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UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Physiological Role of the GRASP Proteins in Unconventional Secretion and Mitotic Golgi Fragmentation

A Dissertation in partial satisfaction of the
Requirements for the degree Doctor of Philosophy

in

Biology

by

Matthew A. Kinseth

Committee in charge:

Professor Vivek Malhotra, Chair
Professor Don Cleveland
Professor Russell F. Doolittle
Professor Scott Emr
Professor Randy Hampton
Professor William F. Loomis

2007

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

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Chair

University of California, San Diego

2007

DEDICATION

This is dedicated to the memory of my mom, Juli Pouncil. You taught me the true meaning of love and sacrifice. I am happy you were able to meet Amy and that you knew I would be okay without you. I love you and miss you.

I will continue to LIVESTRONG for you, always and forever!

Juli Pouncil
1952-2002

EPIGRAPH

It is reasonable to believe that just as the structure of the Golgi is conserved across the eukaryotic kingdom, the proteins involved in mediating the stacking of Golgi cisternae are conserved too.

Barr et al, Cell, 91, 1997

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I would like to acknowledge and thank all members of my committee for their support and help in directing the story of my dissertation. All members challenged my thinking and understanding of the scientific process while also encouraging me to develop my dissertation into the story it has become.

I would also like to thank Danny Fuller, Christophe Anjard, and William F. Loomis from the Loomis lab without whom my study of the GRASP homolog in *Dictyostelium* would ever have been possible. Their expertise and willingness to perform many of the experiments were crucial for the success of this story and in my understanding of *Dictyostelium* as a model organism.

I would like to acknowledge Justin Kollman and Russell F. Doolittle for their help in both obtaining and understanding the GRASP phylogenetic data. I greatly appreciate the countless hours spent looking over sequences and compiling the data with me to better appreciate the conserved nature of these proteins as they relate to Golgi membrane structure.

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Chapter 2, in full, is a reprint of the material as it appears in Cell 2007. Matthew A. Kinseth, Christophe Anjard, Danny Fuller, Gianni Guizzunti, William F. Loomis, Vivek Malhotra, *The Golgi-Associated GRASP is Required for Unconventional Proteins Secretion during Development.,* (2007) **Cell**, 130:524-534. I, Matthew A. Kinseth, am the primary author of this paper.

Chapter 3, in part, will be submitted for publication of the material as it may appear. I, Matthew A. Kinseth, will be the primary author of this paper.

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ABSTRACT OF THE DISSERTATION

The Physiological Role of the GRASP Proteins in Unconventional Secretion and Mitotic Golgi Fragmentation

by

Matthew A. Kinseth

Doctor of Philosophy in Biology

University of California, San Diego, 2007

Professor Vivek Malhotra, Chair

The GRASP proteins were identified as factors involved in the stacking of Golgi cisternae in mammalian cells thereby creating a protein regulated mechanism of mitotic Golgi fragmentation. Although these proteins are well conserved, the structure of Golgi membranes and its fragmentation during mitosis is species specific. Phylogenetic analysis of the GRASP proteins compared with the organization of Golgi membrane across eukaryotes suggest these proteins cannot be conserved on the sole

basis of Golgi structural proteins. Experimental evidence, in both mammalian tissue culture cells and *Dictyostelium discoideum*, reveals these proteins do not play a role in Golgi structure or conventional protein secretion; the two biochemical functions initially assigned to them. Surprisingly, the GRASP homolog in *Dictyostelium* was found to be essential for the unconventional secretion of a protein required during the terminal differentiation of spore cells. This is the first physiologically relevant function found for the GRASP proteins in eukaryotes and suggests a new function of the Golgi membranes on their role in unconventional protein secretion. Furthermore, the mammalian GRASP homolog, GRASP55, is sequentially phosphorylated during mitosis, a necessary step in the fragmentation of Golgi membranes and the entry of cells into mitosis.

Introduction

The Golgi complex represents the central organelle along the secretory pathway. Proteins entering the Golgi complex are intended for one of many destinations, including secretion out of the cell, cell surface expression, or transport to the endosomal/lysosomal membranes. Alternatively, some proteins are either recycled back to the endoplasmic reticulum (ER) or remain in the Golgi as residents (Farquhar and Palade, 1998).

Proteins destined for the Golgi apparatus are folded in the ER and then packaged into protein coated vesicles (COPII) for transport to the highly fenestrated cis-face of the Golgi membranes (cis cisternae, cis-Golgi network or CGN). The proteins then traverse the subsequent medial cisternae by either continual maturation of the cisternae themselves, or with the use of vesicles (COPI) fusing to static cisternal membranes. The trans-Golgi network is the last and highly fenestrated cisternal compartment where the major sorting of proteins take place. This polarized series of cis-, medial- and trans-Golgi membranes are stacked upon each other, defining a single Golgi unit. A cell can contain anywhere from 80-150 Golgi stacks, each stack consisting of 3-7 individual cisternae (Shorter and Warren, 2002; Warren and Malhotra, 1998).

The Golgi is capable of maintaining its structure of stacked, flattened membranes throughout the barrage of vesicles continually fusing and breaking away from it, carrying away cargo proteins while also retaining resident proteins needed at

the Golgi complex. As a result, the structure of the Golgi is closely linked to the transport process from it, and any deregulation of one may have a profound effect on the other.

The evidence of Golgi stacking in any organism can only be visualized by electron microscopy (EM). Immunofluorescence is unable to tell whether Golgi membranes are fragmented or in small, individual stacks. Early EM studies revealed the presence of electron dense regions between Golgi cisternae that was sensitive to protease digestion suggesting that proteins may play a role in its structure (Cluett and Brown, 1992). The Golgi stack is the central structure seen in most eukaryotes, and therefore there has been little question of attention paid to the conservation of these apparent structural Golgi membrane proteins. The identification of these proteins introduced a potential protein-regulated mechanism for maintaining and controlling Golgi membrane structure.

The mammalian cell contains Golgi stacks that are interconnected via membrane tubules to form a single compact ribbon-like structure (Glick and Malhotra, 1998). This “Golgi ribbon,” or Golgi complex, is localized in a microtubule dependent manner adjacent to the nucleus and centrioles, the base of the microtubule organizing center (MTOC). This pericentriolar localization is not unique to mammalian cells. *Dictyostelium* also localize their Golgi membranes perinuclear, although the organization of these membranes is less understood (Schneider et al., 2000).

The focal point in understanding the regulation of Golgi membrane structure is found in mitotic mammalian cells. Electron microscopy of mitotic mammalian cells revealed that Golgi membranes are fragmented into tubulo-reticular and vesicular

elements dispersed throughout the cell (Lucocq et al., 1988). The fragmentation of Golgi membranes has a biological significance. Inhibiting mitotic Golgi fragmentation away from its perinuclear localization leads to the prevention of cells proceeding to mitosis (Sutterlin et al., 2002).

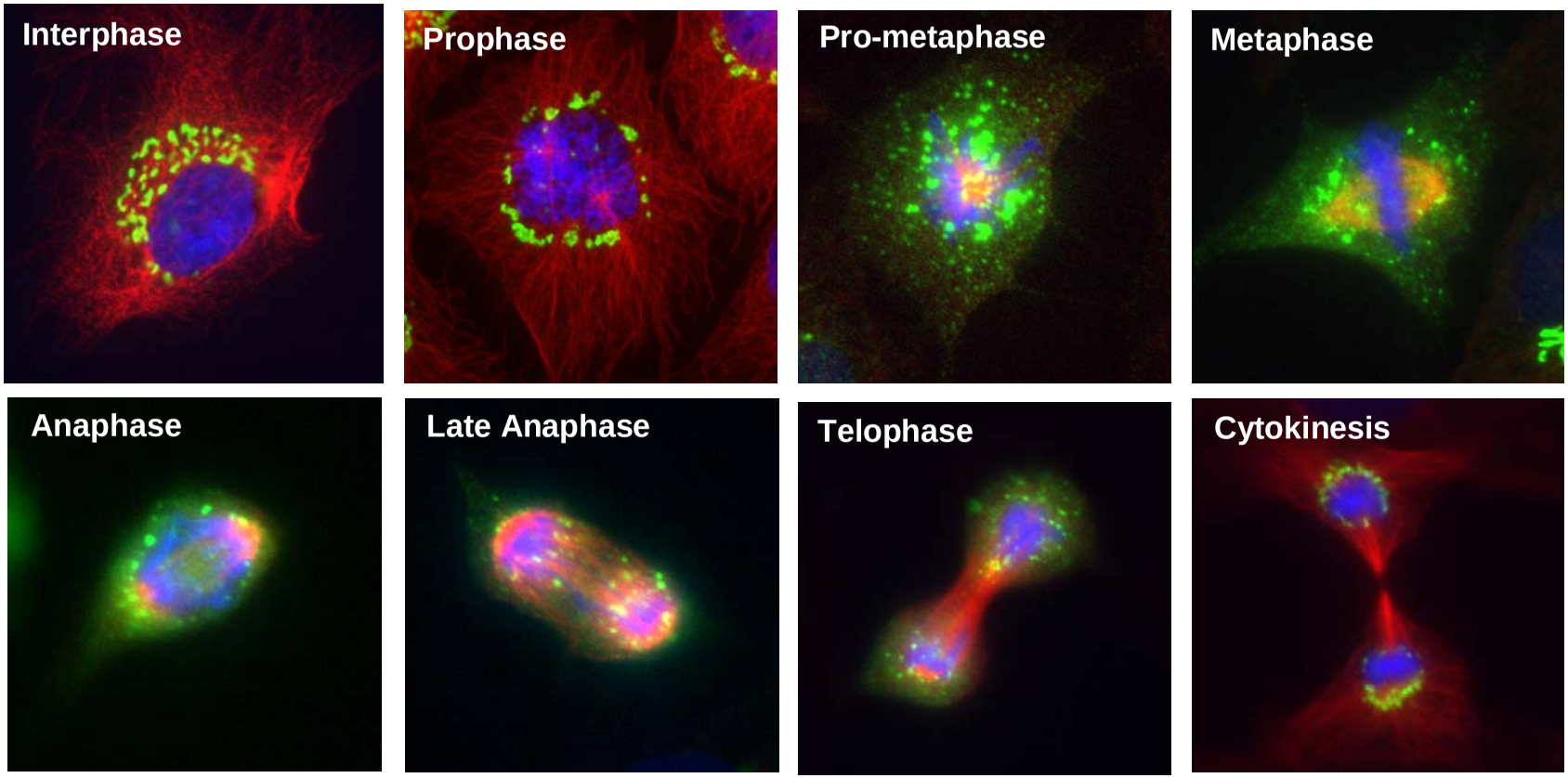
Mitosis-Specific Fragmentation of the Golgi Complex

The mitosis-specific fragmentation of the Golgi was once considered as a way for guaranteeing the equal partitioning of Golgi membranes to the daughter cells (Warren et al., 1995). Fragmentation of the Golgi membranes during mitosis can be witnessed at the immunofluorescence level. At the onset of prophase, the Golgi complex first breaks the inter-membrane tubules holding the individual Golgi stacks together, causing dispersal around the nucleus (Lucocq and Warren, 1987). Further fragmentation occurs once the microtubule network reorganizes to form the spindles. The Golgi membranes lose their perinuclear position, disperse throughout the cytosol and continue fragmenting into clusters of Golgi “blobs” and a dense haze (Lucocq and Warren, 1987). At the onset of late telophase/early cytokinesis the Golgi membrane re-organizes their membranes and re-localizes them to its perinuclear position in a microtubule dependent manner (Figure i-1). This mitosis-specific event established the basis for finding Golgi structural proteins.

Graham Warren and Francis Barr, using purified Golgi membranes established an EM assay in which they monitored the reformation of Golgi membranes after its incubation with purified mitotic cytosol (Rabouille et al., 1995).

Figure i-1: Fragmentation of the Golgi Complex During Mitosis

Immunofluorescence microscopy of Normal Rat Kidney (NRK) cells show that Golgi membranes begin away from each other and disperse around the nucleus at the onset of prophase. The microtubule network is responsible for the localization of Golgi membranes adjacent to the nucleus. Once the microtubules network reorganizes to form the spindle poles the Golgi "blobs' disperse throughout the cytosol. Further fragmentation occurs through metaphase and anaphase until a haze is seen throughout the cell. Cytosolic kinases, cdc2, Pkl-1, Raf-1, MEK-1 are known to be required for this process. Golgi proteins, GRASP65, GRASP55, GM130, p115 have been suggested to play a role in regulating Golgi fragmentation.


Interphase
Prophase
Pro-metaphase
Metaphase
Anaphase
Late Anaphase
Telophase
Cytokinesis

Using this assay, Warren and colleagues identified the major structural components and they were named for their requirement to restack Golgi membranes after mitotic breakdown. These proteins are called the Golgi Re-Assembling Stacking Proteins of 65 and 55 kD, or GRASP65 and GRASP55 respectively (Barr et al., 1997; Shorter et al., 1999). A homolog of these proteins was identified in fungi, and therefore it was assumed they were conserved as structural proteins used to stack Golgi cisternal membranes in all eukaryotes (Barr et al., 1998; Shorter et al., 1999).

The Mechanism of Mitotic Golgi Fragmentation

Graham Warren and colleagues have proposed the cyclin dependent kinase 1 (cdk1/cdc2) as the key Golgi regulator during mitosis. *In vitro*, cdc2 mitotically phosphorylates GM130, a member of the Golgin class of structural proteins associated with GRASP65 and the Golgi membranes (Lowe et al., 1998). This phosphorylation event is suggested to inhibit COPI vesicles from tethering to the Golgi cisternae for fusion (Nakamura et al., 1997; Nakamura et al., 1995). As a consequence, fusion is inhibited while vesicle production continues resulting in complete vesiculation of Golgi membranes. This COPI-dependent mechanism is thought to account for the loss of 60-70% of cisternal membranes during mitosis. The remaining 30% undergoes fragmentation by a COPI-independent pathway that is less well characterized and is not resolved to date (Misteli and Warren, 1995; Warren et al., 1995).

GM130 was initially thought to associate with Golgi membranes by interacting with GRASP65, although recent evidence places GM130 on the Golgi independent of GRASP65, and depletion of GM130 has no effect on Golgi structure (Barr et al., 1998;

Barr et al., 1997; Puthenveedu et al., 2006; Sutterlin et al., 2005). GRASP65 is anchored to the cytosolic face of the Golgi membrane via myristoylation and binds GM130 at its N-terminus (Barr et al., 1998). Likewise, cdc2 can also mitotically phosphorylate GRASP65, and these combined phosphorylations are thought to disrupt the GRASP65-GM130 trans-dimers to unstack Golgi cisternal membranes (Wang et al., 2005; Yoshimura et al., 2005). Further studies revealed these cdc2 dependent phosphorylations then also allow the binding of polo-like kinase to further phosphorylate GRASP65 (Preisinger et al., 2005).

Therefore, a model of mitotic Golgi fragmentation had developed, relying heavily on the assumption that the GRASP proteins interact with the Golgins, forming a trans-dimer used to stack adjacent Golgi membranes (Short et al., 2005; Shorter and Warren, 2002; Wang et al., 2005). The model seemed clear and precise: phosphorylation of the GRASP-Golgin complex would disrupt Golgi structure in two ways: 1) disruption between adjacent stacks allowing for the Golgi cisternae to detach from one another, and 2) inhibiting further fusion of incoming vesicles while allowing vesicle fission to continue. This second mechanism implied that the GRASP-Golgin complex was also required for general protein secretion. Therefore, the disruption of these complexes allowed for the mitotic specific fragmentation and subsequent Golgi membrane dispersal (Short et al., 2005; Shorter and Warren, 2002).

Alternatively, the Malhotra lab has identified different components of the Golgi fragmentation pathway. Mitotic Golgi fragmentation has been reconstituted in a semi-intact cell system leading to the identification of additional kinases; Raf-1 and the Mitogen Activated Protein Kinase Kinase (MEK1) of the MAP Kinase pathway,

along with Polo-like Kinase (Plk) (Acharya and Malhotra, 1995; Acharya et al., 1998; Colanzi et al., 2003; Sutterlin et al., 2001). EM analysis of Golgi membranes from the semi-intact assay and *in vivo* pre-metaphase Golgi membranes shows similar levels of mitosis specific fragmentation (Acharya et al., 1998).

These two independent assays were combined to form a single model by using a semi-intact assay for mitotic Golgi fragmentation in MDCK (Madin Darby Canine Kidney) cells, showing that the MAP kinase pathway and Plk1 is involved with early mitotic breakdown of Golgi membranes followed by cdc2 (Kano et al., 2000). Although no downstream Golgi targets were identified by the Malhotra lab, the current model of GRASP-mediated Golgi fragmentation implies that these kinases are upstream of any such Golgi structural proteins.

The GRASP Proteins and the Golgi cisternal stacking problem

Single and double labeling EM shows that the two GRASP proteins are arranged differently across the Golgi stack (Shorter et al., 1999). GRASP65 is mainly localized on the cis-cisternae, whereas GRASP55 has a bell-shape curve distribution across all cisternal membranes. Therefore, it was suggested that GRASP65 helped tether vesicles entering Golgi membranes while also stacking these newly forming cisternal membranes, whereas GRASP55 would continue and maintain the organization of the Golgi stack while also serving as a tethering complex for subsequent vesicle fusion across the stack (Barr and Short, 2003; Marra et al., 2001; Short et al., 2001; Weide et al., 2001; Yoshimura et al., 2004).

Since GRASP65 was initially identified as a stacking factor and is a substrate for both cdc2 and polo-like kinase, it was reasonable to question what role GRASP65 played in mitotic Golgi fragmentation (Barr et al., 1997; Lin et al., 2000; Preisinger et al., 2005). Interestingly, further characterization of this protein only complicated its apparent function. Knockdown of GRASP65 by siRNA did not break down Golgi structure, nor inhibit its ability to reform Golgi membranes after treatment with either nocodazole, a microtubule depolymerizing drug, or Brefeldin A (BFA), a drug that fuses Golgi membranes with the ER (Sutterlin et al., 2005). Although Golgi stacks had fewer cisternae, all Golgi proteins maintained their localization, including GM130. More recent evidence, however, suggests that knockdown of GRASP65 disrupts the lateral membrane connections between Golgi stacks in mammalian cells (Puthenveedu et al., 2006). Protein secretion, mitotic entry and Golgi fragmentation were all normal in the absence of GRASP65; however, GRASP65-depleted HeLa cells became arrested at pre-metaphase due to aberrant spindle formation (Sutterlin et al., 2005).

Using our semi-intact assay, we have shown further that addition of GRASP65 specific reagents, including the anti-GRASP65 antibody or peptides of GRASP65, was capable of inhibiting Golgi fragmentation (Sutterlin et al., 2002; Sutterlin et al., 2001). Likewise, when the GRASP65 peptides (or antibody) were microinjected into intact cells, the mitotic index, or number of mitotic cells, decreased. If the Golgi membranes are artificially removed from their perinuclear position, through either the addition of nocodazole or BFA, in the presence of the GRASP65 specific reagents, the mitotic index recovered (Sutterlin et al., 2002). This new evidence suggested that

fragmentation or dispersal of Golgi membranes from its perinuclear position was required for mitotic progression. Other labs later confirmed the finding that the inhibition of Golgi fragmentation prevents mitotic progression (Colanzi et al., 2007; Kondylis et al., 2007; Yoshimura et al., 2005).

GRASP55 was discovered shortly after GRASP65 by sequence homology and was confirmed using the same *in vitro* Golgi reconstitution assay (Shorter et al., 1999). Since its discovery, very few papers have dealt with its function. Similar to the GRASP65-GM130 complex, GRASP55 is thought to bind Golgin45, once thought to be the transcription factor JEM-1 (Short et al., 2001). Therefore, it has been assumed that like GRASP65, its homolog would have a similar mechanism for unstacking Golgi membranes during mitosis. Although evidence suggests that GRASP55 is a mitotic substrate for the MAP kinase, ERK2, the Malhotra lab has ruled out the requirement of both ERK1 and ERK2 on mitotic Golgi fragmentation using their semi-intact assay (Acharya et al., 1998; Jesch et al., 2001). No further work has revealed a mechanism for the role GRASP55 during mitotic Golgi fragmentation.

The assumption of the conserved nature of GRASP as a stacking factor also does not hold up to the current understanding of Golgi membrane structure throughout eukaryotes. There are instances where Golgi stacking does not occur. For instance, the fungi *S. cerevisiae* have isolated Golgi cisternae dispersed throughout the cytosol, and some early diverging organisms (protists) are thought to have maturing vesicles that emerge from the ER and move toward the plasma membrane (Becker and Melkonian, 1996; Preuss et al., 1992). These apparently “Golgi lacking” organisms are not well understood even though some forms of glycosylation, the major protein modification

of Golgi enzymes, are known to occur (Dacks et al., 2003). Evidence also suggest that some Golgi membranes, not only are continuous with themselves, but also are found continuous with the endoplasmic reticulum (Beznoussenko et al., 2007; Mogelsvang et al., 2004). Many other factors have also been shown to control and maintain Golgi cisternal stacking, all of which further complicated the dynamics of Golgi membrane structure (Ayscough et al., 1993; Derganc et al., 2006). Therefore, the question becomes, is the maintenance and organization of Golgi membranes dependent on the GRASP proteins, and if so, what function of these proteins would account for these structural differences? If the GRASP proteins are not required for Golgi cisternal stacking and Golgi membrane organization, then what functions do they serve throughout eukaryotes? These inconsistencies are the basis for my dissertation research.

Dissertation focus

The lack of experimental evidence and the controversial functions of GRASPs as Golgi stacking factor led me to question the true physiological function of these proteins. Since GRASP65 plays a role regulating mitotic Golgi fragmentation it would seem likely that GRASP55 may play a similar role. Therefore, I have separated my dissertation into three chapters.

The first chapter looks at the conserved nature of the GRASP proteins throughout eukaryotic evolution and compares their presence in relationship to Golgi membrane structure. This also serves as a means to identify important regions within

the proteins which may help resolve which domains are critical for their conserved nature.

Chapter two takes a 180-degree turn from the current focus of the GRASP proteins by looking at its function in the developing organism, *Dictyostelium discoideum*. Here I explain the first physiologically relevant function found for the GRASP proteins and explain a potential role in unconventional secretion during cellular development, a mechanism never associated with Golgi membranes or their proteins in the past.

Chapter three returns to mammalian mitotic Golgi fragmentation to further understand the role that GRASP55 serves in this process.

Chapter I

A Phylogenetic and Evolutionary Analysis of the GRASP Proteins and Golgi membrane Stacking

Introduction

Protein trafficking is an essential function in all eukaryotic cell types. The Golgi apparatus is the central organelle along the secretory pathway; it not only is responsible for the correct localization of the proteins that enter, but also for protein glycosylation needed for the proper structure and function of proteins traversing within it (Farquhar and Palade, 1998). Homologs of all proteins involved in secretion have sequences that are strongly conserved. These proteins have been well studied, and their roles are clearly defined. Even in some protists, where no static Golgi are found, there exist large membrane bodies that carry proteins destined for either secretion or cell surface localization (Becker and Melkonian, 1996; Dacks et al., 2003). These too have the fundamental homologs of the secretory proteins, although the system is more rudimentary (Marti et al., 2003).

