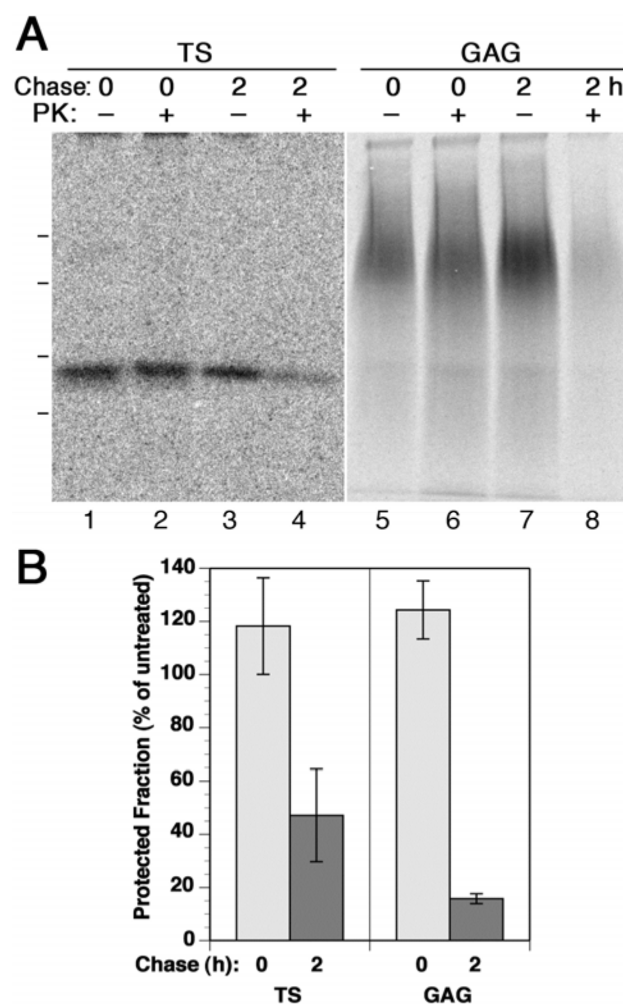


***Fig. 11: GAGs affect the surface-to-intracellular distribution of H1.*** *(A) HeLa cells stably expressing H1-TS (lanes 1-4) or H1-GAG (lanes 5-8) were pulse-labeled for 7 min with [$^{35}$S]sulfate, chased for 0 or 2 h, and then incubated at 4°C with or without proteinase K (PK), before immunoprecipitation. (B) Autoradiographs were quantified and the protected signals of digested samples were plotted as a fraction of the total signals in the undigested ones (average with standard deviation of at least triplicate determinations).*

## GAG-attachement inhibits receptor endocytosis, but does not affect recycling

To determine the rate of internalization, H1-GAG or H1-TS expressing cells were labeled for 2 h with [$^{35}$S]sulfate and chased for 2 h to allow the labeled receptor proteins to reach their steady-state distribution. At 4°C, the cells were then surface biotinylated with the impermeant and cleavable reagent sulfo-NHS-SS-biotin. After incubation for up to 30 min at 37°C to allow endocytosis, surface biotin was released at 4°C by reduction with glutathione. H1 was immuno-precipitated, and the biotinylated fraction was isolated by reprecipitation with avidin beads and analyzed by gel electophoresis and autoradiography (Fig. 12). As expected, H1-TS was rapidly internalized at a rate consistent with its published internalization rate of ~6%/min (Fuhrer et al., 1991) reaching a steady-state plateau of ~50% intracellular protein within less than 10 min (Fig. 12, left panel). In contrast, only ~10% of H1-GAG was protected from glutathione stripping after 10 min (Fig. 12 right panel). With a protected fraction of ~16%, the distribution reached steady-state levels within 30 min. The GAG-carrying form of H1 thus is internalized considerably more slowly than H1-TS or wild-type H1.

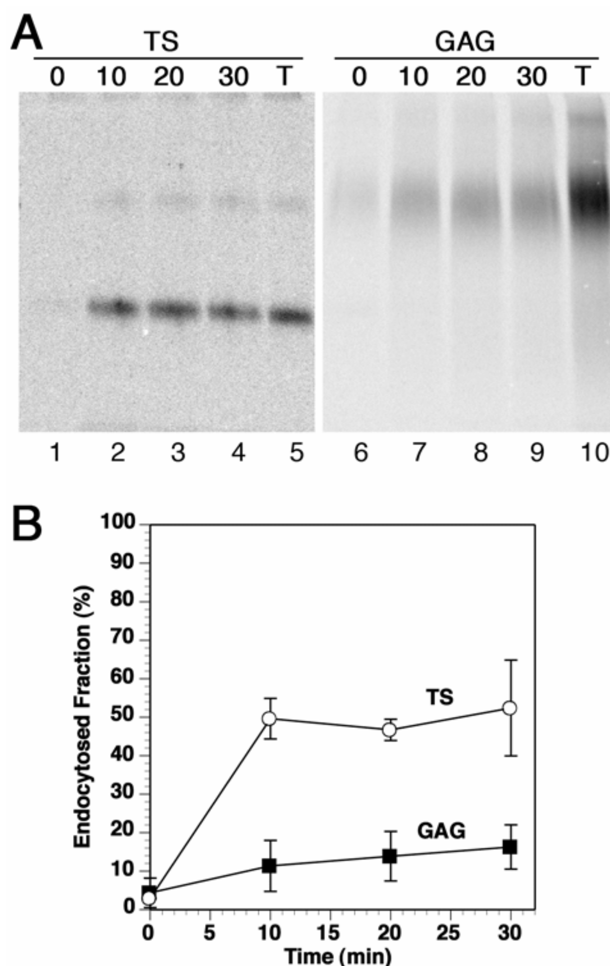


***Fig. 12: GAGs inhibit endocytosis.*** *(A) HeLa cells stably expressing H1-TS (lanes 1-5) or H1-GAG (lanes 6-10) were labeled for 2 h with [$^{35}$S]sulfate and chased for 2 h. They were subsequently surface biotinylated at 4°C and allowed to endocytose for 0, 10, 20, or 30 min at 37°C. After glutathione treatment at 4°C to release surface biotin, protected (i.e. intracellular) H1 was isolated by successive immuno- and avidin-precipitation. As a standard, 50% of total biotinylated material before glutathione stripping was analyzed (T). (B) Autoradiographs were quantified and the protected signals were plotted as a fraction of the total biotinylated protein (average with standard deviation of at least triplicate determinations). H1-GAG, filled squares; H1-TS, open circles.*

To distinguish whether this is a consequence of the glycans, for example by interaction of the GAG chains with the extracellular matrix, or an effect of the added protein tag itself, the experiment was repeated with an additional incubation of the cells with or without chondroitinase ABC at 4°C before biotinylation. Indeed, upon digestion of GAGs, endocytosis was largely restored (Fig. 13).

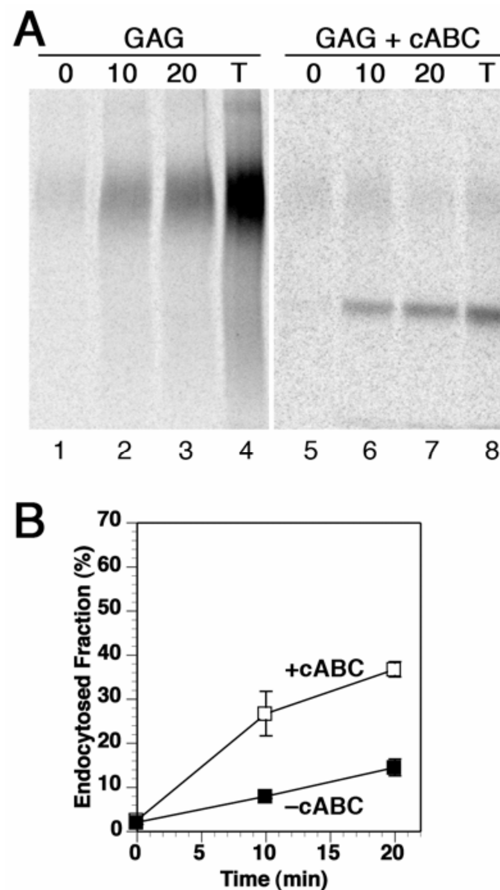


*Fig. 13: **Endocytosis of H1 is largely restored upon removal of GAG chains.** (A) HeLa cells stably expressing H1-TS (lanes 1-4) or H1-GAG (lanes 5-8) were labeled for 2 h with [$^{35}$S]sulfate, chased for 2 h, and incubated at 4°C with or without chondroitinase ABC (cABC). Endocytosis was then monitored by surface biotinylation at 4°C, internalization for 0, 10 and 20 min at 37°C, glutathione stripping of surface biotin and analysis as in Fig. 12. (B) Autoradiographs were quantified and the protected signals were plotted as a fraction of the total biotinylated protein (average with standard deviation of duplicate determinations).*

To analyze the rate of recycling from the endosomal compartment to the cell surface, cells expressing H1-GAG or H1-TS were labeled, chased to equilibrium, biotinylated and allowed to internalize for 30 min as before. Cells were then stripped of surface biotin by glutathione washes at 4°C and incubated for up to 20 min at 37°C to allow recycling. Recycled biotin was again released with glutathione at 4°C. Remaining intracellular receptor was isolated by successive immuno- and avidin-precipitations and analyzed by gel electrophoresis and autoradiography. Both versions of H1 were recycled with a similar rate of ~4%/min (Fig. 14). The attached GAG chains thus did not affect the transport step from endosomes to the plasma membrane.

