MOLECULAR INSIGHTS INTO THE TRANSPORT LECTIN FUNCTION OF ERGIC-53

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von
Christian Appenzeller-Herzog
aus Oberengstringen und Zürich, ZH

Basel, 2004

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
auf Antrag von

Prof. Dr. Hans-Peter Hauri und Prof. Dr. Martin Spiess

Basel, den 8. Juni 2004

Prof. Dr. Marcel Tanner
Dekan

Acknowledgments

In the end, this thesis has resulted in a collection of some meaningful data, but looking back into the past four years, I remember periods – actually three years of work – loaded with nothing but frustration. I am emphasizing this, because the world of science tends to conceal this part of our work leaving behind many high-quality scientists without any measurably referenced success. I am also saying this, because it was my personal belief in God that helped me a lot during these times of failure, and I am grateful for this. Furthermore, I would like to thank Him for the findings that I could document in the Results part of this thesis. I know that it was not only my and my advisors' rationality that turned around the development of this thesis.

I am also grateful to Prof. Dr. Hans-Peter Hauri, because his way of approaching science will always accompany me as a positive example. I could profit a lot from him and it was fun to team up with him to do scientific work, especially to write papers. Also, I would like to thank Käthy Bucher for her positive contributions to this work, her advice and the cheerful atmosphere that she is spreading. My thank also belongs to all my colleagues in the lab and the collaborators that are listed in the Results part. The advice of all of them helped me a lot. Especially, I would like to point out Oliver Nufer and Peter Burkhard. Furthermore, I would like to thank the floormanagers Roger Jenny and Markus Meier, the administrators Iny Wittker and Marianne Liechti, and the lab staff Angèle Klein and Monika Furrer for maintaining the establishment in a good way. Last, but not least, I am very grateful to my wonderful wife Karin, my family and friends for all the support that they gave me. My work really relied upon your continuous backup – thank you very much!

Schlussendlich ist diese Doktorarbeit doch noch zu einer Sammlung von aussagekräftigen Daten zusammengewachsen, aber im Rückblick auf die vergangenen vier Jahre erinnere ich mich vor allem an schwere, frustrierende Zeiten (drei Jahre davon). Ich betone dies hier, weil die Wissenschaft diesen Teil von unserer Arbeit tendenziell verschweigt, was dazu führt, dass sehr gute Wissenschafter ohne messbaren, zu Buche getragenen Erfolg zurückgelassen werden. Ich erwähne das aber auch, weil es mein persönlicher Glaube an Gott war, der mir sehr durch diese Zeit des Misserfolgs geholfen hat, und dafür bin ich Ihm dankbar. Ich möchte Ihm auch danken für alle Ergebnisse, die ich im Resultatteil dieser Doktorarbeit

zusammentragen konnte. Ich weiss, dass es nicht nur meine und meiner Berater Vernunft war, die dieser Arbeit die positive Wendung gegeben hat.

Ich möchte mich auch bei Prof. Dr. Hans-Peter Hauri bedanken, denn seine Art, an die Wissenschaft heranzugehen, wird mir immer ein Vorbild bleiben. Ich konnte viel von ihm profitieren und es hat richtig Spass gemacht, im Team mit ihm zu forschen und, ganz im Speziellen, Publikationen zu verfassen. Ebenfalls möchte ich Käthy Bucher danken für ihre positiven Beiträge zu dieser Arbeit, für ihren Ratschlag und für die fröhliche Atmosphäre, die sie am Arbeitsplatz verbreitet hat. Mein Dank gehört auch allen meinen Kollegen im Labor und auswärtigen Kollaboratoren, die im Resultatteil erwähnt sind. Ihre Ratschläge haben mir sehr geholfen. Speziell hervorheben möchte ich dabei Oliver Nufer und Peter Burkhard. Ausserdem möchte ich mich bei den Departements-Managern Roger Jenny und Markus Meier, den Administratorinnen Iny Wittker und Marianne Liechti und den Labor-Helferinnen Angèle Klein und Monika Furrer bedanken für die gute Aufrechterhaltung des Betriebs. Zu guter letzt, und mit besonderer Betonung, möchte ich meinen Dank aussprechen an meine wunderbare Frau Karin, meine Familie und meine Freunde für ihre Unterstützung. Meine Arbeit hing wirklich von Eurem immerwährenden Beistand ab – vielen, herzlichen Dank!

Summary

Secretion of proteins is an essential function of eukaryotic cells. The secretory proteins' journey along the organelles of the exocytic pathway is initiated by the exit from the endoplasmic reticulum (ER), which defines a major rate-limiting step for protein secretion. ER-exit is subject to tight quality control. Selective, receptor-mediated cargo capture is one of the mechanisms thought to contribute to the elaborate proof-reading system of the ER.

The recycling mannose lectin ERGIC-53 operates as an ER-export receptor of a subset of secretory glycoproteins. The required signals for this transport step, however, remain poorly described. Experiments in this thesis show that ERGIC-53 assisted ER-exit of procathepsin Z depends on a novel transport motif that is composed of a high-mannose type oligosaccharide and a peptide β-hairpin loop. Deletion of either determinant compromises ERGIC-53 association and slows procathepsin Z transport. An equivalent carbohydrate/hairpin structure is identified in cathepsin C, another cargo of ERGIC-53, reflecting the general nature of this ER-export signal. Further experiments reveal that the N-glycans of loop-deficient procathepsin Z become efficiently mannose 6-phosphorylated, but undergo increased carbohydrate processing in the Golgi including complex glycosylation. Strikingly, cathepsin Z lacking the peptide loop is not targeted to its normal destination, the lysosome, suggesting that it lacks the correct carbohydrate signal for lysosomal delivery. The presented data describe the first ER-exit signal on a secretory protein and establish an unexpected link between lectin-mediated export from the ER and post-Golgi sorting.

This thesis also provides the molecular basis for ERGIC-53/cargo dissociation in the ERGIC. *In vitro* mannose binding experiments reveal that the lectin only displays its full activity at pH 7.4 – the pH of the ER – but not at slightly lower pH. The acid-sensitivity is modulated by the calcium concentration indicating a molecular link between pH-sensing and calcium complexation. This link is spotted by the identification of His178 that is conserved throughout the family of animal L-type lectins and – in its deprotonated form – binds a calcium ion in the carbohydrate recognition domain (CRD) of ERGIC-53. pH-induced inactivation of ERGIC-53 is also shown in cell culture. Glycoprotein binding is inhibited, if the ER is acidified, and the kinetics of glycoprotein dissociation are slowed, if the ERGIC is neutralized. The results establish the ERGIC as the earliest acid compartment of the secretory pathway and suggest that pH-induced glycoprotein dissociation may be backed by a mechanism that maintains lower levels of free calcium in the ERGIC.

The organelles of the secretory pathway operate as intracellular calcium stores. High concentrations of calcium have been measured in the lumen of the ER and the Golgi, but the calcium concentration in the ERGIC is not known. Therefore, a strategy was developed to quantitatively assess the free calcium concentration of the ERGIC *in vivo* using the green fluorescent protein-based calcium-indicator yellow cameleon. Targeting of the indicator to the ERGIC is achieved by fusing it to an inert variant of the ER-Golgi SNARE Sec22b. The fusion protein dynamically localizes to the ERGIC without disturbing the function of the endogenous SNARE machinery. It will in the future provide a valuable tool for calcium measurements in the ERGIC.

Table of Contents

1. Introduction

A fundamental property of the eukaryotic cell is to maintain an elaborate morphological system of membrane-enclosed, extracellular space within its own boundaries. These membrane-enclosed spaces are topologically equivalent to the outside of the cell and differ from the cytosol with respect to ion balance and redox conditions. Many cellular functions, including intracellular protein maturation and extracellular signal transduction, critically depend on such compartmental organization within the cell. A graphical outline on the intracellular network of extracytosolic space, called the secretory/lysosomal (vacuolar) and endocytic pathways, is given in Figure 1. The present thesis focuses on the secretory/lysosomal pathway.

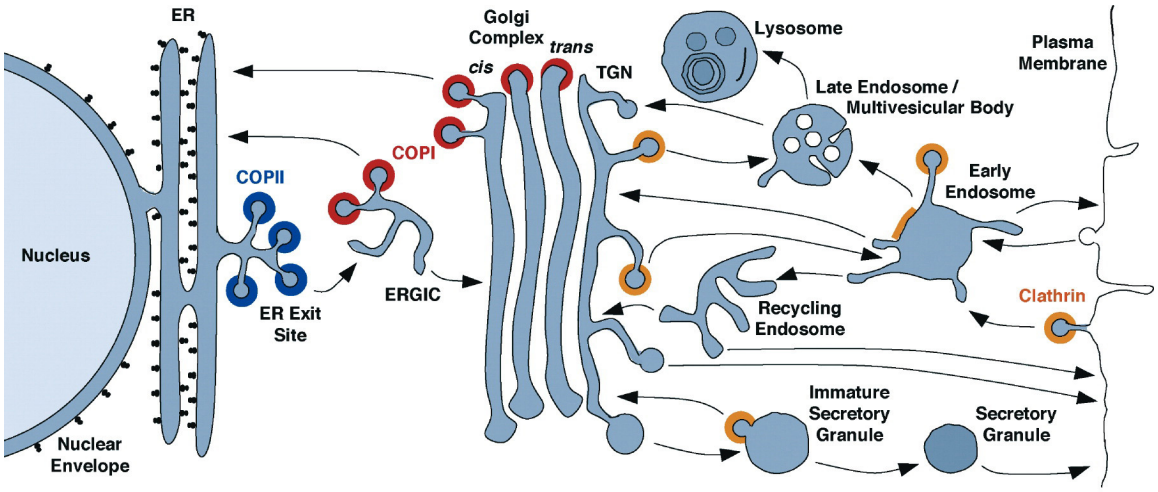


Figure 1

The secretory/lysosomal and endocytic pathways.
The scheme represents the compartments of the secretory/lysosomal and endocytic pathways that mediate protein transport out of the cell or into the cell. Transport steps are indicated by arrows. Colours indicate the locations of the protein coats COPII (blue), COPI (red), and clathrin (orange). The compartments relevant in this thesis are the ER (and ER exit site), the ERGIC, the Golgi Complex, the TGN, and the Lysosome. The figure was reproduced from Bonifacino and Glick (2004).

1.1 PROTEIN TRAFFICKING AND THE CONCEPT OF VESICULAR TRANSPORT

The secretory/lysosomal (or exocytic) pathway is subdivided into a series of different membrane compartments of defined molecular composition. Newly synthesized exocytic proteins enter the pathway at the rough endoplasmic reticulum (ER) and are subsequently sorted to different organelles including Golgi apparatus, plasma membrane, endosomes, and lysosomes. Research in the field of protein trafficking deals with the elucidation of the mechanisms, by which itinerant proteins find their proper place within the cell without compromising the molecular identity of the individual organelles along the pathway. These mechanisms are intimately connected to the vesicular transport hypothesis, which states that the transfer of cargo molecules between organelles of the secretory pathway is mediated by shuttling transport vesicles (Palade, 1975). According to this hypothesis, vesicles bud from a donor compartment ("vesicle budding") by a process that allows selective incorporation of cargo into the forming vesicles, while retaining resident proteins in the donor compartment ("protein sorting"). The vesicles are subsequently targeted to a specific acceptor compartment ("vesicle targeting"), into which they unload their cargo upon fusion of their limiting membranes ("vesicle fusion"). To balance this cargo movement, organelle homeostasis requires the retrieval of transport machinery components and escaped resident proteins from the acceptor compartments back to the corresponding donor compartments, presumably also occuring by vesicular transport.

The molecular elucidation of the secretory pathway combining yeast genetics, morphology, and *in vitro* complementation assays identified the predicted key components of the vesicular transport hypothesis that we today take for granted (Balch et al., 1984; Bonifacino and Glick, 2004; Novick et al., 1980). We now know that membrane trafficking is organized and specified by many protein complexes and protein families. Among these, coat proteins mediate protein sorting and vesicle budding (Bonifacino and Lippincott-Schwartz, 2003; Kirchhausen, 2000) (see also Figure 1), tethering proteins mediate vesicle targeting (Whyte and Munro, 2002), SNARE proteins mediate vesicle fusion (Chen and Scheller, 2001), and Rab GTPases (Segev, 2001) as well as Sec1/Munc18 proteins (Gallwitz and Jahn, 2003) are involved in multiple and diverse aspects of transport. Our standard of knowledge on the generality of these mechanistic principles, but also a summary on the puzzling issues in the molecular understanding of vesicular transport, that are to be resolved in the future, are extensively discussed in the above cited reviews.

Despite the groundbreaking advances in the identification of the vesiclular transport machinery, more recent work may add some variations to the general model that is outlined above. First, live cell imaging studies using fluorescent marker proteins have challenged the idea that the small vesicles (or empty coat cages) that have been produced *in vitro* using the protein coats COPII (Antonny et al., 2003; Matsuoka et al., 1998; Rowe et al., 1996), COPI (Bremser et al., 1999; Spang et al., 1998), and clathrin (Drake et al., 2000; Kirchhausen and Harrison, 1981) are the true transport intermediates within the cellular context (Hirschberg et al., 1998; Presley et al., 1997; Scales et al., 1997; Toomre et al., 1999). It is now generally accepted that at least some membrane carriers are larger and more pleimorphic than conventional vesicles (Bonifacino and Lippincott-Schwartz, 2003; Stephens and Pepperkok, 2001). As it is well conceivable that these large membrane carriers arise by the same protein coat-driven mechanisms that have been defined *in vitro*, we may have to refine our image on how big "vesicles" can be. Second, the paradigm that protein trafficking through the secretory pathway is achieved by bi-directional vesicle flow between stable compartments has been questioned by the formulation of the cisternal maturation hypothesis. In this model, that has been put forth for the Golgi apparatus, but can be applied to the secretory apparatus as a whole, membrane recycling is the driving force for membrane balance as cisternae continuously form out of the ER and move in a *cis* to *trans* direction (Storrie and Nilsson, 2002). Thus, budding transport vesicles would only be necessary for retrograde traffic, while the secretory anterograde traffic itself would occur by progressive maturation of the initial membrane entities ("cisternae"). Third, evidence is accumulating that at multiple levels of the secretory pathway, protein and membrane trafficking involves long membrane tubules (Blum et al., 2000; Keller et al., 2001; Klumperman et al., 1998; Marra et al., 2001; White et al., 1999). These tubules were essentially unstudied until the discovery that the fungal metabolite brefeldin A, known to release the COPI coat from membranes, stimulates their formation. It has been suggested that the extent of tubulation within any compartment can be viewed by the extent of coat protein assembly/disassembly (Klausner et al., 1992). Furthermore, it has been shown that membrane tubulation depends on the activity of cytoplasmic phospholipase A_2 enzymes (Brown et al., 2003). It is possible that one function of tubular transport is to efficiently compensate unequal membrane delivery created by vesicular trafficking.

Because of the focus in this work on transport between the ER and the ER-Golgi intermediate compartment (ERGIC, Figure 1) I will further go into molecular details on this early transport step in the following chapter. Of the various protein coats that have been

identified to date, COPII – involved in ER-to-ERGIC transport – due to its relatively simple architecture is one of the best understood and serves as an ideal example of vesicle budding.

1.2 VESICULAR TRANSPORT MECHANISMS FROM THE ER TO THE ERGIC

Budding

COPII-dependent exit of proteins from the ER occurs at transitional elements or ER-exit sites (ERES). These sites by electron microscopy appear as morphologically characteristic structures devoid of ribosomes (Bannykh et al., 1996). As determined by live cell imaging, ERES are long-lived membrane subdomains from which COPII vesicle budding occurs repeatedly (Hammond and Glick, 2000; Stephens et al., 2000). The COPII coat assembles by the stepwise deposition of Sar1p, Sec23p-Sec24p, and Sec13p-Sec31p onto the ERES. First, the small GTPase Sar1p is recruited from the cytosol by the ER-localized Sec12p guanine nucleotide exchange factor (GEF) (Barlowe and Schekman, 1993), whereby Sar1p-GTP becomes anchored to the membrane by a conserved hydrophobic patch at its N-terminus (Huang et al., 2001). This binding of Sar1p to the ERES leads to acute membrane deformation and has been suggested to contribute to the formation of ERES (Aridor et al., 2001). Activated Sar1p then binds the Sec23p-Sec24p complex. The resulting triple protein complex constitutes the so-called pre-budding complex, which has recently been analyzed by electron microscopy (Lederkremer et al., 2001; Matsuoka et al., 2001) and X-ray crystallography (Bi et al., 2002). Sec23p makes direct contact with Sar1p-GTP, while Sec24p participates in cargo recognition (see below). Once assembled onto membranes, the pre-budding complex recruits the Sec13p-Sec31p subcomplex, which then drives coat polymerization and membrane curvature into a bud (Figure 2). The concave surface of the pre-budding complex is thought to contribute to the membrane deformation activity of the COPII coat (Bi et al., 2002). It is possible that concave coat polymerization *per se* can also provide the mechanistic driving force for the final fission of the vesicle from the donor membrane (Antonny et al., 2003).

The activity of Sec23p as a GTPase-activating protein (GAP) of Sar1p (Yoshihisa et al., 1993) is augmented approximately ten-fold by addition of Sec13p-Sec31p (Antonny et al., 2001). It is thought that, after GTP hydrolysis, Sar1p is released, leading to uncoating of the vesicle and concomitant unmasking of the vesicle associated targeting machinery (see below). Thus, COPII coat assembly is tightly coupled to its disassembly. It has been suggested that GTP hydrolysis occurs already during vesicle coat formation, and that Sar1p-GTP is

dispensable for the integrity of the central area of the forming coat (owing to the kinetic stability of the polymerized coat subunits), but is required to stabilize the propagating coat edges (Antonny and Schekman, 2001).

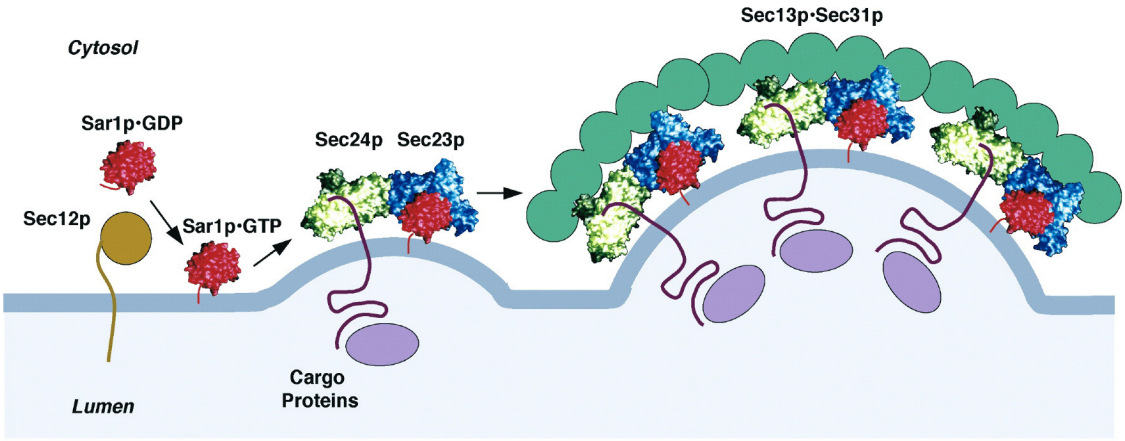


Figure 2

Assembly of COPII.
Cytosolic Sar1p-GDP is bound to the ER membrane by the ER GEF protein Sec12p. Sar1p-GTP in turn recruits the Sec23p-Sec24p subcomplex by binding to Sec23p, which results in the formation of the ternary pre-budding complex. Transmembrane cargo proteins gather at the site of coat assembly by binding to Sec24p. As a next step, the elongated Sec13p-Sec31p subcomplex that appears as a five-globular domain structure in electron microscopy polymerizes onto and thereby crosslinks the pre-budding complexes. The surface structure representation of the ternary pre-budding complex is derived from crystallographic data (Bi et al., 2002). Membrane curvature and bud formation are thought to be initiated by the concave structure of the pre-budding complex and further stimulated by coat polymerization. The figure was reproduced from Bonifacino and Glick (2004).

Targeting

To insure that ER-derived vesicles will recognize their acceptor compartment (the ERGIC in mammalian cells or the Golgi in yeast cells), they are provided with a unique set of targeting molecules that are enriched on the forming COPII bud. These include the long coiled-coil protein p115 and the ER-Golgi SNARE proteins that will be discussed below.

P115 (called Uso1p in yeast) is an essential protein that forms an elongated homodimer with two globular heads and an extended tail formed by the N-terminal coiled-coil domain (Sapperstein et al., 1995; Yamakawa et al., 1996). It is involved in multiple transport events including ER-to-ERGIC, ERGIC-to-Golgi and intra-Golgi transport. For the later transport steps it interacts via its N-terminus with other coiled-coil proteins of the Golgi ("golgins"), namely GM130 and giantin (Linstedt et al., 2000; Seemann et al., 2000; Sonnichsen et al., 1998). During ER-exit, it is specifically recruited to nascent COPII vesicles

by GTP-bound Rab1 (Allan et al., 2000). This interaction is released on the budded vesicle, where p115 binds to ER-Golgi SNARE proteins (see below). P115 is essential for the initial docking of ER-derived vesicles to the yeast Golgi (Cao et al., 1998) and presumably to the ERGIC in mammalian cells (Alvarez et al., 1999). This docking again requires activated Rab1 (called Ypt1p in yeast), but this time on the target membrane (Cao and Barlowe, 2000).

Another interesting question that has been raised only recently concerns the inability of budded COPII vesicles to undergo back-fusion with their donor compartment, the ER. As the COPII coat is thought to be instantly released upon fission of the vesicle (see above), there must be a mechanism to prevent the naked vesicle, fully equipped with the membrane fusion machinery, from fusing back. Interestingly, the protein that has been identified as a back-fusion barrier, Tip20p (Kamena and Spang, 2004), is also part of the supposed membrane tethering complex for Golgi/ERGIC-to-ER retrograde vesicles. This complex is built at least by the three protein subunits Dsl1p-Tip20p-Sec20p identified so far and interacts with the retrograde coat COPI (Andag et al., 2001; Andag and Schmitt, 2003; Reilly et al., 2001). Hence, it may be assumed that the ER selectively consumes vesicles that have not yet or only partially shedded their COPI coat, while COPII vesicles will be rejected in any case.

Fusion

Fusion of intracellular membranes is mediated in many, if not all, cases by SNARE proteins (Chen and Scheller, 2001). The final stage of fusion involves the formation of a bundle of four parallel core SNARE domains in a tetrameric coiled-coil, one contributed by the vesicle and three contributed by the target membrane (Sutton et al., 1998). Such a *trans* SNARE complex bridges the two membranes, and its formation is thought to overcome the energy barrier preventing two membranes from fusing with each other. The specific co-factors N-ethylmaleimide sensitive factor (NSF) and NSF-attachment proteins (SNAPs) are then required to render the SNARE proteins competent for another round of fusion (Mayer et al., 1996), presumably by unwinding the SNARE bundels that arise after fusion is accomplished (termed *cis* SNARE complexes). Concerning anterograde transport in the early secretory pathway, the relevant SNARE complex is formed by four individual transmembrane SNAREs, namely Syntaxin 5, Sec22b, Membrin, and Bet1 (Sed5p, Sec22p, Bos1p, Bet1p in yeast) (McNew et al., 2000; Xu et al., 2000). These proteins cycle between ER and Golgi (Chao et al., 1999). Their specific incorporation into COPII vesicles is driven by direct interaction with the vesicle coat component Sec24p (Liu et al., 2004; Miller et al., 2003; Mossessova et al., 2003; Peng et al., 1999; Springer and Schekman, 1998).

ER-Golgi *trans* SNARE complex assembly is catalized and specified by other accessory factors: The two low-molecular-weight proteins LMA1 and GATE-16 are suggested to keep the cis-SNAREs that have been dissociated by NSF/SNAP apart from each other and, thus, competent for fusion (Elazar et al., 2003). Furthermore, the association of the tethering protein p115 with the SNARE proteins on ER-derived vesicles (Allan et al., 2000) (see above) that occurs via a SNARE motif-related domain within p115 catalizes the assembly of fusogenic *trans* SNARE complexes (Sapperstein et al., 1996; Shorter et al., 2002). Of note, it is this SNARE-related function that marks the p115 activity that is essential for Golgi biogenesis (Puthenveedu and Linstedt, 2004). Finally, the tight binding of the Sec1/Munc18 protein Sly1p to Syntaxin 5 contributes to the specificity of *trans* SNARE complex formation by preventing the assembly of non-physiologic (non-cognate) *trans* SNARE complexes (Peng and Gallwitz, 2002).

1.3 PROTEIN SORTING DURING ER-EXIT

Transmembrane Cargo

As already outlined for the ER-Golgi SNARE proteins, transmembrane cargo proteins can have access to a budding vesicle by means of direct interaction with the vesicle forming coat. Transport of the vesicular stomatitis virus glycoprotein (VSV-G) has served as a model to study the export of transmembrane proteins from the ER. VSV-G, a type I transmembrane protein that traffics to the cell surface, is abundantly expressed in VSV-infected cells and concentrated into ER-derived transport vesicles (Balch et al., 1994). VSV-G possesses a cytoplasmically exposed C-terminal tail sequence of 29 residues that is required for transport from the ER. Within this tail sequence, a conserved YTDIEM motif is necessary for efficient ER-export of VSV-G (Nishimura and Balch, 1997; Sevier et al., 2000). Similar motifs have been found in other membrane proteins that are efficiently exported from the ER, including the Kir potassium channel proteins (Ma et al., 2001; Stockklausner et al., 2001) and the yeast membrane proteins Sys1p (Votsmeier and Gallwitz, 2001) and Gap1p (Malkus et al., 2002). These so-called di-acidic motifs have been shown to bind to the same site in Sec24p as the ER-exit motif in the SNARE protein Bet1p (Miller et al., 2003).

Shortly after the discovery of the di-acidic motif in VSV-G, another class of ER-exit signals in membrane cargo has been identified. The founding member of the membrane proteins that bind COPII by a di-hydrophobic motif is ERGIC-53 that carries a FF motif on its

very C-terminus (Kappeler et al., 1997). The di-hydrophobic motifs generally consist of a pair of bulky hydrophobic residues, whose position within the cytosolic tail of the cargo protein can vary. Other cargo proteins carrying a di-hydrophobic motif for COPII-association are members of the p24 protein family (Belden and Barlowe, 2001a; Dominguez et al., 1998) and the Erv41p-Erv46p complex (Otte and Barlowe, 2002).

More recent studies have described further COPII-binding ER-export motifs on transmembrane cargo, underlining the general principle of this mechanism. A very powerful COPII-binding ER-export motif is represented by a C-terminal valine residue that can be found in many membrane cargoes including multispanning plasma membrane receptors (Mu et al., 2003; Nufer et al., 2002). Rather surprizingly, it was reported that Golgi glycosyltransferases efficiently exit the ER by means of a di-basic motif with the consensus sequence [RK](X)[RK] that – at least in some cases – directly binds to Sar1p (Giraudo and Maccioni, 2003). Although di-basic motifs are present in the N-terminal tail of many Golgi enzymes, this class of targeting motifs has previously been ascibed rather to ER-retention than to ER-exit of transmembrane proteins (Nufer et al., 2003b; Xia et al., 2001; Zerangue et al., 1999).

Interestingly, many of these transmembrane cargo proteins, including ERGIC-53, VSV-G, and the p24 familiy, form oligomeric complexes, such that a given exported protein would presumably display multiple signals to the COPII budding machinery. The impact of oligomerization on ER-export efficiency has been studied in detail for ERGIC-53, for which a complete map of ER-exit determinants has been obtained (Nufer et al., 2003a). The results suggest an ER-export mechanism in which transmembrane and luminal determinants mediate oligomerization required for efficient recruitment of ERGIC-53 into budding vesicles via the C-terminal COPII-binding di-hydrophobic motif.

Soluble Secretory Cargo

How are soluble secretory proteins that are not directly accessible to COPII subunits sorted away from ER-resident proteins? Two non-exclusive models, known as the “bulk flow” and “receptor-mediated” or “cargo capture” export models, have been described in studies addressing export of soluble cargo from the ER (Figure 3).

First, a passive or bulk flow process (Wieland et al., 1987) appears to operate in the export of amylase and chymotrypsinogen from the ER of pancreatic exocrine cells (Martinez-Menarguez et al., 1999). Concentration of these soluble secretory proteins was not detected in COPII buds, but was observed in tubular structures corresponding to the ERGIC. A

concentration by exclusion model has been proposed to explain this result such that, after soluble proteins exit the ER, cargo is excluded from retrograde-directed COPI vesicles that bud from the observed tubular membrane compartment.

Second, the receptor-mediated or cargo capture model hypothesizes that export of soluble cargo from the ER is an active process that insures the incorporation of transport-competent cargo into ER-derived vesicles. In this model, transmembrane cargo receptors would be needed to link luminal cargo to the COPII coat. Several specific cargo receptor/cargo interactions have been identified in the past few years, namely ERGIC-53/catZr (Appenzeller et al., 1999), Emp24p/Gas1p (Muniz et al., 2000), Erv29p/glyco-pro-α-factor (Belden and Barlowe, 2001b), and Erv14p/Axl2p (Powers and Barlowe, 2002), whereas Axl2p atypically represents a transmembrane cargo protein that is transported by a receptor-mediated mechanism out of the ER. It is now generally assumed that each of the cargo receptors accounts for efficient ER-exit of a limited set of soluble secretory cargo proteins (Belden and Barlowe, 2001b; Nichols et al., 1998; Schimmoller et al., 1995; Vollenweider et al., 1998). The receptors are proposed to recognize and bind to specific export signals contained within distinct soluble cargo molecules, but so far, no such signals have been identified. Furthermore, it has been postulated that the binding is regulated such that only fully folded secretory proteins are recognized by the receptor (Barlowe, 2003). In some cases, however, cargo receptors also bind and transport incompletely folded proteins from the ER (Caldwell et al., 2001). Nevertheless, the principal function of these receptors presumably is to filter out transport-competent cargo from the folding and quality control machinery of the ER (reviewed in (Ellgaard and Helenius, 2003)) by providing the cytosolic signals for intracellular transport. Such positive cargo selection would represent a mechanism of secondary quality control. It must be emphasized though that ER-export by cargo capture can (Malkus et al., 2002; Mizuno and Singer, 1993), but does not *per se* imply the concentration of secretory cargo at the ERES.

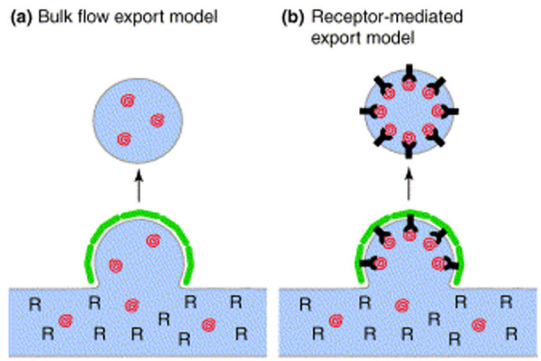


Figure 3

Bulk-flow and receptor-mediated ER-export models. (a) In the bulk-flow model, soluble cargo molecules depart in vesicles by random incorporation at a concentration equal to that found in the ER lumen. (b) The receptor-mediated export model results from selective sorting of soluble cargo during vesicle formation that can involve cargo concentration and relies on receptor proteins to link cargo to the membrane coat complex. It is possible that ER-resident proteins ('R') fail to be packaged into the transport vesicles because of some retention/exclusion mechanism.
The figure was reproduced from Barlowe (2003).

1.4 N-GLYCOSYLATION AND LECTINS WITHIN THE SECRETORY PATHWAY

Many secretory and membrane proteins acquire N-linked oligosaccharides (glycans) during their synthesis in the ER. This process, called N-linked glycosylation, occurs co-translationally by oligosaccharyltransferase-catalized *en bloc* transfer of a pre-assembled carbohydrate structure ($Glc_3Man_9GlcNAc_2$) (where Glc is glucose, Man is mannose, and GlcNAc is N-acetylglucosamine) from a lipid intermediate to a consensus-site asparagine side chain in the nascent glycoprotein. The protein-linked carbohydrate structure may subsequently be processed by numerous glycosidases and glycosyltransferases in ER and Golgi (Kornfeld and Kornfeld, 1985). Many structural intermediates generated during oligosaccharide maturation are thought to serve as signaling tags that are decoded by specific intracellular lectins. Lectin-mediated decoding is accomplished by their non-enzymatic binding affinity toward a particular carbohydrate substrate and implies diverse processes, such as quality control, degradation, ER-export, Golgi-to-plasma membrane transport or lysosomal delivery. This section will summarize our knowledge on the intracellular lectins calnexin/calreticulin, EDEM, ERGIC-53, Vip36 or the mannose 6-phospate (M6P) receptors that are all functioning within the secretory pathway.

Two homologous lectins are known to be localized in the ER, the type I membrane protein calnexin and the soluble protein calreticulin. Their interaction with glycans occurs through a binding site in their globular lectin domain (Kapoor et al., 2004; Schrag et al., 2001), which is structurally related to legume lectins (Loris, 2002). The specificity of calnexin and calreticulin for binding monoglucosylated glycan ($Glc_1Man_9GlcNAc_2$) (Kapoor et al., 2003; Vassilakos et al., 1998) leads to the transient association of one or both of these chaperones with almost all of the glycoproteins that are synthesized in the ER. This association is responsible for promoting the folding and ER-retention of non-native glycoproteins (Helenius et al., 1997) and, in some cases, the targeting of misfolded glycoproteins for degradation (Liu et al., 1999) (see below). Although not fully interchangeable during assistance of glycoprotein folding, calnexin and calreticulin can work independently and appear to cover largely the same spectrum of folding substrates (Molinari et al., 2004). It has also become clear by now that, beyond the glycan, which is thought to be crucial but for the initial recognition, there are further protein-protein contacts between substrate (glyco)proteins and the lectin chaperones (Danilczyk and Williams, 2001; Ihara et al., 1999; Leach and Williams, 2004; Saito et al., 1999; Ware et al., 1995). Furthermore, both calnexin and calreticulin form complexes via an extended, arm-like domain with ERp57

(Frickel et al., 2002), a thiol-disulphide oxidoreductase that is known to form transient disulphide bonds with calnexin- and calreticulin-bound glycoproteins (Molinari and Helenius, 1999). In this way, a protected space is formed for the bound substrate between ERp57 and the lectin domain.

Two functionally independent ER enzymes mediate the on- and off-cycle in this chaperone system (Figure 4). Glucosidase II is responsible for dissociating the substrate glycoprotein from calnexin or calreticulin by hydrolysing the glucose from the monoglucosylated glycan. UDP-glucose:glycoprotein glucosyltransferase (GT), on the other hand, is responsible for re-glucosylating the substrate so that it can re-associate with calnexin or calreticulin. GT works as the folding sensor in this quality control cycle (Parodi, 2000). It recognizes glycoproteins in partially folded, molten globule-like conformations (Caramelo et al., 2003), but ignores native or random-coil conformations (Trombetta and Helenius, 2000). A glycoprotein substrate can only exit the quality control cycle, when GT fails to re-glucosylate it. The cycles of glucosylation and de-glucosylation continue until the glycoprotein has either reached its native conformation or is targeted for degradation.

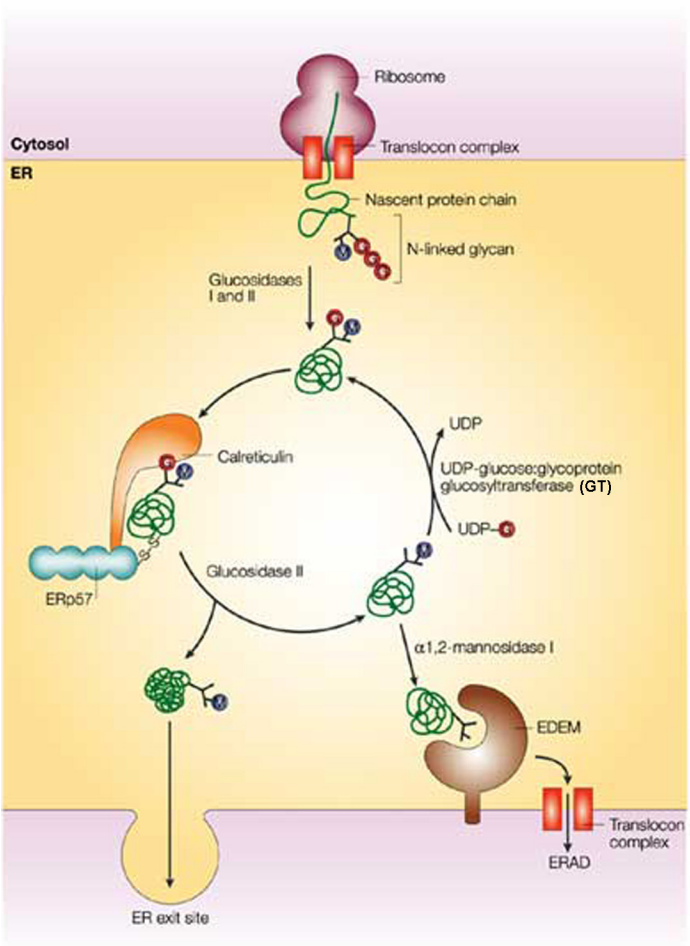


Figure 4

The calnexin/calreticulin cycle.
After transfer of the N-linked glycan to the nascent chain of the protein, two glucoses are removed by glucosidase I an II. This generates a monoglucosylated glycoprotein that can interact with calnexin and calreticulin (for simplicity, only calreticulin is depicted). Both chaperones associate with the thiol-disulphide-oxidoreductase ERp57 through an extended arm-like domain, thereby collaborating in glycoprotein folding assistance. Cleavage of the remaining glucose by glucosidase II terminates the interaction with calnexin and calreticulin. On their release, correctly folded glycoproteins can exit the ER. By contrast, non-native glycoproteins are substrates for GT, which places a single glucose back on the glycan and thereby promotes the renewed association with the ER chaperones. If the glycoprotein is permanently misfolded, the mannose residue in the middle branch of the N-glycan is removed by ER α1,2-mannosidase I. This leads to recognition by EDEM, which probably targets glycoproteins for ER-associated degradation (ERAD) in the cytosol.
The figure was reproduced from Ellgaard and Helenius (2003).

For the degradation of glycoproteins, trimming of a single mannose by the ER α1,2-mannosidase I in the middle branch of the oligosaccaride is required in both yeast (Jakob et al., 1998) and mammalian cells (Hosokawa et al., 2003; Liu et al., 1999), which is followed by the action of the mannosidase-like transmembrane protein EDEM (Hosokawa et al., 2001; Jakob et al., 2001) (Figure 4). EDEM is thought to display lectin activity toward glycans in the $Man_8GlcNAc_2$ or the $Glc_1Man_8GlcNAc_2$ configuration, even though direct proof for this notion is still missing. The inferred interaction of the glycoprotein substrate with EDEM then diverts the glycoprotein from the calnexin/calreticulin cycle and promotes its degradation (Molinari et al., 2003; Oda et al., 2003).

Another important group of intracellular lectins is the family of animal L-type lectins that does not only share structural (Loris, 2002; Velloso et al., 2002), but also sequence homology to legume lectins (plant L-type lectins) (Fiedler and Simons, 1994; Nufer et al., 2003b). Best known among animal L-type lectins are the ERGIC-marker cargo receptor protein ERGIC-53 and the, likewise, recycling protein Vip36, whose functions may be more diverse (see below). Recently, two related proteins have been discovered, ERGL (Yerushalmi et al., 2001) and VIPL (Neve et al., 2003; Nufer et al., 2003b), both of which localize to the ER without any known function (Neve et al., 2003; Nufer et al., 2003b) (L. Liang and H.-P. Hauri unpublished observations). All animal L-type lectins described so far are type I membrane proteins with a relatively short cytosolic tail and a luminal domain with known or inferred carbohydrate binding activity.