Golgi enzymes are responsible for glycosylation. This process varies in protists but is essential for higher eukaryotes. Likewise, functional homologs are readily

identified in the many organisms that require this protein modification (Lehle et al., 2006; Martin and Russell, 2003).

Protein trafficking through the Golgi and maintenance of Golgi structure are dependent processes. The static appearance of the Golgi complex as a cellular organelle, together with its dynamic instability, has produced much interest in the field of structural organization of Golgi membranes that define its compartmentalization and dynamics. The identification of the Golgi structural proteins, the GRASPs, has introduced a potential mechanism of how Golgi structure may be regulated.

The Golgi Re-Assembling Stacking Proteins of 65 and 55 kDa, or GRASP65 and GRASP55 respectively, were originally identified in a cell free assay using purified rat liver Golgi membranes to study the reformation of Golgi after their mitosis specific fragmentation (Barr et al., 1997; Shorter et al., 1999). It has been presumed that since the GRASP proteins are conserved from yeast to man, and Golgi stacks are seen in most cell types, that Golgi cisternal stacking is their conserved function (Barr et al., 1998; Shorter et al., 1999; Wang et al., 2005).

A closer look at the experimental evidence of these proteins challenges the assumptions drawn from the biochemical assay used to study and identify the GRASP proteins. Previously published results on mammalian GRASP65, *Drosophila*, yeast and *Dictyostelium* GRASP homologs have clearly shown that the GRASP proteins play no direct role in Golgi cisternal stacking and conventional protein secretion (Behnia et al., 2007; Kinseth et al., 2007; Kondylis et al., 2005; Sutterlin et al., 2005).

Likewise, mitotic Golgi fragmentation is not conserved in all cells. Although many cell types increase the population of Golgi membranes during mitosis, few are

known to fragment them to the extent of mammalian cells (Gerisch et al., 2004; Kondylis et al., 2007; Stanley et al., 1997). The study of the GRASP proteins only as Golgi structural regulators during mitosis has not considered the conserved nature of these proteins.

If we are to assume the GRASP proteins play a role in cisternal stacking and vesicle tethering, as are the major functions studied to date, a few questions must be addressed:

1) Is GRASP sequence conservation consistent with a role in mitotic Golgi fragmentation?

2) Does the presence of the GRASP gene dictate the presence of Golgi cisternal stacking?

3) If neither mitotic Golgi fragmentation nor Golgi stacking is conserved, what is the major role of the GRASP proteins?

To help address some of these questions a phylogenetic and evolutionary analysis of the GRASP proteins has been performed. The presence of GRASP homologs was then compared with published data on Golgi membrane stacking and organization.

Our results indicate that all GRASP orthologs can be defined by two distinct PDZ-like domains that evolved separately from each other. We also suggest that Golgi membrane organization is dependent, not on the presence of GRASP gene, but rather on the specific complexity of the endomembrane system as found in an individual cellular/organism basis.

Results

Identification of GRASP orthologs reveal three distinct domains

BLAST-P analysis (www.ncbi.nlm.nih.gov/BLAST/) of either mammalian full length rat GRASP55 or GRASP65, or sub-domains within the protein, was capable of finding many of the homologs whose gene sequences are known. As published previously, no sequence homologs were found in any plant genome searches, even though several plant genomes are complete (Kinseth et al., 2007). This is of great interest as plant Golgi membranes are stacked. To study the conservation of the GRASP proteins throughout eukaryotic evolution I have compared a small subset of thirteen different homologs of these genes. Since the degree of similarity is much higher among the vertebrates, I chose two distantly related animals to compare: zebrafish (*Danio rerio*) and rat (*Rattus norvegicus*) (Figure 1).

BLAST-P searches identify the N-terminus of the GRASP proteins to have two different PDZ-like domains which have similarities with bacterial serine- and metallo-proteases. It is common for many higher eukaryotic PDZ domains to resemble these bacterial domains but it is unknown why the similarity exists (personal communication with Russell F. Doolittle)(Fan and Zhang, 2002). Therefore, each protein was separated into three distinct regions. From previously published results, GRASP have been identified with two PDZ-like domains at the N-terminus of the protein with a

Organism	Gene or Protein Name	Accession Number
R. norvegicus	GRASP65, GRASP55	O35254, Q9R064
D. rerio	GRASP1, GRASP2	NP_ 001007412, NP_956997
C. intestinalis	cDNA clone AK113161	AK113161
D. melanogaster	CG7809-PA	NP_649160
C. elegans	Y42H9AR.1	NP_501354.1
E. cunuliculi	ECU01_0530	NP_586663
S. pombe	SPAC1D4.02c	NP_593015
S. cerevisiae	Grh1p (Ydr517wp)	NP_01085
P. pastoris	-	-
M. grisea	MG05757.4	XP_360383
N. crassa	-	CAD79671
D. discoideum	GrpA	XP_629220
P. falciparum	PF10 _0168	NP_700642

Figure 1-1: List of GRASP homologs used in the phylogenetic analysis

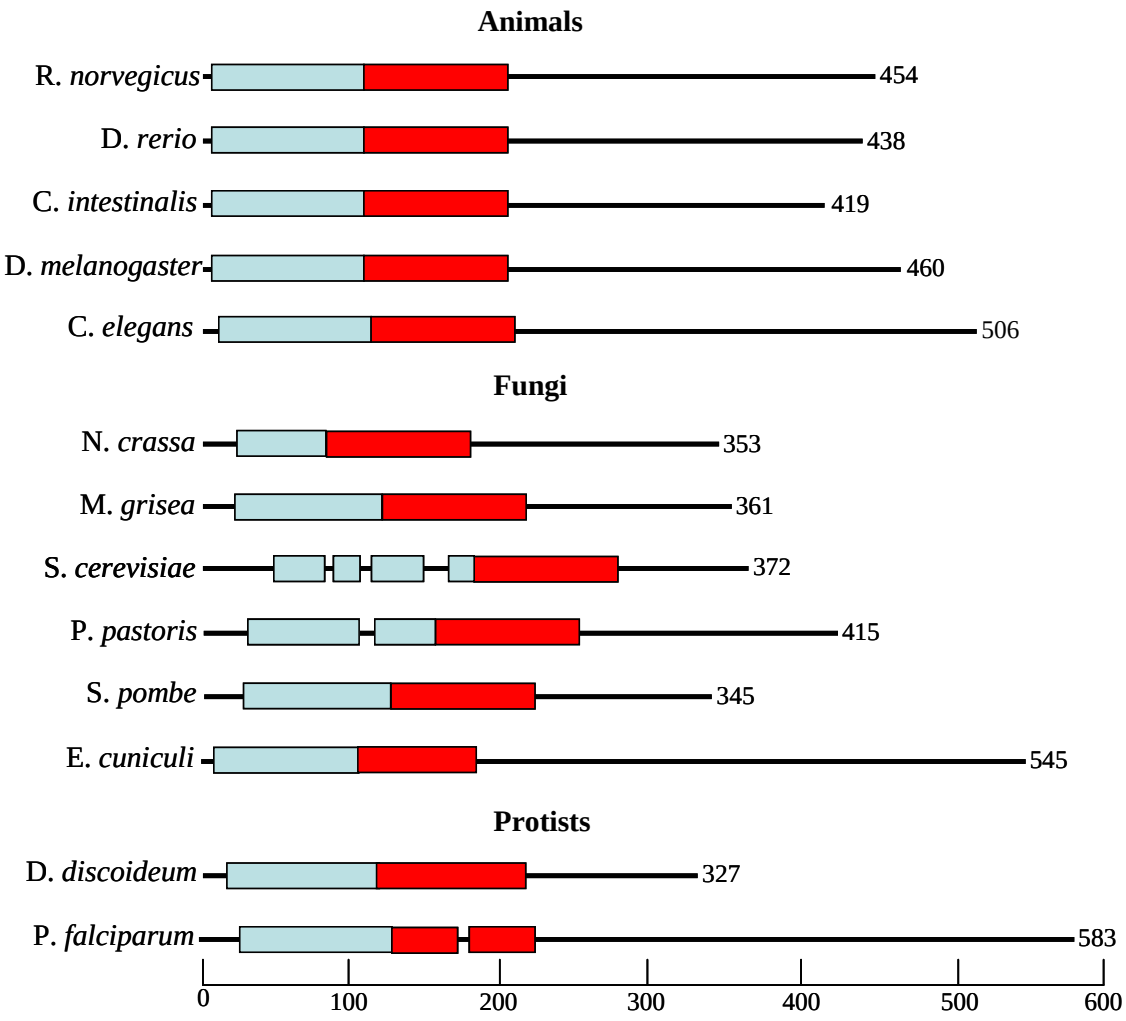


Figure 1-2: The GRASP proteins contain three distinct regions.

The GRASP proteins have two conserved PDZ-like domains at the N-terminal region. PDZ-1 (blue) is slightly less conserved in eukaryotes, especially the fungi. PDZ-2 (red) is very well conserved in all GRASP homologs. The C-terminal regions of the GRASP proteins have low complexity and vary in size.

variable C-terminus (Kinseth et al., 2007; Shorter et al., 1999; Wang et al., 2005). The 13 homologs used were broken into these three regions based on sequence alignment. Each region from the 13 GRASP proteins was compared for percent identity, and a phylogenic tree was constructed, where appropriate. to help understand the evolution of these domains, the origins of these proteins, and their relationship, if any, to Golgi membrane structure (Figure 2).

The GRASP PDZ domains are evolving at different rates

The boundaries of the PDZ-1 and PDZ-2 domains were identified based on sequence alignment. From this alignment it is evident that the second PDZ-like domain of the GRASP proteins is the more conserved region within all of the GRASP proteins. For instance, comparison of rat55 and zebrafish55 PDZ-2 domains shows a 95% identity where rat65 and zebrash65 are only 79% identical. A sequence alignment of all PDZ-2 domains produces an evolutionary tree with the GRASP duplication event occurring at the dawn of the vertebrates. Thus, all invertebrate, fungi and protists show only one copy of this gene, regardless of which homolog is used for searching the databases (Figures 3, 4, 5).

Sequence alignment of the first PDZ domain (PDZ-1) of all GRASP orthologs shows slightly less conservation than PDZ-2. Comparisons of Rat55 and zebrafish55 PDZ-1 domains show an 90% identity, where the same comparison with the GRASP65 domains are only 79% identical. Interestingly, all fungi PDZ-1 domains have changed at a dramatically faster rate than protists and animal PDZ-1 domains.

For instance, rat55 PDZ-1 vs *S. cerevisaie* PDZ-1 is only 21% identical as compared with rat55 PDZ-2 vs *S. cerevisaie* PDZ-2, which is 45% identical. This rapid rate of change for PDZ-1 suggests its function is either lost or is substantially different from other non-fungi homologs. Therefore, all fungi GRASP PDZ-1 domains were excluded from the alignment (Figure 6, 7). Based on this inconsistent evolutionary rate of change, a phylogenic tree of all PDZ-1 domains has been excluded. However, we have found that by weighting the alignment by including more GRASP sequences from animals and fewer sequences from fungi, we were able to create a phylogenic tree that shows a similar duplication event seen in the PDZ-2 alignment (Figure 8). From this analysis, we also identify the duplication of the GRASP gene occurring early in chordate evolution at the dawn of the vertebrates.

Once the gene duplication occurred, another interesting observance is found: the rates of change of vertebrate GRASP65 vs GRASP55 differ greatly. The question arises, does vertebrate GRASP65 speed up, or does GRASP55 slow down? Interestingly, in the mammalian proteins, both show a very slow rate of change. This is seen most dramatically in the GRASP65 line. Differing rates of change are common after gene duplications and may indicate the presence of a new function. Whether there are additional functions gained by both proteins after the duplication will require further experimental evidence comparing the physiological function of an early diverging GRASP homolog vs. its mammalian counterpart. To date, no experiments have been performed to test any functional homology between species.

```
R.norvegicus65    112 WHVLDV-EPSSPAALAGLRPYTDYIVGSDQILQE---SEDFFTLIES-----------HE
D.rerio65         114 WHVLDV-ESNSPAALAGLQEHSDFIVGADQVLQD---SEDFFSLIEA-----------HE
R.norvegicus55    113 WHVLEV-ESNSPAALAGLRPHSDYIIGADTVMNE---SEDLFSLIET-----------HE
D.rerio55         113 WHVLEV-EPNSPAALAGLRPHTDYIIGADTVMNE---SEDLFSLIET-----------HE
C.intestinalis    113 WHVLDV-EPNAPAYLAGLRPFSDYIIGADSLLSE---QDDLFTLIDS-----------HD
D.melanogaster    113 WHILEV-HPNSPAELAGLRAYSDYVIGADAIRHE---NDDLFTLIET-----------HE
C.elegans         113 WHIISV-QPNSPASLAGLIADTDYILGAESVLHQ---ADDLIALVQA-----------NE
P.pastoris        154 WHVLDT-LPNSPGHSANLLPGSDYIIGAQDGLLAMGGEELLGQTLYSV------YSTNRE
S.cerevisiae      190 YHILNVNIPDGPAQSAGLIPDEDYIIGCQDGLLATGGETLLQDIVR-----------SRA
N.crassa           90 WHVLDV-PANSPADVAGLLPYSDYILGTPEGVLH--GESGLSELVED-----------HI
M.grisea          118 WHVLDV-PANSPADQAGLLPYSDYILGSPDGVLH--GEGGLGELVED-----------HI
S.pombe           120 WHILNV-IDDSPVARASLVPYEDYIVGTPEGMMT--GEKALSDLIES-----------HL
D.discoideum      117 WHVLDV-YLNSPAHEAELQTRLDYIVGTPDLILN--EQEDFFTLINN-----------NM
P.falciparum      121 VRILEI-LENSPAYQSQLIEYEDFIIGYDKGIFR--NQDEFMSYINMNNIIKENSHDKKV
E.cuniculi        104 --VLRV-NPESPAEKAGMVVG-DYILGIENIYSK--DEEDLLRFLEMN-----------R
consensus             ......  ...*. ...... .*...* . ..      ... ....            ..


R.norvegicus65        GKPLKLMVYNSESDSCREVTVTPNAAWGGEGSLGCGIGYGYLHRIP 202
D.rerio65             GKPLKLLIYNTETDKCREVVVTPNGAWGGEGSLGCGIGYGYLHRIP 204
R.norvegicus55        AKPLKLYVYNTDTDNCREVIITPNSAWGGEGSLGCGIGYGYLHRIP 203
D.rerio55             GKGLKLYVYNTDTDNCREVVITPNSAWGGEGSLGCGIGYGYLHRIP 203
C.intestinalis        GRQLKLFVYNKDTDASREVIITPNSAWGGEGSMGCGIGYGYLHRIP 203
D.melanogaster        QQPLKMYVYNLDDDACREVTIKPNTAWGGEGALGCGIGFGYLHRIP 203
C.elegans             GKPLKLYVYNVDTDVVREVSLTPNSAWGGEGCLGCDIGYGYLHRIP 203
P.pastoris            NATLVLYVYNQIHNVVRPVTIYPNRNWGGIGLLGCDVGYGFLHRIP 252
S.cerevisiae          NYDLVLYVYNKVSDCVRPITVH----IGPDGRLGCNVGYGFLHRIP 280
N.crassa              DRPLRLYIYNNEYNVTREVTIQPSRDWGGEGALGCVLGYGALHRLP 181
M.grisea              GRPLRVWVYNNEYDVTRELSIQPSRDWGGQGALGCVLGYGALHRLP 209
S.pombe               NRPLRLYIYNHYRDSTRQVTIVPNRHWGGNGAIGCGVGHGVLHRLP 211
D.discoideum          YRPIQLYVYSSLTEQIRLVTITPNKNWGGSGSLGCDIGFGLLHRIP 208
P.falciparum          LFNTILYVYNYKHENIRKVQIQLNDSWGGKGLLGCNVATGYLHKIP 223
E.cuniculi            RRVVPLLVYNSDLEYVRAVSLQ----VGVDVLLGCQIGMGELYKVP 189
consensus             .......*. ...  *.... ..  .*... .** ...*.*...*
```

Figure 1-3: Sequence Alignment for GRASP PDZ-2 like domains

	Zf55-2	Rt65-2	Zf65-2	Dict-2	Plas-2	Cint-2	Dros-2	Cele-2	Neur-2	Magn-2	cere-2	pich-2	pomb-2	Ecun-2
Rt55-2	94.5	74.7	79.1	56.0	40.7	74.7	72.5	64.8	53.9	52.8	44.7	53.9	49.5	38.1
Zf55-2		76.9	78.0	55.0	40.7	74.7	71.4	67.0	51.7	51.7	45.9	53.9	48.4	39.3
Rt65-2			79.1	59.3	36.3	70.3	67.0	60.4	52.8	53.9	48.2	48.4	50.6	39.3
Zf65-2				55.0	35.2	65.9	60.4	61.5	52.8	51.7	38.8	46.2	45.1	35.7
Dict-2					38.0	55.0	51.7	48.4	46.7	43.5	37.2	48.9	43.5	38.1
Plas-2						35.2	39.6	36.3	33.7	31.5	35.6	37.4	33.7	31.8
Cint-2							67.0	62.6	50.6	51.7	48.2	51.7	45.1	35.7
Dros-2								61.5	51.7	50.6	43.5	49.5	48.4	36.9
Cele-2									52.8	51.7	47.1	49.5	41.8	41.7
Neur-2										85.9	41.9	52.2	59.8	38.1
Magn-2											41.9	48.9	52.2	36.9
cere-2												56.7	46.5	34.6
pich-2													44.6	34.1
pomb-2														32.1

Figure 1-4: Similarity (Percent Identity) of PDZ-2 like domains for the GRASP homologs

Represented organisms: Rt (rat), Zf (*Zebrafish*), Dict (*Dictyostelium*), Plas (*P. falciparum*), Cint (*C. intestinalis*), Dros (*Drosophila*), Cele (*C. elegans*), Neur (*Neurospora*), Mag (*M. grisea*), cere (*S. cerevisiae*), pich (*P. pastoris*), pom (*S. pombe*), Ecun (*E. cuniculi*)

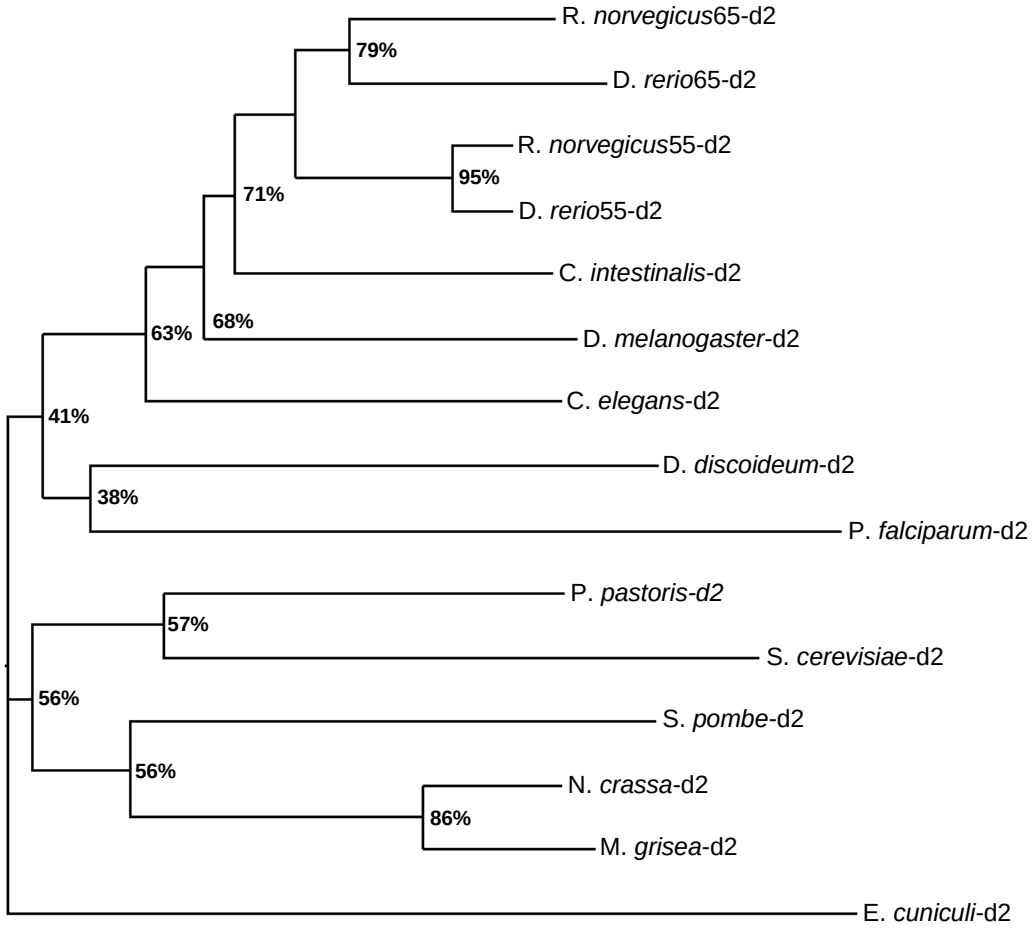


Figure 1-5: Phylogenetic Tree of GRASP PDZ-2 like domains with percent identities

```
R.norvegicus55    15 EGYHVLRVQENSPGHRAGLEPFFDFIVSISGSRLNKDNDTLKDLLKANVEKP
R.norvegicus65    14 EGFHLHGVQENSPAQQAGLEPYFDFIITIGHSRLNKENDTLKALLKANVEKP
G.gallus55        15 EGYHVLRVQENSPGHRAGLEPFFDFIVSINGSRLNKDNDTLKDLLKANVEKP
G.gallus65        15 EGFHVHGVQENSPAQQGGLEPFFDFIIAIGHTRLNKENNMLKDLLKANAEKA
X.laevis55        15 EGYHVLRVQENSPGHRAGLEPFFDFIVSINGIRLNKDNDTLKDLLKANVEKP
X.laevis65        17 EGYHVHGIQENSPAQSAGLEPFFDFIIAIGHARLNKENSMFKDLLKANAEKP
D.rerio55         15 EGYHVLRVQENSPGHRAGLEPFFDFIVSINNTRLNKDNDTLKDILKASVEKP
D.rerio65         16 EGYHVHGVQEDSPAERAGLEPFFDFIISIGHNRLNQENDMLKDLLKANVEKP
C.intestinalis    15 EGYHVLRVQENSPGYKAGLEPFFDFIVMIGNTRLNKDDDRLKDLLRANVEKP
D.melanogaster    15 EGYHVLKVQDNSPGQKAGLEAFFDFIVAIAGTRLDQDNDMLKELLRQNVDKP
C.elegans         15 EGYHVLRVQENSPGAVAGLEPFFDFIVSIGNIRLDKDNDTMKEVLKQHIEKP
D.discoideum      20 KGYHVLQVHPNSPS-TGKLVPFFDFIVAANQVIFEKEDSRFSDTFKSNIGKS
consensus            :*:*:  :: :**.  . * .:****:      :::::. :.  ::    *.


R.norvegicus55       KTLELREASVTPSNLWGGQGLLGVSIRFCSFDGANENV 112
R.norvegicus65       KTMRVREVEVVPSNMWGGQGLLGASVRFCSFRRASEHV 111
G.gallus55           KTLELRETSVTPSNMWGGQGLLGVSIRFCSFDGANENV 112
G.gallus65           KTMKIREVEVVPSNMWGGQGLLGASVRFCSFQGANEHV 112
X.laevis55           KTLELRETSVTPSNMWGGQGLLGVSIRFCSFEGANENV 112
X.laevis65           KSMKVREVEVTPSNMWGGQGLLGASVRFCSFQGANEHV 114
D.rerio55            KTLELREATVTPSNMWGGQGLLGVSIRFCSFEGANENV 112
D.rerio65            KTMRMRELEVVPSNMWGGQGLLGASVRFCSFQGANENV 113
C.intestinalis       KTLKIREVTLTPSSIWGGQGLLGVSIRFCSFDGANENV 112
D.melanogaster       KTQTVRELTLTPSNNWGGQGLLGVSIRFCSFEGANESV 112
C.elegans            KSQAVRQTSIVPSQNWGGQGLLGVSIRFCSFDGASQHV 112
D.discoideum         KSDTTREVSITPSTTWGGQGLAGISIRYCSWEKTLENV 116
consensus            *:   *:  :.**  ****** * *:*:**:  : : *
```

Figure 1-6: Sequence alignment of the GRASP PDZ-1 like domains

	Zf55-1	Rt65-1	Zf65-1	Dict-1	Plas-1	Cint-1	Dros-1	Cele-1	Neur-1	Magn-1	cere-1	pich-1	pomb-1	Ecun-1
Rt55-1	89.8	66.3	74.5	47.4	34.1	80.2	71.4	68.4	26.6	27.0	20.6	28.9	24.2	25.6
Zf55-1		65.3	74.5	48.5	33.0	81.3	72.5	68.4	26.6	25.8	19.6	29.9	25.3	25.6
Rt65-1			78.6	41.2	29.6	62.5	59.2	59.2	23.4	24.7	16.5	26.8	29.7	23.2
Zf65-1				42.3	28.4	70.8	65.3	58.2	25.0	24.7	18.6	26.8	26.4	26.8
Dict-1					33.3	47.4	46.4	45.4	23.8	22.7	19.8	25.0	23.3	25.9
Plas-1						33.7	30.7	36.4	18.6	14.1	16.1	20.9	17.9	22.1
Cint-1							75.0	65.6	25.0	25.3	20.6	30.2	28.0	21.4
Dros-1								66.3	25.0	28.1	18.6	33.0	30.8	24.4
Cele-1									28.1	23.6	18.6	28.9	24.2	22.0
Neur-1										75.7	20.8	21.9	27.4	22.4
Magn-1											23.0	27.5	30.2	15.3
cere-1												28.5	17.4	18.2
pich-1													29.7	17.4
pomb-1														20.3

Figure 1-7: Degree of Similarity **(**Sequence Identity) of PDZ-1 like domains of GRASP homologs.