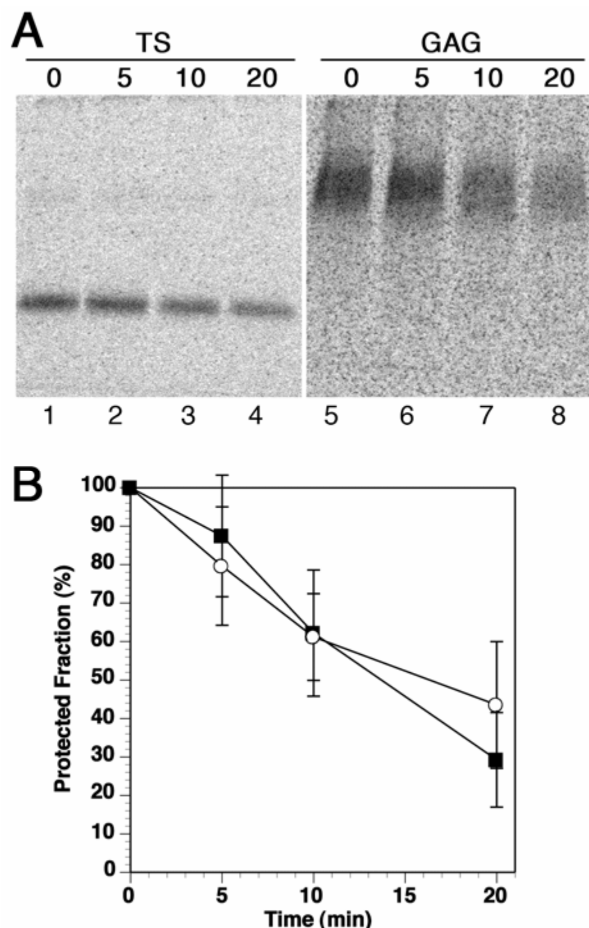


***Fig.14: Endosomal recycling is not affected by GAGs.*** *(A) HeLa cells stably expressing H1-TS (lanes 1-4) or H1-GAG (lanes 5-8) were labeled for 2 h with [$^{35}$S]sulfate, chased for 2 h, surface biotinylated at 4°C, and allowed to endocytose for 30 min at 37°C. After glutathione treatment at 4°C to release surface biotin, cells were allowed to recycle proteins from endomembranes at 37°C for 0–20 min before restripping with glutathione at 4°C. Biotinylated H1 still protected within the cells was analyzed by successive immuno- and avidin-precipitations as in Fig. 12. (B) Autoradiographs were quantified and the protected signals were plotted as a fraction of the initial values (average with standard deviation of at least triplicate determinations). H1-GAG, filled squares; H1-TS, open circles.*

## H1-GAG exits the TGN more rapidly than H1-TS

To measure the exocytosis rate from the site of sulfation (i.e. the TGN) to the cell surface, cells expressing H1-GAG or H1-TS were pulse-labeled for 7 min with [$^{35}$S]sulfate, chased for up to 30 min, and then surface-shaved at 4°C with proteinase K. After inactivation of the protease, protected receptor was immuno-precipitated and analyzed by gel electrophoresis and autoradiography (Fig. 15). H1-GAG arrived at the cell surface more rapidly, since after 10 min already more than 40% was accessible to external protease. In contrast, H1-TS was still fully protected at this time. The slight increase in signal may be due to continued incorporation of radioactivity after

switching to the chase medium; as seen in lanes 5 and 7 in Fig. 11, this occurred for H1-GAG as well. H1-TS appears to experience a lag phase of ~10 min before arrival at the surface, which is not detectable for H1-GAG. After 30 min, the protected fraction of both proteins reached levels corresponding to their steady-state levels. The distinct TGN-to-plasma membrane transport characteristics of the two receptor forms suggest different transport carriers or pathways.

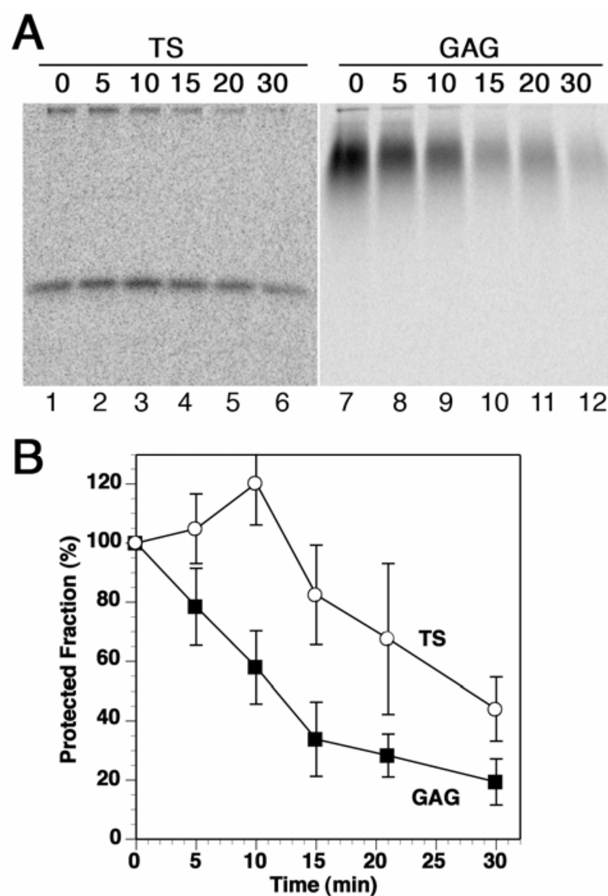


***Fig. 15: GAGs accelerate biosynthetic exocytosis.*** *(A) HeLa cells stably expressing H1-TS (lanes 1-6) or H1-GAG (lanes 7-12) were labeled for 7 min with [$^{35}$S]sulfate and chased for 0, 5, 10, 20, and 30 min before proteinase K digestion of surface proteins and immuno-precipitation. (B) Autoradiographs were quantified and the protected signals were plotted as a fraction of the initial values (average with standard deviation of triplicate determinations). H1-GAG, filled squares; H1-TS, open circles.*

# Discussion

The role of GAGs has so far been studied mainly with respect to their function in signaling and development (Bishop et al., 2007) or their role in sorting of other proteins interacting with them (Kolset et al., 2004). The sorting information inherent

to GAGs was mostly investigated with respect to polarized sorting. CS chains were found to favor apical sorting (Kolset et al., 1999), whereas HS chains were shown to promote basolateral transport in a manner that is dominant over GPI-mediated apical sorting (Mertens et al., 1996).

Here, we have transplanted a glycosaminoglycan attachment site to a recycling receptor in order to investigate its influence on post-Golgi trafficking. CS attachment was found to affect TGN-to-plasma membrane transport and endocytosis, but not recycling from endosomes. For the two affected transport steps, the effect of the GAG modification was obviously dominant over existing sorting signals in the H1 receptor protein. This is in contrast to the effects of other glycans. N- and O-glycosylation were found to act as apical sorting determinants (Potter et al., 2006), but were generally recessive to cytosolic basolateral signals (Monlauzeur et al., 1995). Also the basolateral delivery of APLP2, the CS attachment site of which we have used here, depends on a tyrosine signal (Kuan et al., 2006) and was not affected by its CS chain (Lo et al., 1995).

The effect of GAG-attachment on H1 endocytosis is inhibitory. We propose that the lowered rate of internalization is due to interaction of the CS chain with, and assembly into the extracellular matrix of the cell, rather than interaction with a specific sorting machinery. This may result in reduced mobility of the protein within the plasma membrane and reduced availability for interaction of its cytoplasmic tyrosine motif with AP-2 adaptors in clathrin coats. This is supported by the fact that upon removal of the GAG, the endocytosis rate is largely restored (Fig. 13). Consistent with this model, recycling was not affected from endosomes, where there is no matrix to interact with.

Most interestingly, however, we observed that GAG chain affected the kinetics of TGN-to-cell surface transport, not by inhibiting it, but rather by accelerating its kinetics. We have previously shown that H1-TS is transported from the TGN to the plasma membrane via endosomes in MDCK cells (Leitinger et al., 1995). Similarly, this has been demonstrated for the transferrin receptor (Futter et al., 1995), the polymeric immunoglobulin receptor (Orzech et al., 2000) and more recently evidence was provided for an endosomal route of vesicular stomatitis virus G protein in live

cells (Ang et al., 2004). Acceleration of transport may be accomplished by the use of an alternative route, for example via direct secretory vesicles. Indeed, GAG chains have been implicated in secretory granule formation (Forsberg et al., 1999; Humphries et al., 1999; Kolset et al., 2004), where the glycans potentially associate or coaggregate with granule cargo. In HeLa cells lacking regulated secretory granules, GAGs may associate with newly synthesized extracellular matrix proteins already in the Golgi apparatus and thereby be directed to constitutive secretory carriers, circumventing the detour through endosomes. It is conceivable that the protein modified with a GAG chain, by interaction with newly synthesized extracellular matrix proteins, is hindered from entering carriers destined to endosomes and enters constitutive secretory vesicles together with secretory proteins.

Furthermore, our results have interesting implications for part-time proteoglycans, proteins which are only partially modified with a GAG chain such as the amyloid precursor protein. Our findings suggest that the two forms are likely to have different distribution and trafficking properties within the cell with potential physiological function.

Taken together, our results indicate that (1) endosomal sorting is unaffected by glycosaminoglycans, (2) that endocytosis is impaired presumably due to interaction with the extracellular matrix and (3) that the sorting information in GAGs is dominant over the sorting information in the amino acid sequence of the asialoglycoprotein receptor subunit H1 in the TGN. The sorting machinery involved in this sorting step remains to be elucidated.

# *Materials and methods*

## Cloning procedures

The H1-TS construct was described in (Leitinger et al., 1994). To generate the 3 H1-GAG constructs, H1-TS in pCB6 was cut with KpnI and EcoRI to excise the TS tag sequence. The complementary oligonucleotide sequences GTACCGAAGATGAGGCTAGTGGTATCGGACCTGAGTG and AATTCACTCA GGTCCGATACCACTAGCCTCATCTTCG, GTACCTTCATGCTCGAAGATGAG GCTAGTGGTATCGGACCTGAGGTCCCCGATGACTG and AATTCAGTCATC

GGGGACCTCAGGTCCGATACCACTAGCCTCATCTTCGAGCATGAAG, GTA CCGCCCTTCCTGAAAATGAGGGTTCTGGCGTTGGAGAACAGGACTG and AATTCAGTCCTGTTCTCCAACGCCAGAACCCTCATTTTCAGGAAGGGCG were pair wise annealed and ligated into the vector plasmid to generate the decorin short, decorin long, and APLP2 tag, respectively, as shown in Fig. 1.

## Transient and stable transfection

Transient transfections were performed with lipofectine according to the manufacturer's protocol. Stable HeLa cell lines were generated using polyethylenimine. Clones resistant to 1 mg/ml G418 were isolated and expression was assessed by Western analysis.

## Labeling, chase, proteinase digestion, chondroitinase digestion and detection.

Cells were cultured in Dulbecco's minimal essential medium (DMEM; from Invitrogen, Carlsbad, CA) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum at 37 °C with 7.5% CO2. For labeling, cells were grown in 6-well cell culture dishes to 90% confluence and incubated with 0.5 ml sulfate-free or methionine-free medium for 1 h at 37°. Labeling was performed with 0.5 ml sulfate-free medium reconstituted with 1 mCi/ml [$^{35}$S]sulfate or methionine-free medium reconstituted with 100 μCi/ml [$^{35}$S]methionine for indicated times. Chase was performed with DMEM at 37°C.

To digest cell surface proteins, cells were treated with 1 mg/ml proteinase K in phosphate-buffered saline (PBS) with 0.5 mM EDTA at 4°C with rocking for 30 min. Digestion was stopped phenylmethylsulfonyl fluoride (PMSF) and 4 mg/ml bovine serum albumin (BSA). The cells were gently pelleted at 800×g and lysed in 0.5 ml 1% Triton, 0.5% deoxycholate in PBS. H1 was immuno-precipitated using a polyclonal rabbit anti-H1 antiserum and protein A-sepharose (from Zymed Laboratories, South San Francisco, CA), boiled in SDS-sample buffer, and analyzed by 12.5%-polyacrylamide SDS-gel electrophoresis and autoradiography. Signals were quantified by phophorimager.

