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UNIVERSITY OF CALIFORNIA, SAN DIEGO

A declaration of independence: The Golgi apparatus is here to stay

A Dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Matthew Y. Pecot

Committee in charge:

Professor Vivek Malhotra, Chair
Professor Scott Emr, Co-Chair
Professor James Feramisco
Professor Randolph Hampton
Professor Michael Karin

2006

The Dissertation of Matthew Y. Pecot is approved, and it is acceptable in quality and form for publication on microfilm:

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Co-Chair

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Chair

University of California, San Diego

2006

This Dissertation is dedicated to my parents, Justin and Karen Pecot who have sacrificed much of their happiness so that I would have all the opportunities in life they never had.

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VITA

1999 BS, Biochemistry and Cell Biology, University of California, San Diego

2006 PhD., Biomedical Sciences, University of California, San Diego

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ABSTRACT OF THE DISSERTATION

A declaration of independence: The Golgi apparatus is here to stay

by

Matthew Y. Pecot

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2006

Professor Vivek Malhotra, Chair

Professor Scott Emr, Co-Chair

How does the Golgi apparatus maintain its organization amidst the constant flow of traffic through the secretory pathway? It was previously believed that cells maintain biochemically distinct membranes rather than constantly reproducing them, and that daughter cells inherit differentiated membrane from the mother. However, recent

evidence suggests that the entire Golgi apparatus rapidly cycles through the Endoplasmic Reticulum (ER), indicating that Golgi membranes are constantly formed from ER membranes. We have tested this idea with a procedure that traps Golgi proteins in the ER when they visit there. Rapamycin induces a specific association between FKBP and FRAP. Golgi enzymes fused to FKBP can be captured in the ER when they visit there by an ER protein fused to FRAP in the presence of rapamycin. With this method the rate at which Golgi proteins associate with the ER can be measured while the secretory pathway remains intact.

In Chapter I the ER-trapping procedure is utilized to test whether Golgi membranes fuse with the ER during cell division in mammalian cells. In mitotic cells Golgi membranes are broken down into small elements and then reformed in daughter cells. It had been reported that these small elements fuse with the ER, indicating that the Golgi is made de novo from the ER of each daughter cell. A sialyltransferase-FKBP reporter was not captured in the ER of dividing cells demonstrating that Golgi membranes remain separate from the ER during mitosis.

Chapter II investigates the behavior of Golgi proteins in non-dividing cells. We found that, unlike a component of the ER-Golgi Intermediate Compartment (ERGIC) both early and late Golgi enzymes do not constitutively cycle through the ER. This combined with our

findings from Chapter I indicate that Golgi membranes are maintained independent of the ER.

In Chapter III the roles of GRASP65 in mitotic Golgi fragmentation and cell cycle progression are investigated. We discovered that phosphorylation of GRASP65 is required to initiate Golgi fragmentation and mitotic progression. We have also identified a 75 amino acid region of the protein that interacts with the factor(s) responsible for these events.

INTRODUCTION

A. History of the Golgi apparatus

In the late 1800's Camillo Golgi developed a complex staining procedure that allowed individual neurons to be visualized in their entirety for the first time (Golgi *et al.*, 2001). In his observations he consistently noticed the presence of a peri-nuclear structure he called "apparato reticulare interno", which we presently refer to as the Golgi apparatus, the Golgi complex, or oftentimes just the Golgi. We now know that the Golgi apparatus is an intracellular membrane-bound compartment that is involved in a variety of cellular activities including protein secretion and glycosylation. However, in the decades following Golgi's discovery the existence of the Golgi apparatus was challenged and considered by many to be an artifact of his staining procedure. The advent of the electron microscope in the early 1950's allowed intracellular structures to be resolved with high resolution and in short time, mostly due to the work of Dalton and Felix (Dalton and Felix, 1954, 1956), the existence of the Golgi apparatus was generally accepted. It was found that Golgi's staining procedure reproducibly labeled membrane structures called lamellae (cisternae), and in the years that followed the morphological characteristics of the Golgi apparatus in many different cell types were documented. It became generally

accepted that the Golgi complex consisted of cisternae (the most prominent Golgi structure), small vesicles, and vacuoles. It was also understood that cisternal elements were curved, resembling arcs with narrow bodies and dilated rims, and that they packed together forming polarized stacks such that one side was convex and the other was concave. In most cases the convex side (*cis*) of cisternal stacks was found to associate with small vesicles and face the Endoplasmic Reticulum (ER), while the concave side (*trans*) was affiliated with vacuoles and faced the plasma membrane.

The development of phosphatase chemistry in the early 60's revealed the heterogeneity of Golgi membranes. The activities of specific enzymes, as visualized by electron microscopy (EM), were found to differ between cisternae of the same stack, and in some cases were observed exclusively in cisternal rims, demonstrating that enzymes are heterogeneously distributed between cisternae of a given stack as well as within individual cisternae (Farquhar *et al.*, 1974; Cheng and Farquhar, 1976). The asymmetric organization of enzymes within the Golgi apparatus hinted that the latter may contain specialized compartments for the modification of specific substrates.

In the mid to late 60's, the application of auto-radiographic techniques, which allowed researchers to label and follow newly synthesized proteins, revealed the majority of what we currently understand regarding Golgi function. Previously, light microscopy

experiments illustrated the close association of Golgi membranes with secretory granules suggesting a role for the Golgi in protein secretion. Electron micrographs confirmed this as they clearly displayed the contents of secretory granules within the dilated rims of Golgi cisternae (Farquhar and Rinehart, 1954; Sjostrand and Hanzon, 1954). The revolutionary experiments performed by Palade and colleagues mapped the intracellular route taken by newly synthesized radio-labeled secretory proteins, including the position of the Golgi apparatus along this pathway (Jamieson and Palade, 1967, 1971). From their work it became clear that newly synthesized secretory proteins first travel to the rough ER, and are transported to the Golgi via small vesicles. Although the mode of transport through the Golgi was not determined (and is still controversial), it was shown that secretory cargo are shipped to condensing vacuoles (which associate with the *trans*-Golgi), or are directly concentrated in the rims of the *trans*-most Golgi cisternae. In the latter case the concentrated protein is thought to bud from the Golgi in vesicles which fuse to form mature secretory granules.

Neutra and LeBlond utilized autoradiography to study intracellular sugar metabolism. In their experiments animals were treated with radio-labeled hexose, and the regions within cells that were associated with the auto-radiographic grains at certain times after hexose treatment were observed. They discovered that, for the cell types tested, the vast majority of the autoradiography was found in the Golgi apparatus

(Neutra and Leblond, 1966). These findings provided the first evidence for the Golgi's involvement in the biogenesis of glyco-proteins. More in depth analyses performed by LeBlond and colleagues demonstrated that core glycosylation (mannose) occurs in the rough ER, while terminal glycosylation (galactose, fucose, sialic acid) occurs in the Golgi apparatus (Whur *et al.*, 1969; Haddad *et al.*, 1971), documenting the first example of intracellular separation of labor (Farquhar and Palade, 1981). Similarly, Godman and Lane performed autoradiography experiments in which they observed the initial distribution of radiolabeled sulfate when it was introduced into goblet and cartilage cells (Godman and Lane, 1964). They found that the radioactive grains immediately and explicitly labeled Golgi membranes. It is now understood that many molecules including lipids, glycoproteins, and steroid hormones become sulfated within cells, and it is believed that this reaction takes place exclusively in the Golgi apparatus.

In the late 60's it became known that many secretory and membrane proteins undergo proteolysis as a means for activation. One example of such a protein is pro-insulin, an inactive insulin precursor that becomes active upon cleavage. In 1970 Steiner and colleagues found that inhibiting membrane transport from the ER to the Golgi apparatus prevented the production of insulin from proinsulin (Steiner *et al.*, 1980). This was the first clue that pro-secretory proteins are activated via proteolysis in the Golgi complex. Today, it is known that

Golgi-specific proteases (e.g. furin) regulate many aspects of physiology by controlling the biogenesis of growth factors, chemokines, and cell adhesion molecules.

Even though much had been learned about Golgi function in the 60's Golgi-specific components were yet to be identified. Enzymes known to be associated with Golgi membranes were also found to localize to other regions of the cell, and as a result preparations of Golgi membranes could not be confirmed unambiguously. However, in 1969 Fleischer and Ozawa discovered that a galactosyltransferase activity responsible for terminal glycosylation was highly concentrated in Golgi membranes purified from bovine liver (Fleischer *et al.*, 1969). This discovery revealed for the first time a marker of the Golgi apparatus that could be used to specifically identify the organelle. Over the years reagents that detect glycosyltransferases have been indispensable to researchers striving to understand the particular behavior of Golgi membranes. To this day observing galactosyltransferases is still the most accurate way to identify the Golgi apparatus.

B. The Golgi apparatus, Endoplasmic Reticulum, and the secretory pathway

The secretory pathway describes the biogenesis of secretory proteins inside the cell. Proteins that are inserted into membranes or secreted from the cell traverse this pathway to achieve biological maturity (become active), and reach their respective destinations. Proteins that make up the cell surface and extracellular matrix, as well those that signal between cells or function in the extracellular space are examples of secretory proteins. These molecules are essential for intercellular communication and play important roles in development and organizmal homeostasis. In addition, proteins that facilitate protein trafficking and maintain the organization of the secretory pathway are also referred to as secretory proteins.

Nascent secretory proteins (secretory cargo) are translated into the Endoplasmic Reticulum (ER), a vast network of membrane and protein that surrounds the nucleus and extends into the cell periphery (see Figure 1 for an illustration of the secretory pathway). Inside the ER secretory cargoes undergo folding and core glycosylation. When the proteins are ready to leave the ER they are packaged into COPII coated transport carriers, which bud from the ER (Barlowe *et al.*, 1994) and deliver the cargoes to the Golgi apparatus, a membrane-bound compartment localized near the centrioles in mammalian cells. Currently, there are

two models for protein transport between the ER and Golgi. The first model called the Transport Complex (TC) model, predicts that COPII coated transport carriers fuse homotypically to generate the ER-Golgi Intermediate Compartment (ERGIC) (Bannykh *et al.*, 1998). In this model the ERGIC consists of large membrane clusters that contain secretory cargoes and stain positive for the lectin ERGIC-53, a cargo-receptor that cycles between the ER and Golgi. These ERGIC elements are thought to travel to the Golgi through their association with microtubules, and either fuse with or give rise to Golgi membranes. However, recent evidence supports a different model for ER-Golgi transport. Live imaging studies investigating the dynamics of secretory cargo and ERGIC-53 indicate that ERGIC elements are stationary compartments that receive secretory cargo from the ER in a COPII dependent process and sort the cargo into transport carriers that travel to the Golgi apparatus (Ben-Tekaya *et al.*, 2005).

The Golgi apparatus is a ribbon of connected stacks of flattened membrane called cisternae. The cisternae in each Golgi stack are organized cis-trans (early-late) such that the cis-face is near the ER, and the trans-side faces the plasma membrane. Secretory cargoes enter the cis-Golgi and leave through the trans-Golgi. As they move through the stack they are modified in a sequential manner by the polarized array of Golgi enzymes (enzymes that specifically localize to Golgi membranes). Types of Golgi enzymes include; glycosylation enzymes that attach

sugar residues to secretory proteins, sulfation enzymes that attach sulfate groups to cargo, and proteolytic enzymes that cleave and activate precursor proteins. These enzymes act sequentially and rely on the actions of earlier enzymes to generate the appropriate substrates. Thus, by regulating the activity of Golgi enzymes the cell can control the molecular composition of the cell surface (glycosylation pattern) which is important for intercellular interactions and responses to extracellular signals. Cargoes move through the Golgi stack until they reach the Trans-Golgi Network (TGN), where they are sorted into distinct transport carriers and delivered to their respective destinations. The movement of secretory cargo through the Golgi is not well understood. Thus far, two models have been proposed to describe traffic through Golgi membranes. These models are not mutually exclusive, and it is likely that both are correct to a certain degree. At present the 'cisternal maturation' model (Glick *et al.*, 1997) is the most accepted of these. This model predicts that secretory cargoes are transported through the Golgi within individual cisterna that mature cis-trans by the retrograde (backward) transport of Golgi enzymes. This mode of transport explains how very large secretory cargoes (larger than the size of vesicles) reach the cell surface without leaving Golgi cisternae (Becker and Melkonian, 1996). Alternatively, the 'vesicle shuttle model' (Malhotra and Mayor, 2006) predicts that secretory cargoes travel between Golgi cisternae via COPI coated vesicles. In this model Golgi cisternae are stable

compartments that retain Golgi enzymes within their boundaries, allowing cargo molecules to advance while maintaining the polarized organization of Golgi membranes. Much of the evidence supporting this model comes from in vitro assays that demonstrate the transport of secretory cargoes between cisternae from different populations of Golgi membranes (Fries and Rothman, 1981). However, as was discussed earlier this model cannot account for the secretion of cargo molecules much too large to fit into COPI coated vesicles (Mironov *et al.*, 2001). Even so, it is likely that cisternal maturation and vesicle shuttling occur simultaneously, depending on the secretory activity required and the types of cargo being secreted.

From the TGN secretory cargoes can be; transported out of the cell, expressed on the cell surface, or delivered to another membrane bound compartment called an endosome. Endosomes are dynamic structures that play major roles in the recycling of proteins from the cell surface and endocytosis.

C. Golgi fragmentation and a novel cell cycle checkpoint

In mammalian cells the Golgi apparatus is a ribbon of connected stacks that are anchored to the pericentriolar region. During mitosis Golgi membranes are disassembled into small elements that become dispersed throughout the cell (Warren, 1993). This process occurs in two steps (Colanzi *et al.*, 2003a). First, the Golgi is fragmented into large pieces that move away from the pericentriolar region. Second, these large fragments are further broken down into vesiculo-tubular elements that appear as a 'haze' by fluorescence microscopy (Pecot and Malhotra, 2004). As daughter cells separate Golgi membranes reform around the centrioles of each new cell (Warren, 1993).

There has been much interest in understanding mitotic Golgi fragmentation on a molecular level because it would reveal how Golgi membranes are organized under normal conditions. Two in vitro assays that reconstitute this process have been used to identify proteins involved in Golgi fragmentation. One assay developed by Malhotra and colleagues involves incubating permeabilized Normal Rat Kidney (NRK) cells with mitotic extract. Under these conditions step 1 of Golgi fragmentation (large fragments) is reproduced (Acharya *et al.*, 1998; Colanzi *et al.*, 2000). With this approach it has been shown that the MAPK pathway, Polo-like Kinase 1 (Plk1), and the Golgi associated proteins GRASP55 and GRASP65 are involved in Golgi disassembly

during mitosis (Acharya *et al.*, 1998; Colanzi *et al.*, 2000; Sutterlin *et al.*, 2001; Sutterlin *et al.*, 2002). GRASP65 is a substrate of Plk1 in vivo (Lin *et al.*, 2000) and it has been shown that ERK2 specifically phosphorylates GRASP55 during mitosis (Jesch *et al.*, 2001). The other approach involves incubating Golgi membranes purified from rat liver with mitotic extract. With this method it was found that phosphorylation of the peripheral Golgi protein GM130 by cdc2 was required for Golgi fragmentation (Lowe *et al.*, 1998). The main theme that can be extracted from these in vitro studies is that Golgi disassembly occurs through the phosphorylation of structural components. GRASP65 and GRASP55 were both discovered as essential proteins for the stacking of Golgi cisternae in vitro (Barr *et al.*, 1997). GM130 was identified as a component of a detergent-resistant matrix that shares morphological characteristics with the Golgi apparatus (Nakamura *et al.*, 1995). It has been reported that GRASP65 is the binding partner for GM130 on Golgi membranes (reference) and that this interaction is required to maintain the organization of the Golgi (reference). The phosphorylation of structural Golgi proteins may be analogous to weakening the supports of a large building. When a building is destroyed explosives take out the supporting structures that hold the building together, causing the building to collapse. Proteins like GRASP65, GRASP55, and GM130 may play a similar role for Golgi membranes. However, unlike fallen

buildings the Golgi apparatus can be quickly rebuilt by reversing the phosphorylation of these proteins.

Interestingly, reagents that antagonize GRASP65 and GRASP55 prevent Golgi fragmentation and arrest cells in G2 (reference). Cells enter mitosis normally in the presence of these reagents when the Golgi is artificially fragmented by the addition of Brefeldin A (BFA), a fungal metabolite, or nocodazole, a microtubule de-polymerizing agent (Sutterlin *et al.*, 2002). This suggests that Golgi fragmentation must occur for cells to enter mitosis, and that cells monitor Golgi structure, possibly through a cell cycle checkpoint. It is not clear why Golgi fragmentation is required for mitotic entry, but there are many possibilities. Perhaps, in addition to its function as a secretory organelle the Golgi also acts as a scaffold to regulate the activity of proteins that act at the G2/M transition. Golgi fragmentation would release such components, allowing them to travel to their respective sites of action and drive cell cycle progression. GRASP65 and GMAP210 have been shown to be involved in the formation of the mitotic spindle (Sutterlin *et al.*, 2005). Additionally, other Golgi localized proteins appear to play roles in chromosome segregation and cytokinesis (reference). Another possibility is that the physical presence of the Golgi in the pericentriolar region prevents cell cycle progression. Mammalian cells keep Golgi membranes anchored to the centrioles, but Golgi membranes in *Drosophila*, *cerevesiae*, and plants are randomly dispersed (referecen).

Additionally, *cerevesiae* and plants do not fragment their Golgi membranes (reference), and preventing mitotic disassembly of the Golgi in *drosophila* has no effect on cell cycle progression (reference). Perhaps mammalian cells maintain the Golgi in the pericentriolar region to possess additional control over ell division.

D. De novo biogenesis of the Golgi apparatus: fact or fiction?

For over a decade it has been debated whether Golgi membranes fuse with the ER or remain as discrete elements during cell division. Specifically, the morphology of the Golgi 'haze' (step 2 of mitotic Golgi fragmentation) has been sought after. If Golgi membranes fuse with the ER during mitosis then they must form de novo from the ER of each daughter cell. If Golgi elements remain separate from the ER then the Golgi is inherited from pre-existing elements. Biochemical methods indicate that the Golgi remains separate from the ER during mitosis (Jesch and Linstedt, 1998). However, the population of mitotic cells collected for this study could have been enriched for cells in which the Golgi is in the form of large chunks, before formation of the 'haze'. Fluorescence microscopy has also revealed that Golgi membranes remain independent of the ER during cell division (Shima *et al.*, 1998). However, a photo-bleaching study has demonstrated that the mobility of Golgi enzymes during mitosis is the same as the mobility of Golgi enzymes in the ER in cells treated with BFA (Zaal *et al.*, 1999). Thus the issue remains controversial.

The concept of de novo Golgi biogenesis is supported by studies examining the behavior of Golgi enzymes in non-dividing cells. In cells treated with BFA, which inhibits the recruitment of Arf1 to Golgi membranes, or a dominant-negative form of sar1 (sar1dn), a GTPase

required for ER exit, protein transport from the ER is inhibited (Fujiwara *et al.*, 1988; Aridor *et al.*, 1995). Since, under these conditions Golgi enzymes rapidly accumulate in the ER it was proposed that Golgi enzymes constitutively cycle through the ER. Further analysis through use of sar1dn revealed that all Golgi proteins recycle through the ER with t ½ ~1h (Miles *et al.*, 2001). Based on these findings the entire Golgi apparatus cycles through the ER every couple of hours, indicating that Golgi membranes undergo constant biogenesis from the ER (Miles *et al.*, 2001; Ward *et al.*, 2001). The recycling of Golgi membranes has been proposed to account for Golgi fragmentation in response to microutubule de-polymerizing agents (Storrie *et al.*, 1998). Under these conditions the Golgi appears as individual stacks that localize adjacent to ER exit sites (Thyberg and Moskalewski, 1985; Cole *et al.*, 1996). A similar situation has been suggested to occur during cell division when microtubules are de-polymerized to form the mitotic spindle (Altan-Bonnet *et al.*, 2006), supporting the idea that the Golgi membranes fuse with the ER during cell division.

It seems clear that Golgi enzymes have the capacity to rapidly relocate into the ER under artificial conditions, but what happens when the secretory pathway is not perturbed?

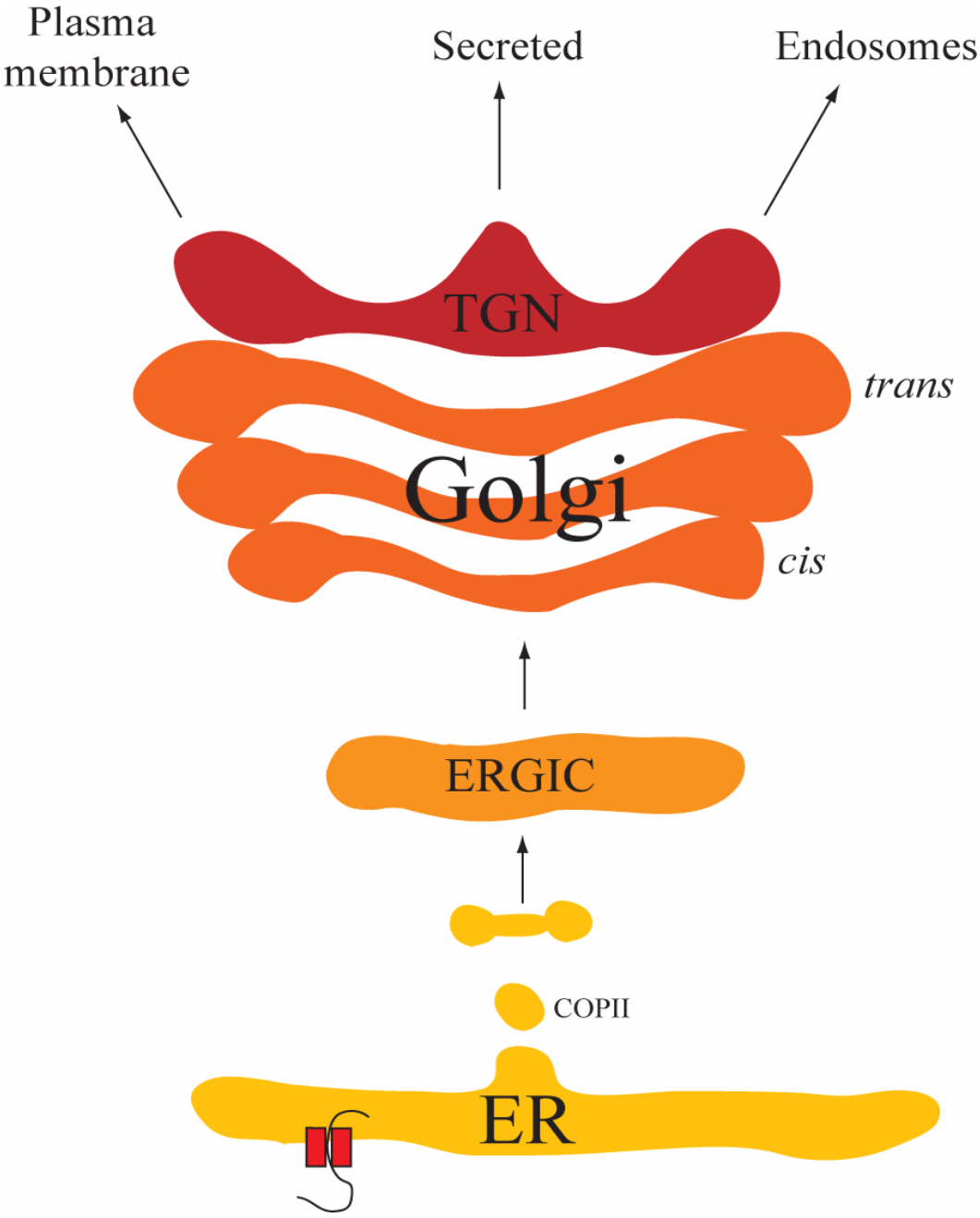


Figure 1. Newly synthesized secretory proteins are translated into the Endoplasmic Reticulum and shipped to the Golgi apparatus via COPII coated transport carriers by way of the ER-Golgi Intermediate Compartment (ERGIC). Inside Golgi membranes secretory proteins are modified *cis-trans* by the array of Golgi enzymes. When the cargos have moved through the Golgi stack into the *Trans*-Golgi Network (TGN) they are segregated into specific transport carriers that will shuttle them out of the cell, to the cell surface, or to the endosomes.