ERGIC-53 is a mannose-specific lectin that requires calcium for binding to immobilized mannose *in vitro* (Itin et al., 1996) or to glycoprotein substrates *in vivo* (Appenzeller et al., 1999). This activity, in conjunction with COPII- and COPI-dependent cycling between ER and ERGIC/cis-Golgi (Klumperman et al., 1998; Nufer et al., 2003a; Tisdale et al., 1997), mediates the transport lectin function of ERGIC-53, which is to capture secretory glycoproteins in the ER and guide them through the COPII pathway to the ERGIC, where dissociation occurs (Appenzeller et al., 1999) (see above). Up to now, we know that catZr, a cathepsin Z-related protein (Appenzeller et al., 1999), and apparently another protein of the same family, cathepsin C (Vollenweider et al., 1998), are efficiently transported out of the ER by this lectin domain-dependent glycoprotein shuttling mechanism. Similarly, loss of functional ERGIC-53 expression in humans causes combined deficiency of coagulation factors V and VIII (Nichols et al., 1998), a secretory defect that can be mimicked in cell culture by expression of dominant negative ERGIC-53 (Moussalli et al., 1999). A subsequent

study has identified a second protein implicated in this disease, MCFD2, a soluble, luminal EF-hand protein that co-purifies with ERGIC-53 in a calcium-dependent way (Zhang et al., 2003). Whether or not the transport lectin function and the MCFD2-dependent activity of ERGIC-53 are identical, remains to be shown.

Vip36, on the other hand, has initially been thought to recycle between the trans-Golgi network (TGN) and the plasma membrane (Fiedler et al., 1994). A later publication though reported its localization to the cis-Golgi and some ERGIC elements, while the cell surface localization of exogenous Vip36 was proposed to be caused by overexpression (Fullekrug et al., 1999). Nevertheless, recent studies point to the involvement of endogenous Vip36 in both pre-Golgi (Dahm et al., 2001; Shimada et al., 2003b) and post-Golgi (Hara-Kuge et al., 2002; Hara-Kuge et al., 2004; Shimada et al., 2003a) transport of glycoproteins, possibly depending on the cell type. Carbohydrate-dependent binding of Vip36 to the TGN-to-plasma membrane cargo glycoproteins clusterin (Hara-Kuge et al., 2002) and α-amylase (Hara-Kuge et al., 2004) has also been demonstrated. The data also indicate that Vip36 is responsible for the targeting of specific glycoproteins to the apical face of the plasma membrane in polarized cells (Hara-Kuge et al., 2002). Concerning the lectin properties of Vip36, again some ambiguity exists. Originally, Vip36 was thought to recognize GalNac residues (Fiedler and Simons, 1996). This proposal, however, was challenged by the finding that Vip36 shows specificity for glycans of the $Man_{6-9}GlcNAc_2$ structure that are linked to an α-substituted asparagine residue (Hara-Kuge et al., 1999). Interestingly, binding of glycoproteins was optimal at pH 6.0 that would correspond to the pH of the TGN. Curiously, however, although purified Vip36 binds two moles of calcium (Fiedler and Simons, 1996), its ability to bind glycoproteins appeared not to depend on the presence of divalent ions (Hara-Kuge et al., 1999).

The last class of intracellular lectins, the P-type lectins, consists of two family members: the ~46-kDa cation-dependent M6P receptor (CD-MPR) and the ~300-kDa insulin-like growth factor II/MPR (IGF-II/MPR). Delivery of ~50 different newly synthesized lysosomal enzymes to lysosomes is dependent upon their acquisition of M6P residues that act as recognition signals for high-affinity binding to the MPRs. Generation of the M6P signal occurs by a two-step enzymatic process during transit of the lysosomal enzymes through the ER–Golgi biosynthetic pathway. First, a phosphotransferase attaches a phosphorylated GlcNAc moiety to the C-6 hydroxyl group of mannose to form the M6P–OGlcNAc phosphodiester intermediate (Reitman and Kornfeld, 1981; Waheed et al., 1982). Next, in the TGN, the "uncovering" enzyme removes the GlcNAc moiety, thereby revealing the M6P

signal (Rohrer and Kornfeld, 2001; Varki and Kornfeld, 1980). Following M6P recognition by the MPRs in the TGN, the enzyme-bound receptors are transported to acidified, prelysosomal compartments where the low-pH environment induces release of the enzymes from the receptors. Whereas the lysosomal enzymes are further packaged into lysosomes, the MPRs recycle back to the TGN to repeat this process or move to the cell surface where the IGF-II/MPR, but not the CD-MPR, binds and internalizes a diverse population of extracellular ligands. The two MPRs have been studied in detail in terms of their lectin properties and structures, the cellular components that mediate their transport through numerous intracellular compartments, and their M6P-unrelated functions. The wealth of information on MPRs has been subject to excellent reviews (Dahms and Hancock, 2002; Ghosh et al., 2003).

1.5 AIMS OF THE THESIS

It has been established that ERGIC-53 binds anterograde-directed glycoproteins in the ER by a lectin-interaction that requires Ca^{2+} and that dissociation and segregation occurs in the ERGIC. Likewise, the trafficking cycle of ERGIC-53 between the ER and the ERGIC has been explored in detail in terms of the molecular motifs that determine ERGIC-53's association with cytosolic coat proteins. The aim of this thesis was to elucidate the transport lectin function of ERGIC-53 on the molecular level by identifying the luminal signals governing the recognition of glycoproteins in the ER and their release in the ERGIC. Therefore, the structural requirements for the binding of glycoprotein to ERGIC-53 were investigated by rational mutagenesis of the model substrate procathepsin Z. This issue is particularly interesting because signals in secretory proteins that guide their receptor-mediated ER-exit have not been described so far. Another open question was the mechanism by which ERGIC-53 releases glycoprotein cargo in the ERGIC. To this end, calcium-dependent mannose binding by purified ERGIC-53 was studied *in vitro*. This approach was chosen to potentially discriminate between proteinaceous and environmental dissociation factors such as low pH. In addition, the notion of acid-induced lectin inactivation was tested by site-directed mutagenesis of supposed acid-sensor residues in the CRD of ERGIC-53 and by applying different strategies of pH-manipulation in cell culture. To address the fascinating question whether changes in the availability of calcium from ER to ERGIC may also contribute to the compartmental regulation of ERGIC-53 activity, an approach was developed to quantitatively determine the calcium concentration of the ERGIC *in vivo*.

Characterization of the molecular mechanisms underlying the transport lectin function of ERGIC-53 in particular and the role of the ERGIC as a molecular sorting station in general may not only provide new insights into the enthralling inner life of a cell, but also help us understand the basis of various transport defects.

REFERENCES

Allan, B.B., Moyer, B.D. and Balch, W.E. (2000) Rab1 recruitment of p115 into a cis-SNARE complex: programming budding COPII vesicles for fusion. *Science*, **289**, 444-448.

Alvarez, C., Fujita, H., Hubbard, A. and Sztul, E. (1999) ER to Golgi transport: Requirement for p115 at a pre-Golgi VTC stage. *J Cell Biol*, **147**, 1205-1222.

Andag, U., Neumann, T. and Schmitt, H.D. (2001) The coatomer-interacting protein Dsl1p is required for Golgi-to-endoplasmic reticulum retrieval in yeast. *J Biol Chem*, **276**, 39150-39160.

Andag, U. and Schmitt, H.D. (2003) Dsl1p, an essential component of the Golgi-endoplasmic reticulum retrieval system in yeast, uses the same sequence motif to interact with different subunits of the COPI vesicle coat. *J Biol Chem*, **278**, 51722-51734.

Antonny, B., Gounon, P., Schekman, R. and Orci, L. (2003) Self-assembly of minimal COPII cages. *EMBO Rep*, **4**, 419-424.

Antonny, B., Madden, D., Hamamoto, S., Orci, L. and Schekman, R. (2001) Dynamics of the COPII coat with GTP and stable analogues. *Nat Cell Biol*, **3**, 531-537.

Antonny, B. and Schekman, R. (2001) ER export: public transportation by the COPII coach. *Curr Opin Cell Biol*, **13**, 438-443.

Appenzeller, C., Andersson, H., Kappeler, F. and Hauri, H.P. (1999) The lectin ERGIC-53 is a cargo transport receptor for glycoproteins. *Nat Cell Biol*, **1**, 330-334.

Aridor, M., Fish, K.N., Bannykh, S., Weissman, J., Roberts, T.H., Lippincott-Schwartz, J. and Balch, W.E. (2001) The Sar1 GTPase coordinates biosynthetic cargo selection with endoplasmic reticulum export site assembly. *J Cell Biol*, **152**, 213-229.

Balch, W.E., Dunphy, W.G., Braell, W.A. and Rothman, J.E. (1984) Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of N-acetylglucosamine. *Cell*, **39**, 405-416.

Balch, W.E., McCaffery, J.M., Plutner, H. and Farquhar, M.G. (1994) Vesicular stomatitis virus glycoprotein is sorted and concentrated during export from the endoplasmic reticulum. *Cell*, **76**, 841-852.

Bannykh, S.I., Rowe, T. and Balch, W.E. (1996) The organization of endoplasmic reticulum export complexes. *J Cell Biol*, **135**, 19-35.

Barlowe, C. (2003) Signals for COPII-dependent export from the ER: what's the ticket out? *Trends Cell Biol*, **13**, 295-300.

Barlowe, C. and Schekman, R. (1993) SEC12 encodes a guanine-nucleotide-exchange factor essential for transport vesicle budding from the ER. *Nature*, **365**, 347-349.

Belden, W.J. and Barlowe, C. (2001a) Distinct roles for the cytoplasmic tail sequences of Emp24p and Erv25p in transport between the endoplasmic reticulum and Golgi complex. *J Biol Chem*, **276**, 43040-43048.

Belden, W.J. and Barlowe, C. (2001b) Role of Erv29p in collecting soluble secretory proteins into ER-derived transport vesicles. *Science*, **294**, 1528-1531.

Bi, X., Corpina, R.A. and Goldberg, J. (2002) Structure of the Sec23/24-Sar1 pre-budding complex of the COPII vesicle coat. *Nature*, **419**, 271-277.

Blum, R., Stephens, D.J. and Schulz, I. (2000) Lumenal targeted GFP, used as a marker of soluble cargo, visualises rapid ERGIC to Golgi traffic by a tubulo-vesicular network. *J Cell Sci*, **113 (Pt 18)**, 3151-3159.

Bonifacino, J.S. and Glick, B.S. (2004) The mechanisms of vesicle budding and fusion. *Cell*, **116**, 153-166.

Bonifacino, J.S. and Lippincott-Schwartz, J. (2003) Coat proteins: shaping membrane transport. *Nat Rev Mol Cell Biol*, **4**, 409-414.

Bremser, M., Nickel, W., Schweikert, M., Ravazzola, M., Amherdt, M., Hughes, C.A., Sollner, T.H., Rothman, J.E. and Wieland, F.T. (1999) Coupling of coat assembly and vesicle budding to packaging of putative cargo receptors. *Cell*, **96**, 495-506.

Brown, W.J., Chambers, K. and Doody, A. (2003) Phospholipase A2 (PLA2) enzymes in membrane trafficking: mediators of membrane shape and function. *Traffic*, **4**, 214-221.

Caldwell, S.R., Hill, K.J. and Cooper, A.A. (2001) Degradation of endoplasmic reticulum (ER) quality control substrates requires transport between the ER and Golgi. *J Biol Chem*, **276**, 23296-23303.

Cao, X., Ballew, N. and Barlowe, C. (1998) Initial docking of ER-derived vesicles requires Uso1p and Ypt1p but is independent of SNARE proteins. *Embo J*, **17**, 2156-2165.

Cao, X. and Barlowe, C. (2000) Asymmetric requirements for a Rab GTPase and SNARE proteins in fusion of COPII vesicles with acceptor membranes. *J Cell Biol*, **149**, 55-66.

Caramelo, J.J., Castro, O.A., Alonso, L.G., De Prat-Gay, G. and Parodi, A.J. (2003) UDP-Glc:glycoprotein glucosyltransferase recognizes structured and solvent accessible hydrophobic patches in molten globule-like folding intermediates. *Proc Natl Acad Sci U S A*, **100**, 86-91.

Chao, D.S., Hay, J.C., Winnick, S., Prekeris, R., Klumperman, J. and Scheller, R.H. (1999) SNARE membrane trafficking dynamics in vivo. *J Cell Biol*, **144**, 869-881.

Chen, Y.A. and Scheller, R.H. (2001) SNARE-mediated membrane fusion. *Nat Rev Mol Cell Biol*, **2**, 98-106.

Dahm, T., White, J., Grill, S., Fullekrug, J. and Stelzer, E.H. (2001) Quantitative ER <--> Golgi transport kinetics and protein separation upon Golgi exit revealed by vesicular integral membrane protein 36 dynamics in live cells. *Mol Biol Cell*, **12**, 1481-1498.

Dahms, N.M. and Hancock, M.K. (2002) P-type lectins. *Biochim Biophys Acta*, **1572**, 317-340.

Danilczyk, U.G. and Williams, D.B. (2001) The lectin chaperone calnexin utilizes polypeptide-based interactions to associate with many of its substrates in vivo. *J Biol Chem*, **276**, 25532-25540.

Dominguez, M., Dejgaard, K., Fullekrug, J., Dahan, S., Fazel, A., Paccaud, J.P., Thomas, D.Y., Bergeron, J.J. and Nilsson, T. (1998) gp25L/emp24/p24 protein family members of the cis-Golgi network bind both COP I and II coatomer. *J Cell Biol*, **140**, 751-765.

Drake, M.T., Zhu, Y. and Kornfeld, S. (2000) The assembly of AP-3 adaptor complex-containing clathrin-coated vesicles on synthetic liposomes. *Mol Biol Cell*, **11**, 3723-3736.

Elazar, Z., Scherz-Shouval, R. and Shorer, H. (2003) Involvement of LMA1 and GATE-16 family members in intracellular membrane dynamics. *Biochim Biophys Acta*, **1641**, 145-156.

Ellgaard, L. and Helenius, A. (2003) Quality control in the endoplasmic reticulum. *Nat Rev Mol Cell Biol*, **4**, 181-191.

Fiedler, K., Parton, R.G., Kellner, R., Etzold, T. and Simons, K. (1994) VIP36, a novel component of glycolipid rafts and exocytic carrier vesicles in epithelial cells. *Embo J*, **13**, 1729-1740.

Fiedler, K. and Simons, K. (1994) A putative novel class of animal lectins in the secretory pathway homologous to leguminous lectins. *Cell*, **77**, 625-626.

Fiedler, K. and Simons, K. (1996) Characterization of VIP36, an animal lectin homologous to leguminous lectins. *J Cell Sci*, **109 (Pt 1)**, 271-276.

Frickel, E.M., Riek, R., Jelesarov, I., Helenius, A., Wuthrich, K. and Ellgaard, L. (2002) TROSY-NMR reveals interaction between ERp57 and the tip of the calreticulin P-domain. *Proc Natl Acad Sci U S A*, **99**, 1954-1959.

Fullekrug, J., Scheiffele, P. and Simons, K. (1999) VIP36 localisation to the early secretory pathway. *J Cell Sci*, **112 (Pt 17)**, 2813-2821.

Gallwitz, D. and Jahn, R. (2003) The riddle of the Sec1/Munc-18 proteins - new twists added to their interactions with SNAREs. *Trends Biochem Sci*, **28**, 113-116.

Ghosh, P., Dahms, N.M. and Kornfeld, S. (2003) Mannose 6-phosphate receptors: new twists in the tale. *Nat Rev Mol Cell Biol*, **4**, 202-212.

Giraudo, C.G. and Maccioni, H.J. (2003) Endoplasmic reticulum export of glycosyltransferases depends on interaction of a cytoplasmic dibasic motif with Sar1. *Mol Biol Cell*, **14**, 3753-3766.

Hammond, A.T. and Glick, B.S. (2000) Dynamics of transitional endoplasmic reticulum sites in vertebrate cells. *Mol Biol Cell*, **11**, 3013-3030.

Hara-Kuge, S., Ohkura, T., Ideo, H., Shimada, O., Atsumi, S. and Yamashita, K. (2002) Involvement of VIP36 in intracellular transport and secretion of glycoproteins in polarized Madin-Darby canine kidney (MDCK) cells. *J Biol Chem*, **277**, 16332-16339.

Hara-Kuge, S., Ohkura, T., Seko, A. and Yamashita, K. (1999) Vesicular-integral membrane protein, VIP36, recognizes high-mannose type glycans containing alpha1-->2 mannosyl residues in MDCK cells. *Glycobiology*, **9**, 833-839.

Hara-Kuge, S., Seko, A., Shimada, O., Tosaka-Shimada, H. and Yamashita, K. (2004) The binding of VIP36 and {alpha}-amylase in the secretory vesicles via high mannose-type glycans. *Glycobiology*.

Helenius, A., Trombetta, E.S., Hebert, D.N. and Simons, J.F. (1997) Calnexin, calreticulin and the folding of glycoproteins. *Trends Cell Biol*, **7**, 193-200.

Hirschberg, K., Miller, C.M., Ellenberg, J., Presley, J.F., Siggia, E.D., Phair, R.D. and Lippincott-Schwartz, J. (1998) Kinetic analysis of secretory protein traffic and characterization of golgi to plasma membrane transport intermediates in living cells. *J Cell Biol*, **143**, 1485-1503.

Hosokawa, N., Tremblay, L.O., You, Z., Herscovics, A., Wada, I. and Nagata, K. (2003) Enhancement of endoplasmic reticulum (ER) degradation of misfolded Null Hong Kong alpha1-antitrypsin by

human ER mannosidase I. *J Biol Chem*, **278**, 26287-26294.

Hosokawa, N., Wada, I., Hasegawa, K., Yorihuzi, T., Tremblay, L.O., Herscovics, A. and Nagata, K. (2001) A novel ER alpha-mannosidase-like protein accelerates ER-associated degradation. *EMBO Rep*, **2**, 415-422.

Huang, M., Weissman, J.T., Beraud-Dufour, S., Luan, P., Wang, C., Chen, W., Aridor, M., Wilson, I.A. and Balch, W.E. (2001) Crystal structure of Sar1-GDP at 1.7 A resolution and the role of the NH2 terminus in ER export. *J Cell Biol*, **155**, 937-948.

Ihara, Y., Cohen-Doyle, M.F., Saito, Y. and Williams, D.B. (1999) Calnexin discriminates between protein conformational states and functions as a molecular chaperone in vitro. *Mol Cell*, **4**, 331-341.

Itin, C., Roche, A.C., Monsigny, M. and Hauri, H.P. (1996) ERGIC-53 is a functional mannose-selective and calcium-dependent human homologue of leguminous lectins. *Mol Biol Cell*, **7**, 483-493.

Jakob, C.A., Bodmer, D., Spirig, U., Battig, P., Marcil, A., Dignard, D., Bergeron, J.J., Thomas, D.Y. and Aebi, M. (2001) Htm1p, a mannosidase-like protein, is involved in glycoprotein degradation in yeast. *EMBO Rep*, **2**, 423-430.

Jakob, C.A., Burda, P., Roth, J. and Aebi, M. (1998) Degradation of misfolded endoplasmic reticulum glycoproteins in Saccharomyces cerevisiae is determined by a specific oligosaccharide structure. *J Cell Biol*, **142**, 1223-1233.

Kamena, F. and Spang, A. (2004) Tip20p prohibits back-fusion of COPII vesicles with the endoplasmic reticulum. *Science*, **304**, 286-289.

Kapoor, M., Ellgaard, L., Gopalakrishnapai, J., Schirra, C., Gemma, E., Oscarson, S., Helenius, A. and Surolia, A. (2004) Mutational analysis provides molecular insight into the carbohydrate-binding region of calreticulin: pivotal roles of tyrosine-109 and aspartate-135 in carbohydrate recognition. *Biochemistry*, **43**, 97-106.

Kapoor, M., Srinivas, H., Kandiah, E., Gemma, E., Ellgaard, L., Oscarson, S., Helenius, A. and Surolia, A. (2003) Interactions of substrate with calreticulin, an endoplasmic reticulum chaperone. *J Biol Chem*, **278**, 6194-6200.

Kappeler, F., Klopfenstein, D.R., Foguet, M., Paccaud, J.P. and Hauri, H.P. (1997) The recycling of ERGIC-53 in the early secretory pathway. ERGIC-53 carries a cytosolic endoplasmic reticulum-exit determinant interacting with COPII. *J Biol Chem*, **272**, 31801-31808.

Keller, P., Toomre, D., Diaz, E., White, J. and Simons, K. (2001) Multicolour imaging of post-Golgi sorting and trafficking in live cells. *Nat Cell Biol*, **3**, 140-149.

Kirchhausen, T. (2000) Three ways to make a vesicle. *Nat Rev Mol Cell Biol*, **1**, 187-198.

Kirchhausen, T. and Harrison, S.C. (1981) Protein organization in clathrin trimers. *Cell*, **23**, 755-761.

Klausner, R.D., Donaldson, J.G. and Lippincott-Schwartz, J. (1992) Brefeldin A: insights into the control of membrane traffic and organelle structure. *J Cell Biol*, **116**, 1071-1080.

Klumperman, J., Schweizer, A., Clausen, H., Tang, B.L., Hong, W., Oorschot, V. and Hauri, H.P. (1998) The recycling pathway of protein ERGIC-53 and dynamics of the ER-Golgi intermediate compartment. *J Cell Sci*, **111 (Pt 22)**, 3411-3425.

Kornfeld, R. and Kornfeld, S. (1985) Assembly of asparagine-linked oligosaccharides. *Annu Rev Biochem*, **54**, 631-664.

Leach, M.R. and Williams, D.B. (2004) Lectin-deficient calnexin is capable of binding class I histocompatibility molecules in vivo and preventing their degradation. *J Biol Chem*, **279**, 9072-9079.

Lederkremer, G.Z., Cheng, Y., Petre, B.M., Vogan, E., Springer, S., Schekman, R., Walz, T. and Kirchhausen, T. (2001) Structure of the Sec23p/24p and Sec13p/31p complexes of COPII. *Proc Natl Acad Sci U S A*, **98**, 10704-10709.

Linstedt, A.D., Jesch, S.A., Mehta, A., Lee, T.H., Garcia-Mata, R., Nelson, D.S. and Sztul, E. (2000) Binding relationships of membrane tethering components. The giantin N terminus and the GM130 N terminus compete for binding to the p115 C terminus. *J Biol Chem*, **275**, 10196-10201.

Liu, Y., Choudhury, P., Cabral, C.M. and Sifers, R.N. (1999) Oligosaccharide modification in the early secretory pathway directs the selection of a misfolded glycoprotein for degradation by the proteasome. *J Biol Chem*, **274**, 5861-5867.

Liu, Y., Flanagan, J.J. and Barlowe, C. (2004) Sec22p export from the endoplasmic reticulum is independent of SNARE pairing. *J Biol Chem*.

Loris, R. (2002) Principles of structures of animal and plant lectins. *Biochim Biophys Acta*, **1572**, 198-208.

Ma, D., Zerangue, N., Lin, Y.F., Collins, A., Yu, M., Jan, Y.N. and Jan, L.Y. (2001) Role of ER export signals in controlling surface potassium channel numbers. *Science*, **291**, 316-319.

Malkus, P., Jiang, F. and Schekman, R. (2002) Concentrative sorting of secretory cargo proteins into COPII-coated vesicles. *J Cell Biol*, **159**, 915-921.

Marra, P., Maffucci, T., Daniele, T., Tullio, G.D., Ikehara, Y., Chan, E.K., Luini, A., Beznoussenko, G., Mironov, A. and De Matteis, M.A. (2001) The GM130 and GRASP65 Golgi proteins cycle through and define a subdomain of the intermediate compartment. *Nat Cell Biol*, **3**, 1101-1113.

Martinez-Menarguez, J.A., Geuze, H.J., Slot, J.W. and Klumperman, J. (1999) Vesicular tubular clusters between the ER and Golgi mediate concentration of soluble secretory proteins by exclusion from COPI-coated vesicles. *Cell*, **98**, 81-90.

Matsuoka, K., Orci, L., Amherdt, M., Bednarek, S.Y., Hamamoto, S., Schekman, R. and Yeung, T. (1998) COPII-coated vesicle formation reconstituted with purified coat proteins and chemically defined liposomes. *Cell*, **93**, 263-275.

Matsuoka, K., Schekman, R., Orci, L. and Heuser, J.E. (2001) Surface structure of the COPII-coated vesicle. *Proc Natl Acad Sci U S A*, **98**, 13705-13709.

Mayer, A., Wickner, W. and Haas, A. (1996) Sec18p (NSF)-driven release of Sec17p (alpha-SNAP) can precede docking and fusion of yeast vacuoles. *Cell*, **85**, 83-94.

McNew, J.A., Parlati, F., Fukuda, R., Johnston, R.J., Paz, K., Paumet, F., Sollner, T.H. and Rothman, J.E. (2000) Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. *Nature*, **407**, 153-159.

Miller, E.A., Beilharz, T.H., Malkus, P.N., Lee, M.C., Hamamoto, S., Orci, L. and Schekman, R. (2003) Multiple cargo binding sites on the COPII subunit Sec24p ensure capture of diverse membrane proteins into transport vesicles. *Cell*, **114**, 497-509.

Mizuno, M. and Singer, S.J. (1993) A soluble secretory protein is first concentrated in the endoplasmic reticulum before transfer to the Golgi apparatus. *Proc Natl Acad Sci U S A*, **90**, 5732-5736.

Molinari, M., Calanca, V., Galli, C., Lucca, P. and Paganetti, P. (2003) Role of EDEM in the release of misfolded glycoproteins from the calnexin cycle. *Science*, **299**, 1397-1400.

Molinari, M., Eriksson, K.K., Calanca, V., Galli, C., Cresswell, P., Michalak, M. and Helenius, A. (2004) Contrasting functions of calreticulin and calnexin in glycoprotein folding and ER quality control. *Mol Cell*, **13**, 125-135.

Molinari, M. and Helenius, A. (1999) Glycoproteins form mixed disulphides with oxidoreductases during folding in living cells. *Nature*, **402**, 90-93.

Mossessova, E., Bickford, L.C. and Goldberg, J. (2003) SNARE selectivity of the COPII coat. *Cell*, **114**, 483-495.

Moussalli, M., Pipe, S.W., Hauri, H.P., Nichols, W.C., Ginsburg, D. and Kaufman, R.J. (1999) Mannose-dependent endoplasmic reticulum (ER)-Golgi intermediate compartment-53-mediated ER to Golgi trafficking of coagulation factors V and VIII. *J Biol Chem*, **274**, 32539-32542.

Mu, Y., Otsuka, T., Horton, A.C., Scott, D.B. and Ehlers, M.D. (2003) Activity-dependent mRNA splicing controls ER export and synaptic delivery of NMDA receptors. *Neuron*, **40**, 581-594.

Muniz, M., Nuoffer, C., Hauri, H.P. and Riezman, H. (2000) The Emp24 complex recruits a specific cargo molecule into endoplasmic reticulum-derived vesicles. *J Cell Biol*, **148**, 925-930.

Neve, E.P., Svensson, K., Fuxe, J. and Pettersson, R.F. (2003) VIPL, a VIP36-like membrane protein with a putative function in the export of glycoproteins from the endoplasmic reticulum. *Exp Cell Res*, **288**, 70-83.

Nichols, W.C., Seligsohn, U., Zivelin, A., Terry, V.H., Hertel, C.E., Wheatley, M.A., Moussalli, M.J., Hauri, H.P., Ciavarella, N., Kaufman, R.J. and Ginsburg, D. (1998) Mutations in the ER-Golgi intermediate compartment protein ERGIC-53 cause combined deficiency of coagulation factors V and VIII. *Cell*, **93**, 61-70.

Nishimura, N. and Balch, W.E. (1997) A di-acidic signal required for selective export from the endoplasmic reticulum. *Science*, **277**, 556-558.

Novick, P., Field, C. and Schekman, R. (1980) Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell*, **21**, 205-215.

Nufer, O., Guldbrandsen, S., Degen, M., Kappeler, F., Paccaud, J.P., Tani, K. and Hauri, H.P. (2002) Role of cytoplasmic C-terminal amino acids of membrane proteins in ER export. *J Cell Sci*, **115**, 619-628.

Nufer, O., Kappeler, F., Guldbrandsen, S. and Hauri, H.P. (2003a) ER export of ERGIC-53 is controlled by cooperation of targeting determinants in all three of its domains. *J Cell Sci*, **116**, 4429-4440.

Nufer, O., Mitrovic, S. and Hauri, H.P. (2003b) Profile-based data base scanning for animal L-type lectins and characterization of VIPL, a novel VIP36-like endoplasmic

reticulum protein. *J Biol Chem*, **278**, 15886-15896.

Oda, Y., Hosokawa, N., Wada, I. and Nagata, K. (2003) EDEM as an acceptor of terminally misfolded glycoproteins released from calnexin. *Science*, **299**, 1394-1397.

Otte, S. and Barlowe, C. (2002) The Erv41p-Erv46p complex: multiple export signals are required in trans for COPII-dependent transport from the ER. *Embo J*, **21**, 6095-6104.

Palade, G. (1975) Intracellular aspects of the process of protein synthesis. *Science*, **189**, 347-358.

Parodi, A.J. (2000) Protein glucosylation and its role in protein folding. *Annu Rev Biochem*, **69**, 69-93.

Peng, R. and Gallwitz, D. (2002) Sly1 protein bound to Golgi syntaxin Sed5p allows assembly and contributes to specificity of SNARE fusion complexes. *J Cell Biol*, **157**, 645-655.

Peng, R., Grabowski, R., De Antoni, A. and Gallwitz, D. (1999) Specific interaction of the yeast cis-Golgi syntaxin Sed5p and the coat protein complex II component Sec24p of endoplasmic reticulum-derived transport vesicles. *Proc Natl Acad Sci U S A*, **96**, 3751-3756.

Powers, J. and Barlowe, C. (2002) Erv14p directs a transmembrane secretory protein into COPII-coated transport vesicles. *Mol Biol Cell*, **13**, 880-891.

Presley, J.F., Cole, N.B., Schroer, T.A., Hirschberg, K., Zaal, K.J. and Lippincott-Schwartz, J. (1997) ER-to-Golgi transport visualized in living cells. *Nature*, **389**, 81-85.

Puthenveedu, M.A. and Linstedt, A.D. (2004) Gene replacement reveals that p115/SNARE interactions are essential for Golgi biogenesis. *Proc Natl Acad Sci U S A*, **101**, 1253-1256.

Reilly, B.A., Kraynack, B.A., VanRheenen, S.M. and Waters, M.G. (2001) Golgi-to-endoplasmic reticulum (ER) retrograde traffic in yeast requires Dsl1p, a component of the ER target site that interacts with a COPI coat subunit. *Mol Biol Cell*, **12**, 3783-3796.

Reitman, M.L. and Kornfeld, S. (1981) UDP-N-acetylglucosamine:glycoprotein N-acetylglucosamine-1-phosphotransferase. Proposed enzyme for the phosphorylation of the high mannose oligosaccharide units of lysosomal enzymes. *J Biol Chem*, **256**, 4275-4281.

Rohrer, J. and Kornfeld, R. (2001) Lysosomal hydrolase mannose 6-phosphate uncovering enzyme resides in the trans-Golgi network. *Mol Biol Cell*, **12**, 1623-1631.

Rowe, T., Aridor, M., McCaffery, J.M., Plutner, H., Nuoffer, C. and Balch, W.E. (1996) COPII vesicles derived from mammalian endoplasmic reticulum microsomes recruit COPI. *J Cell Biol*, **135**, 895-911.

Saito, Y., Ihara, Y., Leach, M.R., Cohen-Doyle, M.F. and Williams, D.B. (1999) Calreticulin functions in vitro as a molecular chaperone for both glycosylated and non-glycosylated proteins. *Embo J*, **18**, 6718-6729.

Sapperstein, S.K., Lupashin, V.V., Schmitt, H.D. and Waters, M.G. (1996) Assembly of the ER to Golgi SNARE complex requires Uso1p. *J Cell Biol*, **132**, 755-767.

Sapperstein, S.K., Walter, D.M., Grosvenor, A.R., Heuser, J.E. and Waters, M.G. (1995) p115 is a general vesicular transport factor related to the yeast endoplasmic reticulum to Golgi transport factor Uso1p. *Proc Natl Acad Sci U S A*, **92**, 522-526.

Scales, S.J., Pepperkok, R. and Kreis, T.E. (1997) Visualization of ER-to-Golgi transport in living cells reveals a sequential mode of action for COPII and COPI. *Cell*, **90**, 1137-1148.

Schimmoller, F., Singer-Kruger, B., Schroder, S., Kruger, U., Barlowe, C. and Riezman, H. (1995) The absence of Emp24p, a component of ER-derived COPII-coated vesicles, causes a defect in transport of selected proteins to the Golgi. *Embo J*, **14**, 1329-1339.

Schrag, J.D., Bergeron, J.J., Li, Y., Borisova, S., Hahn, M., Thomas, D.Y. and Cygler, M. (2001) The Structure of calnexin, an ER chaperone involved in quality control of protein folding. *Mol Cell*, **8**, 633-644.

Seemann, J., Jokitalo, E.J. and Warren, G. (2000) The role of the tethering proteins p115 and GM130 in transport through the Golgi apparatus in vivo. *Mol Biol Cell*, **11**, 635-645.

Segev, N. (2001) Ypt and Rab GTPases: insight into functions through novel interactions. *Curr Opin Cell Biol*, **13**, 500-511.

Sevier, C.S., Weisz, O.A., Davis, M. and Machamer, C.E. (2000) Efficient export of the vesicular stomatitis virus G protein from the endoplasmic reticulum requires a signal in the cytoplasmic tail that includes both tyrosine-based and di-acidic motifs. *Mol Biol Cell*, **11**, 13-22.

Shimada, O., Hara-Kuge, S., Yamashita, K., Tosaka-Shimada, H., Yanchao, L., Einan, L., Atsumi, S. and Ishikawa, H. (2003a) Localization of VIP36 in the post-Golgi secretory pathway also of rat parotid acinar cells. *J Histochem Cytochem*, **51**, 1057-1063.

Shimada, O., Hara-Kuge, S., Yamashita, K., Tosaka-Shimada, H., Yanchao, L.,

Yongnan, L., Atsumi, S. and Ishikawa, H. (2003b) Clusters of VIP-36-positive vesicles between endoplasmic reticulum and golgi apparatus in GH3 cells. *Cell Struct Funct*, **28**, 155-163.

Shorter, J., Beard, M.B., Seemann, J., Dirac-Svejstrup, A.B. and Warren, G. (2002) Sequential tethering of Golgins and catalysis of SNAREpin assembly by the vesicle-tethering protein p115. *J Cell Biol*, **157**, 45-62.

Sonnichsen, B., Lowe, M., Levine, T., Jamsa, E., Dirac-Svejstrup, B. and Warren, G. (1998) A role for giantin in docking COPI vesicles to Golgi membranes. *J Cell Biol*, **140**, 1013-1021.

Spang, A., Matsuoka, K., Hamamoto, S., Schekman, R. and Orci, L. (1998) Coatomer, Arf1p, and nucleotide are required to bud coat protein complex I-coated vesicles from large synthetic liposomes. *Proc Natl Acad Sci U S A*, **95**, 11199-11204.

Springer, S. and Schekman, R. (1998) Nucleation of COPII vesicular coat complex by endoplasmic reticulum to Golgi vesicle SNAREs. *Science*, **281**, 698-700.

Stephens, D.J., Lin-Marq, N., Pagano, A., Pepperkok, R. and Paccaud, J.P. (2000) COPI-coated ER-to-Golgi transport complexes segregate from COPII in close proximity to ER exit sites. *J Cell Sci*, **113 (Pt 12)**, 2177-2185.

Stephens, D.J. and Pepperkok, R. (2001) Illuminating the secretory pathway: when do we need vesicles? *J Cell Sci*, **114**, 1053-1059.

Stockklausner, C., Ludwig, J., Ruppersberg, J.P. and Klocker, N. (2001) A sequence motif responsible for ER export and surface expression of Kir2.0 inward rectifier K(+) channels. *FEBS Lett*, **493**, 129-133.

Storrie, B. and Nilsson, T. (2002) The Golgi apparatus: balancing new with old. *Traffic*, **3**, 521-529.

Sutton, R.B., Fasshauer, D., Jahn, R. and Brunger, A.T. (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. *Nature*, **395**, 347-353.

Tisdale, E.J., Plutner, H., Matteson, J. and Balch, W.E. (1997) p53/58 binds COPI and is required for selective transport through the early secretory pathway. *J Cell Biol*, **137**, 581-593.

Toomre, D., Keller, P., White, J., Olivo, J.C. and Simons, K. (1999) Dual-color visualization of trans-Golgi network to plasma membrane traffic along microtubules in living cells. *J Cell Sci*, **112 (Pt 1)**, 21-33.

Trombetta, E.S. and Helenius, A. (2000) Conformational requirements for glycoprotein reglucosylation in the endoplasmic reticulum. *J Cell Biol*, **148**, 1123-1129.

Varki, A. and Kornfeld, S. (1980) Identification of a rat liver alpha-N-acetylglucosaminyl phosphodiesterase capable of removing "blocking" alpha-N-acetylglucosamine residues from phosphorylated high mannose oligosaccharides of lysosomal enzymes. *J Biol Chem*, **255**, 8398-8401.

Vassilakos, A., Michalak, M., Lehrman, M.A. and Williams, D.B. (1998) Oligosaccharide binding characteristics of the molecular chaperones calnexin and calreticulin. *Biochemistry*, **37**, 3480-3490.

Velloso, L.M., Svensson, K., Schneider, G., Pettersson, R.F. and Lindqvist, Y. (2002) Crystal structure of the carbohydrate recognition domain of p58/ERGIC-53, a protein involved in glycoprotein export from the endoplasmic reticulum. *J Biol Chem*, **277**, 15979-15984.

Vollenweider, F., Kappeler, F., Itin, C. and Hauri, H.P. (1998) Mistargeting of the lectin ERGIC-53 to the endoplasmic reticulum of HeLa cells impairs the secretion of a lysosomal enzyme. *J Cell Biol*, **142**, 377-389.

Votsmeier, C. and Gallwitz, D. (2001) An acidic sequence of a putative yeast Golgi membrane protein binds COPII and facilitates ER export. *Embo J*, **20**, 6742-6750.

Waheed, A., Hasilik, A. and von Figura, K. (1982) UDP-N-acetylglucosamine:lysosomal enzyme precursor N-acetylglucosamine-1-phosphotransferase. Partial purification and characterization of the rat liver Golgi enzyme. *J Biol Chem*, **257**, 12322-12331.

Ware, F.E., Vassilakos, A., Peterson, P.A., Jackson, M.R., Lehrman, M.A. and Williams, D.B. (1995) The molecular chaperone calnexin binds Glc1Man9GlcNAc2 oligosaccharide as an initial step in recognizing unfolded glycoproteins. *J Biol Chem*, **270**, 4697-4704.

White, J., Johannes, L., Mallard, F., Girod, A., Grill, S., Reinsch, S., Keller, P., Tzschaschel, B., Echard, A., Goud, B. and Stelzer, E.H. (1999) Rab6 coordinates a novel Golgi to ER retrograde transport pathway in live cells. *J Cell Biol*, **147**, 743-760.

Whyte, J.R. and Munro, S. (2002) Vesicle tethering complexes in membrane traffic. *J Cell Sci*, **115**, 2627-2637.

Wieland, F.T., Gleason, M.L., Serafini, T.A. and Rothman, J.E. (1987) The rate of bulk flow from the endoplasmic reticulum to the cell surface. *Cell*, **50**, 289-300.

Xia, H., Hornby, Z.D. and Malenka, R.C. (2001) An ER retention signal explains

differences in surface expression of NMDA and AMPA receptor subunits. *Neuropharmacology*, **41**, 714-723.

Xu, D., Joglekar, A.P., Williams, A.L. and Hay, J.C. (2000) Subunit structure of a mammalian ER/Golgi SNARE complex. *J Biol Chem*, **275**, 39631-39639.