Chondroitinase ABC (from Sigma, Saint Louis, MO) treatment on beads was described previously (Dumermuth et al., 2002). Cell surface chondroitin digestion was performed for 30 min at 4°C with 0.5 ml of 2 U/ml chondroitinase solution.

## Exocytosis, endocytosis and recycling assays

For exocytosis assays, cells were grown in 10-cm dishes, pulse-labeled with 2 ml of 1 mCi/ml [$^{35}$S]sulfate labeling medium for 7 min at 37°C and chased for the indicated times. Membrane traffic was stopped with ice-cold PBS and cells were shaved with proteinase K and analyzed as above. 2 ml of 1 mg/ml proteinase solution was used.

For endocytosis assays, cells were grown on 6-well plates, labeled with 0.5 ml of 1 mCi/ml [$^{35}$S]sulfate labeling medium for 1 h and chased for 2 h. Surface-biotinylation with 1 mg/ml sulfosuccinimidyl-2-(biotinamido)-ethyl-1,3-dithiopropionate (from Pierce Chemical, Rockford, IL) in PBS was performed at 4°C for 30 min. The reaction was quenched by washing the cells two times with PBS, by a 5 min incubation with 50 mM glycine in PBS, and by another two washes with PBS. The cells were then incubated in prewarmed serum-free medium containing 20mMHEPES (pH 7.4) for indicated times at 37°C to allow internalization of biotinylated surface proteins. Cells were rinsed with ice-cold PBS. Biotin at the cell surface was stripped by two 20 min incubations with 50 mM reduced glutathione in 75 mM NaCl, 75 mM NaOH, 1 mM EDTA, with 1% BSA. The cells were rinsed twice with PBS and incubated for 5 min with 5 mg/ml iodoacetamide in PBS to quench any residual glutathione (Pagano et al., 2004). H1 was immuno-precipitated and released from the beads by boiling in 2% SDS. Biotinylated material was recovered by reprecipitation with avidin sepharose (from Pierce Chemical, Rockford, IL).

For recycling assays, cells were grown, labeled and biotinylated as above. Biotinylated proteins were internalized for 20 min, but, after glutathione stripping, cells were allowed to recycle in the same medium as for internalization. Also, no iodoacetamide was used to quench glutathione; cells were rinsed thoroughly with PBS instead. Surface biotin was then again removed with glutathione, and the latter was quenched with iodoacetamide. Detection as in endocytosis assays.

# Results-Part II

## *In vitro* reconstitution of *trans*-Golgi exit

## An assay to reconstitute TGN exit

To reconstitute the formation of transport carriers from the TGN, we developed an *in vitro* assay mainly based on the classical procedures first introduced by the groups of Balch and Huttner. Beckers et al. (Beckers et al., 1987) first introduced semi-intact cells (or "broken cells") to reconstitute formation of ER-derived vesicles. Tooze and Huttner (Tooze and Huttner, 1992) used sulfation as a method to specifically label proteins in the TGN to define the starting compartment for the *in vitro* formation of secretory granules. Our adapted procedure is schematically summarized in Figure 16.

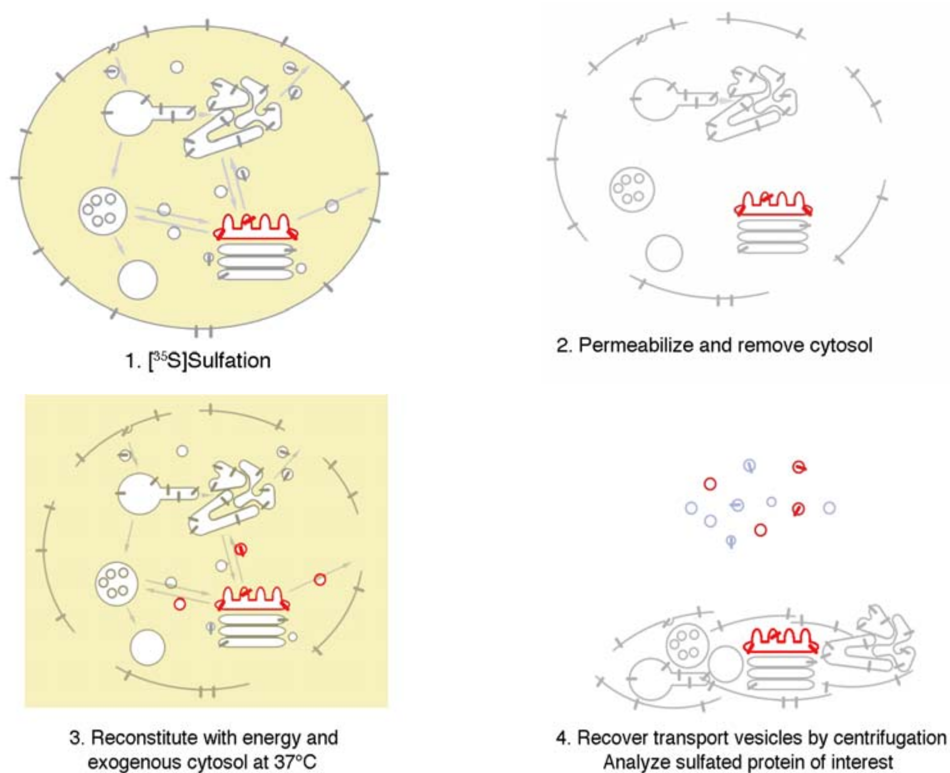


***Fig. 16: The TGN carrier formation assay.*** *(1) Cells expressing a sulfatable reporter protein are pulse labeled with [$^{35}$S]sulfate for 7 min at 37°C or for 2 h at 19°C. Labeled compartments are indicated in red. (2) Cells are permeabilized by incubation in a hypo-osmotic buffer and cytosol and peripheral membrane proteins are removed by a high-salt wash. (3) Exogenous cytosol, energy, and an energy regenerating system are mixed with membranes and the mixture is transferred to 37°C to allow budding to occur. (4) Post-Golgi carriers are separated from donor membranes by low speed centrifugation and lysed. The reporter is immuno-precipitated and analyzed by SDS-gel electrophoresis and autoradiography.*

Cells were grown on poly-l-lysine in order to keep them firmly attached to the dish to improve cell recovery as well as permeabilization upon swelling and scraping. They were then labeled with [$^{35}$S]sulfate for 2 h at 19°C, a temperature at which TGN

export is blocked. Cells were transferred to 4°C and premeabilized by addition of a hypo-osmolar buffer to swell them and by scraping them off the substratum. Thus generated semi-intact cells were gently pelleted and the supernatant containing most of the cytosol was removed. To remove residual cytosol and peripheral membrane proteins the broken cells were carefully resuspended in high-salt buffer and repelleted. This "high-salt wash" was performed twice, and the remaining membranes were suspended in the reaction mix. This included cytosol derived from calf brain, ATP, GTP and an energy regenerating system consisting of creatin phosphate and creatin kinase. The budding reaction was allowed to take place at 37°C and the broken cells (including the donor membranes) were separated by low-speed centrifugation (800×g) from post-Golgi carriers that might have been generated. From these, the reporter protein was immuno-precipitated and analyzed by autoradiography. In this procedure, the donor compartment is defined by the method of labeling the proteins, by sulfation. The destination of the vesicles is determined by the protein to be analyzed, e.g. secretory proteins leave the TGN in secretory vesicles destined for the plasma membrane, MPR and H1 in carriers to endosomes.

A limitation of sulfation is that only a minority of natural proteins carry this modification (Huttner, 1988). To extend the range of proteins that can be analyzed in the way described above, they can be tagged with a sequence specifying a tyrosine-sulfation motif. This has previously been done by Leitinger et al. (Leitinger et al., 1994), adding a nonapeptide of pro cholecystokinin (proCCK) to the lumenal C-terminus of H1 and of the secretory protein A1Pi. Itin et al. (Itin et al., 1997) inserted the same sequence together with a myc epitope and a 6-His tag between the signal sequence and the mature part of MPR.

Initial experiments using H1-TS performed in stably expressing MDCK cells is depicted in Fig. 17. Approximately 15% of total reporter protein was recovered in budded fraction in the presence of cytosol and energy vs. only ~5% in the control reaction. This suggested cytosol-dependent release of H1-containing membranes. However, the result also highlighted technical limitations of the assay. First, the signals obtained in a reaction corresponding to 5 million cells were very low, requiring exposure times of several weeks. Second, the background signal in the absence of added cytosol was relatively high and the signal therefore only three-fold

above background. This background might represent unspecific processes like partial disassembly of the TGN or alternatively, was the result of inefficient removal of cytosolic proteins, some of which may already have been recruited to the TGN membrane at the time of permeabilization. To establish a useful assay, we first concentrated (1) on increasing the incorporation of radioactivity into the reporter proteins and (2) on improving the assay conditions, addressing in particular mechanical breakage of membranes, high-salt stripping of cytosolic proteins, and the use of different cytosols.

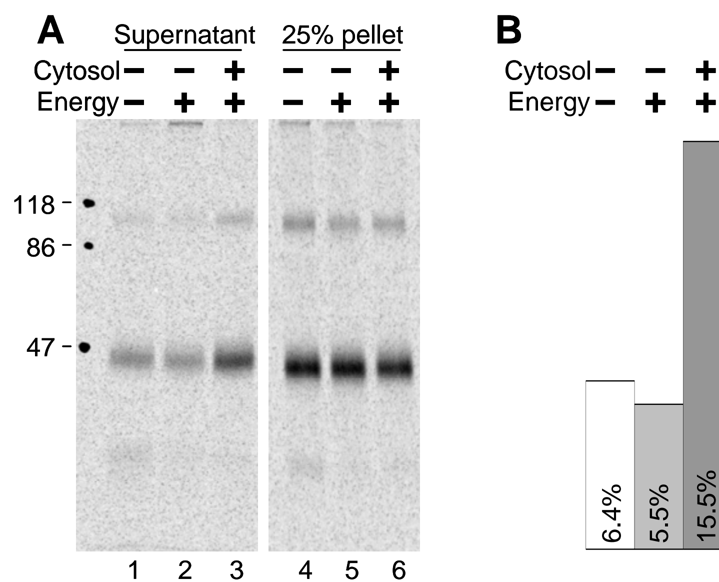


***Fig. 17: H1 carrier formation on the TGN is dependent on cytosol and energy.*** *(A) A budding assay was performed on MDCK cells stably expressing H1-TS. Cells were labeled for 2 h at 19°C and cytosol at a final concentration of 12 mg/ml was used. Supernatant (left panel) and 25% of pellet (right panel) of the low speed centrifugation were subjected to H1 immuno-precipitation. (B) Quantification of lanes 1-3 in (A).*

## *Improving the sulfation signal*

The tyrosine sulfation tag used so far consisted of the C-terminal nonapeptide of the cholecystokinin precursor (Leitinger et al., 1994) and contained two potentially sulfatable tyrosine residues. It was determined, however, that on average only a single sulfate was attached per protein. To increase the sulfation efficiency and thus the signal in the reconstitution assay we explored two strategies: (1) to tag the protein with two or three copies of the proCCK tag, and (2) to introduce a GAG-attachment

sequence for carbohydrate sulfation. Since GAGs are multiply sulfated, this approach carried the potential of improving the signal manyfold.