CHAPTER I

The Golgi apparatus remains segregated from the Endoplasmic Reticulum during mitosis in mammalian cells

1.1 ABSTRACT

What happens to organelles during mitosis, and how they are apportioned to each of the daughter cells, is not completely clear. We have devised a procedure to address whether Golgi membranes fuse with the Endoplasmic Reticulum (ER) during mitosis via the detection of interactions between ER and Golgi proteins. This procedure involves coexpressing an FKBP-tagged Golgi enzyme with an ER-retained protein fused to FRAP in COS cells. Since treatment with rapamycin induces a tight association between FKBP and FRAP, one would expect rapamycin to trap the FKBP-fused Golgi protein in the ER if it ever visits the ER during mitosis. However, after the doubly transfected cells progress through mitosis in the presence of rapamycin, we find the Golgi protein in the newly formed Golgi stacks and not in the ER. Based on these results, we conclude that Golgi membranes remain separate from the ER during mitosis in mammalian cells

1.2 INTRODUCTION

In mammalian cells the Golgi apparatus is anchored to the pericentriolar region and is organized as a ribbon of connected stacks. In organisms such as plants, yeast, and *Drosophila*, the Golgi is randomly dispersed throughout the cytosol (Preuss *et al.*, 1992; Stanley *et al.*, 1997; Nebenfuhr and Staehelin, 2001). It is not clear why mammalian cells keep their Golgi membranes near the centrioles. During mitosis, mammalian cells sequentially fragment their pericentriolar Golgi into small pieces (Warren, 1993; Colanzi *et al.*, 2003a). In general it is understood that this occurs via a two-step process (Colanzi *et al.*, 2003a). In prophase pericentriolar Golgi membranes are fragmented into small elements referred to as "Golgi blobs" (first step). These blobs undergo further fragmentation and appear as a haze by fluorescent microscopy sometime between prophase and anaphase (second step). As daughter cells begin to separate, the Golgi haze is converted into larger Golgi fragments that eventually reform in the pericentriolar region of each daughter cell. For over a decade, it has been debated whether the Golgi haze represents small Golgi fragments or Golgi membranes fused with the ER (Puthenveedu and Linstedt, 2001). The lack of conclusive evidence either way has polarized the field into two ways of thinking about how Golgi membranes are inherited. In one view, the Golgi is inherited

independent of the ER (Shima *et al.*, 1997; Jesch and Linstedt, 1998). The other proposal is that during mitosis, Golgi membranes fuse with the ER and are partitioned into daughter cells through the ER (Thyberg and Moskalewski, 1992; Zaal *et al.*, 1999).

We have designed an assay to address the issue of whether Golgi membranes fuse with the ER during mitotic progression in mammalian cells. This assay relies on the ability of two proteins to conditionally bind in the presence of ligand. Rapamycin, a small molecule, binds to the FK506 binding protein (FKBP) (Wiederrecht *et al.*, 1991). The FKBP-rapamycin associated protein (FRAP) binds to the FKBP-rapamycin complex (Brown *et al.*, 1994; Sabatini *et al.*, 1994), and only in the presence of rapamycin do these molecules interact. We have fused FKBP to a Golgi enzyme of the medial to trans Golgi cisternae, sialyltransferase (ST, ST-FKBP), and FRAP to an ER-retained version of the human invariant chain protein (Ii, Ii-FRAP). If Golgi membranes are redistributed into the ER with Brefeldin A, Ii-FRAP quickly and efficiently captures ST-FKBP in the presence of rapamycin. When pre-mitotic COS-7 cells expressing ST-FKBP and Ii-FRAP are treated with rapamycin, we find that, after cell division, ST-FKBP is found in reforming Golgi membranes and is not retained in the ER. We conclude that, in mammalian cells, the Golgi apparatus is inherited independent of the ER.

1.3 RESULTS

A Procedure for Trapping Golgi Enzymes in the ER

We have constructed a fusion protein (ST-FKBP) containing the FKBP coding region fused to the Golgi localization domain of sialyltransferase (ST). Green fluorescent protein (GFP) is appended to the C-terminus of this construct (Figure 1Aa). By fluorescence microscopy this construct localizes to the Golgi when expressed in COS7 cells (Figure 1Ac). Western blot analysis of immunoprecipitations and transfected lysate show proteins of the predicted size (Figure 1Ab). Another construct, named Ii-FRAP, consists of the FKBP-rapamycin binding domain fused between two regions of the human invariant γ chain protein (Ii) and the HA epitope (Figure 1Ba). The invariant γ chain protein contains an arginine-based ER retention motif (Schutze *et al.,* 1994) that allows it to remain in the ER until it associates with the β chain of the MHC class II molecule (Khalil *et al.*, 2003). In COS7 cells, the MHC class II β chain is not expressed, and the invariant γ chain region of Ii-FRAP is sufficient to restrict it to the ER, where it colocalizes with calreticulin (Figure 1Bc). This construct is also expressed at the expected molecular weight as determined by Western blots of lysate from transfected cells and immunoprecipitations (Figure 1Bb). When electroporation is used to introduce ST-FKBP and Ii-FRAP cDNAs into COS7 cells, we find that,

by fluorescence microscopy, a high percentage of cells (approximately 60%) are cotransfected. Under these conditions ST-FKBP and Ii-FRAP remain separate as visualized by fluorescent microscopy, and are found in the Golgi and ER, respectively (Figure 1C).

When membrane-permeant rapamycin is added to cells coexpressing ST-FKBP and Ii-FRAP it should bind to FKBP in the lumen of the Golgi. Ii-FRAP would be prevented from binding the ST-FKBP-rapamycin complex because it is retained in the ER. However, if, in the presence of rapamycin, Golgi membranes were induced to fuse with the ER, then Ii-FRAP should bind to ST-FKBP bound to rapamycin. Importantly, once formed in the ER, the ST-FKBP-Ii-FRAP complex should remain in the ER even after Golgi membranes are allowed to relocate to the pericentriolar region, because of the ER-retention motif present in Ii-FRAP. To test whether Ii-FRAP can trap ST-FKBP in the ER, we designed an experiment in which cotransfected COS7 cells are treated with Brefeldin A (BFA), a drug that induces the Golgi to fuse with the ER (Lippincott-Schwartz *et al.*, 1989), for 1 hr at 37ºC. This incubation is carried out in the presence or absence of rapamycin. After 1 hr, BFA and excess rapamycin are washed out (rapamycin bound to FKBP is not washed out) and the cells are incubated in fresh medium for 90 min. At this time we determine by fluorescence microscopy if ST-FKBP is found at the pericentriolar region or in the ER bound to Ii-FRAP (Figure 2A). After a 1 hr BFA treatment ST-FKBP is in the ER

and colocalizes with Ii-FRAP. When the cells are released from BFA in the absence of rapamycin ST-FKBP relocalizes to the pericentriolar region. In the presence of rapamycin following BFA release ST-FKBP remains in the ER and colocalizes extensively with Ii-FRAP. Under these conditions, the integrity of the Golgi is unaffected, as observed by fluorescence microscopy with an antibody specific for GM130, a Golgi associated protein (data not shown). To establish the efficiency and kinetics with which ST-FKBP is captured by Ii-FRAP, we determined the percentage of cotransfected cells that have ST-FKBP either at the pericentriolar region or in the ER after BFA release in the presence or absence of rapamycin for assorted periods of time (Figure 2B). To fuse the Golgi with the ER, BFA with or without rapamycin was added to electroporated COS7 cells for 1 hr at 37ºC. The cells were then washed with PBS and incubated in fresh media for 90 min, or incubated with rapamycin for 2.5, 5, or 10 min in the presence of BFA and then washed and incubated with fresh media for 90 min. At this time the cells were fixed and prepared for fluorescence microscopy. After BFA release in the absence of rapamycin, 97% of cotransfected cells have ST-FKBP at the pericentiolar region and 3% contain it in the ER (cotransfected cells expressing high levels of Ii-FRAP were scored). Of cotransfected cells treated with BFA *and* rapamycin, after drug release only 30% show ST-FKBP at the pericentriolar region while 70% display it in the ER. This trapping efficiency is the same if cells are treated with rapamycin for 10,

5, or just 2.5 min prior to drug washout. From these results, we conclude that Ii-FRAP quickly and efficiently traps ST-FKBP in the ER in the presence of rapamycin when Golgi-ER fusion is induced by BFA.

Do Golgi Membranes Fuse with the ER during Mitosis?

To visualize the dynamics of Golgi membranes during mitosis, COS7 cells expressing ST-FKBP and Ii-FRAP were plated on glass coverslips coated with pronectin F and arrested in S-phase overnight with aphidicolin as described previously (Acharya *et al.*, 1998; Sutterlin *et al.*, 2002). 8.5 hr after aphidicolin release a significant percentage of cells are found in various stages of mitosis. At this time, the cells were fixed and prepared for fluorescence microscopy (Figure 3). In interphase cells, ST-FKBP and GM130 (data not shown) localize at the pericentriolar region and Ii-FRAP forms a reticular network around the nucleus. During prophase, ST-FKBP staining is almost completely converted into a haze that persists through anaphase. GM130 also becomes largely diffuse although a significant percentage exists as small fragments. Ii-FRAP maintains a reticular pattern throughout mitosis, and this observation is in agreement with published results on the behavior of ER proteins during cell division in mammalian cells (Ellenberg *et al.*, 1997). In telophase, small ST-FKBP fragments that partially colocalize with GM130 begin to emerge from the haze. After cytokinesis, these proteins are found in large fragments situated next to the centrosomes

(Figure 4A). Even though deconvolution microscopy clearly shows that the Golgi haze is separate from the reticular ER (Figure 3), it is possible that Golgi proteins transiently relocate to the ER and quickly reemerge. This is an issue because while it is known that transport out of the ER is blocked during mitosis (Featherstone *et al.*, 1985), the exact stage at which this block is induced and then alleviated is not known. Thus, the Golgi haze could represent Golgi membranes that have already fused with the ER and reemerged or Golgi membranes that have yet to do so. To address this issue, we employed our trapping assay to mitotic COS 7 cells. Cotransfected cells were treated as before except 7 hr after aphidicolin release, rapamycin was added for 90 min. This time period was chosen to allow rapamycin to bind and remain bound to ST-FKBP throughout mitosis. Cells were then fixed and prepared for fluorescence microscopy. Our working hypothesis is the following: if Golgi membranes fuse with the ER at any time during mitosis, we expect ST-FKBP to be associated with Ii-FRAP in the ER and excluded from reforming Golgi membranes after cell division. Conversely, if Golgi membranes remain independent of the ER during cell division, then ST-FKBP will be found with reforming Golgi membranes in daughter cells after mitosis. Fluorescence microscopy of rapamycin-treated cells just completing or in the process of cytokinesis (when the Golgi and ER are prominently distinguishable) clearly illustrates ST-FKBP's presence in reforming Golgi fragments that also contain GM130 (GM130 data not

shown, (Figure 4A). In support of this, the percentage of newly divided cells containing ST-FKBP in reforming Golgi elements (98% ± 2%) was the same in the presence or absence of rapamycin (Figure 4C). Thus Golgi membranes do not cycle through the ER during mitosis.

To further strengthen our conclusion, we asked if ST-FKBP would be captured in the ER by Ii-FRAP if Golgi membranes were artificially fused with the ER under mitotic conditions. COS 7 cells were arrested in S phase with aphidicolin treatment, released from this block for 6 hr, and then incubated with BFA for 1 hr to fuse Golgi membranes with the ER, in the presence or absence of rapamycin. The cells were released from BFA for 90 min to allow for progression into and through mitosis. Cells in or just after cytokinesis were visualized by fluorescence microscopy. Under these conditions, Golgi-specific ST-FKBP was found trapped in the ER in the presence of rapamycin and was associated with reforming Golgi fragments in its absence (Figure 4B). Thus, when Golgi membranes are fused with the ER and the cells are allowed to progress through mitosis, Golgi-specific proteins can be trapped in the ER through use of the rapamycin-based procedure. In other words, if Golgi proteins were to cycle through the ER during mitosis, our procedure would have captured and retained Golgi-specific ST-FKBP in the ER. Based on our findings, we conclude that during normal progression through mitosis in mammalian cells, Golgi membranes remain separate from the ER.

1.4 DISCUSSION

The fact that Golgi membranes are fragmented during mitosis has been known since the 60s (Robbins and Gonatas, 1964; Maul and Brinkley, 1970). But in these early studies the morphology of mitotic Golgi membranes was not revealed. It wasn't until Warren and colleagues used antibodies to Golgi-specific enzymes in the early 80s that it became apparent that Golgi membranes are diffusely distributed in cells during mitosis (haze) (Burke *et al.*, 1982). Electron microscopy (EM) performed by Warren and colleagues revealed that the Golgi haze is made up of membranes in the form of small vesicles (Lucocq and Warren, 1987; Sonnichsen *et al.*, 1996). Contrastly, EM performed by Thyberg and Moskalewski in 1992 showed Golgi enzymes in the ER of mitotic cells (Thyberg and Moskalewski, 1992), suggesting that the haze represents the relocation of Golgi enzymes into the ER. However, it can be argued that the ER localization of Golgi enzymes was due to the ER retention of newly synthesized proteins and proteins arriving via retrograde transport in cells chronically arrested in mitosis by nocodazole treatment.

More recently, Warren and colleagues have reported that Golgi membranes do not appear as a haze but remain as large and discrete elements (132 ± 5) composed of tubulo-vesicular membranes (Shima *et al.*, 1997; Shima *et al.*, 1998). However, work from our lab and the labs

of Adam Lindstedt, Jennifer Lippincott-Schwartz, and Benjamin Glick reveals that Golgi membranes are first converted into small elements (Golgi blobs), which, in a cell type-specific manner, are further processed and appear diffusely dispersed (Golgi haze) (Jesch and Linstedt, 1998; Zaal *et al.*, 1999; Colanzi *et al.*, 2000; Hammond and Glick, 2000). But what is the molecular description of this haze? Does it represent Golgi membranes in the form of small vesicles or the relocation of Golgi enzymes into the ER? Linstedt and colleagues have reported that Golgi membranes can be isolated away from the ER in nocodazole-arrested mitotic cells (Jesch and Linstedt, 1998). However, the cells used for the isolation of Golgi membranes could have easily been enriched in early mitotic stages, when Golgi membranes are in the form of large blobs and therefore, not surprisingly, separated from the ER. Lippincott-schwartz and colleagues have reported that the Golgi haze is due to relocation of Golgi proteins into the ER (Zaal *et al.*, 1999). They expressed a chimera consisting of a Golgi-specific Galactosyltransferase fused to GFP (GalT-GFP) and found that it localized to the ER in mitotic cells by EM. However, it appears that the majority of the GalT-GFP protein used in this study was retained in the ER in an unfolded form (Jokitalo *et al.*, 2001). In addition, the Lippincott-Schwartz group observed the presence of endogenous Galactosyltransferase in the ER. However, this pool may have represented newly synthesized protein arrested in the ER due to a mitotic

block in ER exit (Featherstone *et al.*, 1985). In their report, Lippincott-Schwartz and colleagues also used photo bleaching-based analysis to show that, during mitosis, Golgi membranes are found in a continuous, reticular environment like the ER. However, it is unclear whether this approach would have distinguished between Golgi membranes in the form of vesicles and Golgi membranes fused with the ER.

Golgi Membranes Fragment during Mitosis but Do Not Fuse with the ER

As reported here, Golgi-specific ST-FKBP expressed in COS 7 cells is found as a haze from prophase through anaphase. Interestingly, the Golgi haze containing ST-FKBP is separate from the reticular ER network containing Ii-FRAP, as they do not colocalize by fluorescence microscopy (Figure 3). Thus even at this level of analysis, Golgi membranes are likely not contained in ER membranes. However, it could be argued that Golgi membranes are en route to fusion with the ER or have exited the ER and are in the process of reassembly.

Our scheme of trapping Golgi enzymes in the ER through use of rapamycin and rapamycin binding proteins was designed to address this issue. Soon after cytokinesis in COS 7 cells, Golgi membranes are found in the pericentriolar region and are close to complete assembly. If Golgi membranes had fused with the ER, then, at this time, in the presence of rapamycin, the Golgi protein ST-FKBP should be trapped in the ER

through binding to Ii-FRAP. However, this is clearly not the case, as, after cell division, we find that ST-FKBP is excluded from the ER network and associates with reforming Golgi membranes (Figures 4A and 4C). We have ruled out trivial possibilities that rapamycin is ineffective during mitosis. Cells treated with BFA, to fuse the Golgi with the ER, and then allowed to progress through mitosis in the presence of rapamycin reveal Golgi-specific ST-FKBP arrested in the ER (Figures 4B and 4C). On the basis of these results, we conclude that Golgi membranes remain separate from the ER during mitotic progression in mammalian cells.

Why Mammalian Cells Fragment the Golgi Apparatus So Extensively during Mitosis?

During mitosis in mammalian cells, Golgi membranes are sequentially fragmented, first into large blobs (step1) that are subsequently converted into smaller elements (Golgi haze, step 2) (Colanzi *et al.*, 2003a). A Golgi apparatus that has been fragmented into large blobs, in principle, should be sufficient for partitioning into daughter cells. The Golgi membranes in plants, early *Drosophila* embryos, and yeast (cerevisiae) are distributed throughout the cytoplasm and during cell division are partitioned into daughter cells without a major change in organization (Preuss *et al.*, 1992; Stanley *et al.*, 1997; Nebenfuhr and Staehelin, 2001). Why then do mitotic Golgi blobs in

mammalian cells undergo conversion into smaller elements? Is this necessary for partitioning into daughter cells, or do cells benefit from Golgi fragmentation in more ways than currently obvious? We have shown that if the pericentriolar Golgi membranes are not fragmented into large blobs, cells remain arrested in G2 and do not enter mitosis (Sutterlin *et al.,* 2002). Thus cells have a means to monitor the initiation of Golgi fragmentation and a defect in this process prevents mitotic entry. However, the significance of the second step of mitotic Golgi fragmentation remains unknown. Kondo and colleagues have recently reported that injection of reagents that prevent inhibition of a protein called p47 prevents conversion of Golgi blobs into the haze in metaphase, but has no effect on progression of cells through the later stages of mitosis (Uchiyama *et al.*, 2003). This finding suggests that the second step of Golgi fragmentation is not essential for mitotic progression. Thus, the reason for converting Golgi blobs into smaller elements remains elusive.

In conclusion, it is our opinion that our findings have resolved a long-standing controversy regarding the fate of Golgi membranes during mitotic progression. We conclude from our results that mitotic Golgi fragments remain separate from the ER and thus propose that biogenesis of the Golgi apparatus in daughter cells is from preexisting Golgi elements rather than de novo.

1.5 METHODS

Antibodies

From Covance: monoclonal anti-HA, polyclonal anti-HA, monoclonal anti-GFP. Anti-GM130 was a gift from Dr. M.A. Matteis (Mario Negri Sud Institute, Italy). Rabbit polyclonal anti-Calreticulin was purchased from Affinity Bioreagents. Secondary antibodies from Molecular Probes: Rhodamine red anti-mouse, Alexa Fluor 555 anti-mouse, Alexa Fluor 647 anti-rabbit. Secondary antibodies from Jackson Laboratories: (HRP conjugated) anti-mouse, anti rabbit. Hoechst was purchased from Molecular Probes.

Cloning and Constructs

pCMV3-ST-GFP was a gift from Ian Trowbridge. The FKBP coding region (Ho *et al.*, 1996), a gift from Stefan Ho (UCSD Department of Molecular Medicine), was cloned into the EcoRI site of pCMV3-ST-GFP (ST-FKBP). The human invariant γ chain cDNA was a gift from Lars Karlsson (Johnson and Johnson Pharmaceutical Research and Development). Human FRAP cDNA was also a gift from Stefan Ho. The FKBP-rapamycin binding domain of FRAP (AA 2026-2114) and

the HA epitope were cloned into the XhoI and SalI sites of the vector containing the human invariant γ chain gene.

Cell Culture

COS 7 cells were grown at 37°C with 5% CO2 in complete DMEM with 10% FBS, 100 U/ml penicillin/streptomycin, and 2 mM L-glutamine.

Deconvolution Microscopy

Images were captured with a Delta Vision deconvolution microscope system (Applied Precision, Inc., Issaquah, Washington). The system includes a photometrics CCD mounted on a Nikon TE-200 inverted epi-fluorescence microscope. In general, 50 optical sections spaced by 0.2 μm were taken. The best optical section for each data set was selected for publication. Exposure times were set such that the camera response was in the linear range for each fluorophore. A 100× (NA 1.4) lens was used. The data sets were deconvolved and analyzed using SoftWorx software (Applied Precision, Inc) on a Silicon Graphics Octane workstation.

Rapamycin Trapping Assay

5 × 106 COS 7 cells were electroporated (960 F, 0.22 V) with ST-FKBP and Ii-FRAP cDNAs (15 μg each) and plated into 6 cm culture dishes with glass coverslips coated with pronectin F (approximately 1/4-2/5 of

cell population/6 cm dish). After 24 hr of expression, the cells were treated with BFA (2 μg/ml) ± rapamycin (200 nM) for 1 hr at 37°C (at this time, prepare cells for fluorescence microscopy to visualize ST-FKBP in the ER). The cells were then washed ×2 with PBS and incubated with fresh media for 90 min at 37°C. After this, the cells were fixed with 4% formaldehyde for 15 min at room temperature (RT) and blocked with buffer containing 0.1% TX-100, 2% horse serum, and 0.05% NaN3 for 30 min at room temperature. ST-FKBP was visualized by GFP fluorescence. Ii-FRAP was observed with a monoclonal antibody against the HA epitope (1:1K in PBS, 1 hr RT) followed by secondary detection with Alexa Fluor 555 or rhodamine red (1:1K in PBS, 30 min RT). GM130 was stained with polyclonal anti-GM130 antibody (1:1K in PBS, 1 hr RT) and was detected secondarily with Alexa Fluor 647 (1:1K in PBS, 30 min RT). ST-FKBP was observed via GFP fluorescence and the DNA was stained with Hoechst (1:50K in PBS). Images were taken as described above (Deconvolution microscopy).

Quantitation of Trapping Efficiency

COS 7 cells were treated as described above (Rapamycin trapping assay). After BFA release in the presence or absence of rapamycin, cotransfected cells were scored for having ST-FKBP (GFP) at the Golgi or in the ER (Ii-FRAP, anti-HA). Cotransfected cells were selected

based on overexpression of Ii-FRAP to ensure enough capturing protein to trap ST-FKBP in each cell counted. At least 150 cells were scored per experiment. The data are presented in the form of a histogram.

Establishing Trapping Kinetics

COS 7 cells, growing on glass coverslips coated with pronectin F expressing ST-FKBP and Ii-FRAP were treated with BFA (2 μg/ml) for 1 hr at 37°C followed by the addition of rapamycin (200 nM) for 0, 2.5, 5, or 10 min in the presence of BFA. The cells were released from BFA and excess rapamycin and incubated in fresh medium for 90 min at 37°C. At this point, coverslips were prepared for fluorescence microscopy (Rapamycin trapping assay) and cotransfected cells were scored as described above (Quantitation of Trapping Efficiency section).

Immunofluorescence of Golgi Membranes during Mitosis in COS 7 Cells

COS 7 cells were electroporated (Rapamycin trapping assay) and plated in 6 cm dishes with glass coverslips coated with pronectin F (1/4-2/5 cell population). The cells were treated overnight with media containing aphidicolin (2.5 μg/ml) to obtain a more synchronized cell population. After this, incubation the cells were washed ×2 with PBS and incubated in fresh media for 8.5 hr (COS 7 cells become mitotic 6-9 hr after

aphidicolin release). At this time, the cells were prepared for fluorescence microscopy as previously described (Rapamycin trapping assay). Images were acquired as described above (Deconvolution microscopy).