Represented organisms: Rt (rat), Zf (*Zebrafish*), Dict (*Dictyostelium*), Plas (*P. falciparum*), Cint (*C. intestinalis*), Dros (*Drosophila*), Cele (*C. elegans*), Neur (*Neurospora*), Mag (*M. grisea*), cere (*S. cerevisiae*), pich (*P. pastoris*), pom (*S. pombe*), Ecun (*E. cuniculi*)

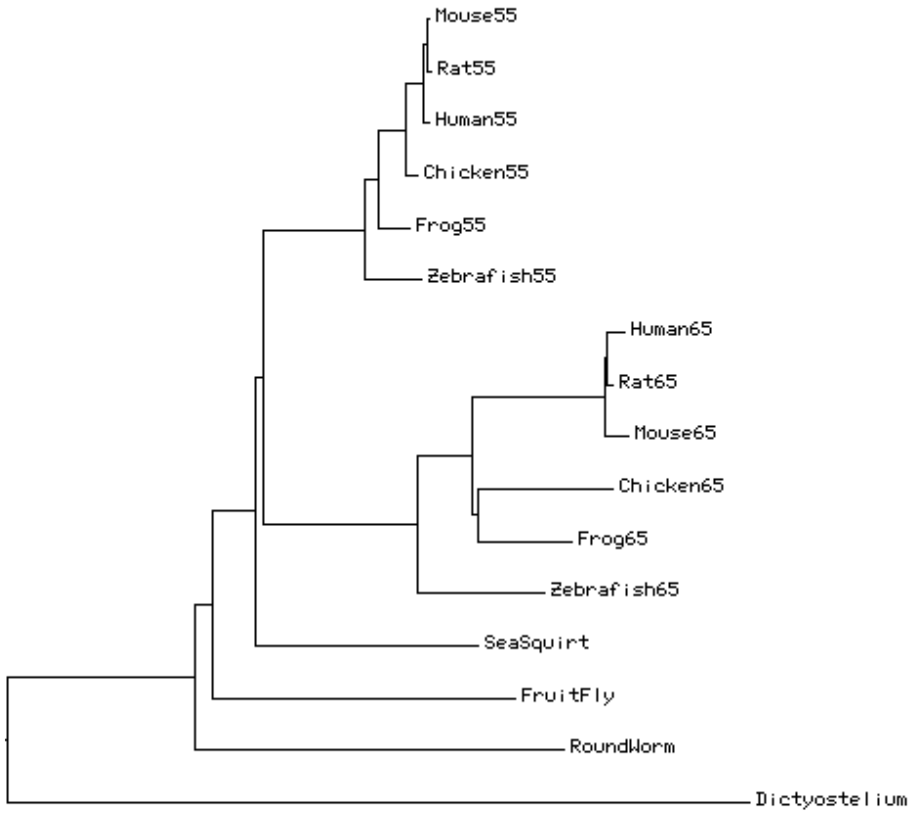


Figure 1-8: Phylogenetic tree of the conserved N-terminus of GRASP homologs

Comparisons of the two PDZ domains can give us an indication of their origins as well. An alignment of all PDZ-1 with all PDZ-2 domains of the available GRASP proteins, except fungi, gives a similarity around 35% (Figure 9). Generally speaking, proteins come from other proteins, and it appears that the first duplication event led to the appearance of a second PDZ domain, which occurred early in eukaryotic evolution. The origins of this gene is unclear since very few genomes from earlier diverging organisms have been completely sequenced. It follows that these domains have changed independently of each other. This may explain why the identity between any pair of PDZ-1 and PDZ-2 domain remains constant throughout evolution. Further experimental evidence about these domains may ultimately reveal why these are the only conserved regions of the GRASP proteins.

The majority of experiments to determine the function of the PDZ domains have been mainly performed on GRASP65. The first PDZ domain of GRASP65 is a potential docking site for the mitotic protein Polo-like kinase (Lin et al., 2000; Sutterlin et al., 2001). Plk is required for mitotic Golgi fragmentation and is capable of phosphorylating the C-terminus of GRASP65 that mitosis specific Golgi fragmentation and mitotic entry (Preisinger et al., 2005; Sutterlin et al., 2001; Yoshimura et al., 2005). The majority of GRASP proteins are anchored to the cytoplasmic face of the Golgi via an N-myrstoylation site at the second amino acid, glycine (Kinseth et al., 2007; Kondylis et al., 2005; Struck et al., 2005; Wang et al., 2005). This glycine is required for proper Golgi localization. Interestingly, in all fungi, the N-terminal glycine has been lost and replaced with an amphipathic helix that is acetylated for proper Golgi membrane localization (Behnia et al., 2007). This

shift in anchor type correlates with the rapid rate of change seen in the fungi PDZ-1 domains. What selective advantage led to fungi changing this localization motif? Further experimental evidence may determine additional differences in the fungi GRASP homolog pertaining to the rapid rate of change of PDZ-1.

The second PDZ domain is a potential dimerization motif, as well as a docking site for the second class of structural Golgi proteins the Golgins; GM130 for GRASP65 and Golgin45 for GRASP55 (Barr et al., 1998; Barr et al., 1997; Short et al., 2001). Alanine mutants within the second PDZ domain of GRASP65 have shown the protein acts as a monomer on gel filtration columns, where the wild-type protein runs as a dimer (Barr et al., 1998). These Ala mutants have also been shown to inhibit binding to GM130 which may also play a role in the correct localization of GRASP65 to the Golgi. It is still not understood what role these protein interactions play on Golgi structure and function, and new *in vivo* studies have shown this model to be more complicated than previously thought (Kinseth et al., 2007; Puthenveedu et al., 2006; Sutterlin et al., 2005).

The potential for the PDZ-2 domain to dimerize and possibly help in maintaining Golgi localization would explain why it has remained tightly conserved in all organisms. This structural requirement for the proteins function would therefore restrict any mutations that would occur over time and would suggest a common role in all species. Therefore, it is possible that GRASP proteins exist as a higher ordered complex in all species containing the protein.

	Rt55-2	Zf55-2	Rt65-2	Zf65-2	Dict-2	Plas-2	Cint-2	Dros-2	Cele-2	Neur-2	Magn-2	cere-2	pich-2	pomb-2	Ecun-2
Rt55-1	37.5	38.6	37.5	37.5	36.8	34.5	40.0	38.6	37.5	20.0	18.6	21.4	19.3	20.0	18.8
Zf55-1	36.4	37.5	36.4	36.4	39.1	34.5	38.9	37.5	36.4	20.0	19.8	22.5	20.5	20.0	18.8
Rt65-1	37.5	38.6	35.2	34.1	41.4	35.6	38.9	37.5	38.6	18.3	17.4	21.4	19.3	22.4	18.8
Zf65-1	43.2	39.8	37.5	37.5	37.9	37.9	41.1	38.6	37.5	20.0	18.6	23.6	19.3	22.4	17.5
Dict-1	38.2	38.2	31.5	32.6	34.1	29.6	39.6	41.6	34.8	19.7	19.5	21.1	21.4	22.1	22.2
Plas-1	26.1	27.2	32.6	28.3	33.0	33.3	29.4	27.2	28.3	18.0	16.9	19.6	19.0	21.8	15.9
Cint-1	35.2	34.1	34.1	33.0	39.1	34.5	40.0	34.1	34.1	18.3	19.8	21.4	19.3	22.4	15.0
Dros-1	31.8	33.0	33.0	33.0	33.3	32.2	35.6	33.0	33.0	18.3	15.1	19.1	15.9	17.7	16.3
Cele-1	39.8	36.4	42.1	36.4	36.8	34.5	42.2	40.9	34.1	15.0	17.4	22.5	22.7	17.7	15.0
Neur-1	34.8	33.7	34.8	32.6	34.1	30.7	35.2	38.2	38.2	19.7	17.2	21.1	21.4	25.6	17.3
Magn-1	36.0	34.8	36.0	36.0	36.4	29.6	35.2	38.2	40.5	21.3	18.4	23.3	23.6	23.3	17.3
cere-1	26.1	29.6	29.6	29.6	27.6	29.4	27.9	28.4	28.4	13.3	13.3	18.0	19.3	21.2	20.0
pich-1	29.4	30.4	30.4	28.3	31.9	30.1	31.5	32.6	33.7	21.3	18.0	17.5	16.8	18.4	15.9
pomb-1	29.2	28.1	28.1	29.2	33.0	28.4	28.6	29.2	31.5	21.3	14.9	20.0	16.9	18.6	19.8
Ecun-1	34.2	31.7	30.5	31.7	30.9	22.0	36.9	36.6	32.9	14.0	13.6	18.1	22.0	21.0	17.1

Figure 1-9: PDZ-1 vs PDZ-2 similarities (percent identity) of the GRASP homologs.

Represented organisms: Rt (rat), Zf (*Zebrafish*), Dict (*Dictyostelium*), Plas (*P. falciparum*), Cint (*C. intestinalis*), Dros (*Drosophila*), Cele (*C. elegans*), Neur (*Neurospora*), Mag (*M. grisea*), cere (*S. cerevisiae*), pich (*P. pastoris*), pom (*S. pombe*), Ecun (*E. cuniculi*)

The C-terminus of the GRASP Proteins Has No Apparent Structure

Apart from the two PDZ domains, the sequence resemblance between GRASP homologs deteriorates dramatically to the point where it could be argued that no identity exists. The C-terminus is defined as the sequence immediately following the second PDZ domain and therefore differs from protein to protein (Figure 10). Consistent with GRASP55 vs. GRASP65, the C-termini of vertebrate GRASP55 proteins are more conserved than the C-termini of vertebrate GRASP65 proteins. C-terminal comparsions of zebrafish to rat proteins show a 53% for the GRASP55 sequence, but only 20% identity for GRASP65 vertebrate sequences. Such low numbers in the GRASP65 C-termini as well as the other GRASP homologs is a strong indication of no selective constraints to conserve a particular function.

The lack of conservation may also explain why the size of the GRASP protein varies among all species. If only the presence of the C-terminus is required - and not a particular conserved sequence - then stop codons can occur without consequence to the organism. This would help explain why the GRASP proteins vary in length from 327-583aa amongst all species studied. These low-complexity regions are considered to be useful sites for protein stability, protein-protein interactions and protein regulations and current data supports this claim (Sim and Creamer, 2004).

Based on functional data, the phosphorylation of the mammalian C-terminus of GRASP65 regulates Golgi fragmentation, spindle dynamics and mitotic entry (Sutterlin et al., 2001; Sutterlin et al., 2005; Wang et al., 2005). Although more conserved than GRASP65, the C-terminus of GRASP55 is very Pro/Ser/Thr rich and shows no sign of secondary structure. Similarly to its homolog, the C-terminus of

GRASP55 may also be used to regulate mitotic Golgi fragmentation and mitotic entry (Feinstein and Linstedt, 2007; Jesch et al., 2001). Since mitotic Golgi fragmentation is cell specific and appears to be regulated by the C-terminal region of the GRASP proteins, it would appear as if only the presence of this region is needed to act as a scaffold for protein-protein interactions.

	Ch55-c	Xp55-c	Zf55-c	Rt65-c	Ch65-c	Xp65-c	Zf65-c	Dict-c	Plas-c	Cint-c	Dros-c	Cele-c	Neur-c	Mag-c	cere-c	pich-c	pom-c	Ecun-c
Rt55-c	62.3	55.8	52.5	23.1	24.6	22.1	25.2	13.3	10.0	35.6	22.1	14.4	23.8	22.5	22.7	15.7	17.5	11.3
Ch55-c		48.8	53.5	18.1	22.1	21.4	21.5	12.8	10.5	37.7	19.1	13.6	21.0	19.4	16.9	17.5	19.7	11.0
Xp55-c			46.7	23.0	21.6	22.4	23.1	12.0	13.2	35.3	22.1	15.0	20.0	20.2	18.6	19.1	20.3	12.4
Zf55-c				20.2	22.8	20.0	26.8	12.5	9.1	40.0	17.1	15.0	19.4	18.6	13.6	14.5	17.5	12.2
Rt65-c					22.4	17.8	17.2	7.6	7.2	15.0	11.2	12.9	17.5	17.1	13.6	15.9	15.0	8.9
Ch65-c						31.1	19.3	10.2	10.3	18.4	12.3	11.0	16.6	10.4	13.5	11.1	16.3	11.7
Xp65-c							18.1	11.3	9.0	19.8	14.7	14.8	15.2	17.5	18.0	10.8	18.9	9.5
Zf65-c								11.9	9.4	18.2	14.1	12.1	15.3	15.0	14.6	13.3	13.8	7.7
Dict-c									6.7	12.2	12.5	13.9	9.9	8.9	5.7	12.8	16.1	3.4
Plas-c										5.6	7.4	5.6	8.1	10.5	9.8	11.7	13.4	8.5
Cint-c											14.0	18.0	16.1	15.6	15.0	14.6	12.5	8.8
Dros-c												15.7	15.4	17.6	12.0	9.2	9.9	10.9
Cele-c													8.2	8.0	13.0	9.4	11.3	9.0
Neur-c														38.7	16.1	10.3	20.8	9.3
Magn-c															20.0	12.5	22.5	13.8
cere-c																4.7	19.2	13.0
pich-c																	11.1	12.3
pomb-c																		8.2

Figure 1-10: Degree of Similarity **(**Sequence Identity) of C-termini of GRASP homologs.

Represented organisms: Rt (rat), Xp (*Xenopus*), Zf (*Zebrafish*), Ch (Chicken), Dict (*Dictyostelium*), Plas (*P. falciparum*), Cint (*C. intestinalis*), Dros (*Drosophila*), Cele (*C. elegans*), Neur (*Neurospora*), Mag (*M. grisea*), cere (*S. cerevisiae*), pich (*P. pastoris*), pom (*S. pombe*), Ecun (*E. cuniculi*)

The Conservation of the GRASP Proteins and Golgi Stacking

Whether Golgi membranes are seen in its classical structure of a stack of flattened membrane cisternae, or as individual cisternae dispersed throughout the cell, or merely large budding vesicles, the function is always the same; the transport of protein to the cell surface for exit or localization. From evolutionary, experimental and phylogenic data, the conservation of protein secretion process occurs in three forms: 1) vesicle formation and budding, 2) the movement of vesicle from donor membrane to target membrane and 3) vesicle docking and fusion to target membrane (Becker and Melkonian, 1996). These essential steps of protein secretion are found in all eukaryotic organisms. Likewise the proteins required for these actions are conserved amongst all species. Through BLAST and genome analysis, homologs have been identified for all the factors involved in these three essential steps (Becker and Melkonian, 1996; Koumandou et al., 2007).

Why, then, do Golgi cisternal membranes stack in some organisms and not others? It is not a clear evolutionary development. In some protists Golgi stacks appear, and in others they do not (Becker and Melkonian, 1996). Since secretion studies and Golgi morphology is limiting in such organisms there is little experimental evidence to base assumptions on. Biochemically, it appears as if all organisms have a method of protein transport, whether a clear morphological Golgi apparatus can be determined or not (Dacks et al., 2003).

The evolution of the endomembrane system sheds light on the origins of the Golgi but not its organization. Golgi membranes organizes itself in a cell dependent manner depending on what proteins it secrets and the efficiency of secretion

(Beznoussenko et al., 2007; Hehl and Marti, 2004; Kappe et al., 2004; Koumandou et al., 2007; Moreau et al., 2007; Segui-Simarro and Staehelin, 2006). This would explain why Golgi membranes are not always in a “recognizable” form and why certain Golgi proteins are conserved while others are lost. Studies in fungi have given the experimental evidence why certain Golgi membranes may stack their cisternae, all relying on the organization and efficiency of transport and exit from the ER (Ayscough et al., 1993; Beznoussenko et al., 2007; Hashimoto et al., 2002; Losev et al., 2006; Rossanese et al., 1999).

Possibly the most intriguing and compelling evidence is the complete absence of the GRASP gene from plants despite their well described stacked Golgi membranes. From these combined studies, and the evolutionary data on GRASP proteins, it is clear the mere presence or absence of a GRASP protein has no specific bearing on the structure of Golgi membranes, and other variables may very well be at play with regards to this dynamic organelle.

Discussion

Phylogenic analysis has been used to find the important domains of the GRASP proteins and to determine which questions need to be addressed from a functional and experimental level. Based on these comparisons we can conclude that the presence of GRASP proteins and the appearance of Golgi stacks are not defined by any domain or presence of the protein.

Trafficking through the Golgi is a highly complex process involving recycling of membranes from parent compartments (the ER, endosomes and plasma membrane), protein regulation (G-proteins, SNAREs SNAPs, NSF, coatamers), cytoskeletal contributions, acidification and ion pumps, and lipid metabolism. Any alteration of these can disrupt Golgi dynamics. These are not structural proteins but rather a multiple variable step process that is capable the achieving dynamic equilibrium we call the static Golgi complex. Therefore, any single group of proteins may not be solely responsible for the proper stacking and order of Golgi membranes when so many elements contribute to its existence. From an evolutionary perspective, as well as the experimental evidence to date, the presence of the GRASP protein does not predict a function in Golgi cisternal stacking.

The Loss of the Plant GRASP Gene

Why is GRASP gene absent in plant and algae? As mentioned previously, plant and algae have some of the most elaborate Golgi stacks in all organisms (daSilva

et al., 2004; Moreau et al., 2007). Plant Golgi membranes do not fragment to the extent of mammalian Golgi membranes during mitosis but rather are required for membrane depositing during cytokinesis (Segui-Simarro and Staehelin, 2006). The lack of the GRASP gene in plants sets up an interesting question. Evolutionarily speaking, the protists branched off earlier than those of the metazoan/plants. Therefore a protein that exists with an identity of 40% (PDZ-2) in protists should theoretically appear in plants. There is always the possibility that Plasmodium picked up the gene by horizontal transfer from an animal host, but given the percent identity number this is unlikely. It should also be noted that a simple BLAST-P analysis all other essential Golgi proteins are easily identifiable within many plant and algae genomes.

Another explanation is conceivable in which plant GRASP proteins changed at a faster rate than other species, and therefore their homolog cannot be found by sequence comparison. For this to be so, one would have to assume a relaxation of selective constraints on the gene which would imply these genes are not very important in the function they hold. Either way, a look at plant Golgi clearly show a stacked and ordered cisternae with no GRASP sequence homolog existing.

It still cannot be dismissed that there may be a plant GRASP gene that exists in an unrecognizable form that does the same job but one must assume that job is being done differently. It should be noted that all proteins involved in secretion and sugar transport are conserved in plants, protists, fungi and vertebrates, regardless of a stacked Golgi membrane. Why would selective constraints in plants be less than those of other organisms?

A further understanding of the GRASP proteins in multiple biological systems may help reveal not only their physiological importance, but why these domains are conserved in this manner as well as why plant and algae have lost this controversial gene.

Acknowledgments

I would like to acknowledge Justin Kollman and Russell F. Doolittle for their help in both obtaining and understanding the GRASP phylogenetic data. I greatly appreciate the countless hours spent looking over sequences and compiling the data with me to better appreciate the conserved nature of these proteins as they relate to Golgi membrane structure. I also thank Russell F. Doolittle for his help in the preparing and editing this chapter in my dissertation.

Chapter II

The Golgi associated protein GRASP is required for unconventional protein secretion during development.

Summary

During *Dictyostelium* development, prespore cells secrete Acyl CoA binding protein (AcbA). Upon release, AcbA is processed to generate a peptide called SDF-2, which triggers terminal differentiation of spore cells. We have found that cells lacking GRASP, a protein attached peripherally to the cytoplasmic surface of Golgi membranes, fail to secrete AcbA, and thus produce inviable spores. Surprisingly, AcbA lacks a signal sequence and is not secreted via the conventional secretory pathway [endoplasmic reticulum-Golgi-cell surface]. GRASP is not required for conventional protein secretion, growth, and the viability of vegetative cells. Our findings reveal a physiological role of GRASP and provide a means to understand unconventional secretion and its role in development.