## Multiple tyrosine sulfation tag

One or two additional sulfation sequences were fused to the original construct of H1-TS (H1 with a tyrosine sulfation motif at the C-terminus) resulting in H1-TS2 and H1-TS3. When analyzed by immuno-fluorescence in COS-1 cells, the construct with the largest tag exhibited surface staining, indicating that it was properly folded (Fig 18). However, after radiolabeling transfected COS-1 cells with [$^{35}$S]sulfate and immuno-precipitation, the autoradiograph did not show significant differences between the 3 constructs (Fig. 19A, lanes 4-6, and 19B).

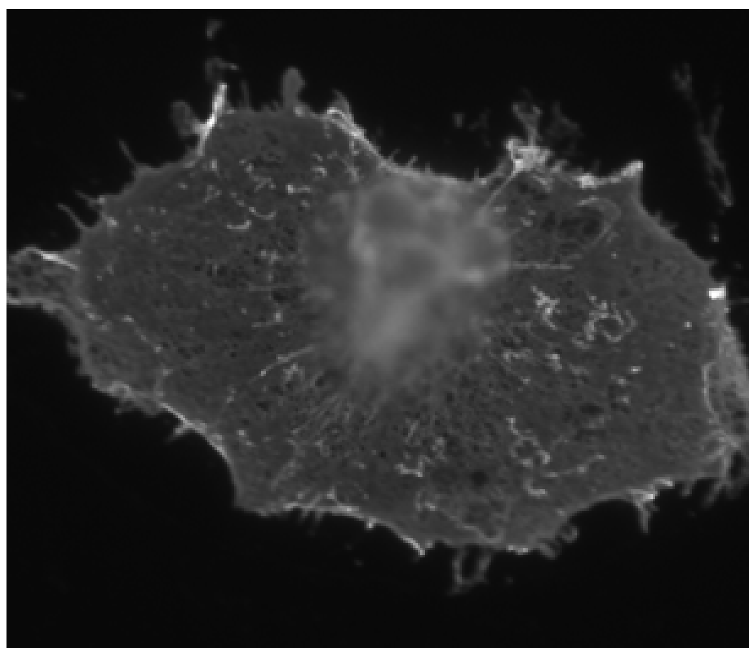

***Fig. 18: H1-TS3 is transported to the cell surface.*** *COS-1 cells transiently transfected with H1-TS3 were immuno-labeled according to materials and methods and analyzed by fluorescence-microscopy.*

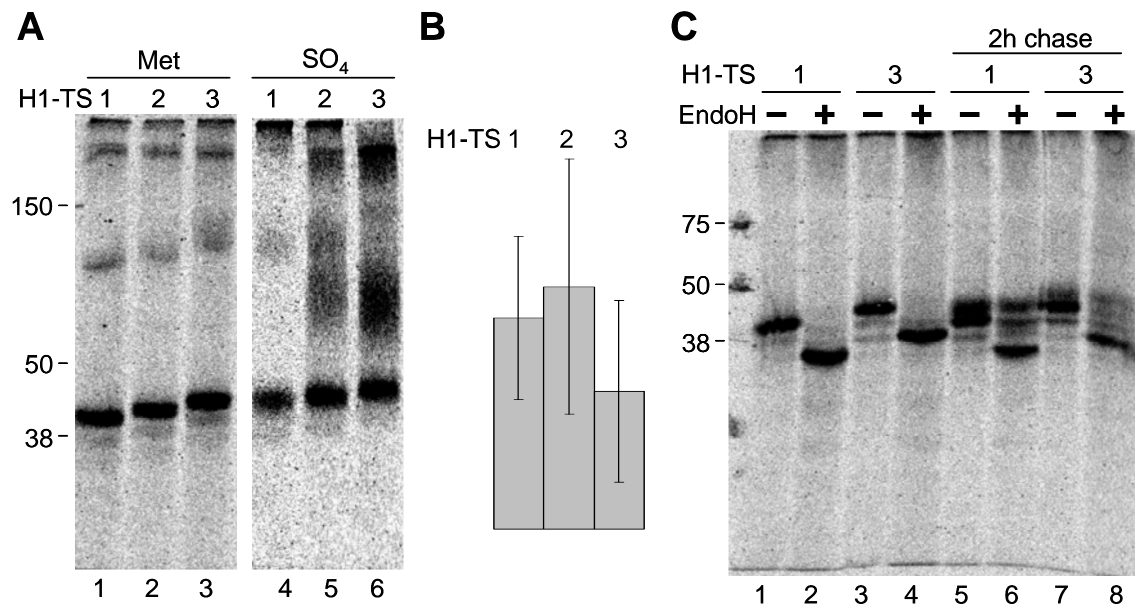


***Fig. 19: H1 tagged with multiple TS sites does not produce stronger signals than H1-TS, because it does not efficiently exit the ER.*** *(A) COS-1 cells transiently transfected with H1-TS (lanes 1 and 4), H1-TS2 (lanes 2 and 5) or H1-TS3 (lanes 3 and 6) were labeled for 30 min with either [$^{35}$S]methionine (lanes 1-3) or [$^{35}$S]sulfate (lanes 4-6). (B) The experiment from (A) was performed three times and the mean of [$^{35}$S]sulfate to [$^{35}$S]methionine signal ratios was plotted for each H1 construct. Error bars indicate standard deviation. (C) COS-1 cells transiently transfected with either H1-TS (lanes 1, 2, 5, and 6) or H1-TS3 (lanes 3, 4, 7, and 8) were labeled with [$^{35}$S]methionine for 30 min, either not chased (lanes 1-4) or chased for 2 h (lanes 5-8) and H1 was immuno-precipitated. Precipitates from lanes 2, 4, 6, and 8 were incubated with endoglycosidase H (EndoH) as described in materials and methods.*

When transfected COS-1 cells expressing H1-TS and H1-TS3 were [$^{35}$S]methionine labeled for 30 min, the constructs were expressed as a ~47 and ~50 kDa band respectively (Fig 19C), corresponding to the expected molecular weight of these proteins modified with the high-mannose N-glycan precursor. To confirm that these were not the mature, complex glycosylated species, samples were digested with endoglycosidase H (endoH). Upon this treatment, both bands shifted to a lower migrating band that represented the proteins devoid of N-glycan. Thus, 30 min were not enough to exit the ER for neither of the constructs. After a 2 h chase however, a significant portion of H1-TS shifted towards a higher migrating, complex glycosylated form, while most of H1-TS3 did not. Complex glycosylation was confirmed with endoH resistance. This indicated that H1-TS3 did not exit the ER as efficiently as H1-TS; this explained why [$^{35}$S]sulfate signal was comparable for both H1 variants.

Thus, tag repetition was not a viable strategy to improve the sulfation signal. Proteins modified with a single TS-tag were used to generate stably transfected cell lines needed to perform the budding assay. Next to H1, the TGN-to-endosome traffic reporter, A1Pi, a constitutive secretory pathway (TGN-to-PM) marker (Leitinger et al., 1995) was stably transfected. The cell lines used were HeLa and human embryonal kidney (HEK293) cells; both are non-polarized. MDCK cells from the preliminary experiment were omitted, because their polarized sorting machinery could complicate the interpretation of data obtained with the assay.

## GAG-tag

Attachment of a GAG to the reporter was the second attempt to improve the [$^{35}$S]sulfate signal. As described in part I, H1 was C-terminally tagged with a GAG attachment site. Indeed, the radioactive signal was greatly improved, but the tag altered trafficking properties of the reporter. While stability and endosomal recycling of the protein remained unchanged, the rate of endocytosis was reduced, presumably due to interaction of the glycan with the ECM. Most importantly however, TGN-to-PM transport was accelerated, indicating that GAG-tagged H1 was sorted by a different mechanism than tyrosine sulfated protein. Thus, attachment of glycosaminoglycan was not suitable for investigation of wild type behavior. Nevertheless, the assay could be applied to GAG-tagged reporter and due to the strong sulfation signal, TGN accumulation could be omitted and cells were pulse-labeled for 7 min at 37°C instead. H1-GAG budding was energy and cytosol dependent (Fig 20A). Interestingly, budding of free GAGs, synthesized on β-D-Xyloside was also energy and cytosol dependent (Fig. 20B).

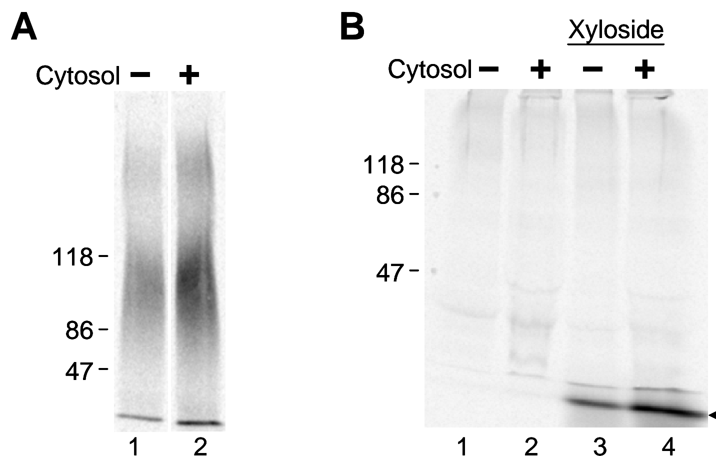


***Fig. 20: H1-GAG or free GAGs can serve as a reporter in the TGN carrier formation assay.*** *(A) A standard budding assay was performed on HeLa cells stably expressing H1-GAG. Cells were labeled for 7 min at 37°C and final cytosol concentration was 12 mg/ml. (B) Wild type HeLa cells were treated without (lanes 1 and 2) or with (lanes 3 and 4) xyloside to initiate free GAG synthesis and used in a budding assay with 7 min labeling at 37°C and 12 mg/ml cytosol. Arrowhead indicates short, free GAGs that migrated at the front of the gel.*

The GAG-tag was also fused to A1Pi. Similarly to modified H1, while A1Pi-TS produced a band of defined size (Fig. 21A, lane 2), GAG-tagged protein generated a broad band upon SDS-gel electrophoresis due to the heterogeneous size of the GAG chains (Fig. 21A, lane 1). In order to characterize basic trafficking properties, secretion efficiency was assessed by a pulse-chase experiment with subsequent immuno-isolation of protein from the chase medium. In transiently transfected HeLa cells, all A1Pi-GAG that was intracellular after 30 min labeling, was recovered from chasing medium after 2 h of chase. HeLa clones stably expressing A1Pi-GAG were generated. These will allow investigation of the influence of GAG attachment on trafficking of a secretory protein.