Trapping ST-FKBP in the ER during Mitosis

COS 7 cells expressing ST-FKBP and Ii-FRAP were grown on glass coverslips and arrested with aphidicolin (2.5 μg/ml) overnight. Six hours after release from aphidicolin, BFA (2 μg/ml) was added ± rapamycin (200 nM) for 1 hr at 37°C. The cells were washed x2 with PBS and incubated for 90 min with fresh media at 37°C to allow them to progress through mitosis. At this time, coverslips were fixed and prepared for fluorescence microscopy as previously described (Rapamycin trapping assay).

Quantitation of Trapping Efficiency in Newly Divided COS 7 Cells

Recently divided cells (in or just completing cytokinesis, see Figure 4 legend for cell selection criteria) were selected at 100× magnification. Cells were scored for having ST-FKBP either in reforming Golgi fragments or in the reticular ER bound to Ii-FRAP. Thirty-five cells were scored for each experiment.

Figure 2. Generation of Rapamycin Binding Golgi- and ER-Specific ProteinsImages represent single, deconvolved Z sections (see Experimental Procedures).(Aa) ST-FKBP consists of the FKBP coding region fused between the Golgi localization domain of Sialyltransferase (ST) and GFP.(Ab) COS 7 cells were electroporated with ST-FKBP cDNA. After 24 hr of expression, the cells were trypsinized and collected via centrifugation. Following lysis with RIPA buffer membrane, free lysates were subjected to Western blot analysis or incubated with protein A and protein G sepharose beads conjugated with an anti-GFP antibody overnight. The beads were then washed and incubated with sample buffer. Following sample buffer incubation, samples were boiled and SDS-PAGE was performed to separate proteins. Western blot analysis of transfected lysate (T) and immunoprecipitations with an anti-GFP antibody reveals proteins of the expected molecular weight. (NT) = Nontransfected. The bands present in the GFP IP with nontransfected lysate represent IgGs.(Ac) COS 7 cells were electroporated with ST-FKBP cDNA and plated on glass coverslips coated with pronectin F. After 24 hr of expression, cells were fixed and prepared for fluorescence microscopy. ST-FKBP was visualized via GFP fluorescence and the DNA is stained with Hoechst and seen in blue. In interphase cells, ST-FKBP (green) localizes to the pericentriolar region with GM130 (red).(Ba) Ii-FRAP is composed of the FKBP-rapamycin interacting domain of FRAP fused between two regions of the human invariant γ chain protein (Ii) and the HA epitope.(Bb) COS 7 cells were electroporated with Ii-FRAP cDNA. Transfected lysate (T) and immunoprecipitations with an anti-HA antibody were prepared as in (Ab). Western blot analysis (with an anti-HA antibody) of transfected lysate (T) and immunoprecipitations shows proteins of the predicted molecular weight. Note that both ST-FKBP and Ii-FRAP migrate at about 45 kDa. (NT) = Nontransfected.(Bc) COS 7 cells were electroporated with Ii-FRAP cDNA and plated onto glass coverslips coated with pronectin F. After 24 hr of expression, the cells were fixed and prepared for fluorescence microscopy. Ii-FRAP was visualized with a monoclonal antibody recognizing the HA epitope, and endogenous calreticulin is stained with a polyclonal antibody. Ii-FRAP (red) colocalizes with calreticulin (green) in the ER.(C) COS 7 cells were electroporated with ST-FKBP and Ii-FRAP cDNAs and plated on glass coverslips coated with pronectin F. After 24 hr of expression, cells were fixed and prepared for fluorescence microscopy. The image shows an interphase COS 7 cell expressing ST-FKBP (green, GFP) and Ii-FRAP (red, anti-HA). The DNA is seen in blue (Hoechst). ST-FKBP and Ii-FRAP remain separate in interphase cells.

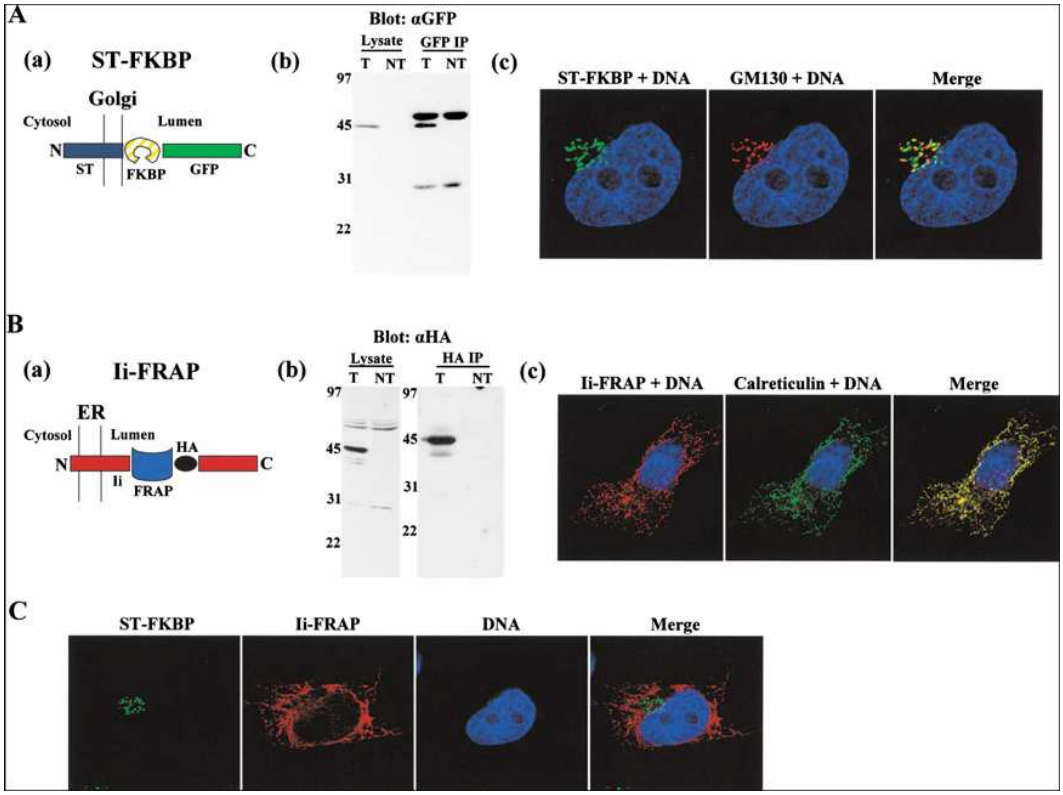
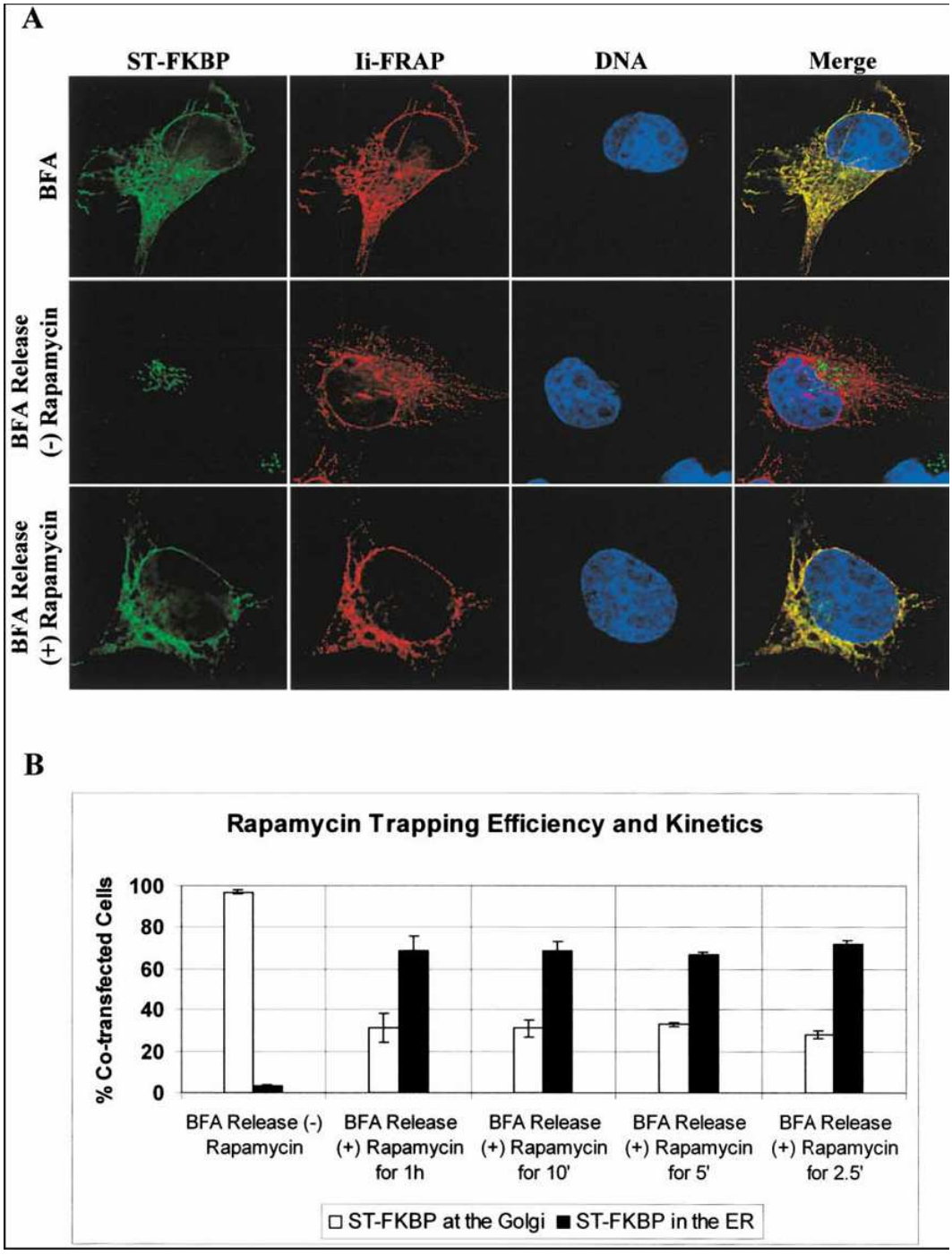

A
(a) ST-FKBP
Golgi
Cytosol
Lumen
N
C
ST
FKBP
GFP
(b)
Blot: αGFP
Lysate
GFP IP
T NT T NT
97
45
31
22
(c)
ST-FKBP + DNA
GM130 + DNA
Merge
B
(a) Ii-FRAP
ER
Cytosol
Lumen
HA
N
C
Ii
FRAP
(b)
Blot: αHA
Lysate
HA IP
T NT T NT
97
45
31
22
(c)
Ii-FRAP + DNA
Calreticulin + DNA
Merge
C
ST-FKBP
Ii-FRAP
DNA
Merge

Figure 3. BFA-Induced Relocation of ST-FKBP into the ER and Trapping by RapamycinImages represent single, deconvolved Z sections (see Experimental Procedures).(A) Cotransfected (electroporated) COS 7 cells were treated with BFA to fuse Golgi membranes with the ER. After 1 hr, BFA treatment ST-FKBP (green, GFP) colocalizes with Ii-FRAP (red, anti-HA) in the ER (yellow). When cells are released from BFA for 90 min in the absence of rapamycin, ST-FKBP is found in the pericentriolar region. When BFA is washed out in the presence of rapamycin, ST-FKBP remains in the ER and colocalizes extensively with Ii-FRAP (yellow). The DNA is visualized in blue (Hoechst).(B) The percentage of cotransfected cells exhibiting ST-FKBP (GFP) at the pericentriolar region or in the ER (Ii-FRAP, anti-HA) was determined after BFA release in the absence or presence of rapamycin for assorted periods of time. Cotransfected cells overexpressing Ii-FRAP (based on fluorescence intensity) were scored. Cells were either treated with BFA ± rapamycin for 1 hr or treated with BFA for 1 hr followed by incubation with rapamycin in the presence of BFA for 2.5, 5, or 10 min. The cells were then washed and incubated in fresh media for 90 min. At least 150 cotransfected cells were counted for each experiment/condition and the histogram represents the average of two (Rap for 2.5, 5, or 10 min) or five (Rap for 0 or 60 min) independent experiments. If cells are treated with rapamycin for just 2.5 min prior to drug washout, ST-FKBP is efficiently trapped in the ER.


A
ST-FKBP
Ii-FRAP
DNA
Merge
BFA
BFA Release (-) Rapamycin
BFA Release (+) Rapamycin
B
Rapamycin Trapping Efficiency and Kinetics
% Co-transfected Cells
100
80
60
40
20
0
BFA Release (-) Rapamycin
BFA Release (+) Rapamycin for 1h
BFA Release (+) Rapamycin for 10'
BFA Release (+) Rapamycin for 5'
BFA Release (+) Rapamycin for 2.5'
□ ST-FKBP at the Golgi ■ ST-FKBP in the ER

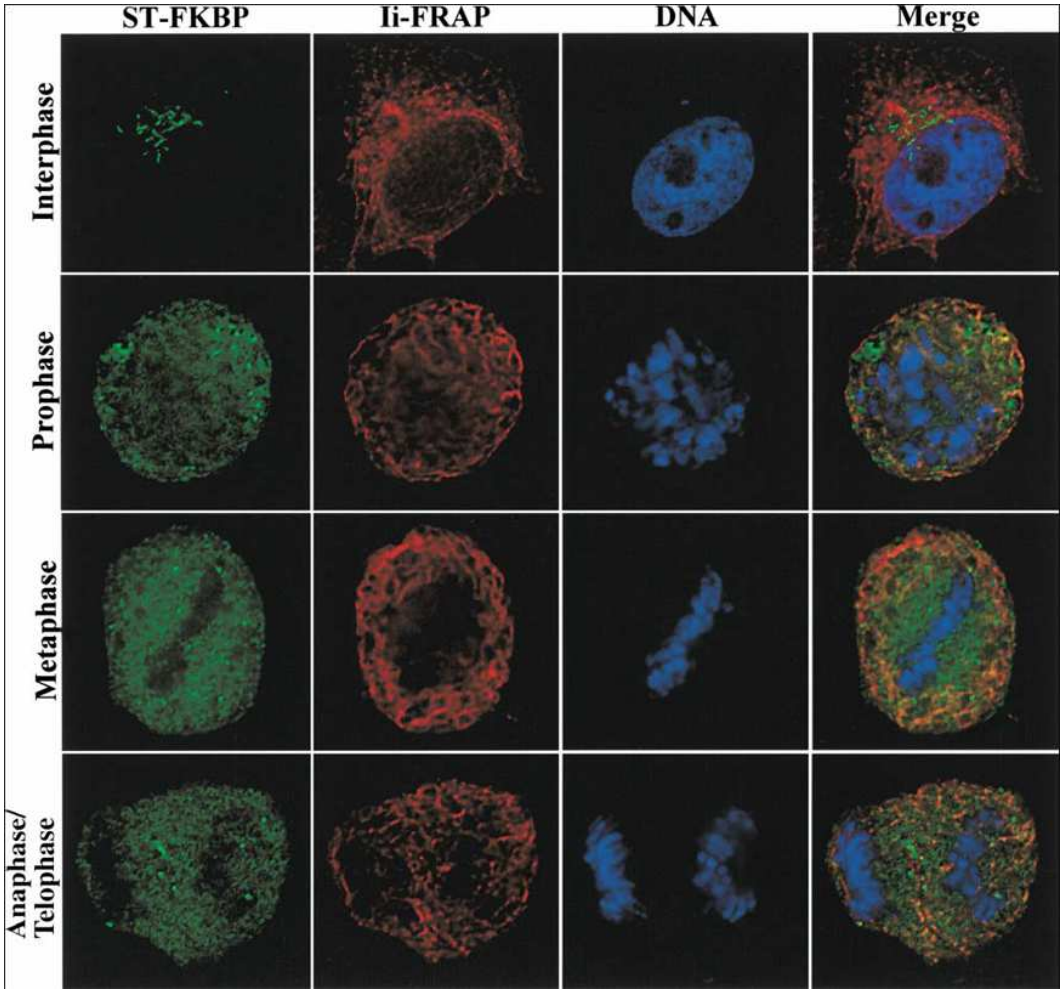
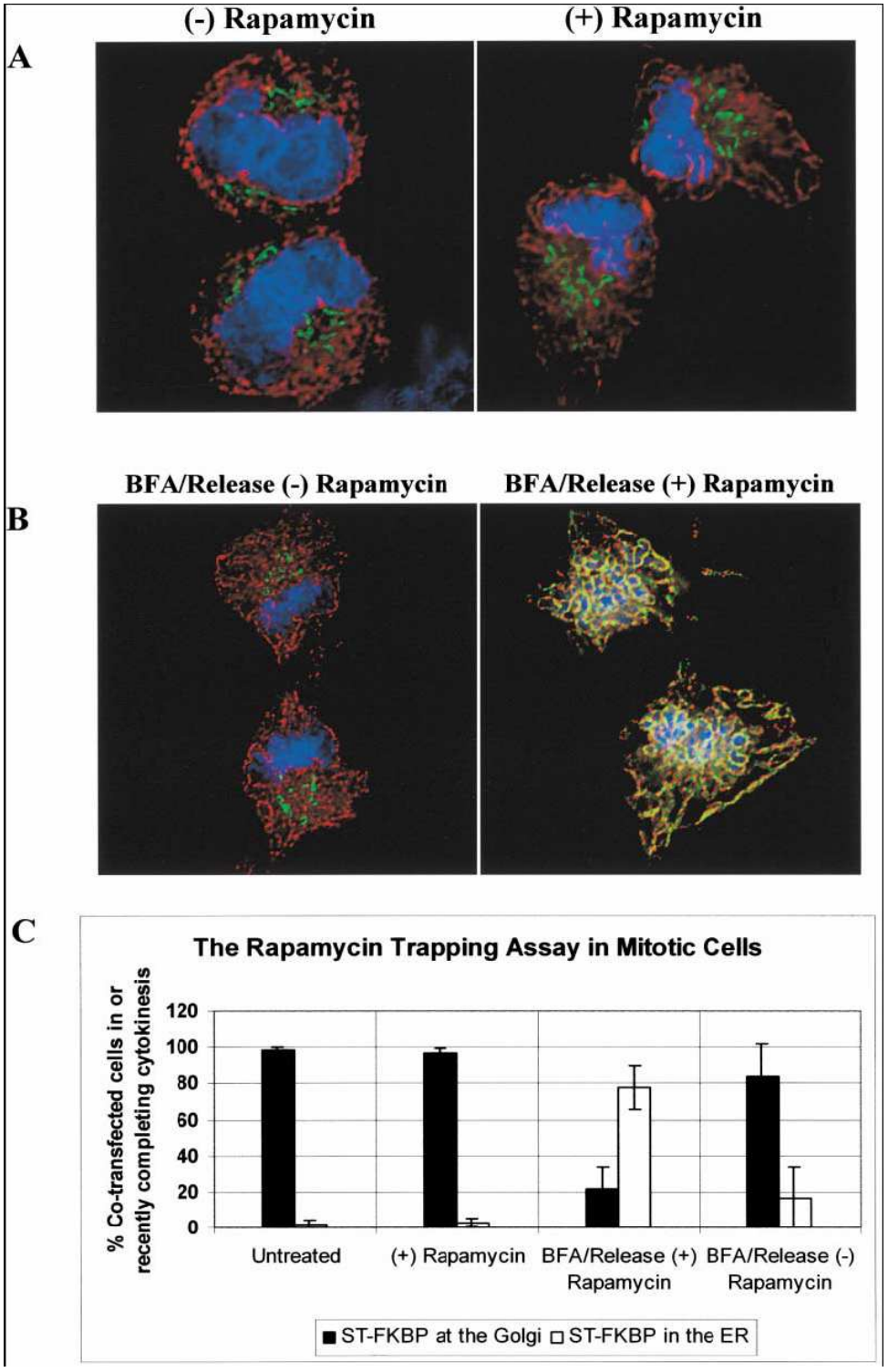


Figure 4. Dynamics of the Golgi Apparatus during Cell Division in COS 7 CellsImages represent single, deconvolved Z sections (see Experimental Procedures).COS 7 cells expressing ST-FKBP and Ii-FRAP were arrested overnight with aphidicolin and released for 8.5 hr in fresh medium. The cells were then fixed and prepared for fluorescence microscopy. ST-FKBP was visualized with GFP fluorescence (green), and Ii-FRAP was observed with an antibody against the HA epitope (red). The DNA is seen in blue (Hoechst). The Golgi is broken down into a haze in prophase that persists through anaphase. In telophase, small pieces containing ST-FKBP begin emerging from the haze. The ER remains reticular throughout mitosis and appears to be separate from the Golgi haze.

Figure 5. ST-FKBP Is Not Trapped in the ER after Cell Division in the Presence of RapamycinAll images represent single, deconvolved Z sections (see Experimental Procedures) of cells expressing ST-FKBP and Ii-FRAP that have just completed cytokinesis. Criteria for cell selection: (1) decondensing DNA of closely opposed cells (Hoechst); (2) coexpression of ST-FKBP and Ii-FRAP (overexpression of Ii-FRAP); (3) appearance of a bridge that once connected closely opposed cells (Ii-FRAP, anti-HA).(A) COS 7 cells expressing ST-FKBP and Ii-FRAP were arrested overnight with aphidicolin and released for 7 hr in fresh medium. Next, rapamycin was added (or not) for 90 min. Cells were then fixed and prepared for fluorescence microscopy. In the presence and absence of rapamycin, ST-FKBP (green, GFP) is found in reforming Golgi elements that are separate from reticular Ii-FRAP (red, anti-HA).(B) COS 7 cells were arrested overnight as in (A). Six hours after aphidicolin release, BFA ± rapamycin was added for 1 h and then washed out with PBS. The cells were incubated in fresh media for 90 min and then prepared for fluorescence microscopy. ST-FKBP (green, GFP) is found in the Ii-FRAP (red, anti-HA) reticulum in the presence of rapamycin. With BFA alone, ST-FKBP is found in reforming Golgi membranes.(C) The percentage of newly divided cells (in or just finishing cytokinesis) having ST-FKBP in reforming Golgi elements or in the ER was determined by fluorescence microscopy. Cells were selected as described above. Bars 1 and 2 represent untreated cells and cells treated with rapamycin for 90 min, respectively, as in Figure 4A. The third and fourth bars represent cells treated with BFA and released for 90 min in the presence or absence of rapamycin, respectively, as in Figure 4B. In each experiment, 35 cells were counted per condition and the histogram represents the average of two (± rapamycin) or three (BFA ± rapamycin) independent experiments. ST-FKBP is trapped in the ER after cell division in COS 7 cells only if the Golgi is first redistributed into the ER by BFA.


(-) Rapamycin
(+) Rapamycin
A
BFA/Release (-) Rapamycin
BFA/Release (+) Rapamycin
B
C
The Rapamycin Trapping Assay in Mitotic Cells
% Co-transfected cells in or recently completing cytokinesis
120
100
80
60
40
20
0
Untreated
(+) Rapamycin
BFA/Release (+) Rapamycin
BFA/Release (-) Rapamycin
ST-FKBP at the Golgi
ST-FKBP in the ER

Chapter I is as it appears in Cell 2004, Pecot, Matt Y; Malhotra, Vivek. I am the primary investigator and author of this paper.

CHAPTER II

The Golgi apparatus maintains its organization independent of the Endoplasmic Reticulum

2.1 ABSTRACT

Under artificial conditions Golgi enzymes have the capacity to rapidly accumulate in the Endoplasmic Reticulum (ER). These observations prompted the idea that Golgi enzymes constitutively recycle through the ER. We have tested this hypothesis under physiological conditions through use of a procedure that captures Golgi enzymes in the ER. In the presence of rapamycin, which induces a tight association between FKBP and FRAP, an FKBP-tagged Golgi enzyme can be trapped when it visits the ER by an ER retained protein fused to FRAP. We find that whilst FKBP-ERGIC-53 of the ER-Golgi Intermediate Compartment (ERGIC) rapidly cycles through the ER (30 min), FKBP-Golgi enzyme chimeras remain stably associated with Golgi membranes. We also demonstrate that Golgi dispersion upon nocodazole treatment mainly occurs through a mechanism that does not involve the recycling of Golgi membranes through the ER. Our findings

suggest that the Golgi apparatus, as defined by its collection of resident enzymes, exists independent of the ER.