Introduction

The **G**olgi **R**e-**A**ssembly **S**tacking **P**roteins were identified through *in vitro* assays as factors required for the stacking of Golgi cisternae and the tethering of vesicles destined to fuse with the Golgi apparatus (Barr et al., 1997; Shorter et al., 1999). There are two isoforms in vertebrates (GRASP55 and GRASP65) and a single gene in other organisms, although the gene has been lost in plants. Despite the proposed functions based on *in vitro* studies, siRNA depletion of GRASP in *Drosophila* and mammalian cells (GRASP65), as well as gene knockout in *S. cerevisiae*, have no significant effect on cisternal stacking or general protein secretion (Behnia et al., 2007; Kondylis et al., 2005; Puthenveedu et al., 2006; Short et al., 2001; Shorter et al., 1999; Sutterlin et al., 2005). Moreover, plant cells have perfectly stacked Golgi cisternae despite having lost the GRASP gene. GRASPs are also reportedly required for Golgi fragmentation during mitosis in mammalian cells, and mitotic progression in both yeast and mammalian cells (Colanzi et al., 2003; Norman et al., 1999; Preisinger et al., 2005; Stanley et al., 1997; Sutterlin et al., 2002; Wang et al., 2005). In sum, GRASPs have been proposed to carry out many functions but their precise physiological role remains elusive. This might be a reflection of the systems and the assays used thus far to investigate the role of GRASPs. We therefore chose to study the role of GRASP in a developmentally regulated organism *Dictyostelium discoideum*, which is amenable to genetic manipulation.

Development in *Dictyostelium* generates two major cells types: prespore and prestalk cells (Anjard et al., 1998; Shaulsky and Loomis, 1996). Spore formation is activated by the release of the protein AcbA from prespore cells (Anjard and Loomis, 2005). Once secreted, AcbA is cleaved to produce the spore differentiation factor-2 (SDF-2) by the proteolytic activity of an ABC transporter/serine protease, TagC, located on the surface of stalk cells. SDF-2 along with other activating peptides leads to sporulation (Anjard and Loomis, 2005; Anjard et al., 1997; Anjard et al., 1998).

We have found that deletion of the single gene encoding GRASP in *Dictyostelium* inhibits secretion of AcbA from prespore cells and thus dramatically reduces spore viability. Interestingly, AcbA lacks a signal sequence necessary for translocation into the endoplasmic reticulum and its subsequent traffic along the conventional secretory pathway. Transport of the SDF-2 receptor, DhkA to the cell surface is unaffected in the GRASP-null strain. Likewise, the conventional secretion of Apr, lysosomal hydrolases, and the spore coat proteins are normal in GRASP null-cells. Our findings on the involvement of GRASP in unconventional protein secretion during cellular development are discussed.

Results

Characterization of *Dictyostelium discoideum* GRASP protein

It has been shown previously that the GRASP proteins contain two PDZ-like domains followed by a variable C-terminal region (Barr et al., 1998; Wang et al., 2005). A BLAST search using both GRASP55 and GRASP65 proteins revealed that only the two N-terminal PDZ domains are conserved in all eukaryotic GRASP proteins. The single GRASP homolog in *Dictyostelium*, which we named GrpA, contains the two conserved N-terminal PDZ domains and a very short C-terminus. Sequence comparisons reveal that the first PDZ domain is 47% identical to rat GRASP55 PDZ-1. The second PDZ domain is relatively more conserved with a 56% sequence identity with rat GRASP55 PDZ-2 domain. Sequence comparison of all the available genomes revealed that plants lack a bona fide GRASP homolog, even though early diverging organisms (protists) contain a highly homologous GRASP protein (Figure 1A).

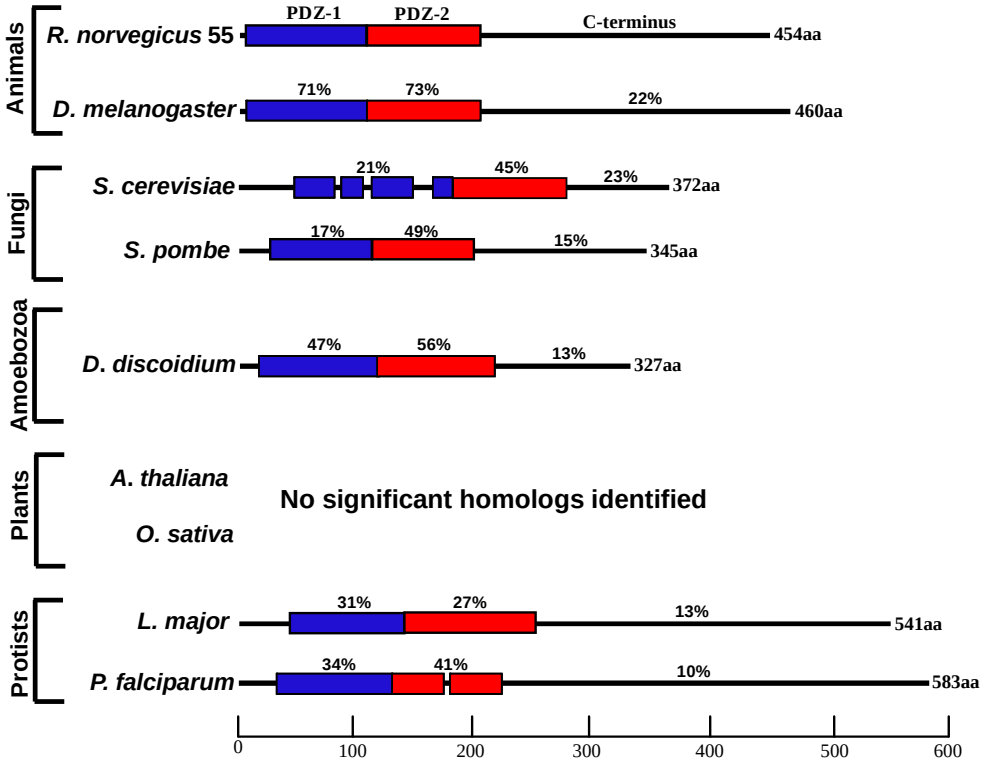
GRASPs are localized to the Golgi membranes through their N-terminus; in mammalian cells, the N-terminal glycine is myristoylated, whereas in *S. cerevisiae* and other fungi, an N-terminal amphipathic helix forms and is acetylated for its attachment to the cis-Golgi membranes (Barr et al., 1997; Behnia et al., 2007; Shorter et al., 1999). GrpA contains the conserved N-terminal glycine, as do the protists *P.y. yoelii* and *L. major* (Struck et al., 2005) (Figure 1B). Interestingly, the C-terminus of

Figure 2-1: Identification of a *Dictyostelium* GRASP homolog

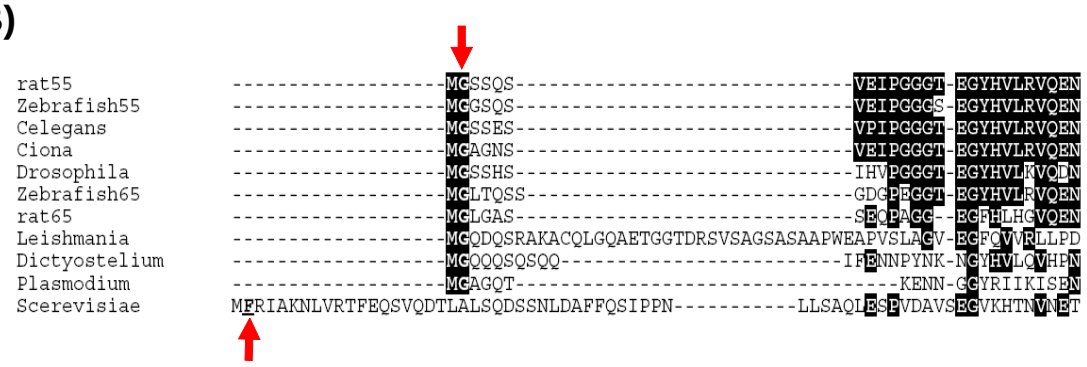
(A) The rat GRASP65 (AF015264) and rat GRASP55 (AF110267) PDZ domains (PDZ-1 and PDZ-2) were used to identify GRASP homologs in *D. rerio* (NP_956997 and NP_001007412), *C. elegans* (NP_501354), *D.melanogaster* (AAF49092), S. *cerevisiae* (NP_010805), *S. pombe* (NP_593015), *D. discoideum* (EAL60823), *L. major* (CAJ06649), *P. falciparum* (AAN35366) and *P. y. yoelii* (EAA21406). Percent identities were obtained by Clustal-W analysis. The N-terminal PDZ domains of GRASPs are highly conserved, whilst the C-terminal region is highly divergent in the corresponding GRASP homologs. The *Dictyostelium* C-terminal region is truncated in comparison with the mammalian GRASP proteins, and is not rich in potential phosphorylation sites, which are thought to regulate mammalian GRASP function during mitosis. Percent identity is listed above each domain and the amino acid length of each homolog immediately follows the protein. Only rat GRASP55 is used in percent identity comparisons with other orthologs.

(B) An N-terminal sequence alignment of vertebrate, invertebrate, fungi and protists GRASP orthologs reveal that the N-terminal glycine (arrow), responsible for its anchoring to the Golgi via mrystoylation, is conserved in *D. discoideum*. The *S. cerevisiae* ortholog lacks the N-terminal myristoylation but instead folds into an amphipathic helix, which is acetylated at its second amino acid (arrow)

A)


Animals
R. norvegicus 55
PDZ-1
PDZ-2
C-terminus
454aa
D. melanogaster
71%
73%
22%
460aa
Fungi
S. cerevisiae
21%
45%
23%
372aa
S. pombe
17%
49%
15%
345aa
Amoebozoa
D. discoidium
47%
56%
13%
327aa
Plants
A. thaliana
O. sativa
No significant homologs identified
Protists
L. major
31%
27%
13%
541aa
P. falciparum
34%
41%
10%
583aa
0
100
200
300
400
500
600

B)


rat55
Zebrafish55
Celegans
Ciona
Drosophila
Zebrafish65
rat65
Leishmania
Dictyostelium
Plasmodium
Scerevisiae

eukaryotic GRASP proteins vary in size and have no conserved sequence homology with any known protein. Therefore, GRASP proteins are identified on the basis of their homologous N-terminal PDZ domains followed by a low complexity C-terminal region. It has been reported that such low complexity regions have multiple functions including but not limited to protein-protein interactions, substrate binding or to maintain the structural integrity of a protein (Sim and Creamer, 2002). The C-terminal region of GrpA compared with other GRASP proteins is truncated and contains a high percentage (34%) of the amino acid glutamine. Interestingly, the mammalian GRASP proteins have longer C-termini and are enriched instead in Ser/Thr and prolines. This could potentially explain the involvement of mammalian GRASPs in mitosis specific events through it C-terminus, which is a substrate for mitotic kinases (Feinstein and Linstedt, 2007; Preisinger et al., 2005; Sutterlin et al., 2002; Wang et al., 2005).

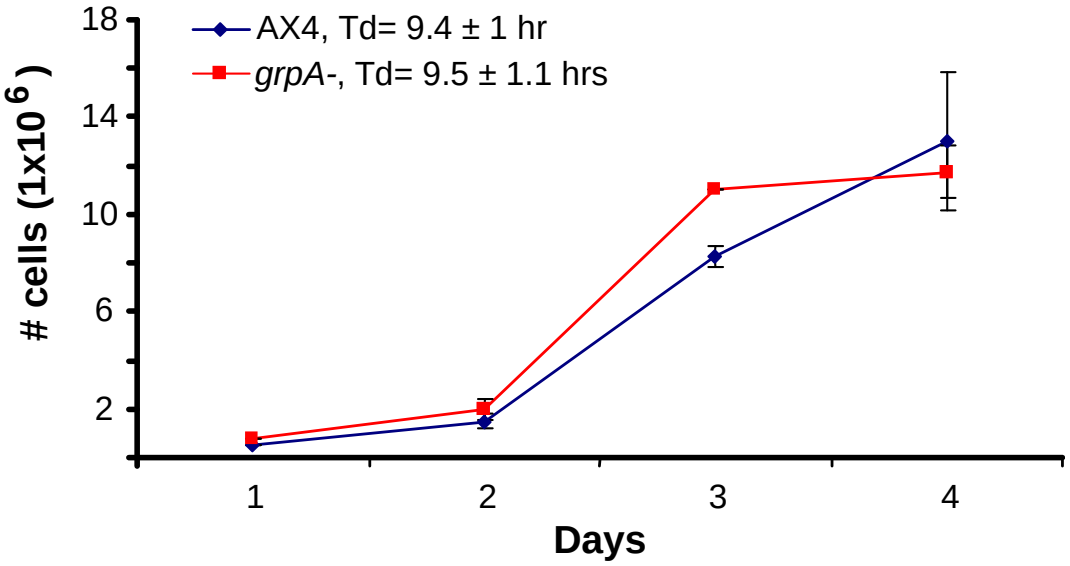
Based on the Dictybase gene sequence (DDB0191849), primers were generated to PCR the *grpA* gene from wild type AX4 genomic DNA. 400bp flanking regions were used for homologous recombination to disrupt *grpA* with the Blasticidin gene as the selective marker. Clones were screened using PCR, Southern blot analysis, and confirmed with restriction enzyme digestion of genomic DNA from both wt and *grpA*- cells.

Figure 2-2: Deletion of GRASP does not affect cell growth or Golgi organization in *Dictyostelium*

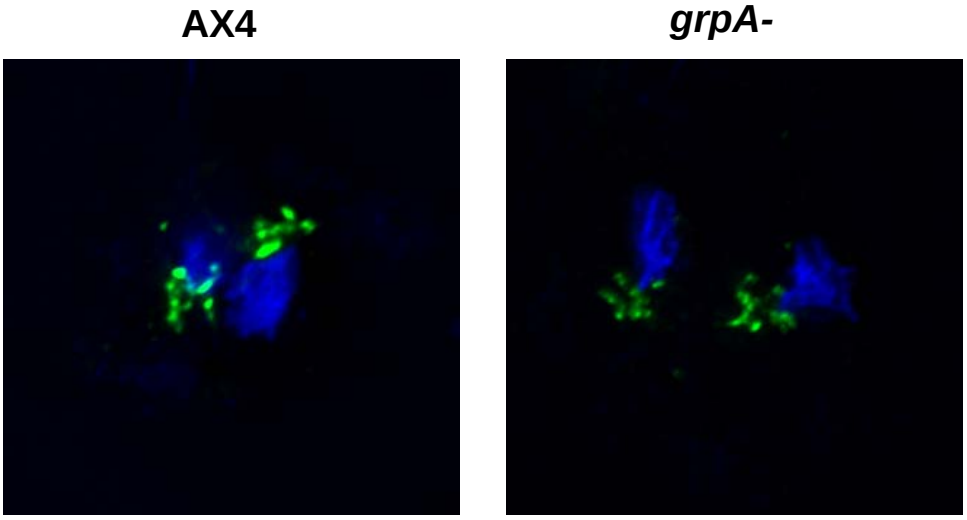
(A) 10^6 cells/ml were seeded in suspension culture and counted for 4 successive days. Doubling time was calculated using the equation: $T_2 = (t \ln 2)/\ln (N/N_0)$, where t is the elapsed time in hrs, N and N_0 are the final initial cell count, respectively

(B) Golvesin-GFP was stably transformed into both AX4 and *grpA*- cells. Cells were plated on glass coverslips, fixed and stained with Hoechst 33342 to visualize DNA. Deconvolution microscopy revealed that GRASP deletion does not affect the organization of Golgi membranes in *Dictyostelium*.

A)


AX4, Td= 9.4 ± 1 hr
grpA-, Td= 9.5 ± 1.1 hrs
cells (1x10^6)
18
14
10
6
2
1
2
3
4
Days

B)


AX4
grpA-

GRASP is not required for cell growth in *Dictyostelium*

To test the role of GRASP in cell growth and viability, wild type AX4 and *grpA* mutant cells were grown in suspension and counted each day in triplicate for 4 days. The loss of GrpA had no effect on the doubling time of axenically growing *Dictyostelium* cells when compared to wild type AX4 (Figure 2A).

The GRASP proteins were originally identified *in vitro* as factors involved in regulating Golgi structure in mammalian cells. To test the involvement of GRASP in Golgi structure and organization, we stably transformed a Golvesin-GFP marker previously shown to localize to the Golgi apparatus (Schneider et al., 2000) into both wild type AX4 and the *grpA*- strain. Immunofluorescence microscopy revealed that Golvesin-GFP staining of a perinuclear Golgi is similar in both strains. Based on immunofluorescence data, we suggest that the Golgi membranes maintain their overall organization in *grpA*- cells (Figure 2B).

GRASP is required for *Dictyostelium* spore viability

Following starvation, *Dictyostelium* cells aggregate and differentiate into two major cell types, prestalk and prespore cells, which ultimately form fruiting bodies containing mature spores. To test if GrpA has any role in cellular development, both AX4 and *grpA*- strains were allowed to develop for 24hrs before fruiting bodies were collected, and treated with a mild detergent. Spores produced from both strains were isolated, counted and a fixed number were plated together with a *K. aerogenes* bacterial suspension as a food source. After 4 days, individual plaques were counted to

determine percent spore viability. We found that only 25% of spores plated from the *grpA*- strain were viable as compared with 100% of wild type AX4 spores (Table 1).

GRASP is required for the production of spore differentiation factor-2 peptide (SDF-2)

Proper spore formation requires two major peptide factors, spore differentiation factors 1 (SDF-1) and 2 (SDF-2) (Anjard et al., 1998). To measure secreted levels of SDF-1 and SDF-2, we isolated these peptides based on their ability to tightly bind either cation (C-50) or anion (A-25) exchange resins (Anjard et al., 1997). The enriched SDF-1 and SDF-2 fractions were incubated with KP cells to test for their ability to activate spore formation (Anjard et al., 1998). As reported previously, KP cells are a *Dictyostelium* AX2 cell line which over-expresses protein kinase A (PKA) under the control of its own promoter. When developed at low density monolayers for 18 hours these KP cells are capable of producing spores within 1-2 hours and, therefore, provide a suitable assay to monitor the ability of cells to produce, secrete and process AcbA, as well as other factors involved in sporulation (Anjard et al., 1998). To investigate the significance of GRASP in spore viability we measured the levels of SDF-1 and SDF-2 production during development in wt and *grpA*- fruiting bodies. Interestingly, we found that *grpA*- cells produce normal levels of SDF-1 but no measurable SDF-2 (Table 1).

AcbA is a 10kD protein which lacks a signal sequence for targeting to the conventional secretory pathway (Anjard and Loomis, 2005). AcbA, upon release from

Table 2-1: GRASP is required for the unconventional secretion of AcbA in prespore cells

As described previously, spores from each strain were collected and plated on SM-plates together with a *K. aerogenes* bacterial suspension to test spore viability (Anjard and Loomis, 2005). The amount of SDF-1 and SDF-2 was normalized in units per 10^3 cells. Priming cells with SDF-2 alone does not rescue SDF-2 levels in the *grpA*- strain but the addition of SDF-2 + AcbA rescues levels of SDF-2 indicating that both DhkA and TagC are present and active in *grpA*- cells. Priming cells with 10nM GABA in place of SDF-2 gave similar results. The intracellular AcbA was determined by treating cell lysates with trypsin to convert it into SDF-2. No SDF-2 (less than 0.2 units) was detected from lysates of *acbA*$^-$ null cells.

Addition of 10% wild type (AX4) cells to *grpA*- cells was able to rescue *grpA*- spore viability, but was not able to recover SDF-2 levels. A mixture with 10% *acbA*- reveals that SDF-2 is not generated from the *grpA*- strain. Therefore, the *grpA*- phenotype is cell autonomous for SDF-2 production and non cell autonomous for spore viability. All experiments were carried out at least three times except for the GrpA-Flag over expression experiment (n=1). ND: not determined.

Strain	**% viable spore**	**SDF-1 in fruiting body**	**SDF-2 in fruiting body**	**SDF-2 upon priming**	**SDF-2 upon priming + AcbA**	**SDF-2 from Intracellular AcbA**
AX4	100%	10^3	10^4	10^4	10^4	10^5 –2x10^5
grpA-	25±5%	10^3	<0.2	<10	2x10^3-10^4	10^5 –2x10^5
90% *grpA*- 10% Ax4	94 ±12%	ND	7 x 10^2	ND	ND	ND
90% *grpA*- 10% *acbA*-	55±10%	ND	<0.2	ND	ND	ND
grpA- + GrpA-Flag	93%	ND	10^4	ND	ND	ND

the prespore cell is cleaved by the stalk specific protease TagC to generate the peptide SDF-2. SDF-2 binds to the histidine kinase receptor, DhkA, thus generating a feedback loop that promotes further AcbA release from prespore cells, a burst in SDF-2 production, and ultimately terminal differentiation of spore cells (Anjard and Loomis, 2005). Therefore, generation of SDF-2 requires that AcbA be produced, secreted, and cleaved by TagC, all of which requires the presence of the receptor DhkA for downstream signaling.

GRASP is required for the unconventional secretion of AcbA

To test whether *grpA*- cells are capable of producing AcbA, *grpA*- and AX4 cells were harvested at 0hr and 22hrs during development. Cell pellets were resuspended in buffer and lysed by passing through a Nuclepore® Track-Etch membrane. Samples were analyzed on a 10-20% Tris-tricine gradient gel and transferred to PVDF membrane. Western blot analysis revealed that the *grpA*- fruiting bodies produce normal levels of AcbA within the cell both during vegetative growth and during development (Figure 3A). Fractionation of cells revealed that AcbA is almost entirely present in a soluble pool (not membrane associated) and this distribution is unaltered in *grpA*- cells both during vegetative growth and development (Figure 3B).

Since *grpA*- cells are capable of producing AcbA, we next tested for their ability to secrete AcbA. Purified SDF-2 added exogenously at low levels to wild type cells stimulates AcbA release, a process termed priming (Anjard et al., 1997). Priming cells with SDF-2 both promotes the release of AcbA from prespore cells as well as the

presentation and activation of TagC on stalk cells (Anjard and Loomis, 2005). Both of these responses are triggered by binding of SDF-2 to the receptor DhkA which must be present on both cell types (Wang et al., 1999). Therefore, an increase in SDF-2 production due to AcbA processing by the protease TagC is a measure of AcbA release. The presence of AcbA/SDF-2 in the secreted medium was measured using the KP cell sporulation assay (Anjard and Loomis, 2005). We found that priming *grpA*- cells with low levels of SDF-2 failed to increase SDF-2 levels, whereas wild type cells produced high levels of SDF-2 (Table 1).