Yamakawa, H., Seog, D.H., Yoda, K., Yamasaki, M. and Wakabayashi, T. (1996) Uso1 protein is a dimer with two globular heads and a long coiled-coil tail. *J Struct Biol*, **116**, 356-365.

Yerushalmi, N., Keppler-Hafkemeyer, A., Vasmatzis, G., Liu, X.F., Olsson, P., Bera, T.K., Duray, P., Lee, B. and Pastan, I. (2001) ERGL, a novel gene related to ERGIC-53 that is highly expressed in normal and neoplastic prostate and several other tissues. *Gene*, **265**, 55-60.

Yoshihisa, T., Barlowe, C. and Schekman, R. (1993) Requirement for a GTPase-activating protein in vesicle budding from the endoplasmic reticulum. *Science*, **259**, 1466-1468.

Zerangue, N., Schwappach, B., Jan, Y.N. and Jan, L.Y. (1999) A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane K(ATP) channels. *Neuron*, **22**, 537-548.

Zhang, B., Cunningham, M.A., Nichols, W.C., Bernat, J.A., Seligsohn, U., Pipe, S.W., McVey, J.H., Schulte-Overberg, U., de Bosch, N.B., Ruiz-Saez, A., White, G.C., Tuddenham, E.G., Kaufman, R.J. and Ginsburg, D. (2003) Bleeding due to disruption of a cargo-specific ER-to-Golgi transport complex. *Nat Genet*, **34**, 220-225.

A common motif for lectin-mediated ER-export and lysosomal delivery

Submitted to *EMBO Journal*

[1]Christian Appenzeller-Herzog, [2]Peter Burkhard, [3,4]Inigo Santamaria, [3]Carlos Lopez-Otin and [1*]Hans-Peter Hauri

[1]Department of Pharmacology and Neurobiology, Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland.
[2]M.E. Müller Institute for Structural Biology, Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland.
[3]Departamento de Bioquimica y Biologia Molecular, Instituto Universitario de Oncologia, Universidad de Oviedo, 33006-Oviedo, Spain.
[4]Present address: Bone and Mineral Research Unit, Hospital Universitario Central de Asturias, 33006 Oviedo, Spain.

* Correspondence:
Phone: +41 61 267 2222
FAX: +41 61 267 2208
E-mail: Hans-Peter.Hauri@unibas.ch

Running title: Dual decoding of a novel transport signal
Character count: 54’959
Subject Category: Membranes & Transport

Abstract

Some secretory proteins leave the endoplasmic reticulum (ER) by a receptor-mediated cargo capture mechanism, but the signals required for the cargo-receptor interaction are largely unknown. Here, we describe a novel targeting motif that is composed of a high-mannose type oligosaccharide and a peptide β-hairpin loop. The motif accounts for lectin ERGIC-53-assisted ER-export of the lyososomal enzyme procathepsin Z. An equivalent carbohydrate/hairpin structure is present in cathepsin C, another cargo of ERGIC-53, reflecting the general nature of this ER-export signal. Surprisingly, the peptide loop is also essential for the final targeting of cathepsin Z to lysosomes, establishing an unexpected link between ER-export and post-Golgi sorting. We present evidence for an indirect effect of the peptide loop on lysosomal delivery by protecting carbohydrate chains from irregular processing in the Golgi. Our data demonstrate an unprecedented dual decoding of a sorting motif within the secretory pathway.

endoplasmic reticulum / glycosylation / lectins / lysosomes / protein sorting signals

Introduction

A complex interplay between the glycosylation machinery and the deciphering of carbohydrate signals by lectins ensures proper synthesis and targeting of nascent glycoproteins in the secretory pathway of eukaryotic cells. N-linked oligosaccharides are cotranslationally attached to a consensus-site asparagine side chain during translocation of the polypeptide into the ER and may subsequently be processed by numerous glycosidases and glycosyltransferases in ER and Golgi (Kornfeld and Kornfeld, 1985). N-glycans are initially trimmed in the ER from $Glc_3Man_9GlcNAc_2$ to the high-mannose configuration $Man_{7-9}GlcNAc_2$. Later conversion to hybrid- or complex-type oligosaccharides, initiated by the action of Golgi mannosidase I, can occur during passage through the Golgi. A special subset of glycoproteins, the lysosomal enzymes, undergoes highly specific mannose phosphorylation that is catalyzed by GlcNAc-phosphotransferase of the cis-Golgi and the phosphodiesterase uncovering enzyme of the trans-Golgi.

Many N-glycan structural intermediates generated during oligosaccharide maturation are thought to serve as signaling tags for quality control, degradation, ER-export, Golgi-to-plasma membrane transport or lysosomal delivery. The tags are decoded by specific intracellular lectins such as calnexin/calreticulin, EDEM, ERGIC-53, Vip36 or the mannose 6-phospate (M6P) receptors (Dahms and Hancock, 2002; Ellgaard and Helenius, 2003; Hauri et al., 2000a). Most of these lectins direct their binding solely against a defined carbohydrate motif on the surface of the glycoprotein. In contrast, the fact that transport of only a subset of glycoproteins depends on the mannose-specific lectin ERGIC-53 suggests the presence of a pivotal proteinaceous determinant in these glycoproteins for selective recognition (Hauri et al., 2000b). So far, the search for such additional motifs has remained without success.

One of the functions of ERGIC-53 is to capture transport-competent secretory glycoproteins in the ER and guide them through the COPII pathway to the ERGIC where dissociation occurs triggered by a pH-switch (Appenzeller et al., 1999; Appenzeller-Herzog et al., 2004). While the glycoproteins proceed through the secretory pathway, ERGIC-53 is recycled back to the ER. This capture mechanism is thought to accelerate the delivery of a number of glycoproteins to post-ER compartments, although its physiological relevance has been demonstrated but for the secretion of blood coagulation factors V and VIII (Nichols et al., 1998).

The lysosomal glycoprotein cathepsin Z (catZ; also named cathepsin X, -P or -Y) is ubiquitously expressed and belongs to the papain family of cysteine proteases. It is synthesized in the ER as a preproprotein before the hydrophobic signal peptide is cleaved to

release procathepsin Z (pro-catZ). Pro-catZ is a monomer featuring an unusually short propeptide (Santamaria et al., 1998) that is linked into the active site of the enzyme by a disulfide bond (Sivaraman et al., 2000) and cleaved upon lysosomal maturation to produce the active enzyme that remains monomeric. Activated catZ has been purified from human liver (Klemencic et al., 2000). It functions as a carboxymonopeptidase, although in some cases it also exhibits dipeptidase and endopeptidase activity (Sakamoto et al., 1999; Therrien et al., 2001).

In a previous study we have identified a specific cargo protein for ERGIC-53 that was dubbed cathepsin Z-related protein (catZr) based on short peptide sequences matching the protein sequence of human pro-catZ (Appenzeller et al., 1999). In the current study we show that catZr is the Chinese hamster orthologue of human catZ and describe a novel targeting motif in catZ. Teaming up with specific oligosaccharides, this peptide motif is essential for both efficient ERGIC-53-mediated ER-export and lysosomal targeting.

Results

Pro-catZ is a cargo for ERGIC-53 and is processed by Golgi mannosidase I.

A previous effort to isolate cargo proteins of the ER-export receptor ERGIC-53 from Chinese hamster ovary (CHO)-derived GMAA cells (Kappeler et al., 1997) resulted in the identification of catZr (Appenzeller et al., 1999). To characterize this protein in more detail, we screened a CHO-cDNA library as described in Supplemental Data, Fig. S1A. A partial cDNA of 1334 bp length was obtained that includes the start methionine and a poly-A tail and encodes a protein of 306 amino acids with a predicted MW of ~34 kDa and 82% identity to human preprocathepsin Z (Fig. 1A). The sequence has two potential N-glycosylation sites. We refer to this protein as to the Chinese hamster ortholog of pro-catZ. An anti-peptide rabbit antiserum (Fig. 1A, (Appenzeller-Herzog et al., 2004)) readily immunoprecipitated exogenous as well as endogenous catZ (Fig. S1B).

Is the hamster pro-catZ indeed identical to catZr? To test this, we immunoprecipitated ERGIC-53 from GMAA cells that were treated with or without the cleavable crosslinker dithiobis(succinimidylpropionate) (DSP) and probed for the presence of pro-catZ by immunoblotting using affinity purified anti-catZ. As shown in Fig. 1B, pro-catZ as well as mature catZ (after the proteolytic removal of its propeptide, Figs. 1A, S1B) was detected in cell homogenates, but primarily pro-catZ was co-isolated with GMAA-ERGIC-53 in a DSP-dependent way suggesting a specific interaction between these two proteins (the minor fraction of cleaved pro-catZ is most likely due to a post-lysis event). Previously, we have shown that exit of catZr from the early secretory pathway is delayed in cells expressing a transport-impaired, dominant negative KKAA-mutant (GMAA) of ERGIC-53 (Kappeler et al., 1997) compared to cells expressing GM-ERGIC-53, a tagged wild-type (wt) protein (Appenzeller et al., 1999). With this in mind, we studied pro-catZ maturation in GMAA and GM cells by pulse-chase analysis followed by DSP-treatment (to reduce background, see Fig. S1B) and anti-catZ immunoprecipitation. Fig. 1C shows that pro-catZ transport is indeed inefficient in GMAA cells. Collectively, these results demonstrate that catZr is identical to the Chinese hamster ortholog of pro-catZ.

Besides propeptide cleavage, we noted another, earlier processing event that was apparent after 30 to 60 min of chase in GM cells and led to a slight increase in mobility of pro-catZ (Fig. 1C). To test the possibility that this shift reflects oligosaccharide trimming by an α1,2-mannosidase, we performed pulse-chase experiments with CHO cells treated with the α1,2-mannosidase inhibitor kifunensin. Kifunensin prevented the mobility shift of pro-catZ

and increased the apparent Mr of mature catZ (Fig. 2A) indicating that the shift was due to an α1,2-mannosidase. There are at least four kifunensin-sensitive mannosidases: ER α-mannosidase I and Golgi α1,2-mannosidases IA, IB or IC (Herscovics, 2001). To further characterize pro-catZ maturation and transport, we used the fungal drug brefeldin A (BFA) that is known to relocalize Golgi enzymes to the ER and to block secretion. As shown by the pulse-chase experiment in Fig. 2B, BFA-treatment led to premature mannosidase trimming of pro-catZ (and interfered with its maturation, as expected). This outcome argues that pro-catZ is a substrate for an α1,2-mannosidase only after its transport to the Golgi. Interestingly, no Golgi mannosidase I-shift was observed in GMAA cells (Fig. 1C). To investigate whether a Golgi α1,2-mannosidase is present at all in GMAA cells, we analyzed the electrophoretic mobility of ERGIC-53-bound pro-catZ after metabolic labeling and treatment of the cells with BFA and kifunensin. Fig. 2C shows that BFA caused a slight mobility shift of pro-catZ (showing up as a DSP-caused doublet after crosslinking (Appenzeller et al., 1999)) that could be suppressed by kifunensin. We conclude that the relevant Golgi α1,2-mannosidase I is expressed in GMAA cells and that its action on pro-catZ is indirectly inhibited by ER-restricted ERGIC-53. We further conclude that mannose trimming of pro-catZ occurs after its dissociation from GMAA-ERGIC-53 (Fig. 2C) and GM-ERGIC-53 (Fig. S2), which is consistent with our previous studies using 1-deoxymannojirimycin (Appenzeller et al., 1999).

Efficient binding of pro-catZ to ERGIC-53 depends on a high-mannose-glycan/ β-hairpin-loop motif.

To explore the interaction between pro-catZ and ERGIC-53 in more detail, we switched to the human enzyme (Santamaria et al., 1998) and generated the following glycosylation-site mutations tagged with a HA-epitope at their N-terminus: N184Q (N1-site), N224Q (N2-site) and N184+224Q for double mutation. First, we studied the secretion and the stability of these mutants when transiently expressed in CHO cells. The cells were pulsed with ^{35}S-methionine, chased for different times and recombinant pro-catZ was immunoprecipitated from the cell lysate or the culture medium using anti-HA. As shown in Fig. 3A, the different mutants displayed markedly distinct properties. While wt-pro-catZ and N224Q were efficiently secreted and immunoprecipitated, N184Q and N184+224Q were poorly transported, and N184+224Q was barely detectable after the chase. To distinguish if these mutants were degraded or inefficiently recognized by the antibody, we repeated the experiment using denaturing immunoprecipitation and found efficient recovery of all pro-catZ mutants under

these conditions (Fig. 3B and data not shown). Thus, the N-linked oligosaccharides of pro-catZ are required for efficient transport, but not for the stability of the glycoprotein.

To study the interaction of the three glycosylation mutants of pro-catZ with ERGIC-53, we tested to what extent the two partners can still be crosslinked by DSP. To this end, we transfected GMAA cells with the mutants, treated them with DSP, immunoprecipitated ERGIC-53 and visualized the mutant proteins by Western blotting using anti-HA. Fig. 4A shows that, relative to wt-pro-catZ, the binding of N184Q to ERGIC-53 was decreased, while that of N224Q was enhanced. Interestingly, some weak, but reproducible interaction of N184+224Q with ERGIC-53 was observed even without crosslinking. To exclude the trivial possibility that these differences were due to a sterical effect of the N-terminal HA-tag we repeated the crosslinking experiments using a pro-catZ construct in which the HA-tag was inserted distal to the N-terminus by replacement of a stretch of flexible amino acids in the propeptide. Co-immunoprecipiations with these alternative mutants gave identical results (Fig. S3). These results suggest that ERGIC-53 recognizes the N184-linked glycan (N1-glycan) on pro-catZ and that this interaction is enhanced in the absence of the N224-linked glycan (N2-glycan).

The data obtained with the glycosylation mutants also point to a carbohydrate-independent motif that contributes to the recognition of pro-catZ. This prompted us to look more closely into the structural features of pro-catZ. We were intrigued by a β-hairpin loop in close proximity of the N1-site ((Sivaraman et al., 2000), Fig. 1B), the function of which is unknown. Fig. 4B depicts this well-exposed peptide motif which comprises a disulfide linkage and the high-mannose ($Man_9GlcNAc_2$) N1-glycan that is likely to contact the polar amino acid side chains of the hairpin loop. To study the function of this loop it was deleted by mutagenesis, resulting in the constructs Δloop* and Δloop (Fig. 4B). Crosslinking experiments showed a reduction in the binding of Δloop to ERGIC-53, while binding of Δloop* appeared unaffected (Fig. S4). We therefore directed our further efforts to Δloop and, in addition, constructed the combined mutant N184Q+Δloop. As shown in Fig. 4C, removal of the β-hairpin loop neither affected the stability nor the glycosylation of pro-catZ (as indicated by the mobility shift between Δloop and N184Q+Δloop, see also Fig. 6D). We noted, however, a slight retardation in the secretion of the two mutants relative to wt-pro-catZ (compare Figs. 3A and 4C) and in the case of Δloop more pronounced electrophoretic mobility shifts (see below).

The above approach to measure binding of pro-catZ to ERGIC-53 yielded a steady-state read-out that was critically influenced by mutant-specific transport speeds, rendering it

impossible to quantify this interaction. To circumvent this limitation, we studied newly synthesized wt and mutant pro-catZ in GMAA cells by pulse-chase. Since maximal binding of endogenous pro-catZ to ERGIC-53 occurs after a lag-period of 15 min (Appenzeller et al., 1999), the analysis was performed either immediately after the pulse or after a 15 min chase. The cells were subjected to crosslinking followed by immunoprecipitation using covalently coupled anti-ERGIC-53 (Fig. 4D, upper panel). Immunocomplexes were released under denaturing conditions and reprecipitated using anti-HA to recover only recombinant pro-catZ crosslinked to GMAA-ERGIC-53 (Fig. 4D, lower panel). Control anti-HA immunoprecipitations from the initial cell lysate (using denaturing conditions) showed equal expression levels for all pro-catZ variants (not shown). Quantification of co-immunoprecipitated glycoproteins revealed a 80% reduction in ERGIC-53 binding of both N184Q and Δloop relative to wt-pro-catZ that was not further compromised if both mutations were combined, suggesting a single carbohydrate/peptide binding motif. Interestingly, with none of the mutants deficient in this motif we observed the descibed lag-phase in respect of ERGIC-53 association (see Discussion). Moreover, as already seen at steady-state (Figs. 4A, S2), the N224Q mutation proved beneficial for the binding of pro-catZ to ERGIC-53.

To corroborate these findings by a different, crosslinking-independent approach, we analyzed the influence of dominant negative ERGIC-53 on the secretion of pro-catZ mutants. GMAA or Lec1 cells (Lec1 is the parental line of GMAA (Kappeler et al., 1997)) were transfected with wt-, N184Q-, N224Q- and Δloop-pro-catZ cDNAs, pulse-labeled with ^{35}S-methionine and chased for 1 h followed by immunoprecipitation of intracellular or secreted pro-catZ using anti-HA. (Note that pro-catZ secreted from these cells lacks complex glycosylation (Fig. 6) because of the absence of Golgi GlcNAc transferase I (Kappeler et al., 1997)). Fig. 4E shows that only the transport of pro-catZ mutants capable of binding to ERGIC-53 is delayed by ER-localized GMAA-ERGIC-53 in a way similar to endogenous pro-catZ (see Fig. 1C).

A high-mannose-glycan/β-hairpin-loop motif in cathepsin C

Is the combined carbohydrate/peptide motif also present in other glycoproteins that are recognized by ERGIC-53, such as procathepsin C (Vollenweider et al., 1998)? Like catZ, cathepsin C is a lysosomal papain protease. Maturation of procathepsin C involves splicing of the propeptide so that the very N-terminal segment, the exclusion domain, is part of the active, tetrameric structure. Strikingly, a characteristic β-hairpin loop has been found in the cathepsin C structure (Turk et al., 2001) that shares many features with the ER-exit motif in

pro-catZ. Like a forefinger, both hairpin loops project out of the protease structure (Fig. 5) without any spacial influence on the active site of the protease. Remarkably, also the cathepsin C hairpin teams up with a proximal N-glycan (Turk et al., 2001) and the linear order of strand-loop-strand-glycosylation-site is identical to pro-catZ (Fig. 1A) including the number of spacing amino acids. The fact that cathepsin C lacks an intra-loop disulfide bridge suggests that this modification is not required for ERGIC-53 binding. The carbohydrate-adjacent β-strand backbones can be superimposed with a rms deviation of 0.92 Å (Fig. 5) suggesting that the exact 3D structure of this peptide may be important for lectin association.

The β-hairpin loop motif is required for correct oligosaccharide processing of pro-catZ. Next we focused on subsequent events in the maturation of pro-catZ. An interesting observation from the pulse-chase experiments (Figs. 3A, 4C) was the characteristic electrophoretic mobilities of the glycosylation-site mutants. We hypothesized that the mobility shifts associated with wt- and N184Q-pro-catZ may reflect oligosaccharide processing of the N2-glycan. To test this we probed for the action of Golgi mannosidase I (a prerequisite for complex glycosylation) by using kifunensin or for complex glycosylation by endoglycosidase H (endoH), an enzyme that can only cleave N-glycans before Golgi mannosidase II-trimming. First, we found the small shift of intracellular pro-catZ after 1 h of chase to be prevented by kifunensin treatment (Fig. 6A, compare also Fig. 2). Second, secreted pro-catZ (after a 3 h-chase) was partially resistant to endoH, but sensitive to PNGase F, that recognizes all forms of N-glycans, indicating that the decreased mobility of secreted wt- and N184Q-pro-catZ is due to complex glycosylation of a subpopulation of the proteins on the N2-glycan (Fig. 6B). Conversely, N224Q appeared to largely resist complex modification in the Golgi revealing that the major fraction of N1-glycans remained in the high-mannose form.

Pro-catZ is also known to acquire the M6P modification (Appenzeller et al., 1999) that is used as a signal for lysosomal targeting (Dahms and Hancock, 2002). To determine whether M6phosporylation occurred on the N1-, the N2- or on both glycans, we metabolically labeled pro-catZ with ^{32}P-orthophosphate. To minimize the impact of mutant-specific secretion rates (see above), we collected pro-catZ secreted from CHO cells during an 18 h labeling period. Culture media were subjected to anti-HA immunoprecipiation. The isolated proteins were treated with endoH, to release the carbohydrate-linked phosphate label, and analyzed by SDS-PAGE/phosphorimaging. A parallel experiment using ^{35}S-methionine revealed poor recovery of N184Q under these conditions. This was a result of its instability after secretion (not

shown). Fig. 6C shows that both glycosylation-site mutants carried the ^{32}P-label to a similar extent, if normalized to the ^{35}S-signal, revealing robust M6phosphorylation of both oligosaccharides. As expected, the M6P tag was almost completely removed by endoH-treatment indicating that phosphorylation essentially occurs on the endoH-sensitive pool of pro-catZ. Interestingly though, a small portion of ^{32}P-labeled wt-pro-catZ was endoH-resistant, suggesting complex modifications on some M6phosphorylated oligosaccharides.

If the β-hairpin loop – as proposed in fig. 4B – was indeed used like a crook to anchor the highly flexible high-mannose N1-glycan by a network of hydrogen bonds, one would expect that its deletion exposes the N1-glycan to abnormal processing. To test this prediction, we analyzed the oligosaccharide processing of the loop deletion mutants. As illustrated in Fig. 6D, Δloop* and, even more drastically, Δloop were far better substrates for the complex glycosylation machinery than the wt enzyme. Unexpectedly, the increased complex glycosylation arose from additional modifications on both oligosaccharides as evidenced by the appearance of a fully endoH-resistant species on the one hand (Fig. 6D), and the increase of complex modified N2-glycan on N184Q+Δloop comparing to N184Q on the other hand (Fig. 6E). Consistent with this, the prominent mobility shift of intracellular Δloop after a 1 h-chase (Fig. 4C) could be attributed to mannosidase trimming of apparently both N-glycans by its inhibition with kifunensin (not shown). We also probed for M6phosphorylation on pro-catZ lacking the hairpin loop. As shown in Fig. 6F, the M6P signal was present on both Δloop and Δloop*, and the fraction of fully endoH-resistant, phosphorylated pro-catZ was increased comparing to wt-pro-catZ. Thus, the β-hairpin loop protects both N-glycans of pro-catZ from irregular processing, but does not appear to be involved in M6phosphorylation.

The β-hairpin loop motif is essential for lysosomal targeting of pro-catZ.

What are the structural requirements for lysosomal delivery of pro-catZ? It must be emphasized that, in the experiments presented in the previous paragraphs, the mature lysosomal form of exogenous catZ was not visible because the proteolytic cleavage of the N-terminal propeptide (Fig. 1A) would remove the HA-epitope. To visualize the mature protein, we raised an antiserum against a fusion protein of GST and mature human catZ (Santamaria et al., 1998) which minimally crossreacted with hamster pro-catZ (Fig. 7A, mock). To study the appearance of the mature form of HA-pro-catZ, a pulse-chase experiment was performed with transiently transfected CHO cells followed by immunoprecipitation with anti-GST/catZ. As shown in Fig. 7A, an additional band appeared on the fluorogram after 2 h of chase with the expected size of mature, lysosomally processed catZ. Moreover, the band was absent from the

culture medium (not shown). Strikingly, no such band was seen in extracts from cells transfected with Δloop (Fig. 7A) or Δloop* cDNA (not shown), even when the chase was extended to 4 h (not shown). To confirm lysosomal processing of recombinant pro-catZ, we studied the effect of the weak base chloroquine that potently inhibits the proteolytic maturation of endogenous pro-catZ (Appenzeller-Herzog et al., 2004). As shown in Fig. 7B, this drug prevented the appearance of the presumed mature catZ and increased the signal of pro-catZ after 2 h of chase. These results suggest that exogenous wt- but not Δloop-pro-catZ is correctly targeted to the lysosomal pathway.

To gain further insight into the mechanism of pro-catZ transport to the lysosomes, we included the glycosylation-site mutants into our analysis. Both N184Q and N224Q reached the site where their proteolytic maturation takes place (Fig. 7C) suggesting that the M6P signal on either of the glycans of pro-catZ is sufficient for effective recognition by a M6P receptor. The fact that N184Q is retained for a longer time in the secretory pathway than N224Q (Fig. 3) renders it impossible to quantitatively compare their rate of lysosomal delivery. As expected, N184+224Q was not proteolytically activated confirming its retention early in the secretory pathway (not shown). Collectively, these experiments document a role of pro-catZ's hairpin loop in lysosomal targeting.

Discussion

An ER-exit signal on soluble secretory cargo

Based on their finding that the inhibition of ER glucosidases slows the transport of secretory glycoproteins, Lodish and Kong postulated that high-mannose glycans, after removal of glucose residues, may form part of the recognition site for a hypothetical ER-export receptor (Lodish and Kong, 1984). Although the subsequent discovery of N-glycan-dependent folding in the ER has led to the notion that sugar-dependent transport of glycoproteins in many instances reflects ER quality control (Ellgaard and Helenius, 2003), the present study, 20 years later, finally confirms the Lodish and Kong prediction. So far, knowledge on transport signals of secretory proteins required for the recognition by specific receptors has remained poor (Barlowe, 2003). The secretory proteins invertase and Hsp150 of yeast appear to possess active sorting signals (Fatal et al., 2004; Gaynor and Emr, 1997), but no cargo receptor has been identified yet. In the case of the yeast pheromone precursor glyco-pro-α-factor Erv29p acts as an ER-export receptor (Belden and Barlowe, 2001). A number of structural features including N-glycosylation are required for efficient intracellular transport of pro-α-factor (Caplan et al., 1991), but no link of these putative targeting motifs to Erv29p has been established.

In the present work, we have characterized a transport motif on pro-catZ that is composed of sugar and peptide determinants. It is obvious that the distinct secretion kinetics of the glycosylation-site- and Δloop-mutants of pro-catZ (Figs. 3A and 4C) go together with their ability to bind ERGIC-53 (Fig. 4D). Since export from the ER is the rate-limiting step for most secretory glycoproteins during exocytosis (Lodish, 1988), the secretion rate of pro-catZ is an accurate measure of its efficiency of ER-export. It is generally difficult, however, to attribute the transport phenotype of a given mutant to the absence of positive transport signals and to exclude its retention by the quality control machinery. Several considerations argue that we have indeed defined a positive transport signal in conjunction with its molecular decoding by the ER-export lectin ERGIC-53. First, we noted an increase in cargo-lectin binding after an initial lag phase that is characteristic for pro-catZ mutants harboring the full carbohydrate/peptide motif. This increase is consistent with the notion that only correctly folded pro-catZ after release from the quality control machinery binds ERGIC-53 and consequently leaves the ER. Second, glycoprotein binding to ERGIC-53 is affected by untrimmed glucose residues (Appenzeller et al., 1999) which are known to play a major role in the calnexin/calreticulin cycle (Ellgaard and Helenius, 2003). Third, our discovery that

ERGIC-53 recognizes a tertiary structure motif that is stabilized by an intrinsic disulfide linkage indicates that non-native conformers of pro-catZ cannot present the proper ticket for efficient ER-exit. Interestingly, the N1-glycosylation site is conserved from *C. elegans* to man (Fig. 1A) suggesting a pivotal targeting role for this oligosaccharide throughout the animal kingdom.

Moreover, we have identified an equivalent motif in cathepsin C, another substrate for ERGIC-53-mediated ER-export (Vollenweider et al., 1998). It is worth noting that despite the close relationship of the two cathepsins their β-hairpin loops appear to be evolutionary unconnected. While in pro-catZ the hairpin is contained in the papain domain, it is cathepsin C's exclusion domain that harbors this 3D motif. Moreover, the two amino acid sequences reveal no similarity apart from a tendency for charged side chains (Fig. 5). These observations argue that the two recognition motifs arose independently by convergence. Accordingly, the search for additional ERGIC-53 cargoes will require 3D information. Furthermore, the notion that some glycoproteins have independently adapted to lectin-assisted transport raises the question of whether the transport lectin activity of ERGIC-53 is a developmentally late function of this ubiquitously expressed and conserved protein. Such thinking gains additional support by the fact that yeast homologues lack critical carbohydrate binding residues (Nufer et al., 2003b) as well as a HIS-loop (Appenzeller-Herzog et al., 2004) within the carbohydrate recognition domain.

Our findings that the glycan-less mutant N184+224Q efficiently co-precipitates with ERGIC-53 (Fig. 4A) and that still ~20% binding to ERGIC-53 remains in absence of the functional N1-glycan/hairpin loop motif (Fig. 4D) point to the presence of a second binding motif on pro-catZ that needs to be characterized in detail. Two observations indicate that this motif localizes proximal to N224. First, binding to ERGIC-53 increases in the absence of the N2-glycan. Second, an artificial ER-overload with ERGIC-53 causes indirect inhibition of Golgi mannosidase I trimming of the N2-glycan in the Golgi (Figs. 1C, 2 and 6A). We propose that this inhibition is caused by the steric hindrance of ER α-mannosidase I which normally produces the $Man_8GlcNAc_2$ isomer B (Herscovics, 2001). High-mannose substrates other than this isomer are poorly recognized by α1,2-mannosidases in the Golgi (Lal et al., 1998). The observations that this second ERGIC-53/cargo interaction is seen with N184+224Q and is already maximal after a short ^{35}S-methionine pulse (and does not increase during the chase) may hint at some sort of mannose-independent quality control event. Interestingly, carbohydrate- and lectin domain-independent binding to ERGIC-53 has also been noted for coagulation factor VIII (Cunningham et al., 2003).

The β-hairpin of pro-catZ is essential for correct processing and targeting beyond the early secretory pathway.

The present study also revealed a second function of the β-hairpin loop of pro-catZ. Strikingly, the deletion of the loop completely abolished lysosomal cleavage of pro-catZ. The theoretical possibility that the loop is required for the proteolytic activation itself appears extremely unlikely given the good accessibility and the flexible nature of the propeptide cleavage site and its structural distance from the hairpin motif (Sivaraman et al., 2000). The results rather support a model whereby the β-hairpin loop functions by protecting pro-catZ from irregular oligosaccharide processing, which in turn would indirectly affect sorting to the lysosomes by the M6P machinery.

Do the loop amino acids serve as a phosphotransferase-binding site? Indeed, β-hairpin loops have been implicated in such activity (Jain et al., 1996; Lukong et al., 1999). Several lines of evidence, however, argue against this notion. First, the pro-catZ hairpin structure does not resemble the phosphotransferase-binding hairpins of β-glucuronidase, cathepsins A or D, as these are several residues longer, protrude less into the solvent and do not superimpose with the pro-catZ motif (not shown). Second, recognition of lysosomal enzymes by phosophotransferase occurs at a certain distance from the oligosaccharide to be phosphorylated, and in β-glucuronidase, the site 1 glycan in the extreme proximity of the β-hairpin is hardly phosphorylated (Jain et al., 1996). In pro-catZ, however, we observed robust phosphorylation of the hairpin-associated N1-glycan. Third, with the penta-peptide $I_{107}KRKG_{111}$ that is conserved among vertebrates (Fig. 1A) we are quite confident to have identified at least one of the phosphotransferase binding sites of pro-catZ. It aligns with the phosphotransferase-binding peptide B2 of the papain protease cathepsin B (IHTNG, (Lukong et al., 1999)) in both its primary and tertiary structure (rms deviation$_{C\alpha}$ = 0.16 Å), and additionally contains positively charged surface residues that have been assigned phosphotransferase-binding activity ((Warner et al., 2002) and refs. therein). The Cα-distance of R109 to N184 and N224 is 22 Å and 36 Å, respectively, which is in good agreement with the spacial array in other lysosomal proteins. Fourth and most importantly, we have shown that Δloop is efficiently phosphorylated (Fig. 6F). We conclude that inefficient M6P tagging does not account for the lysosomal transport defect of this mutant.

How else can the transport defect be explained? By two independent readouts, that is, altered electrophoretic mobility during pulse-chase (reflecting Golgi mannosidase I trimming and complex glycosylation, Figs. 3A, 4C) and resistance to endoH (Fig. 6D), we demonstrate increased Golgi processing in Δloop (as compared to wt) on both glycans. Thus, it is

conceivable that lysosomal delivery of Δloop fails because it lacks the correct carbohydrate signal. At least part of the abnormally processed glycans was phosphorylated (compare double glycosylated bands of Δloop ± endoH in Figs. 6D and F) revealing that the increased activity of Golgi mannosidase I toward this mutant does not cleave the M6P residues from the glycans (M6phosphorylation can occur on two mannose residues that are not subject to α1,2-mannosidase trimming (Varki and Kornfeld, 1980)). Of note, the degree of endoH-resistance does not reflect the full range of Golgi processing. Indeed, although the majority of phosphoglycans on secreted Δloop are removed by endoH-treatment (Fig. 6F), they are more extensively modified than wt-pro-catZ glycans (Figs. 3A, 4C). The mechanism underlying the lysosomal delivery phenotype of the Δloop mutants may involve inefficient uncovering and/or recognition by the M6P receptors in the late Golgi. Although the size of oligosaccharide and the nature of the protein backbone have little effect on the activity of the uncovering enzyme toward phosphodiesters (Varki and Kornfeld, 1981), to our knowledge, there are no data on the impact of Golgi-mediated abnormal processing including complex glycosylation. The same holds true for M6P receptor recognition (Dahms and Hancock, 2002).

A common motif for optimal presentation of two different carbohydrate signals

The common structural motif in pro-catZ conveying both its ER-export and lysosomal delivery is the β-hairpin loop. In both instances the loop appears to play a rather indirect mechanistic role. Regarding ERGIC-53 binding, the lack of amino acid identity between the hairpins of pro-catZ and procathepsin C renders it unlikely that these peptides directly associate with the lectin. We suggest that the loop motif functions by supporting an optimal conformation of the adjacent high-mannose glycan, which serves as ERGIC-53 recognition motif. Similarly, the hairpin loop helps maintaining the glycans of pro-catZ in correct configuration for efficient lysosomal targeting by the M6P receptors. We speculate that such conformational support of carbohydrate chains by adjacent proteinaceous determinants may be a general mechanism that is fundamental for proper lectin-recognition. Many glycoproteins may need to present their oligosaccharide signals in a similar manner. Interestingly, for accurate Golgi processing of pro-catZ the full peptide loop is required, while recognition by ERGIC-53 appears not to depend on the outermost amino acids of the hairpin (see Δloop*, Fig. S4). Furthermore, the hairpin seems to assist only the N1-glycan during ER-exit, but both glycans during passage through the Golgi.

In summary, we have identified the first ER-export motif on soluble secretory cargo. Consistent with previous functional studies on the transport receptor ERGIC-53, this motif

consists of both carbohydrate and peptide determinants and is found in precise structural analogy on two glycoproteins, both of which require functional ERGIC-53 for efficient transport. Furthermore, for pro-catZ we unambiguously show that the same peptide loop that confers efficient ER-export plays an additional role in lysosomal delivery. To our knowledge this is the first example of dual interpretation of a targeting motif within the eukaryotic secretory pathway.

Materials and Methods

Antibodies

The following antibodies were used: mAb 9E10.2 against a c-myc epitope (ATCC CRL 1729), mAb G1/93 against human ERGIC-53 (Schweizer et al., 1988), mAb 16B12 against the HA.11-epitope (Covance Research Products, Berkeley CA, USA) and pAb against Chinese hamster pro-catZ (anti-catZ, (Appenzeller-Herzog et al., 2004)). For affinity purification of anti-catZ serum, a 1:1 mix of the 2 immunogenic peptides (Fig. 1A) including a N-terminal cysteine were coupled to thiol activated Sepharose 4B (Amersham) as described (Kappeler et al., 1997). Crude serum was subjected to ammonium sulfate precipitation, dialyzed against PBS and bound to the peptide column overnight. After washing with 20 volumes of PBS and 10 volumes of 10 mM Tris/HCl, pH 7.4, 500 mM NaCl, bound antibodies were eluted sequentially with 10 volumes each of 100 mM glycine/HCl, pH 2.8 and 100 mM triethylamine, pH 11.5. OD_{280} peak-fractions were neutralized with Tris, dialyzed against PBS and concentrated in a Centriplus YM-30 filter device (Millipore, Bedford MA, USA). An antiserum against GST/catZ was produced in a rabbit.

Recombinant DNAs

Insertions or deletions in human preprocathepsin Z were generated using PCR-based splicing by overlap extension. The 5'-end primer contained a *Bam*HI site before the start ATG, the 3'-end primer a *Xho*I site after the stop codon. The HA-epitope (YPYDVPDYA) was either introduced downstream of the signal peptide cleavage site flanked by two glycins (corresponding to G30) or into the propeptide region by replacing amino acids 50-58. Δloop and Δloop* were produced as depicted in Fig. 4B using the HA-tagged cDNAs as template. The resulting cDNAs were cloned into pcDNA 3.1 vector (Invitrogen) via *Bam*HI and *Xho*I sites. Glycosylation-site mutations (changing the codons 184 and/or 224 from AAC to CAG) were introduced by PCR. N184Q+Δloop was generated by the QuikChange method (Stratagene) using the Δloop-cDNA as template.

Cell culture, transfections, drug treatment, and in situ crosslinking

CHO-KI, lec1, GM and GMAA cells were cultured as descibed (Appenzeller-Herzog et al., 2004) and transfected with FuGENE 6 (Roche). Kifunensin (100 µM / Toronto Research Chemicals, North York, Canada) and BFA (10 µg/ml / Epicentre Technologies, Madison WI, USA) were added 60 or 30 min, respectively, before and during the entire pulse-chase period.

Chloroquine (Sigma) treatment (Appenzeller-Herzog et al., 2004) as well as *in situ* crosslinking with DSP (Pierce, (Appenzeller et al., 1999)) have been descibed.

Metabolic labeling, immunoprecipitation, and endoglycosidase digest

Pulse-chase experiments with ^{35}S-methionine (PerkinElmer) or ^{32}P-orthophosphate (Amersham) and native immunoprecpitations from cell lysates were performed as described (Appenzeller et al., 1999; Nufer et al., 2003a). Secreted HA-pro-catZ was recovered by immunoprecipitation from cell media that, after clearance by a 10 min centrifugation step (20,000 g), had been supplemented with 1% TX-100. Immunoprecipitations using anti-catZ or anti-GST/catZ were done after antigen denaturation (Appenzeller-Herzog et al., 2004). For the experiments presented in Figs. 1B, 4A, 4D, S3 and S4, we used anti-ERGIC-53 immunobeads that had been chemically coupled with dimethyl pimelimidate (Sigma). For ERGIC-53/HA sequential immunoprecipitations, crosslinked anti-ERGIC-53 immunocomplexes were released from the beads and denatured by boiling them for 10 min in 30 mM triethanoamine/HCl pH 8.1, 100 mM NaCl, 5 mM EDTA, 1.6% SDS. The resulting supernatant was then split for direct analysis (10%) or for subsequent anti-HA immunoprecipitation (90%) after diluting and adjusting the buffer to 2% TX-100. EndoH (Roche) digestion of immunoprecipitates has been described (Kappeler et al., 1997). For PNGase F digestion, immunobeads were boiled for 3 min in 100 mM $NaPO_4$, pH 7.2, 1% ß-mercaptoethanol, 10 mM EDTA and 0.1% SDS. An equal volume of the same buffer containing 1% TX-100 instead of SDS and protease inhibitors was added, and the samle digested with 1 U of PNGase F (Roche) at 37°C overnight. Proteins were separated by 10% reducing SDS-PAGE and visualized by Western blotting, phosphorimaging or fluorography as descibed (Appenzeller-Herzog et al., 2004).

Acknowledgments

We thank Käthy Bucher for technical assistance, Franziska Kuhn for helpful suggestions, and Lorenza Bordoli for advice in biocomputing. The study was supported by the Swiss National Science Foundation (H.P.H.), the University of Basel (H.P.H), the European Union (C.L-O.) and the CICYT-Spain (C.L-O.). I.S. is recipient of a Research Contract from Fondo de Investigaciones Sanitarias (FIS 00/3161).