2.2 INTRODUCTION

In mammalian cells the Golgi apparatus is composed of dozens of connected stacks of cisternae that are anchored to the peri-centriolar region. Each stack is compartmentalized by the *cis-trans* (early-late) distribution of the different Golgi-specific enzymes (Farquhar and Palade, 1981). Integral membrane proteins and proteins that are secreted from the cell are translated into the ER, where they are properly folded, and transported to the Golgi apparatus. Within Golgi membranes proteins are sequentially modified by the polarized array of Golgi enzymes, and then transported to their respective destinations. Brefeldin A (BFA), a fungal metabolite that blocks ER-Golgi transport (Fujiwara *et al.*, 1988) induces the relocation of Golgi enzymes into the ER (Lippincott-Schwartz *et al.*, 1989). Preventing ER exit through the over-expression of a dominant-negative form of sar1, a GTPase that recruits the COPII coat to ER exit sites (Barlowe *et al.*, 1994), also relocates Golgi enzymes into the ER (Storrie *et al.*, 1998; Zaal *et al.*, 1999; Miles *et al.*, 2001; Ward *et al.*, 2001). These studies demonstrate that Golgi enzymes have the capacity to quickly redistribute into the ER under

artificial conditions, but they may not depict what happens normally, when the organization of the secretory pathway is not perturbed. Nonetheless, based on these findings it was proposed that Golgi enzymes constitutively cycle through the ER (Zaal *et al.*, 1999; Miles *et al.*, 2001; Ward *et al.*, 2001). Further investigation through use of dominant-negative sar1 reagents has revealed that ER-recycling is a general property of all Golgi proteins (Miles *et al.*, 2001). Implicit to this idea is the dependence of Golgi function on the perpetual retrieval of Golgi components from the ER, which opposes the status of the Golgi as an independent organelle.

The dynamics of the Golgi apparatus are determined by the collective behavior of its resident enzymes. Thus, the relationship between the Golgi and the ER can be ascertained by determining the rate at which Golgi enzymes associate with the ER. Previously, to achieve this researchers have employed the use of fluorescence recovery after photo-bleaching techniques (Zaal *et al.*, 1999; Miles *et al.*, 2001; Ward *et al.*, 2001). However, in these studies conclusions were based on the analysis of very few cells [n = 3 cells (Miles *et al.*, 2001)]. Moreover, in some studies a clearly detectable ER specific pool of a fluorescently tagged Golgi reporter was required in order to measure ER recycling with this method (Zaal *et al.*, 1999; Miles *et al.*, 2001). In our experiments Golgi enzymes are only detected in the ER if highly

overexpressed. Since the behavior of highly overexpressed Golgi enzymes may not accurately emulate that of endogenous enzymes we made a point to execute the experiments presented in this report under more physiological expression levels. We have investigated the ER-recycling of resident Golgi proteins through use of a procedure that captures Golgi enzymes in the ER (Pecot and Malhotra, 2004). This method exploits the conditional interaction of two proteins, and thus allows the dynamics of Golgi enzymes to be observed without photo-bleaching cells or disrupting the secretory pathway. The FK506 Binding Protein (FKBP) and the FKBP-Rapamycin Associated Protein (FRAP) only interact in the presence of rapamycin, a small molecule. Rapamycin specifically binds to FKBP (Wiederrecht *et al.*, 1991) and FRAP binds to the FKBP-rapamycin complex (Brown *et al.*, 1994; Sabatini *et al.*, 1994). In our procedure, FKBP is fused to a Golgi enzyme, and FRAP is attached to an ER-retained protein. If the FKBP-tagged Golgi enzyme ever visits the ER it can be trapped there in the presence of rapamycin by the ER-FRAP chimera (Figure 1A). We have shown previously that a Golgi enzyme (sialyl-transferase) fused to FKBP can be trapped quickly and efficiently in the ER of BFA treated cells via this method (Pecot and Malhotra, 2004). Through use of this procedure we demonstrated that Golgi membranes remain segregated from the ER during mitosis in mammalian cells (Pecot and Malhotra,

2004). Here we use the ER-trapping method to investigate the relationship between the Golgi apparatus and the ER in non-dividing cells.

2.3 RESULTS

Generation and expression of ER-Trapping Constructs

Our strategy was to compare the rate at which Golgi enzymes associate with the ER to that of a protein known to rapidly cycle through the ER. To accomplish this we fused the FKBP coding region to the Golgi localization domain of mannosidase II (M2, M2-FKBP) and to full length ERGIC-53 (E53, FKBP-E53) (Figure 1B). M2 is a resident enzyme of early Golgi cisternae (Dunphy *et al.*, 1981) and E53 resides in the ERGIC (Schweizer *et al.*, 1988). E53 rapidly cycles through the ER as it is a cargo receptor for the transport of glycoproteins between the ER and ERGIC (Hauri *et al.*, 2000). For this reason E53 is an excellent molecule with which to compare the ER-association of Golgi enzymes. When expressed in HeLa cells M2-FKBP and FKBP-E53 localize to the Golgi and ERGIC, respectively (Figure 1B). The GFP-E53 construct, from which FKBP-E53 is derived, was previously constructed and utilized to investigate the dynamics of the ERGIC in living cells, and is thus a proven marker of the ERGIC (Ben-Tekaya *et*

al., 2005). Ii-FRAP and ST-FKBP (Figure 1B) have been previously described (Pecot and Malhotra, 2004). Briefly, the FKBP-rapamycin binding domain of FRAP (Chen *et al.*, 1995) was fused to an ER-retained version of the invariant chain protein [Iip33, (Schutze *et al.*, 1994)] (Ii-FRAP), and the Golgi localization domain of sialyl-transferase (a late Golgi enzyme) was fused to FKBP and GFP (ST-FKBP). Iip33 cannot be detected in post-ER compartments with biochemical methods (Schutze *et al.*, 1994) or by fluorescence microscopy (Schutze *et al.*, 1994) (Figure 1B) indicating that it does not leave the ER. In addition, unlike Sec31 Ii-FRAP does not accumulate in proliferating ERGIC elements at 15ºC (Figure 2). At this temperature ERGIC elements undergo proliferation (Hauri and Schweizer, 1992; Saraste and Kuismanen, 1992) and accumulate molecules that cycle between the ER and Golgi (Schweizer *et al.*, 1990; Jackson *et al.*, 1993; Tang *et al.*, 1993; Nickel *et al.*, 1997; Hay *et al.*, 1998) including components of ER exit sites (Hammond and Glick, 2000).

FKBP-E53 Rapidly Cycles through the ER

To determine the kinetics with which FKBP-E53 cycles through the ER, FKBP-E53 and Ii-FRAP were co-expressed in HeLa cells. The cells were then treated or not with rapamycin for assorted periods of time in the presence of cycloheximide to prevent contamination from

newly synthesized proteins. Cycloheximide has been shown to inhibit the peptidyl prolyl isomerase activity of the FKBP protein (Christner *et al.*, 1999), but does not hinder the efficiency of ER-trapping in our experiments. At designated time-points the cells were fixed and the localization of FKBP-E53 was observed by fluorescence microscopy. We discovered that the recycling of FKBP-E53 to the ER takes place in three stages (Figure 3A). Stage I is depicted by the loss of a compact peri-nuclear structure and the formation of an ER-like reticular pattern. Greater than 90% of observed cells display this phenotype after 5 minutes (Figure 3B). In stage II, the entire visible pool of FKBP-E53 has been converted into a reticular ER pattern. At this point the cycling of FKBP-E53 into the ER is complete as there is no longer evidence of any other structure by de-convolution microscopy. After 15 minutes, more than 60% of the observed cells exhibit this staining for FKBP-E53 (Figure 3B). Interestingly, although at this time FKBP-E53 is completely in the ER, it has not yet spread throughout the entire ER network as observed by Ii-FRAP staining. Stage III signifies the complete dispersal of FKBP-E53 throughout the ER network. In 30 minutes approximately half of the observed cells are stage III, and by one hour nearly all the cells display this phenotype (Figure 3B). These results confirm that FKBP-E53 rapidly cycles through the ER and that the ER-trapping procedure is capable of capturing such proteins.

In BFA Treated Cells M2-FKBP is Rapidly and Efficiently Captured in the ER in the Presence of Rapamycin

After establishing the rate of FKBP-E53 recycling we tested whether M2-FKBP, when in the ER, can be rapidly and proficiently trapped there in the presence of rapamycin. HeLa cells co-expressing M2-FKBP and Ii-FRAP were treated with BFA to fuse Golgi membranes with the ER. After 10 minutes with a high concentration of BFA (10 µg/mL) M2-FKBP can be completely relocated into the ER where it co-localizes with Ii-FRAP (Figure 4A). The effects of BFA are reversible, and when the BFA treated cells are washed and incubated with fresh media at 37º C for 2 hours M2-FKBP returns to the peri-centriolar region (Figure 4A). However, if rapamycin and BFA are added together for 10 minutes, M2-FKBP remains trapped in the ER bound to Ii-FRAP after BFA release (rapamycin bound to FKBP cannot be washed out) (Figure 4A). To establish the efficiency of ER-trapping we determined the percentage of co-transfected cells containing the *entire* pool of M2-FKBP trapped in the ER after BFA release in the presence of rapamycin. Since in many cells BFA treatment failed to completely redistribute M2-FKBP into the ER, it was necessary to normalize the trapping efficiency to the percentage of cells in which BFA worked to completion in each experiment. This lessened the

probability of recording false negative results by adjusting for cells that were scored negative for trapping due to an incomplete ER-redistribution of M2-FKBP. We found that, of the co-transfected cells in which BFA completely relocated M2-FKBP into the ER, approximately 80% had trapped the entire pool of M2-FKBP in the ER after BFA release in the presence of rapamycin (Figure 4B). These results, combined with the data obtained for FKBP-E53 demonstrate that the ER-trapping procedure is efficient and capable of capturing rapidly recycling proteins, thereby justifying its use as a method to determine if Golgi enzymes constitutively cycle through the ER.

Golgi enzymes do not Constitutively Cycle through the ER

If Golgi enzymes recycle through the ER then, in the presence of rapamycin, our Golgi enzyme reporters (M2-FKBP, ST-FKBP) should get captured there overtime in cells expressing Ii-FRAP. HeLa cells co-expressing M2-FKBP and Ii-FRAP were treated with cycloheximide in the presence or absence of rapamycin for various periods of time. As a positive control BFA-trapping experiments were performed in parallel. At specific time-points the cells were fixed and the localization of M2-FKBP was determined by fluorescence microscopy. The percentage of co-transfected cells at each time point in which M2-FKBP had recycled to the ER was determined and the results are presented as a graph in

Figure 5A. Recycling was deemed complete when the *entire* visible Golgi-pool of M2-FKBP was converted into the ER. Based on previous reports it was expected that M2-FKBP would completely recycle after a couple of hours (Zaal *et al.*, 1999; Miles *et al.*, 2001). However, after 16 hours in the presence of rapamycin 90% of the observed cells still displayed a clearly visible Golgi-specific pool of M2-FKBP (Figure 4A, Table 1). Similar results were recorded for cells co-expressing Ii-FRAP and ST-FKBP (Table 1). When M2-GFP (lacking FKBP coding region) was substituted for M2-FKBP in recycling experiments no background recycling was observed (data not shown). Time points longer than 16 hours could not be taken because long term cycloheximide treatment induces apoptosis.

Although complete recycling was not observed in the vast majority of cells it is still possible that a significant amount of our Golgi reporters relocate to the ER in our recycling experiments. We therefore quantified the percentage of co-transfected cells displaying partial trapping of M2-FKBP or ST-FKBP in the ER after 8 or 16 h of treatment with rapamycin (Figure 5B, Table 1). Partial trapping was scored as weak (weak ER staining) or strong (strong ER staining) and was normalized for background at time t=0. After 8 h 17% of cells co-expressing Ii-FRAP and M2-FKBP displayed partial ER-trapping of M2-FKBP (7% weak and 10% strong), 9% of the cells had completely

trapped M2-FKBP in the ER, and 74% of the cells had no visible ER staining for M2-FKBP (Table 1). After 16 h in the presence of rapamycin 8% of co-transfected cells exhibited partial trapping of M2-FKBP (5% weak and 3% strong), 4% displayed complete ER-trapping of M2-FKBP, and 88% showed no visible ER pool of M2-FKBP (Table 1). Similar results were observed for cells co-expressing Ii-FRAP and ST-FKBP (Table 1). The small population of co-transfected cells displaying partial ER-trapping of our Golgi reporters strongly indicates that these do not recycle through the ER. The partial trapping that is observed in our experiments may be attributed to missorting of our reporters back to the ER overtime, or an incomplete block in protein synthesis. These findings demonstrate that early and late Golgi enzymes remain stably associated with Golgi membranes.

Nocodazole Induced Golgi Dispersion does not Result from the Recycling of Golgi Membranes through the ER

In cells treated with nocodazole, a microtubule de-polymerizing agent, the Golgi apparatus becomes fragmented into individual stacks of cisternae that are dispersed throughout the cell (Thyberg and Moskalewski, 1985; Cole *et al.*, 1996). It is thought that this fragmentation occurs via the recycling of Golgi membranes through the ER and their re-emergence at areas adjacent to ER exit sites (Cole *et al.*,

1996; Storrie *et al.*, 1998) (Figure 6A). We have tested this idea through use of the ER-trapping procedure. If it is true that nocodazole induces the fragmentation of Golgi membranes through ER-recycling, then trapping Golgi enzymes in the ER should prevent the appearance of the scattered Golgi fragments (Figure 6A). If the ER-trapping procedure cannot prevent the formation of small Golgi elements then nocodazole induced Golgi dispersion must transpire through an alternative mechanism. HeLa cells expressing M2-FKBP and Ii-FRAP were treated with nocodazole (1μg/ml) and at assorted time-points the cells were fixed and analyzed by fluorescence microscopy. After 1.5 hours the typical Golgi-pattern represented by M2-FKBP began to breakdown into small, scattered elements that localized near ER exit sites (Figure 6B). When rapamycin was added with nocodazole and cycloheximide for 1.5-2 hours the appearance of small scattered fragments was prevented in approximately 14% of co-transfected cells (Figure 6C). In the vast majority of cells (86%) small Golgi elements were clearly visible, indicating their appearance was not due to ER-recycling (Figure 6B and C). Our results suggest that the initial fragmentation and dispersal of the Golgi apparatus in response to nocodazole treatment primarily occurs through a mechanism that does not involve the recycling of Golgi membranes through the ER.

2.4 DISCUSSION

How does the Golgi apparatus maintain its organization amidst the constant flux of traffic through the secretory pathway? Recent reports suggest that Golgi proteins constitutively cycle through the ER at a rapid rate, implying that the Golgi undergoes constant biogenesis from the ER (Zaal *et al.*, 1999; Miles *et al.*, 2001; Ward *et al.*, 2001). Exposing cells to BFA or dominant-negative forms of sar1 inhibits protein traffic out of the ER (Fujiwara *et al.*, 1988; Storrie *et al.*, 1998) and induces the accumulation of Golgi enzymes in the ER (Lippincott-Schwartz *et al.*, 1989; Storrie *et al.*, 1998; Miles *et al.*, 2001; Ward *et al.*, 2001), suggesting the existence of a constitutive Golgi-ER recycling pathway. However, both reagents may act non-specifically due to the large amounts that must be added to cells to observe the desired effects. In support of this, BFA mediated Golgi recycling requires microtubules (Lippincott-Schwartz *et al.*, 1990) while sar1 induced recycling is microtubule independent (Storrie *et al.*, 1998).

Flourescence recovery after photo-bleaching techniques have been applied to demonstrate the rapid ER-cycling of Golgi enzymes under physiological conditions (Zaal *et al.*, 1999; Miles *et al.*, 2001; Ward *et al.*, 2001). However, as was discussed earlier few cells can be examined with this method, and in some reports the cells that are examined must

over-express the observed Golgi reporter in the ER (Zaal *et al.*, 1999; Miles *et al.*, 2001). We feel that these limitations make it difficult to make conclusions about the behavior of endogenous Golgi enzymes. Researchers have also utilized the temperature sensitive features of the Vesicular Stomatitis Virus G-protein (VSVG) to show that Golgi proteins recycle to the ER under normal conditions (Cole *et al.*, 1998). The thermo-sensitive form of VSVG (VSVGtsO45) contains a temperature sensitive mutation that causes it to aggregate and be retained in the ER at temperatures above 39.8º C (Gallione and Rose, 1985). Golgi proteins that have been fused with VSVGtsO45 have been shown to accumulate in the ER under these conditions (Cole *et al.*, 1998) providing evidence for their constitutive recycling to the ER. However, it has been reported that at temperatures sufficient to arrest VSVGtsO45 in the ER the budding of COPII coated vesicles from the ER is prevented (Aridor *et al.*, 1999) and general protein transport is inhibited (Trucco *et al.*, 2004), demonstrating that these conditions do not represent a physiological situation. Furthermore, this study excluded Golgi enzymes and focused instead on Golgi-associated proteins known to behave dynamically as a result of their function in membrane trafficking (KDEL Receptor, TGN-38).

Due to the difficulty in accurately studying the behavior of Golgi enzymes, the relationship between the Golgi and the ER has been a

contentious topic. For this reason, we developed the ER-trapping procedure (Figure 1A), which represents a simple, straightforward method to observe the association of Golgi enzymes with the ER under physiological circumstances. Previously we established that a late Golgi reporter (ST-FKBP) can be efficiently trapped in the ER when it visits there (Pecot and Malhotra, 2004). Here we have done the same for an early Golgi marker (M2-FKBP, Figure 4A and B). We then determined if these reporters relocate to the ER overtime. We discovered that while the entire pool of an ERGIC-53 reporter (FKBP-E53) is rapidly captured in the ER (30 min, Figure 3A and B), our early and late Golgi markers remain stably associated with Golgi membranes (Figure 5, Table 1). Partial relocation of these proteins into the ER occurred in only a small percentage of cells (Figure 5B, Table 1) indicating that they do not constitutively cycle through the ER.

In this study we have also investigated the mechanism underlying nocodazole induced Golgi fragmentation. Prior reports have claimed that Golgi fragmentation under these conditions occurs via the constitutive recycling of Golgi membranes through the ER (Cole *et al.*, 1996; Storrie *et al.*, 1998) (Figure 6A). Based on these findings it was also suggested that ER-recycling is responsible for Golgi inheritance during cell division (Zaal *et al.*, 1999; Altan-Bonnet *et al.*, 2006), when Golgi membranes breakdown during spindle formation and reassemble

in daughter cells (Warren, 1993). We have demonstrated that the appearance of nocodazole dependent Golgi fragments cannot be prevented by trapping Golgi proteins in the ER (Figure 6B and C), suggesting that fragmentation occurs through an alternative mechanism. These results support our prior finding that Golgi membranes remain segregated from the ER during mitosis in mammalian cells (Pecot and Malhotra, 2004).

We have demonstrated that both early and late Golgi enzymes remain stably associated with Golgi membranes and do not constitutively cycle through the ER. Based on this we propose that the Golgi apparatus is a stable compartment that does not rely on the ER for its constant biogenesis. The results presented in this report combined with work regarding the fate of Golgi membranes during cell division (Jesch and Linstedt, 1998; Rossanese and Glick, 2001; Axelsson and Warren, 2004; Pecot and Malhotra, 2004) indicate that the Golgi apparatus remains independent from the ER throughout the life of the cell.

2.5 METHODS

Antibodies. The monoclonal HA antibody (HA.11) was purchased from Covance Inc., and monoclonal Sec31A antibody was purchased from

Transduction Laboratories. Secondary antibodies were purchased from Molecular Probes: Rhodamine Red anti-mouse, Alexa fluor 647. Hoechst was also purchased from Molecular Probes.

Cloning and Constructs. ST-FKBP and Ii-FRAP were previously described (Pecot and Malhotra, 2004). GFP-ERGIC-53 was a gift from Dr. Hauri (Ben-Tekaya *et al.*, 2005). One tandem repeat of full-length FKBP was cloned into the BglII restriction site of GFP-ERGIC-53, generating GFP-FKBPx2-ERGIC-53 (FKBP-E53). M2-FKBP was constructed by cloning the first 100 amino acids of mouse alpha-mannosidase II (bp 88-307) into the HindIII and EcoRI restriction sites of pEGFP-N3. The entire FKBP coding region was then cloned into the EcoRI and BamHI restriction sites of this vector, leaving a C-terminal GFP tag.

Cell Culture. HeLa cells (ATCC) were grown at 37º C with 7% CO2 in DMEM supplemented with 10% FBS, and 2mm L-glutamine.

De-convolution Microscopy. Images were captured with a Nikon Eclipse TE 2000-U inverted microscope and a Photometrics CoolSNAP HQ camera (A.G. Heinz). Image acquisition was run by Metamorph software, and images were de-convolved by AutoDeblur &

AutoVisualize 9.3 software. In general, using a 60x objective lens (NA 1.4), 21 optical sections per cell spaced by 0.1 um were taken. The best optical section for each set of data was used for publication. Exposure times were set such that the camera response was in the linear range for each fluorophore.

Transfections. HeLa cells were split into 6 cm plates the night before (600-900K cells/plate). In the morning the cells were between 80-95% confluent. Transfections were performed in the morning via Lipofectamine 2000 Reagent (Invitrogen). 20 μL of Reagent plus 10 μg of total DNA (FKBP-E53, M2-FKBP, or ST-FKBP + Ii-FRAP) was added to each 6 cm plate, and transfections were allowed to proceed for at least 6 h. In the evening the cells were split onto coverslips in 6 cm plates, and allowed to attach for 12-18 hours.

ER-Trapping Procedure. After transfection, cells were treated with media only, media + cycloheximide (CHX, 100ug/ml) + BFA (10 ug/mL), or media + CHX + BFA + rapamycin (Rap, 200 nM) for 10 min at 37º C. At this point coverslips were fixed to determine the percentage of cells that had completely redistributed M2-FKBP into the ER. Co-transfected cells expressing high amounts of Ii-FRAP were counted (~ 400 cells / experiment). The remaining coverslips were washed x3 with

PBS and incubated for 2 h at 37º C with media + CHX, or media + CHX + Rap. The coverslips were then fixed (4% formaldehyde in PBS) for 10 min at room temperature, followed by treatment with blocking buffer (2% FBS, 0.1% TX-100, and 0.05% NaN3 in PBS) for 30 min at room temperature. Ii-FRAP was labeled with a monoclonal HA antibody (1:1K in PBS, 1 h room temp). All secondary antibodies (Molecular Probes) were used at a dilution of 1:2K in PBS (20 min room temp). After antibody staining, co-transfected cells were visualized by fluorescence microscopy (GFP constructs were visualized via GFP fluorescence). ER-trapping was determined in cells expressing large amounts of Ii-FRAP. Only the complete re-distribution of M2-FKBP into the ER was counted as trapping. Approximately 300 cells were scored per experiment (3 independent experiments).

15ºC Experiment. HeLa cells expressing Ii-FRAP and FKBP-E53 were incubated at 37ºC or 15ºC for 3 h in sealed 24-well plates with culture medium containing 25 mM Hepes. The cells were then fixed and prepared for fluorescence microscopy as described above.