SDF-2 levels are dependent upon the activity of the protease TagC to process secreted AcbA. Therefore we next tested whether TagC is active and present on stalk cells in *grpA*- fruiting bodies. When *grpA*- cells were primed with SDF-2 in the presence of recombinant AcbA we found that SDF-2 levels were restored to normal as compared with wild type cells (Table 1). This illustrates that both DhkA and TagC are active and present on the surface of *grpA*- cells.

AcbA is also released in response to GABA (Gamma-aminobutyric acid) acting through its receptor GrlE (Anjard and Loomis, 2006). Similar to SDF-2 priming, priming cells with GABA leads to AcbA secretion and TagC mediated cleavage to produce SDF-2. The GrlE pathway is independent of the DhkA pathway and both independently control the unconventional secretion of AcbA. To determine whether GABA is capable of inducing AcbA expression in *grpA*- cells, we performed the experiment described above but instead of SDF-2, GABA was used to prime the cells. We found that in *grpA*- cells, GABA did not induce the release of AcbA, but SDF-2 levels were restored with the addition of GABA and recombinant AcbA (data

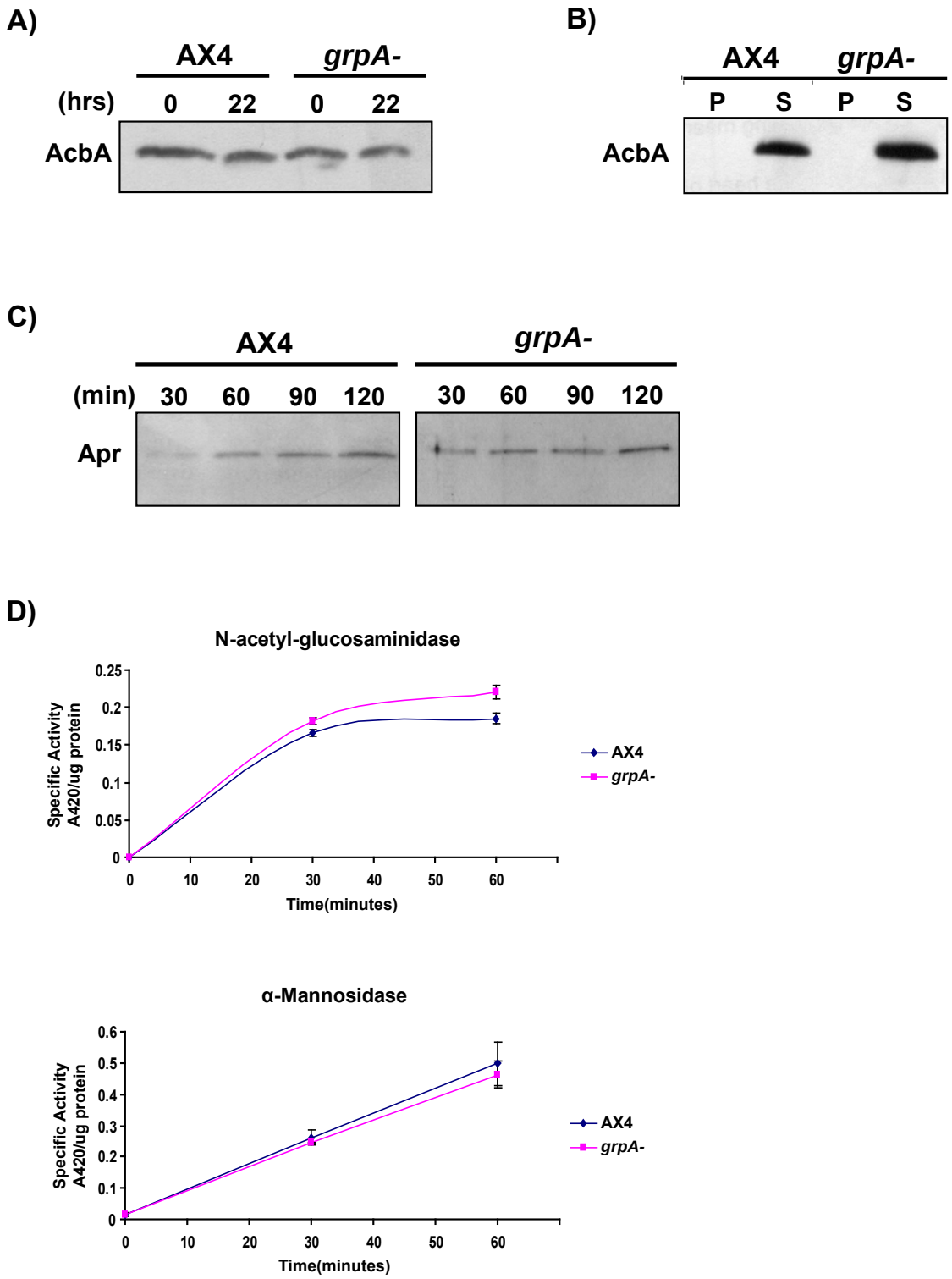
Figure 2-3: *GrpA*- cells contain normal levels of AcbA protein levels and are not defective in conventional protein secretion.

(A) A total of 10^7 cells for each strain were cultured for 24h. Fruiting bodies were collected and AcbA protein levels analyzed on a 10-20% Tris-tricine gradient gel. Western blot using anti-AcbA antibody indicates that AcbA proteins levels are normal in *grpA*- cells compared with wt AX4 at both vegetative growth (0 hr) as well as during development (22hrs).

(B) Cell lysates of fruiting bodies collected after 22 h of development were centrifuged at 12K for 10min followed by centrifugation of the supernatants at 55k for 1hr. The 55K pellet (P) and the supernatant (S) were analyzed on a 10-20% Tris-tricine gel and transferred to PVDF membrane. Anti-AcbA immunostaining indicates that AcbA is almost entirely in a soluble pool in both wild type and grpA- cells.

(C) Growth medium from vegetative wild type and *grpA*- cells grown at the same density was collected at the times shown to measure Apr secretion by western blotting with an anti-Apr antibody. There is no obvious difference in the amount and the kinetics of Apr secretion between grpA- cells and wild type cells.

(D) Wild type and *grpA*- cells grown at the same cell density were transferred to phosphate buffer and incubated for 4h, followed by washing and continued incubation in phosphate buffer for additional 2h. After 6hrs of starvation, the cells were harvested and the supernatant tested for the presence of N-acetyl-glucosaminidase and α-mannosidase enzymatically at the times shown. There is no defect in the secretion of these lysosomal hydrolases, as measured by their activity, in *grpA*- cells compared with the wild type cells.


A)
AX4
grpA-
(hrs)
0
22
0
22
AcbA
B)
AX4
grpA-
P
S
P
S
AcbA
C)
AX4
grpA-
(min)
30
60
90
120
30
60
90
120
Apr
D)
N-acetyl-glucosaminidase
Specific Activity
A420/ug protein
0.25
0.2
0.15
0.1
0.05
0
0
10
20
30
40
50
60
Time(minutes)
AX4
grpA-
α-Mannosidase
Specific Activity
A420/ug protein
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
Time(minutes)
AX4
grpA-

not shown). From these studies we conclude that GRASP is not required for the cell surface expression of DhkA, GrlE or the presence and activation of TagC, but is required for the release of AcbA from the prespore cell, whether they are activated by GABA or SDF-2.

Spore viability is a direct consequence of the GRASP dependent secretion of AcbA

To test the cell autonomy of the *grpA*- phenotype, AX4 were mixed with *grpA*- cells at a ratio of 1:10 and allowed to develop for 24 hrs. Interestingly, spore viability dramatically increased to 93% (Table 1). To determine the strain responsible for the production of viable spores, the spores were isolated, plated and selected for Blasticidin resistance, the gene used to disrupt *grpA*. Approximately 90% of the viable spores were found to originate from the *grpA*- strain (data not shown). This indicates that the addition of AX4 cells was able to rescue spore viability of the *grpA*- mutant strain.

To determine which strain was responsible for AcbA secretion and hence SDF-2 production in the same experiment, fruiting bodies were collected and SDF-2 levels measured as before. The levels of SDF-2 were only 7% compared with the normal (Table 1). This suggests that SDF-2 is produced by the wild type cells, which were present at 10%, and not *grpA*- cells. Therefore, in the presence of SDF-2, spore viability of *grpA*- cells is completely rescued. In comparison, the secretion of AcbA by wild type cells was unable to rescue AcbA secretion in *grpA*- cells (Table 1). This is

consistent with our previous experiments of priming *grpA*- cells with SDF-2. We conclude that spore inviability is a non-cell autonomous process, and is rescued by SDF-2 signaling pathway, whereas AcbA secretion is a cell autonomous process for *grpA*- cells, which cannot be rescued by wild type cells. Interestingly, spore viability recovery also indicates that the conventional secretion of the spore coat proteins is normal in *grpA*- cells. Therefore, the decreased spore viability is a direct consequence of the inhibition of AcbA secretion.

To further test that *grpA*- cannot produce SDF-2, *acbA*- cells were mixed with *grpA*- cells at a ratio of 1:10 and allowed to develop for 24 hrs. Although a partial increase in spore viability was seen, SDF-2 levels were not rescued (Table 1). These combined results show that the inability of *grpA*- cells to secrete AcbA from prespore cells is directly involved in the dramatic loss of spore viability of these cells.

Although no posttranslational modifications have been found, it is possible that GRASP has a role in the intracellular processing/maturation of AcbA, which may be necessary for its secretion during development. To test this possibility, intracellular AcbA from wild type and *grpA*- cells was collected and tested for its ability to produce SDF-2. Developed wild type and *grpA*- cells were lysed; extracts were treated with trypsin; SDF-2 was purified on anionic beads and tested on KP cells. Extracts from both the wild type and *grpA*- cells produced comparable levels of SDF-2 (Table 1). All together these findings strengthen our proposal that *grpA*- cells produce functionally active AcbA but fail to secrete it.

It is well established that defects in the conventional secretion of a number of factors results in aberrant cell growth and development for *Dictyostelium* (Devine et

al., 1983; Metcalf et al., 2003; West, 2003). The fact that addition of AcbA to *grpA*- cells restores the spore viability is a strong indication that conventional secretion is not inhibited by GRASP deletion. To further test this proposal we monitored the secretion (ER-Golgi-cell surface) of a protein called Apr, which regulates the growth of *Dictyostelium* (Brock and Gomer, 2005). Vegetative cells were collected and the medium was analyzed by western blotting for the presence of Apr from both wild type and *grpA*- cells. We found no obvious change in the kinetics of Apr secretion between wt and *grpA*- cells (Figure 3C). In addition, we have monitored the conventional secretion of two lysosomal hydrolases, N-acetylglucosaminidase and α-Mannosidase that are synthesized following the initiation of development. Under starvation conditions, lysosomal hydrolases are secreted by *Dictyostelium* cells (Mierendorf et al., 1985; Wood and Kaplan, 1985). We found that *grpA*- cells show no defect in the release and activity of these secreted enzymes when compared with wild type cells (Figure 3D). All together our findings reveal that GrpA depletion does not affect the conventional secretory pathway.

Over expression of GRASP rescues SDF-2 and spore viability in *grpA*- cells

To determine if the phenotype of *grpA*- cells is a direct result in the loss of the GRASP gene only, *grpA* was cloned from AX4 genomic DNA, fused with a Flag tag sequence, and transformed in *grpA*- cells. The reintroduction of wt GrpA-Flag rescued both SDF-2 levels and spore viability (Table 1). Therefore we can conclude that

GRASP is directly responsible for the unconventional secretion of AcbA during the terminal differentiation of spore cells during *Dictyostelium* development.

To test whether GRASP binds directly to AcbA during development, cells over expressing GrpA-Flag were allowed to develop and fruiting bodies were collected at 20, 22 and 24 hrs of development. Cells were lysed and GrpA-Flag was immunoprecipitated with an anti-Flag antibody conjugated to agarose beads. Samples were analyzed on a 10-20% gel as described above and stained for AcbA. Although we were able to immunoprecipitate GrpA-Flag, AcbA failed to co-precipitate (data not shown). This suggests that GRASP, although essential for its secretion, does not appear to directly bind AcbA during *Dictyostelium* development.

The unconventional secretion of AcbA is not through ABC membrane transporters

A number of routes have been proposed for the release of proteins lacking a signal sequence (Nickel, 2005). These include release through membrane (ABC) transporters, encapsulation of cargo into vesicles that ultimately fuse with the cell surface, as well as other unidentified schemes. 68 ABC transporter have been identified in *Dictyostelium* (Anjard and Loomis, 2002). It has been reported that pharmacological inhibition of the ABC transporter/TagC blocks the generation of SDF-2 as a result of lack of the protease activity (Anjard and Loomis, 2006; Good et al., 2003). To test the involvement of ABC transporters in the release of AcbA we primed KP cells with low levels of GABA and measured the release of AcbA in the

presence or absence of pharmacological inhibitors (Table 2). To measure the activity of released AcbA we made use of the fact that it contains trypsin sensitive sites where cleavage produces SDF-2. Our results show that in the presence of ABC transport inhibitors, AcbA is released but not converted into SDF-2 because of the inactivation of the TagC mediated proteolysis (Table 2). These results suggest that unconventional secretion of AcbA is not through an ABC transporter in *Dictyostelium* (Figure 4).

Table 2-2: Effect of ABC transporter inhibitors on AcbA release

Developed KP cells were treated in the absence or presence vanadate, verapamil or corticosterone for 1 hr before addition of 10 nM GABA. Ten minutes later 100µl of supernatant was collected and incubated with 1µl of trypsin (5 mg/ml) to convert AcbA into SDF-2 independent of the endogenous TagC protease. All experiments were repeated at least 2 times. As a control, we tested the ability of the ABC Transporter fusion protease TagC, whose proteolysis of AcbA is dependent on ABC activity, to produce SDF-2. 1nM recombinant AcbA was added to the media after the induction by GABA as described above. After an incubation of one hour at room temperature, the supernatants were tested for the presence of SDF-2 by serial dilution on fresh KP test cells. We conclude that the release of AcbA is independent of the general activity of ABC transporters.

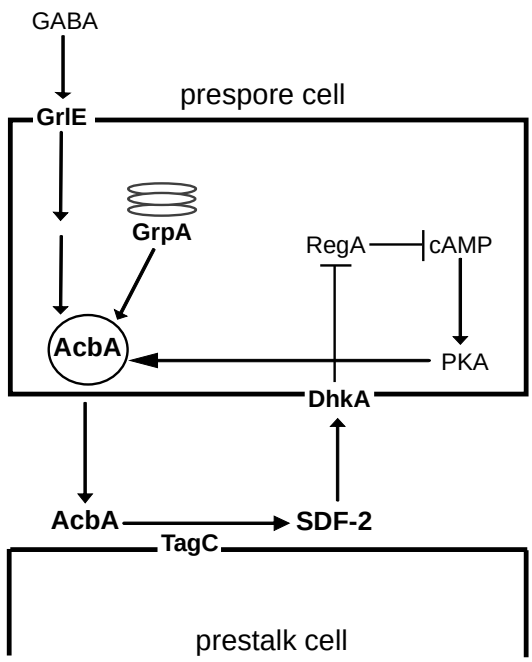
Incubations	AcbA released	TagC activity
None	<10 units	<10 units
10 nM GABA	$5x10^3$-10^4 units	>10^4 units
10 nM GABA + 2 mM vanadate	$5x10^3$-10^4 units	<10 units
10 nM GABA + 100 µM verapamil	$5x10^3$-10^4 units	<10 units
10 nM GABA + 200 µM corticosterone	$5x10^3$-10^4 units	<10 units

Figure 2-4: Known components of AcbA release from prespore cells and the possible routes.

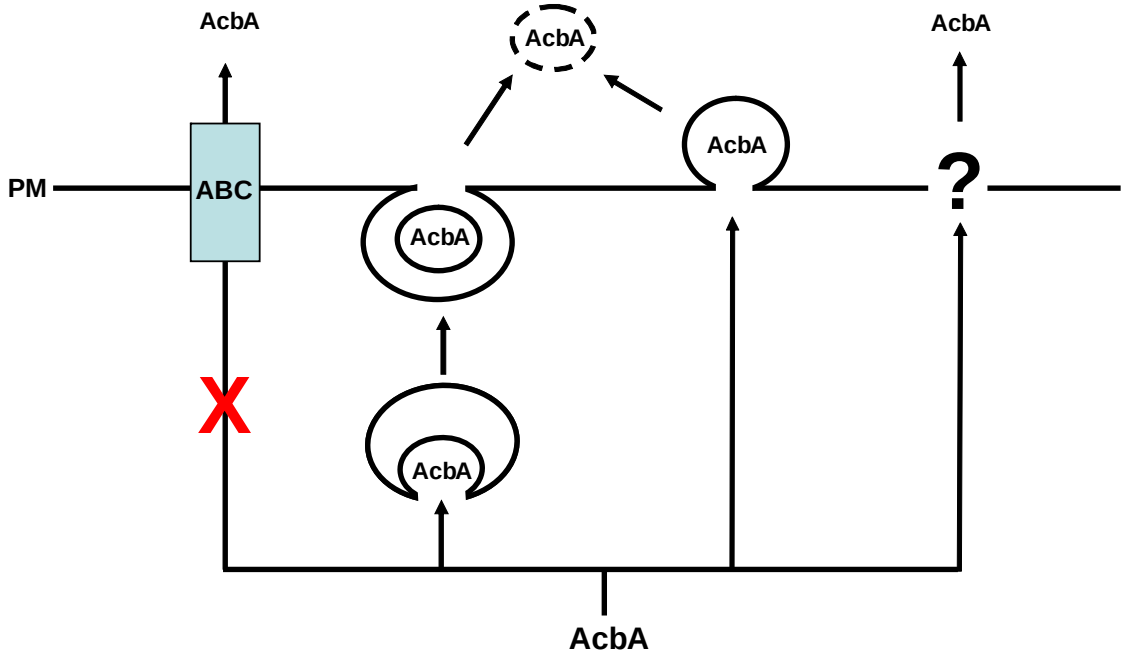
(A) The terminal differentiation of spore cells requires the production and signaled release of AcbA, proteolysis of AcbA by the stalk specific TagC to generate SDF-2, and binding of SDF-2 to the histidine kinase receptor, DhkA. DhkA activates a feedback loop by inhibiting the cAMP phosphodiesterase, RegA, to promote further AcbA secretion and subsequent spore encapsulation. Independently of DhkA, GABA can stimulate the release of AcbA through its receptor GrlE.

(B) A number of routes for the unconventional secretion have been described thus far (Nickel, 2005). The unconventional secretion of AcbA may involve the use of vesicular or non-vesicular routes. Based on our experimental findings we suggest that the unconventional secretion of AcbA is not via ABC transporters.

A)


GABA
prespore cell
GrlE
GrpA
RegA
cAMP
AcbA
PKA
DhkA
AcbA
SDF-2
TagC
prestalk cell

B)


AcbA
AcbA
AcbA
PM
ABC
AcbA
?
AcbA
AcbA

Since the GRASP proteins are well conserved, we decided to test whether GRASP is required for unconventional secretion of a-factor in *S. cerevisiae*. The unconventional secretion of a-factor through the ABC transporter Ste6p requires Ste14p (Huyer et al., 2006). This is necessary for the growth arrest and proper mating of MATa and MATα strains. Therefore, quantitative bioassays (halo assays) were used to monitor a-factor secretion by pheromone-induced growth arrest (Sprague, 1991). A lawn of MATα sst2 tester cells was grown on YPD plates. These tester strains are sensitive to the presence of a-factor causing the cells to undergo a G1 arrest. The G1 arrest appears as a clear zone termed a halo around a-factor secreting cells. MATa cells, which secrete a-factor, were spotted at three different concentrations onto the MATα tester lawns. Our findings revealed that after 48h, wild type yeast secreting a-factor resulted in the formation of a halo around the spotted MATa cells. As a negative control, we tested the *Ste14p* mutant strain, which in unable to secrete a-factor. As expected, MATa cells lacking Ste14p did not form the halo. Conversely, the GRASP (Grh1) deleted MATa cells formed a halo like their wild type counterparts (Figure 5). These results reveal that Grh1 in *S. cerevisiae* is not involved in the secretion of a-factor via the membrane transporter. Taken together our findings suggest that GRASP dependent release of proteins lacking a signal sequence is not via the cell surface ABC transporters.

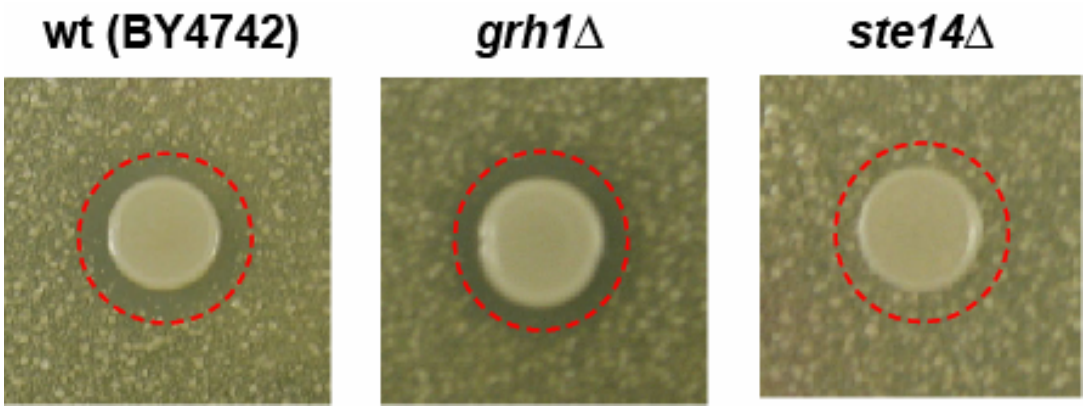


Figure 2-5: *S. cerevisiae* GRASP (Grh1p) is not involved in secretion of signal sequence lacking a-factor via its ABC transporter.

Quantitative bioassays (halo assays) were used to monitor a-factor secretion by pheromone-induced growth arrest. Cells were grown in rich culture media (YPD). MATα sst2 tester cells were top-spread onto YPD plates and MATa cells were spotted onto the MATα tester lawns. The relative ability of MATa cells to secrete a-factor was determined by comparing halo diameters in the lawn of MATα sst2 tester cells (dotted red circle). The MATa, *ste14*, *grh1* mutant cells and isogenic wild type control cells (BY4741) were tested side by side using three separate dilutions of which one spot of each is shown above.