References

Appenzeller, C., Andersson, H., Kappeler, F. and Hauri, H.P. (1999) The lectin ERGIC-53 is a cargo transport receptor for glycoproteins. *Nat Cell Biol*, **1**, 330-334.

Appenzeller-Herzog, C., Roche, A.C., Nufer, O. and Hauri, H.P. (2004) pH-induced conversion of the transport lectin ERGIC-53 triggers glycoprotein release. *J Biol Chem*, **279**, 12943-12950.

Barlowe, C. (2003) Signals for COPII-dependent export from the ER: what's the ticket out? *Trends Cell Biol*, **13**, 295-300.

Belden, W.J. and Barlowe, C. (2001) Role of Erv29p in collecting soluble secretory proteins into ER-derived transport vesicles. *Science*, **294**, 1528-1531.

Caplan, S., Green, R., Rocco, J. and Kurjan, J. (1991) Glycosylation and structure of the yeast MF alpha 1 alpha-factor precursor is important for efficient transport through the secretory pathway. *J Bacteriol*, **173**, 627-635.

Cunningham, M.A., Pipe, S.W., Zhang, B., Hauri, H.P., Ginsburg, D. and Kaufman, R.J. (2003) LMAN1 is a molecular chaperone for the secretion of coagulation factor VIII. *J Thromb Haemost*, **1**, 2360-2367.

Dahms, N.M. and Hancock, M.K. (2002) P-type lectins. *Biochim Biophys Acta*, **1572**, 317-340.

Ellgaard, L. and Helenius, A. (2003) Quality control in the endoplasmic reticulum. *Nat Rev Mol Cell Biol*, **4**, 181-191.

Fatal, N., Karhinen, L., Jokitalo, E. and Makarow, M. (2004) Active and specific recruitment of a soluble cargo protein for endoplasmic reticulum exit in the absence of functional COPII component Sec24p. *J Cell Sci*, **117**, 1665-1673.

Gaynor, E.C. and Emr, S.D. (1997) COPI-independent anterograde transport: cargo-selective ER to Golgi protein transport in yeast COPI mutants. *J Cell Biol*, **136**, 789-802.

Hauri, H., Appenzeller, C., Kuhn, F. and Nufer, O. (2000a) Lectins and traffic in the secretory pathway. *FEBS Lett*, **476**, 32-37.

Hauri, H.P., Kappeler, F., Andersson, H. and Appenzeller, C. (2000b) ERGIC-53 and traffic in the secretory pathway. *J Cell Sci*, **113**, 587-596.

Herscovics, A. (2001) Structure and function of Class I alpha 1,2-mannosidases involved in glycoprotein synthesis and endoplasmic reticulum quality control. *Biochimie*, **83**, 757-762.

Jain, S., Drendel, W.B., Chen, Z.W., Mathews, F.S., Sly, W.S. and Grubb, J.H. (1996) Structure of human beta-glucuronidase reveals candidate lysosomal targeting and active-site motifs. *Nat Struct Biol*, **3**, 375-381.

Kappeler, F., Klopfenstein, D.R., Foguet, M., Paccaud, J.P. and Hauri, H.P. (1997) The recycling of ERGIC-53 in the early secretory pathway. ERGIC-53 carries a cytosolic endoplasmic reticulum-exit determinant interacting with COPII. *J Biol Chem*, **272**, 31801-31808.

Klemencic, I., Carmona, A.K., Cezari, M.H., Juliano, M.A., Juliano, L., Guncar, G., Turk, D., Krizaj, I., Turk, V. and Turk, B. (2000) Biochemical characterization of human cathepsin X revealed that the enzyme is an exopeptidase, acting as carboxymonopeptidase or carboxydipeptidase. *Eur J Biochem*, **267**, 5404-5412.

Kornfeld, R. and Kornfeld, S. (1985) Assembly of asparagine-linked oligosaccharides. *Annu Rev Biochem*, **54**, 631-664.

Lal, A., Pang, P., Kalelkar, S., Romero, P.A., Herscovics, A. and Moremen, K.W. (1998) Substrate specificities of recombinant murine Golgi alpha1, 2-mannosidases IA and IB and comparison with endoplasmic reticulum and Golgi processing alpha1,2-mannosidases. *Glycobiology*, **8**, 981-995.

Lodish, H.F. (1988) Transport of secretory and membrane glycoproteins from the rough endoplasmic reticulum to the Golgi. A rate-limiting step in protein maturation and secretion. *J Biol Chem*, **263**, 2107-2110.

Lodish, H.F. and Kong, N. (1984) Glucose removal from N-linked oligosaccharides is required for efficient maturation of certain secretory glycoproteins from the rough endoplasmic reticulum to the Golgi complex. *J Cell Biol*, **98**, 1720-1729.

Lukong, K.E., Elsliger, M.A., Mort, J.S., Potier, M. and Pshezhetsky, A.V. (1999) Identification of UDP-N-acetylglucosamine-phosphotransferase-binding sites on the lysosomal proteases, cathepsins A, B, and D. *Biochemistry*, **38**, 73-80.

Nichols, W.C., Seligsohn, U., Zivelin, A., Terry, V.H., Hertel, C.E., Wheatley, M.A., Moussalli, M.J., Hauri, H.P., Ciavarella, N., Kaufman, R.J. and Ginsburg, D. (1998) Mutations in the ER-Golgi intermediate compartment protein ERGIC-53 cause

combined deficiency of coagulation factors V and VIII. *Cell*, **93**, 61-70.

Nufer, O., Kappeler, F., Guldbrandsen, S. and Hauri, H.P. (2003a) ER export of ERGIC-53 is controlled by cooperation of targeting determinants in all three of its domains. *J Cell Sci*, **116**, 4429-4440.

Nufer, O., Mitrovic, S. and Hauri, H.P. (2003b) Profile-based data base scanning for animal L-type lectins and characterization of VIPL, a novel VIP36-like endoplasmic reticulum protein. *J Biol Chem*, **278**, 15886-15896.

Sakamoto, E., Sakao, Y., Taniguchi, Y. and Yamafuji, K. (1999) Cathepsin Y (a novel thiol enzyme) produces kinin potentiating peptide from the component protein of rat plasma. *Immunopharmacology*, **45**, 207-214.

Santamaria, I., Velasco, G., Pendas, A.M., Fueyo, A. and Lopez-Otin, C. (1998) Cathepsin Z, a novel human cysteine proteinase with a short propeptide domain and a unique chromosomal location. *J Biol Chem*, **273**, 16816-16823.

Schweizer, A., Fransen, J.A., Bachi, T., Ginsel, L. and Hauri, H.P. (1988) Identification, by a monoclonal antibody, of a 53-kD protein associated with a tubulo-vesicular compartment at the cis-side of the Golgi apparatus. *J Cell Biol*, **107**, 1643-1653.

Sivaraman, J., Nagler, D.K., Zhang, R., Menard, R. and Cygler, M. (2000) Crystal structure of human procathepsin X: a cysteine protease with the proregion covalently linked to the active site cysteine. *J Mol Biol*, **295**, 939-951.

Therrien, C., Lachance, P., Sulea, T., Purisima, E.O., Qi, H., Ziomek, E., Alvarez-Hernandez, A., Roush, W.R. and Menard, R. (2001) Cathepsins X and B can be differentiated through their respective mono- and dipeptidyl carboxypeptidase activities. *Biochemistry*, **40**, 2702-2711.

Turk, D., Janjic, V., Stern, I., Podobnik, M., Lamba, D., Dahl, S.W., Lauritzen, C., Pedersen, J., Turk, V. and Turk, B. (2001) Structure of human dipeptidyl peptidase I (cathepsin C): exclusion domain added to an endopeptidase framework creates the machine for activation of granular serine proteases. *Embo J*, **20**, 6570-6582.

Varki, A. and Kornfeld, S. (1980) Structural studies of phosphorylated high mannose-type oligosaccharides. *J Biol Chem*, **255**, 10847-10858.

Varki, A. and Kornfeld, S. (1981) Purification and characterization of rat liver alpha-N-acetylglucosaminyl phosphodiesterase. *J Biol Chem*, **256**, 9937-9943.

Vollenweider, F., Kappeler, F., Itin, C. and Hauri, H.P. (1998) Mistargeting of the lectin ERGIC-53 to the endoplasmic reticulum of HeLa cells impairs the secretion of a lysosomal enzyme. *J Cell Biol*, **142**, 377-389.

Warner, J.B., Thalhauser, C., Tao, K. and Sahagian, G.G. (2002) Role of N-linked oligosaccharide flexibility in mannose phosphorylation of lysosomal enzyme cathepsin L. *J Biol Chem*, **277**, 41897-41905.

Figure legends

Fig 1: *CatZr is the Chinese hamster ortholog of pro-catZ.*

(A) Sequence alignment of prepro-catZ from different species. Sequences are available from GenBank/EMBL/DDBJ under accession numbers Q9EPP7(hamster), Q9WUU7(mouse), Q9R1T3(rat), Q9UBR2(human), NP_491023(worm). Signal peptides are underlined; the propeptide cleavage site is marked with a triangle; peptides used for immunizing rabbits are boxed; identified catZr-peptides are shaded; glycosylation sites are bold and marked with an asterisk; the two strands of the β-hairpin loop and the S-S linkage within the loop are highlighted; the putative phosphotransferase-binding peptide is shown in italic.

(B) GMAA cells were treated with or without DSP and subjected to anti-ERGIC-53 immunoprecipitation (IP). H-lanes: 1% of the total homogenate. Shown are Western blots with anti-myc or affinity purified anti-catZ. Note that pro-catZ can be crosslinked to ERGIC-53.

(C) Maturation of endogenous pro-catZ in GM and GMAA cells. The pulse(15 min)-chase(as indicated) analysis illustrates the KKAA-ERGIC-53 induced retardation of pro-catZ transport (fluorogram). Arrows indicate (from bottom to top) immature-, intermediate- and mature catZ. Note that in GMAA cells the transport of catZ is slowed and no intermediate form is generated.

Fig 2: *Pro-catZ is a substrate for Golgi mannosidase I.*

(A) Pro-catZ time-course as in Fig. 1C, but in CHO cells treated with or without kifunensin (KIF). Kifunensin ablates production of the intermediate form (fluorogram).

(B) Same experiment as in (A) in cells pretreated with BFA. Note that BFA causes premature mannosidase trimming of pro-catZ and inhibits its proteolytic maturation (fluorogram).

(C) GMAA cells treated with or without kifunensin and BFA were pulsed for 3 h with ^{35}S-methionine, crosslinked with DSP and ERGIC-53-associated pro-catZ isolated by anti-ERGIC-53 immunoprecipitation (fluorogram). Kifunensin-treatment prevents the BFA-induced mobility shift.

Fig 3: *Secretion of pro-catZ differentially depends on its two N-glycan chains.*

(A) CHO cells were transfected with wt or glycosylation-site mutant pro-catZ cDNAs, pulsed for 5 min and chased for the indicated times. Intracellular and secreted pro-catZ was recovered by anti-HA immunoprecipitation (fluorogram). c, cell lysate; m, medium.

(B) Analogous experiment as in (A) but using denaturing immunoprecipitation. Note that under these experimental conditions N184Q+N224Q is readily detectable.

Fig 4: *Efficient binding of pro-catZ to ERGIC-53 requires a mixed carbohydrate/peptide motif.*

(A) Crosslinking and co-immunoprecipitation of GMAA-ERGIC-53 and pro-catZ mutants. Shown are pro-catZ bands visualized by anti-HA immunoblotting. Note that DSP-dependent co-isolation is decreased by N184Q, but favoured by N224Q. H, homogenate (1% input); IP, anti-ERGIC-53 immunoprecipitation.

(B) Graphical representation of the β-hairpin loop (cyan) and the high-mannose N184-linked glycan (yellow). Selected amino acid side chains that are likely to anchor the adjacent oligosaccharide by hydrogen bonds and the disulfide linkage are shown. As depicted in single letter code, mutations to eliminate the hairpin to the level of the S-S bridge (Δloop*) or completely (Δloop) were engineered by introducing a DG-turn (corresponding to pro-catZ turns at position 72/73, 252/253 or 282/283).

(C) Stability and secretion of Δloop and N184Q+Δloop were assessed as in Fig. 3A (fluorogram).

(D) Sequential immunoprecipitation of GMAA-ERGIC-53 and recombinant pro-catZ (2^{nd}, lower gel) after a 10 min-pulse with or without 15 min-chase and DSP-treatment. The upper gel shows 10% of the first (anti-ERGIC-53) immunoprecipitate. The diagram represents densitometric pro-catZ levels normalized against ERGIC-53 (recovered in the first immunoprecipitation) and expressed as percentage of wt/15 min chase (mean ± SD, n = 3). Pro-catZ mutants lacking the full carbohydrate/peptide motif characteristically show weak binding without a boost after the chase.

(E) Comparison of the percentage of secretion after 5 min of pulse and 1 h of chase from GMAA versus Lec1 cells. The KKAA-ERGIC-53 induced delay on the secretion of pro-catZ mutants was recorded by fluorography and densitometric quantification. Shown is a typical experiment (left) and the plot of GMAA delays in percent of 3

independent experiments (mean ± SD, right). Note that only in the case of wt and N224Q the delay is statistically significantly different from zero ($P < 0.05$, Student's *t* test). c, cell lysate; m, medium.

Fig 5: *Superposition of the hairpin loop motifs of pro-catZ and cathepsin C.*

The two highlighted hepta-peptides of pro-catZ (red) and cathepsin C (orange) were superimposed using the *Swiss PDB viewer* software (available at www.expasy.ch). Shown are the peptide backbones of pro-catZ (white, PDB ID: 1DEU) and cathepsin C (CatC, gray, PDB ID: 1K3B), the side chains of glycosylation-site asparagines 184 and 95 and the disulfide bridge within the pro-catZ hairpin. Note that the overlay is restricted to the β-hairpin loops. The full sequence of the cathepsin C motif is $\underline{K_{82}YKE}EGS\underline{KVTT}YCNET_{97}$ (β-strands are underlined, numbering according to (Turk et al., 2001)).

Fig 6: *Golgi oligosaccharide processing of pro-catZ mutants.*

(A) Wt and glycosylation-site mutant pro-catZ was labeled for 5 min in CHO cells and chased for the indicated times prior to immunoprecipitation (fluorogram). Where indicated, cells had been treated with kifunensin (KIF).

(B, D and E) Pro-catZ protein secreted from transfected CHO cells after a 5 min-pulse and a 3 h-chase was isolated by immunoprecipitation and subjected to endoH or PNGase F digest (fluorogram). Note that only in the presence of the loop the endoH-resistance is limited to the N2-glycan.

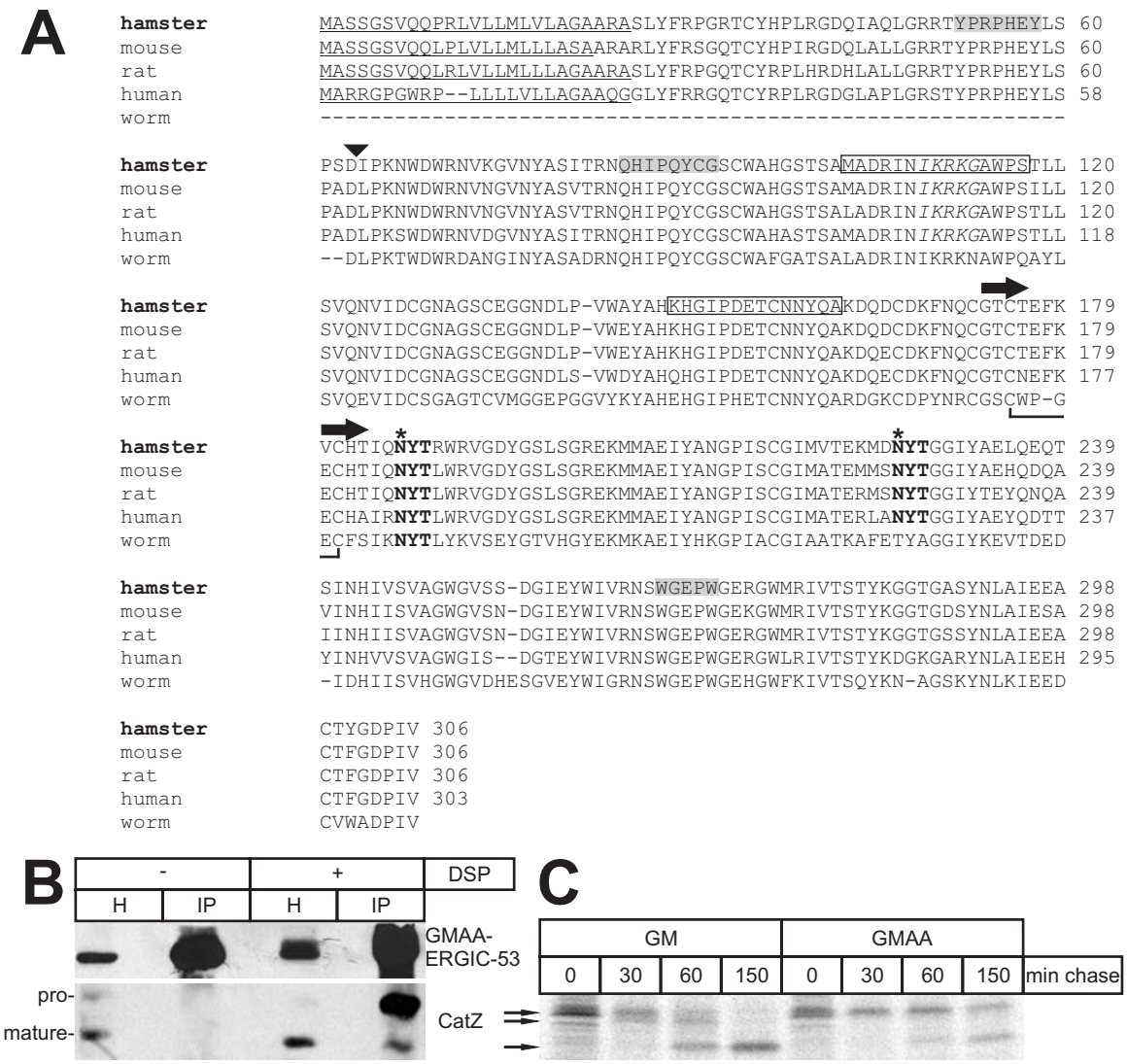
(C and F) CHO cells were transfected with pro-catZ cDNAs as indicated and labeled for 18 h with ^{32}P-orthophosphate or ^{35}S-methionine. Secreted pro-catZ was immunoisolated from the cell media and treated with or without endoH (autoradiogram). Numbers indicate doubly-, singly-, and non-glycosylated pro-catZ. Note in (F) that fully deglycosylated pro-catZ is invisible and that the singly-glycosylated species carries only half of the label.

Fig 7: *Δloop is not targeted to the lysosomes.*

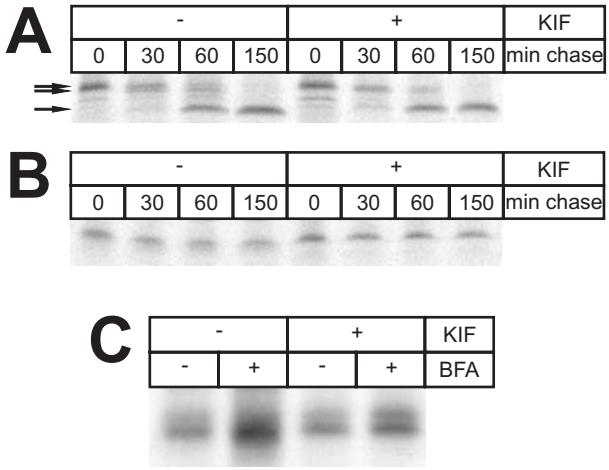
(A) Pulse(15 min)-chase(as indicated) analysis of mock (empty vector), wt- or Δloop-pro-catZ transfected CHO cells followed by anti-GST/catZ immunoprecipitation (fluorograms). The mature form of wt catZ is marked with a filled arrowhead. Note that Δloop does not become proteolytically activated (empty arrowhead), but is more extensively trimmed in the Golgi than wt (cross). Asterisk, nonspecific degradation product; circled asterisk, non-glycosylated pro-catZ.

(B) Analogous experiment as in (A) with chloroquine-treated cells. This drug ablates catZ activation (arrowheads) and leads to accumulation of pro-catZ (cross).

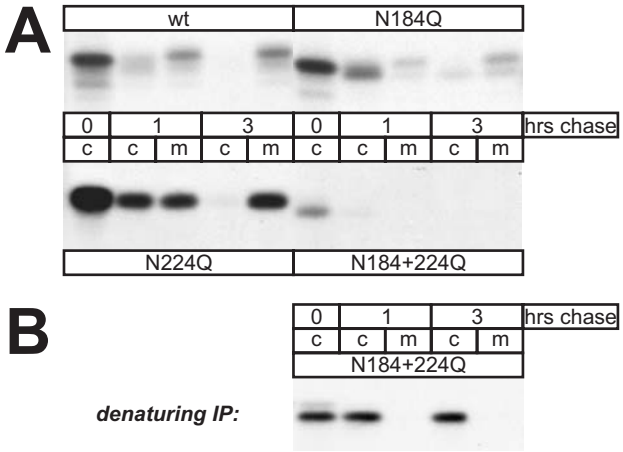
(C) Lysosomal delivery of pro-catZ glycosylation-site mutants was assessed as in (A) and (B). Note that only N184Q is mannosidase-modified (cross). Arrowheads, mature catZ.



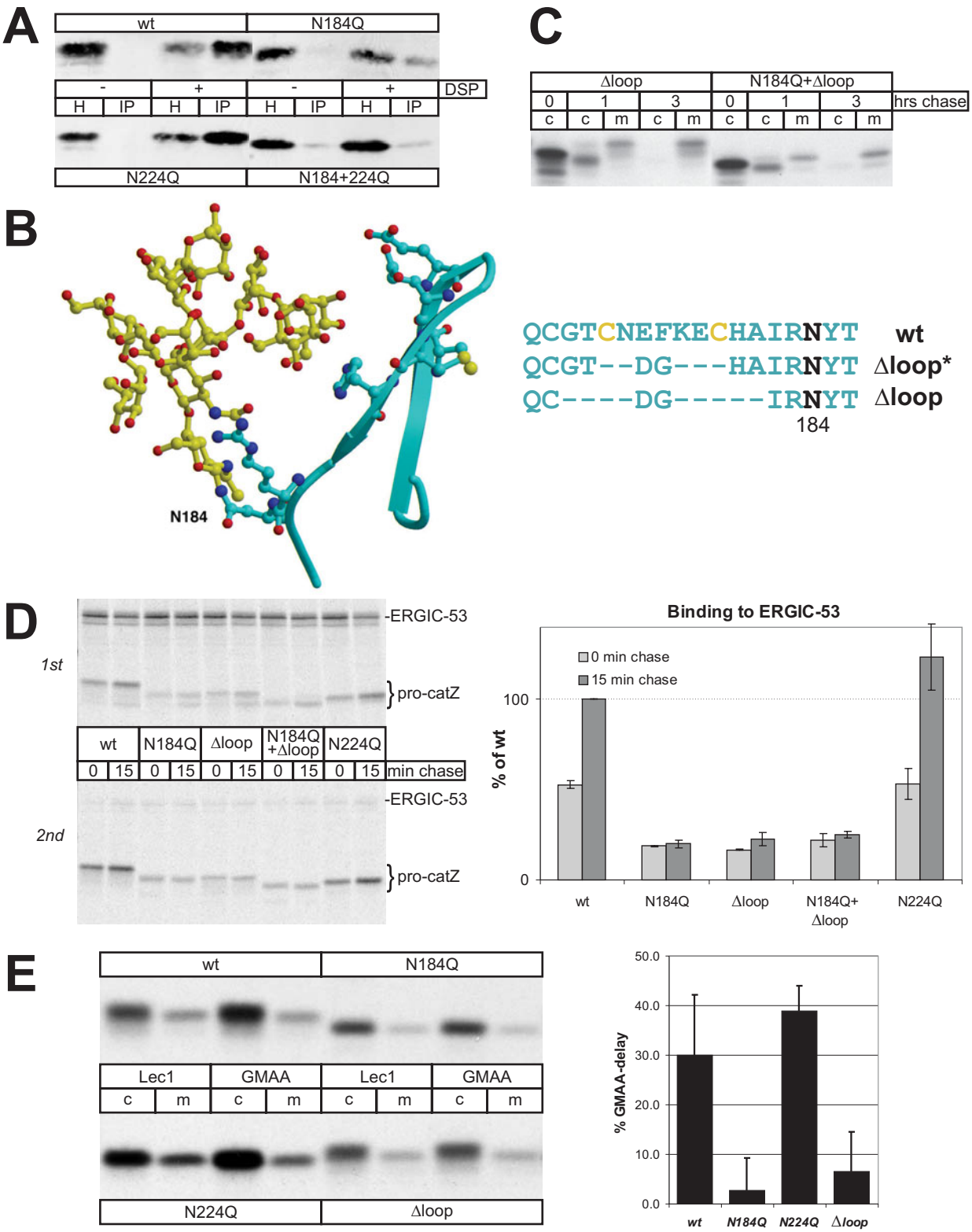
Appenzeller-Herzog et al. Figure 1



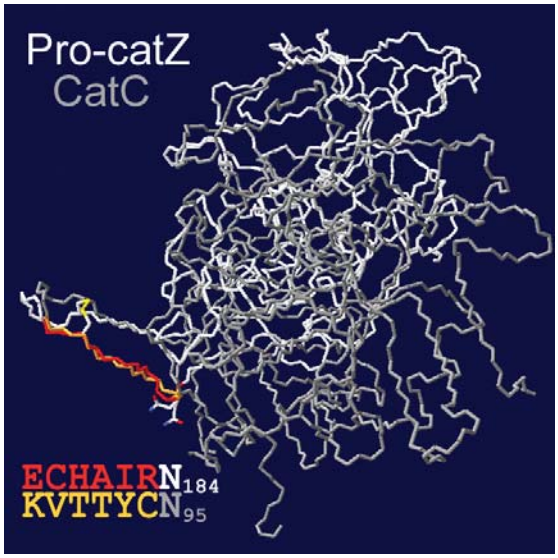
Appenzeller-Herzog et al. Figure 2



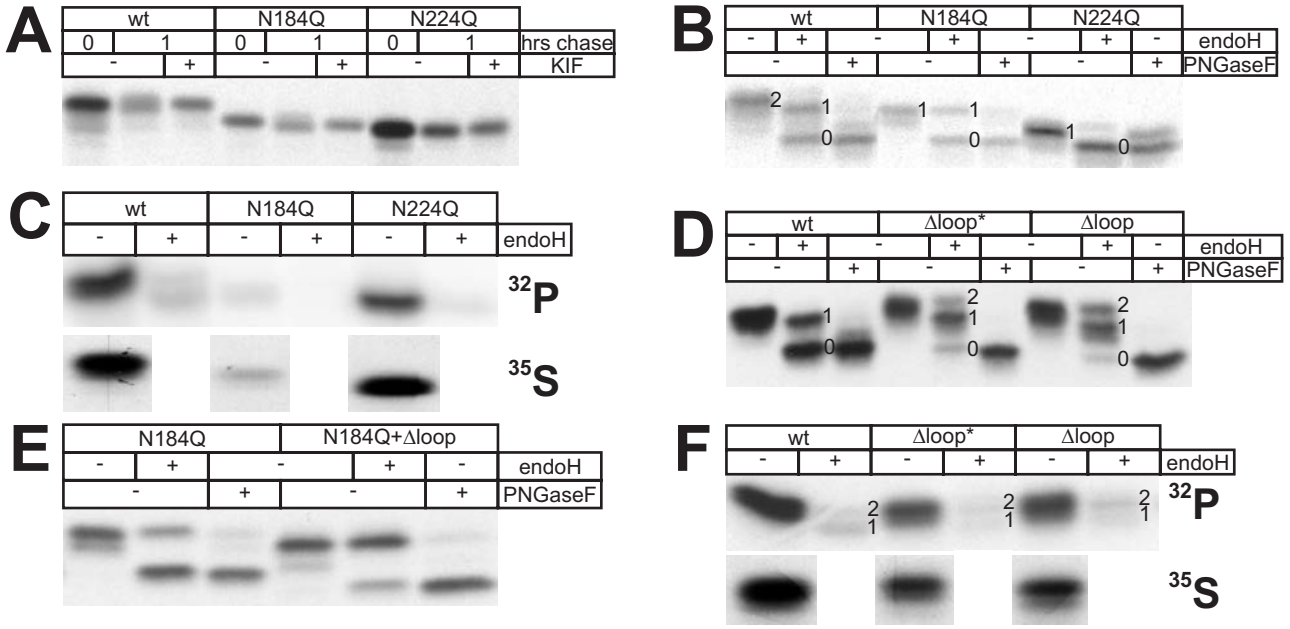
Appenzeller-Herzog et al. Figure 3



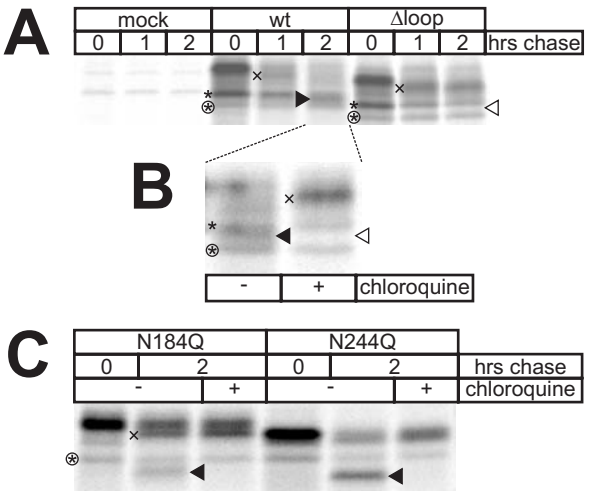
Appenzeller-Herzog et al. Figure 4



Appenzeller-Herzog et al. Figure 5



Appenzeller-Herzog et al. Figure 6



Appenzeller-Herzog et al. Figure 7

Supplemental data

Screening of a CHO cDNA library

We designed two pairs of degenerate primers corresponding to the microsequenced catZr-peptides (Appenzeller et al., 1999) for subsequent RT-PCR on total RNA from GMAA cells. We obtained two fragments of approximately 650 and 550 bp length that were further used as probes to screen a CHO Lambda cDNA library (Fig. S1A). On a Northern blot, both DNA-probes revealed a single, sharp RNA-band of equal size (not shown) and we obtained several Lambda clones containing the corresponding cDNA. Sequence analysis yielded a partial cDNA encoding Chinese hamster prepro-catZ (Fig. 1A). The sequence information has been deposited at NCBI (accession number: AJ303074).

Degenerate primers corresponding to the catZr peptide sequences were: Coding strand primers 5'-TATCCTCGGCCGCARGAGTACC-3' and 5'-CAGCAYATCCCACAGTAC-3' and the template strand primer 5'-CCCCAGGGYTCGCCCCATG-3'. Total RNA of GMAA cells was extracted with TRIzol (Invitrogen), reverse transcibed with MMLV-reverse transcriptase and oligo-dT (Roche) and used as a template for 2 PCR reactions with the above primer pairs. The resulting products were [^{32}P]ATP-labeled using a Random Primed DNA labeling kit (Roche) and used to screen a CHO Uni-ZAP XR Lambda cDNA library (Stratagene) according to the manufacturer's instructions and to isolate a cDNA encoding hamster prepro-catZ.

Fig S1: *Cloning of Chinese hamster prepro-catZ*

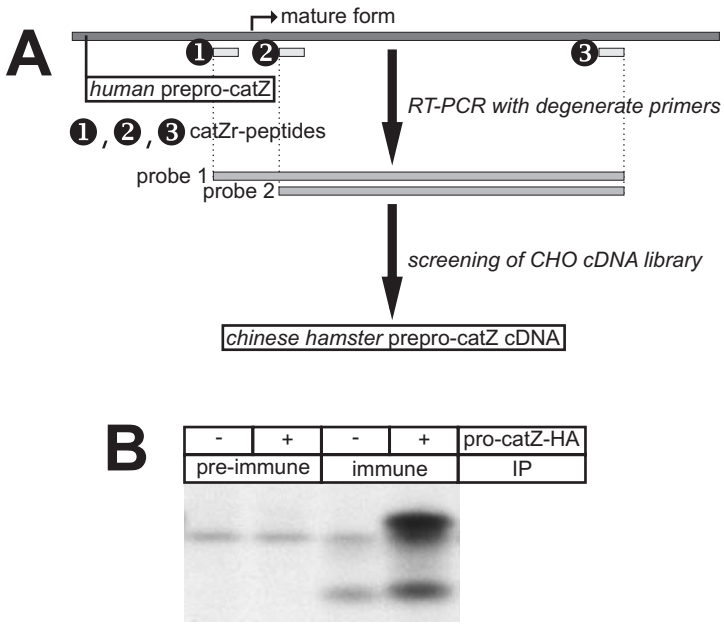
(A) Schematic representation of the cloning of Chinese hamster prepro-catZ.

(B) HA-tagged hamster pro-catZ was expressed in GM cells {Kappeler, 1997 #2} and, after metabolic labeling with ^{35}S-methionine for 3 h, isolated by immunoprecipitation with anti-HA (not shown) or a rabbit antiserum (immune) raised against two peptides derived from hamster pro-catZ (Fig. 1A). In non-transfected cells a band was detected that represents endogenous catZ after lysosomal maturation, i.e. after the proteolytic removal of its propeptide (Fig. 1A, see also Fig. 1C). Pro-catZ is hidden by a contaminating band that is also present in precipitates obtained with pre-immune serum but can be removed by prior treatment of the cells with DSP (not shown).

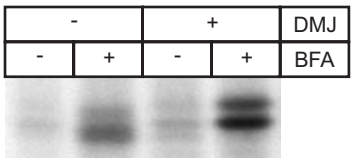
Fig S2: *Pro-catZ release in GM cells occurs prior to Golgi mannosidase I cleavage.*
GM cells treated with or without 1-deoxymannojirimycin (DMJ) and BFA were pulsed for 3 h with ^{35}S-methionine, crosslinked with DSP and ERGIC-53-associated pro-catZ isolated by anti-ERGIC-53 immunoprecipitation (fluorogram). Note that 1-deoxymannojirimycin-treatment prevents the BFA-induced mobility shift and that in untreated cells pro-catZ dissociates from ERGIC-53 before being trimmed by Golgi mannosidase I.

Fig S3: *The distinct binding intensities of HA-pro-catZ glycosylation-site mutants to ERGIC-53 are not a result of sterical hindrance by the HA-epitope.*
Crosslinking and co-immunoprecipitation of GMAA-ERGIC-53 and alternatively tagged pro-catZ glycosylation-site mutants (see Experimental Procedures). Shown are pro-catZ bands visualized by anti-HA immunoblotting. Note that DSP-dependent co-isolation is decreased by N184Q, but favoured by N224Q. H, homogenate (1% input); IP, anti-ERGIC-53 immunoprecipitation; asterisk, sample loss.

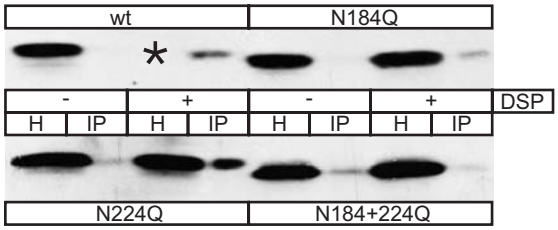
Fig S4: *Δloop, but not Δloop*, affects ERGIC-53-association of pro-catZ.*
Crosslinking and co-immunoprecipitation experiment as in Fig. S3 after transfection of wt-, Δloop*- or Δloop-pro-catZ cDNA. While Δloop* is indistinguishable from wt, Δloop shows a stronger steady-state signal (H) but still weaker binding to ERGIC-53 (IP + DSP). H, homogenate (1% input); IP, anti-ERGIC-53 immunoprecipitation.



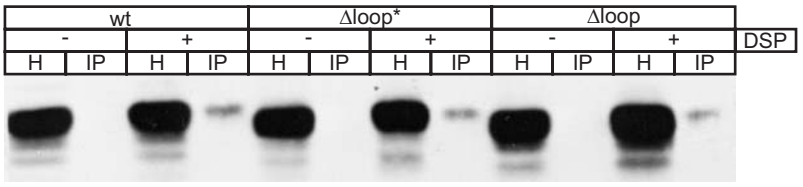
Appenzeller-Herzog et al. Figure S1



Appenzeller-Herzog et al. Figure S2



Appenzeller-Herzog et al. Figure S3



Appenzeller-Herzog et al. Figure S4

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 13, Issue of March 26, pp. 12943–12950, 2004
Printed in U.S.A.

pH-induced Conversion of the Transport Lectin ERGIC-53 Triggers Glycoprotein Release*

Received for publication, December 4, 2003, and in revised form, January 8, 2004
Published, JBC Papers in Press, January 12, 2004, DOI 10.1074/jbc.M313245200

Christian Appenzeller-Herzog‡, Annie-Claude Roche§¶, Oliver Nufer‡, and Hans-Peter Hauri‡‖

From the ‡Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland and §Glycobiologie, Centre de Biophysique Moleculaire, CNRS, F-45071 Orléans, France

The recycling mannose lectin ERGIC-53 operates as a transport receptor by mediating efficient endoplasmic reticulum (ER) export of some secretory glycoproteins. Binding of cargo to ERGIC-53 in the ER requires Ca^{2+}. Cargo release occurs in the ERGIC, but the molecular mechanism is unknown. Here we report efficient binding of purified ERGIC-53 to immobilized mannose at pH 7.4, the pH of the ER, but not at slightly lower pH. pH sensitivity of the lectin was more prominent when Ca^{2+} concentrations were low. A conserved histidine in the center of the carbohydrate recognition domain was required for lectin activity suggesting it may serve as a molecular pH/Ca^{2+} sensor. Acidification of cells inhibited the association of ERGIC-53 with the known cargo cathepsin Z-related protein and dissociation of this glycoprotein in the ERGIC was impaired by organelle neutralization that did not impair the transport of a control protein. The results elucidate the molecular mechanism underlying reversible lectin/cargo interaction and establish the ERGIC as the earliest low pH site of the secretory pathway.

After translocation into the ER,[1] soluble secretory proteins, here termed cargo proteins, start their journey along the organelles of the secretory pathway. This process requires correct folding and ongoing sorting from resident proteins of the compartments through which they move. The first such separation occurs during transport from the ER to the Golgi apparatus. The molecular nature of this sorting event has been extensively studied and led to two models that may coexist and each apply for a distinct subset of cargo proteins (1). According to the bulk-flow model, ER-exit occurs by default and requires no transport signals. Retention signals would be required for keeping ER resident proteins in the ER and retrieval signals would salvage those few that inadvertently escape. Conversely, the receptor-mediated model positions the targeting information on the cargo proteins themselves that would carry positive sorting signals for ER exit. These signals are recognized by membrane spanning transport receptors that couple the cargo proteins to the cytosolic vesicle budding machinery and cycle between the ER and post-ER compartments. Several transport receptors have been identified in the past few years, including ERGIC-53 (2), Emp24p (3), and Erv29p (4), each acting as an ER export receptor for a subset of cargo proteins.

The type I transmembrane protein ERGIC-53 is ubiquitously expressed and constitutively cycles between ER and ER-Golgi intermediate compartment (ERGIC) (5). In the ERGIC the protein segregates from anterograde-directed protein traffic and returns to the ER largely bypassing the Golgi apparatus (6). ERGIC-53 is a lectin. In its luminal part it carries a carbohydrate recognition domain (CRD) with a β-sandwich-fold (7) that shares significant sequence similarity and many structural details with the carbohydrate binding sites of plant L-type lectins (8–10). It preferentially binds to D-mannose (11) and recognizes protein-linked high mannose-type oligosaccharides *in vivo* (2). The lack of functional ERGIC-53 leads to inefficient secretion of the glycoproteins pro-cathepsin C (12) and blood coagulation factors V and VIII (13).