Recycling Experiments. HeLa cells were transfected as described above. Transfected cells were treated with media + CHX (100 ug/mL), or media + CHX + Rap (200 nM) for various periods of time. At

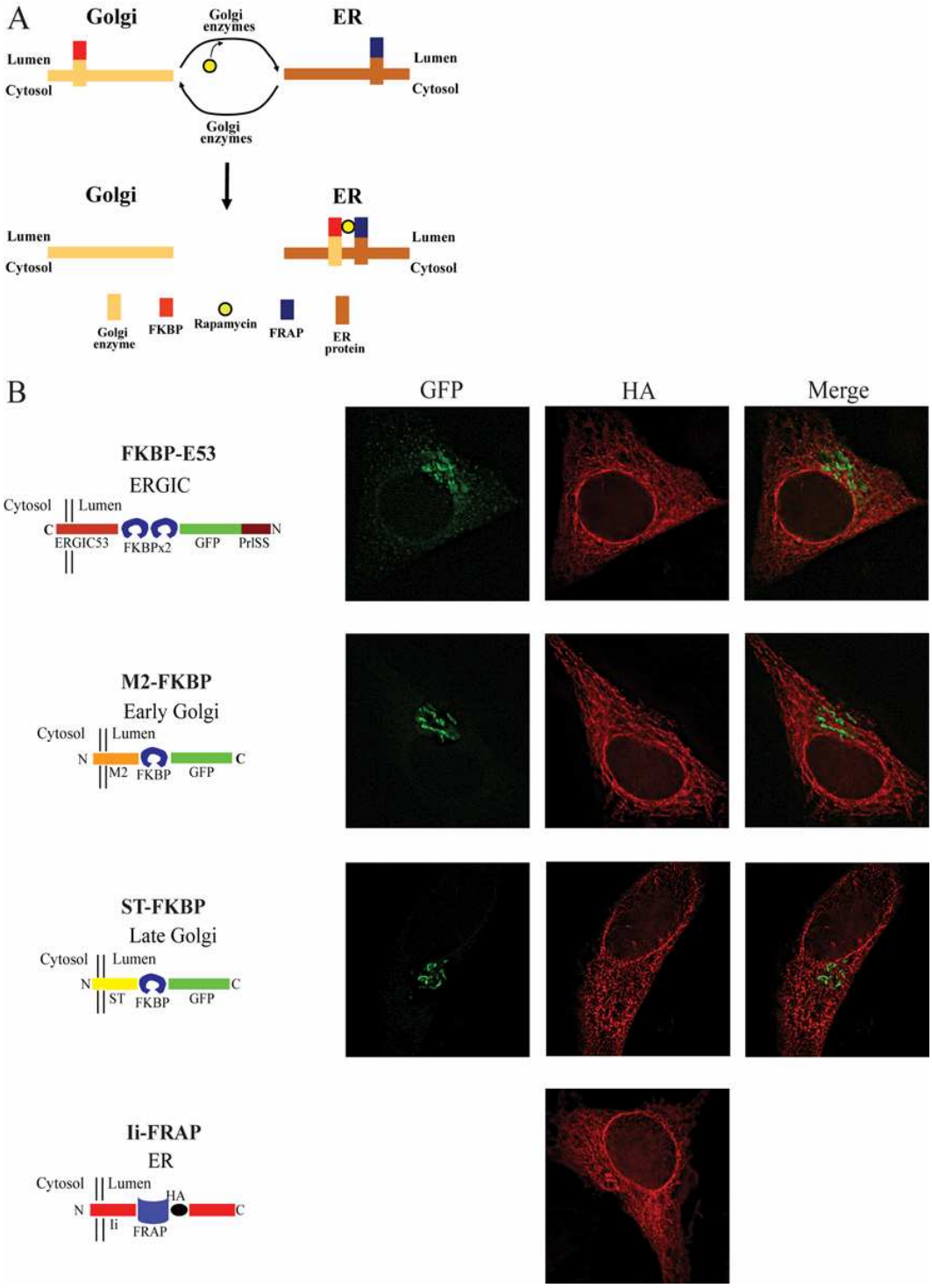
designated time-points the cells were fixed and prepared for fluorescence microscopy (as described above). Co-transfected cells expressing high amounts of Ii-FRAP were scored for the recycling of Golgi chimeras to the ER. Only complete relocation into the ER was counted as recycling. Approximately 200 cells were analyzed per experiment with M2-FKBP (at least 3 independent experiments), ~ 55 cells per experiment with FKBP-E53 (at least 2 independent experiments), and ~ 80 cells per experiment with ST-FKBP (2 independent experiments).

Quantitation of partial trapping for recycling experiments. Recycling experiments were performed as described above. The percentage of co-transfected cells displaying complete Golgi localization (No Trapping), complete ER localization (Complete Trapping), weak partial ER localization (weak ER staining), or strong partial ER localization (strong ER staining) for M2-FKBP or ST-FKBP after 8 h or 16 h in the presence of CHX +/- Rap was determined.

Nocodazole Experiments. Cells expressing Ii-FRAP and M2-FKBP were treated with media + CHX (100ug/mL), media + CHX + nocodazole (Noc, 1uM), or media + CHX + Noc + Rap (200 nM) at 37º C for 1.5-2 hours. Cells were prepared for fluorescence microscopy as

described above, except that Hoechst (1:25K) was added to the secondary antibody incubation. The effects of ER-trapping on the appearance of nocodazole induced Golgi fragments were observed in co-transfected cells expressing large amounts of Ii-FRAP. Cells were scored based on their having or not having small Golgi fragments. Approximately 100 cells were analyzed per experiment (4 independent experiments).

Figure 6. Scheme of the ER-trapping procedure and description and localization of trapping constructs. (A) Diagram of the trapping procedure. FKBP is fused to a Golgi enzyme and FRAP is fused to an ER protein. When the FKBP-Golgi chimera visits the ER it can be captured there in the presence of rapamycin. (B) Description and localization of trapping constructs. FKBP-E53 is made up of FL ERGIC-53 fused to x2 FKBP coding regions, GFP, and the prolactin signal sequence (PrlSS). For the construction of M2-FKBP the Golgi localization domain of mouse α-mannosidase II was fused to the FKBP coding region and GFP. ST-FKBP consists of the Golgi localization domain of sialyl-transferase appended to the FKBP coding region and GFP. Ii-FRAP is an ER-retained version of the invariant chain protein fused to the FKBP-rapamycin binding domain of FRAP and the HA epitope. All constructs were designed so that FKBP and FRAP domains are in the lumen of the Golgi or ER. In HeLa cells expressing Ii-FRAP FKBP-E53, M2-FKBP, and ST-FKBP (green, GFP) localize to the ERGIC, early Golgi, and late Golgi, respectively, and remain separate from ER-specific Ii-FRAP (red, HA).


A
Golgi
Golgi enzymes
ER
Lumen
Cytosol
Lumen
Cytosol
Golgi enzymes
Golgi
ER
Lumen
Cytosol
Lumen
Cytosol
Golgi enzyme
FKBP
Rapamycin
FRAP
ER protein
B
GFP
HA
Merge
FKBP-E53
ERGIC
Cytosol
Lumen
C
N
ERGIC53
FKBPx2
GFP
PrlSS
M2-FKBP
Early Golgi
Cytosol
Lumen
N
C
M2
FKBP
GFP
ST-FKBP
Late Golgi
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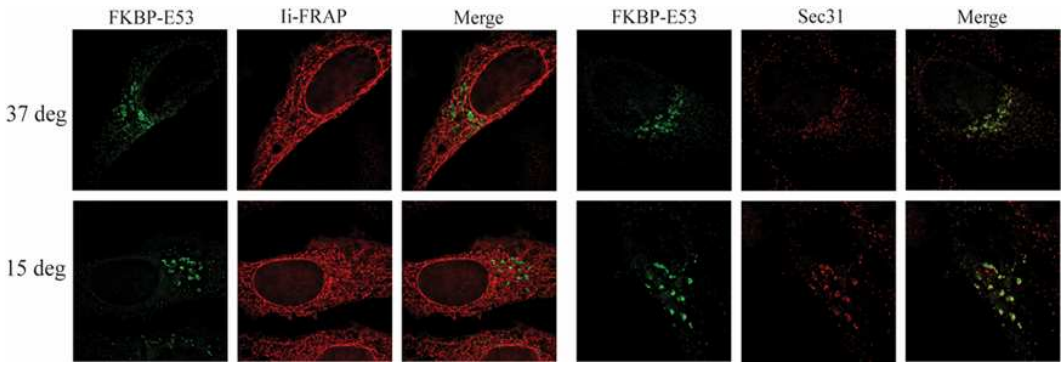


Figure 7. Ii-FRAP does not accumulate in ERGIC elements at 15ºC. HeLa cells expressing Ii-FRAP (red, 1st panel) and FKBP-E53 (green) were incubated at 37ºC or 15ºC for 3 h. At 15ºC ERGIC elements proliferate and accumulate Sec31 (red, 2nd panel) but not Ii-FRAP. FKBP-E53 was visualized by GFP fluorescence, Ii-FRAP with an αHA antibody, and Sec31 was labeled with a monoclonal αSec31 antibody.

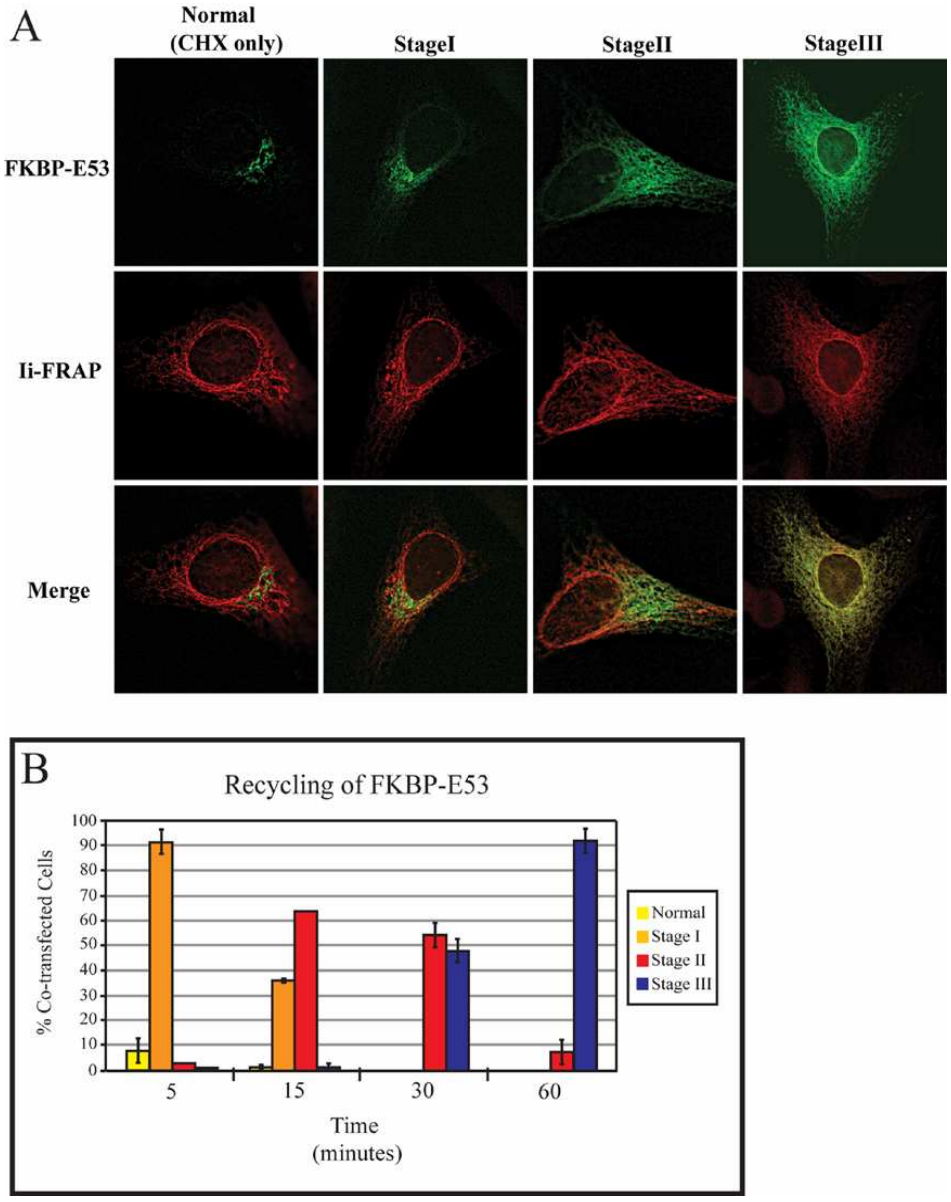
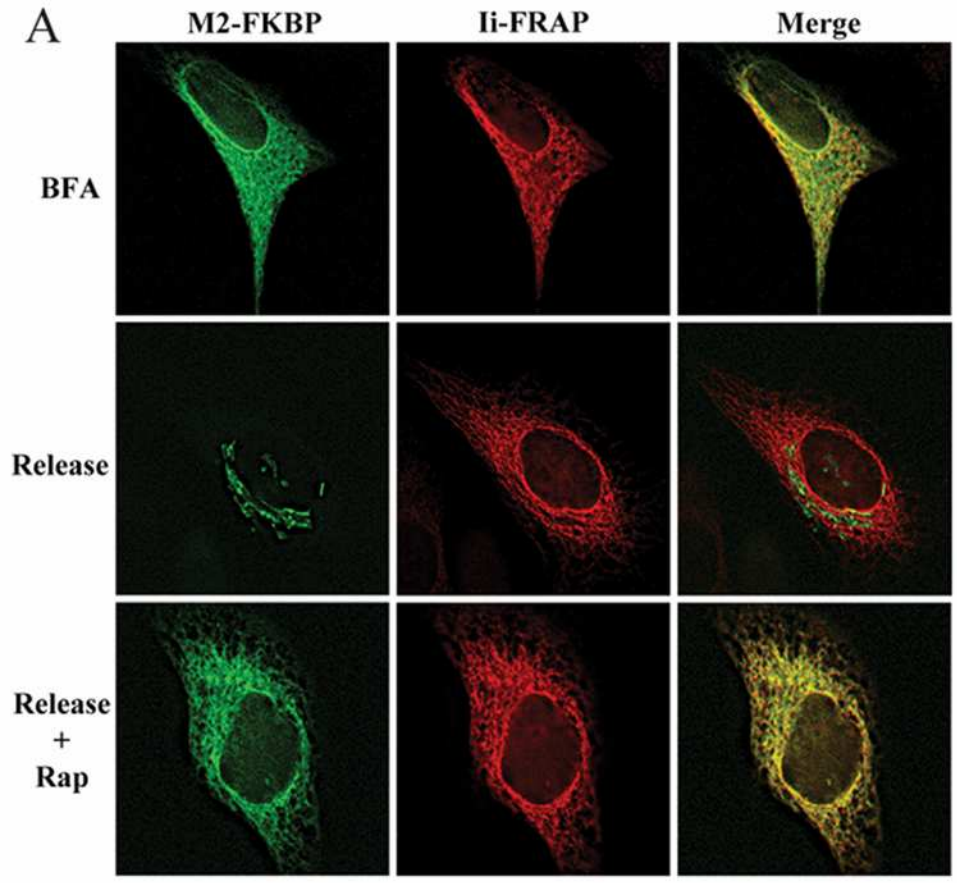
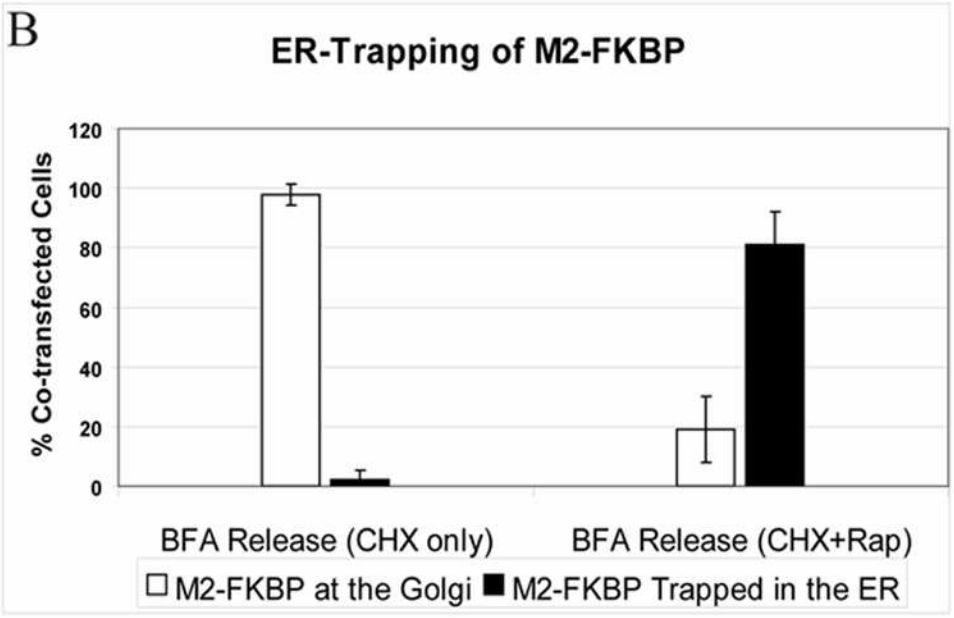


Figure 8. Recycling of FKBP-E53. (A) Stages of FKBP-E53 recycling. HeLa cells expressing FKBP-E53 (green) and Ii-FRAP (red) were treated with cycloheximide +/- rapamycin for assorted periods of time. FKBP-E53 recycles to the ER in III stages. By stage II the entire pool of FKBP-E53 has been converted into the ER. FKBP-E53 was visualized by GFP fluorescence and Ii-FRAP was stained with an αHA antibody. (B) Time-course of FKBP-E53 recycling. Cells were treated as in (a) and the percentage of co-transfected cells displaying each stage of FKBP-E53 recycling was determined at various time points. After 30 min in the presence of rapamycin FKBP-E53 has recycled to the ER in nearly all co-transfected cells.

Figure 9. M2-FKBP can be quickly and efficiently trapped in the ER in the presence of BFA. (A) BFA induced M2-FKBP trapping in the ER. HeLa cells co-expressing M2-FKBP (green) and Ii-FRAP (red) were treated with BFA (to fuse Golgi membranes with the ER) and cycloheximide (CHX) +/- rapamycin (Rap) for 10 min. After BFA treatment M2-FKBP co-localizes extensively with Ii-FRAP in the ER. Cells were then washed and incubated with fresh media and CHX +/- Rap for 2 h. With CHX only, after BFA release M2-FKBP relocates to the peri-centriolar region. However, in the presence of Rap M2-FKBP is trapped in the ER bound to Ii-FRAP after BFA is washed out. M2-FKBP was visualized by GFP fluorescence and Ii-FRAP was stained with an αHA antibody. (B) Efficiency of ER-trapping. Cells were treated as in (A) and the percentage of cells displaying the entire pool of FKBP in the ER was determined after BFA release in the presence or absence of Rap. This value was normalized to the percentage of cells in which BFA completely redistributed M2-FKBP into the ER. In the presence of Rap approximately 80% of co-transfected cells in which BFA worked to completion displayed absolute trapping of M2-FKBP in the ER after BFA release.


A
M2-FKBP
Ii-FRAP
Merge
BFA
Release
Release
+
Rap


B
ER-Trapping of M2-FKBP
% Co-transfected Cells
120
100
80
60
40
20
0
BFA Release (CHX only)
BFA Release (CHX+Rap)
M2-FKBP at the Golgi
M2-FKBP Trapped in the ER

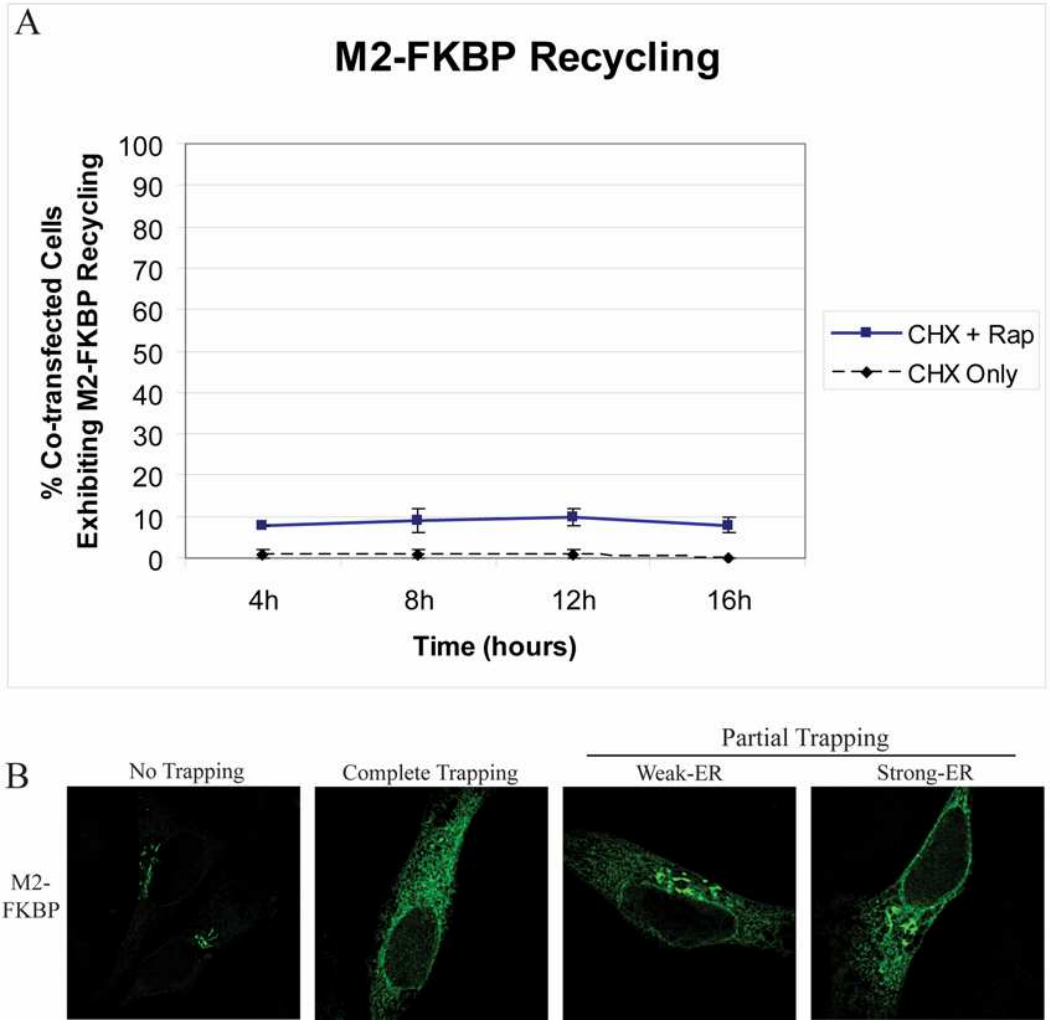


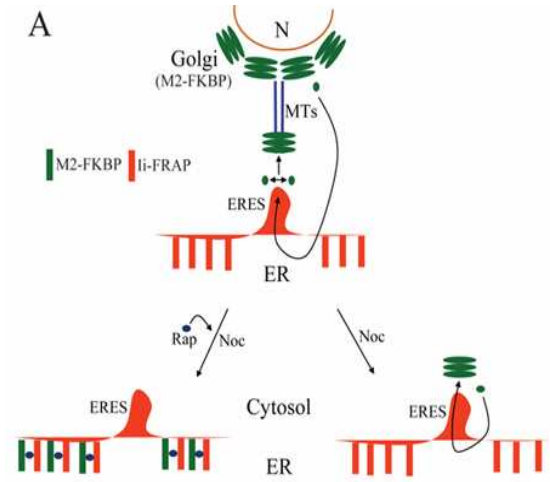
Figure 10. Golgi enzymes do not constitutively cycle through the ER. (A) M2-FKBP does not recycle through the ER. HeLa cells co-expressing M2-FKBP and Ii-FRAP were treated with cycloheximide (CHX) +/- rapamycin (Rap) for assorted periods of time. After 16 h in the presence of Rap 90% of co-transfected cells display a clearly visible Golgi-specific pool of M2-FKBP. (B) Phenotypes of M2-FKBP during recycling experiments. Cells expressing Ii-FRAP and M2-FKBP (green) were treated as in (A) and the following patterns for M2-FKBP were observed to varying degrees; No Trapping (complete Golgi localization), Complete Trapping (complete ER localization), Weak-ER (partial trapping, weak ER staining), Strong-ER (partial trapping, strong ER staining).