Discussion

Our findings reveal that GrpA is essential for differentiation of spore cells during development through its direct requirement in the unconventional secretion of AcbA. AcbA belongs to a group of proteins that are secreted from cells yet lack a signal sequence essential for targeting to the conventional ER-Golgi secretion pathway. Among these proteins are the fibroblast growth factors FGF1 and FGF2, the inflammatory cytokine interleukin 1ß, macrophage migration inhibitory factor (MIF), and a-factor in *S. cerevisiae* (Flieger et al., 2003; Nickel, 2005; Schafer et al., 2004; Seelenmeyer et al., 2005; Sprague, 1991; Stegmayer et al., 2005). Similar to AcbA release in *Dictyostelium*, the secretion of these components is a regulated process. Unconventional secretion routes can be independent of each other and may include either vesicular or non-vesicular pathways (Nickel, 2005). Very little is known about AcbA release, but we have shown that the inhibition of ABC transporter activity does not block the secretion of AcbA from prespore cells. Moreover, deletion of the GRASP gene (*grh1*) in *S. cerevisiae* does not affect the unconventional secretion of a-factor from its ABC transporter. We therefore suggest that GRASP is not involved in secretion by an ABC transporter mediated pathway. Whether GRASP mediated secretion of AcbA involves a mechanism akin to formation of an autophagosome, direct flipping of the protein across the plasma membrane or a yet to be identified mode of release from the cells are now possibilities to explore.

GRASP Function: conserved or cell type specific?

The GRASP proteins are localized to Golgi membranes in all eukaryotes, from *Plasmodium* to mammals (Barr et al., 1997; Behnia et al., 2007; Kondylis et al., 2005; Shorter et al., 1999; Struck et al., 2005). These proteins have mainly been studied in the context of Golgi structure, general protein secretion, and in Golgi fragmentation during mitosis in mammalian cells. But, as mentioned above, its function *in vivo* with regards to these processes remains unclear. Our result show that GRASP is essential for the unconventional secretion of AcbA during *Dictyostelium* cellular development. While this is the first definitive role of GRASP *in vivo*, our findings raise an obvious question. Is GRASP involved in unconventional protein secretion in [all] eukaryotes or does its function vary depending upon the cell type? In order to resolve this issue we first need to identify the role of GRASPs in other cell types and organisms. Once this function is known, its mechanism of action can be deciphered. Furthermore, there are two GRASP proteins in mammalian cells; are both involved in unconventional secretion, and are their functions developmentally regulated? As mentioned earlier, the N-terminal region of GRASPs are highly homologous. The C-terminal regions are diverse. The mammalian GRASPs have a longer C-terminal region, enriched in Ser/Thr and are a substrate of mitotic kinases (Feinstein and Linstedt, 2007; Preisinger et al., 2005; Wang et al., 2005; Yoshimura et al., 2005). The C-terminal of *Dictyostelium* GRASP is truncated and enriched in glutamine. It is therefore important to test whether the mammalian GRASPs are also involved in unconventional secretion, which of the two forms closely resemble the *Dictyostelium* GRASP functionally, and whether these forms are interchangeable.

AcbA is highly similar to the mammalian protein ACBP, which is released by glial cells and processed into the neuropetide DBI (benzodiazepine binding inhibitor). DBI binds to GABA-A ionotrophic receptor on the post-synaptic neurons and regulates the effects of GABA (Guidotti et al., 1983). The mechanism of ACBP secretion (lacking a signal sequence) is not known. Sequence comparison reveals that GrpA is relatively more homologous to GRASP55 than GRASP65. We predict that GRASP proteins, especially GRASP55, in neurons are likely involved in secretion of ACBP. Therefore, further dissection of the role of GRASP in unconventional secretion will not only help understand the processes of angiogenesis, immune reaction but also in the development of a drug target for various neuropsychiatric ailments.

Golgi membranes and their new physiological functions

The role of Golgi membranes in intracellular sorting and transport of proteins has long been confirmed (Farquhar and Palade, 1981). More recently however, it has been shown that its organization regulates progression of cells into mitosis; its fragmentation during mitosis releases the attached protein ACBD3 to regulate cell fate; and our new findings reveal that GRASP regulates the unconventional secretion of proteins such as AcbA in a development specific manner (Colanzi et al., 2007; Feinstein and Linstedt, 2007; Sutterlin et al., 2005; Zhou et al., 2007). Thus Golgi membranes, through their localization, organization and protein composition regulate cellular functions in addition to the sorting and transport of conventionally secreted proteins. A better understanding of the organizational complexities of Golgi membranes will help reveal insights into their new and important functions.

Experimental Procedures

Disruption of *grpA* by Blasticidin S gene disruption

The GRASP gene was identified using the PDZ-2 domain of rat GRASP55 as the BLAST query in Dictybase. The UTR region was very A-T rich and unsuitable for PCR. Primers were designed to clone regions of the gene to serve as the homologous recombination sites for the gene disruption. The first 400kb and last 400kb regions of the gene were cloned using the following primers; 5'-GGCCGAATTCATGGGACAACAACAATCACAAAG-3', 5'-TTATGGATCCGAAGTTCTGCTTCATGTGCTGG-3' primers were used to clone the 5' 400bp region of the gene. 5'-TTATGGATCCGGTAGTTTAGGTTGTGATATTGG-3', 5'-GGCCTCTAGATTACAATTGAACTTTACTAAACTGG-3' primers were used to clone the 3' 400bp region. The PCR products were inserted together into the pBSII (KS+) transport vector (Stratagene). Blasticidin was excised from the pBSR19 vector using BamHI. This digest was inserted between the 5' and 3' flanking regions of the *grpA* gene using the BamHI site. Correct orientation of the Blasticidin insert was confirmed by restriction digest and sequencing. The GrpA gene disruption construct was linearized by digestion before transformation. Transformation and selection for Blasticidin S resistance were carried out as previously described (Shaulsky and Loomis, 1996). Resistant clones were screened by PCR, using the 5' and 3' primers of grpA, 5'-GGCCGAATTCATGGGACAACAACAATCACAAAG-3', -GGCCTCTAGATTACAATTGAACTTTACTAAACTGG-3'

followed by Southern blot analysis of genomic DNA. Southern blots of restriction digested genomic DNA was also performed (Vollrath et al., 1988). Each selection process was based on an upward band shift of 1.6kb indicating the presence of the Blasticidin gene within the GRASP locus.

Growth Curve and doubling time calculation

AX4 and *grpA*- strains were grown in flasks starting with $1x10^6$ cells/ml in HL5 media. Cells were collected and counted each succeeding day until the cell density became static. Doubling time was calculated using the following equation: T_2= (t ln2)/ln (N/N_0), where t is the elapsed time in hrs, N is the current cell count and N_0 is the initial cell count.

Overexpression of wt GrpA in *grpA*- cells

The 5' and 3' grpA flanking primers were used to clone grpA from genomic AX4 DNA; 5'-GGCCGAATTCATGGGACAACAACAATCACAAAG-3', 5'-GGCCTCTAGATTACAATTGAACTTTACTAAACTGG-3'. The PCR product was inserted into pBSII (Stratagene) for sequencing then transferred to pA15. Primers were designed to insert a Flag tag at the 3' end of the gene using the MunI restriction site at the end of the native *grpA* sequence. Colonies were picked and screened for the expression of grpA-Flag by Western Blot analysis.

Spore Viability assay, SDF measurements

The KP strain is a previously described derivative of strain Ax2 over-expressing PKA under the control of its endogenous promoter (Anjard et al., 1992). This strain was used for the sporogenous bio-assays as described in Anjard et al., (1998). Vegetative cells were harvested during exponential growth and resuspended in 1ml buffer (20mM MES pH 6.2, 20mM NaCl, 20mM KCl, 1mM $MgSO_4$, 1mM $CaCl_2$). $4.5x10^4$ KP cells were added to 12.5 ml buffer containing 5 mM camp. 500 µl aliquots were distributed in each well of a 24 well dish, resulting into a density of $2x10^3$ cells/cm^2. The cells were incubated overnight at 23°C before addition of test samples or defined products. Induction of spore formation was scored by counting spores and undifferentiated cells one hour (SDF-2) or two hours (SDF-1) after addition of the samples. SDF-1 and SDF-2 activity was determined by serial dilution of the sample before addition to KP cells. One unit corresponds to the lowest dilution giving full induction of spore formation. The number of units in the sample were standardize to 10^3 producing cells when applicable.

To test SDF production in fruiting bodies, a total of 10^7 cells of each strain or mixture was developed on a filter saturated with PDF (20mM phosphate buffer pH 6.5, 20mM KCl, 1.2mM $MgSO_4$) for 24h as previously described (Anjard and Loomis, 2005). Fruiting bodies were dissociated in 1ml buffer and centrifuged at high speed. Aliquots of the supernatant were harvested to measure the level of SDF-1 and SDF-2 produced while the pellet was kept to determine spore viability. Supernatants were incubated with either cation (C-50) or anion (A-25) exchange resins to trap SDF-1 and SDF-2 respectively before testing on the KP cells.

To measure AcbA inside cells, 10^7 cells of each strain were developed on a filter saturated with PDF (20mM phosphate buffer pH 6.5, 20mM KCl, 1.2mM $MgSO_4$) for 22 hours and then dissociated in 1ml buffer containing 0.5% triton X-100. The cells were lysed and incubated with protease inhibitors. 25µl of trypsin (5 mg/ml) was added to the sample and incubated at 37°C for over 3h. Trypsin was inactivated by incubation at 95°C for 15 minutes and the lysates were briefly spun to remove insoluble material. The supernatant was incubated with 50µl anion (A-25) exchange resins which binds SDF-2. The resin was washed twice with 1 ml buffer and SDF-2 was eluted in 1 ml buffer containing 1M NaCl. The amount of SDF-2 was then determined by serial dilution as described before with developed KP cells.

Spore viability was determined by resuspending the fruiting body pellets in 1ml PDF containing 1% tritonX-100. After 5 minutes of incubation, the detergent resistant spores were collected by centrifugation, resuspended in 1ml buffer and counted. Roughly 50 spores were plated on SM-plates together with a *K. aerogenes* suspension. Two plates were made for each sample and at least three experiments were performed for each strain and synergy assay. The number of plaques was counted after incubation for 4 days at room temperature.

Golvesin-GFP Immunofluorescence and Western Blot analyses

Cells were grown in HL-5 medium supplemented with 5µg/ml G418. For immunofluorescence, cells were plated on poly-lysine treated glass coverslips for 15min. Cells were fixed in -20ºC Methanol for 15min, washed with Hoechst 33342 in PBS and mounted for microscopy. For Western blotting, cells were plated for

development, as described above, and fruiting bodies were collected at various time points. To monitor AcbA levels, cells were resuspended in 10mM Tris-Cl pH 7.4, 1mM EDTA, 1mM PMSF, plus a protease inhibitor cocktail of aprotinin, leupeptin, pepstatin. Cells were lysed by passing through a Nuclepore® Track-Etch membrane (Whatman). The lysate was centrifuged at 12K for 10min; the supernatants were normalized for equal protein loading and analyzed on a 10-20% Tris-tricine gradient gel (BioRad). Samples were transferred to a PVDF membrane, fixed in 1% glutaraldehyde and immunoblotted with anti-AcbA. For Flag immunoprecipitation, fruiting bodies were collected at 0, 20, 22 and 24 hrs of development, cells were lysed as described above, proteins levels were normalized and loaded onto anti-Flag M2 agarose beads (Sigma). The lysate-bead slurry incubated at 4ºC for 2hrs. The beads were centrifuged at low speed and washed with lysis buffer. The beads were boiled in sample buffer including SDS and were analyzed on a 10-20% Tris-tricine gradient gel (BioRad) and processed as described above. The transfer was immunoblotted with both anti-Flag M2 antibody (Sigma) and anti-AcbA. For AcbA membrane fractionation, cell pellets were lysed as mentioned above. Cell lysis was centrifuged at 12K for 10min. The supernatant was transferred to a new tube and centrifuged at 55k for 1hr. Protein concentrations were normalized for equal loading. The 55K pellet (P) and the supernatant (S) were analyzed on a 10-20% Tris-tricine gel, transferred to PVDF and immunoblotted with anti-AcbA.

ABC transport activity and AcbA release

Developed KP cells were treated in the absence or presence of vanadate, verapamil or corticosterone for 1hr before addition of 10 nM GABA for 10 minutes. Supernatants were then collected and incubated with 1µl of trypsin (5 mg/ml) to convert AcbA into SDF-2 independent of TagC. After 2 hrs at 37ºC samples were tested for SDF-2 activity at various dilutions on KP cells (Anjard and Loomis, 2006). The activity of TagC in the presence of the ABC transporter inhibitors were carried out in parallel. The addition of 1nM recombinant AcbA was added to the media after the induction by GABA. After an incubation of 1h at room temperature, the supernatants were tested for the presence of SDF-2 by serial dilution on fresh KP test cells.

Lysosomal Enzyme Assay

Vegetative AX4 and *grpA*- cells were grown separately in HL-5 medium and collected, centrifuged at low speed and washed in PDF. 10^7cells/ml was shaken in PDF at 23ºC to activate lysosomal enzyme synthesis and release. After 4 hrs, the cells were washed and continued shaking in PDF for an additional 2 hrs. AX4 and *grpA*- cells were centrifuged at low speed and the supernatant was further centrifuged at high speed (12K for 1min) to remove possible cell debris. The supernatant of each sample was tested for activity using p-nitrophenyl substrates to their corresponding lysosomal hydrolases. 100µl of each sample was added to 500µl of 5mM sodium acetate pH 5, followed by either 80µl of 50mM p-nitrophenyl-N-acetyl glucoasminidine or p-nitrophenyl-α-D-mannopyranoside. Samples were incubated at 35ºC for 0, 30, and 60

minutes. The reactions were stopped with the addition of 700µl of 1M Na_2CO_3 and the absorbency was measured at 420nm. The absorbency of each sample was normalized with total protein concentration of each sample and plotted as specific activity over time. n=3.

Secretion of Apr and western blotting

Wild type and *grpA*- at 10^6cells/ml were allowed to shake at room temperature. Samples were collected and centrifuged at low speed. The supernatant was centrifuged at high speed to remove potential cell debris. Sample buffer was added to the high speed supernatant and analyzed by western blotting with anti-Apr antibody at 0.4ug/ml in blocking buffer.

Pheromone Response Assay

Quantitative bioassays (halo assays) were used to monitor a-factor secretion by pheromone-induced growth arrest (Sprague, 1991). Cells were grown in rich culture media (YPD). MATα sst2 tester cells (provided by Dr. Jeremy Thorner, UC-Berkeley) were top-spread onto YPD plates. Next, MATa cells were spotted onto the MATα tester lawns. The relative ability of MATa cells to secrete a-factor was determined by comparing halo diameters in the lawn of MATα sst2 tester cells. The MATa, *ste14*, *grh1* mutant cells and isogenic wild type control cells (BY4741) were obtained from Resgen.

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Chapter III

Sequential phosphorylation of GRASP55 is required for Golgi fragmentation and entry of cells into mitosis.

Abstract

Mitotic Golgi fragmentation in mammalian cells is an essential step before the entry of cells into mitosis. We have found, using an *in vitro* reconstitution assay that GRASP55 plays an important regulatory role in this process. Addition of GRASP55-specific peptides is capable of inhibiting both Golgi fragmentation and mitotic entry. Phosphopeptide mapping of full length GRASP55 revealed two mitotic specific sites within the C-terminus of the protein. Only when both of these sites are mutated to mimic phosphorylated residues is the block in Golgi fragmentation and mitotic entry rescued. Interestingly, depletion of GRASP55 by siRNA shows no defect in Golgi structure, protein secretion or cell growth. Our results suggest that sequential phosphorylation of GRASP55 is required to break Golgi membranes away from its perinuclear positioning, an essential step for the entry of cells into mitosis.

Introduction

In mammalian cells, the Golgi apparatus consists of 40-100 individual Golgi stacks, which are connected by membrane tubules to form a ribbon-like structure that is adjacent to the nucleus. The perinuclear organization is unique in mammalian cells, as Golgi membranes in plants, yeast, and Drosophila, although may be stacked, are dispersed throughout the cytosol (Nebenfuhr et al., 2000; Preuss et al., 1992; Ripoche et al., 1994). The positioning of the mammalian Golgi complex, which is dependent on microtubules, has a physiological relevance. Inhibiting the fragmentation and dispersal of Golgi membranes from its perinuclear position prevents cells from entering mitosis (Preisinger et al., 2005; Sutterlin et al., 2001; Yoshimura et al., 2005). Therefore, the position and organization of the mammalian Golgi apparatus defines a novel cell-cycle specific checkpoint.

To reveal the mechanism of the mitosis specific Golgi fragmentation we have reconstituted this process *in vitro*. Using this assay we have previously identified the involvement of polo-like kinase (Plk) and the Raf1-MEK1 pathway (Acharya et al., 1998; Colanzi et al., 2000; Colanzi et al., 2003b; Sutterlin et al., 2001). Cdc2 kinase is also involved in Golgi fragmentation *in vitro* (Kano et al., 2000; Lowe et al., 1998; Wang et al., 2005; Yoshimura et al., 2005). Both polo-like kinase and cdc2 phosphorylate the Golgi membrane associated protein GRASP65 during mitosis (Preisinger et al., 2005; Wang et al., 2005; Wang et al., 2003). Similarly, the MAP-Kinase, ERK2, phosphorylates GRASP55 during mitosis both *in vitro* and *in vivo* (Jesch et al., 2001). Recently a new isoform of ERK1, called ERK1c, was found to localize to the Golgi membranes and shown to be required for MEK1 dependent Golgi

fragmentation (Aebersold et al., 2004). Thus, the pathways, Raf1-MEK1-ERK (2 or 1c)-GRASP55 along with Plk (Cdc2)-GRASP65 are the proposed mechanism for fragmentation of the Golgi apparatus during mitosis.

In vitro analysis revealed a role for GRASP65 and GRASP55 in the stacking of Golgi cisternae (Barr et al., 1997; Shorter et al., 1999; Wang et al., 2003). Further studies of GRASP65 have suggested functions contrary to the biochemical findings. For instance, depletion of GRASP65 by siRNA has no effect on stacking of Golgi cisternae, or protein secretion, although it has been suggested that the lateral connections between Golgi stacks may be disrupted (Puthenveedu et al., 2006; Sutterlin et al., 2005). Interestingly, cells depleted of GRASP65 results in multiple mitotic spindles. As a result, the GRASP65-depleted cells arrest in premetaphase and undergo apoptosis (Sutterlin et al., 2005). Many reports have indicated that GRASP65 phosphorylation through its C-terminal domain is responsible for fragmenting Golgi membranes during mitosis (Barr et al., 1997; Puthenveedu et al., 2006; Wang et al., 2005; Wang et al., 2003; Yoshimura et al., 2005). Furthermore, inhibiting the phosphorylation of GRASP65 inhibits Golgi fragmentation and hence mitotic entry which originally introduced the idea of a Golgi-specific cell cycle checkpoint (Sutterlin et al., 2002; Wang et al., 2003; Yoshimura et al., 2005).

The emerging role of GRASP65 in Golgi fragmentation, mitotic entry and spindle dynamics, prompted us to examine the significance of its homolog GRASP55. We have found that GRASP55 is sequentially phosphorylated within its C-terminal region and these phosphorylations are required for mitotic Golgi membrane fragmentation allowing for entry of cells into mitosis. This work furthers our

understanding and the continuing mechanism of how the position of the Golgi influences mitotic progression in mammalian cells.

Results

Cloning of GRASP55 and the production of a GRASP55-specific antibody

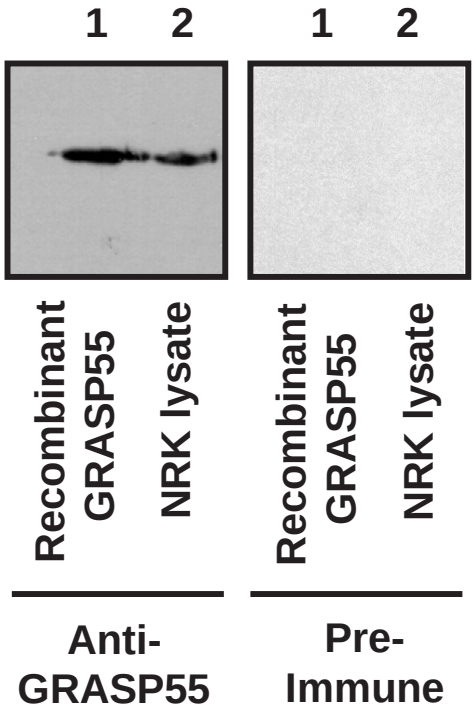
The GRASP55 gene was cloned from a rat C6-glioma cDNA library and transformed into bacterial cells for recombinant protein expression. The full length epitope tagged protein was used to generate polyclonal antiserum. The crude anti-GRASP55 serum was affinity-purified on a GRASP55 antigen column. To test the specificity of the affinity-purified antibody, we performed Western blot analysis on both purified recombinant GRASP55 and total Normal Rat Kidney (NRK) cell lysate. The GRASP55 antiserum, in contrast to the pre-immune IgG serum, recognized the recombinant protein as well as a single polypeptide of 55kDa in the total NRK cell lysate (Figure 1A). Immunofluorescence microscopy of NRK cells with the affinity purified antibody, but not the pre-immune IgG, revealed staining of the Golgi apparatus (Figure 1B). Based on this analysis we believe that the anti-GRASP55 antibody recognizes specifically GRASP55 and is therefore a suitable reagent for further analyses.

Figure 3-1: The GRASP55 antibody recognizes a single-polypeptide in NRK cell extracts.

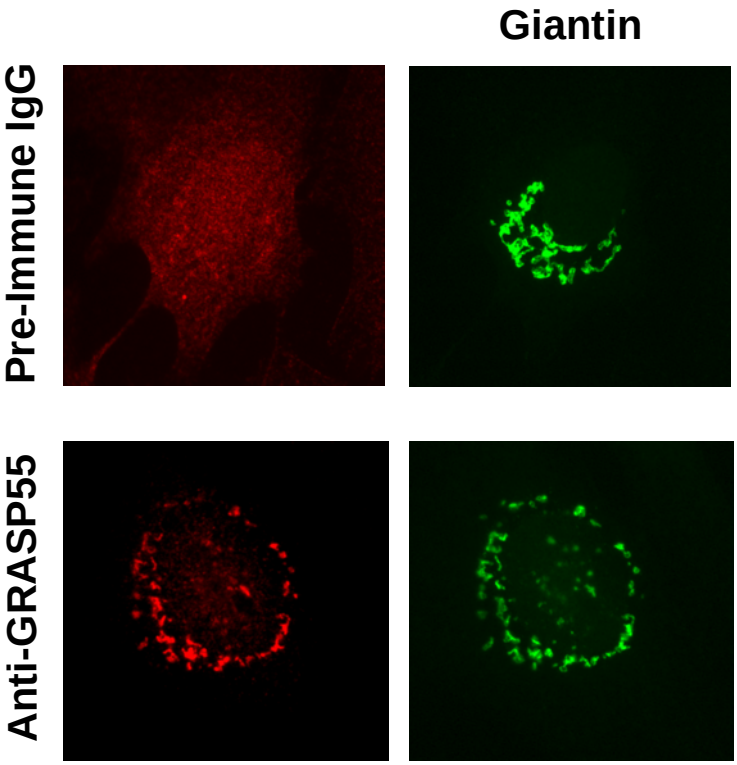
(A) The GRASP55 cloned from a rat C6-glioma cDNA library, subcloned into the BamH1/HindIII sites of pQE30 plasmid (Qiagen). The epitope-tagged full-length GRASP55 (N-terminal 6 Histidine tag) was expressed in bacteria, and the recombinant protein was purified by affinity chromatograph on a Nickel-agarose column. The pure protein was used for immunization of rabbits. Pre-immune serum and affinity-purified antiserum were tested for specificity by Western blotting on either purified recombinant protein (lane1) or total NRK lysate (lane 2).