Cross-linking studies have established a cathepsin Z-related protein (catZr) as a model glycoprotein cargo for ERGIC-53 and documented that cargo capture starts in the ER and cargo release occurs in the ERGIC (2). Although such a differential binding of the transport receptor to its cargo is fundamental, the molecular nature of this process has remained elusive. Based on the finding that lectin activity of ERGIC-53 strictly depends on Ca^{2+} (2, 11), we have speculated earlier that a drop of calcium levels along the ER to ERGIC pathway may trigger glycoprotein dissociation (5). Indeed, an imaging approach that measures *total* Ca^{2+} in ultrathin cryosections revealed positive signals for both ER and Golgi, but ERGIC elements remained below detection levels (14). Nevertheless, calcium deprivation as the sole determinant for cargo release appears unlikely, as the concentration gradient of *free* Ca^{2+} from ER to ERGIC may be subtle. Apart from that, a pH-driven sorting mechanism, in analogy to the endosomal system, has been suggested based on *in vitro* studies (15, 16). Organellar pH is determined by the presence of active H^+ v-ATPase pumps, a 10^6-kDa complex consisting of 13 polypeptides, and of opposed H^+ leak rates (17). Whereas the progressive acidification from the ER (pH 7.1–7.4) to the trans-Golgi network (pH 5.9–6.3) has been established, it is still largely questioned, however, if significant proton pumping and organelle acidification occurs already in the early secretory pathway, *i.e.* the ERGIC/cis-Golgi region (for review see Ref. 18). The evidence is rather sketchy and includes the observations that the v-ATPase proteolipid subunit co-fractionates with ERGIC membranes, the v-ATPase inhibitor bafilomycin A1 produces a cell-type specific Golgi to ER retrograde transport defect, and there is minor overlap of DAMP staining with ERGIC-53 (19, 20). The

* This work was supported by the Swiss National Science Foundation and the University of Basel. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Research Director of Inserm.
‖ To whom correspondence should be addressed. Tel.: 41-61-267-2222; Fax: 41-61-267-2208; E-mail: Hans-Peter.Hauri@unibas.ch.

[1] The abbreviations used are: ER, endoplasmic reticulum; catZr, cathepsin Z-related protein; CRD, carbohydrate recognition domain; DSP, dithiobis(succinimidylpropionate); endo D, endoglycosidase D; endo H, endoglycosidase H; ERGIC, endoplasmic reticulum-Golgi intermediate compartment; HIS, histidine ion sensor; mAb, monoclonal antibody; CHO, Chinese hamster ovary; MES, 4-morpholineethanesulfonic acid.

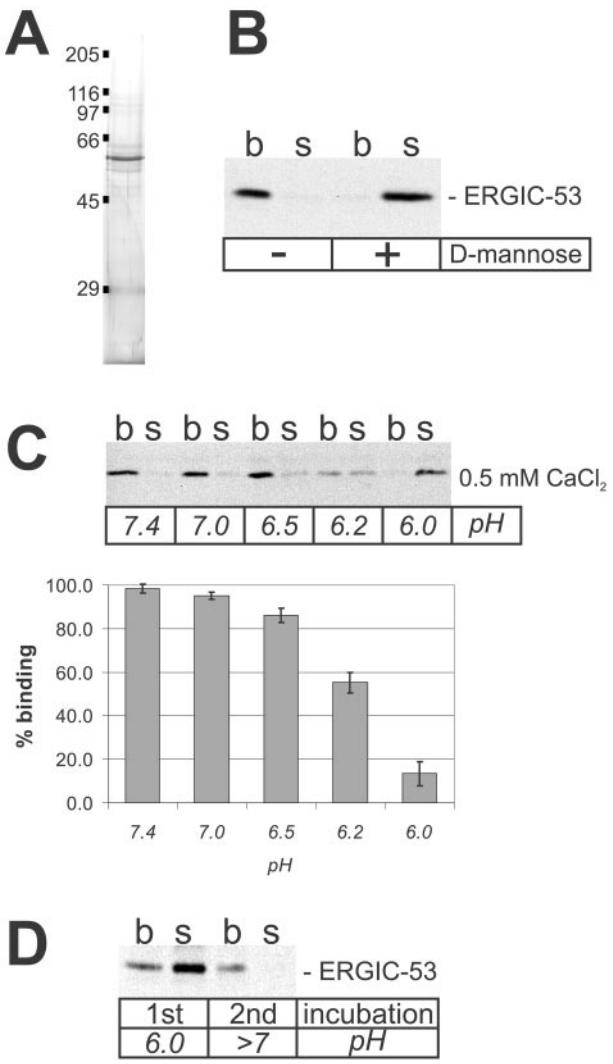


FIG. 1. **Binding of purified ERGIC-53 to D-mannose is acid sensitive.** *A*, recombinant, myc/His_6-tagged ERGIC-53 was expressed in COS-1 cells and purified on a Ni^{2+} resin. Purified ERGIC-53 forms disulfide-linked dimers and hexamers (Ref. 11 and data not shown). Under reducing conditions, it shows a single band of 53 kDa on a silver-stained gel. *B*, 50 ng of ERGIC-53 was incubated with D-mannose Affi-Gel beads in the presence of 0.5 mM $CaCl_2$. Soluble (*s*) and bound protein (*b*) was visualized by immunoblotting using anti-myc. Note that lectin binding can be competed with free D-mannose. *C*, ERGIC-53 was bound to mannose beads in buffers with gradually decreased pH (mean ± S.D., n = 3). *D*, the supernatant of a binding assay at pH 6.0 (0.5 mM $CaCl_2$) was split and one-half was neutralized with 30 mM Tris buffer and reincubated with mannose beads. B- and S-fractions of the first and second incubation are shown. Note that neutralization can reactivate ERGIC-53.

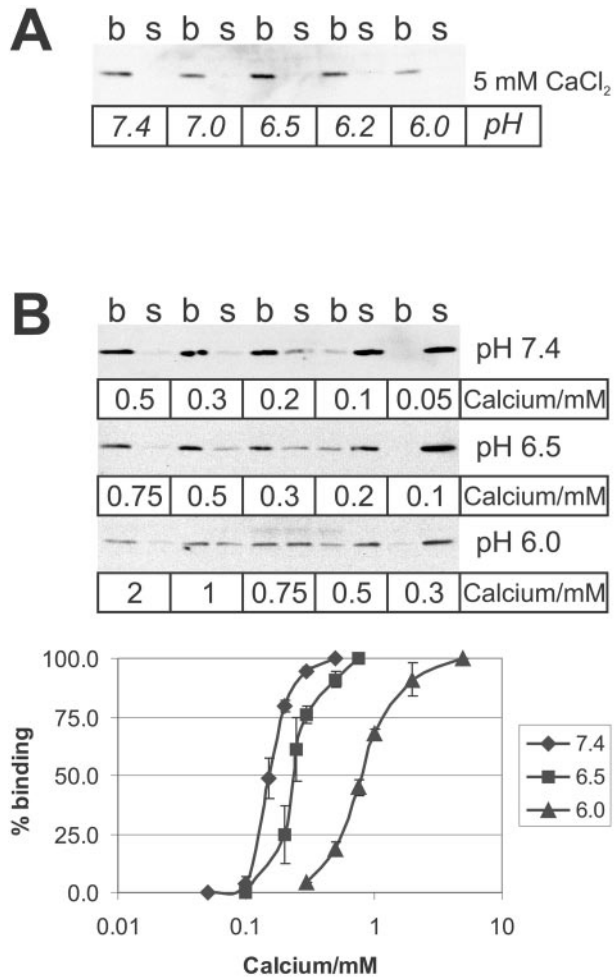


FIG. 2. **Acid sensitivity of ERGIC-53 is modulated by Ca^{2+}.** *A*, binding of ERGIC-53 as described in the legend to Fig. 1C, but in the presence of 5 mM $CaCl_2$. Under these conditions lectin activity is clearly restored at low pH. *B*, mannose binding at pH 7.4, 6.5, or 6.0 in the presence of different concentrations of $CaCl_2$. A typical experiment and the data of three independent experiments are shown (mean ± S.D., $n = 3$).

notion of pre-Golgi acidification is far from being established and requires further evidence to be proven.

By studying the lectin properties of ERGIC-53 in more detail, we report here that low pH modulates the activity of ERGIC-53 *in vitro* and *in vivo*. The data suggest a molecular scenario underlying reversible lectin inactivation that involves protonation of a conserved histidine sensor residue and loss of Ca^{2+}. In conjunction with our previous studies, the results establish the ERGIC as the earliest acid compartment of the secretory pathway.

EXPERIMENTAL PROCEDURES

Reagents—The following antibodies were used: mAb 9E10.2 against a c-myc epitope (ATCC CRL 1729), mAb G1/93 against human ERGIC-53 (21), and polyclonal antibody against horse fibronectin (kindly provided by Matthias Chiquet, University of Bern). A rabbit antiserum against catZr was obtained by keyhole limpet hemocyanin-coupled immunization of the following peptide sequences: CMADRINIKRKGAWPS and CKHGIPDETCNNYQA. 4-Isothiocyanatophenyl α-D-mannopyranoside and Affi-102 beads (Bio-Rad) were used to prepare immobilized D-mannose (22). D-Mannose and chloroquine were from Sigma, endoglycosidase D (endo D) and monensin from Calbiochem, and endoglycosidase H (endo H) from Roche Diagnostics. Nigericin was kindly provided by Jean Pieters (Biozentrum, Basel).

Recombinant DNAs—myc/His_6 (11) was mutated using the QuikChange method (Stratagene) and the following primers (coding strand): D152S, 5′-CTGTGGAATGGTGTTGGAATATTTTTTTCTTCTTTTGACAATGATGGAAAG-3′; H175Q, 5′-GGACAAATCCAGTATGACCATCAAAATGACGGGG-3′; H178Q, 5′-GGACAAATCCATTATGACCAGCAAAATGACGGGG-3′.

Cell Culture, Transfections, Drug Treatment, and in Situ Cross-linking—COS-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 1 μg ml^{-1} amphotericin B (Sigma). Cells were transfected using the DEAE-dextran method (23) (10 μg of DNA/10-cm dish). CHO-KI cells and Lec1-derived stable clones (GM or GMAA (24)) were grown in α-minimal essential medium supplemented with 10% fetal bovine serum, 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 1 μg ml^{-1} amphotericin B. Chloroquine (100 μM) was added 1 h before metabolic labeling and was present throughout the pulse-chase period. For pH-clamp experiments, cells were incubated for 15 min at 37 °C in 10 mM HEPES, 10 mM MES (pH 6.0, 6.5, 7.0), 60 mM NaCl, 60 mM KCl, 1.5 mM K_2HPO_4, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM methionine, and 10 μM each of nigericin and monensin (adapted from Ref. 25). AlF_4^- was used at the following final concentra-

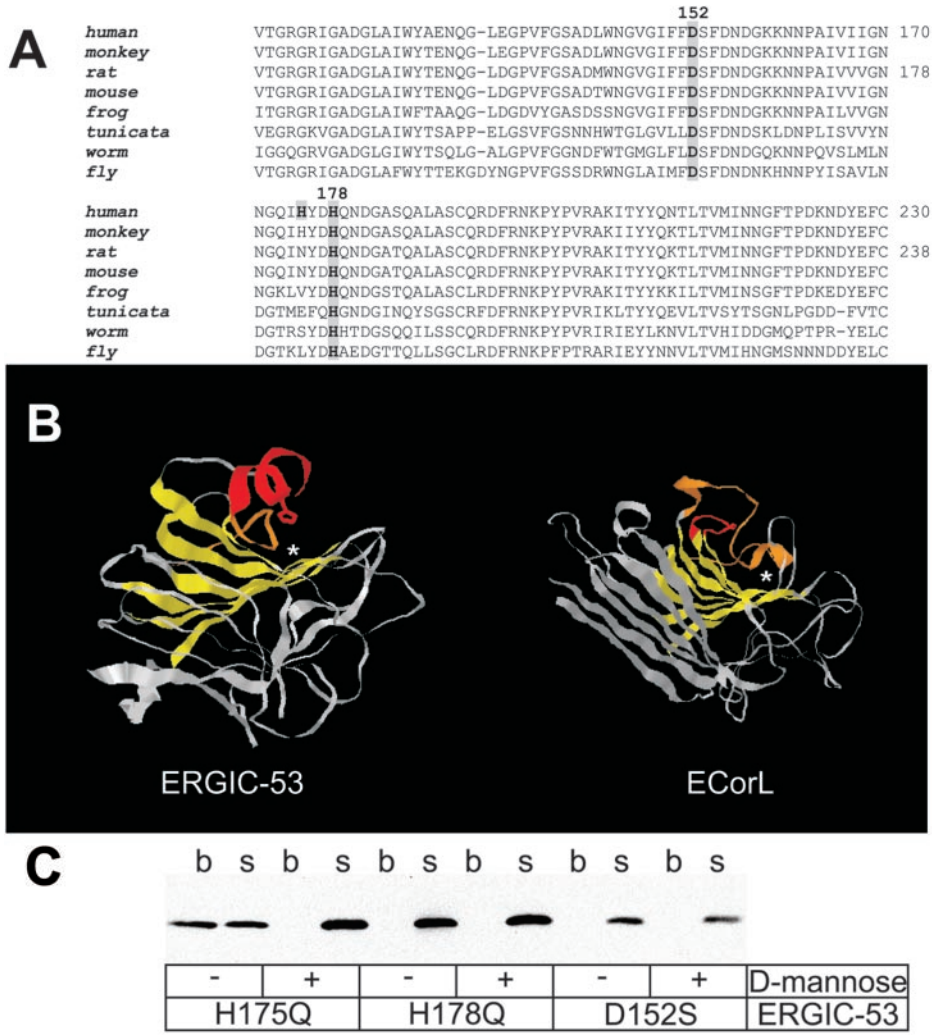


FIG. 3. **Histidine 178 is conserved and essential for lectin activity.** *A*, sequence alignment of the CRDs of ERGIC-53 orthologs (ClustalW 1.82, using default settings). Amino acid numbering is shown for the human and rat sequence. These sequence data are available from GenBank™/EMBL/DDBJ under accession numbers P49257 (*human*), Q9TU32 (*monkey*), Q62902 (*rat*), Q9D0F3 (*mouse*), Q91671 (*frog*), Q9GR90 (*tunicata*), P90913 (*worm*), and Q9V3A8 (*fly*). *B*, structural comparison of animal and plant L-type lectins. Shown are the closely related β-sandwich folds of the CRD of rat ERGIC-53 (7) and *E. corallodendron* lectin (*ECorL* (37)). The inner concave β-sheet oriented toward the sugar binding pocket (*asterisk*) is colored in *yellow*. The imidazole ring of His-178 in the mannose binding pocket is shown in *red*. Note that the α-helical loop comprising His-178 is replaced by a shorter loop in ECorL (*red* colored backbone), and that this space instead is filled by an extended amino acid insertion relative to the ERGIC-53 structure (*orange* colored backbone). Figures were designed using RasMol V2.5. *C*, ERGIC-53 (myc/His_6) variants carrying the H175Q, H178Q, or D152S mutation were purified and probed for *in vitro* lectin activity in the presence of 5 mM $CaCl_2$. Note that only ERGIC-53(H175Q) specifically binds to mannose beads.

tions: 30 mM NaF and 50 μM $AlCl_3$. *In situ* cross-linking with dithiobis(succinimidylpropionate) (DSP) (Pierce) was performed on intact cells (2).

Mannose Binding Assay—myc/His_6-ERGIC-53 was purified from transiently transfected COS-1 cells solubilized in 10 mM Tris (pH 7.4), 150 mM NaCl, 20 mM imidazole, 1% Triton X-100. Cleared lysate (100,000 × *g*, 1 h) from 10 10-cm dishes was incubated for 1 h at room temperature with 100 μl of Ni^{2+}-nitrilotriacetic acid-agarose (Qiagen) under constant agitation. The beads were washed in the same buffer. Bound protein was eluted in 10 mM Tris (pH 7.4), 150 mM NaCl, 0.4 M imidazole, 0.04% Triton X-100 and dialyzed against the same buffer lacking imidazole. Purified ERGIC-53 was shock frozen and stored in aliquots at −80 °C. Mannose binding was performed for 4 h at 4 °C in 10 mM HEPES, 10 mM MES (pH 7.4) (or as indicated; pH was set at 4 °C), 150 mM NaCl, 0.5 mM $CaCl_2$ (or as indicated), 0.04% Triton X-100 in a volume of 100 μl using 50 ng of protein and 20 μl (dry volume) of D-mannose beads. After removing the supernatant, beads were washed twice with binding buffer and bound protein was eluted in 100 μl of Tris (pH 7.4), 150 mM NaCl, 10 mM EDTA, 0.04% Triton X-100 for 5 min at 4 °C. B- and s-fractions were analyzed by Western blotting using anti-myc and the ECL detection system (Amersham Biosciences) and quantified with a ChemImager™ device and AlphaEase™ software (Alpha Inotech Corporation).

Pulse-Chase Experiments, Immunoprecipitation, and Endoglycosidase Digest—Cells were labeled with [^{35}S]methionine, chased in α-minimal essential medium containing 10 mM unlabeled methionine, and subjected to immunoprecipitation (26) using mAb G1/93 or polyclonal antibody against fibronectin and protein A-Sepharose (Amersham Biosciences). Immunoprecipitation of intracellular catZr was performed after antigen denaturation at 95 °C in 30 mM triethanoamine/HCl (pH 8.1), 100 mM NaCl, 5 mM EDTA containing 1.6% SDS followed by addition of an excess of Triton X-100 (2% final concentration) in the same buffer. Secreted catZr was methanol-chloroform precipitated (27) and then subjected to the same procedure. Where indicated, cells were treated with DSP prior to solubilization, or immunoprecipitates were digested with endo D or endo H as previously described (24). Gels were analyzed and quantified on a STORM 820 PhosphorImager (Amersham Biosciences) or by fluorography.

Immunofluorescence Microscopy—GM-Lec1 cells were cultured in 8-well multichamber Permanox slides (Milian, Plan-les-Ouates, Switzerland), fixed with 3% paraformaldehyde, permeabilized with phosphate-buffered saline, 0.1% saponin, and processed for indirect immunofluorescence using mAb G1/93 and GAM IgG(H+L)-Alexa Fluor 488 (Molecular Probes, Leiden, Netherlands). Confocal laser scanning images were acquired on a Leica microscope (TCS NT) with a 63× objective (NA 1.32).

RESULTS

Lectin Activity of ERGIC-53 Is Modulated by pH and Ca^{2+}—To gain insight into the molecular mechanism of the ERGIC-53/cargo interaction, we designed an *in vitro* lectin assay. Full-length oligomeric ERGIC-53 carrying a NH_2-terminal myc epitope and a His_6 tag at the C terminus (myc/His_6 (11)) was purified from COS-1 cells (Fig. 1*A*) and incubated with immobilized D-mannose in the presence of 0.5 mM $CaCl_2$. Bound and unbound protein was visualized by immunoblotting using anti-myc. Binding of 50 ng of protein was complete, required Ca^{2+}, and could be competed with 0.2 M free D-mannose (Fig. 1*B*), indicating specificity.

This assay was used to further study the lectin properties of ERGIC-53. First, we tested the possibility that mannose binding was sensitive to acid. To this end, binding experiments were performed in buffers of pH 7.4, corresponding to the pH of

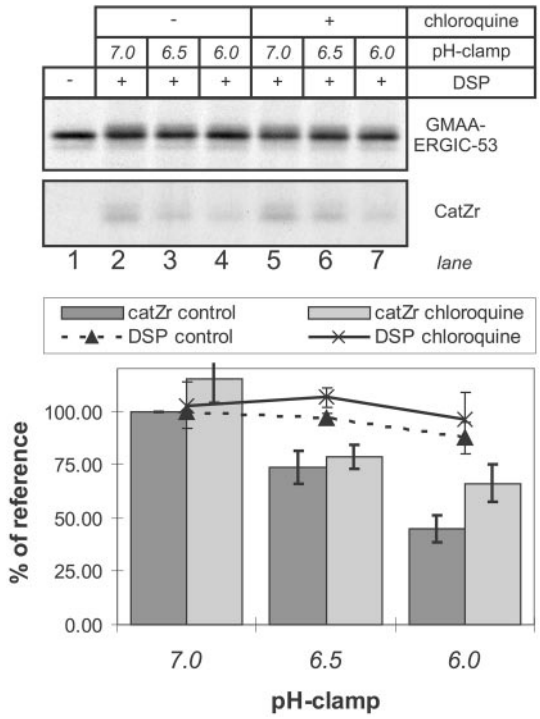


FIG. 4. **Intracellular binding of ERGIC-53 to a glycoprotein substrate is impaired by acidification of cells.** *Upper panel*, Lec1 cells stably expressing GMAA-ERGIC-53 were labeled with [^{35}S]methionine for 2 h, pH clamped as indicated, cross-linked by DSP in the presence or absence of 0.2 mM chloroquine, and subjected to immunoprecipitation with anti-ERGIC-53. Two different exposures of the same gel are shown for GMAA-ERGIC-53 and catZr. *Lower panel*, quantification of cross-linked catZr (*bars*) and cross-linking efficiency (*lines*), i.e. DSP-"cap" on ERGIC-53 bands both normalized against total ERGIC-53 (mean ± S.D., $n = 3$). Levels of control cells with pH clamp 7.0 were set to 100%. The decrease of catZr signals is statistically significant, variations in cross-linking efficiency are not ($p < 0.05$, Student's t test).

the ER (18), down to pH 6.0. Fig. 1*C* shows that the fraction of active ERGIC-53 was gradually reduced to 13 ± 6% at pH 6.0. Inactivation by pH 6.0 treatment could be readily reversed by neutralization (Fig. 1*D*). Interestingly, the sensitivity of the lectin to low pH was linked to Ca^{2+} as it was completely suppressed if [Ca^{2+}] was raised to 5 mM (Fig. 2*A*). In a next set of experiments the binding of ERGIC-53 was assayed at constant pH over a range of [Ca^{2+}] (Fig. 2*B*). Half-maximal binding was observed in the presence of 150 μM Ca^{2+} at pH 7.4, 230 μM Ca^{2+} at pH 6.5, and 800 μM Ca^{2+} at pH 6.0. We conclude that pH sensitivity of ERGIC-53 is at least in part because of pH-induced changes in Ca^{2+} affinity raising the possibility that pH sensing and Ca^{2+} complexation may be mediated by the same amino acid(s).

Histidine 178 Has the Hallmarks of a Molecular pH Sensor—In search of the molecular mechanism underlying acid sensitivity we scanned the CRD of ERGIC-53 for potentially titratable residues. Histidine is the only basic amino acid that can be considerably protonated in moderately acidic solutions as used above. His-178 is both conserved in ERGIC-53 orthologs and positioned in a characteristic α-helix in the active site of ERGIC-53 (Fig. 3, *A* and *B* (7)). To mimic and freeze the unprotonated state, His-178 was mutated to glutamine and the mutant lectin purified. According to our expectations, H178Q would abrogate or at least reduce pH sensitivity of mannose binding. However, we found ERGIC-53(H178Q) to be inactive (Fig. 3*C*). In contrast, mutating the non-conserved His-175 only minimally affected the binding. Taken together with the central position in the CRD, the fact that a neutral mutation of His-178 inactivates lectin function indicates that this residue may be directly involved in mannose binding or, more likely, Ca^{2+} complexation (see Fig. 2*B*). To further support this idea, we probed the mannose binding of a Ca^{2+}-binding mutant (D152S) that was designed on the basis of the close homology of ERGIC-53 and plant lectins (8, 28). As expected, D152S abrogated lectin activity comparable with H178Q (Fig. 3*C*), consistent with a role of His-178 in Ca^{2+} binding. These findings support the notion that His-178 serves as a molecular pH sensor of ERGIC-53. We call the α-helical loop comprising His-178 the histidine ion sensor (HIS)-loop.

ERGIC-53/Glycoprotein Association Is Sensitive to Acidic pH—Does the binding of ERGIC-53 to a known glycoprotein ligand also depend on neutral pH? We studied intracellular binding of catZr to ERGIC-53 by *in situ* cross-linking (2) in cells that had been subjected to a pH-clamp procedure. pH clamping adapts all cellular compartments to the pH of the applied buffer, irrespective of organelle-specific ion concentrations and permeabilities (25). Because this procedure perturbs transport along the biosynthetic pathway (18),[2] we made use of GMAA cells that stably express a transport-impaired ERGIC-53 mutant that is restricted to the ER (24), but still efficiently cross-links to catZr by DSP (2). This cross-linker covalently links the two proteins and after reductive cleavage slightly increases their apparent molecular mass. We chose to assess the effect of pH 6.5 and 6.0 treatment in relation to neutral pH taking into account pK_a(His) of ~6.5. Because chemical coupling of amino groups by DSP works poorly below pH 7, the cells were cross-linked at pH 7.4 after metabolic labeling with [^{35}S]methionine and pH clamping. ERGIC-53 immunoprecipitates were then analyzed by SDS-PAGE and phosphorimaging. Cross-linking efficiency, as determined by quantification of the DSP-induced gel-mobility shift of GMAA-ERGIC-53 (Fig. 4, compare *lane 1* to other lanes), only minimally changed upon acid treatment indicating that incubation at neutral pH after the pH clamp allowed the cells to re-establish neutral ER pH (Fig. 4, *lanes 2–4*, *GMAA-ERGIC-53*). To confirm this, neutralization during cross-linking was imposed by inclusion of the lysosomotropic agent chloroquine that is known to raise intraorganellar pH within seconds (18). As shown in the *lower panel* of Fig. 4 (*DSP control/chloroquine*), the variations in cross-linking efficiency with or without addition of chloroquine remained statistically insignificant. The amount of the catZr-doublet (2) cross-linked to GMAA-ERGIC-53 on the other hand was significantly reduced with decreasing pH (Fig. 4, *lanes 2–4*, *catZr*). During the cross-linking procedure at neutral pH some reassociation may occur, but this process seems to be slow at 4 °C in the viscous environment of the ER lumen. Consistent with this, binding of catZr to ERGIC-53 after acidic pH clamping was only partially restored if cross-linking was performed in the presence of chloroquine (Fig. 4, *lanes 5–7*, *catZr*). Because pH-induced misfolding of catZr, a predicted lysosomal enzyme, is unlikely, we conclude that an artificially applied acidic pH in the lumen of the ER drives dissociation of catZr from ERGIC-53.

Neutralization of the ERGIC Inhibits Cargo Release—We wondered if pH-driven glycoprotein dissociation from ERGIC-53 also occurs in living cells without acidic manipulation. Our previous studies have localized the site of catZr cargo release from ERGIC-53 to the ERGIC (2). If indeed an endogenous acidification mechanism of the ERGIC is the driving force for the dissociation, we expect that neutralization of the intra-organellar pH would delay this process. We chose to use the dibasic compound chloroquine that elevates the pH within acidic organelles by a weak base mechanism at micromolar concentrations (minimizing osmotic swelling) and by the lack of

[2] C. Appenzeller-Herzog and H.-P. Hauri, unpublished observations.

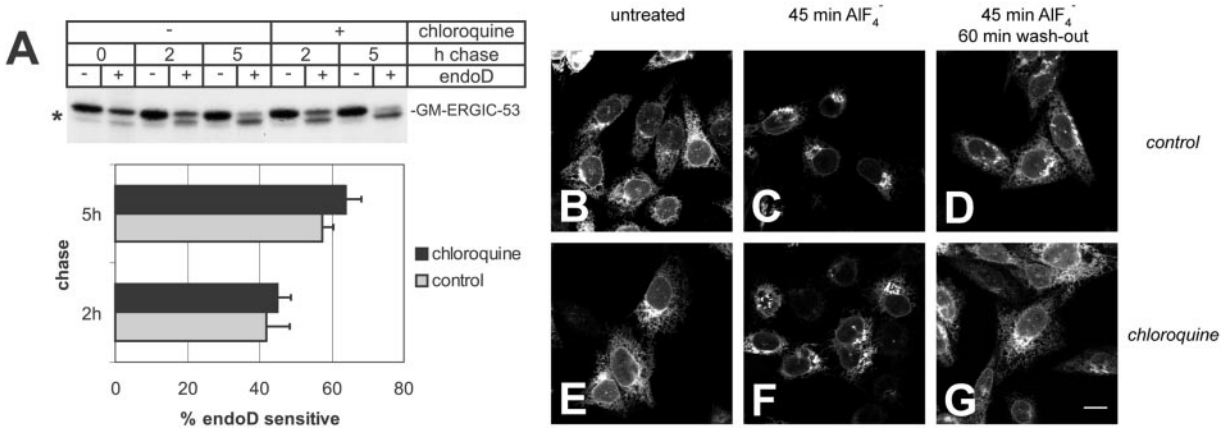


FIG. 5. **ERGIC-53 recycling is not impaired by chloroquine treatment.** *A*, GM-Lec1 cells were pulsed for 20 min with [^{35}S]methionine and chased as indicated. Immunoprecipitated ERGIC-53 was probed for endo D sensitivity and analyzed on 7–10% gradient gels. Where indicated, chloroquine was added to the culture medium. *Asterisk*, endogenous non-glycosylated ERGIC-53. Control and chloroquine kinetics are statistically indistinguishable ($n = 3$, $p > 0.1$, Student's t test). *B–G*, immunofluorescence localization of ERGIC-53 in GM-Lec1 cells treated with AlF_4^- as indicated. For organelle neutralization, chloroquine was added 1 h before AlF_4^- and was present until fixation. Note that AlF_4^- reversibly concentrates ERGIC-53 to the Golgi area irrespective of the presence or absence of chloroquine. *Bar*, 10 μm.

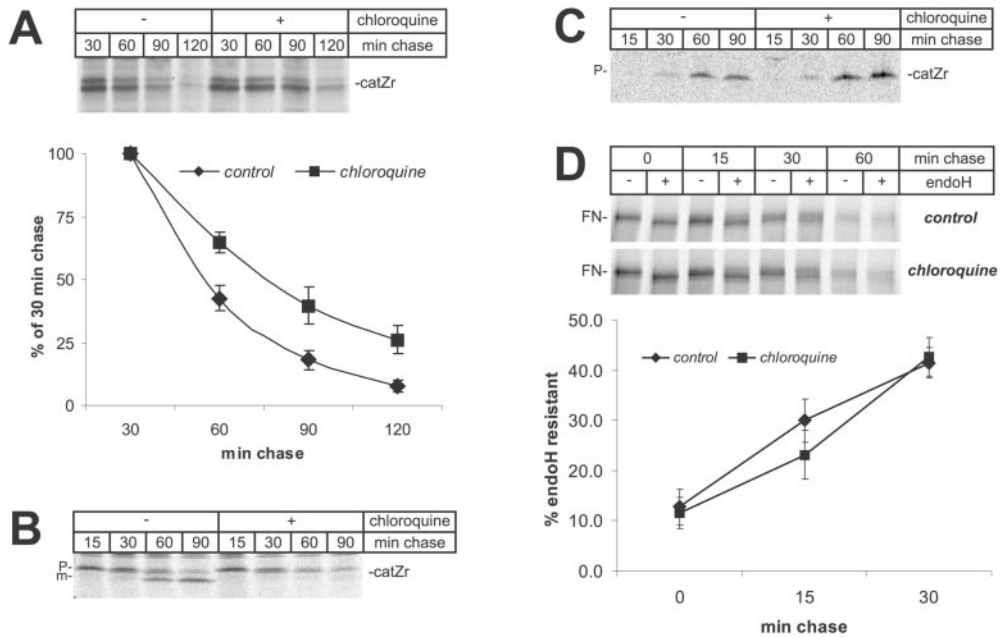


FIG. 6. **Dissociation of glycoprotein cargo from ERGIC-53 is delayed upon organelle neutralization.** *A*, pulse-chase analysis of catZr release. GM cells were pulsed for 10 min and chased as indicated with or without addition of chloroquine. After DSP treatment, ERGIC-53 was immunoprecipitated and co-precipitated catZr was analyzed by phosphorimaging. The diagram shows the decrease of catZr signals normalized against ERGIC-53 and expressed as percentage of the value after 30 min of chase (mean ± S.D., n = 3; Student's t test for *chloroquine* versus *control* at time points 60, 90, and 120: $p < 0.02$, $p < 0.01$, and $p < 0.02$). *B*, maturation of catZr in the presence or absence of chloroquine as seen with a pulse (15 min)-chase experiment. *P*, proform; *m*, mature form. *C*, secretion time course of pulse-labeled (15 min) catZr from control- and chloroquine-treated GM cells. *D*, CHO-KI cells were metabolically labeled for 20 min and chased for the indicated times. Fibronectin (*FN*) immunoprecipitates were treated with or without endo H and resolved on a 4–10% gradient gel. Quantification of three independent experiments is shown (mean ± S.D.).

primary amino groups does not interfere with DSP cross-linking (see above).

To verify that such organelle neutralization does not generally affect membrane traffic in the early secretory pathway, which would lead to misinterpretations, we assayed the recycling of ERGIC-53 by two different methods. First, pulse-chase experiments were performed in the presence or absence of 100 μM chloroquine using CHO-KI-derived GM-Lec1 cells (24), and immunoprecipitated ERGIC-53 was probed for sensitivity to endo D. Lec1 cells lack the Golgi enzyme *N*-acetylglucosamine transferase I, and as a consequence *N*-glycosylated proteins, upon passage through the cis-Golgi, become and remain endo D-sensitive by the action of Golgi mannosidase I. As the major recycling route of ERGIC-53 largely bypasses the cis-Golgi (6), the acquisition of endo D sensitivity is a slow process (24) and an exact measure of ERGIC-53 trafficking. Fig. 5*A* shows that chloroquine treatment does not slow down the recycling of glycosylated ERGIC-53. As a second read-out for recycling, we studied the localization of ERGIC-53 by immunofluorescence microscopy in GM-Lec1 cells that had been treated with AlF_4^-. This drug accumulates recycling proteins in the ERGIC in a reversible manner (24). Very much like in control cells, in cells pretreated with chloroquine, ERGIC-53 was concentrated in a juxtanuclear area and the reticular ER fluorescence diminished after AlF_4^- treatment (Fig. 5, *C* and *F*). After AlF_4^- washout, these effects were reversed in both neutralized and control

cells (Fig. 5, *D* and *G*). These results suggest that recycling kinetics of ERGIC-53 as well as morphology of the ER-Golgi interface remain undisturbed in the presence of chloroquine.

Therefore, we used chloroquine to study the role of luminal pH in the kinetics of catZr release from ERGIC-53. It has been shown that cargo binding by ERGIC-53 persists as far as to the ERGIC, where dissociation takes place (2). To measure the rate of catZr dissociation, GM-Lec1 cells were pulse-labeled with [^{35}S]methionine and chased for the indicated times with or without addition of chloroquine. After cross-linking the amount of catZr co-immunoprecipitated with anti-ERGIC-53 was analyzed. Chloroquine indeed delayed the release of catZr (Fig. 6*A*). We reasoned that this observation could reflect: (i) enhanced cross-linking in the presence of chloroquine; (ii) an unspecific block in secretion that would slow down transport of catZr at the level of ER exit, possibly followed by its degradation; or (iii) impaired dissociation from ERGIC-53 in the ERGIC. An effect of chloroquine on the efficiency of DSP treatment can be excluded (Fig. 4 and *30 min chase* in Fig. 6*A*). To test the second possibility we performed a pulse-chase analysis in GM-Lec1 cells with an antiserum that specifically recognizes catZr.[2] As shown in Fig. 6*B*, in untreated cells, catZr gradually converted from the proform to the mature enzyme indicating its targeting to the lysosomal pathway. In contrast, upon treatment with chloroquine, catZr remained unprocessed and the proform persisted slightly longer, which is consistent with its slower transit through the early secretory pathway. Furthermore, we analyzed the appearance of the metabolically labeled, unprocessed catZr-proenzyme in the culture medium from chloroquine-treated or control cells. Regardless of neutralization the cells secreted catZr (Fig. 6*C*). In chloroquine-treated cells the inhibition of intracellular maturation (Fig. 6*B*), which may arise from the impaired function of acid hydrolases and/or from transport defects, caused hypersecretion of catZr, a phenomenon known for lysosomal proteins. This renders it impossible to quantitatively compare the rate of secretion with or without neutralization and most likely accounts for the seeming paradox of retention in the ERGIC *versus* enhanced secretion of catZr. Collectively, these findings demonstrate that chloroquine neither initiates the degradation nor blocks the secretion of catZr, but causes some setback in its early transport through the secretory pathway. To discriminate whether under these conditions secretory transport is generally affected or if this delay is specific for catZr, we sought to measure the transport of an independent secretory marker. To this end, we examined glycosylation modifications on fibronectin, a ubiquitous component of the extracellular matrix. Because we failed to detect the accumulation of endo D-sensitive fibronectin in GM-Lec1 cells (data not shown), we used endo H and wt CHO-KI cells. Contrary to Lec1 cells, fibronectin secreted from this cell line was resistant to endo H as expected (data not shown). To study ER to medial-Golgi transport of fibronectin we performed pulse-chase/endo H experiments and assessed the acquisition of endo H resistance. Fig. 6*D* shows that intracellular fibronectin was readily lost with or without chloroquine and that this drug did not slow down fibronectin trafficking from the ER to the medial-Golgi. We conclude that chloroquine treatment specifically retards the dissociation of catZr from ERGIC-53 indicating that pH-induced conversion of the lectin domain of ERGIC-53 in the ERGIC contributes to efficient release of its glycoprotein cargo.

DISCUSSION

In this study we have uncovered the molecular mechanism of reversible lectin binding of ERGIC-53 to its substrates. Subtle pH changes inactivate and reactivate the CRD of ERGIC-53 *in vitro* and *in vivo*. We propose a model for cargo release by pH-induced loss of Ca^{2+} (Fig. 7). Ca^{2+}-complexed ERGIC-53

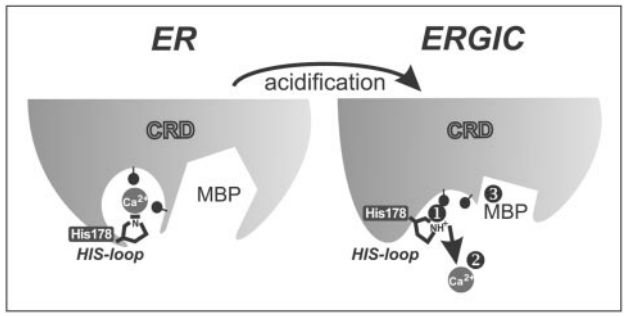


FIG. 7. **Model for cargo release by pH-induced loss of Ca^{2+}.** Upon arrival in the ERGIC, His-178 in the HIS-loop of ERGIC-53 is protonated because of lowered luminal pH (*1*) leading to the loss of one (or more) Ca^{2+} (*2*). Loss of Ca^{2+} as a cofactor inactivates the mannose binding pocket (*3*) and triggers cargo release. *MBP*, mannose binding pocket.

binds cargo in the ER at neutral pH. Upon arrival in the ERGIC, His-178 is protonated because of lowered luminal pH and Ca^{2+} is lost. Because Ca^{2+} is required for the lectin activity of ERGIC-53 (Refs. 2 and 11, and this study), its loss leads to the inactivation of the mannose binding pocket resulting in the release of glycoprotein cargo. Subsequently, the transport receptor is recycled back to the ER, where, with a deprotonated and reactivated CRD, it can start a new round of cargo capture. Interestingly, a similar mechanism has been reported for C-type lectins, such as asialoglycoprotein receptor, and their glycoprotein cargo upon endocytosis from the plasma membrane (29, 30). In that case, however, the acidification ranges from extracellular pH 7.3 to endosomal pH 5.4 and, as a consequence, pH sensing by the asialoglycoprotein receptor can be achieved by a cluster of amino acids that includes an aspartic acid and an arginine residue (31). It is important to note that the [Ca^{2+}] range we found to allow pH-sensitive mannose binding of ERGIC-53 (Fig. 2*B*) corresponds well to physiological Ca^{2+} levels in the ER (32). Accordingly, a mechanism that maintains lower levels of *free* Ca^{2+} in the ERGIC would further promote pH-induced loss of Ca^{2+} on ERGIC-53 and thereby contribute to efficient cargo release in a moderately acidic milieu. In quantitative terms, still 89 ± 4% binding would occur in the presence of 0.5 mM Ca^{2+} assuming a pH_{ERGIC} of 6.5. A more prominent decrease in the fraction of active ERGIC-53 at that pH (25 ± 12%), however, would be seen, if $[Ca^{2+}]_{ERGIC}$ came to 0.2 mM (Figs. 1 and 2). Such thinking does not only demonstrate how the potentiation of these two parameters, pH and [Ca^{2+}], can fully describe the molecular process of cargo dissociation, but also provides the theoretical basis for our observation, that elevating solely the pH of the ERGIC leads to a relatively moderate defect (80 ± 2% binding at pH 7.4, 0.2 mM [Ca^{2+}]) in glycoprotein release (Fig. 6*A*). Although high concentrations of *free* Ca^{2+} have been measured within both the ER (~0.4 mM) and the Golgi (~0.3 mM) (32–34), we are still lacking quantitative records of $[Ca^{2+}]_{ERGIC}$. The *in vivo* demonstration of differential Ca^{2+} regulation along the secretory pathway will be one of our challenges in the future.