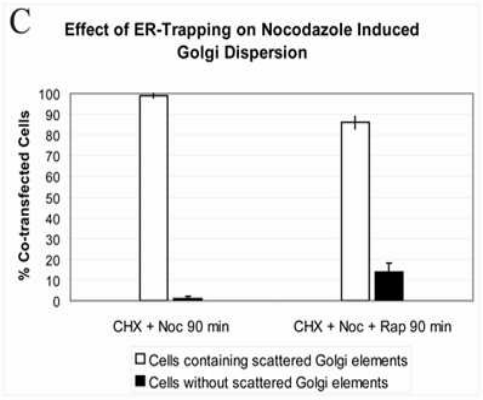
Table 1. Quantitation of partial trapping in recycling experiments. Cells expressing Ii-FRAP and M2-FKBP or ST-FKBP were treated with cycloheximide (CHX) +/- rapamycin (Rap) for 8 or 16 h as in Figure 5. The percentage of co-transfected cells displaying the phenotypes shown in Figure 5B for the Golgi reporters was determined. Percentages were normalized against background at t=0. Partial trapping occurs in only a small percentage of cells.

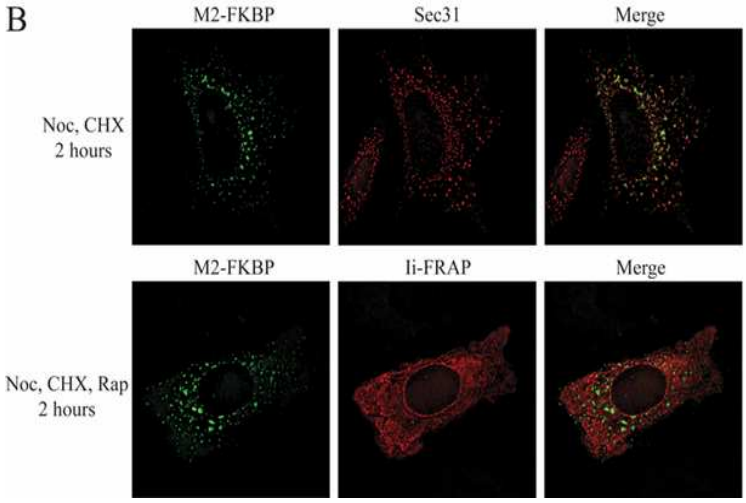
Table 1. Percentage of co-transfected HeLa cells

Experiment	No Trapping	Complete Trapping	Partial Trapping		
			Weak ER staining	Strong ER staining	Total
(M2-FKBP) CHX + Rap 8 h	74 +/- 7	9 +/- 0	7 +/- 2	10 +/- 5	17 +/- 7
(M2-FKBP) CHX + Rap 16 h	88 +/- 1	4 +/- 2	5 +/- 3	3 +/- 2	8 +/- 1
(ST-FKBP) CHX + Rap 16 h	72 +/- 10	2 +/- 1	20 +/- 10	6 +/- 1	26 +/- 10

Figure 11. Nocodazole induced Golgi dispersion does not occur via the recycling of Golgi membranes through the ER. (A) Diagram of the current model for nocodazole (Noc) dependent Golgi fragmentation and the expected effect of the ER-trapping procedure under these circumstances. Currently it is thought that Golgi proteins recycle through the ER via a constitutive pathway. From peri-centriolar Golgi stacks Golgi proteins are transported to the ER in small vesicles that do not require microtubules for movement. Inside the ER Golgi proteins move to ER Exit Sites (ERES) and exit the ER in membrane bound transport carriers. The transport carriers fuse together, forming a Golgi stack which is subsequently directed to the peri-centriolar region via a microtubule dependent process. In the presence of Noc, which depolymerizes microtubules, Golgi proteins continue to recycle through the ER. However, Golgi stacks that form from the fusion of transport carriers harboring Golgi proteins fail to move to the peri-centriolar region. This results in Golgi fragmentation in the form of numerous dispersed, individual Golgi stacks that reside near ERESs. When cells are treated with Noc in the presence of rapamycin, recycling M2-FKBP should be captured in the ER by Ii-FRAP. This would prevent Golgi fragmentation as visualized by M2-FKBP. (B) The ER-trapping procedure does not prevent the formation of Noc induced Golgi fragments. HeLa cells expressing M2-FKBP (green) and Ii-FRAP (red, bottom panel) were treated with Noc and cycloheximide (CHX) +/- rapamycin (Rap) for 2 h. Under these circumstances M2-FKBP was found in punctate structures adjacent to ERES, as visualized with an antibody against Sec 31 (red, top panel). In cells co-expressing M2-FKBP and Ii-FRAP Golgi fragmentation was not prevented in the presence of Rap. M2-FKBP was visualized by GFP fluorescence and Ii-FRAP was observed through staining with an αHA antibody. (C) Quantitation of nocodazole induced Golgi fragmentation in Hela cells co-expressing M2-FKBP and Ii-FRAP in the presence and absence of Rap. Co-transfected cells were treated with Noc and CHX +/- Rap for 1.5-2 h. In the presence of Rap the formation of small Golgi fragments is inhibited in a small percentage of cells, indicating that Golgi fragmentation mainly occurs through a mechanism that does not involve ER-recycling.


A
N
Golgi
(M2-FKBP)
MTs
M2-FKBP
Ii-FRAP
ERES
ER
Rap
Noc
Noc
ERES
Cytosol
ERES
ER


C
Effect of ER-Trapping on Nocodazole Induced Golgi Dispersion
% Co-transfected Cells
100
90
80
70
60
50
40
30
20
10
0
CHX + Noc 90 min
CHX + Noc + Rap 90 min
Cells containing scattered Golgi elements
Cells without scattered Golgi elements


B
M2-FKBP
Sec31
Merge
Noc, CHX
2 hours
M2-FKBP
Ii-FRAP
Merge
Noc, CHX, Rap
2 hours

Chapter II has been submitted for publication in Molecular Biology of the Cell 2006, Pecot, Matthew Y.; Malhotra, Vivek. I am the primary investigator and author of this manuscript.

CHAPTER III

Phosphorylation of the Golgi-associated protein GRASP65 regulates Golgi fragmentation and mitotic progression

3.1 ABSTRACT

During mitosis in mammalian cells the Golgi apparatus becomes fragmented into small pieces and dispersed away from the pericentriolar region. Preventing this event through use of reagents that antagonize the Golgi-associated protein GRASP65 also blocks mitotic progression. Here we show that GRASP65 is hyper-phosphorylated during mitosis and that a mitotic form of GRASP65 can be visualized with a phospho-antibody for the duration of Golgi fragmentation. We also demonstrate that this signal requires Polo-like kinase 1. Additionally, we have mapped the major mitotic phosphorylation sites within GRASP65 and have identified an adjacent 75 amino acid region of the protein that is required for phosphorylation at these sites. When present in excess this domain of GRASP65 inhibits mitotic progression. Our findings suggest that phosphorylation of GRASP65 is important for the progression of mitotic event

3.2 INTRODUCTION

In mammalian cells the Golgi apparatus is organized into connected stacks of cisternae that are anchored to the pericentriolar region. During mitosis the Golgi is broken down into small elements and dispersed away from the cell center. This process is likely involved in the partitioning of the Golgi into daughter cells but may serve additional purposes (Colanzi *et al.*, 2003a). Mitotic Golgi fragmentation was previously reconstituted in permeabilized Normal Rat Kidney (NRK) cells (Acharya *et al.*, 1998). Through use of this assay the MAPK pathway (Acharya *et al.*, 1998; Colanzi *et al.*, 2000; Colanzi *et al.*, 2003b), Polo-like kinase 1 (Sutterlin *et al.*, 2001), and the Golgi-associated proteins GRASP65 (Sutterlin *et al.*, 2002) and GRASP55 (Kinseth *et al.*, submitted) have been implicated in the breakdown of Golgi membranes during mitosis.

The MAPK pathway is a signaling cascade that serves to activate ERKs (Extracellular Regulated Kinases) resulting in the phosphorylation of ERK targets and changes in cellular physiology (reference). ERK has been shown to localize to Golgi membranes (Acharya *et al.*, 1998) where it likely phosphorylates GRASP55 during mitosis (Jesch *et al.*, 2001). GRASP55 is required for the connections between cisternal stacks and is involved in mitotic Golgi fragmentation (Kinseth *et al.*,

submitted). We hypothesize that during mitosis phosphorylation of GRASP55 by ERK severs the connections between cisternal stacks resulting in the initiation of Golgi fragmentation. In support of this idea, stimulation of the MAPK pathway in non-dividing cells leads to Golgi disassembly (Cha and Shapiro, 2001).

Polo-like kinase 1 (Plk1) regulates cell cycle events through phosphorylation of its target substrates. It initiates mitosis by activating cdk1 and is also required for centrosome duplication and bipolar spindle formation (Nigg, 1998). Plk1 was found to interact with and phosphorylate GRASP65 in vivo (Lin *et al.*, 2000) and was later shown to be required for Golgi fragmentation (Sutterlin *et al.*, 2001). These findings indicated that GRASP65 could be an effector of Plk1-dependent Golgi fragmentation. To determine if GRASP65 has a role in Golgi fragmentation, reagents that antagonize GRASP65 were tested for their effects on Golgi disassembly in permeabilized NRK cells (Sutterlin *et al.*, 2002). When added to the semi-intact cells, a polyclonal antibody and the C-terminal portion of GRASP65 to which the antibody binds (Figure 1A) inhibited Golgi fragmentation (Sutterlin *et al.*, 2002). It was hypothesized that, in these experiments, both reagents acted to prevent endogenous GRASP65 from interacting with a factor(s) that is required to initiate Golgi fragmentation. The antibody could prevent such an interaction by directly binding to GRASP65, while the C-terminal

portion of GRASP65 could sequester the interacting factor(s). Since these reagents inhibited Golgi fragmentation by antagonizing a Golgi protein and not a multi-functional kinase (MAPK cascade, Plk1) they could be used to specifically test the function of mitotic Golgi fragmentation in cells. It was found that when either reagent was added to dividing cells Golgi fragmentation was prevented and the cells arrested in G2 (Sutterlin *et al.*, 2002). The block in cell cycle progression was alleviated by pre-fragmenting the Golgi through use of the drugs Brefeldin A and nocodazole, which induce Golgi-ER fusion and microtubule depolymerization, respectfully. This suggests that cells can sense Golgi fragmentation and that this event must occur for cells to continue dividing.

In this report we investigate the mechanism by which GRASP65 initiates Golgi fragmentation. We provide evidence for a phosphorylation induced, mitotic form of GRASP65 that regulates Golgi fragmentation and mitotic progression.

3.3 RESULTS

GRASP65 is hyper-phosphorylated during mitosis

To determine if GRASP65 becomes phosphorylated during mitosis we performed in vitro phosphorylation experiments. Full length rat

GRASP65 (FL) and the C-terminal portion of the protein to which αGRASP65 binds (the last 250 amino acids (Δ200)) were expressed in *e.coli* and purified from lysate by Ni2+ - agarose beads. The recombinant proteins were incubated with either interphase or mitotic extract obtained from Normal Rat Kidney (NRK) cells for 45 min at 32ºC, in the presence of ATP. After the incubation, the GRASP65 constructs were isolated from the reactions and examined for phosphorylation by autoradiography (Figure 1B). Both FL and Δ200 were phosphorylated by interphase extract. However, the level of phosphorylation increased for both (as determined with a phosphorimager) when exposed to mitotic extract (Figure 1B) indicating the existence of a mitosis-specific phosphorylation event.

A mitotic form of GRASP65

Previously it had been reported that an antibody made against ERK mono-phosphorylated on the tyrosine residue in its activation loop (α-pYERK) stained Golgi membranes during mitosis for a period that correlated with Golgi fragmentation and reassembly (Cha and Shapiro, 2001). We have reconstituted this signal biochemically with purified Golgi membranes and recombinant protein and found that the mitotic-Golgi labeling observed with this antibody results from the staining of GRASP65 and not ERK. To recapitulate the mitotic signal observed

with α-pYERK we incubated purified rat liver Golgi membranes with interphase or mitotic extract and ATP. After 30 min at 32ºC a protein of 65 KDa was labeled by α-pYERK with mitotic but not interphase extract (Figure 2A). This protein was not detected in mitotic extract alone and is most likely associated with Golgi membranes (Figure 2A). We postulated that the 65 KDa Golgi associated protein could be GRASP65. To test this idea we pre-incubated Golgi membranes with increasing amounts of α-GRASP65 and then treated the membranes with mitotic extract and ATP as before. Under these conditions, the 65 KDa signal observed with α-pYERK was diminished in a dose dependent manner (Figure 2B). To further demonstrate that α-pYERK labels a mitotis-specific form of GRASP65 we incubated recombinant FL GRASP65 with mitotic or interphase extract and ATP for 30 min at 32ºC. We found that FL GRASP65 was labeled by α-pYERK when incubated with mitotic but not interphase extract (data not shown). We also demonstrate that α-pYERK recognizes Δ200 (Figure 2C), which is the region of GRASP65 that interacts with α-GRASP65 and is involved in Golgi fragmentation and mitotic progression (Sutterlin *et al.*, 2002). These findings strongly suggest that α-pYERK recognizes a mitotic form of GRASP65.

Plk1 is required for mitotic GRASP65

Since GRASP65 is a known substrate of Plk1 and both are involved in Golgi fragmentation we tested whether the α-pYERK signal observed on GRASP65 requires Plk1. Plk1 was immunodepleted from mitotic extract (Figure 3). After two rounds of immunodepletion Plk1 could not be detected in mitotic extract. Δ200 incubated with mitotic extract lacking Plk1 was not recognized by α-pYERK, but Δ200 treated with mock depleted mitotic extract yielded a robust signal (Figure 3). This demonstrates that Plk1 is required to generate the mitotic form of GRASP65 that is recognized by the α-pYERK antibody.

Mapping the major mitotic phosphorylation sites in GRASP65

We next wished to discover which residues in GRASP65 are phosphorylated during mitosis. To achieve this we performed phospho-peptide mapping. Recombinant FL GRASP65 was incubated with interphase or mitotic extract for 30 min at 32ºC. After the incubation recombinant GRASP65 was purified from the reactions and trypsinized to completion. The resulting protein fragments were spotted onto Thin Layer Chromatography plates and separated in two dimensions based on charge and hydrophobicity. Phosphorylated peptides were visualized by autoradiography (Figure 4A). In both interphase and mitosis the

majority of the phosphorylation on GRASP65 appears to occur within the same peptide(s) (Figure 4A). However, phosphorylation within this peptide(s) was increased with mitotic extract (Figure 4A). These results are consistent with our prior observation that FL GRASP65 is hyper-phosphorylated during mitosis (Figure 1B). The major spots generated by mitotic phosphorylation were collected from the TLC plates and the phosphorylated peptides were extracted from this material. The peptides were hydrolyzed into individual amino acids, which were spotted onto new TLC plates and separated based on charge and hydrophobicity. Phosphorylated amino acids were visualized by autoradiography and an amino acid standard was observed with a dye. Phospho-amino acid analysis clearly demonstrated that each spot contained phosphorylated serine and threonine residues (Figure 4B). Serine was the major phospho-residue in both spots, and each spot contained approximately equal amounts of phospho-threonine (pThr). A phosphorimager was used to determine the ratio of pSer to pThr in each spot. It was found that pSer was present 3:1 and 2:1 over pThr in spots A and B respectively.

The addition of a phosphate residue to a peptide affects the overall charge and hydrophobicity of the peptide and induces a characteristic shift in the phospho-peptide map. Thus, a peptide that harbors multiple phosphorylations can exist as multiple spots in a phospho-peptide map if

a significant population of the peptide is only partially phosphorylated. These peptides display a characteristic distribution in a phospho-peptide map, and as a result they can often be identified. The position of the two major spots in the mitotic phospho-peptide map of GRASP65 hinted that the spots may represent a single peptide that exists in two different phosphorylation states. The location of these spots within the phospho-peptide map, the results of the phospho-amino acid analysis, and the composition of the predicted peptides generated from a theoretical tryptic digestion of GRASP65 indicated that the spots may correspond to a peptide made up of amino acids 211-223 (Figure 4C). This peptide is located within Δ200, which is hyper-phosphorylated during mitosis (Figure 1B), and contains multiple serine residues and a threonine residue. Among these residues are a ser-pro (217-218) and a thr-pro (220-221) which are potential targets of mitotic kinases (Yaffe *et al.*, 1997).

To verify that the major mitotic phosphorylation sites in GRASP65 lay within the predicted peptide (211-223) a fragment of GRASP65 (195-305) encompassing this peptide was purified from bacteria and tested for its ability to reproduce the phosphorylation pattern observed for FL GRASP65. We found that, when incubated with mitotic extract 195-305 did in fact reproduce the major mitotic phosphorylations in GRASP65 (Figure 5A). We next made several different combinations of

alanine substitutions for the possible phosphorylated ser and thr residues within amino acids 211-223 in the background of 195-305. We found that when either thr 220 or ser 216 was mutated to alanine one of the major spots was lost from the mitotic phospho-peptide map (data not shown). When both thr 220 and ser 216 were mutated to alanine in the same GRASP65 fragment both major spots were lost from the phospho-peptide map (Figure 5B). This indicates that the two major spots observed in the mitotic phospho-peptide map of GRASP65 come from a single peptide and result from phosphorylation on thr 220 and ser 216.

Excess GRASP65 195-305 prevents Golgi fragmentation and mitotic Progression

If phosphorylation of GRASP65 on ser 216 and thr 220 is required for mitotic Golgi fragmentation, then an excess of GRASP65 195-305 should prevent Golgi fragmentation by sequestering the kinase(s) responsible. To test this, permeabilized NRK cells were incubated with mitotic extract +/- recombinant GRASP65 195-305 and an ATP regenerating system for 60 min at 32ºC. Approximately 70% (67 +/- 8%) of cells incubated with mitotic extract only displayed fragmented Golgi membranes typical of mitotic cells (Figure 6A). Contrastly, only 25% (25 +/- 4%) of the cells treated with mitotic extract *and* excess 195-305 displayed Golgi fragmentation (Figure 6A). To determine if excess

195-305 also blocks mitotic entry recombinant 195-305 was microinjected into NRK cells that had been arrested in S-phase overnight by the presence of aphidicolin, an inhibitor of DNA polymerase (Sutterlin *et al.*, 2002). We found the mitotic index was reduced from 100% to 56 +/- 10% in cells that were microinjected with 195-305 versus bacterial lysate (Figure 6B). These findings indicate that the region of GRASP65 corresponding to amino acids 195-305 are important for Golgi fragmentation and mitotic progression.

GRASP65 230-305 is important for phosphorylation and mitotic progression

We next tested constructs of 195-305 that either contained alanines or glutamic acid residues for S216, T220 for their effects on Golgi fragmentation in vitro. Recombinant forms of the constructs were added to the semiintact Golgi fragmentation assay and the percentage of cells displaying fragmented Golgi membranes was determined in each case. We found that all the mutants inhibited Golgi fragmentation to a level similar to wild type 195-305 (data not shown). We hypothesized that these constructs could still interact with the kinase(s) responsible for phosphorylation, and so we generated GRASP65 230-305 which lacks the region containing S216 and T220. 230-305 strongly inhibited the phosphorylation of 195-305 but is itself unphosphorylated (Figure 6C),

indicating that it interacts with the kinase(s) responsible for phosphorylation on S216 and T220.

We next tested if 230-305, when microinjected into NRK cells, has an effect on cell cycle progression. Cell synchronization/microinjection experiments were performed as before and it was determined that, in the presence of 230-305 the mitotic index dropped from 91% (Bacterial lysate) to 16% (Figure 6B). This inhibition of mitotic entry was much stronger than was observed with α-GRASP65 antibody, Δ200, and 195-305.

3.4 CONCLUSIONS

Recent evidence indicates that the G2/M transition is controlled by Golgi fragmentation (Sutterlin *et al.*, 2002), suggesting the existence of a cell cycle checkpoint that monitors Golgi structure. Thus far GRASP65 is the only known component of this pathway and the nature of its role is not clear. Reagents that antagonize the C-terminus of GRASP65 inhibit Golgi fragmentation and arrest cells in G2. We hypothesized that these reagents prevent components required for these events from interacting with endogenous GRASP65. Specifically, we speculated that GRASP65 becomes phosphorylated at the G2/M transition initiating Golgi fragmentation and mitotic progression

Here we show that GRASP65 is hyper-phosphorylated during mitosis (Figure 1B), and that a mitosis-specific form of the protein can be visualized, in a Plk1 dependent manner (Figure 3), with a phospho-antibody (Figure 2). This signal is apparent at the beginning of Golgi fragmentation and lasts until Golgi membranes begin to reform in daughter cells (Cha and Shapiro, 2001). In addition, we have mapped the major mitotic phosphorylation sites in GRASP65 to S216 and T220 (Figure 5). A small fragment of GRASP65 (195-305) that harbors these residues inhibits Golgi fragmentation and mitotic progression to an extent similar to α-GRASP65 antibody and Δ200 (Figure 6B). Interestingly, constructs of 195-305 mutant for S216 and T220 still prevented Golgi fragmentation. However, we determined that these constructs were still able to bind the kinase(s) responsible for phosphorylation as GRASP65 230-305, which is not phosphorylated, completely prevents phosphorylation of 195-305, and strongly inhibits mitotic progression (Figure 6B and C). Thus the region of GRASP65 between amino acids 230 and 305 interacts with a factor(s) that is required for phosphorylation of GRASP65, which in turn initiates Golgi fragmentation and mitotic progression.

The obvious question is, “what does this region bind?” One possibility is Plk1. GRASP65 is an in vivo substrate of Plk1 (Lin *et al.*,

2000) and Plk1 has been shown to be required for Golgi fragmentation (Sutterlin *et al.*, 2001). In addition, Plk1 is required for the mitotic form of GRASP65 observed with the α-pYERK antibody (Figure 3). However, the α-pYERK antibody does not recognize phosphorylated 195-305 (data not shown) which contains 230-305 and S216 and T220, the major sites of phosphorylation in GRASP65. Nevertheless, it is highly likely that Plk1 directly phosphorylates GRASP65 during G2/M (S372 consensus site), but this phosphorylation event may be different from those involving 230-305.

3.5 METHODS

Reagents. α-GRASP65 is described in (Sutterlin *et al.*, 2002). α-pYERK was purchased from SIGMA. 8847 polyclonal Plk1 antibody was a gift from Doug Ferris. Monoclonal α-Plk1 was purchased from Zymed. 2º antibodies were purchased from Jackson Laboratories.

Phosphorylation Experiments. Recombinant GRASP65 constructs were purified from *e.coli* as in (Sutterlin *et al.*, 2002), and mitotic and interphase extracts were prepared as in (Acharya *et al.*, 1998). In general, 3ug of recombinant GRASP65 protein was incubated with 5uL

of interphase or mitotic extract (13 ug/mL), phosphorylation buffer (10 mM MGCl2, 50 mM Hepes pH 7.4, 100 uM ATP [cold], 1 mM DTT, 5 uM microcystin, 100 uM PMSF), and P32-ATP in a total volume of 20 uL. The reactions were carried out at 32ºC for 45 min. GRASP65 proteins were isolated from the reactions with Ni2+-agarose beads (His-tag) and subjected to SDS PAGE. Phosphorylation was observed by autoradiography.

Reconstituting the pYERK signal with purified Golgi membranes. Golgi membranes were purified as in (Lowe *et al.*, 1998). 50 ug of rat liver Golgi membranes was incubated with 5 uL mitotic extract, ATP (500 uM), and buffer B (Lowe *et al.*, 1998) for 30 min at 32ºC. Sample buffer containing SDS was added to the reactions and the samples were subjected to SDS PAGE. The samples were then transferred to nitrocellulose blot paper, washed (3% milk in PBST), and incubated with α-pYERK (1:1000, 3% BSA, PBST) for 1h at room temperature. After incubation with 2º ab conjugated to HRP proteins were visualized via ECL.

Immunodepletion of Plk1 from mitotic extract. Rabbitt polyclonal α-Plk1 (8847, Doug Ferris) was incubated with protein G sepharose beads overnight at 4ºC. The beads were washed and incubated with 6uL

mitotic extract and incubated at 4ºC 90 min (round 1). The mitotic extract was isolated from the beads and incubated with a second set of protein G beads conjugated to rabbit α-Plk1 for 90 min at 4ºC (round 2). The mitotic extract was isolated from the beads and treated with sample buffer, as were both sets of beads from rounds 1 and 2. The resulting samples were subjected to SDS PAGE and the pYERK signal was observed as described above, or Plk1 was observed with a monoclonal antibody (Zymed).