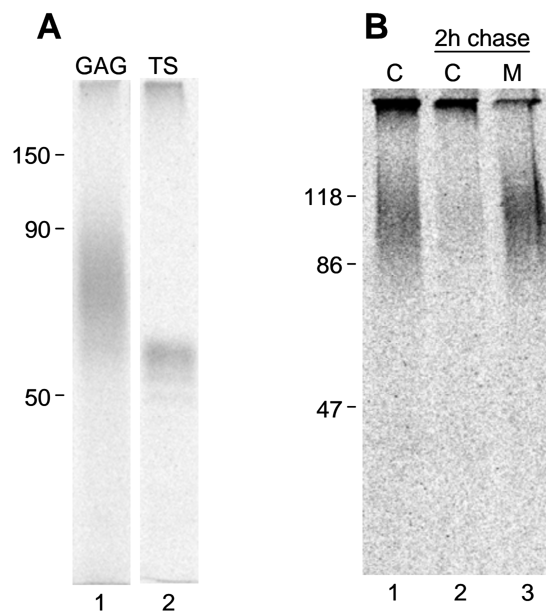


***Fig. 21: A1Pi can be tagged with a glycosaminoglycan and this does not inhibit efficient secretion.*** *(A) HeLa cells transiently expressing A1Pi-GAG (lane 1) or A1Pi-TS (lane 2) were labeled with [$^{35}$S]sulfate for 30 min and subjected to A1Pi immuno-precipitation. (B) HeLa cells transiently expressing A1Pi-GAG were labeled with [$^{35}$S]sulfate for 30 min and not chased (lane 1) or chased for 2 h. A1Pi immuno-precipitation was performed on cells (C; lanes 1 and 2) or chase medium (M; lane 3).*

## Optimizing assay conditions

Since improving the signal through multiple TS sites or GAG attachment was not possible, the assay had to be modified. Encouraged by the signal-to-noise ratio in initial findings, budding experiments were performed without the 20°C TGN block, but only a 7 min pulse at 37°C. 4 different reporters exhibited cytosol and energy dependence, although the signal was weak (Fig. 22). H1, A1Pi and MPR budding in HeLa cells depended on cytosol and energy. Formation of TGN carriers for aminopeptidase N (APN; a marker of TGN to apical PM in polarized cells (Renold et al., 2000)), was only poorly reconstituted in this single experiment.

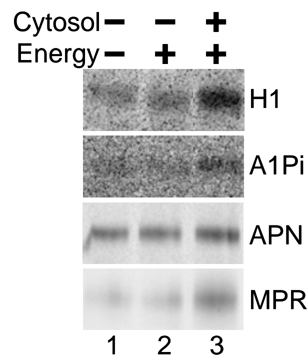


***Fig. 22: The TGN carrier formation assay with 7 min of pulse labeling is applicable to multiple reporter proteins.*** *H1, A1Pi, and MPR in HeLa cells and APN in MDCK cells were subjected to a budding assay with 7 min labeling and a cytosol concentration of 12 mg/ml for H1, A1Pi, and APN, or 6 mg/ml for MPR.*

## Mechanical stress as a source of artifacts

Even though cytosol dependence was reproducible, the observed variability in the strength of this effect was very disturbing and rendered the assay qualitative but not quantitative. Experiments performed in HEK293 cells were not conclusive and this was presumably due to their fragility: upon resuspension in high-salt buffer, cells became viscous most likely due to disruption of nuclear envelope and subsequent DNA release. Fortunately, nuclear disruption was never observed in HeLa or MDCK cells, but it could not be excluded that TGN membranes are also disrupted by resuspension.

To test this, membranes from HeLa cells stably expressing MPR were treated very carefully during resuspension or mixed vigorously. Indeed, mechanical stress was a source of inter-experimental variation: while experiments from gently treated membranes produced cytosol and energy dependent signals, budding from harshly resuspended membranes caused recovery of reporter in the supernatant independently of cytosol and energy (Fig. 23).

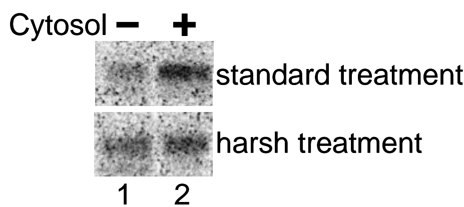


***Fig. 23: Harsh treatment of donor membranes disrupts them and causes their recovery in low speed supernatant.*** *HeLa cells stably expressing MPR were subjected to a budding assay with 7 min labeling and a cytosol concentration of 12 mg/ml. After high-salt washing, donor membranes were either gently (standard treatment) or harshly (harsh treatment) resuspended in transport buffer.*

This issue could be solved by reducing the number of washing and resuspension steps (and extremely gentle handling of membranes). To elucidate which washing step could be omitted, membranes from MDCK cells expressing H1-TS were prepared like in a budding experiment and individual buffers were analyzed by western blotting (not shown). The hypo-osmotic buffer used to perforate membranes (swelling buffer) contained no H1-TS. Upon scraping the cells into the transport buffer some reporter was released, but the largest portion of protein that would contribute to unspecific budding was removed by high-salt washing. The supernatant from the second high-salt washing step did not contain significant amounts of H1 and hence was omitted in later experiments.

## Optimizing the cytosol conditions

In early experiments the clathrin antibody that inhibited endosomal budding of H1 did not inhibit MPR budding from the TGN, despite previous findings claiming that, at least in part, MPR leaves TGN in CCVs (Ghosh et al., 2003). The concern was that the concentration of clathrin was too high to be inhibited. While endosomal budding was reliable with a cytosol concentration of 1.2 mg/ml (Pagano et al., 2004), 12 mg/ml were needed for TGN carrier formation in initial experiments. The improvement of the protocol however, prompted the repetition of cytosol titration to estimate which concentration was necessary. As depicted in Fig. 24A, the concentration of 6 mg/ml was needed, which was still relatively high compared to endosomal budding, but lower compared to early TGN budding experiments.

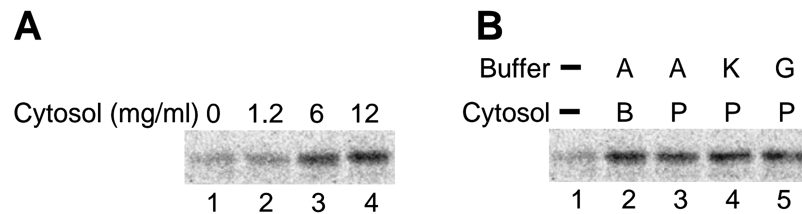


***Fig. 24: TGN carrier formation requires 6 mg/ml cytosol and can be performed with bovine and porcine cytosol in different buffers.*** *(A) HeLa cells stably expressing MPR were subjected to a budding assay with 7 min labeling and a cytosol concentrations of 1.2, 6, and 12 mg/ml of cytosol (lanes 2, 3, and 4 respectively). (B) HeLa cells stably expressing MPR were subjected to a budding assay with 7 min labeling and a cytosol concentration of 12 mg/ml using either bovine cytosol (B; lane 2), or porcine cytosol (P; lanes 3-5) in buffer A (A; lanes 2 and 3), buffer K (K; lane 4), or buffer A with 10% glycerol (G; lane 5).*

In order to increase the sensitivity of the assay, cytosols from different species in different buffers were tested for their efficiency in promoting budding of MPR in HeLa cells. Bovine and porcine cytosols at 6 mg/ml were equally efficient in standard buffer (buffer A, see methods; Fig. 24B). The same was true for cytosol extracted in buffer K, which is more physiological due to cytosolic concentration of potassium, and cytosol extracted in buffer A with 10% glycerol as a cryoprotectant. Thus, the assay was very robust, but none of the cytosol preparations was more efficient in promoting budding than the others.

## Elimination of broken membranes by proteinase digestion

Since mechanical stress disrupts membranes, one would expect fragments of donor membranes to contaminate intact carriers in the supernatant. To examine this, a standard budding assay with H1-TS was performed and supernatants were split into 2 tubes, and treated with or without 0.5 mg/ml proteinase K (Fig. 25A, lanes 4-6). Upon this treatment bands generated by H1-TS, were shifted towards lower molecular weight, representing the loss of the cytoplasmic portion of the protein. The intensity of the background budding signal was reduced to very low levels, while the signal of cytosol dependent budding was reduced by a similar amount. This was a very important finding, since it showed that the majority of the background budding signal was composed of H1-TS not protected by membranes and could thus be removed.

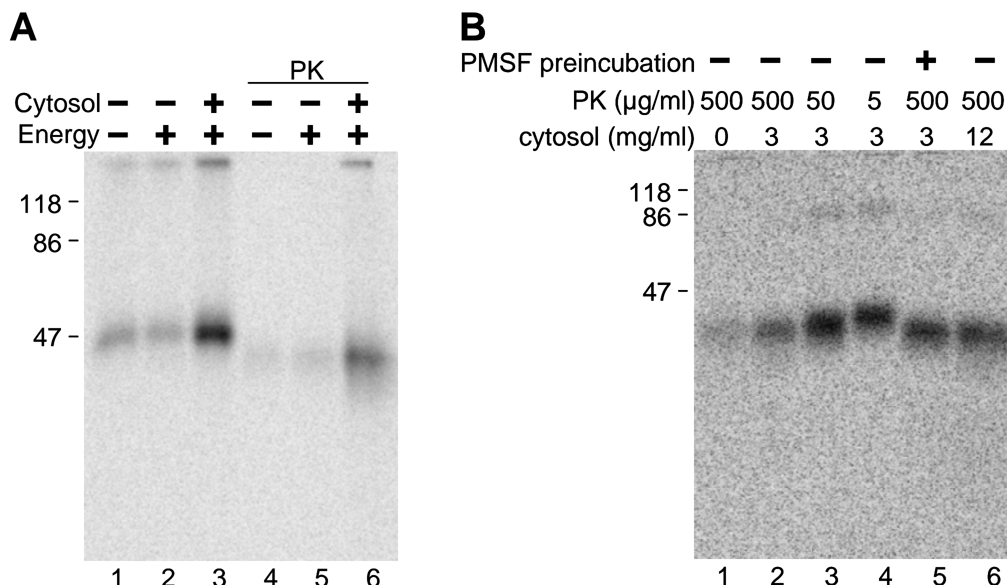


***Fig. 25: A large portion of radioactive signal in budding assay supernatants is derived from broken membranes and can be removed by proteinase K treatment.*** *(A) Standard budding procedure with 7 min labeling and 12 mg/ml cytosol was applied to HeLa cells stably expressing MPR. Low speed supernatants were treated without (lanes 1-3) or with (lanes 4-6) 0.5 mg/ml proteinase K (PK) for 30 min at 4°C. (B) Budding assay was performed as in (A), but cytosol concentration could be reduced to 3 mg/ml (lanes 2-5). Proteinase K was titrated and the concentration of 500 µg/ml was needed (lane 2), as concentrations of 50 (lane 3) or 5 (lane 4) µg/ml did not completely remove accessible proteins. Protease was stopped with PMSF in lysis buffer (lanes 1-4 and 6) or by 5 min preincubation with PMSF in PBS prior to lysis (lane 5).*

The proteinase was titrated on a standard MPR budding assay (Fig. 25B). The high concentration of 0.5 mg/ml was necessary to efficiently remove the artificial signal. It was also tested whether stopping of digestion with 8 mM PMSF in lysis buffer was sufficient. To test this, a preincubation with PMSF was performed prior to lysis. Comparing the signal in lane 5 (with preincubation) with lane 2 (PMSF coincident with lysis) of Fig. 25B, shows that a large portion of the signal can be rescued.