The presented model implies a molecular link between pH sensing and Ca^{2+} complexation. Indeed, our *in vitro* mannose binding studies indicated a modulation in the affinity of ERGIC-53 to Ca^{2+} ions in a slightly acidified environment (Fig. 2*B*). Furthermore, we identified His-178 to be essential for lectin activity, presumably in its deprotonated state. Glutamine cannot mimic the function of His-178, suggesting that this amino acid may be part of a precise ligand binding pocket. These findings led us to propose that the pH sensor residue His-178, directly or indirectly, is involved in Ca^{2+} binding, and that its protonation would trigger loss of the cation by a repul-

sive interaction (Fig. 7). Whether such a mechanism would involve one or more Ca^{2+} ions is not known. It is worth noting that the closely related lectin Vip36 has been shown to bind two Ca^{2+} ions (35). Co-crystallization of ERGIC-53 with Ca^{2+} and a sugar ligand will help to address this issue and verify our prediction. In the calcium-free crystal structure of ERGIC-53 (7) His-178 is located in an α-helical loop next to the mannose binding site that we termed the HIS-loop. It must be emphasized that in the crystal, notably the central metal/sugar binding loop C (36), was disordered in the absence of Ca^{2+} (7). Hence, the structure presented in Fig. 3*B* might resemble the inactive form of the CRD with protonated His-178 and the conformation of the HIS-loop in the presence of Ca^{2+} may significantly differ. Moreover, we illustrate in Fig. 3*B* that the HIS-loop is characteristic for the animal L-type lectin ERGIC-53 and may functionally replace the B loop (Ref. 36, shown in *orange*) of the plant L-type lectin family (represented here by *Erythrina corallodendron* lectin (37), for alignments see Ref. 28).[3] Consequently, although His-178 is conserved among animal L-type lectins, leguminous lectins as well as yeast homologues of ERGIC-53 lack a central histidine sensor suggesting that these proteins perform functions unrelated to those known for ERGIC-53.

By which mechanism and in which compartment of the secretory pathway v-ATPase starts H^+ pumping and, as a consequence, organelle acidification occurs, is still a matter of debate (18). Herein, we have used the weak base chloroquine that elevates the pH within acidic compartments. This treatment delays the dissociation of catZr from ERGIC-53 indicating that the site of cargo release, the ERGIC, is acidified and serves as a target for neutralizing agents. Importantly, we have found that catZr is not nonspecifically trapped in the ER in chloroquine-treated cells. Additionally, we demonstrated that this drug neither generally affects the secretory transport from the ER to the medial-Golgi nor retards the recycling pathway of ERGIC-53. The latter observation stands in sharp contrast to a study that concluded that pre-Golgi acidification by v-ATPase is required for retrograde transport to the ER (20). Using the v-ATPase inhibitor bafilomycin A1 the authors showed tubulation of the ERGIC, redistribution of β-COP, and some retardation of brefeldin A-stimulated retrograde transport of Golgi mannosidase II that seems to be highly cell type-specific (18). In the light of our quantitative recycling studies in the presence of chloroquine (Fig. 5*A*), however, it appears likely that these observations were not a result of organelle neutralization but rather because of other effects caused by bafilomycin A1 treatment (18). Although the staining pattern of ERGIC-53 showed some minor overlap with acidic compartments that were trapped (and neutralized) with the weak base DAMP, consistent with our chloroquine experiments (Fig. 5*B*), no tubulation phenotype was seen in these cells and the obvious negative control using bafilomycin A1 was omitted (20). Other suggestions in the literature for an acidification mechanism of the ERGIC or early Golgi were deduced from pH-dependent *in vitro* processes such as the binding of ADP-ribosylation factor to microsomes (38), the dissociation of RAP from LDL receptor-related protein (15), and the binding of KDEL peptides to KDEL receptor (16). None of these pH dependences, however, could be (i) demonstrated *in situ* or (ii) attributed to the action of titratable sensor amino acids. Finally, a proteolytic pre-Golgi event that remained unclassified appeared to depend on organelle acidification (39, 40). The data presented here, however, combine the *in vitro* characterization of a pH-driven molecular mechanism with the documentation of its *in vivo* relevance and thus, to our knowledge, add the highest level of evidence for pre-Golgi acidification. As targeting of a pH probe (25, 41) exclusively to the ERGIC is impossible because of the lack of a marker protein that does not recycle through the ER, a reliable measurement of pH_{ERGIC} has never been reported and may hardly be expected. Even so, the conclusions presented herein are of particular biomedical interest given the fact that multiple diseases including cystic fibrosis (42) and Alzheimer's disease (43) have been associated with molecular processes in the ERGIC.

Loss of ERGIC-53 expression in humans causes combined deficiency of coagulation factors V and VIII (13). A recent study has revealed a second protein implicated in this disease, MCFD2, an EF-hand protein that co-purifies with ERGIC-53 in a Ca^{2+}-dependent way (44). Our data show that ERGIC-53 purified in the absence of Ca^{2+} (see "Experimental Procedures") or from Ca^{2+}-depleted cells (data not shown) still efficiently binds mannose upon readdition of Ca^{2+}. Thus, this activity appears to be independent on complex formation with accessory proteins. The requirement for MCFD2 may be restricted to specific substrates such as coagulation factors V and VIII and the molecular role of this protein remains to be characterized. In summary, our study provides evidence that the function of ERGIC-53 as a transport lectin is determined by molecular switches in its substrate binding site itself that are triggered by changes in the ion composition between subcellular compartments involving pH and, presumably, calcium.

Acknowledgments—We thank Pete Burkhard and Markus Meier for help in lectin structure analysis, Käthy Bucher for technical assistance, and Matthias Chiquet for kindly providing us with antibodies to fibronectin.

[3] After submission of this manuscript, Velloso and colleagues (45) published the crystal structure of ERGIC-53 in complex with Ca^{2+}. His-178 complexes one of the two calcium ions by its Nδ-1 atom via a water molecule, whereas Nϵ-2 may be directly involved in ligand binding. The side chain of Asp-152 directly coordinates the same Ca^{2+}.

REFERENCES

1. Warren, G., and Mellman, I. (1999) *Cell* **98,** 125–127
2. Appenzeller, C., Andersson, H., Kappeler, F., and Hauri, H. P. (1999) *Nat. Cell Biol.* **1,** 330–334
3. Muniz, M., Nuoffer, C., Hauri, H. P., and Riezman, H. (2000) *J. Cell Biol.* **148,** 925–930
4. Belden, W. J., and Barlowe, C. (2001) *Science* **294,** 1528–1531
5. Hauri, H. P., Kappeler, F., Andersson, H., and Appenzeller, C. (2000) *J. Cell Sci.* **113,** 587–596
6. Klumperman, J., Schweizer, A., Clausen, H., Tang, B. L., Hong, W., Oorschot, V., and Hauri, H. P. (1998) *J. Cell Sci.* **111,** 3411–3425
7. Velloso, L. M., Svensson, K., Schneider, G., Pettersson, R. F., and Lindqvist, Y. (2002) *J. Biol. Chem.* **277,** 15979–15984
8. Loris, R., Hamelryck, T., Bouckaert, J., and Wyns, L. (1998) *Biochim. Biophys. Acta* **1383,** 9–36
9. Schrag, J. D., Procopio, D. O., Cygler, M., Thomas, D. Y., and Bergeron, J. J. (2003) *Trends Biochem. Sci.* **28,** 49–57
10. Loris, R. (2002) *Biochim. Biophys. Acta* **1572,** 198–208
11. Itin, C., Roche, A. C., Monsigny, M., and Hauri, H. P. (1996) *Mol. Biol. Cell* **7,** 483–493
12. Vollenweider, F., Kappeler, F., Itin, C., and Hauri, H. P. (1998) *J. Cell Biol.* **142,** 377–389
13. Nichols, W. C., Seligsohn, U., Zivelin, A., Terry, V. H., Hertel, C. E., Wheatley, M. A., Moussalli, M. J., Hauri, H. P., Ciavarella, N., Kaufman, R. J., and Ginsburg, D. (1998) *Cell* **93,** 61–70
14. Pezzati, R., Bossi, M., Podini, P., Meldolesi, J., and Grohovaz, F. (1997) *Mol. Biol. Cell* **8,** 1501–1512
15. Bu, G., Geuze, H. J., Strous, G. J., and Schwartz, A. L. (1995) *EMBO J.* **14,** 2269–2280
16. Scheel, A. A., and Pelham, H. R. (1996) *Biochemistry* **35,** 10203–10209
17. Wu, M. M., Grabe, M., Adams, S., Tsien, R. Y., Moore, H. P., and Machen, T. E. (2001) *J. Biol. Chem.* **276,** 33027–33035
18. Weisz, O. A. (2003) *Int. Rev. Cytol.* **226,** 259–319
19. Ying, M., Flatmark, T., and Saraste, J. (2000) *J. Cell Sci.* **113,** 3623–3638
20. Palokangas, H., Ying, M., Vaananen, K., and Saraste, J. (1998) *Mol. Biol. Cell* **9,** 3561–3578
21. Schweizer, A., Fransen, J. A., Bachi, T., Ginsel, L., and Hauri, H. P. (1988) *J. Cell Biol.* **107,** 1643–1653
22. Pimpaneau, V., Midoux, P., Monsigny, M., and Roche, A. C. (1991) *Carbohydr. Res.* **213,** 95–108
23. Cullen, B. R. (1987) *Methods Enzymol.* **152,** 684–704
24. Kappeler, F., Klopfenstein, D. R., Foguet, M., Paccaud, J. P., and Hauri, H. P. (1997) *J. Biol. Chem.* **272,** 31801–31808
25. Wu, M. M., Llopis, J., Adams, S. R., McCaffery, J. M., Teter, K., Kulomaa,

M. S., Machen, T. E., Moore, H. P., and Tsien, R. Y. (2000) *Methods Enzymol.* **327,** 546–564
26. Itin, C., Schindler, R., and Hauri, H. P. (1995) *J. Cell Biol.* **131,** 57–67
27. Wessel, D., and Flugge, U. I. (1984) *Anal. Biochem.* **138,** 141–143
28. Nufer, O., Mitrovic, S., and Hauri, H. P. (2003) *J. Biol. Chem.* **278,** 15886–15896
29. Feinberg, H., Torgersen, D., Drickamer, K., and Weis, W. I. (2000) *J. Biol. Chem.* **275,** 35176–35184
30. Feinberg, H., Park-Snyder, S., Kolatkar, A. R., Heise, C. T., Taylor, M. E., and Weis, W. I. (2000) *J. Biol. Chem.* **275,** 21539–21548
31. Wragg, S., and Drickamer, K. (1999) *J. Biol. Chem.* **274,** 35400–35406
32. Solovyova, N., and Verkhratsky, A. (2002) *J. Neurosci. Methods* **122,** 1–12
33. Pinton, P., Pozzan, T., and Rizzuto, R. (1998) *EMBO J.* **17,** 5298–5308
34. Griesbeck, O., Baird, G. S., Campbell, R. E., Zacharias, D. A., and Tsien, R. Y. (2001) *J. Biol. Chem.* **276,** 29188–29194
35. Fiedler, K., and Simons, K. (1996) *J. Cell Sci.* **109,** 271–276
36. Sharma, V., and Surolia, A. (1997) *J. Mol. Biol.* **267,** 433–445
37. Elgavish, S., and Shaanan, B. (1998) *J. Mol. Biol.* **277,** 917–932
38. Zeuzem, S., Feick, P., Zimmermann, P., Haase, W., Kahn, R. A., and Schulz, I. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 6619–6623
39. Bonifacino, J. S., Lippincott-Schwartz, J., Chen, C., Antusch, D., Samelson, L. E., and Klausner, R. D. (1988) *J. Biol. Chem.* **263,** 8965–8971
40. Antusch, D., Bonifacino, J. S., Burgess, W. H., and Klausner, R. D. (1990) *J. Immunol.* **145,** 885–890
41. Miesenbock, G., De Angelis, D. A., and Rothman, J. E. (1998) *Nature* **394,** 192–195
42. Gilbert, A., Jadot, M., Leontieva, E., Wattiaux-De Coninck, S., and Wattiaux, R. (1998) *Exp. Cell Res.* **242,** 144–152
43. Annaert, W. G., Levesque, L., Craessaerts, K., Dierinck, I., Snellings, G., Westaway, D., George-Hyslop, P. S., Cordell, B., Fraser, P., and De Strooper, B. (1999) *J. Cell Biol.* **147,** 277–294
44. Zhang, B., Cunningham, M. A., Nichols, W. C., Bernat, J. A., Seligsohn, U., Pipe, S. W., McVey, J. H., Schulte-Overberg, U., de Bosch, N. B., Ruiz-Saez, A., White, G. C., Tuddenham, E. G., Kaufman, R. J., and Ginsburg, D. (2003) *Nat. Genet.* **34,** 220–225
45. Velloso, L. M., Svensson, K., Pettersson, R. F., and Lindqvist, Y. (2003) *J. Mol. Biol.* **334,** 845–851

A green fluorescent protein-based approach to measure free calcium in the ER-Golgi intermediate compartment

Christian Appenzeller-Herzog and Hans-Peter Hauri

Summary

In the ER-Golgi intermediate compartment (ERGIC) anterograde protein traffic segregates from the recycling transport machinery of the early secretory pathway. This process includes physical dissociation of specific cargo proteins from their ER-export receptors. Although sorting is thought to result in part from changes in calcium concentration along the ER-to-ERGIC pathway, the ion composition of the ERGIC is not known. Here, we present a possible approach to measure $[Ca^{2+}]_{ERGIC}$ *in vivo* using a chimeric reporter protein targeted to this compartment. An inert variant of the ER-Golgi SNARE Sec22b, that does not interfere with the endogenous SNARE machinery, was engineered and fused to the green fluorescent protein-based calcium-indicator yellow cameleon. The fusion protein is shown to dynamically localize to the ERGIC and to provide an ideal tool for future fluorescence resonance energy transfer-calcium measurements within the ERGIC.

Introduction

Protein traffic from the ER to the Golgi involves the vesiculo tubular clusters of the ER-Golgi intermediate compartment (ERGIC). While the morphological mechanisms underlying the transport from ER-exit site to ERGIC and from ERGIC to Golgi are still a matter of debate (Ben-Tekaya et al., 2004; Mironov et al., 2003), the functionality of the ERGIC is less disputed. It acts as a protein sorting station. Several investigators have localized the segregation of anterograde-directed- from recycling protein flow to the ERGIC (Appenzeller et al., 1999; Aridor et al., 1995; Ben-Tekaya et al., 2004; Bu et al., 1995; Klumperman et al., 1998; Martinez-Menarguez et al., 1999; Shima et al., 1999), demonstrating that this compartment functions as a major crossroad of the biosynthetic protein traffic. In the case of cargo proteins that have been bound to a recycling transport receptor for selective ER-exit, sorting in the ERGIC requires receptor-cargo dissociation. Insofar, the functionality of the ERGIC may be best compared to that of the early endosome, a sorting station for endocytosed receptor-cargo complexes.

One of the determinants eliciting protein release in the ERGIC is luminal acidification. This is perhaps best exemplified by functional studies on the transport receptor ERGIC-53 that looses its affinity for cargo molecules as soon as a histidine sensor residue has been protonated in the acidified ERGIC (Appenzeller-Herzog et al., 2004). Binding of ERGIC-53 to its substrates requires calcium (Appenzeller et al., 1999; Itin et al., 1996). Interestingly, *in vitro* studies revealed that pH-sensitivity of ERGIC-53 was increased, when calcium concentrations were low (Appenzeller-Herzog et al., 2004). Accordingly, a mechanism that maintains lower calcium levels in the ERGIC would promote pH-induced cargo release.

While both the ER (Barrero et al., 1997; Miyawaki et al., 1997) and the Golgi (Griesbeck et al., 2001; Pinton et al., 1998) account for high-capacity storage of intracellular calcium, we are still lacking quantitative records of $[Ca^{2+}]_{ERGIC}$. Of note, an imaging approach that measures *total* calcium in ultrathin cryosections revealed positive signals for both ER and Golgi, but ERGIC elements remained below detection level (Pezzati et al., 1997). This drop in *total* calcium from ER to ERGIC may hint at, but does not prove an analogous concentration gradient of *free* calcium ($[Ca^{2+}]_{free}$), which is the physiologically relevant term for calcium-dependent processes. $[Ca^{2+}]_{free}$ integrates the rate of calcium pumping, the rate of calcium leakage and the calcium buffering capacity of a given compartment. Calcium housekeeping has been studied in detail for the ER (Meldolesi and Pozzan, 1998a; 1998b). Concerning the ERGIC, however, little is known regarding these mechanisms. The evidence of cell

fractionation experiments was taken to propose the presence of sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pumps in the ERGIC (Ying et al., 2002). Furthermore, the abundant and high-affinity Ca^{2+}-binding protein CALNUC, that in conjunction with SERCA and IP_3-receptor is involved in the formation of Golgi Ca^{2+}-stores (Lin et al., 1999), has been found to accumulate within the perinuclear ERGIC by immunoelectron microscopy (Lin et al., 1998).

Several tools have been developed to quantitatively assess organellar $[Ca^{2+}]_{free}$. These are synthetic dyes trapped into organelles or else genetically encoded probes that can be targeted by way of standard molecular biology methods (Rudolf et al., 2003). The latter include the cameleons (Demaurex and Frieden, 2003; Miyawaki et al., 1997), which rely on the fluorescence resonance energy transfer (FRET) between two GFP mutants of different colors that can be quantified and calibrated into $[Ca^{2+}]_{free}$. Here, we describe the targeting of cameleon probes to the lumen of the ERGIC by means of attaching them to the transmembrane protein Sec22b. The resulting fusion protein proves to be a promising probe for *in vivo* measurements of $[Ca^{2+}]_{free}$ in the ERGIC.

Results and Discussion

To target the Ca^{2+}-sensor protein yellow cameleon (Miyawaki et al., 1997) to the ERGIC, we added the C-terminally membrane-anchored protein Sec22b as a N-terminal targeting moiety. Sec22b (Hay et al., 1998) as well as a Sec22b-GFP fusion protein (Chao et al., 1999) have been shown to accumulate in ERGIC membranes, while continuously cycling between ER and ERGIC. The protein belongs to the SNARE familiy and accounts for membrane fusion events at the ER-Golgi interface (Zhang et al., 1999) by the regulated assembly with other ("cognate") SNARE proteins (for review see: (Chen and Scheller, 2001)). Ectopic expression of Sec22b in tissue culture, however, has been shown to interfere with the localization of endogenous ER-Golgi SNAREs including Syntaxin 5 (Hay et al., 1997), indicating some general disturbance of the early secretory pathway. To avoid these unwanted dominant trafficking effects, we sought to express a rationally designed, functionally impaired form of Sec22b attached to YC.

The interaction between cognate SNAREs is mediated by the membrane-proximal SNARE-domains. These domains characteristically associate in a tetrameric coiled-coil (Burkhard et al., 2001), the structure of which has been resolved for the synaptic SNARE complex (Sutton et al., 1998). Interestingly, the main structural features (derived from protein sequence), including an ionic middle layer consisting of an arginine and three glutamine residues each provided by one of the four α-helices, are highly conserved among related SNARE complexes. This led to the reclassification of the SNARE proteins into R-SNAREs, such as Sec22b, and Q-SNAREs (Fasshauer et al., 1998). R to G mutation in this ionic layer abolished *in vitro* binding of yeast Sec22b to a cognate SNARE in the presence of detergent (Sacher et al., 1997), but showed only minor effects, if any, in a co-immunoprecipitation experiment (not shown), which is in line with genetic and biochemical data on the yeast exocytic SNARE complex (Katz and Brennwald, 2000; Ossig et al., 2000). Therefore, we introduced an additional mutation in a neighbouring, apolar layer to generate the double mutant R159G/L166E (Fig. 1).

To test for the contribution of HA-tagged variants of wt Sec22b or R159G/L166E (see *Experimental Procedures*) into endogenous SNARE complexes, we transiently expressed these variants in HepG2 cells followed by indirect double immunofluorescence using anti-HA and anti-syntaxin 5. As shown in Fig. 2A-C, exogenous Sec22b induced expression level-dependent relocalization of Syntaxin 5, thus confirming previous observations (Hay et al., 1997). Conversely, R159G/L166E expression did not alter Syntaxin 5 trafficking despite the

colocalization of the two markers in the Golgi region (Fig. 2D-F), suggesting that the mutations in the SNARE domain efficiently disturbed coiled-coil formation with the endogenous SNARE machinery. To verify this, we performed a co-immunoprecipitation experiment. HepG2 cells transiently expressing wt or coiled-coil impaired Sec22b constructs were subjected to anti-HA immunoprecipitation and subsequent Western blotting with anti-syntaxin 5. Expression levels of the recombinant Sec22b variants were equal (not shown). As expected, the two isoforms of Syntaxin 5 (Hui et al., 1997) were present in wt-, but absent from mock- or R159G/L166E-immunoprecipitates (Fig. 3).

The finding that R159G/L166E was excluded from endogenous SNARE complexes, but was still correctly targeted to the early secretory pathway (Fig. 2 and see below) turned this mutant into an ideal, inert, and monomeric ERGIC-targeting tag for FRET-probes. Furthermore, it revealed that the dynamic localization of Sec22b does not depend on its ability to interact with cognate SNARE partners. Interestingly, the same has been observed for the ER-Golgi SNARE rbet1, whereas the targeting information could be specified to the SNARE domain (Joglekar et al., 2003). Unlike rbet1, Sec22b contains an independent N-terminal domain that is thought to interact with the SNARE domain in a folded back conformation and to exert an autoinhibitory effect (Gonzalez et al., 2001; Tochio et al., 2001). Although the data suggest that the cytosolic domain as a whole mediates the correct localization of Sec22b, the precise transport signals presented to the vesicle budding machinery (Miller et al., 2003; Mossessova et al., 2003; Rein et al., 2002) remain to be analyzed. In addition, targeting information may also be comprised within its transmembrane domain, as is the case for syntaxin 5 (Banfield et al., 1994).

With the aim to measure $[Ca^{2+}]_{free}$ in the ERGIC, the following cDNAs encoding medium-affinity (YC3) and low-affinity (YC4) Ca^{2+}-probes (Miyawaki et al., 1997) were synthesized: Sec22b(R159G/L166E)-YC3 (YC3ergic), Sec22b(R159G/L166E)-YC4 (YC4ergic), and YC3ergic/YC4ergic carrying the citrine mutations V68L/Q69M in the EYFP moiety (YC3^{cit}ergic and YC4^{cit}ergic) to reduce the sensitivity to acid (Griesbeck et al., 2001). The intracellular targeting of these probes was assessed in HepG2 cells. To this end, cells were transfected with the different YCergic cDNAs, fixed and stained with anti-ERGIC-53 to compare YC-fluorescence with the staining of the ERGIC-marker protein ERGIC-53. The fluorescence pattern of all YCergic-probes was indistiguishable (not shown). As exemplified with YC3ergic (Fig. 4A), they were concentrated in peripheral spots as well as in the perinuclear Golgi region, which corresponded to peripheral and perinuclear ERGIC clusters (Klumperman et al., 1998) as revealed by colocalization with ERGIC-53. Notably, the ER-

staining by anti-ERGIC-53 was far more prominent than with YC, indicating efficient post-ER concentration of the Ca^{2+}-probe. Likewise, the YCergic- and the reticular YCer-pattern (Miyawaki et al., 1997) were apparently different in HepG2 cells (not shown). To find out, whether YCergic, like Sec22b, recycles between ER and ERGIC and to exclude its partial penetration into the Golgi, we treated the cells with brefeldin A prior to immunofluorescence analysis. Brefeldin A induces the relocalization of Golgi proteins to the ER as well as the accumulation of recycling proteins to peripheral ERGIC-like structures (Breuza et al., 2004; Lippincott-Schwartz et al., 1990). Fig. 4B shows the punctate fluorescence pattern of YC4ergic after brefeldin A-treatment that is typical for recycling proteins of the early secretory pathway such as ERGIC-53, demonstrating the dynamic localization of the Ca^{2+}-probe particularly to the ERGIC.

These YCergic probes will in the future provide a valuable tool for $[Ca^{2+}]_{free}$ measurements in the ERGIC. The choice of a monomeric, coiled-coil impaired, and C-terminally anchored (i.e. lacking potentially harming luminal domains) targeting moiety reduces the harm toward the FRET-indicator to a minimum. Despite continuous recycling through the ER, YCergic (or: at least the correctly folded pool of YCergic) is nicely concentrated in post-ER compartments, that is, the vesiculo tubular clusters of the ERGIC (Fig. 4, (Hay et al., 1998)). It must be emphasized that so far – even though the ERGIC has proven to consist a stationary compartment (Ben-Tekaya et al., 2004) – there is no marker protein that exquisitely localizes to the ERGIC and does not recycle through the ER. Therefore, it will be of importance to set the FRET-signal obtained with YCergic in relation with a concomitant YCer read-out. Some background FRET-signal arising from the ER pool of YCergic is to be expected. The ER background yet could potentially be reduced by ERGIC to ER transport inhibition such as 15°-incubation (Ben-Tekaya et al., 2004), 1,3-Cyclohexanebis(methylamine)- (Hu et al., 1999), bafilomycin A1- (Palokangas et al., 1998) or brefeldin A-treatment (Fig. 4B). Moreover, reversible ER-exit blockage using the kinase inhibitor H89 (Ben-Tekaya et al., 2004) or the phospholipase D inhibitor 1-butanol (Pathre et al., 2003) will be useful to transiently turn YCergic into an ER-Ca^{2+}-probe and to follow the presumable changes in FRET. To dissect the contributions to these changes by $[Ca^{2+}]_{free}$ and/or by the deleterious effect of low pH on the EYFP acceptor chromophor, the inclusion of YC^{cit}ergic-probes will be helpful.

Taken together, we have developed a strategy to target reporter proteins to the ERGIC by means of a standard molecular biology/transfection approach. Our strategy may prove to be useful for the targeting of a variety of proteinaceous probes including pH-indicators

(Kneen et al., 1998; Llopis et al., 1998; Miesenbock et al., 1998; Wu et al., 2000) or rationally fragmented protein-protein interaction reporters (Michnick, 2003).

Experimental Procedures

Reagents

The following antibodies were used: mAb G1/93 against human ERGIC-53, mAb 12CA5 against the HA-epitope and affinity purified pAb against recombinant Syntaxin 5 ((Hui et al., 1997), kindly provided by G. Warren). Antimycin A and N-ethylmaleimide were from Sigma. Brefeldin A was purchased from Epicentre Technologies (Madison WI, USA).

Recombinant DNAs

The cDNAs for Sec22b and a 5'-fragment of YC (5'YC) were fused by PCR-based splicing by overlap extension (Horton et al., 1989). Initial amplifications were performed with 5'-GAGAGAGGTACCGATGGTGCTGCTGACGATGATCGCCCGTG-3' (underlined: Asp718 restriction site) and 5'-CTCACCATTGTAGCGATAGAT-CCCAGCCACCAAAACCGCACATACACTATCAGCATG-3' for Sec22b, and 5'-GTG-GCTGGGATCTATCGCTACAATGGTGAGCAAGGGCGAGGAGCTGTTCACC-3' and 5'-CGGAGCTGGAGATCTTCTTGAACCG-3' for 5'YC (using YC3er or YC4er (Miyawaki et al., 1997) as template). The fusion PCR-product was digested by Asp718 and AgeI. For 3'YC-amplification the following primer-pair was used: 5'-GGGAAGCAGATATCGATGGTGATGGC-3' and 5'-GTTACAGCTCGTCCATGCCG-AGAGTGATCCCG-3'. The resulting product was subcloned into pCR2.1-TOPO (Invitrogen) and re-excised by AgeI/XhoI. Sec22b-5'YC and 3'YC were double-ligated into Asp718/XhoI-linearized pcDNA3 (Invitrogen). The cDNA encodes Sec22b protein fused to the N-terminus of YC by the linker peptide GSIAT.

Amino acid substitutions were introduced by the QuikChange method (Stratagene) using the following primers (only coding strand): 5'-GAAGT-CCTACAGGGGGGAGAAGCACTCTCAG-3' (R159G), 5'-GGGAGAAGCACTCTCA-GCAGAGGATTCAAAGGCTAAC-3' (L166E), 5'-CCTTCGGCTACGGCCTGATGTG-CTTCGCCC-3' (V68L/Q69M).

For the characterization of R159G/L166E (Figs. 2, 3) we used Sec22b carrying a luminal HA-epitope fused to aequorin (Brini et al., 1995).

Cell culture and transfection

HepG2 cells were cultured as previously described (Klumperman et al., 1998). Transfections were carried out with FuGENE 6 (Roche).

SNARE-SNARE co-immunoprecipitation

The protocol of Xu et al. (2000) was adapted as follows: For ATP-depletion and NSF-inhibition, transfected HepG2 cells were incubated for 10 min at 37° in 25 mM HEPES, pH 7.3, 70 mM sucrose, 130 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.2 mM N-ethylmaleimide, and 0.1 µM antimycin A, prior to cell lysis in 100 mM $NaPO_4$, pH 8.0, and 3% TX-100. Lysates were cleared (100,000 g, 1 h) and subjected to immunoprecipitation against HA-tagged Sec22b with 12CA5-protein A-sepharose that had been chemically coupled with dimethyl pimelimidate (Sigma). Immunoprecipitates were resolved by 10% SDS-PAGE and co-immunoprecipitated Syntaxin 5 revealed by Western blotting.

Immunofluorescence microscopy

HepG2 cells were cultured in 8-well multi-chamber glass slides (Milian, Plan-les-Ouates, Switzerland) that had been coated with 1 mg/ml poly-L-lysine (MW 30,000 - 70,000, Sigma), fixed with 3 % paraformaldehyde, permeabilized with PBS/0.1 % saponin, and processed for indirect immunofluorescence using the indicated primary antibodies followed by GAM IgG(H+L)-AlexaFluor 568 and, for double staining, GAR IgG(H+L)-AlexaFluor 488 (Molecular Probes, Leiden, Netherland). YC fluorescence was recorded with AlexaFluor 488-settings. Confocal laser scanning images were acquired on a Leica microscope (TCS NT) with a 63x objective (NA 1.32).

Acknowledgements

We thank Käthy Bucher for technical assistance, Pete Burkhard for advice in coiled-coil manipulation, Wanjin Hong and Nicolas Demaurex for providing the Sec22b- and YC3er/YC4er cDNAs, and Graham Warren for the gift of anti-syntaxin 5 antiserum.

References

Appenzeller, C., Andersson, H., Kappeler, F. and Hauri, H.P. (1999) The lectin ERGIC-53 is a cargo transport receptor for glycoproteins. *Nat Cell Biol*, **1**, 330-334.

Appenzeller-Herzog, C., Roche, A.C., Nufer, O. and Hauri, H.P. (2004) pH-induced conversion of the transport lectin ERGIC-53 triggers glycoprotein release. *J Biol Chem*, **279**, 12943-12950.

Aridor, M., Bannykh, S.I., Rowe, T. and Balch, W.E. (1995) Sequential coupling between COPII and COPI vesicle coats in endoplasmic reticulum to Golgi transport. *J Cell Biol*, **131**, 875-893.

Banfield, D.K., Lewis, M.J., Rabouille, C., Warren, G. and Pelham, H.R. (1994) Localization of Sed5, a putative vesicle targeting molecule, to the cis-Golgi network involves both its transmembrane and cytoplasmic domains. *J Cell Biol*, **127**, 357-371.

Barrero, M.J., Montero, M. and Alvarez, J. (1997) Dynamics of [Ca2+] in the endoplasmic reticulum and cytoplasm of intact HeLa cells. A comparative study. *J Biol Chem*, **272**, 27694-27699.

Ben-Tekaya, H., Miura, K., Pepperkok, R. and Hauri, H.P. (2004) ER-Golgi intermediate compartment clusters define a stationary sorting compartment, *in preparation*.

Breuza, L., Halbeisen, R., Jenö, P., Otte, S., Barlowe, C., Hong, W. and Hauri, H.P. (2004) Proteomics of ERGIC membranes identifies ERGIC-32, a new cycling protein interacting with Erv46, *in preparation*.

Brini, M., Marsault, R., Bastianutto, C., Alvarez, J., Pozzan, T. and Rizzuto, R. (1995) Transfected aequorin in the measurement of cytosolic Ca2+ concentration ([Ca2+]c). A critical evaluation. *J Biol Chem*, **270**, 9896-9903.

Bu, G., Geuze, H.J., Strous, G.J. and Schwartz, A.L. (1995) 39 kDa receptor-associated protein is an ER resident protein and molecular chaperone for LDL receptor-related protein. *Embo J*, **14**, 2269-2280.

Burkhard, P., Stetefeld, J. and Strelkov, S.V. (2001) Coiled coils: a highly versatile protein folding motif. *Trends Cell Biol*, **11**, 82-88.

Chao, D.S., Hay, J.C., Winnick, S., Prekeris, R., Klumperman, J. and Scheller, R.H. (1999) SNARE membrane trafficking dynamics in vivo. *J Cell Biol*, **144**, 869-881.

Chen, Y.A. and Scheller, R.H. (2001) SNARE-mediated membrane fusion. *Nat Rev Mol Cell Biol*, **2**, 98-106.

Demaurex, N. and Frieden, M. (2003) Measurements of the free luminal ER Ca(2+) concentration with targeted "cameleon" fluorescent proteins. *Cell Calcium*, **34**, 109-119.

Fasshauer, D., Sutton, R.B., Brunger, A.T. and Jahn, R. (1998) Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. *Proc Natl Acad Sci U S A*, **95**, 15781-15786.

Gonzalez, L.C., Jr., Weis, W.I. and Scheller, R.H. (2001) A novel snare N-terminal domain revealed by the crystal structure of Sec22b. *J Biol Chem*, **276**, 24203-24211.

Griesbeck, O., Baird, G.S., Campbell, R.E., Zacharias, D.A. and Tsien, R.Y. (2001) Reducing the environmental sensitivity of yellow fluorescent protein. Mechanism and applications. *J Biol Chem*, **276**, 29188-29194.

Hay, J.C., Chao, D.S., Kuo, C.S. and Scheller, R.H. (1997) Protein interactions regulating vesicle transport between the endoplasmic reticulum and Golgi apparatus in mammalian cells. *Cell*, **89**, 149-158.

Hay, J.C., Klumperman, J., Oorschot, V., Steegmaier, M., Kuo, C.S. and Scheller, R.H. (1998) Localization, dynamics, and protein interactions reveal distinct roles for ER and Golgi SNAREs. *J Cell Biol*, **141**, 1489-1502.

Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K. and Pease, L.R. (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene*, **77**, 61-68.

Hu, T., Kao, C.Y., Hudson, R.T., Chen, A. and Draper, R.K. (1999) Inhibition of secretion by 1,3-Cyclohexanebis(methylamine), a dibasic compound that interferes with coatomer function. *Mol Biol Cell*, **10**, 921-933.

Hui, N., Nakamura, N., Sonnichsen, B., Shima, D.T., Nilsson, T. and Warren, G. (1997) An isoform of the Golgi t-SNARE, syntaxin 5, with an endoplasmic reticulum retrieval signal. *Mol Biol Cell*, **8**, 1777-1787.

Itin, C., Roche, A.C., Monsigny, M. and Hauri, H.P. (1996) ERGIC-53 is a functional mannose-selective and calcium-dependent human homologue of leguminous lectins. *Mol Biol Cell*, **7**, 483-493.

Joglekar, A.P., Xu, D., Rigotti, D.J., Fairman, R. and Hay, J.C. (2003) The SNARE motif contributes to rbet1 intracellular targeting

and dynamics independently of SNARE interactions. *J Biol Chem*, **278**, 14121-14133.

Katz, L. and Brennwald, P. (2000) Testing the 3Q:1R "rule": mutational analysis of the ionic "zero" layer in the yeast exocytic SNARE complex reveals no requirement for arginine. *Mol Biol Cell*, **11**, 3849-3858.

Klumperman, J., Schweizer, A., Clausen, H., Tang, B.L., Hong, W., Oorschot, V. and Hauri, H.P. (1998) The recycling pathway of protein ERGIC-53 and dynamics of the ER-Golgi intermediate compartment. *J Cell Sci*, **111 (Pt 22)**, 3411-3425.

Kneen, M., Farinas, J., Li, Y. and Verkman, A.S. (1998) Green fluorescent protein as a noninvasive intracellular pH indicator. *Biophys J*, **74**, 1591-1599.

Lin, P., Le-Niculescu, H., Hofmeister, R., McCaffery, J.M., Jin, M., Hennemann, H., McQuistan, T., De Vries, L. and Farquhar, M.G. (1998) The mammalian calcium-binding protein, nucleobindin (CALNUC), is a Golgi resident protein. *J Cell Biol*, **141**, 1515-1527.

Lin, P., Yao, Y., Hofmeister, R., Tsien, R.Y. and Farquhar, M.G. (1999) Overexpression of CALNUC (nucleobindin) increases agonist and thapsigargin releasable Ca2+ storage in the Golgi. *J Cell Biol*, **145**, 279-289.

Lippincott-Schwartz, J., Donaldson, J.G., Schweizer, A., Berger, E.G., Hauri, H.P., Yuan, L.C. and Klausner, R.D. (1990) Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an ER recycling pathway. *Cell*, **60**, 821-836.

Llopis, J., McCaffery, J.M., Miyawaki, A., Farquhar, M.G. and Tsien, R.Y. (1998) Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. *Proc Natl Acad Sci U S A*, **95**, 6803-6808.

Martinez-Menarguez, J.A., Geuze, H.J., Slot, J.W. and Klumperman, J. (1999) Vesicular tubular clusters between the ER and Golgi mediate concentration of soluble secretory proteins by exclusion from COPI-coated vesicles. *Cell*, **98**, 81-90.

Meldolesi, J. and Pozzan, T. (1998a) The endoplasmic reticulum Ca2+ store: a view from the lumen. *Trends Biochem Sci*, **23**, 10-14.

Meldolesi, J. and Pozzan, T. (1998b) The heterogeneity of ER Ca2+ stores has a key role in nonmuscle cell signaling and function. *J Cell Biol*, **142**, 1395-1398.

Michnick, S.W. (2003) Protein fragment complementation strategies for biochemical network mapping. *Curr Opin Biotechnol*, **14**, 610-617.

Miesenbock, G., De Angelis, D.A. and Rothman, J.E. (1998) Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature*, **394**, 192-195.

Miller, E.A., Beilharz, T.H., Malkus, P.N., Lee, M.C., Hamamoto, S., Orci, L. and Schekman, R. (2003) Multiple cargo binding sites on the COPII subunit Sec24p ensure capture of diverse membrane proteins into transport vesicles. *Cell*, **114**, 497-509.