Phospho-peptide and phospho-amino acid analysis of GRASP65 constructs. All experiments were performed using the Hunter Thin Layer Peptide Mapping Electrophoresis System (Model # HTLE-7002), and the experiments were carried out exactly as is described in the instruction manual. In general, GRASP65 proteins were phosphorylated as described above, and isolated from the reactions. The proteins were then trypsinized to completion and spotted onto TLC plates. The peptides were separated by electrophoresis and by chromatography, and then visualized by autoradiography. For phospho-amino acid analysis peptide spots were scraped and hydrolyzed into individual amino acids, which were spotted onto TLC plates and separated as described.

Golgi fragmentation assays and effects of GRASP65 fragments on cell cycle progression. Experiments were performed as in (Sutterlin *et al.*, 2002).

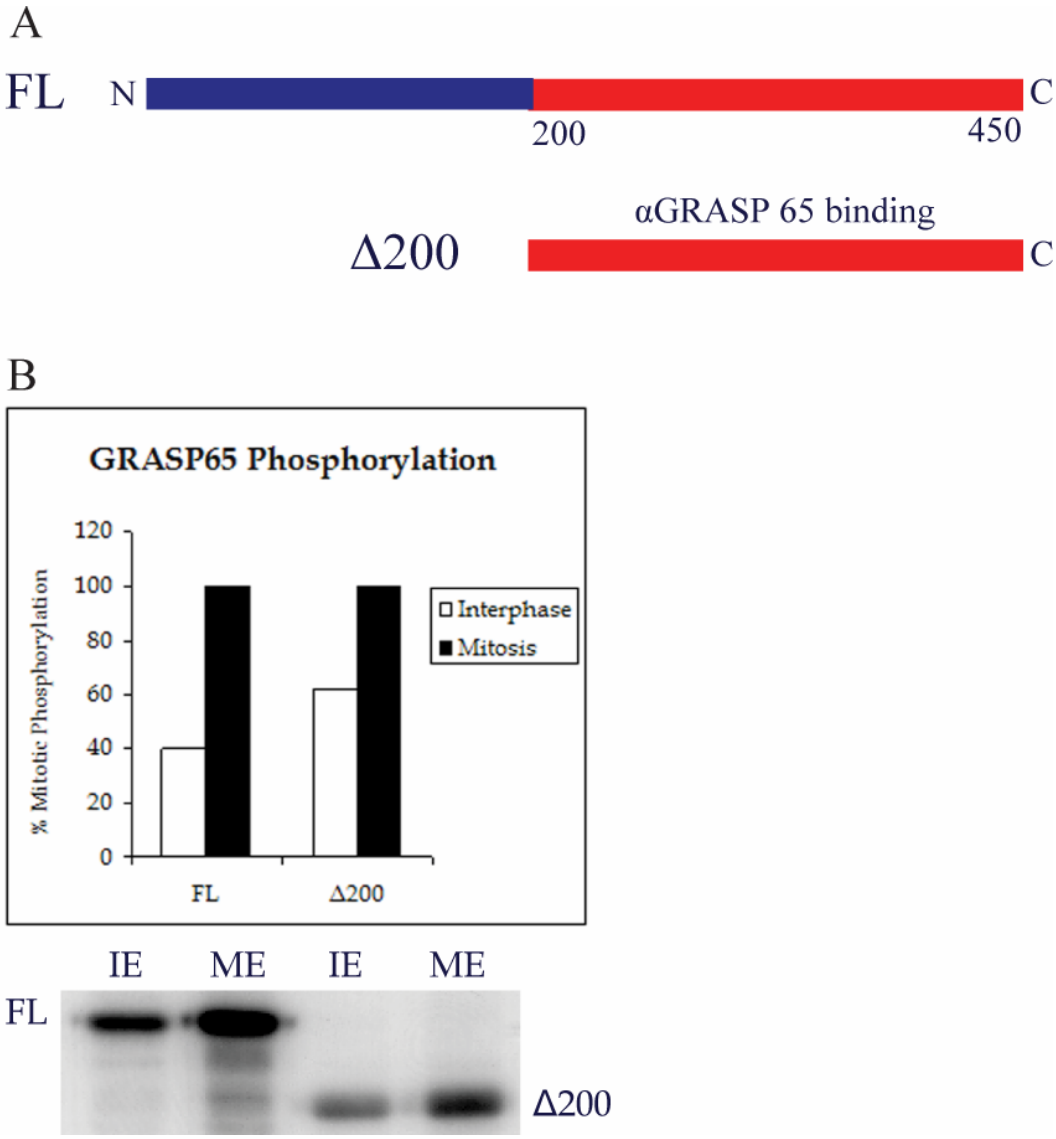


Figure 12. GRASP65 is hyperphosphorylated during mitosis. (A) Diagramof full length GRASP65 (FL) and the C-terminal 250 residues (Δ200). A-GRASP65 binds to Δ200. (B) GRASP65 is hyperphosphorylated during mitosis. Equal amounts of recombinant FL GRASP65 and Δ200 were phosphorylated with interphase or mitotic extract from NRK cells. The proteins were isolated from the reactions and phosphorylation was observed via autoradiography. A phosphorimager was used to determine the extent of phosphorylation for each reaction. The phosphorylation of both FL and Δ200 increases upon incubation with mitotic extract.

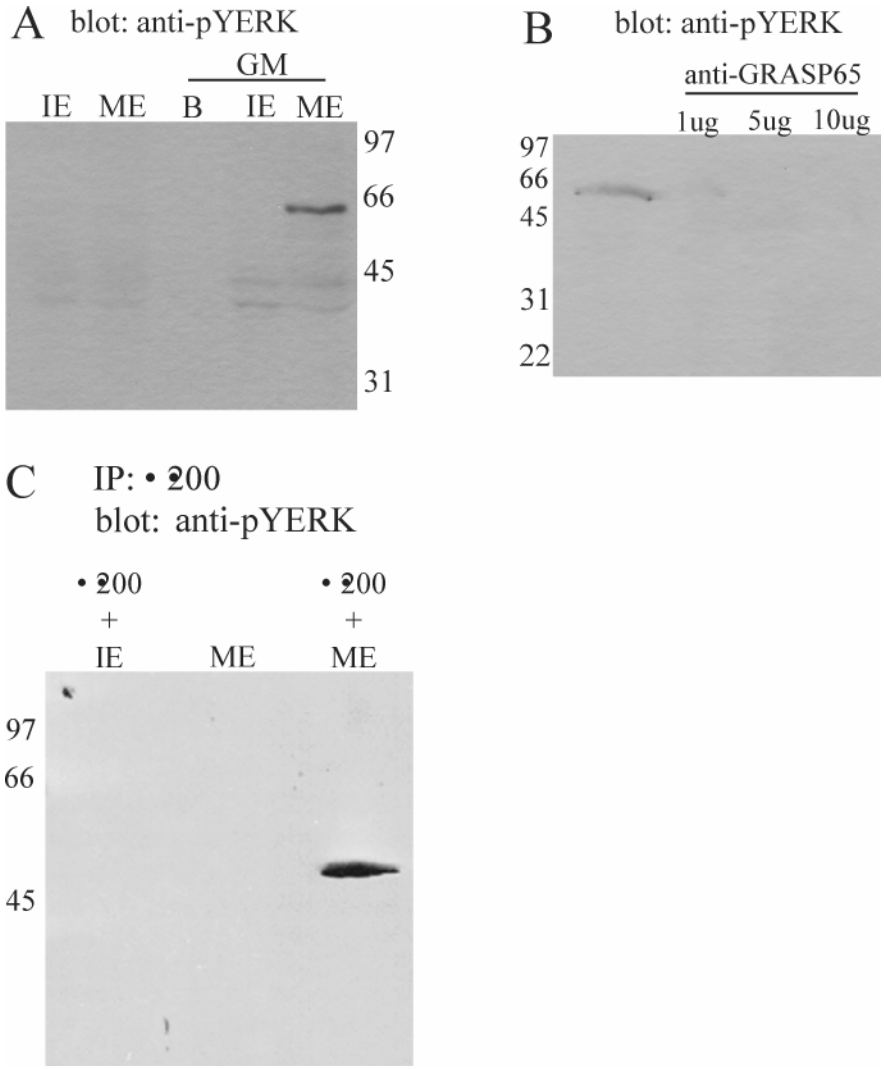


Figure 13. A mitotic form of GRASP65. (A) Rat liver Golgi membranes (GM) were incubated with interphase (IE) or mitotic extract (ME). The α-pYERK antibody specifically labels a protein of 65 KDa for the incubation with mitotic extract. (B) Rat liver Golgi membranes were preincubated with increasing amounts of α-GRASP65 antibody and then incubated with mitotic extract. The 65 KDa signal observed with the α-pYERK antibody is prevented by the presence of α-GRASP65. (C) Recombinant Δ200 was incubated with interphase or mitotic extract. The α-pYERK antibody recognizes Δ200 that is incubated with mitotic extract.

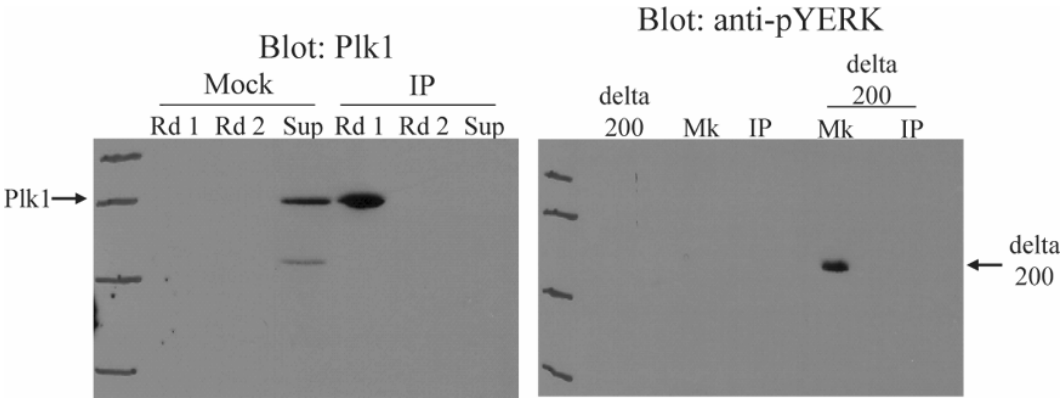
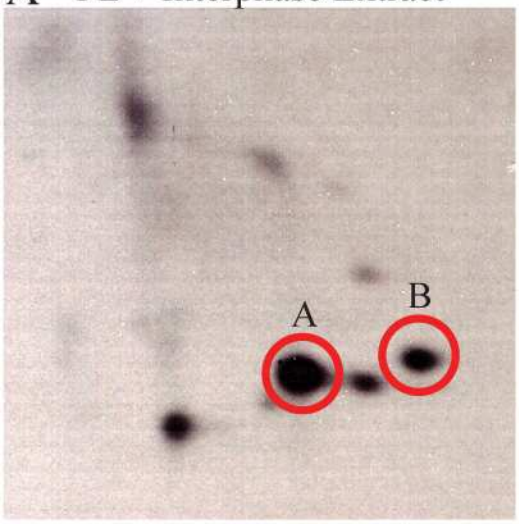


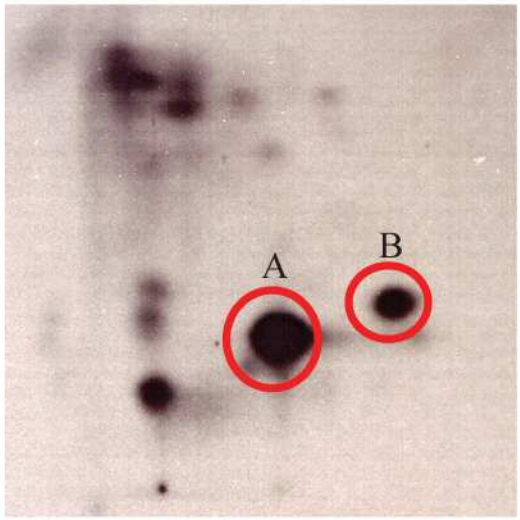
Figure 14. Plk1 is required for the mitotic form of GRASP65 recognized by the pYERK antibody. Mitotic extract was immuno-depleted of PLK1. Delta 200 was incubated with immuno-depleted (IP) or mock (MK) depleted mitotic extract. The pYERK antibody does not recognize delta 200 incubated with mitotic extract lacking PLK1.

Figure 15. Phospho-peptide maps reveal the major mitotic phosphorylation sites in GRASP65. (A) Phospho-peptide mapping of FL GRASP65. FL GRASP65 was incubated with interphase or mitotic extract in the presence of radiolabeled ATP. GRASP65 was isolated from the reactions and trypsinized to completion. The resulting peptide fragments were spotted onto TLC plates and separated in two dimensions. Phosphorylated peptides are visualized by autoradiography. Spots A and B are the major sites of phosphorylation in both interphase and mitosis. Equal counts of radioactivity were spotted onto each TLC plate. (B) Phospho-amino acid analyasis of spots A and B. Spots A and B were scraped from TLC plates and hydrolyzed into individual amino acids. The resulting residues were spotted onto TLC plates and separated in two dimensions. Phosphorylated residues were observed via autoradiography, and a phosphor-amino acid standard was visualized with a dye. Spots A and B contain pSer and pThr. Equal counts of radioactivity were spotted onto each TLC plate. (C) Tryptic peptide of GRASP65 harboring the phosphorylations represented by spots A and B.

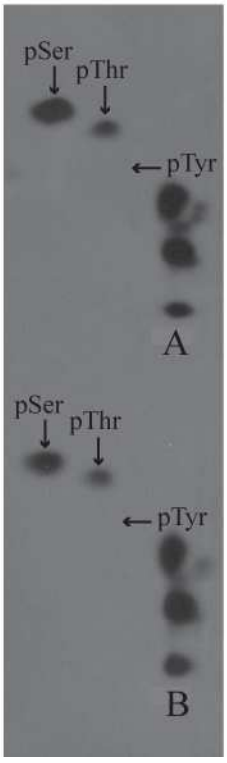
A FL + Interphase Extract



FL + Mitotic Extract



B



C

211 – K P P S A S S P G T P A K - 223

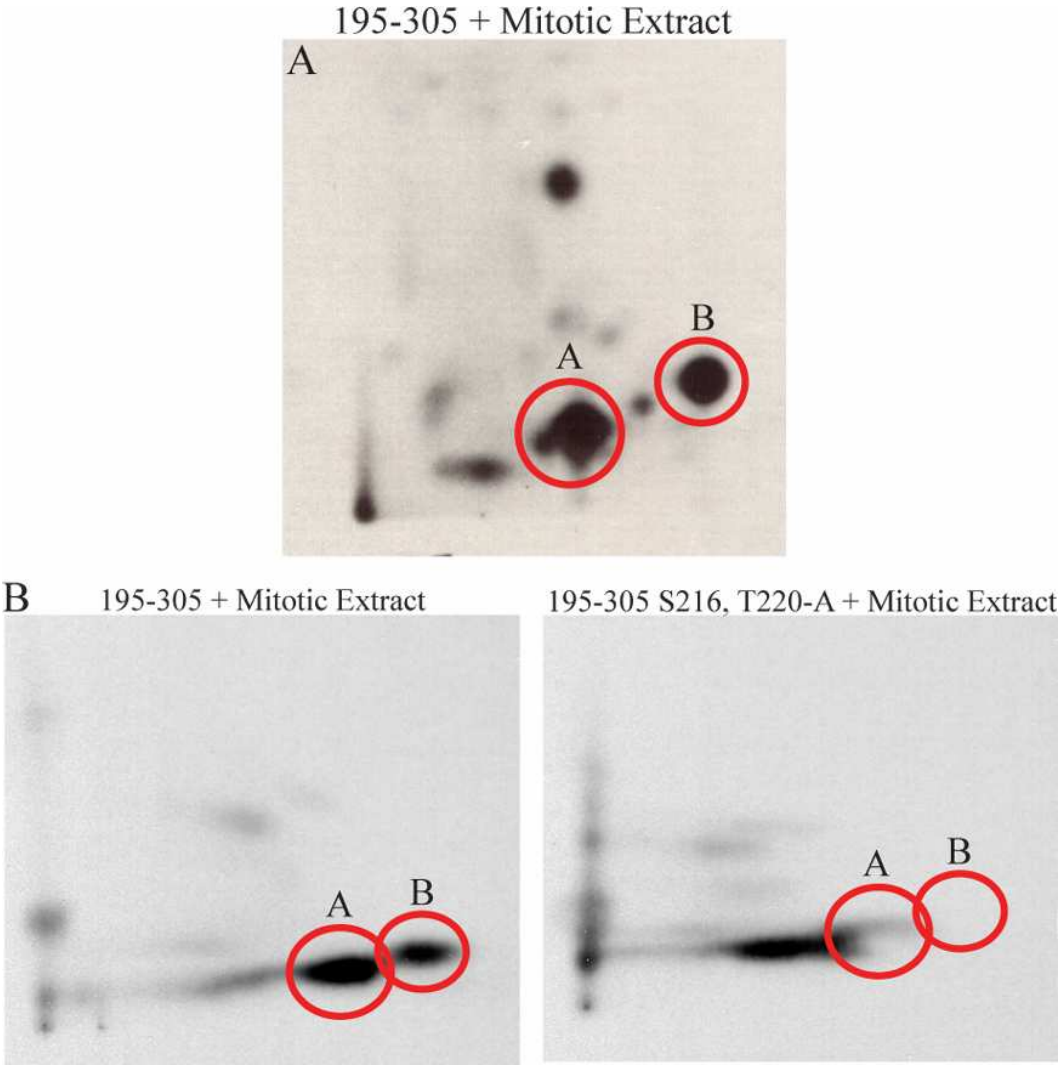
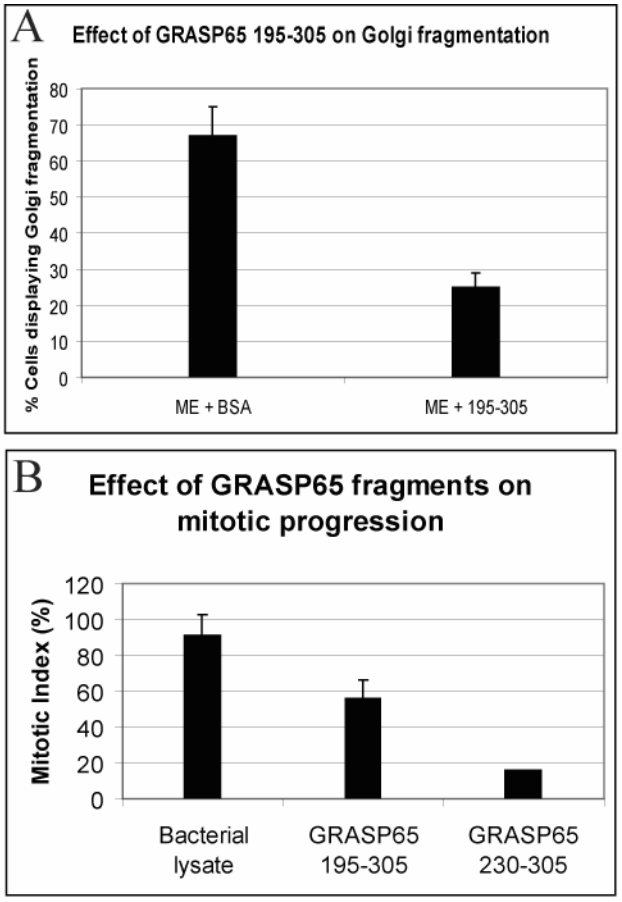


Figure 16. GRASP65 is phosphorylated on S216 and T220 during mitosis. (A) GRASP65195-305 contains the major mitotic phosphorylation sites. GRASP65 195-305 was incubated with mitotic extract and radio-labeled ATP. 195-305 was isolated from the reaction and trypsinized to completion. The peptide fragments were spotted onto a TLC plate and separated in two dimensions. Phosphorylated peptides were visualized with auto-radiography. (B) S216 and T220 are the major phosphorylation sites in GRASP65. 195-305 or 195-305 S216, T220-A was treated as in (A). Phosphorylated peptides were observed via autoradiography. When S216 and T220 are mutated to alanines spots A and B disappear. Equal counts of radioactivity were spotted on to each TLC plate.

Figure 17. Effects of GRASP65 fragments on Golgi fragmentation, mitotic progression, and phosphorylation. (A) Permeablized NRK cells were incubated with mitotic extract (ME) + BSA or GRASP65 195-305 and the percentage of cells displaying Golgi fragmentation after the incubation was determined. 195-305 strongly inhibits Golgi fragmentation. (B) NRK cells were arrested in S-phase, released, and microinjected with bacterial lysate, 195-305, or 230-305. The mitotic index was determined after 7.5 h. Both 195-305 and 230-305 inhibit mitotic progression. (C) Recombinant GRASP65 195-305 was incubated with ME, ME + 230-305, or ME + delta 300 (last 150 amino acids of GRASP65, Figure 7). The phosphorylation state of the proteins in each reaction was determined by autoradiography. 230-305 inhibits phosphorylation of 195-305 but is itself unphosphorylated.


A
Effect of GRASP65 195-305 on Golgi fragmentation
% Cells displaying Golgi fragmentation
80
70
60
50
40
30
20
10
0
ME + BSA
ME + 195-305
B
Effect of GRASP65 fragments on mitotic progression
Mitotic Index (%)
120
100
80
60
40
20
0
Bacterial lysate
GRASP65 195-305
GRASP65 230-305
C
195-305 + ME
delta
-
230-305
300
delta 300
195-305
230-305

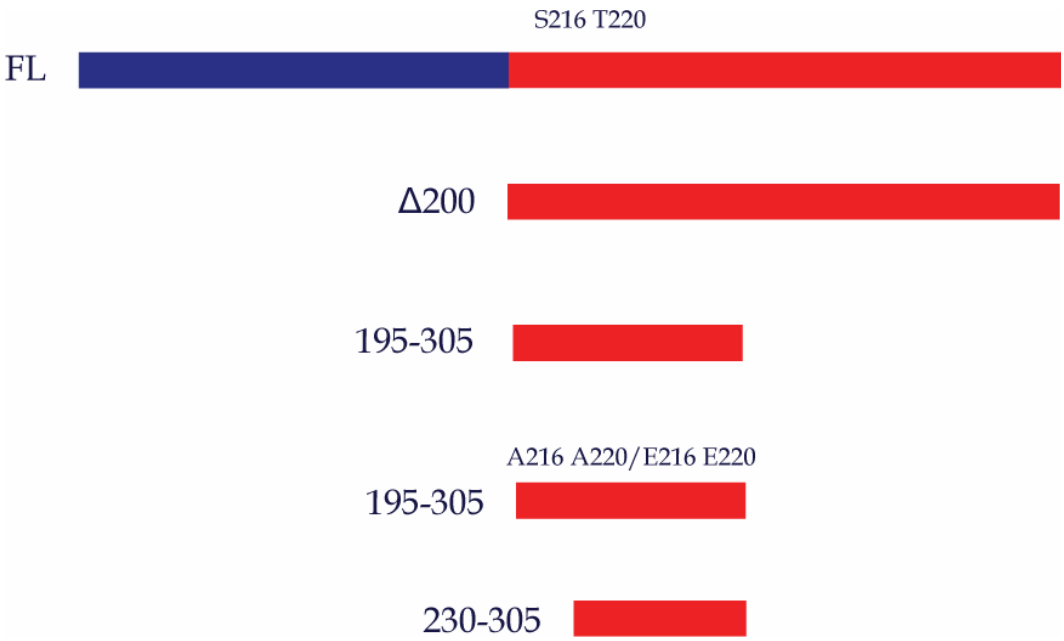


Figure 18. Diagram of GRASP65 constructs.