(B) Pre-immune or affinity-purified anti-GRASP55 antibodies were tested by immunofluorescence microscopy of NRK cells. The pre-immune serum does not show any specific staining. The anti-GRASP55 antibody recognizes the Golgi apparatus as revealed by co-localization with a bona fide protein, Giantin.

A


1
2
1
2
Recombinant GRASP55
NRK lysate
Recombinant GRASP55
NRK lysate
Anti-GRASP55
Pre-Immune

B


Giantin
Pre-Immune IgG
Anti-GRASP55

GRASP55 is not required for Golgi structure or cell growth

HeLa cells were depleted of GRASP55 by transfection of two individual siRNAs (Figure 2A). This procedure resulted in roughly a 90% reduction in the levels of the GRASP55 protein three days after transfection, as determined by fluorescence microscopy and western blotting with an anti-GRASP55 antibody (Figure 2). Immunofluorescence microscopy of GRASP55-depleted cells revealed that Golgi membranes appear normally localized to the perinuclear region. It was previously reported that knockdown of GRASP65 resulted in a mitotic arrest and subsequent cell death due to aberrant spindle formation. In contrast, GRASP55 knockdown had no effect on either mitotic progression or cell viability (Figure 3). To further understand the morphology of the Golgi membranes in GRASP55 depleted cells, the siRNA experiment was repeated and cells were fixed in a glutaraldeyde solution and processed for electron microscopy. Electron microscopy revealed no changed in the architecture of Golgi membranes during GRASP55 depletion (Figure 4). We therefore conclude that depletion of GRASP55 has no effect on Golgi cisternal stacking or mitotic progression.

Figure 3-2: Depletion of GRASP55 by RNAi.

(A) The sequences of the human GRASP55 gene used for designing siRNA probes.

(B) HeLa cells were either transfected with control siRNA, GR55-1 siRNA, GR55-2 siRNA or both GR55-1 and GR55-2 siRNAs. After 72hrs, cells were processed for immunofluorescence and stained with anti-GRASP55, Giantin, and Hoechst 33342.

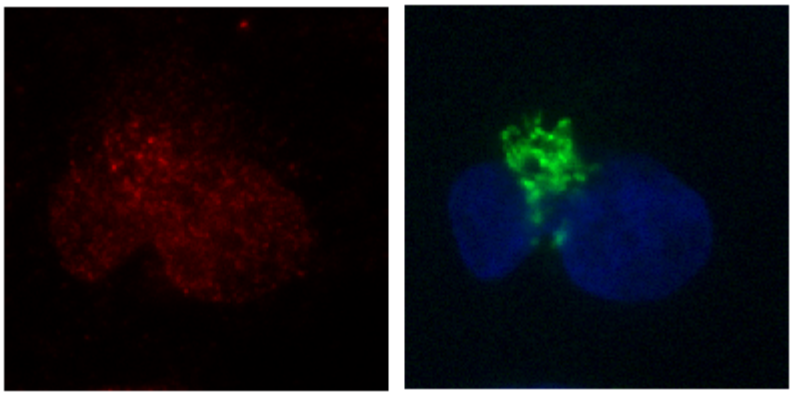
(C) Cells were transfected as before. After 72 hrs, the cells were lysed and their contents analyzed by SDS-PAGE followed by Western blot with antibodies against GRASP55 and β–actin as a loading control.

A)

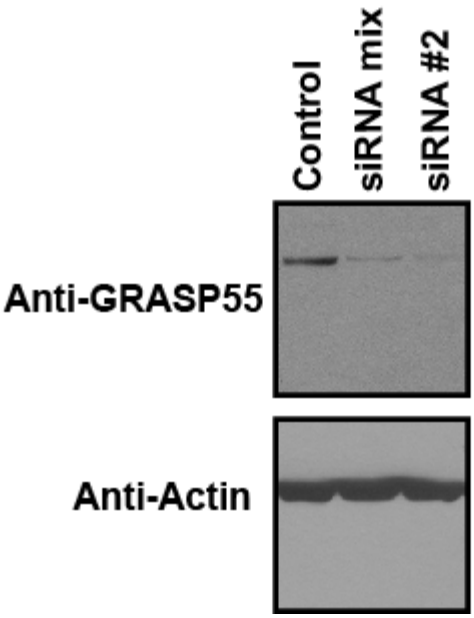
GR55-1: AAGAAGATCTATTCAGCCTTA

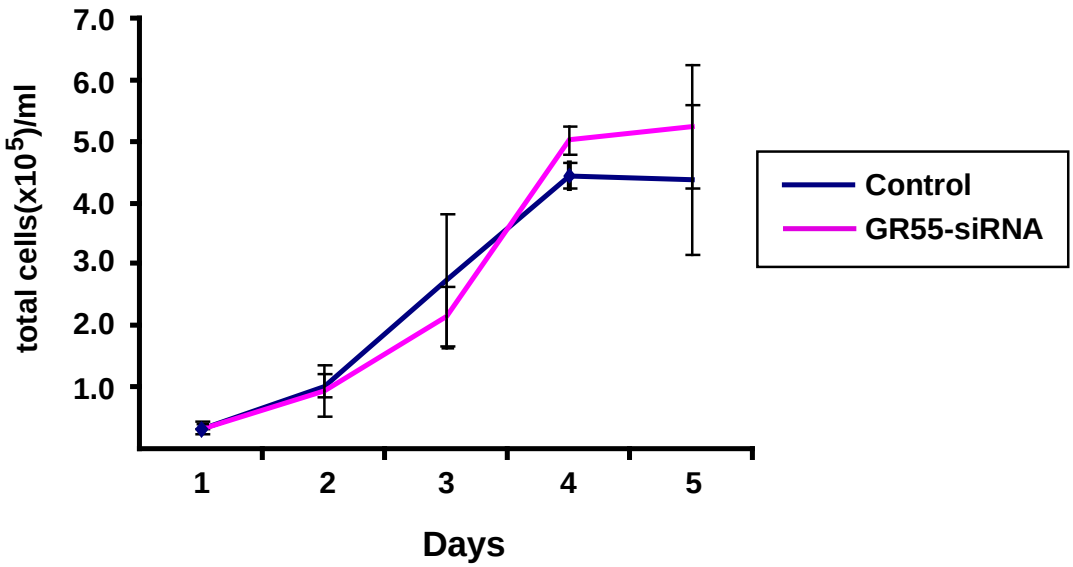
GR55-2: AACTATTCAGCCTTATCGAAA

B)



C)





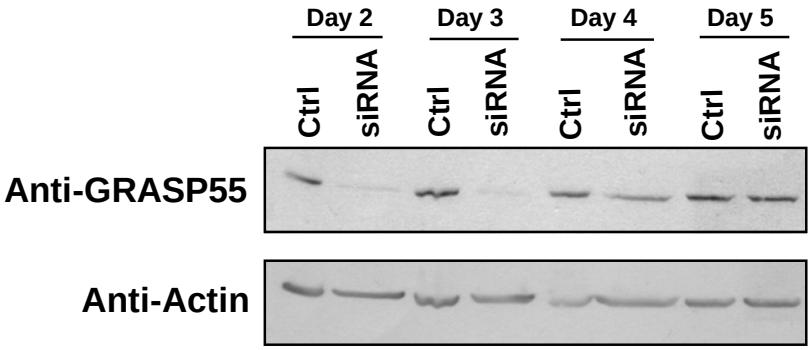


Figure 3-3. GRASP55 depletion does not effect cell growth and viability.

(A) HeLa cells were transfected with either control or GR55-2 siRNA. 24 hrs later, cells were replated on a 6-well dish at 10% density. After 24hrs for a five days total, cells were typsanized and counted, Experiments were repeated three times.

(B) HeLa cells collected from above were centrifuged, lysed and normalized for equal protein loading. Each sample was analysized on a 12% SDS-PAGE, transferred to Nitrocellose and stained for GRASP55 protein levels. β–actin was used as a loading control.

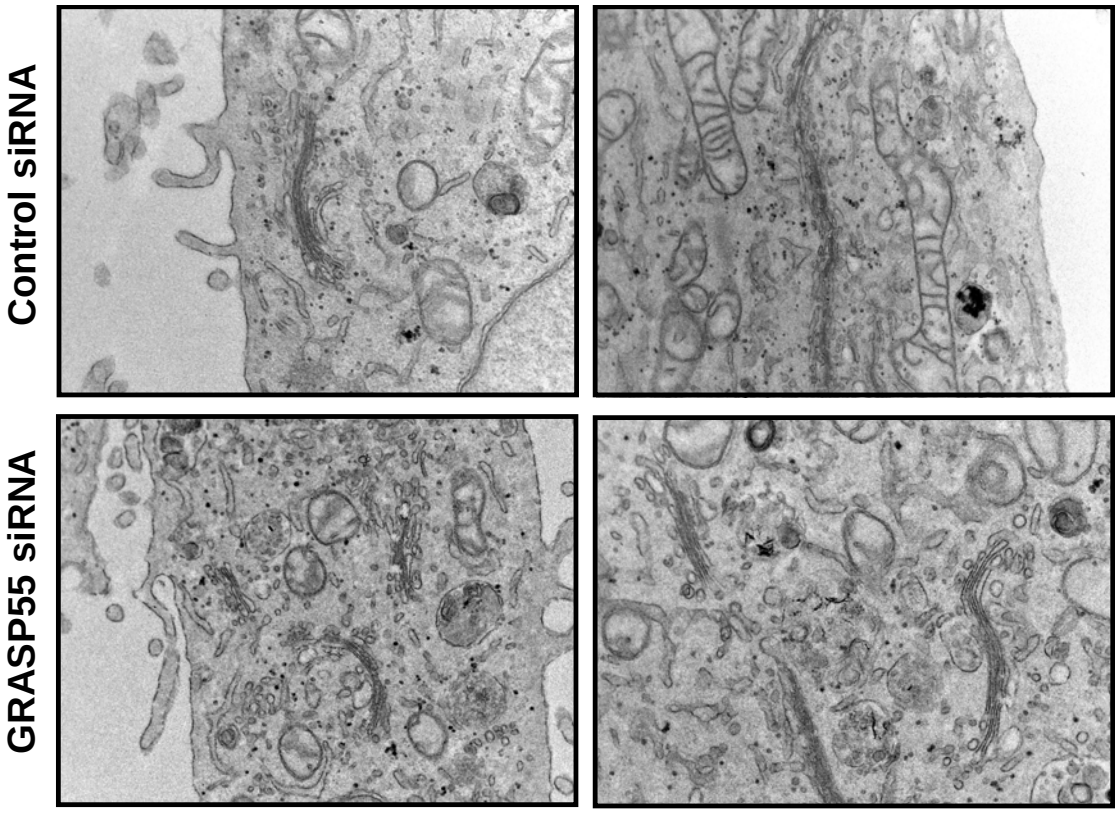


Figure 3-4: GRASP55 is not required for Golgi stacking

As before, HeLa cells were transfected with either control or GRASP55 siRNAs. After 72 hrs, cells were collected and fixed in 1% glutaraldehyde solution and processed for electron microscopy. Analysis reveals that Golgi cisternal stacking is not affected in the absence of GRASP55. Cisternal numbers range from 3-5 cisternae per Golgi stack

GRASP55 is not required for protein secretion of soluble and membrane bound cargo

The GRASP proteins have also been biochemically suggested to help in the tethering of vesicles during protein secretion. Since GRASP55 depletion still retained Golgi stacks and all Golgi markers tested had proper localization we next tested what role GRASP55 played in protein secretion. In order to analyze the effects of GRASP55 knockdown on protein secretion, we performed two separate transport experiments, one looking at the movement of the well characterized VSV-G protein and the other on our recently published transport assay using ss-HRP (Bard et al., 2006). For the ss-HRP assay, we used a mammalian expression plasmid encoding a fusion protein between the signal sequence (SS) of human growth hormone and the enzyme horseradish peroxidase (HRP). The encoded protein also contains a Flag epitope in its C-terminus and will therefore be referred to as SS-HRP-Flag. When synthesized, SS-HRP-Flag is translocated into the ER where its signal sequence is cleaved. The soluble HRP-Flag is transported along the secretory pathway and is eventually secreted into the medium. The release of HRP into the medium is quantitated using an HRP activity assay as described previously (Bard et al., 2006). 24 hours after transfection of the SS-HRP-Flag plasmid in HeLa cells, the measured HRP activity in the extracellular medium was found to be ~20-fold higher than that observed for non-transfected cells (data not shown). When this secretion assay was carried out in cells previously transfected with GRASP55 siRNAs there was no change in secreted HRP activity in the GRASP55-depleted cells when compared to

cells transfected with control siRNA (Figure 5A). We therefore conclude that GRASP55 is not required for the transport of soluble cargo outside the cell.

We next tested the role GRASP55 had on transport of transmembrane associated cargo by using the VSV-G protein. Both control and GRASP55-depleted cells were transfected with VSV-G, blocked at the nonpermissive temperature and then moved to the permissive temperature to measure transport. Transport of VSV-G was monitored using an antibody against the cell surface expression of the protein and measured against total VSVG-GFP. We once again found no loss of VSV-G at the cell surface in GRASP55 depleted cells along similar time points. We conclude that GRASP55 is not required the secretion of membrane bound proteins (Figure 5B).

These experiments are consistent with previous studies of mammalian GRASP65, as well as the *Drosophila*, *S. cerevisiae* and *Dictyostelium* GRASP homologs. From these combined reports we must conclude that the GRASP proteins, although associated with Golgi membranes, play no role in the stacking of Golgi cisternae nor the general transport of proteins through the secretory pathway (Behnia et al., 2007; Kinseth et al., 2007; Kondylis et al., 2005; Sutterlin et al., 2005).

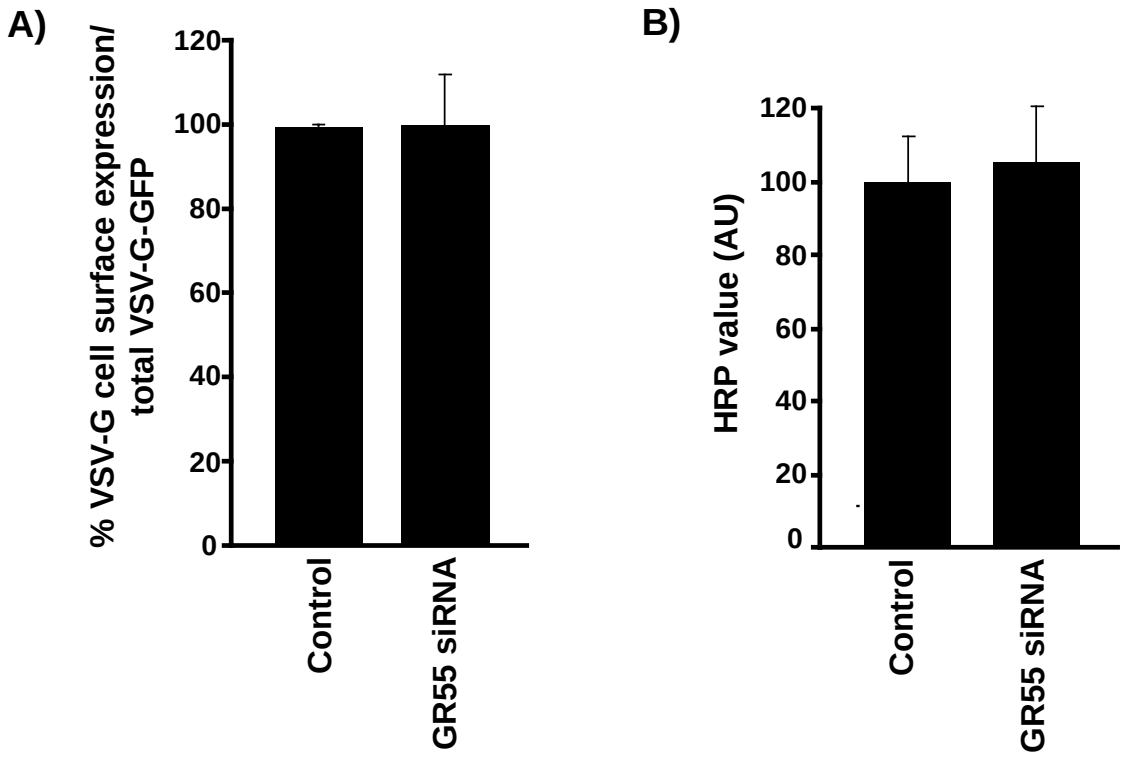


Figure 3-5: GRASP55 is not required for protein secretion.

(A) VSV-G surface expression was not affected in both control and siRNA transfected cells. Cell surfaceVSV-G was determined using the anti-VSV-G mAb 8G5F11, which is specific for the extracellular domain of VSV-G.

(B) The soluble cargo ss-HRP was tested as described previously (Bard, et al., 2006). Secreted HRP was assayed using ECL and found to be at comparable levels at all timepoints tested.

GRASP55 is involved in mitosis specific Golgi fragmentation

As mentioned earlier, the mammalian Golgi apparatus undergoes extensive fragmentation during mitosis. By immunofluorescence microscopy it has been shown that the Golgi apparatus begins to break in early prophase before Golgi membranes disperse away from the nucleus and fragment into vesicular/tubular elements (Shima et al., 1998; Shorter and Warren, 2002). Breakdown of the Golgi complex and removal from its perinuclear location is required for entry into mitosis and so we classify this as the first step in Golgi fragmentation (Colanzi et al., 2003a). Since GRASP65 is required for maintaining the lateral connections between Golgi stacks, it raises the possibility that GRASP55 may be involvement in the mitosis specific Golgi fragmentation (Feinstein and Linstedt, 2007; Puthenveedu et al., 2006). As reported previously, incubation of permeabilized NRK cells with mitotic cytosol and an ATP-regenerating system at 32°C caused fragmentation and dispersal of the Golgi membranes (Acharya et al., 1998). To test whether GRASP55 is involved in mitotic Golgi fragmentation, purified recombinant GRASP55 or an affinity purified anti-GRASP55 antibody was added to the assay reconstituting Golgi fragmentation. Addition of either the purified His-tagged, full length GRASP55 or the anti-GRASP55 antibody inhibited Golgi fragmentation as compared to the addition of purified pre-immune IgG or mitotic cytosol alone as controls (Figure 6). We propose that the addition of proteins to our semi-intact assay could act as a dominant negative reagent, sequestering kinases and/or additional factors required at the Golgi complex for proper fragmentation. Inhibition of Golgi fragmentation by GRASP55-specific reagents would therefore suggest it may plays a role in this process.

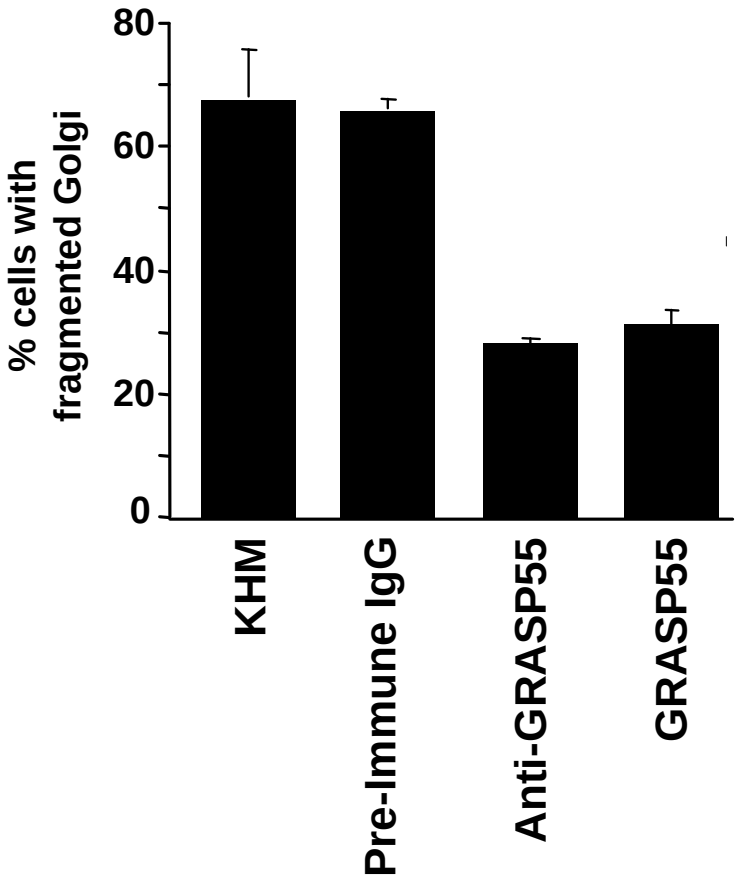


Figure 3-6: Anti-GRASP55 antibody and full-length GRASP55 inhibits mitosis-specific Golgi fragmentation in permeabilized cells.

NRK cells were grown on coverslips and treated with 2mM thymidine for 10-12hrs. The cells were subsequently permeabilized, washed with 1 M KCl-containing buffer, and incubated with 7mg/ml mitotic extract in the absence or presence of affinity purified pre-immune IgG, anti-GRASP55 antibody or purified recombinant GRASP55. After 60min incubation at 32°C, cells were fixed and processed for immunofluorescence using an anti-Giantin or anti-Mannosidase II antibody. The percentage of cells with fragmented Golgi after incubation of cells with mitotic extract containing either KHM buffer, preimmune serum, anti-GRASP55 antibody or recombinant GRASP55 is shown. The data represents the average of individual experiments (buffer, n=10, preimmune, n=2, anti-GRASP55, n=4, recombinant GRASP55, n=4)

To further understand the action by which the anti-GRASP55 antibody inhibits Golgi fragmentation, multiple regions of the protein were cloned, expressed and purified and tested for their ability to be recognized by the anti-GRASP55 antibody (Figure 7A). Western blotting analysis of the recombinant purified GRASP55 peptides reveals that the last 100 amino acids of the C-terminus (termed C100, aa354-454) is recognized by the anti-GRASP55 antibody, (Figure 7B). It should be noted that the antibody does not recognize the more conserved N-terminal region or the internal region of the protein (aa201-304). This suggests the C-terminal regions of GRASP55 may play a regulatory role of Golgi fragmentation during mitosis, consistent with findings of GRASP65 (Sutterlin et al., 2002; Wang et al., 2005; Yoshimura et al., 2005).