## *Effects of manipulated cytosol on budding of MPR and H1*

Having removed most of the background budding, the assay became more sensitive to low cytosol concentrations. 2.5 mg/ml was used and components of the clathrin machinery were tested for their importance in budding of MPR and H1.

### Budding of H1 and MPR is independent of clathrin machinery but dependent on GTP hydrolysis

With the assay in its final revision the ratio between cytosol dependent and background budding grew from, at best, 3:1 (Fig. 17) with cytosolic protein concentration of 12 mg/ml, to 5:1 (Fig .26A) with concentration of 2.5 mg/ml. The assay showed in Fig. 26A also confirms that energy alone is not sufficient for efficient budding (lane 2), but that it is necessary (lane 3).

The TGN carrier formation procedure could now be used to test for importance of candidate proteins in TGN exit of H1 and MPR. Components of the clathrin sorting machinery were tested first. Arf1, the GTPase that regulates CCV assembly can be trapped in its GDP (inactive) state by inhibiting its guanidine exchange factor (GEF) with a fungal compound, brefeldin A (BFA). A budding assay with 100 µg/ml of this drug was performed (Fig. 26B). It had neither an effect on MPR budding, nor on H1 budding (lane 3).

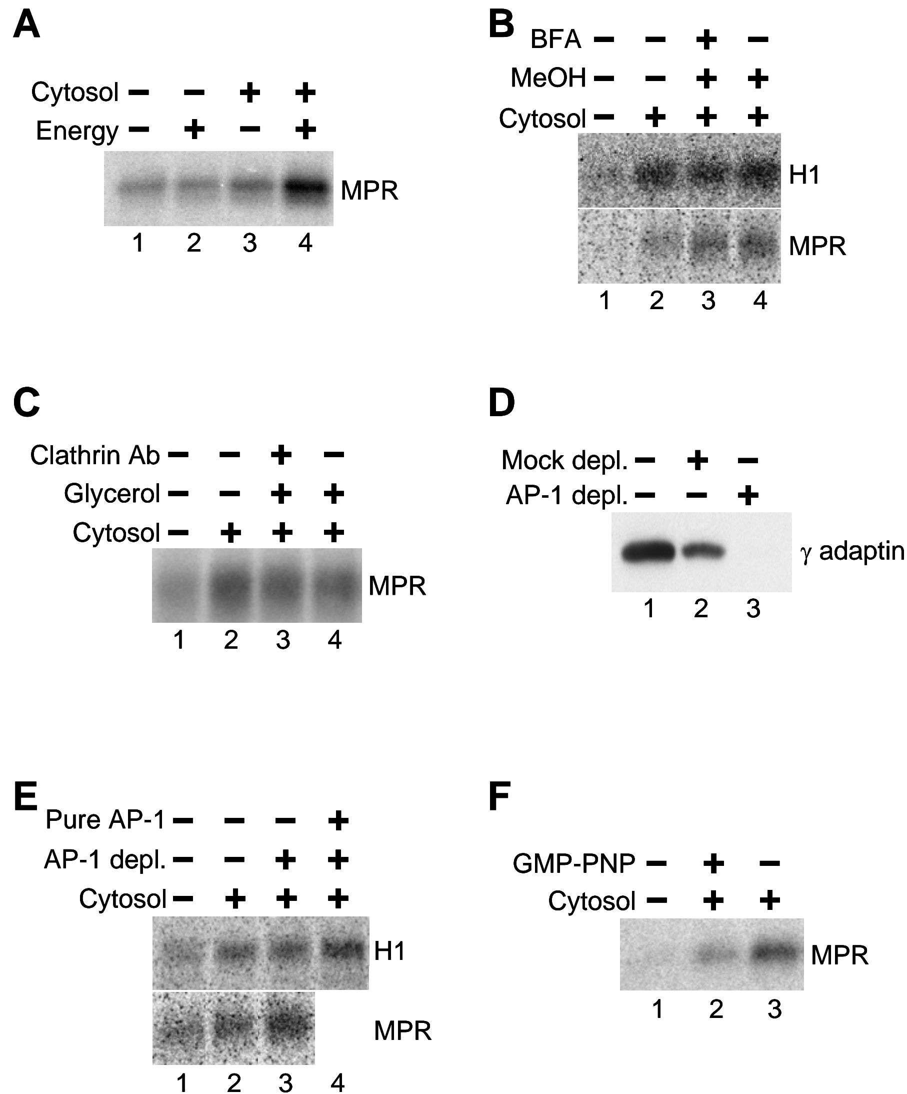


***Fig. 26: MPR and H1 carrier formation at the TGN does not depend on the clathrin machinery, but requires GTP hydrolysis.*** *(A) HeLa cells stably expressing MPR were subjected to a budding assay with 7 min labeling and a cytosol concentration of 2.5 mg/ml. Efficient budding occurred only in presence of cytosol and energy (lane 4), but not in presence of cytosol (lane 3) or energy (lane 2) alone. (B) Budding assay with H1 or MPR was performed as in (A), but cytosol was preincubated for 30 min at 37°C with 100 µg/ml BFA (BFA; lane 3) or equivalent amount of methhanol (MeOH; lane 4), which was the solvent for BFA. (C) MPR budding performed as in (A). Cytosol was preincubated with 80 µg/ml of X22 clathrin inhibitory antibody (Clathrin Ab; lane 3), or equivalent amount of 40% glycerol in PBS (Glycerol; lane 4), which was the solvent for X22, for 30 min at 37°C. (D) Western analysis of AP-1 depletion: AP-1 was depleted from bovine cytosol as described in materials and methods. 300 µg of untreated cytosol (lane 1), 150 µg of mock depleted (Mock depl.; lane 2), or 150 µg of cytosol depleted of AP-1 (AP-1 depl.; lane 3) were loaded on the gel. (E) Budding of H1 and MPR was performed as in (A). Mock depleted (lane 2), AP-1 depleted (lane 3), or AP-1 depleted and supplemented with 12 µg/ml (lane 4) cytosol was used. (F) MPR budding was performed as in (A). GMP-PNP at a final concentration of 4 mM (GMP-PNP; lane 2) was preincubated with the cytosol for 30 min at 37°C and no GTP was added to the budding reaction. Control reaction (lane 3) was performed with cytosol without GMP-PNP, but otherwise treated as above.*

Clathrin itself can be blocked by the use of an antibody (X22, Pagano et al., 2004). When used in a MPR budding experiment, the concentration of 80 μg/ml (twice the amount sufficient to inhibit endosomal recycling) had no effect on TGN carrier formation (Fig. 26C, lane 3). Another component of the clathrin machinery on the TGN is AP-1. But, its interaction with a kinesin (Nakagawa et al., 2000) could allow it to operate independently of clathrin. Hence, its influence on MPR and H1 carrier formation was tested as well (Fig. 26D and E). Even though AP-1 depletion was complete when measured by western blotting (Fig. 26D, lane 3), this cytosol still promoted budding (Fig. 26D, lane 3), indicating that AP-1 had no influence on carrier formation in this experimental setup.

Knowing that GTPases regulate most events in a cell, we tested a non-cleavable GTP analogue, GMP-PNP, for its influence on budding. A standard assay was performed and GMP-PNP was added instead of GTP (Fig. 26F, lane 2). The same concentration was used as for GTP in control lanes (4 mM). GMP-PNP clearly inhibited budding of MPR albeit not to the same level as background.

## Cytosol dependent budding does not represent Golgi disintegration

Since the TGN is a very dynamic organelle, where fission and fusion of membranes are kept in equilibrium, concerns arose whether the independence of clathrin machinery was an artifact caused by a shift of this equilibrium towards fission. To test this, budding was performed on H1-tailless, a version of H1 that lacks the cytosolic sorting determinants. As shown by immuno-fluorescence, this protein accumulates in the TGN (Wahlberg et al., 1995). Hence, H1-TS budding was compared to H1-tailless.

H1-TS carrier formation was clearly cytosol dependent. It was however very weak (Fig. 27A), as measured by comparison of supernatant and pellet, when compared to an early experiment (Fig. 17). This was most probably because of proteinase digestion. But, when compared to H1-tailless carrier formation (Fig. 27B), H1-TS budding was strong. When image levels were adjusted so that 20% of pellet signal was represented by a nearly saturated band, H1-TS cytosol dependence was clearly

seen, while H1-tailless was not. The signal intensity had to be elevated beyond the saturation level of 20% pellet in order to see a cytosol dependent band. Thus, some cytosol dependent TGN disintegration takes place, but not in the signal range of *bona fide* budding.

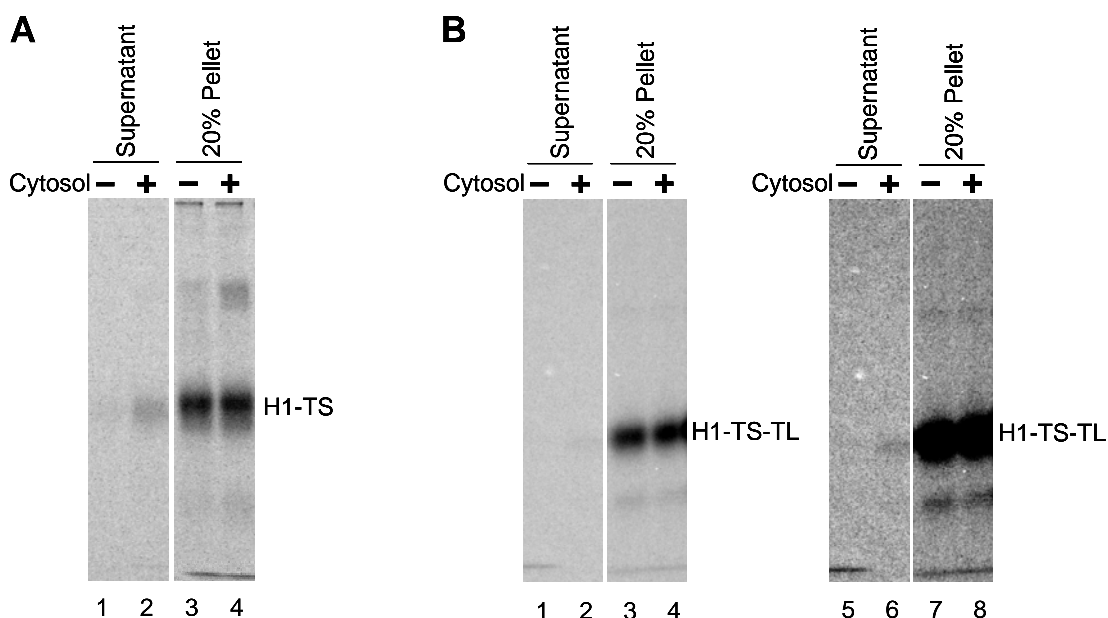


***Fig. 27: Cytosol dependent budding does not represent Golgi disintegration.*** *Standard budding with 7 min of labeling and 2.5 mg/ml cytosol was performed on HeLa cells stably expressing H1-TS (A) or H1-TS-TL (B). While H1-TS was recovered from low speed supernatant in a cytosol dependent manner ((A), lane 2), H1-TS-TL was not ((B), lane 2). Only after severe overexposure ((B), right panel, lanes 5-8) some H1-TS-TL could be detected (lane 6).*

## The new prospect: cytosol from cell cultures

Manipulating cytosol by incubation with drugs or by antibody effects is not the only way to alter its properties. When cytosol is prepared from cell cultures instead of tissue, it can be genetically modified e.g. with a dominant mutation of a candidate protein. This is a very elegant (because most physiological) method for cytosol modification. But tissue culture cytosol had first to be tested for its ability to promote TGN carrier formation.