Mironov, A.A., Mironov, A.A., Jr., Beznoussenko, G.V., Trucco, A., Lupetti, P., Smith, J.D., Geerts, W.J., Koster, A.J., Burger, K.N., Martone, M.E., Deerinck, T.J., Ellisman, M.H. and Luini, A. (2003) ER-to-Golgi carriers arise through direct en bloc protrusion and multistage maturation of specialized ER exit domains. *Dev Cell*, **5**, 583-594.

Miyawaki, A., Llopis, J., Heim, R., McCaffery, J.M., Adams, J.A., Ikura, M. and Tsien, R.Y. (1997) Fluorescent indicators for Ca2+ based on green fluorescent proteins and calmodulin. *Nature*, **388**, 882-887.

Mossessova, E., Bickford, L.C. and Goldberg, J. (2003) SNARE selectivity of the COPII coat. *Cell*, **114**, 483-495.

Ossig, R., Schmitt, H.D., de Groot, B., Riedel, D., Keranen, S., Ronne, H., Grubmuller, H. and Jahn, R. (2000) Exocytosis requires asymmetry in the central layer of the SNARE complex. *Embo J*, **19**, 6000-6010.

Palokangas, H., Ying, M., Vaananen, K. and Saraste, J. (1998) Retrograde transport from the pre-Golgi intermediate compartment and the Golgi complex is affected by the vacuolar H+-ATPase inhibitor bafilomycin A1. *Mol Biol Cell*, **9**, 3561-3578.

Pathre, P., Shome, K., Blumental-Perry, A., Bielli, A., Haney, C.J., Alber, S., Watkins, S.C., Romero, G. and Aridor, M. (2003) Activation of phospholipase D by the small GTPase Sar1p is required to support COPII assembly and ER export. *Embo J*, **22**, 4059-4069.

Pezzati, R., Bossi, M., Podini, P., Meldolesi, J. and Grohovaz, F. (1997) High-resolution calcium mapping of the endoplasmic reticulum-Golgi-exocytic membrane system. Electron energy loss imaging analysis of quick frozen-freeze dried PC12 cells. *Mol Biol Cell*, **8**, 1501-1512.

Pinton, P., Pozzan, T. and Rizzuto, R. (1998) The Golgi apparatus is an inositol 1,4,5-trisphosphate-sensitive Ca2+ store, with functional properties distinct from those of the endoplasmic reticulum. *Embo J*, **17**, 5298-5308.

Rein, U., Andag, U., Duden, R., Schmitt, H.D. and Spang, A. (2002) ARF-GAP-mediated interaction between the ER-Golgi v-SNAREs and the COPI coat. *J Cell Biol*, **157**, 395-404.

Rudolf, R., Mongillo, M., Rizzuto, R. and Pozzan, T. (2003) Looking forward to seeing calcium. *Nat Rev Mol Cell Biol*, **4**, 579-586.

Sacher, M., Stone, S. and Ferro-Novick, S. (1997) The synaptobrevin-related domains of Bos1p and Sec22p bind to the syntaxin-like region of Sed5p. *J Biol Chem*, **272**, 17134-17138.

Shima, D.T., Scales, S.J., Kreis, T.E. and Pepperkok, R. (1999) Segregation of COPI-rich and anterograde-cargo-rich domains in endoplasmic-reticulum-to-Golgi transport complexes. *Curr Biol*, **9**, 821-824.

Sutton, R.B., Fasshauer, D., Jahn, R. and Brunger, A.T. (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. *Nature*, **395**, 347-353.

Tochio, H., Tsui, M.M., Banfield, D.K. and Zhang, M. (2001) An autoinhibitory mechanism for nonsyntaxin SNARE proteins revealed by the structure of Ykt6p. *Science*, **293**, 698-702.

Wu, M.M., Llopis, J., Adams, S.R., McCaffery, J.M., Teter, K., Kulomaa, M.S., Machen, T.E., Moore, H.P. and Tsien, R.Y. (2000) Studying organelle physiology with fusion protein-targeted avidin and fluorescent biotin conjugates. *Methods Enzymol*, **327**, 546-564.

Xu, D., Joglekar, A.P., Williams, A.L. and Hay, J.C. (2000) Subunit structure of a mammalian ER/Golgi SNARE complex. *J Biol Chem*, **275**, 39631-39639.

Ying, M., Sannerud, R., Flatmark, T. and Saraste, J. (2002) Colocalization of Ca2+-ATPase and GRP94 with p58 and the effects of thapsigargin on protein recycling suggest the participation of the pre-Golgi intermediate compartment in intracellular Ca2+ storage. *Eur J Cell Biol*, **81**, 469-483.

Zhang, T., Wong, S.H., Tang, B.L., Xu, Y. and Hong, W. (1999) Morphological and functional association of Sec22b/ERS-24 with the pre-Golgi intermediate compartment. *Mol Biol Cell*, **10**, 435-453.

Figure legends

Figure 1

Amino acid sequences of the four α-helices of the ER/Golgi SNARE complex [Swiss-Prot accession numbers: O75396 (Sec22b), Q13190 (Syntaxin 5), O15155 (Bet1), O14653 (Membrin)]. Layers of contacting residues on the inner surface of the SNARE complex are shown in italic. Coiled-coil impaired Sec22b was engineered by introducing the R159G/L166E double mutation to destabilize the boxed layers.

Figure 2

Double immunofluorescence microscopy of recombinant, HA-tagged Sec22b and endogenous Syntaxin 5. HepG2 cells were transfected, fixed and stained with anti-HA (A and D, red) and anti-syntaxin 5 (B and E, green). Note that overexpression of wt Sec22b disturbs the steady-state localization of Syntaxin 5 (A – C). Conversely, R159G/L166E does not interfere with the perinuclear staining pattern of the endogenous SNARE (D – F).

Figure 3

R159G/L166E prevents the participation of Sec22b into SNARE complexes. Shown is an anti-syntaxin 5 immunoblot of Sec22b-HA immunoprecipitates from transfected HepG2 cells. The two isoforms of co-immunoprecipitated Syntaxin 5, that are absent from mock-precipitates, are marked with filled triangles. Note that the interaction with endogenous Syntaxin 5 is compromised by the R159G/L166E double mutation.

Figure 4

Localization of YCergic in HepG2 cells. (A) Micrograph of YC3ergic-transfected cells, stained with anti-ERGIC-53. Arrows illustrate colocalization of the two proteins in both peripheral and perinuclear ERGIC clusters. While ERGIC-53 also prominently localizes to the ER in HepG2 cells, YC3ergic fluorescence is largely concentrated in the punctate structures of the ERGIC. (B) Colocalization of YC4ergic and ERGIC-53 in brefeldin A-treated HepG2 cells (arrows). Note that the brefeldin A-induced redistribution of both YC4ergic and ERGIC-53 is typical for proteins that continuously recycle between ER and ERGIC.

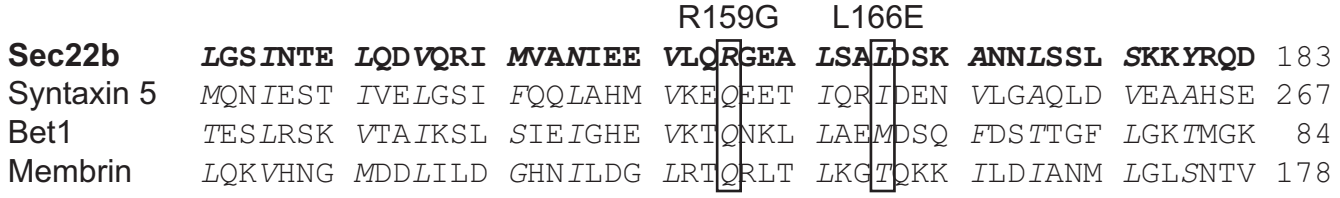

R159G
L166E
Sec22b LGSINTE LQDVQRI MVANIEE VLQRGEA LSALDSK ANNLSSL SKKYRQD 183
Syntaxin 5 MQNIEST IVELGSI FQQLAHM VKEQEET IQRIDEN VLGAQLD VEAAHSE 267
Bet1 TESLRSK VTAIKSL SIEIGHE VKTQNKL LAEMDSQ FDSTTGF LGKTMGK 84
Membrin LQKVHNG MDDLILD GHNILDG LRTQRLT LKGTQKK ILDIANM LGLSNTV 178

Figure 1

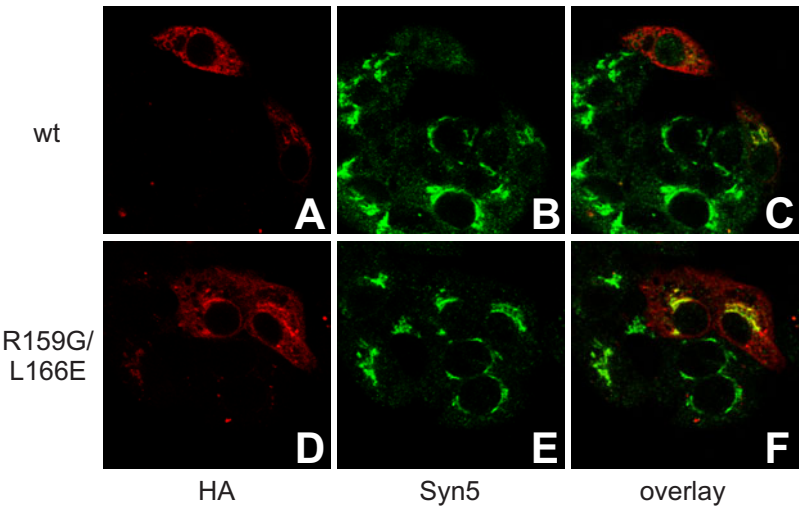


Figure 2

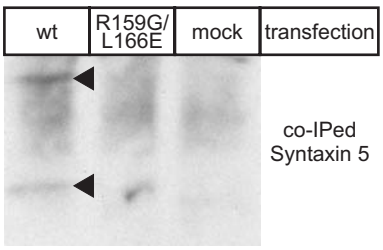


Figure 3

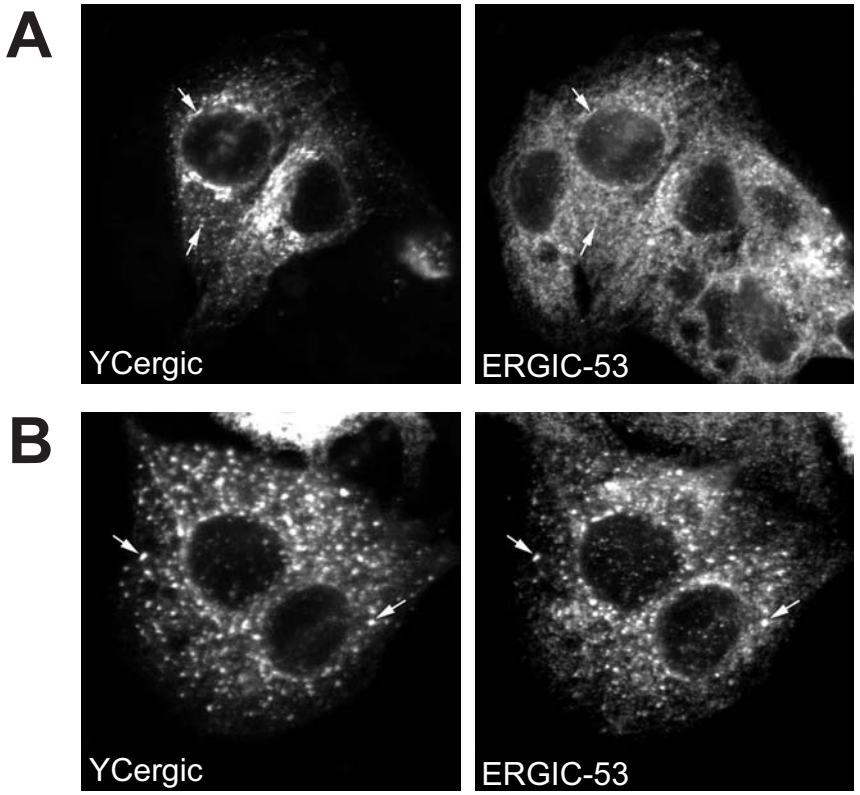


Figure 4

3. The ER-Golgi intermediate compartment: In search of its identity and function

Christian Appenzeller-Herzog and Hans-Peter Hauri

Abstract

Protein traffic from the endoplasmic reticulum (ER) to the Golgi involves the pleiomorphic membrane clusters of the ER-Golgi intermediate compartment (ERGIC). The dynamic nature and role of these membranes has been debated for quite some time. Here, we point out that the available morphological and functional data on the ERGIC can be best accomodated by the stable compartment model. The role of this stationary ERGIC may extend beyond protein sorting and involve protein folding and quality control.

Research activity on the complex membrane system interposed between the rough endoplasmic reticulum (ER) and the stacked Golgi was initiated by the identification of a 53 kDa membrane protein (now called ERGIC-53) that is predominantly localized to this membrane system (Schweizer et al., 1988). Transport studies with the tsO45 mutant G protein of vesicular stomatitis virus (VSV-G) or the E1 glycoprotein of semliki forest virus in conjunction with temperature shift experiments demonstrated that ERGIC-53-positive membranes consist of intermediate elements in ER-to-Golgi transport (Schweizer et al., 1990; Saraste and Svensson, 1991) that are biochemically distinct from its neighbouring compartments (Schweizer et al., 1991). The discovery of these elements that were designated ER-Golgi intermediate compartment (ERGIC) – now also referred to as vesiculo tubular clusters or pre-Golgi intermediates – has led to several models on the structural organisation of the ER-Golgi interface (Hauri and Schweizer, 1992). Inter alia the ERGIC has been suggested to be a specialized domain of the ER (Sitia and Meldolesi, 1992) or the cis-Golgi (Mellman and Simons, 1992). Ultrastructural and three-dimensional reconstruction analysis of pancreatic acinar cells, however, has reinforced the notion that the ERGIC constitutes an independent structure lacking membrane continuity with the ER or the cis-Golgi (Sesso et al., 1994). Several other laboratories have confirmed this finding (Bannykh et al., 1996; Klumperman et al., 1998; Fan et al., 2003), whereas Hong and colleagues, by omitting chemical fixation, recently have challenged the view of the ERGIC as an assembly of convoluted tubular membranes: In rapidly frozen cells, membranes that are positive for ERGIC marker proteins appear as large, pleiomorphic bodies with numerous budding profiles at their rims (Horstmann et al., 2002). This review deals with the dynamic morphology of the ERGIC and discusses the existing models for ER-to-Golgi transport.

3.1 ROLE OF THE ERGIC IN ER-TO-GOLGI TRANSPORT

Numerous genetic and biochemical studies with the yeast *Saccharomyces cerevisiae* that is devoid of ERGIC clusters have established a simple transport model, where COPII vesicles mediate ER-to-Golgi and COPI vesicles Golgi-to-ER transport (Bonifacino and Glick, 2004). Also in higher eukaryotes, transport from the ER to the Golgi is initiated by COPII-mediated budding of vesicular carriers (Barlowe, 2002). COPII-dependent budding activity is restricted to specialized, long-lived subdomains of the ER, the ER-exit sites (ERES) (Bannykh et al., 1996; Hammond and Glick, 2000). It is now firmly recognized that ERGIC membranes

(traditionally defined by ERGIC-53) localize in close proximity to ERES and delineate the subsequent stage in transport to the Golgi, which, however, cannot be fully resolved from the ERES by light microscopy. Unlike the ERES that are marked by COPII, ERGIC membranes are decorated with the unrelated vesicle-budding coat COPI (Aridor et al., 1995; Bannykh et al., 1996; Martinez-Menarguez et al., 1999; Spang, 2002).

What is the dynamic role of the interposed ERGIC membrane clusters in transport from ER to Golgi? The direct visualization of green fluorescent protein (GFP)-tagged marker proteins within the secretory pathway has tremendously inspired and facilitated the examination of trafficking mechanisms and in particular of the role of the ERGIC during ER-to-Golgi transport (Lippincott-Schwartz et al., 2001). Illumination of the early secretory pathway by the transmembrane marker VSV-G-GFP has led to the proposal that the ERGIC is a mobile membrane structure itself carrying secretory material along microtubules from the ER to the Golgi complex (Presley et al., 1997; Scales et al., 1997), while constantly maturing by segregating and returning recycling material to the ER (Shima et al., 1999). The characterization of these carriers that typically appear as vehicles of up to 1 μm in diameter has been integrated to the formulation of the transport complex (TC)-model (Bannykh et al., 1998; Stephens and Pepperkok, 2001). According to the TC-model, the ERGIC consists of a transient cargo container formed de novo from the ER before migrating to and fusing with or giving rise to the cis-Golgi.

A current live cell imaging study on ERGIC-53 (Hauri et al., 2000), that was fluorescently tagged with GFP and expressed at moderate levels, however, has called the TC-model into question (Ben-Tekaya et al., 2004). GFP-ERGIC-53 mainly localizes to long-lived stationary membrane entities that are next to, but distinct from ERES and do not move to the Golgi. Importantly, these stationary membranes are sites of repeated sorting of a fluorescent secretory marker protein, ssDsRed, into large anterograde carriers (ACs), that leave behind the GFP-ERGIC-53 compartment while moving themselves to the Golgi. These findings have revitalized the original model, whereby the ERGIC is a membrane compartment in the true sense that receives cargo from ER-derived COPII carriers and transmits ACs destined for the Golgi.

Both anterograde markers, VSV-G-GFP and ssDsRed, when released from the early secretory pathway, were packed into large membrane carriers that were readily seen by light microscopy to be translocated to the perinuclear Golgi area. It has been suggested that in higher eukaryotes conventional transport vesicles (60-80 nm) would only operate in short distance transport (for example, between ERES and ERGIC), whereas these large membrane

carriers (up to 1 μm) would allow long-range distribution along cytoskeletal tracks through the cytoplasm (Stephens and Pepperkok, 2001; Bonifacino and Lippincott-Schwartz, 2003). But, are these transient membrane carriers that are marked with anterograde cargo identical to the ERGIC, as suggested by the TC-model?

Another eminent question concerns the physical relationship between ERES and ERGIC. Initial models implied that small ER-derived vesicles after shedding their COPII-coat undergo heterotypic fusion with the ERGIC or homotypic fusion to form the ERGIC (Bannykh and Balch, 1997). Indeed, COPII-coated vesicles have been seen by electron microscopy ((Martinez-Menarguez et al., 1999) and J. Klumperman, personal communication) that may be arrested to form chain-like clusters at lower temperature (Horstmann et al., 2002). In an alternative view, the ERES and the ERGIC are in physical continuity and represent a common membrane structure that appears as a grape-like outgrowth of the ER. Hence, the ERGIC would consist of a subdomain of the ERES that is specialized for the transmission of large membrane carriers packed with anterograde cargo, whereby the transition from ERES to ERGIC would require COPII activity. A similar model has been put forward by a recent study that used synchronized ER-exit of two marker proteins, VSV-G and procollagen, in conjuction with correlative video/light electron microscopy to show direct en bloc protrusion, fission and maturation of large saccular carriers (Mironov et al., 2003). These carriers either contained VSV-G or procollagen that were apparently sorted at the level of ER-exit (Stephens and Pepperkok, 2002). Formation of the saccular cargo containers from the ER required functional COPII, but was not prevented by conditions that interfered with membrane fusion (Mironov et al., 2003), suggesting the absence of a budding/fusion cycle between the ER and the saccular container. Nevertheless, there is convincing evidence that ERES and ERGIC are separated membrane entities. For example, the expression of a viral microtubule-binding protein resulted in the full separation of these two structures, as determined by indirect immunofluorescence microscopy (Xu et al., 2000). Another study unveiled that VSV-G that had been misfolded within the 15° compartment (which is the site, equivalent to the ERGIC, where anterograde cargo is blocked by low temperature (Schweizer et al., 1990; Lotti et al., 1992; Klumperman et al., 1998)), can no longer return to the folding machinery in the ER, while VSV-G that had been misfolded in the ERES still can (Mezzacasa and Helenius, 2002).

ERGIC-53-positive membranes could also be co-labelled with anti-VSV-G and were interpreted as carriers in move to the Golgi (Horstmann et al., 2002). On the other hand, the large membrane structures labelled with GFP-ERGIC-53 showed no measurable movement

toward the Golgi, as opposed to VSV-G-GFP (Ben-Tekaya et al., 2004). It is difficult to judge whether the large moving TCs that were discribed using VSV-G-GFP really correspond to the ERGIC membranes that were characterized in ultrastructural studies using antibodies against ERGIC-53. The only evidence that we can find for this is their similar size. We would like to emphasize though that monitoring anterograde marker movement only cannot reveal a stable ERGIC compartment that presumably is left behind by the TC/AC at the ERES. Alternatively, massive VSV-G transport by itself could modulate the membrane dynamics early in the secretory pathway. During the last 20 years, the utility of VSV-G as a marker for the secretory pathway has been manifold. Its exclusive, strong expression and, most importantly, its capacity to be accumulated in and released from the ER, the ERES, the ERGIC or the trans-Golgi network by manipulations as simple as temperature shifts has turned VSV-G into a unique tool for the exploration of the secretory pathway. Nevertheless, it must be considered that massive, synchronized overload of intracellular membranes with a viral transmembrane protein, that is highly specialized for efficient forward transport, i.e. binding the COPII coat (Nishimura and Balch, 1997; Sevier et al., 2000), but not the retrieval coat COPI, may shape the secretory apparatus to best suit viral production. Accordingly, it would seem that these conditions might not faithfully represent normal cell physiology. For example, VSV-G transport as compared to the trafficking of soluble marker proteins might lead to the exaggeration of TC-movement or the suppression of possible post-ER quality control mechanisms (see below). Similarly, synchronization of ER-exit may have the potential to influence the formation and morphology of transport vehicles from the ER membrane. It must be emphasized that both proteins that have been defined as markers for saccular transport, VSV-G and the ~300 nm long procollagen fibers (Mironov et al., 2003), in some respect represent special cases.

Challenging the mechanistically simple TC-model and relaunching the discussion on stationary ERGIC membranes, on the other hand, is bringing back numerous unresolved questions that seem to have fallen into oblivion. What is the molecular machinery that mediates ERGIC-to-Golgi transport? Can COPI vehicles go in anterograde and retrograde direction? What determines the specificity of membrane targeting early in the secretory pathway? What is the physiological relevance of the ERGIC as a stationary compartment? We will try to address these and other issues in the following sections.

3.2 MOLECULAR BASIS OF ANTEROGRADE TRAFFIC IN THE ER-GOLGI INTERFACE

Membrane traffic is organized and specified by a multitude of molecular key players (Bonifacino and Glick, 2004). Transport directionality from a donor to an acceptor compartment is achieved on several levels starting with coat protein-mediated cargo uptake and membrane partition, followed by motor protein-mediated transit, and accomplished by tethering protein- and SNARE protein-mediated delivery. The higher morphological complexity of the ER-Golgi interface in metazoan comparing to yeast cells is also reflected by the severalfold higher complexity of the molecular transport machinery in higher eukaryotes. This may be best illustrated by the Rab GTPases (Ypt proteins in yeast) that act as key membrane organizers involved at all the levels of membrane traffic (Segev, 2001). Yeast contains 11 Rab proteins, 10 of which have an assigned function. Among the 60 Rab proteins found in the human genome (Bock et al., 2001), some share a high degree of both sequence and functional identity to their yeast counterparts, while for many others functional information is still missing. In the following section, we will try to integrate the available mechanistic data on the mammalian ER-Golgi interface into a working model on protein traffic through the ERGIC.

The protein coat COPII is required for ERES-to-ERGIC transport, whereby it is replaced by COPI (Aridor et al., 1995; Scales et al., 1997; Stephens et al., 2000). In the case of COPI, the function in mammals may be somewhat more versatile than in yeast. Although the COPI-mediated retrieval of early resident proteins from ERGIC/Golgi back to the ER that was initially described in yeast (Letourneur et al., 1994) also applies to higher organisms, anti-COPI antibodies have been shown to inhibit transport from the ERGIC to the Golgi (Pepperkok et al., 1993). This may suggest that COPI carriers are additionally involved in anterograde transport. Consistent with this, some membrane carriers labelled by GFP-tagged COPI or by a fluorescent anti-COPI antibody were seen to segregate from ERES and travel to the Golgi (Scales et al., 1997; Stephens et al., 2000; Presley et al., 2002). It can only be speculated on how the same protein coat from the same donor compartment could generate short-range transport back to the ER as well as Golgi-directed AC partition. COPI appears to be a highly adaptable machinery though. Possible modulators of COPI coat formation include transmembrane (Goldberg, 2000) and soluble cargo (Volchuk et al., 2000) as well as membrane curvature (Bigay et al., 2003) and Rab GTPases (Alvarez et al., 2003). It is likely that Rabs play a central role in membrane compartmentalization on the ERGIC. For example,

Rab2 in concert with atypical protein kinase C iota/lambda and glyceraldehyde-3-phosphate dehydrogenase is thought to regulate COPI-dependent retrograde transport from the ERGIC (Tisdale, 2003), while Rab1 that comes in two flavours, Rab1a and Rab1b (Segev, 2001), is involved in membrane tethering (Cao et al., 1998; Allan et al., 2000; Moyer et al., 2001) and influences COPI recruitment (Alvarez et al., 2003). It has also been suggested that the function of protein coats may extend beyond vesicle budding (Klausner et al., 1992; Bonifacino and Lippincott-Schwartz, 2003). Rab GTPase-coordinated COPI-coated membrane domains on the ERGIC (Martinez-Menarguez et al., 1999; Shima et al., 1999) could help organize its partition into a stationary phase and a mobile phase destined for the Golgi.

Another conceivable and non-mutually exclusive mechanism may involve a Rab/motor protein complex in analogy to the Rab6/Rabkinesin6 COPI-independent Golgi-to-ER pathway (Girod et al., 1999; White et al., 1999). Such motor protein-driven anterograde trafficking from the ERGIC might result from the recruitment of the dynein/dynactin complex via BICD (Matanis et al., 2002) to an activated ER-Golgi Rab GTPase that would coordinate membrane segregation. Dynein has been immunolocalized to the ERGIC (Roghi and Allan, 1999) and its displacement arrested the movement of VSV-G-containing TCs (Presley et al., 1997) presumably at the level of the ERGIC. It is important to note that motor protein-mediated membrane transport is strictly dependent on local coat depolymerization on the membrane carrier. Conversely, it is the COPI-coat that is recognized at the ER membrane during the docking of retrograde vesicles (Andag et al., 2001; Reilly et al., 2001; Andag and Schmitt, 2003), suggesting that these vesicles during short range ERGIC-to-ER transport preserve their coat. Therefore, anterograde and retrograde directionality may be determined by the specific modulation of COPI depolymerization kinetics (Goldberg, 2000; Bigay et al., 2003) on distinct membrane carriers. Interestingly, a COPI-dependent tethering mechanism has also been proposed for the docking of retrograde COPI-vesicles to the Golgi (Suvorova et al., 2002).

Can ERES-to-ERGIC and ERGIC-to-Golgi transport additionally be discriminated by the tethering and fusion machineries that accomplish the delivery steps? Concerning the SNARE proteins (Chen and Scheller, 2001), from the available functional data it is impossible to precisely localize the membrane fusion activity of specific SNAREs in the mammalian ER-Golgi interface. ER-Golgi SNAREs generally recycle between ER and Golgi (Chao et al., 1999) via their interactions with COPII (Miller et al., 2003; Mossessova et al., 2003) and COPI (Rein et al., 2002). Nevertheless, there is now wide agreement that – in analogy to yeast

– the major ER-Golgi SNARE complex is formed by Syntaxin 5, Sec22b, Membrin, and Bet1. This complex is thought to be implicated at two membrane fusion interfaces: the fusion of ER-derived vesicles with the ERGIC and the later fusion of ACs at the cis-face of the Golgi. Interestingly, the discovery of a long isoform of Syntaxin 5 in mammalian cells that localizes more prominently to the ERGIC than to the Golgi (Hui et al., 1997) may point to some molecular variation between fusion complexes at the ERGIC and at the cis-Golgi. Alternatively, it has been proposed that a late step in ER-to-Golgi transport may involve a Syntaxin 5-Bet1-GOS-28-Ykt6 SNARE complex (Zhang and Hong, 2001). The full specificity for membrane delivery though does not appear to be encoded by the ER-Golgi SNARE machinery.

Recent research has highlighted the function of so-called tethering complexes that bridge the apposing membranes prior to SNARE complex formation and, therefore, can contribute to the fidelity of membrane fusion (Whyte and Munro, 2002). Beyond this, tethering molecules in the early secretory pathway perform additional key functions such as assistance in SNARE assembly and activation of Rab proteins (see below). A central role in ER-to-Golgi traffic is ascribed to the large coiled-coil protein p115 (Uso1p in yeast). P115 is a Rab1 effector (meaning that it can only interact with GTP-bound Rab1 (Allan et al., 2000)) that is implicated in membrane tethering (Cao et al., 1998; Sonnichsen et al., 1998), catalysis of SNARE complex formation (Sapperstein et al., 1996; Shorter et al., 2002), and presumably COPI-function (Garcia-Mata and Sztul, 2003). Several laboratories have reported that p115 action starts during COPII vesicle biogenesis (Allan et al., 2000; Morsomme and Riezman, 2002; Kondylis and Rabouille, 2003), whereas the guanine nucleotide exchange factor (GEF) that activates Rab1 at the ERES remains to be determined. The finding that the Rab1-p115 interaction during vesicle budding is followed by a p115-SNARE interaction on the vesicle essential for subsequent fusion (Allan et al., 2000) has gained additional mechanistic significance by the discovery that a SNARE motif-related domain within p115 stimulates the formation of fusogenic SNARE complexes (Shorter et al., 2002). It is this SNARE interaction that marks the p115 activity essential for Golgi biogenesis (Puthenveedu and Linstedt, 2004). Antibodies against p115 inhibit ER-to-Golgi transport after ER-exit at the level of the ERGIC (Alvarez et al., 1999), which is consistent with the inability of ER-derived vesicles to complete fusion. Conversely, antibodies against the two p115-interacting golgins GM130 and giantin block transport at the Golgi (Alvarez et al., 2001) suggesting that these tethering proteins act downstream of p115. Kinetic staging experiments revealed that the tethering activity of the Rab1-GTP/GM130 complex (Moyer et al., 2001) preceedes the giantin-requir-

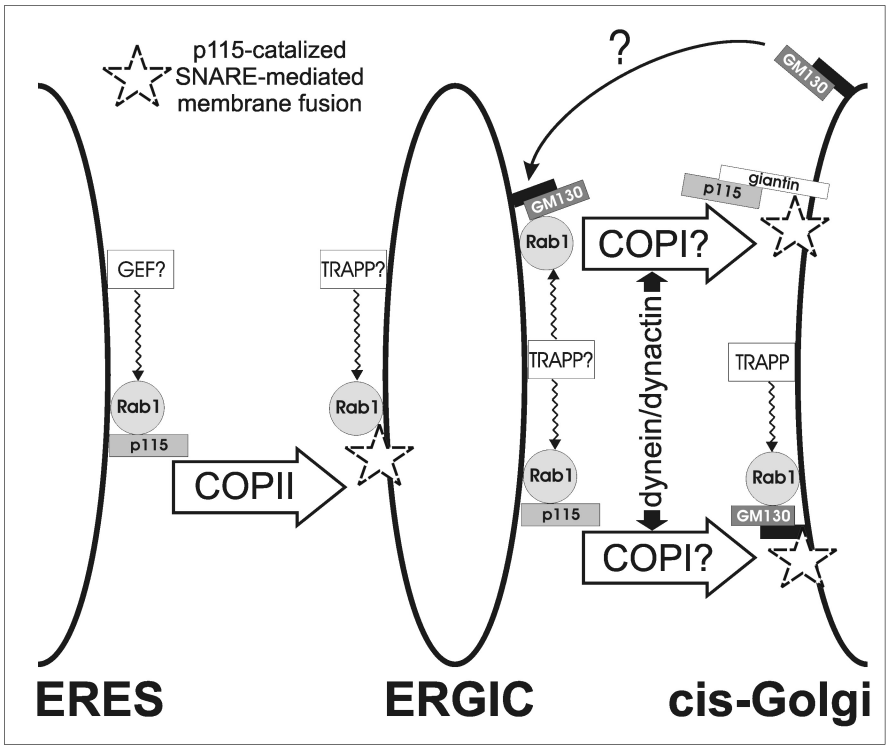


Figure 1

Working model on the molecular basis of membrane transport through the ERGIC.
Shown is a scheme of the early secretory pathway represented by ER-exit site (ERES), ERGIC, and cis-Golgi. Transport from ERES to ERGIC depends on COPII. The transport step from ERGIC to cis-Golgi requires the dynein/dynactin microtubule motor activity and possibly COPI. The targeting of membrane carriers to the correct acceptor compartment is orchestrated by the tethering machinery. First, Rab1 is activated and thereby recruited to the membrane by the action of guanine nucleotide exchange factors (GEFs). The GEF for Rab1 on the ERES is not known, while on the cis-Golgi and presumably on the ERGIC the TRAPP complex exhibits GEF activity toward Rab1 (zigzag-arrows). During ER-exit, activated Rab1 recruits p115 (Allan et al., 2000), which in turn is responsible for the docking of ER-derived vesicles to the ERGIC. This docking event presumably again requires activated Rab1 on the ERGIC (Cao et al., 1998). For ERGIC-to-cis-Golgi transport there are two possible scenarios depicted: In one scenario, p115 is bound to the ERGIC via activated Rab1 in analogy to p115 recruitment during ER-exit. Docking to the cis-Golgi would occur by a p115-GM130 tether, whereas also GM130 that is membrane anchored by binding to the peripheral membrane protein GRASP65 (black rectangel) requires activated Rab1 to display this activity (Moyer et al., 2001). In an alternative scenario, the GM130/GRASP65 complex recycles from the cis-Golgi to the ERGIC (Marra et al., 2001), where it is primed by activated Rab1. Docking to the Golgi would then involve the ternary tether GM130-p115-giantin (Sonnichsen et al., 1998). Membrane docking in all cases is followed by SNARE-mediated membrane fusion that requires catalysis by p115 (Shorter et al., 2002; Puthenveedu and Linstedt, 2004).

ing stage (Alvarez et al., 2001). It has been suggested that the p115-GM130 interaction is needed for the anterograde delivery of ACs into an early Golgi compartment, while the p115-giantin interaction may be essential for vesicular retrograde recycling of Golgi resident proteins to that compartment. Consistent with this, giantin has been localized to the rims of Golgi cisternae that are involved in retrograde vesicle biogenesis (Martinez-Menarguez et al., 2001). Alternatively, GM130 recruitment may take place before the cis-Golgi, where giantin

tethers would then capture ACs via a trimeric GM130-p115-giantin complex (Sonnichsen et al., 1998). Indeed, GFP-tagged GM130 in complex with its membrane anchor GRASP65 (Barr et al., 1998) has been reported to mark a tubular Golgi-to-ERGIC recycling pathway, which has been taken to suggest the existence of a late intermediate compartment in COS7 cells (Marra et al., 2001).

As GM130 and giantin are not present in yeast, we propose that tethering of ER-vesicles to the ERGIC in higher eukaryotes is mechanistically related to the Uso1p/p115-requiring docking of ER-vesicles to the fragmented yeast Golgi. This process has been shown to additionally require Ypt1p (yeast Rab1) (Cao et al., 1998) that presumably has been activated by the GEF activity of the Golgi-localized TRAPP complex (Wang et al., 2000). Somewhat conflicting, TRAPP per se has been shown to be essential (Barrowman et al., 2000) and sufficient (Sacher et al., 2001) for COPII vesicle tethering in another in vitro assay. A subunit of mammalian TRAPP has been localized both to the ERGIC and to the Golgi, when transiently expressed in COS7 cells (Gecz et al., 2000), raising the possibility that TRAPP-catalized Rab1 activation can occur in both compartments. In Fig. 1 we present a working model on the molecular basis of protein transport through the ERGIC.

3.3 POSITIONING OF THE ERGIC WITHIN THE CELL

The importance of the microtubule cytoskeleton on the integrity of the early secretory apparatus is depicted by the repositioning of the ER (Terasaki et al., 1986) and the fragmentation of the Golgi apparatus (Cole et al., 1996) in cells that had been treated with the microtubule depolymerizing agent nocodazole. As to the ERGIC, it has been found that a viral transmembrane microtubule-binding protein relocalizes ERGIC-53-positive membranes away from ERES and arrests anterograde transport from the ERGIC (Xu et al., 2000), indicating that the position and function of the ERGIC in the proximity of ERES critically depends on the dynamic interaction with microtubules. ERGIC membranes do not only associate the microtubule minus-end motor dynein (Roghi and Allan, 1999), but also the plus-end motor kinesin (Lippincott-Schwartz et al., 1995). These two activities may not only contribute to the segregation of ACs from the ERGIC, but also regulate its steady-state positioning on the microtubule cytoskeleton. At 15° ERGIC membranes move closer to the Golgi, raising the possibility that low temperature can modulate the equilibrium of motor protein-activity on the ERGIC. It has also been proposed that both dynein and kinesin motors

remain membrane-connected while recycling between ER and Golgi (Lippincott-Schwartz et al., 1995; Roghi and Allan, 1999). This, however, would require that during transport either activity is to be down-regulated by an as yet unknown mechanism.

3.4 PROTEIN SORTING IN THE ERGIC

The function of the ERGIC as the first sorting station of anterograde- and retrograde-transported proteins is now beyond dispute, presumably owing to the fact that it is independent on the question, of whether the ERGIC consists a stationary compartment or a transient TC. Several investigators have localized the segregation of anterograde-directed- from recycling protein flow to the ERGIC (Aridor et al., 1995; Bu et al., 1995; Klumperman et al., 1998; Appenzeller et al., 1999; Martinez-Menarguez et al., 1999; Ben-Tekaya et al., 2004), demonstrating that this compartment functions as a major crossroad of the biosynthetic protein traffic. In some ways, the ERGIC may be functionally related to the sorting endosome/endocytic recycling compartment system that represents the branch point in the receptor-mediated endocytosis pathway (Maxfield and McGraw, 2004).

The issue of protein sorting in the ERGIC gains complexity in the case of cargo proteins that require the assistance of recycling transport receptors during ER-export (Appenzeller et al., 1999; Muniz et al., 2000; Belden and Barlowe, 2001; Powers and Barlowe, 2002). Such proteins, for successful anterograde delivery from the ERGIC, need to be physically released from their receptors, suggesting that the ERGIC exhibits features different from the ER-environment, where receptor-association occurs. For example, it has been proposed that the ERGIC, contrary to the ER, maintains a low pH environment (Palokangas et al., 1998). In line with this, the efficient release of procathepsin Z from its pH-sensitive transport receptor ERGIC-53 requires organelle acidification (Appenzeller-Herzog et al., 2004). Perhaps, low pH in the ERGIC represents the general trigger for ER-export receptor-cargo dissociation. Furthermore, the acidification of pre-Golgi/Golgi compartments appears to be important for other processes than ER-export receptor function (Fig. 2). pH-dependent binding of the ER-resident protein RAP to LDL receptor-related protein, which prevents premature ligand interactions, is released in the ERGIC, where the two proteins segregate. Other than that, lowered organellar pH is thought to contribute to the effective recognition and ER-retrieval of KDEL ligands from post-ER compartments by the KDEL receptor. Most interestingly, the pH-dependence of substrate binding by the KDEL receptor

(Scheel and Pelham, 1996) and ERGIC-53 (Appenzeller-Herzog et al., 2004) are inversed, which reflects their reciprocal mode of action (Fig. 2). The steady-state localization as well as the recycling pathway of the two receptors, however, slightly differ, with the KDEL receptor distribution shifted preferentially to the Golgi region (Tang et al., 1995). It has been well established that only the binding of ligands will stimulate the COPI-mediated retrograde transport of KDEL receptor (Lewis and Pelham, 1992), possibly via homooligomerization (Aoe et al., 1998) or cytosolic phosphorylation of the receptor (Cabrera et al., 2003). This raises the possibility that acidification along the ER-Golgi pathway may occur gradually, presumably by selective uptake of active H^+ v-ATPase pumps during AC partition from the ERGIC, and that the optimal pH for KDEL ligand binding may be only achieved at the level of the cis-Golgi.