CONCLUSIONS

A. De novo biogenesis of the Golgi apparatus

Protein secretion is one of the most important processes that occur within cells, yet we have a poor understanding of how the secretory pathway is organized and maintained. In general, we know that nascent secretory proteins are translated into the ER where they are folded and modified with core sugar residues. From the ER secretory cargoes are shipped to the Golgi apparatus where they are modified further by Golgi enzymes and transported to the Trans Golgi Network (TGN). In the TGN proteins are sorted into specific transport carriers and delivered to their respective destinations. One issue that remains unresolved is how the Golgi apparatus maintains its organization amidst the constant flow of secretory traffic. In the past, the Golgi has been thought of as a stable structure that proteins traversing the secretory pathway visit to complete their biogenesis and reach their sites of action. Support for this concept was derived from the heterogeneity between Golgi cisternae of a given stack and the biochemical asymmetry within individual cisterna themselves. The major conclusion drawn from these observations was that each daughter cell inherits a full set of completely differentiated membrane from its mother (Farquhar and Palade, 1981). In other words,

cells preserve the integrity of the secretory pathway by maintaining biochemically differentiated membrane rather than constantly changing one type of membrane into another (Farquhar and Palade, 1981). This was thought to be achieved by controlling the trafficking of components between biochemically different membrane structures with vesicular intermediates (Farquhar and Palade, 1981). However, it has recently been proposed that the Golgi apparatus is a transient structure that is constantly reborn from the ER. This idea is based on reports demonstrating that Golgi membranes fuse with the ER during cell division (Zaal *et al.*, 1999; Altan-Bonnet *et al.*, 2006) and accumulating evidence that all Golgi proteins rapidly cycle through the ER (Miles *et al.*, 2001; Ward *et al.*, 2001). This hypothesis argues that the Golgi apparatus is maintained by the constant transformation of ER membranes into Golgi membranes.

The bulk of the evidence supporting this hypothesis was obtained under conditions in which the secretory pathway was perturbed through the use of chemicals or dominant-negative constructs (Lippincott-Schwartz *et al.*, 1989; Storrie *et al.*, 1998; Zaal *et al.*, 1999; Miles *et al.*, 2001; Ward *et al.*, 2001). To investigate the recycling of Golgi proteins to the ER under physiological conditions we developed a procedure that captures Golgi proteins when they visit the ER (Pecot and Malhotra, 2004). This method exploits the conditional interaction between the

FK506 Binding Protein (FKBP) and the FK506-Rapamycin Associated Protein (FRAP). Rapamycin specifically binds to FKBP (Wiederrecht *et al.*, 1991), and FRAP specifically binds to the FKBP-rapamycin complex (Brown *et al.*, 1994; Sabatini *et al.*, 1994). We have shown that an early Golgi enzyme (mannosidaseII, M2-FKBP) and a late Golgi enzyme (sialyltransferase, ST-FKBP), when fused to FKBP can be efficiently captured in the ER of BFA treated cells by an ER protein fused to FRAP (invariant chain [Iip33], Ii-FRAP) in the presence of rapamycin (Pecot and Malhotra, 2004). As an additional control we generated an ERGIC-53-FKBP fusion (FKBP-E53) and found that it rapidly associates with our ER reporter under normal conditions (30 min) (Pecot and Malhotra, submitted). Having established the ER-trapping procedure we tested the binding of our early and late Golgi reporters with our ER reporter under normal conditions, during cell division, and in the presence of a microtubule de-polymerizing agent (nocodazole). We found no significant association between these constructs under these circumstances. Based on our findings we conclude that Golgi membranes remain independent of the ER throughout the lifetime of the cell.

B. The role of GRASP65 in Golgi fragmentation and mitotic progression

During cell division in mammalian cells the Golgi apparatus undergoes a dramatic structural reorganization before reforming in each daughter cell. The ribbon of connected cisternal stacks is completely converted into small elements that represent the partitioning unit of the organelle (Pecot and Malhotra, 2004). This occurs via a two step process in which Golgi membranes are first fragmented into large pieces that eventually breakdown into vesiculo-tubular elements (Colanzi *et al.*, 2003a). Preventing the first step of Golgi fragmentation with reagents that antagonize the Golgi associated protein GRASP65 arrests cells in G2 (Sutterlin *et al.*, 2002). Cells enter mitosis normally in the presence of these reagents if Golgi membranes are artificially fragmented with BFA or nocodazole (Sutterlin *et al.*, 2002), indicating the existence of a cell cycle checkpoint that monitors Golgi structure.

A polyclonal α-GRASP65 antibody and the C-terminal fragment (Δ200, last 250 amino acids) to which it binds both inhibit Golgi fragmentation and mitotic progression. We hypothesized that these reagents might interphere with the phosphorylation of endogenous GRASP65 at the G2/M transition. We demonstrated that GRASP65 is

hyperphosphorylated during mitosis and that a mitotic form of GRASP65 can be visualized with a phospho-antibody in a Plk1 dependent manner. This signal is observed concomitantly with Golgi fragmentation suggesting a correlation between the two. We also determined that the major phosphorylation events in GRASP65 occur on S216 and T220. An adjacent 75 amino acid region of the protein is required for phosphorylation at these sites but is itself not phosphorylated, indicating that it may directly interact with the kinase(s) responsible. When microinjected into cells a peptide corresponding to this region prevented mitotic progression to a much stronger degree than α-GRASP65 or Δ200. Based on these findings we conclude that phosphorylation of GRASP65 is required to initiate Golgi fragmentation and therefore mitotic progression, and that the region corresponding to GRASP65-230-305 likely binds to proteins required for these processes.

REFERENCES

Acharya, U., Mallabiabarrena, A., Acharya, J.K., and Malhotra, V. (1998). Signaling via mitogen-activated protein kinase kinase (MEK1) is required for Golgi fragmentation during mitosis. Cell *92*, 183-192.

Altan-Bonnet, N., Sougrat, R., Liu, W., Snapp, E.L., Ward, T., and Lippincott-Schwartz, J. (2006). Golgi inheritance in mammalian cells is mediated through endoplasmic reticulum export activities. Mol Biol Cell *17*, 990-1005.

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.

Aridor, M., Bannykh, S.I., Rowe, T., and Balch, W.E. (1999). Cargo can modulate COPII vesicle formation from the endoplasmic reticulum. J Biol Chem *274*, 4389-4399.

Axelsson, M.A., and Warren, G. (2004). Rapid, endoplasmic reticulum-independent diffusion of the mitotic Golgi haze. Mol Biol Cell *15*, 1843-1852.

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

Barlowe, C., Orci, L., Yeung, T., Hosobuchi, M., Hamamoto, S., Salama, N., Rexach, M.F., Ravazzola, M., Amherdt, M., and Schekman, R. (1994). COPII: a membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum. Cell *77*, 895-907.

Barr, F.A., Puype, M., Vandekerckhove, J., and Warren, G. (1997). GRASP65, a protein involved in the stacking of Golgi cisternae. Cell *91*, 253-262.

Becker, B., and Melkonian, M. (1996). The secretory pathway of protists: spatial and functional organization and evolution. Microbiol Rev *60*, 697-721.

Ben-Tekaya, H., Miura, K., Pepperkok, R., and Hauri, H.P. (2005). Live imaging of bidirectional traffic from the ERGIC. J Cell Sci *118*, 357-367.

Brown, E.J., Albers, M.W., Shin, T.B., Ichikawa, K., Keith, C.T., Lane, W.S., and Schreiber, S.L. (1994). A mammalian protein targeted by G1-arresting rapamycin-receptor complex. Nature *369*, 756-758.

Burke, B., Griffiths, G., Reggio, H., Louvard, D., and Warren, G. (1982). A monoclonal antibody against a 135-K Golgi membrane protein. Embo J *1*, 1621-1628.

Cha, H., and Shapiro, P. (2001). Tyrosine-phosphorylated extracellular signal--regulated kinase associates with the Golgi complex during G2/M phase of the cell cycle: evidence for regulation of Golgi structure. J Cell Biol *153*, 1355-1367.

Chen, J., Zheng, X.F., Brown, E.J., and Schreiber, S.L. (1995). Identification of an 11-kDa FKBP12-rapamycin-binding domain within the 289-kDa FKBP12-rapamycin-associated protein and characterization of a critical serine residue. Proc Natl Acad Sci U S A *92*, 4947-4951.

Cheng, H., and Farquhar, M.G. (1976). Presence of adenylate cyclase activity in Golgi and other fractions from rat liver. I. Biochemical determination. J Cell Biol *70*, 660-670.

Christner, C., Wyrwa, R., Marsch, S., Kullertz, G., Thiericke, R., Grabley, S., Schumann, D., and Fischer, G. (1999). Synthesis and cytotoxic evaluation of cycloheximide derivatives as potential inhibitors of FKBP12 with neuroregenerative properties. J Med Chem *42*, 3615-3622.

Colanzi, A., Deerinck, T.J., Ellisman, M.H., and Malhotra, V. (2000). A specific activation of the mitogen-activated protein kinase kinase 1 (MEK1) is required for Golgi fragmentation during mitosis. J Cell Biol *149*, 331-339.

Colanzi, A., Suetterlin, C., and Malhotra, V. (2003a). Cell-cycle-specific Golgi fragmentation: how and why? Curr Opin Cell Biol *15*, 462-467.

Colanzi, A., Sutterlin, C., and Malhotra, V. (2003b). RAF1-activated MEK1 is found on the Golgi apparatus in late prophase and is required for Golgi complex fragmentation in mitosis. J Cell Biol *161*, 27-32.

Cole, N.B., Ellenberg, J., Song, J., DiEuliis, D., and Lippincott-Schwartz, J. (1998). Retrograde transport of Golgi-localized proteins to the ER. J Cell Biol *140*, 1-15.

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.

Dalton, A.J., and Felix, M.D. (1954). Cytologic and cytochemical characteristics of the Golgi substance of epithelial cells of the epididymis in situ, in homogenates and after isolation. Am J Anat *94*, 171-207.

Dalton, A.J., and Felix, M.D. (1956). A comparative study of the Golgi complex. J Biophys Biochem Cytol *2*, 79-84.

Dunphy, W.G., Fries, E., Urbani, L.J., and Rothman, J.E. (1981). Early and late functions associated with the Golgi apparatus reside in distinct compartments. Proc Natl Acad Sci U S A *78*, 7453-7457.

Ellenberg, J., Siggia, E.D., Moreira, J.E., Smith, C.L., Presley, J.F., Worman, H.J., and Lippincott-Schwartz, J. (1997). Nuclear membrane dynamics and reassembly in living cells: targeting of an inner nuclear membrane protein in interphase and mitosis. J Cell Biol *138*, 1193-1206.

Farquhar, M.G., Bergeron, J.J., and Palade, G.E. (1974). Cytochemistry of Golgi fractions prepared from rat liver. J Cell Biol *60*, 8-25.

Farquhar, M.G., and Palade, G.E. (1981). The Golgi apparatus (complex)-(1954-1981)-from artifact to center stage. J Cell Biol *91*, 77s-103s.

Farquhar, M.G., and Rinehart, J.F. (1954). Electron microscopic studies of the anterior pituitary gland of castrate rats. Endocrinology *54*, 516-541.

Featherstone, C., Griffiths, G., and Warren, G. (1985). Newly synthesized G protein of vesicular stomatitis virus is not transported to the Golgi complex in mitotic cells. J Cell Biol *101*, 2036-2046.

Fries, E., and Rothman, J.E. (1981). Transient activity of Golgi-like membranes as donors of vesicular stomatitis viral glycoprotein in vitro. J Cell Biol *90*, 697-704.

Fujiwara, T., Oda, K., Yokota, S., Takatsuki, A., and Ikehara, Y. (1988). Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. J Biol Chem *263*, 18545-18552.

Gallione, C.J., and Rose, J.K. (1985). A single amino acid substitution in a hydrophobic domain causes temperature-sensitive cell-surface transport of a mutant viral glycoprotein. J Virol *54*, 374-382.

Glick, B.S., Elston, T., and Oster, G. (1997). A cisternal maturation mechanism can explain the asymmetry of the Golgi stack. FEBS Lett *414*, 177-181.

Godman, G.C., and Lane, N. (1964). On the Site of Sulfation in the Chondrocyte. J Cell Biol *21*, 353-366.

Golgi, C., Bentivoglio, M., and Swanson, L. (2001). On the fine structure of the pes Hippocampi major (with plates XIII-XXIII). 1886. Brain Res Bull *54*, 461-483.

Haddad, A., Smith, M.D., Herscovics, A., Nadler, N.J., and Leblond, C.P. (1971). Radioautographic study of in vivo and in vitro incorporation of fucose-3H into thyroglobulin by rat thyroid follicular cells. J Cell Biol *49*, 856-877.

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

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.

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.

Ho, S.N., Biggar, S.R., Spencer, D.M., Schreiber, S.L., and Crabtree, G.R. (1996). Dimeric ligands define a role for transcriptional activation domains in reinitiation. Nature *382*, 822-826.

Jackson, M.R., Nilsson, T., and Peterson, P.A. (1993). Retrieval of transmembrane proteins to the endoplasmic reticulum. J Cell Biol *121*, 317-333.

Jamieson, J.D., and Palade, G.E. (1967). Intracellular transport of secretory proteins in the pancreatic exocrine cell. I. Role of the peripheral elements of the Golgi complex. J Cell Biol *34*, 577-596.

Jamieson, J.D., and Palade, G.E. (1971). Synthesis, intracellular transport, and discharge of secretory proteins in stimulated pancreatic exocrine cells. J Cell Biol *50*, 135-158.

Jesch, S.A., Lewis, T.S., Ahn, N.G., and Linstedt, A.D. (2001). Mitotic phosphorylation of Golgi reassembly stacking protein 55 by mitogen-activated protein kinase ERK2. Mol Biol Cell *12*, 1811-1817.

Jesch, S.A., and Linstedt, A.D. (1998). The Golgi and endoplasmic reticulum remain independent during mitosis in HeLa cells. Mol Biol Cell *9*, 623-635.

Jokitalo, E., Cabrera-Poch, N., Warren, G., and Shima, D.T. (2001). Golgi clusters and vesicles mediate mitotic inheritance independently of the endoplasmic reticulum. J Cell Biol *154*, 317-330.

Khalil, H., Brunet, A., Saba, I., Terra, R., Sekaly, R.P., and Thibodeau, J. (2003). The MHC class II beta chain cytoplasmic tail overcomes the invariant chain p35-encoded endoplasmic reticulum retention signal. Int Immunol *15*, 1249-1263.

Lin, C.Y., Madsen, M.L., Yarm, F.R., Jang, Y.J., Liu, X., and Erikson, R.L. (2000). Peripheral Golgi protein GRASP65 is a target of mitotic polo-like kinase (Plk) and Cdc2. Proc Natl Acad Sci U S A *97*, 12589-12594.

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.

Lippincott-Schwartz, J., Yuan, L.C., Bonifacino, J.S., and Klausner, R.D. (1989). Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. Cell *56*, 801-813.

Lowe, M., Rabouille, C., Nakamura, N., Watson, R., Jackman, M., Jamsa, E., Rahman, D., Pappin, D.J., and Warren, G. (1998). Cdc2 kinase directly phosphorylates the cis-Golgi matrix protein GM130 and is required for Golgi fragmentation in mitosis. Cell *94*, 783-793.

Lucocq, J.M., and Warren, G. (1987). Fragmentation and partitioning of the Golgi apparatus during mitosis in HeLa cells. Embo J *6*, 3239-3246.

Malhotra, V., and Mayor, S. (2006). Cell biology: the Golgi grows up. Nature *441*, 939-940.

Maul, G.G., and Brinkley, B.R. (1970). The golgi apparatus during mitosis in human melanoma cells in vitro. Cancer Res *30*, 2326-2335.

Miles, S., McManus, H., Forsten, K.E., and Storrie, B. (2001). Evidence that the entire Golgi apparatus cycles in interphase HeLa cells: sensitivity of Golgi matrix proteins to an ER exit block. J Cell Biol *155*, 543-555.

Mironov, A.A., Beznoussenko, G.V., Nicoziani, P., Martella, O., Trucco, A., Kweon, H.S., Di Giandomenico, D., Polishchuk, R.S., Fusella, A., Lupetti, P., Berger, E.G., Geerts, W.J., Koster, A.J., Burger, K.N., and Luini, A. (2001). Small cargo proteins and large aggregates can traverse the Golgi by a common mechanism without leaving the lumen of cisternae. J Cell Biol *155*, 1225-1238.

Nakamura, N., Rabouille, C., Watson, R., Nilsson, T., Hui, N., Slusarewicz, P., Kreis, T.E., and Warren, G. (1995). Characterization of a cis-Golgi matrix protein, GM130. J Cell Biol *131*, 1715-1726.

Nebenfuhr, A., and Staehelin, L.A. (2001). Mobile factories: Golgi dynamics in plant cells. Trends Plant Sci *6*, 160-167.

Neutra, M., and Leblond, C.P. (1966). Synthesis of the carbohydrate of mucus in the golgi complex as shown by electron microscope radioautography of goblet cells from rats injected with glucose-H3. J Cell Biol *30*, 119-136.

Nickel, W., Sohn, K., Bunning, C., and Wieland, F.T. (1997). p23, a major COPI-vesicle membrane protein, constitutively cycles through the early secretory pathway. Proc Natl Acad Sci U S A *94*, 11393-11398.

Nigg, E.A. (1998). Polo-like kinases: positive regulators of cell division from start to finish. Curr Opin Cell Biol *10*, 776-783.

Pecot, M.Y., and Malhotra, V. (2004). Golgi membranes remain segregated from the endoplasmic reticulum during mitosis in mammalian cells. Cell *116*, 99-107.

Preuss, D., Mulholland, J., Franzusoff, A., Segev, N., and Botstein, D. (1992). Characterization of the Saccharomyces Golgi complex through the cell cycle by immunoelectron microscopy. Mol Biol Cell *3*, 789-803.

Puthenveedu, M.A., and Linstedt, A.D. (2001). In search of an essential step during mitotic Golgi disassembly and inheritance. Exp Cell Res *271*, 22-27.

Robbins, E., and Gonatas, N.K. (1964). Histochemical and Ultrastructural Studies on Hela Cell Cultures Exposed to Spindle Inhibitors with Special Reference to the Interphase Cell. J Histochem Cytochem *12*, 704-711.

Rossanese, O.W., and Glick, B.S. (2001). Deconstructing Golgi inheritance. Traffic *2*, 589-596.

Sabatini, D.M., Erdjument-Bromage, H., Lui, M., Tempst, P., and Snyder, S.H. (1994). RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. Cell *78*, 35-43.

Saraste, J., and Kuismanen, E. (1992). Pathways of protein sorting and membrane traffic between the rough endoplasmic reticulum and the Golgi complex. Semin Cell Biol *3*, 343-355.

Schutze, M.P., Peterson, P.A., and Jackson, M.R. (1994). An N-terminal double-arginine motif maintains type II membrane proteins in the endoplasmic reticulum. Embo J *13*, 1696-1705.

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.

Shima, D.T., Cabrera-Poch, N., Pepperkok, R., and Warren, G. (1998). An ordered inheritance strategy for the Golgi apparatus: visualization of mitotic disassembly reveals a role for the mitotic spindle. J Cell Biol *141*, 955-966.

Shima, D.T., Haldar, K., Pepperkok, R., Watson, R., and Warren, G. (1997). Partitioning of the Golgi apparatus during mitosis in living HeLa cells. J Cell Biol *137*, 1211-1228.

Sjostrand, F.S., and Hanzon, V. (1954). Electron microscopy of the Golgi apparatus of the exocrine pancreas cells. Experientia *10*, 367-369.

Sonnichsen, B., Watson, R., Clausen, H., Misteli, T., and Warren, G. (1996). Sorting by COP I-coated vesicles under interphase and mitotic conditions. J Cell Biol *134*, 1411-1425.

Stanley, H., Botas, J., and Malhotra, V. (1997). The mechanism of Golgi segregation during mitosis is cell type-specific. Proc Natl Acad Sci U S A *94*, 14467-14470.

Steiner, D.F., Patzelt, C., Chan, S.J., Quinn, P.S., Tager, H.S., Nielsen, D., Lernmark, A., Noyes, B.E., Agarwal, K.L., Gabbay, K.H., and Rubenstein, A.H. (1980). Formation of biologically active peptides. Proc R Soc Lond B Biol Sci *210*, 45-59.

Storrie, B., White, J., Rottger, S., Stelzer, E.H., Suganuma, T., and Nilsson, T. (1998). Recycling of golgi-resident glycosyltransferases through the ER reveals a novel pathway and provides an explanation for nocodazole-induced Golgi scattering. J Cell Biol *143*, 1505-1521.

Sutterlin, C., Hsu, P., Mallabiabarrena, A., and Malhotra, V. (2002). Fragmentation and dispersal of the pericentriolar Golgi complex is required for entry into mitosis in mammalian cells. Cell *109*, 359-369.

Sutterlin, C., Lin, C.Y., Feng, Y., Ferris, D.K., Erikson, R.L., and Malhotra, V. (2001). Polo-like kinase is required for the fragmentation of pericentriolar Golgi stacks during mitosis. Proc Natl Acad Sci U S A *98*, 9128-9132.

Sutterlin, C., Polishchuk, R., Pecot, M., and Malhotra, V. (2005). The Golgi-associated protein GRASP65 regulates spindle dynamics and is essential for cell division. Mol Biol Cell *16*, 3211-3222.

Tang, B.L., Wong, S.H., Qi, X.L., Low, S.H., and Hong, W. (1993). Molecular cloning, characterization, subcellular localization and dynamics of p23, the mammalian KDEL receptor. J Cell Biol *120*, 325-328.

Thyberg, J., and Moskalewski, S. (1985). Microtubules and the organization of the Golgi complex. Exp Cell Res *159*, 1-16.

Thyberg, J., and Moskalewski, S. (1992). Reorganization of the Golgi complex in association with mitosis: redistribution of mannosidase II to the endoplasmic reticulum and effects of brefeldin A. J Submicrosc Cytol Pathol *24*, 495-508.

Trucco, A., Polishchuk, R.S., Martella, O., Di Pentima, A., Fusella, A., Di Giandomenico, D., San Pietro, E., Beznoussenko, G.V., Polishchuk, E.V., Baldassarre, M., Buccione, R., Geerts, W.J., Koster, A.J., Burger, K.N., Mironov, A.A., and Luini, A. (2004). Secretory traffic triggers the formation of tubular continuities across Golgi sub-compartments. Nat Cell Biol *6*, 1071-1081.

Uchiyama, K., Jokitalo, E., Lindman, M., Jackman, M., Kano, F., Murata, M., Zhang, X., and Kondo, H. (2003). The localization and phosphorylation of p47 are important for Golgi disassembly-assembly during the cell cycle. J Cell Biol *161*, 1067-1079.

Ward, T.H., Polishchuk, R.S., Caplan, S., Hirschberg, K., and Lippincott-Schwartz, J. (2001). Maintenance of Golgi structure and function depends on the integrity of ER export. J Cell Biol *155*, 557-570.

Warren, G. (1993). Membrane partitioning during cell division. Annu Rev Biochem *62*, 323-348.

Whur, P., Herscovics, A., and Leblond, C.P. (1969). Radioautographic visualization of the incorporation of galactose-3H and mannose-3H by rat

thyroids in vitro in relation to the stages of thyroglobulin synthesis. J Cell Biol *43*, 289-311.

Wiederrecht, G., Brizuela, L., Elliston, K., Sigal, N.H., and Siekierka, J.J. (1991). FKB1 encodes a nonessential FK 506-binding protein in Saccharomyces cerevisiae and contains regions suggesting homology to the cyclophilins. Proc Natl Acad Sci U S A *88*, 1029-1033.

Yaffe, M.B., Schutkowski, M., Shen, M., Zhou, X.Z., Stukenberg, P.T., Rahfeld, J.U., Xu, J., Kuang, J., Kirschner, M.W., Fischer, G., Cantley, L.C., and Lu, K.P. (1997). Sequence-specific and phosphorylation-dependent proline isomerization: a potential mitotic regulatory mechanism. Science *278*, 1957-1960.

Zaal, K.J., Smith, C.L., Polishchuk, R.S., Altan, N., Cole, N.B., Ellenberg, J., Hirschberg, K., Presley, J.F., Roberts, T.H., Siggia, E., Phair, R.D., and Lippincott-Schwartz, J. (1999). Golgi membranes are absorbed into and reemerge from the ER during mitosis. Cell *99*, 589-601.