A preliminary cell culture cytosol titration in a budding assay was performed. Concentrations as low as 0.4 mg/ml still promoted budding (Fig. 28, lane 2). This renders the experimental procedure potentially very sensitive. Thus, future experiments will be performed with tissue culture cytosol.

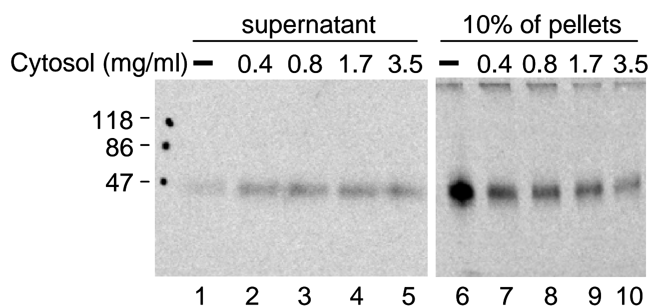


***Fig. 28: TGN carrier formation can be reconstituted with low concentrations of tissue-culture derived cytosol.*** *Standard budding assay with 7 min labeling was performed on HeLa cells stably expressing MPR. Cytosol was titrated from 0.4 to 3.5 mg/ml.*

# Materials and methods

## Reagents

Cell culture media and reagents were obtained from Invitrogen (Carlsbad, CA); protein A-Sepharose was from Zymed Laboratories (South San Francisco, CA). Guanylyl imidodiphosphate (GMP-PNP) ATP, GTP, creatine kinase, and creatine phosphate were from Roche Diagnostics (Indianapolis, IN). BFA was purchased from Sigma-Aldrich (St. Louis, MO). Cell culture flasks were from TPP (Trasadingen, CH), Ni-NTA agarose was from Qiagen (Basel, CH).

Polyclonal rabbit antibody against H1 was used. Rabbit anti-A1Pi antiserum was a kind gift from Jerry L. Brown (University of Colorado Health Sciences Center, Denver, CO). Mouse monoclonal anti-myc (9E10), anti-γ-adaptin (100/3) and anti-clathrin (X22) antibodies were purified using protein A Sepharose from culture media of hybridomas obatained from American Type Culture Collection (Manassas, VA). Horseradish peroxidase-coupled anti-mouse IgG and anti-rabbit IgG antibodies were purchased from Sigma-Aldrich (St. Louis, MO).

## Experimental procedures

### *Cell culture and transfection*

COS-1, HeLa, HEK293 and MDCK II cells were grown in DMEM supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine at 37°C in 7.5% CO2. COS-1 cells were transiently transfected in 6-well

plates using Lipofectine (Life Technologies, Inc.) and used 2–3 days after transfection. To produce stably expressing HEK293 cell lines, the constructs were subcloned into the expression vector pCB6 and transfected using calcium phosphate precipitation. Clonal cell lines resistant to 0.5 mg/ml G418-sulfate were isolated and screened for H1 or A1Pi construct expression by western analysis. Stable transfections in HeLa cells were performed with polyethylenimine (PEI). Clones resistant to 1 mg/ml G418 were isolated and expression was assessed by Western analysis.

### *Radiolabeling and endoglycosidase H treatment*

Transfected cells were grown in 6-well plates and at 90% confluency they were incubated for 30 min at 37°C with medium without sulfate or medium without methionine. Labeling was usually performed for 30 min at 37°C with 1 mCi/ml radioactive sulfate or 0.1 mCi/ml radioactive methionine. The chase was performed in standard DMEM medium and endoglycosidase H digestion was performed according to the manufacturer's protocol (on beads, at 37°C for 1 h with 2 U/ml enzyme).

### *Immunofluorescence*

Cells were grown on 14-mm glass coverslips, fixed with 3% paraformaldehyde for 15 min at room temperature, washed in phosphate-buffered saline (PBS) and quenched with 50 mM $NH_4Cl$ in PBS. Nonspecific antibody binding was blocked with PBS containing 1% bovine serum albumin. The fixed cells were incubated at room temperature with primary antibodies for 1 h, washed with PBS with albumin, and stained with fluorescent secondary antibodies in PBS with albumin for 30 min. After several washes with PBS with albumin, PBS, and water, the coverslips were mounted in Mowiol 488 (Hoechst). Staining patterns were analyzed using a Zeiss Axioplan 2 microscope with a KX Series imaging system (Apogee Instruments).

### *The budding assay*

Cells were grown to sub-confluence in a 150 cm2 flask and one day prior to the experiment transcription was induced with 2 mg/ml sodium butyrate in culture-medium. Cells were starved in medium lacking sulfate for 1 h and pulse-labeled for 7 min at 37°C with starvation medium containing 2 mCi/ml $[^{35}S]SO_4$. Alternatively

cells were labeled for 2 h at 19°C (TGN accumulation). After two rinses with PBS, the cells were permeabilized by incubation in swelling buffer (15 mM HEPES/KOH, pH 7.2, 15 mM KCl) for 15 min at 4°C, scraped into transport buffer (20mM HEPES/KOH, pH 7.2, 90 mM KOAc, 2 mM $Mg(OAc)_2$), and sedimented at 800×g for 5 min. The broken cells were resuspended in stripping buffer (20 mM HEPES/KOH, pH 7.2, 500 mM KOAc, 2 mM $Mg(OAc)_2$) for 10 min on a rocker at 4°C, pelleted again and resuspended in transport buffer. In a standard assay, permeabilized cells were incubated with indicated amount of cytosol and an ATP-regenerating system (2 mM ATP, 4 mM GTP, 12 mM creatine phosphate, 320 mg/ml creatine kinase) in a total volume of 250 µl. The reaction mixture was left on ice for 5 min and then incubated at 37°C for 30 min. Reactions were stopped on ice and centrifuged at 800×g for 5 min. The supernatants were carefully aspirated and solubilized for 1 h at 4°C with lysis buffer (1% Triton X-100, 0.5% deoxycholate in PBS, 4 mM PMSF). Insoluble material was removed by centrifugation in a microcentrifuge at 14,000 rpm for 10 min. Supernatants were recovered and the reporter was immuno-precipitated or, in case of the MPR which was His6-tagged, isolated with Ni-NTA agarose and analyzed by SDS-gel electrophoresis and autoradiography.

### *Cytosol*

Cytosol was obtained from calf brain, pig brain or tissue culture (after detachment from culture dishes with PBS/0.5mM EDTA and gentle pelleting at 800×g) as the high-speed supernatant after homogenization (Campbell et al., 1984) in buffer A (0.1 M MES/NaOH, pH 7.0, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.2 mM DTT), Buffer K (25 mM HEPES-KOH, pH 7.0, 125 mM potassium acetate, 2.5 mM magnesium acetate, 1 mM DTT) or buffer A with 10 % glycerol, supplemented with protease inhibitors. For immuno-depletion of AP-1, protein A-beads, were incubated overnight with anti-γ-adaptin (100/3) in transport buffer. 50 µl of packed beads were then incubated with 100 µl (1.5 mg) of brain cytosol for 2 h at 4°C with gentle rocking. As a control, cytosol was mock treated with beads without antibody. After centrifugation, the supernatant was collected and analyzed by immuno-blotting by using the corresponding specific antibody. BFA (100 µg/ml) and X22 anti-clathrin antibody (80 µg/ml) were incubated with cytosol for 30 min at 37°C prior to the experiment.

### *Protein Purification*

To produce pure AP-1, clathrin-coated vesicles were isolated from calf brains as described previously (Campbell and Rome, 1983). The coats were released and fractionated on a Superose 6 column as described previously (Crottet et al., 2002). AP-1 enriched fractions were collected and pooled.

# Discussion

In the course of this work an *in vitro* assay was developed to study protein exit from the *trans*-Golgi network. Using this assay it was shown that formation of primary post-TGN carriers for MPR and H1 is independent on Arf1 and the clathrin sorting machinery, but dependent on unknown factor(s) in the cytosol and GTP hydrolysis.

## *The assay*

We set out to develop an *in vitro* assay in order to test a single transport step for the requirements of cytosolic factors or other perturbations of the conditions. This is in contrast to *in vivo* manipulation of intact cells by overexpression, knock-down of proteins or addition of inhibitors since the cells will be exposed to the altered conditions in most cases at least several hours if not days (Ang et al., 2004). The alterations may produce indirect effects, like adaptation to the new conditions. At the TGN it is also likely that a protein that is blocked from exit via one pathway may eventually exit by another.

With our experimental procedure, we aimed at minimizing the manipulations that the TGN membranes were exposed to. For this reason, we did not attempt to purify the TGN but used semi-intact cells generated by swelling and scraping. To define the starting compartment we used sulfation which is a modification specific to the TGN. Two similar assays have previously been used to study the generation of secretory granules and the TGN exit of influenza hemagglutinin (Ellis et al., 2004; Ling et al., 1998). However, in both assays [$^{35}$S]methionine was used to label the reporters and extended incubation at 20°C to accumulate them in the TGN was performed. It was previously reported that the 20°C block affects the structure of the TGN (Griffiths et al., 1989; Ladinsky et al., 2002). In addition, proteins labeled with this procedure are

still present in the ER, ERGIC, and early Golgi, so that the origin of carriers is not entirely certain. In our improved assay, sulfation in the TGN is sufficiently efficient to produce a usable signal after only 7 min of labeling at 37°C. The starting compartment is thus well defined and generation of artifacts due to prolonged blocking of TGN exit is circumvented.