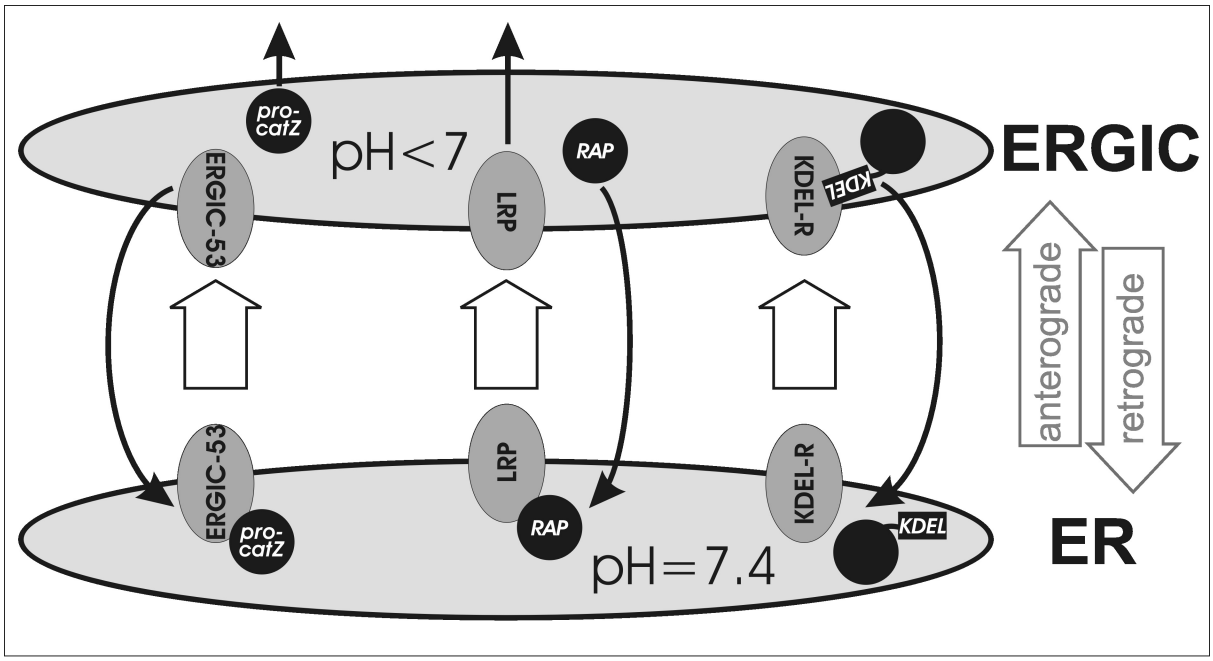


Figure 2

pH-dependent sorting between ER and ERGIC.
Proteins travel from ER to ERGIC in anterograde- and from ERGIC to ER in retrograde direction. The pH of the ER equals 7.4, while the lumen of the ERGIC is acidified. The scheme represents three different pH-dependent sorting mechanisms of anterograde-directed and/or recycling proteins in the early secretory pathway. ERGIC-53 functions as a transport receptor for the secretory/lysosomal protein procathepsin Z (pro-catZ). Association between ERGIC-53 and pro-catZ occurs in the ER at neutral pH, and the interaction is released upon transport to the ERGIC by acid-induced protonation of the ligand-binding site in ERGIC-53 (Appenzeller-Herzog et al., 2004). While ERGIC-53 is recycled back to the ER, pro-catZ proceeds through the secretory pathway. The LDL receptor-related protein (LRP) during transit from ER to ERGIC is complexed by the escort protein RAP (Bu et al., 1995), which inhibits receptor-ligand interactions in the early secretory pathway. Again, low pH in the ERGIC triggers the dissociation of the two proteins. Subsequently, RAP is retrieved to the ER by a C-terminal HNEL motif (presumably involving the KDEL receptor), and LRP travels through the Golgi to the cell surface. Binding of the KDEL receptor (KDEL-R) to escaped ER proteins comprising a C-terminal KDEL sequence, on the other hand, is thought to require the acidified pH in post-ER compartments (Scheel and Pelham, 1996). The ligand binding in turn triggers the retrograde translocation of KDEL receptor-ligand complexes to the ER.

How then can ERGIC-53, whose recycling pathway mostly bypasses the cis-Golgi (Tang et al., 1995; Klumperman et al., 1998), insure proper ligand-release despite the supposedly minimal pH-gradient from ER to ERGIC? Binding of ERGIC-53 to its substrates requires calcium (Itin et al., 1996; Appenzeller et al., 1999), and in vitro binding experiments have shown that the pH-sensitivity of ERGIC-53 was increased, when calcium concentrations were low (Appenzeller-Herzog et al., 2004). Accordingly, a mechanism that maintains lower calcium levels in the ERGIC would promote pH-induced cargo release. Of note, an imaging approach to visualize total calcium (i.e. the sum of free and bound calcium) in quick frozen-freeze dried PC12 cells demonstrated high titers of calcium in ER and Golgi, but ERGIC elements remained below detection level (Pezzati et al., 1997). Although this does not prove an analogous drop in free calcium, which is the physiologically relevant fraction for calcium-dependent processes, it clearly underlines the distinct identity of the ERGIC in relation to ER and Golgi that may provide an optimized platform for ligand-withdrawal from ERGIC-53 and other cargo receptors. It will be interesting to quantitatively assess changes in free calcium and pH from the ER to the ERGIC.

3.5 ADDITIONAL FUNCTIONS OF THE ERGIC

Does the function of the ERGIC go beyond sorting out and retrieval of escaped ER-resident proteins and of components of the trafficking machinery? One additional function of the ERGIC is to mediate an initial concentration step of abundant secretory cargo that is thought to exit the ER by a non-selective process. In this way, amylase and chymotrypsinogen in pancreatic acinar cells were not concentrated in COPII-buds but only at the level of the ERGIC (Martinez-Menarguez et al., 1999). The authors proposed that this concentration of secretory cargo in the ERGIC directly resulted from its exclusion from retrograde-directed COPI vesicles.

In addition to that, evidence is accumulating that the ERGIC might participate in some mechanisms of conformation-based protein quality control and, possibly, protein folding. A proteomic approach to spot proteins cycling in the early secretory pathway, identified – in addition to cargo receptors and membrane trafficking proteins – a number of protein folding chaperones that were enriched in ERGIC-like membranes upon brefeldin A-treatment (Breuza et al., 2004). Likewise, an ultrastructural study using quantitative immunoelectron microscopy localized the highest labelling intensity of the glycoprotein folding sensor UDP-

glucose:glycoprotein glucosyltransferase to the ERGIC (Zuber et al., 2001). If, however, the maturation of a given folding substrate was to partially occur beyond the ER, we would expect that a dead-end folding variant of this substrate is re-routed back to the ER, the major site where terminally misfolded proteins are cleared from the biosynthetic pathway (known as "ER-associated degradation"). This retrieval pathway for ER-associated degradation has been independently discovered by two laboratories in yeast (Caldwell et al., 2001; Vashist et al., 2001). A model has emerged implying that the retrieval pathway is molecularly distinct from the conventional retention pathway and can serve as a backup-system that will be upscaled by the unfolded protein response, when the ER-retention pathway is saturated (Haynes et al., 2002). Moreover, we now know that the post-ER quality control checkpoint is only required for the processing of certain substrate proteins (Vashist and Ng, 2004).

Once again, for most of these implications from yeast studies, we can find evidence in mammalian cells, whereas, not surprisingly, cell-type specificity has been observed. For example, VSV-G, when expressed in Vero cells, blocked in the ERGIC at 15° and then unfolded, did neither become reglucosylated by glucosyltransferase, nor recycled back to the ER for further folding assistance, but rather escaped into the Golgi and beyond (Mezzacasa and Helenius, 2002). If, however, VSV-G protein is expressed in CHO cells at the restrictive temperature that will not allow its folding, instead of being trapped in the ER, it can move to the ERGIC and the cis-Golgi in complex with the soluble folding chaperone BiP, but is then recycled back to the ER (Hammond and Helenius, 1994). Finally, in HepG2 cells, VSV-G is rapidly degraded at the restrictive temperature, but acquires cis-Golgi specific modifications ahead of its withdrawal (Spiro and Spiro, 2001). In the case of unassembled T-cell antigen receptor α-chains, a similar recycling route together with BiP has been demonstrated, whereas the retrieval occurs via the KDEL receptor/COPI pathway, presumably by the recognition of the C-terminal KDEL-motif in BiP (Yamamoto et al., 2001). Other folding substrates that have been suggested to be subject of quality control in the ERGIC include unassembled MHC class I (Hsu et al., 1991), mutant forms of sucrase-isomaltase and lysosomal α-glucosidase (Moolenaar et al., 1997), connexin E186K (VanSlyke et al., 2000), and soluble IgM molecules (Elkabetz et al., 2003). Taken together, incompletely folded proteins that escape the ER-checkpoints because of system overload or failed recognition can be caught by a sequential checkpoint mechanism in the ERGIC.

Albeit speculative, we believe that the ERGIC quality control checkpoint can be seen on the job by expression of GFP-ERGIC-53. Stationary ERGIC structures, when imaged at short time intervals, are connected by rapidly moving, short-lived elements of variable,

sometimes tubular shape (Ben-Tekaya et al., 2004). These elements were more abundant upon rewarming from a 15° transport block, or if GFP-ERGIC-53 was overexpressed, indicating that artificial overload of ERGIC membranes had the potential to induce their appearance. Conversely, they were absent from cells upon microtubule depolymerization. Similar transport intermediates on the move along microtubules were observed with the anterograde marker lum-GFP after release from the 15° block (Blum et al., 2000). Interestingly, these lum-GFP labelled tubules initially colocalized with ERGIC-53, but not with VSV-G positive structures that appeared to be interconnected by GFP-tubules. Likewise, tubular structures emanating from the 15° compartment upon rewarming were seen in fixed cells with endogenous marker proteins (Tang et al., 1995; Klumperman et al., 1998). We propose that these fast moving carriers do not represent anterograde or retrograde carriers, but functionally connect the discontinuous ERGIC system by mediating horizontal transfer of critical components. It is also possible that tubular transport is required to efficiently balance compartmental membrane distribution. It has been shown that the generation of these tubular transport intermediates depends on the activity of cytoplasmic phospholipase A_2 enzymes (Brown et al., 2003).

3.6 CONCLUDING REMARKS

Although the TC-model is now widely accepted to explain the numerous aspects of ER-to-Golgi transport, its formulation was largely based on a single marker protein, VSV-G, and it is incompatible with recently published live cell imaging studies. Irrespectively, the TCs defined by VSV-G-GFP and the ACs that segregate from the stationary GFP-ERGIC-53 structures both move to the Golgi and therefore may represent a common, reconciling theme. There are basically two possibilities: (i) VSV-G TCs really do segregate from the ERGIC, marked by ERGIC-53, by a dissociative process that has never been monitored, or (ii) synchronized VSV-G transport by itself potently modulates the organization of the early secretory pathway leading to the recruitment of the ERGIC as a whole to Golgi-directed movement. It must be emphasized that the latter scenario may simply reflect the capability of the secretory apparatus to adapt to temporary high-throughput conditions. Co-labeling of VSV-G and ERGIC-53 in live cells will be required to resolve this issue. The relevance of a stationary ERGIC sorting compartment may be the necessity to largely extract recycling material and quality control substrates before long range transport to the perinuclear Golgi.

This energy saving mechanism may have only evolved in higher eukaryotes as opposed to small yeast cells.

Another open question is, how ACs are incorporated into the Golgi apparatus. It is conceivable that the cisternal maturation model (Storrie and Nilsson, 2002) may apply to post-ERGIC transport, implying that ACs undergo homotypic fusion to give rise to a new cis-Golgi stack. This stack in turn would further mature by sorting out cis-Golgi proteins and receiving medial/trans-Golgi proteins from later cisternae by COPI vesicular transport. This "cisternal maturation beyond the ERGIC" model may be supported by the fact that ERGIC membranes do not collapse upon brefeldin A-treatment (Breuza et al., 2004). This fungal drug that extracts COPI-components from membranes leads to the spectacular breakdown of the Golgi apparatus, possibly reflecting the COPI-dependent integrity of this organelle that is brought forward by the cisternal maturation hypothesis.

It will be required in the future to reproduce the partition of isolated ERGIC membranes in the test tube. Thereby, it may be important to complement the activity of Rab GTPases and COPI components with microtubules and motor proteins. Other Rab proteins that are lacking an assigned location and function so far could be essential for the proper organisation of ERGIC membranes. Furthermore, it will be required to establish the weight of saccular versus vesicular budding in the early secretory pathway and to precisely localize the subcellular activity and requirement of tethering and SNARE molecules. Another interesting field of research will concern the changed luminal environment of the ERGIC as compared to the ER and the molecular determinants in those ERAD substrates that require transport to the ERGIC to be properly degraded. Although the available data combining live cell imaging and electron microscopy have started to create the basis for our understanding of protein sorting in and transport through the ERGIC, the molecular events that govern these events remain to be elucidated in the future that, for sure, will continue to be controversial, but exiting.

References

Allan, B.B., Moyer, B.D. and Balch, W.E. (2000) Rab1 recruitment of p115 into a cis-SNARE complex: programming budding COPII vesicles for fusion. *Science*, 289, 444-448.

Alvarez, C., Fujita, H., Hubbard, A. and Sztul, E. (1999) ER to Golgi transport: Requirement for p115 at a pre-Golgi VTC stage. *J Cell Biol*, 147, 1205-1222.

Alvarez, C., Garcia-Mata, R., Brandon, E. and Sztul, E. (2003) COPI recruitment is modulated by a Rab1b-dependent mechanism. *Mol Biol Cell*, 14, 2116-2127.

Alvarez, C., Garcia-Mata, R., Hauri, H.P. and Sztul, E. (2001) The p115-interactive proteins GM130 and giantin participate in endoplasmic reticulum-Golgi traffic. *J Biol Chem*, 276, 2693-2700.

Andag, U., Neumann, T. and Schmitt, H.D. (2001) The coatomer-interacting protein Dsl1p is required for Golgi-to-endoplasmic reticulum retrieval in yeast. *J Biol Chem*, 276, 39150-39160.

Andag, U. and Schmitt, H.D. (2003) Dsl1p, an essential component of the Golgi-endoplasmic reticulum retrieval system in yeast, uses the same sequence motif to interact with different subunits of the COPI vesicle coat. *J Biol Chem*, 278, 51722-51734.

Aoe, T., Lee, A.J., van Donselaar, E., Peters, P.J. and Hsu, V.W. (1998) Modulation of intracellular transport by transported proteins: insight from regulation of COPI-mediated transport. *Proc Natl Acad Sci U S A*, 95, 1624-1629.

Appenzeller, C., Andersson, H., Kappeler, F. and Hauri, H.P. (1999) The lectin ERGIC-53 is a cargo transport receptor for glycoproteins. *Nat Cell Biol*, 1, 330-334.

Appenzeller-Herzog, C., Roche, A.C., Nufer, O. and Hauri, H.P. (2004) pH-induced conversion of the transport lectin ERGIC-53 triggers glycoprotein release. *J Biol Chem*, 279, 12943-12950.

Aridor, M., Bannykh, S.I., Rowe, T. and Balch, W.E. (1995) Sequential coupling between COPII and COPI vesicle coats in endoplasmic reticulum to Golgi transport. *J Cell Biol*, 131, 875-893.

Bannykh, S.I. and Balch, W.E. (1997) Membrane dynamics at the endoplasmic reticulum-Golgi interface. *J Cell Biol*, 138, 1-4.

Bannykh, S.I., Nishimura, N. and Balch, W.E. (1998) Getting into the Golgi. *Trends Cell Biol*, 8, 21-25.

Bannykh, S.I., Rowe, T. and Balch, W.E. (1996) The organization of endoplasmic reticulum export complexes. *J Cell Biol*, 135, 19-35.

Barlowe, C. (2002) COPII-dependent transport from the endoplasmic reticulum. *Curr Opin Cell Biol*, 14, 417-422.

Barr, F.A., Nakamura, N. and Warren, G. (1998) Mapping the interaction between GRASP65 and GM130, components of a protein complex involved in the stacking of Golgi cisternae. *Embo J*, 17, 3258-3268.

Barrowman, J., Sacher, M. and Ferro-Novick, S. (2000) TRAPP stably associates with the Golgi and is required for vesicle docking. *Embo J*, 19, 862-869.

Belden, W.J. and Barlowe, C. (2001) Role of Erv29p in collecting soluble secretory proteins into ER-derived transport vesicles. *Science*, 294, 1528-1531.

Ben-Tekaya, H., Miura, K., Pepperkok, R. and Hauri, H.P. (2004) ER-Golgi intermediate compartment clusters define a stationary sorting compartment. *in preparation.*

Bigay, J., Gounon, P., Robineau, S. and Antonny, B. (2003) Lipid packing sensed by ArfGAP1 couples COPI coat disassembly to membrane bilayer curvature. *Nature*, 426, 563-566.

Blum, R., Stephens, D.J. and Schulz, I. (2000) Lumenal targeted GFP, used as a marker of soluble cargo, visualises rapid ERGIC to Golgi traffic by a tubulo-vesicular network. *J Cell Sci*, 113 (Pt 18), 3151-3159.

Bock, J.B., Matern, H.T., Peden, A.A. and Scheller, R.H. (2001) A genomic perspective on membrane compartment organization. *Nature*, 409, 839-841.

Bonifacino, J.S. and Glick, B.S. (2004) The mechanisms of vesicle budding and fusion. *Cell*, 116, 153-166.

Bonifacino, J.S. and Lippincott-Schwartz, J. (2003) Coat proteins: shaping membrane transport. *Nat Rev Mol Cell Biol*, 4, 409-414.

Breuza, L., Halbeisen, R., Jenö, P., Otte, S., Barlowe, C., Hong, W. and Hauri, H.P. (2004) Proteomics of ERGIC membranes identifies ERGIC-32, a new cycling protein interacting with Erv46. *in preparation.*

Brown, W.J., Chambers, K. and Doody, A. (2003) Phospholipase A2 (PLA2) enzymes in membrane trafficking: mediators of membrane shape and function. *Traffic*, 4, 214-221.

Bu, G., Geuze, H.J., Strous, G.J. and Schwartz, A.L. (1995) 39 kDa receptor-associated protein is an ER resident protein and molecular chaperone for LDL receptor-related protein. *Embo J*, 14, 2269-2280.

Cabrera, M., Muniz, M., Hidalgo, J., Vega, L., Martin, M.E. and Velasco, A. (2003) The retrieval function of the KDEL receptor requires PKA phosphorylation of its C-terminus. *Mol Biol Cell*, 14, 4114-4125.

Caldwell, S.R., Hill, K.J. and Cooper, A.A. (2001) Degradation of endoplasmic reticulum (ER) quality control substrates requires transport between the ER and Golgi. *J Biol Chem*, 276, 23296-23303.

Cao, X., Ballew, N. and Barlowe, C. (1998) Initial docking of ER-derived vesicles requires Uso1p and Ypt1p but is independent of SNARE proteins. *Embo J*, 17, 2156-2165.

Chao, D.S., Hay, J.C., Winnick, S., Prekeris, R., Klumperman, J. and Scheller, R.H. (1999) SNARE membrane trafficking dynamics in vivo. *J Cell Biol*, 144, 869-881.

Chen, Y.A. and Scheller, R.H. (2001) SNARE-mediated membrane fusion. *Nat Rev Mol Cell Biol*, 2, 98-106.

Cole, N.B., Sciaky, N., Marotta, A., Song, J. and Lippincott-Schwartz, J. (1996) Golgi dispersal during microtubule disruption: regeneration of Golgi stacks at peripheral endoplasmic reticulum exit sites. *Mol Biol Cell*, 7, 631-650.

Elkabetz, Y., Kerem, A., Tencer, L., Winitz, D., Kopito, R.R. and Bar-Nun, S. (2003) Immunoglobulin light chains dictate vesicular transport-dependent and -independent routes for IgM degradation by the ubiquitin-proteasome pathway. *J Biol Chem*, 278, 18922-18929.

Fan, J.Y., Roth, J. and Zuber, C. (2003) Ultrastructural analysis of transitional endoplasmic reticulum and pre-Golgi intermediates: a highway for cars and trucks. *Histochem Cell Biol*, 120, 455-463.

Garcia-Mata, R. and Sztul, E. (2003) The membrane-tethering protein p115 interacts with GBF1, an ARF guanine-nucleotide-exchange factor. *EMBO Rep*, 4, 320-325.

Gecz, J., Hillman, M.A., Gedeon, A.K., Cox, T.C., Baker, E. and Mulley, J.C. (2000) Gene structure and expression study of the SEDL gene for spondyloepiphyseal dysplasia tarda. *Genomics*, 69, 242-251.

Girod, A., Storrie, B., Simpson, J.C., Johannes, L., Goud, B., Roberts, L.M., Lord, J.M., Nilsson, T. and Pepperkok, R. (1999) Evidence for a COP-I-independent transport route from the Golgi complex to the endoplasmic reticulum. *Nat Cell Biol*, 1, 423-430.

Goldberg, J. (2000) Decoding of sorting signals by coatomer through a GTPase switch in the COPI coat complex. *Cell*, 100, 671-679.

Hammond, A.T. and Glick, B.S. (2000) Dynamics of transitional endoplasmic reticulum sites in vertebrate cells. *Mol Biol Cell*, 11, 3013-3030.

Hammond, C. and Helenius, A. (1994) Quality control in the secretory pathway: retention of a misfolded viral membrane glycoprotein involves cycling between the ER, intermediate compartment, and Golgi apparatus. *J Cell Biol*, 126, 41-52.

Hauri, H.P., Kappeler, F., Andersson, H. and Appenzeller, C. (2000) ERGIC-53 and traffic in the secretory pathway. *J Cell Sci*, 113 (Pt 4), 587-596.

Hauri, H.P. and Schweizer, A. (1992) The endoplasmic reticulum-Golgi intermediate compartment. *Curr Opin Cell Biol*, 4, 600-608.

Haynes, C.M., Caldwell, S. and Cooper, A.A. (2002) An HRD/DER-independent ER quality control mechanism involves Rsp5p-dependent ubiquitination and ER-Golgi transport. *J Cell Biol*, 158, 91-101.

Horstmann, H., Ng, C.P., Tang, B.L. and Hong, W. (2002) Ultrastructural characterization of endoplasmic reticulum--Golgi transport containers (EGTC). *J Cell Sci*, 115, 4263-4273.

Hsu, V.W., Yuan, L.C., Nuchtern, J.G., Lippincott-Schwartz, J., Hammerling, G.J. and Klausner, R.D. (1991) A recycling pathway between the endoplasmic reticulum and the Golgi apparatus for retention of unassembled MHC class I molecules. *Nature*, 352, 441-444.

Hui, N., Nakamura, N., Sonnichsen, B., Shima, D.T., Nilsson, T. and Warren, G. (1997) An isoform of the Golgi t-SNARE, syntaxin 5, with an endoplasmic reticulum retrieval signal. *Mol Biol Cell*, 8, 1777-1787.

Itin, C., Roche, A.C., Monsigny, M. and Hauri, H.P. (1996) ERGIC-53 is a functional mannose-selective and calcium-dependent human homologue of leguminous lectins. *Mol Biol Cell*, 7, 483-493.

Klausner, R.D., Donaldson, J.G. and Lippincott-Schwartz, J. (1992) Brefeldin A: insights into the control of membrane traffic and organelle structure. *J Cell Biol*, 116, 1071-1080.

Klumperman, J., Schweizer, A., Clausen, H., Tang, B.L., Hong, W., Oorschot, V. and Hauri, H.P. (1998) The recycling pathway of protein ERGIC-53 and dynamics of the ER-Golgi intermediate compartment. *J Cell Sci*, 111 (Pt 22), 3411-3425.

Kondylis, V. and Rabouille, C. (2003) A novel role for dp115 in the organization of tER sites in Drosophila. *J Cell Biol*, 162, 185-198.

Letourneur, F., Gaynor, E.C., Hennecke, S., Demolliere, C., Duden, R., Emr, S.D., Riezman, H. and Cosson, P. (1994) Coatomer is essential for retrieval of dilysine-tagged proteins to the endoplasmic reticulum. *Cell*, 79, 1199-1207.

Lewis, M.J. and Pelham, H.R. (1992) Ligand-induced redistribution of a human KDEL receptor from the Golgi complex to the endoplasmic reticulum. *Cell*, 68, 353-364.

Lippincott-Schwartz, J., Cole, N.B., Marotta, A., Conrad, P.A. and Bloom, G.S. (1995) Kinesin is the motor for microtubule-mediated Golgi-to-ER membrane traffic. *J Cell Biol*, 128, 293-306.

Lippincott-Schwartz, J., Snapp, E. and Kenworthy, A. (2001) Studying protein dynamics in living cells. *Nat Rev Mol Cell Biol*, 2, 444-456.

Lotti, L.V., Torrisi, M.R., Pascale, M.C. and Bonatti, S. (1992) Immunocytochemical analysis of the transfer of vesicular stomatitis virus G glycoprotein from the intermediate compartment to the Golgi complex. *J Cell Biol*, 118, 43-50.

Marra, P., Maffucci, T., Daniele, T., Tullio, G.D., Ikehara, Y., Chan, E.K., Luini, A., Beznoussenko, G., Mironov, A. and De Matteis, M.A. (2001) The GM130 and GRASP65 Golgi proteins cycle through and define a subdomain of the intermediate compartment. *Nat Cell Biol*, 3, 1101-1113.

Martinez-Menarguez, J.A., Geuze, H.J., Slot, J.W. and Klumperman, J. (1999) Vesicular tubular clusters between the ER and Golgi mediate concentration of soluble secretory proteins by exclusion from COPI-coated vesicles. *Cell*, 98, 81-90.

Martinez-Menarguez, J.A., Prekeris, R., Oorschot, V.M., Scheller, R., Slot, J.W., Geuze, H.J. and Klumperman, J. (2001) Peri-Golgi vesicles contain retrograde but not anterograde proteins consistent with the cisternal progression model of intra-Golgi transport. *J Cell Biol*, 155, 1213-1224.

Matanis, T., Akhmanova, A., Wulf, P., Del Nery, E., Weide, T., Stepanova, T., Galjart, N., Grosveld, F., Goud, B., De Zeeuw, C.I., Barnekow, A. and Hoogenraad, C.C. (2002) Bicaudal-D regulates COPI-independent Golgi-ER transport by recruiting the dynein-dynactin motor complex. *Nat Cell Biol*, 4, 986-992.

Maxfield, F.R. and McGraw, T.E. (2004) Endocytic recycling. *Nat Rev Mol Cell Biol*, 5, 121-132.

Mellman, I. and Simons, K. (1992) The Golgi complex: in vitro veritas? *Cell*, 68, 829-840.

Mezzacasa, A. and Helenius, A. (2002) The transitional ER defines a boundary for quality control in the secretion of tsO45 VSV glycoprotein. *Traffic*, 3, 833-849.

Miller, E.A., Beilharz, T.H., Malkus, P.N., Lee, M.C., Hamamoto, S., Orci, L. and Schekman, R. (2003) Multiple cargo binding sites on the COPII subunit Sec24p ensure capture of diverse membrane proteins into transport vesicles. *Cell*, 114, 497-509.

Mironov, A.A., Mironov, A.A., Jr., Beznoussenko, G.V., Trucco, A., Lupetti, P., Smith, J.D., Geerts, W.J., Koster, A.J., Burger, K.N., Martone, M.E., Deerinck, T.J., Ellisman, M.H. and Luini, A. (2003) ER-to-Golgi carriers arise through direct en bloc protrusion and multistage maturation of specialized ER exit domains. *Dev Cell*, 5, 583-594.

Moolenaar, C.E., Ouwendijk, J., Wittpoth, M., Wisselaar, H.A., Hauri, H.P., Ginsel, L.A., Naim, H.Y. and Fransen, J.A. (1997) A mutation in a highly conserved region in brush-border sucrase-isomaltase and lysosomal alpha-glucosidase results in Golgi retention. *J Cell Sci*, 110 (Pt 5), 557-567.

Morsomme, P. and Riezman, H. (2002) The Rab GTPase Ypt1p and tethering factors couple protein sorting at the ER to vesicle targeting to the Golgi apparatus. *Dev Cell*, 2, 307-317.

Mossessova, E., Bickford, L.C. and Goldberg, J. (2003) SNARE selectivity of the COPII coat. *Cell*, 114, 483-495.

Moyer, B.D., Allan, B.B. and Balch, W.E. (2001) Rab1 interaction with a GM130 effector complex regulates COPII vesicle cis--Golgi tethering. *Traffic*, 2, 268-276.

Muniz, M., Nuoffer, C., Hauri, H.P. and Riezman, H. (2000) The Emp24 complex recruits a specific cargo molecule into endoplasmic reticulum-derived vesicles. *J Cell Biol*, 148, 925-930.

Nishimura, N. and Balch, W.E. (1997) A di-acidic signal required for selective export from the endoplasmic reticulum. *Science*, 277, 556-558.

Palokangas, H., Ying, M., Vaananen, K. and Saraste, J. (1998) Retrograde transport from the pre-Golgi intermediate compartment and the Golgi complex is affected by the vacuolar H+-ATPase inhibitor bafilomycin A1. *Mol Biol Cell*, 9, 3561-3578.

Pepperkok, R., Scheel, J., Horstmann, H., Hauri, H.P., Griffiths, G. and Kreis, T.E. (1993) Beta-COP is essential for biosynthetic

membrane transport from the endoplasmic reticulum to the Golgi complex in vivo. *Cell*, 74, 71-82.

Pezzati, R., Bossi, M., Podini, P., Meldolesi, J. and Grohovaz, F. (1997) High-resolution calcium mapping of the endoplasmic reticulum-Golgi-exocytic membrane system. Electron energy loss imaging analysis of quick frozen-freeze dried PC12 cells. *Mol Biol Cell*, 8, 1501-1512.

Powers, J. and Barlowe, C. (2002) Erv14p directs a transmembrane secretory protein into COPII-coated transport vesicles. *Mol Biol Cell*, 13, 880-891.

Presley, J.F., Cole, N.B., Schroer, T.A., Hirschberg, K., Zaal, K.J. and Lippincott-Schwartz, J. (1997) ER-to-Golgi transport visualized in living cells. *Nature*, 389, 81-85.

Presley, J.F., Ward, T.H., Pfeifer, A.C., Siggia, E.D., Phair, R.D. and Lippincott-Schwartz, J. (2002) Dissection of COPI and Arf1 dynamics in vivo and role in Golgi membrane transport. *Nature*, 417, 187-193.

Puthenveedu, M.A. and Linstedt, A.D. (2004) Gene replacement reveals that p115/SNARE interactions are essential for Golgi biogenesis. *Proc Natl Acad Sci U S A*, 101, 1253-1256.

Reilly, B.A., Kraynack, B.A., VanRheenen, S.M. and Waters, M.G. (2001) Golgi-to-endoplasmic reticulum (ER) retrograde traffic in yeast requires Dsl1p, a component of the ER target site that interacts with a COPI coat subunit. *Mol Biol Cell*, 12, 3783-3796.

Rein, U., Andag, U., Duden, R., Schmitt, H.D. and Spang, A. (2002) ARF-GAP-mediated interaction between the ER-Golgi v-SNAREs and the COPI coat. *J Cell Biol*, 157, 395-404.

Roghi, C. and Allan, V.J. (1999) Dynamic association of cytoplasmic dynein heavy chain 1a with the Golgi apparatus and intermediate compartment. *J Cell Sci*, 112 (Pt 24), 4673-4685.

Sacher, M., Barrowman, J., Wang, W., Horecka, J., Zhang, Y., Pypaert, M. and Ferro-Novick, S. (2001) TRAPP I implicated in the specificity of tethering in ER-to-Golgi transport. *Mol Cell*, 7, 433-442.

Sapperstein, S.K., Lupashin, V.V., Schmitt, H.D. and Waters, M.G. (1996) Assembly of the ER to Golgi SNARE complex requires Uso1p. *J Cell Biol*, 132, 755-767.

Saraste, J. and Svensson, K. (1991) Distribution of the intermediate elements operating in ER to Golgi transport. *J Cell Sci*, 100 (Pt 3), 415-430.

Scales, S.J., Pepperkok, R. and Kreis, T.E. (1997) Visualization of ER-to-Golgi transport in living cells reveals a sequential mode of action for COPII and COPI. *Cell*, 90, 1137-1148.

Scheel, A.A. and Pelham, H.R. (1996) Purification and characterization of the human KDEL receptor. *Biochemistry*, 35, 10203-10209.

Schweizer, A., Fransen, J.A., Bachi, T., Ginsel, L. and Hauri, H.P. (1988) Identification, by a monoclonal antibody, of a 53-kD protein associated with a tubulo-vesicular compartment at the cis-side of the Golgi apparatus. *J Cell Biol*, 107, 1643-1653.

Schweizer, A., Fransen, J.A., Matter, K., Kreis, T.E., Ginsel, L. and Hauri, H.P. (1990) Identification of an intermediate compartment involved in protein transport from endoplasmic reticulum to Golgi apparatus. *Eur J Cell Biol*, 53, 185-196.

Schweizer, A., Matter, K., Ketcham, C.M. and Hauri, H.P. (1991) The isolated ER-Golgi intermediate compartment exhibits properties that are different from ER and cis-Golgi. *J Cell Biol*, 113, 45-54.

Segev, N. (2001) Ypt and Rab GTPases: insight into functions through novel interactions. *Curr Opin Cell Biol*, 13, 500-511.

Sesso, A., de Faria, F.P., Iwamura, E.S. and Correa, H. (1994) A three-dimensional reconstruction study of the rough ER-Golgi interface in serial thin sections of the pancreatic acinar cell of the rat. *J Cell Sci*, 107 (Pt 3), 517-528.

Sevier, C.S., Weisz, O.A., Davis, M. and Machamer, C.E. (2000) Efficient export of the vesicular stomatitis virus G protein from the endoplasmic reticulum requires a signal in the cytoplasmic tail that includes both tyrosine-based and di-acidic motifs. *Mol Biol Cell*, 11, 13-22.

Shima, D.T., Scales, S.J., Kreis, T.E. and Pepperkok, R. (1999) Segregation of COPI-rich and anterograde-cargo-rich domains in endoplasmic-reticulum-to-Golgi transport complexes. *Curr Biol*, 9, 821-824.

Shorter, J., Beard, M.B., Seemann, J., Dirac-Svejstrup, A.B. and Warren, G. (2002) Sequential tethering of Golgins and catalysis of SNAREpin assembly by the vesicle-tethering protein p115. *J Cell Biol*, 157, 45-62.

Sitia, R. and Meldolesi, J. (1992) Endoplasmic reticulum: a dynamic patchwork of specialized subregions. *Mol Biol Cell*, 3, 1067-1072.

Sonnichsen, B., Lowe, M., Levine, T., Jamsa, E., Dirac-Svejstrup, B. and Warren, G. (1998) A role for giantin in docking COPI vesicles to Golgi membranes. *J Cell Biol*, 140, 1013-1021.

Spang, A. (2002) ARF1 regulatory factors and COPI vesicle formation. *Curr Opin Cell Biol*, 14, 423-427.

Spiro, M.J. and Spiro, R.G. (2001) Release of polymannose oligosaccharides from vesicular stomatitis virus G protein during endoplasmic reticulum-associated degradation. *Glycobiology*, 11, 803-811.

Stephens, D.J., Lin-Marq, N., Pagano, A., Pepperkok, R. and Paccaud, J.P. (2000) COPI-coated ER-to-Golgi transport complexes segregate from COPII in close proximity to ER exit sites. *J Cell Sci*, 113 (Pt 12), 2177-2185.

Stephens, D.J. and Pepperkok, R. (2001) Illuminating the secretory pathway: when do we need vesicles? *J Cell Sci*, 114, 1053-1059.

Stephens, D.J. and Pepperkok, R. (2002) Imaging of procollagen transport reveals COPI-dependent cargo sorting during ER-to-Golgi transport in mammalian cells. *J Cell Sci*, 115, 1149-1160.

Storrie, B. and Nilsson, T. (2002) The Golgi apparatus: balancing new with old. *Traffic*, 3, 521-529.

Suvorova, E.S., Duden, R. and Lupashin, V.V. (2002) The Sec34/Sec35p complex, a Ypt1p effector required for retrograde intra-Golgi trafficking, interacts with Golgi SNAREs and COPI vesicle coat proteins. *J Cell Biol*, 157, 631-643.

Tang, B.L., Low, S.H., Hauri, H.P. and Hong, W. (1995) Segregation of ERGIC53 and the mammalian KDEL receptor upon exit from the 15 degrees C compartment. *Eur J Cell Biol*, 68, 398-410.

Terasaki, M., Chen, L.B. and Fujiwara, K. (1986) Microtubules and the endoplasmic reticulum are highly interdependent structures. *J Cell Biol*, 103, 1557-1568.

Tisdale, E.J. (2003) Rab2 interacts directly with atypical protein kinase C (aPKC) iota/lambda and inhibits aPKCiota/lambda-dependent glyceraldehyde-3-phosphate dehydrogenase phosphorylation. *J Biol Chem*, 278, 52524-52530.

VanSlyke, J.K., Deschenes, S.M. and Musil, L.S. (2000) Intracellular transport, assembly, and degradation of wild-type and disease-linked mutant gap junction proteins. *Mol Biol Cell*, 11, 1933-1946.

Vashist, S., Kim, W., Belden, W.J., Spear, E.D., Barlowe, C. and Ng, D.T. (2001) Distinct retrieval and retention mechanisms are required for the quality control of endoplasmic reticulum protein folding. *J Cell Biol*, 155, 355-368.

Vashist, S. and Ng, D.T. (2004) Misfolded proteins are sorted by a sequential checkpoint mechanism of ER quality control. *J Cell Biol*, 165, 41-52.

Volchuk, A., Amherdt, M., Ravazzola, M., Brugger, B., Rivera, V.M., Clackson, T., Perrelet, A., Sollner, T.H., Rothman, J.E. and Orci, L. (2000) Megavesicles implicated in the rapid transport of intracisternal aggregates across the Golgi stack. *Cell*, 102, 335-348.

Wang, W., Sacher, M. and Ferro-Novick, S. (2000) TRAPP stimulates guanine nucleotide exchange on Ypt1p. *J Cell Biol*, 151, 289-296.

White, J., Johannes, L., Mallard, F., Girod, A., Grill, S., Reinsch, S., Keller, P., Tzschaschel, B., Echard, A., Goud, B. and Stelzer, E.H. (1999) Rab6 coordinates a novel Golgi to ER retrograde transport pathway in live cells. *J Cell Biol*, 147, 743-760.

Whyte, J.R. and Munro, S. (2002) Vesicle tethering complexes in membrane traffic. *J Cell Sci*, 115, 2627-2637.

Xu, A., Bellamy, A.R. and Taylor, J.A. (2000) Immobilization of the early secretory pathway by a virus glycoprotein that binds to microtubules. *Embo J*, 19, 6465-6474.

Yamamoto, K., Fujii, R., Toyofuku, Y., Saito, T., Koseki, H., Hsu, V.W. and Aoe, T. (2001) The KDEL receptor mediates a retrieval mechanism that contributes to quality control at the endoplasmic reticulum. *Embo J*, 20, 3082-3091.

Zhang, T. and Hong, W. (2001) Ykt6 forms a SNARE complex with syntaxin 5, GS28, and Bet1 and participates in a late stage in endoplasmic reticulum-Golgi transport. *J Biol Chem*, 276, 27480-27487.

Zuber, C., Fan, J.Y., Guhl, B., Parodi, A., Fessler, J.H., Parker, C. and Roth, J. (2001) Immunolocalization of UDP-glucose:glycoprotein glucosyltransferase indicates involvement of pre-Golgi intermediates in protein quality control. *Proc Natl Acad Sci U S A*, 98, 10710-10715.