Nevertheless, it is clear that our procedure also results in some damage to the membranes. Proteinase K treatment digested a significant amount of sulfated protein, indicating the presence of broken membranes that allow access of the proteinase to the lumenal side. By routinely performing proteinase digestion after the budding reaction, the background signal from membrane breakage could be eliminated from consideration. High-salt washes have to be used to efficiently remove cytosolic components, and complete removal is probably not possible. It is therefore difficult to ensure that proteins potentially involved in the formation of transport carriers have been sufficiently removed from the membranes. As a consequence, negative results have to be interpreted with great caution.

On the positive side is the finding that sufficient amounts of cytosol at sufficient concentration can be generated from cultured cell lines .This opens the possibility to produce cytosols overexpressing individual proteins without having to purify them. In addition, it should be possible to test the effect of dominant mutant proteins without the need for purification or the use of antibodies which are often difficult or time-consuming to obtain.

The final experimental procedure showed to be robust, as four different reporters (H1, MPR, A1Pi and APN) showed cytosol dependent formation of transport carriers. Also cytosols from three different sources (bovine or porcine brain, and human HeLa cells) could promote budding, and carrier formation could be shown to be dependent on GTP hydrolysis.

## ***TGN exit by tubular carriers?***

Our first application of the assay aimed to confirm the notion that MPR leaves the TGN in a clathrin dependent manner and to test whether H1 exits via the same

mechanism. All our experiments, however, produced a negative answer for both reporters. Neither depletion of AP-1, nor inhibition of clathrin polymerization with an antibody affected TGN carrier formation. Also, perturbation of nucleotide exchange on Arf1 by BFA had no effect on this process. This is surprising as the GTPase was shown to control not only clathrin vesicle formation but also scission of VSVG containing carriers by the concerted action of actin and cortactin and dynamin-2 recruitment (Cao et al., 2005). As VSVG was shown to pass through endosomes en route to the PM (Ang et al., 2004), it is conceivable that MPR and H1 travel to endosomes by the same mechanism. Arf1 controls the GAP of the major actin organizing GTPase, CDC42, which recruits the Arp2/3 complex that itself nucleates actin filaments (Dubois et al., 2005). Also CtBP/BARS, another protein that mediates scission is dependent on Arf1 (Yang et al., 2006). PKD however, could be recruited to TGN membranes in presence of BFA (Liljedahl et al., 2001), thus, it could explain independence of this compound in this assay. But, as mentioned above, negative results have to be interpreted with caution, since for example residual AP-1 on the membranes may still be sufficient for vesicle formation. Arf1 activation may be less dependent on its GEFs and therefore less sensitive to BFA *in vitro*, as it may be activated spontaneously on the membranes during the relatively long incubation (Crottet et al., 2002). The strongest argument against clathrin dependence of MPR carrier formation at the TGN is provided by the clathrin antibody experiment. While formation of endosomal recycling vesicles was completely abolished (Pagano et al., 2004), TGN carrier formation was unaffected in this work.

All our results taken together certainly shed serious doubt on the accepted notion that MPR exits the TGN exclusively by clathrin/AP-1/Arf1 dependent vesicles (Ghosh et al., 2003). The earlier evidence for this is mainly that purified CCVs contain MPR-acid hydrolase complexes (Campbell and Rome, 1983; Schulze-Lohoff et al., 1985) and that CCVs containing MPRs were found by immuno-electron microscopy adjacent to the TGN (Klumperman et al., 1993).

However, there have already been some previous findings that pointed in a different direction. In AP-1 deficient fibroblasts MPR was found accumulated in endosomes and not in the TGN (Meyer et al., 2000). Even after discovery of GGAs that could serve as alternative clathrin adaptors, the problem was not solved, as these cooperate

with AP-1 (Doray et al., 2002b). Using live video microscopy, MPR was shown to exit the TGN, at least in part, in tubular carriers that could fuse with each other and with endosomes (Waguri et al., 2003). Later, these carriers were further characterized by EM tomography (Polishchuk et al., 2006). MPR was predominantly found in non-vesicular carriers (57.1% grape-like, 28.6% tubular, 9.5% saccular; Fig 29), and only 4.8% in vesicular carriers. Yet, roughly half of the surface of these non-vesicular structures was covered with clathrin. To investigate if they were CCV precursors, the authors inhibited fusion with endosomes and found that still, only a minor fraction (7.5%) of MPR was found in vesicular carriers. In the light of these studies and results presented in this thesis, it is unclear which CCVs had previously been analyzed. They may have transported MPR from early to late endosomes or they may have represented the minor fraction of TGN-derived carriers. They could represent sections of partially coated tubular carriers in EM studies, but, in biochemical studies, they could not be retrograde MPR containing CCVs, as acid hydrolases were still present.

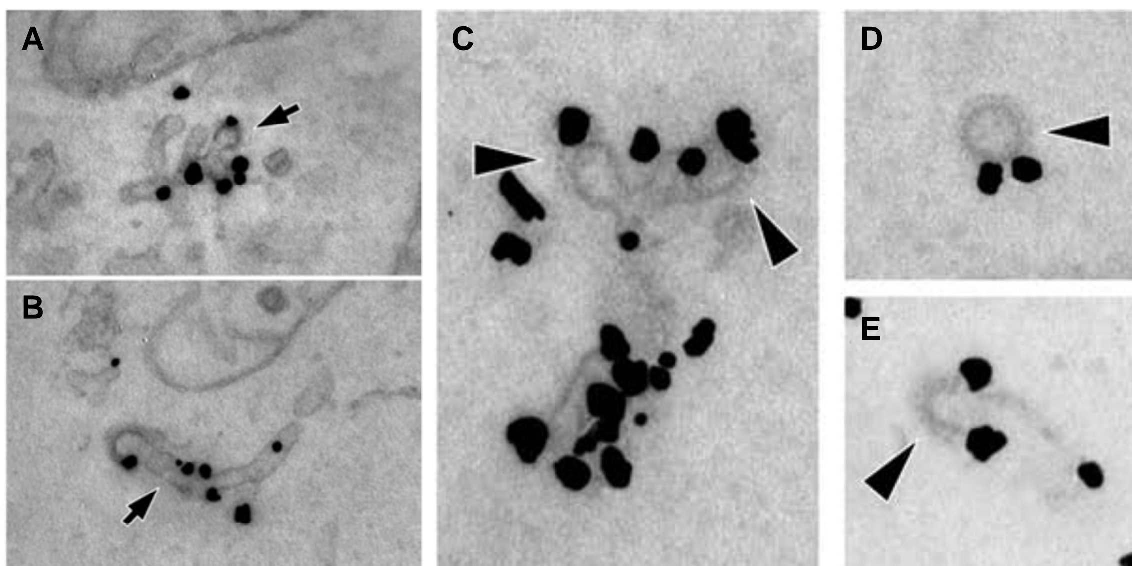


***Fig. 29: TGN exit of MPR and GGA1 clathrin coated structures is largely non-vesicular.*** *MPR (A and B, arrows) and GGA1 (C-E) stain free (as estimated by EM tomography) post-Golgi carriers which are clathrin coated (arrowheads). (A-C and E) show non-vesicular carriers, while (D) shows a typical coated vesicle. Adapted from (Polishchuk et al., 2006).*

## TGN carrier formation depends on GTP hydrolysis

The inhibitory effect of GMP-PNP on TGN carrier formation indicated that hydrolysis of GTP is necessary in this process. Dynamin requires hydrolysis (Roux et al., 2006), but PKD, while dependent on a trimeric G-protein, can be recruited to TGN membranes in the presence of GTPγS, another non-cleavable GTP analogue (Liljedahl et al., 2001). This, together with the fact that PKD did not regulate TGN-to-endosome traffic of the H2-M protein, suggests that this kinase is unimportant in this sorting step. However, basolateral VSVG localization clearly depends on PKD (Yeaman et al., 2004) and this reporter can pass endosomes on the way to the PM (Ang et al., 2004). This raises the question whether H1 and MPR TGN exit could, at least in part, also depend on this kinase. It is supported by the fact that GMP-PNP did not completely abolish budding (Fig. 26F, lane 2).

## GAGs

The GAG-tag was developed initially as a method to improve the sulfation signal for the budding assay. However, as presented in part I of this thesis, GAG attachment was shown to change the trafficking properties of the reporter. H1-GAG, when used in the TGN budding assay also produced lower signals than H1 or MPR lacking GAGs. Similarly low efficiency was also observed for APN and for A1Pi. Since the former is an apical and the latter a secretory protein, both travel directly to the PM. If the GAG redirects the reporter to such a direct carrier, one could speculate that these are formed by a mechanism that is less efficiently reconstituted in the assay or mediated by factors that are not efficiently removed. Indeed, the accelerated biosynthetic exocytosis rate of H1-GAG suggests a sorting mechanism different from that of GAG-free H1.

## Outlook

The good efficiency with which cytosol extracted from cultured cell lines promotes budding led to generation of cell lines stably expressing wild-type, dominant negative and permanently active variants of Rab11 and Rab14. Rab14 is important in traffic between endosomes and TGN, but its precise role is not yet fully established (Junutula et al., 2004; Proikas-Cezanne et al., 2006). Rab11 is important in many trafficking

steps (Zerial and McBride, 2001) including exit of VSVG from the TGN (Chen et al., 1998). Recently, Rab10 was also implicated in this sorting event (Schuck et al., 2007). It will be interesting to see how these mutants affect sorting of reporters used in the TGN budding assay.

Since MPR and H1 carriers generated in this assay are presumably nonvesicular, the most obvious step is to look for a scission machinery. Actin for example can be destabilized with latrunculin or stabilized with phalloidin or jasplakinolide (Bubb et al., 2000). Recently, a compound named dynasore has been identified that specifically blocks the action of dynamin (Macia et al., 2006). Also for inhibition of PKD dependent exit of VSVG a specific compound was isolated (Diaz Anel, 2007). These chemicals are easily applicable in the TGN carrier formation assay. CtBP/BARS would need to be immuno-precipitated and tested for its redundancy with dynamin (Yang et al., 2006).

With respect to carrier shape, it will be interesting to compare migration of different reporters in a velocity gradient. Unfortunately preliminary experiments performed with H1-GAG were not conclusive as the signal was too low.

The accelerated exocytosis of GAG modified proteins requires further investigation, e.g. by comparing surface arrival kinetics of H1-GAG with a typical secretory protein, such as A1Pi. The budding assay could be used to investigate minimal requirements for formation of these carriers with respect to cytosol concentration, energy requirements and time required for carrier formation. Also, it is important to show whether biosynthetic surface delivery of the glycosaminoglycanated protein involves endosomes or not. Finally, it will be interesting to determine the hierarchy between the GAG signal and the basolateral sorting determinant in polarized protein delivery e.g. by transferring the assay into MDCK cells.

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# Curriculum Vitae

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**List of publications**

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