Since the GRASP55 specific antibody blocks Golgi fragmentation, we next tested whether the antibody binding region was the only motif required to inhibit Golgi fragmentation. We found that each recombinant C-terminal fragment of GRASP55, when added to the semi-intact assay, inhibited fragmentation similar to the full length protein where both BSA and recombinant His-tagged GFP protein had no effect on Golgi fragmentation (Figure 7C). To test whether GRASP55-C100 is both necessary and sufficient, the internal peptide not recognized by the GRASP55 specific antibody, termed [201-304] which correlates to the amino acids of the peptide, was tested for its ability to inhibit Golgi fragmentation in the semi-intact assay. This region was previously shown to contain two threonine residues potentially phosphorylated by ERK2 both *in vitro* and *in vivo* (Jesch et al., 2001). As shown, region [201-304] inhibited Golgi fragmentation to the same extent as C100 (Figure 7C). Therefore, it

appears that multiple regions within the C-terminus of GRASP55 (defined as the region after the two putative PDZ domains and denoted amino acids 201-454) are capable of inhibiting Golgi fragmentation.

The entire C-terminal region of GRASP55 is very proline rich, roughly 20% (49 Pro and 50 Ser/Thr in 254 amino acid long peptide) and has no identifiable protein domains or any homology to other proteins, including little homology with GRASP65. Similar results have been shown with GRASP65, where multiple regions within the C-terminus are capable of inhibiting Golgi fragmentation independent of each other. Both Plk and cdc2 phosphorylate GRASP65 and these sites are required for Golgi fragmentation both *in vitro* and *in vivo* (Preisinger et al., 2005; Sutterlin et al., 2001; Wang et al., 2005; Yoshimura et al., 2005). There is no homology between the C-termini of the GRASP proteins and therefore we suggest that independent mechanisms must exist for these homologs. We therefore considered which regions of GRASP55 are phosphorylated specifically during mitosis and could these phosphorylation sites be a requirement for the GRASP55 mediated Golgi fragmentation.

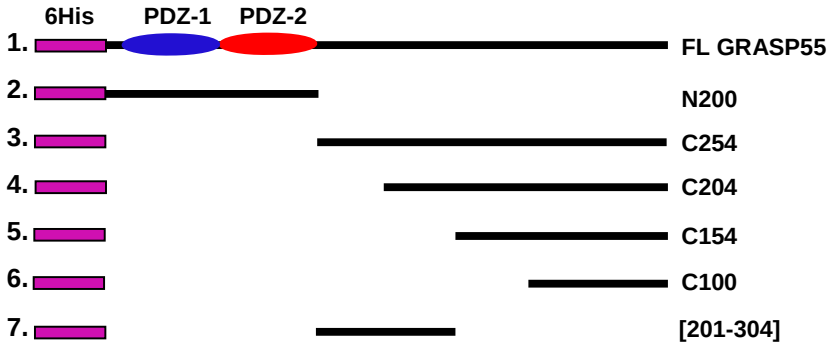
Figure 3-7: Anti-GRASP55 antibody recognizes the C-terminal domain of GRASP55.

(A) A schematic drawing of the recombinant full length GRASP55 protein and the C-terminal fragments, C254, C204, C150, C100, and [201-304] used in this study. All constructs contain an N-terminal 6 Histidine tag (6His). The two putative N-terminal PDZ domains are shown.

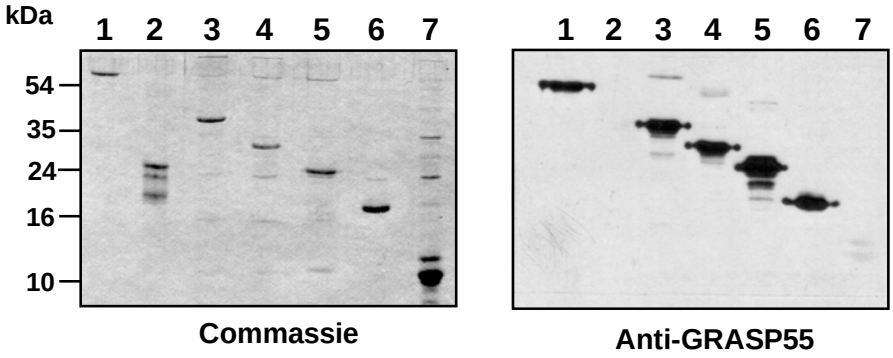
(B) Recombinant full-length GRASP55, the N-terminal fragment (N200 represents amino acids 1-200), C254 (amino acids 201-454), C204 (amino acids 251-354), C150 (amino acids 301-354), C100 (amino acids 351-454), and the internal peptide [201-304] (amino acids 201-304) were generated in bacteria and purified on a Nickel-agarose column. The recombinant proteins were analyized by SDS-PAGE and Western blotting using the affinity purified anti-GRASP55 antibody. A Coomassie stain of a sample gel was run to show total protein in each lane. Anti-GRASP55 antibody recognizes only the C-terminal regions of protein. Note the low affinity for the internal peptide [201-304].

(C) The recombinant C-terminal fragments C254, C204, C150, C100, [201-304], KHM buffer alone, or His-tagged GFP as a control, were incubated with mitotic extract and an ATP-regenerating system for 10min. The mixture was then added to permeabilized, salt washed NRK cells. After 60 min incubation at 32°C, cells were fixed and processed for immunofluorescence using anti-Giantin antibody to visualize the morphology of the Golgi membranes in four independent experiments as shown.

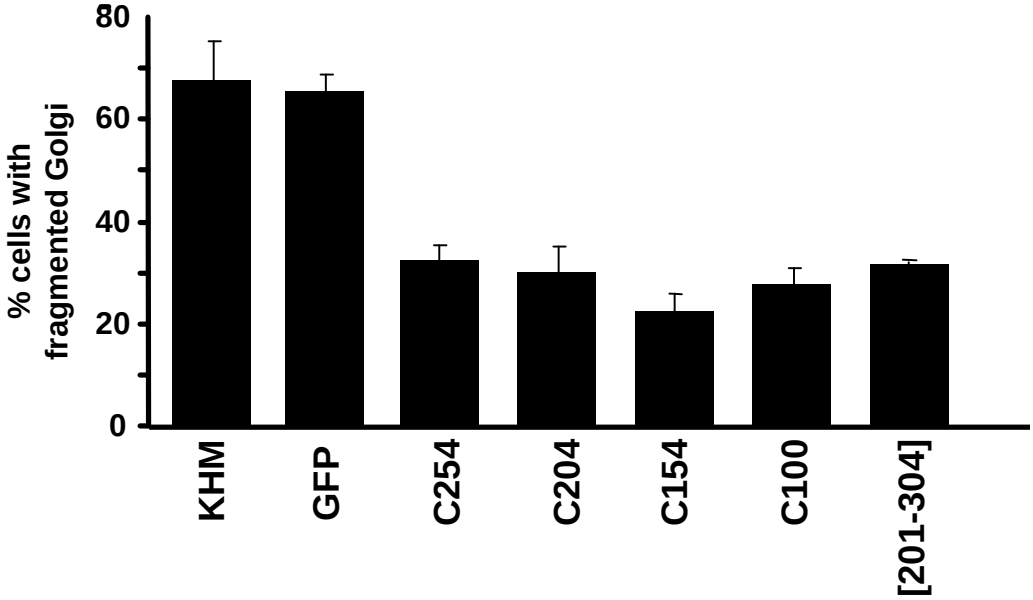
A)


6His
PDZ-1
PDZ-2
1.
2.
3.
4.
5.
6.
7.
FL GRASP55
N200
C254
C204
C154
C100
[201-304]

B)


kDa
1 2 3 4 5 6 7
54
35
24
16
10
Commassie
1 2 3 4 5 6 7
Anti-GRASP55

C)

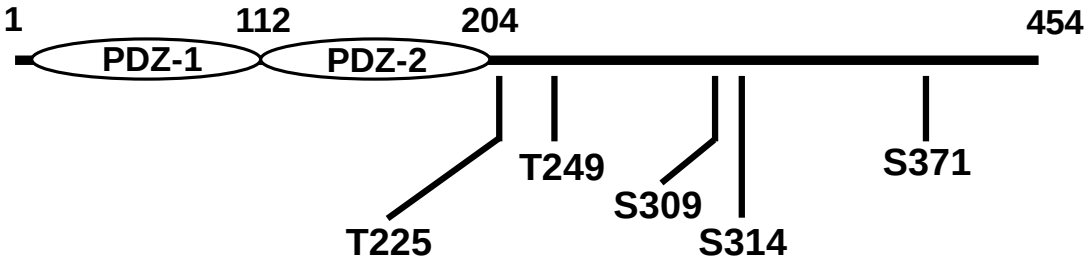

% cells with fragmented Golgi
80
60
40
20
0
KHM
GFP
C254
C204
C154
C100
[201-304]

GRASP55 contains two mitotic specific phosphorylation sites

It has been published by Linstedt and colleagues that GRASP55 is phosphorylated at T222 and/or T225, a predicted MAP kinase (ERK) consensus site, during mitosis (Feinstein and Linstedt, 2007; Jesch et al., 2001). Alanine point mutations at both of these sites have been shown to slow mitotic progression and inhibit the unlinking of Golgi stacks from each other, suggesting that the MAP kinase may be the upstream kinase for GRASP55 during the G2/M transition. We have shown that [201-304], which contains these T222/225 sites, although capable of inhibiting Golgi fragmentation in our semi-intact assay, is not solely required to inhibit Golgi fragmentation as other regions of the C-terminus, the C100 peptide, is also capable of inhibiting Golgi fragmentation independent of this phosphorylation site. It is possible there exist additional phosphorylation sites that were not recognized by the MPM2 antibody used in previous GRASP55 studies (Feinstein and Linstedt, 2007; Jesch et al., 2001).

Therefore we chose to analyze GRASP55 though phosphor-peptide mapping upon incubation of interphase, non-synchronous, and mitotically purified cytosol. Equal amounts of recombinant His-tagged GRASP55 was incubated in each cytosol. After 1hr at 32°C the protein was immunoprecipitated with Ni-Ag beads, washed and eluted. The samples underwent protease digestion and tandem mass spec analysis. Sequencing of the proteolytic fragments revealed only two mitotic specific phosphorylation sites, T225 and T249 (Figure 8). Our analysis confirms that threonine 225, within the ERK consensus site, is phosphorylated during mitosis. In addition, T249, which lacks a known consensus site, was also found to be phosphorylated under

mitotic conditions. Although we found additional phosphorylation sites, one of which was within the C100 peptide, they were not mitotic specific as we saw the same site under non-mitotic conditions. We conclude that C100 may be inhibiting Golgi fragmentation in a mechanism independent of [201-304]. Our findings suggest that the MAP kinases are not the only kinases involved in the phosphorylation of GRASP55, and like GRASP65, multiple kinases may be required for its function (Preisinger et al., 2005; Wang et al., 2005; Yoshimura et al., 2005). These findings also suggest that the C-termini of both GRASP proteins acts as a scaffold containing multiple regions that regulate the unlinking of Golgi stacks from one another before the onset of mitosis.



Sequence	Residue	Interphase	Mitosis
GQMTG**T***PMTPP	T225	No	**Yes**
SPPG**T***AGVEQS	T249	No	**Yes**
MPL**S***AGLP**S***LP	S309/314	Yes	Yes
SDFLP**S***FPLVPE	S371	Yes	Yes

Figure 3-8: Phosphopeptide mapping reveals two mitotic specific phosphorylation sites in GRASP55

Full length recombinant GRASP55 was incubated with either interphase or mitotic arrested cytosol. Proteins were bound to Nickel-Ag beads and eluted. The samples were processed for tandem mass spec analysis and analysized for phosphate addition.

GRASP55 regulates entry into mitosis in a phosphorylation dependent manner

We have previously shown that Golgi membranes must fragment in order for cells to enter mitosis and it has been recorded that the phosphorylation of both GRASP65, by cdc2 and polo-like kinase, and GRASP55, via the MAP kinase pathway, are involved in severing the intercisternal membranes between Golgi stacks to allow entry into mitosis (Feinstein and Linstedt, 2007; Sutterlin et al., 2002; Yoshimura et al., 2005). Since GRASP55-specific reagents inhibit mitotic Golgi fragmentation *in vitro* we tested the effect of these reagents on mitotic progression.

NRK cells were arrested in S-phase to obtain a synchronous population of cells. The cells were released from S-phase and incubated at 37°C for between 5.5 to 9.5hrs. At each time point, cells were fixed and incubated with an anti-phospho-histone H3 antibody and Hoechst 33342 to determine the number of mitotic cells as described previously (Sutterlin et al., 2002). The experiment was then repeated, microinjecting with either the GRASP55 fragment, [201-304], or C100. In both cases, cells microinjected with either peptide had a lower mitotic index than non-injected or cells injected with a control (Figure 9A). In all cases the mitotic index never increased over time and followed similar kinetics with regards to control cells, consistent with our reports of GRASP65-mediated Golgi fragmentation

To test if the decreased mitotic index was due to inhibition of fragmentation, the experiment was repeated, cells were microinjected with GRASP55 specific peptide and at 4.5hrs cells were treated with BFA, a drug that fuses the Golgi membranes with

the ER. Cells were fixed at 7.5hrs and analyzed as before. As with GRASP65, we found that by artificially removing the Golgi membranes away from its perinuclear position, the inhibition by GRASP55 was alleviated and the mitotic index was recovered (data not shown). Therefore, this supports our original hypothesis that the positioning of Golgi membranes acts as a regulator of mitotic entry and any prevention of the Golgi complex to disperse away from this region, either through the mechanism through GRASP65 or GRASP55, prevents or delays entry into mitosis (Sutterlin et al., 2002).

To determine if phosphorylation of GRASP55 at T225 and T249 are necessary for mitotic Golgi fragmentation, these sites were mutated within the [201-304] peptide. Previous reports have shown that mutational analysis of T222A and T225A in full length GRASP55 slowed mitotic entry. Although the MAP kinase sites may be crucial, we have shown that regions outside this domain can inhibit mitotic entry independently of these sites. Therefore, mutations of the phosphorylation sites within the [201-304] peptide, rather than within the entire C-terminus or full length protein, will serve as a way to test the direct role of these sites on Golgi fragmentation and mitotic entry. Since it had been previously published that both T222 and T225 were potential targets, we also included mutations at T222 to remain consistent with previously published reports (Feinstein and Linstedt, 2007; Jesch et al., 2001).

[201-304] mutational constructs were made to either inhibit phosphorylation (T/A), or mimic phosphorylation, T/E. Each peptide was tested for its ability to block entry into mitosis. Once again, cells were released from an S-phase block and incubated at 37°C for 7.5hrs after being microinjected with either with control, wild-

type peptide or the mutated peptides. We found that only when both T222/T225E and T249E phosphorylation mimics were introduced, the peptide no longer blocked entry into mitosis (Figure 9B). Mimicking the phosphorylation of only T249 or the ERK site T222/T225 did not fully rescue the number of mitotic cells entering mitosis. It should be noted that all Ala mutations dramatically decreased the mitotic index independent of each other (data not shown).

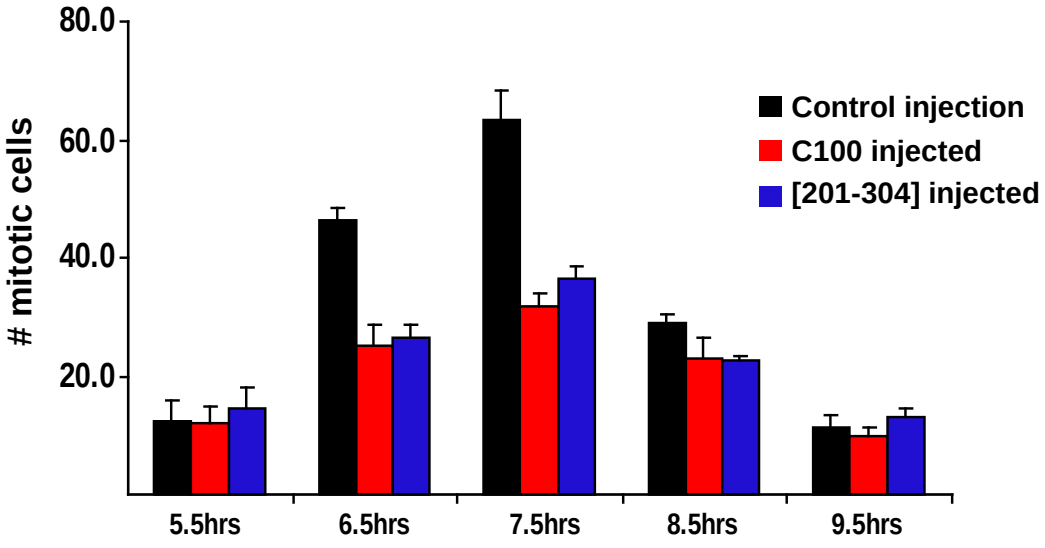
These findings suggest that the both phosphorylation sites are required for GRASP55 to regulate Golgi fragmentation. Based on our report, we also conclude that the depletion of GRASP55 serves as a mimic to phosphorylation and therefore allows cells to enter mitosis. Therefore GRASP55 serves as a regulator of mitotic Golgi fragmentation via the sequential phosphorylations at T225 and T249. It is unclear whether one site is preferred over the other or which site is phosphorylated first as Ala mutations in either site inhibited equally. Based on our analysis, we believe that GRASP55, in conjunction with a number of other Golgi associated proteins, helps to regulate the entry of cells into mitosis via dispersal and fragmentation of the Golgi complex through its sequential phosphorylation.

Figure 3-9: GRASP55 is required for Golgi fragmentation and mitotic entry in a phosphorylation dependent manner.

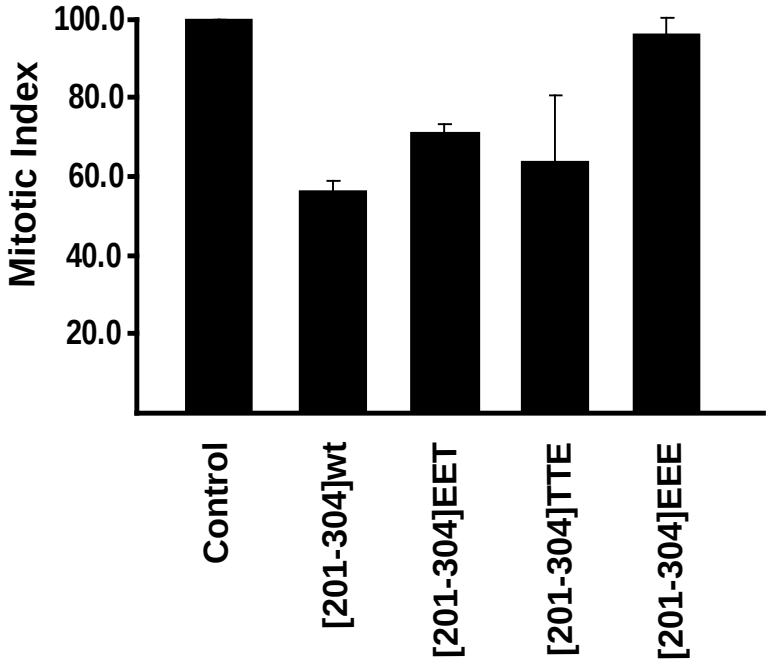
(A) As reported previously (Sütterlin, et al., 2002) NRK cells were arrested in S phase with thymidine. The cells were washed to remove thymidine and at various times were stained with phospho-histone H3 and Hoechst 33342 staining. The number of cells showing condensed DNA and phospho-Histone H3 antibody staining are shown. For each time point 200 cells were counted. At no timepoint do cells microinjected with either C100 or [201-304] rescue normal mitotic index. The data shows an average of four independent experiments.

(B) Injection of either the wild-type [201-304], [201-304]EET, [201-304]TTE decreases the mitotic index as compared to control cells. When both mitotic sites are mutated to glutamate, [201-304]EEE the mitotic index is rescued, indicating that both sites must be phosphorylated in GRASP55 for Golgi fragmentation and entry of cells into mitosis Each sample contained its own control of non-injected cells on the same coverslip. Control samples were normalized to 100% mitotic index and each sample was averaged and normalized based on its own control. The data represents (Control, n=14, C100 injected, n=4, each [201-304] peptide, n=4)

A)


80.0
60.0
40.0
20.0
mitotic cells
Control injection
C100 injected
[201-304] injected
5.5hrs
6.5hrs
7.5hrs
8.5hrs
9.5hrs

B)


100.0
80.0
60.0
40.0
20.0
Mitotic Index
Control
[201-304]wt
[201-304]EET
[201-304]TTE
[201-304]EEE

Discussion

The pericentriolar Golgi apparatus in the mammalian cells is fragmented in mitosis. But when does the process of fragmentation initiate? Inhibition of fragmentation and dispersal of the Golgi apparatus from its pericentriolar localization arrests cells in G2. This block can be alleviated by drugs such as nocodazole and Brefeldin A, which are both capable of disrupting the interconnected stacks and dispersing the Golgi apparatus (Sutterlin et al., 2002). Therefore, the initial signals that induce mitotic Golgi fragmentation must begin whilst the cells are still in G2, although a clear fragmented Golgi phenotype at the light microscopy level is not evident until late prophase. Studies have shown that the Golgi membranes begin to disconnect and encircle the nucleus in early prophase. Subsequent dispersal away from the membrane and further fragmentation is not seen until early metaphase. What changes do these membranes undergo between the G2/M transition to ensure entry of cells into mitosis, and how are cells arrested in G2 upon inhibition of Golgi fragmentation? Previous studies have established the role of GM130, GRASP65 and the MAP kinase pathway as participating in regulating the connections between Golgi stacks and the proper entry of cells into mitosis (Feinstein and Linstedt, 2007; Puthenveedu et al., 2006; Sutterlin et al., 2005; Yoshimura et al., 2005). We describe our current understanding of these issues with regards to a Golgi associated protein called GRASP55.

It has been suggested that the MAP kinase pathway is required for disrupting the tubular connections between the adjacent Golgi stacks, which may be via the Golgi

associated protein, GRASP55 (Feinstein and Linstedt, 2007; Jesch et al., 2001). Although we are unable to see any structural differences in Golgi membranes upon siRNA depletion of GRASP55, we report that two mitotic phosphorylation sites, including the MAP kinase consensus, is required for mitotic Golgi fragmentation and the entry of cells into mitosis.

ERK2 is the proposed kinase of GRASP55 for its role in mitotic Golgi fragmentation (Jesch et al., 2001). We have shown previously that neither ERK1 nor ERK2 activity is required for Golgi fragmentation in our semi-intact assay (Acharya et al., 1998). A possible candidate is an alternatively spliced form of ERK1, ERK1c, which has been shown to localize to the Golgi apparatus in a monoubiquination-dependent manner and involved in fragmenting Golgi membranes in a kinase-activity dependent manner (Aebersold et al., 2004). Although the substrate specificity of ERK1c is not fully understood, it is capable of phosphorylating substrates of ERK1 and ERK2 and therefore makes it another suitable candidate for GRASP55 phosphorylation. Therefore we suggest that the MAP kinase pathway, in conjunction with another unknown kinase, is required for entry of cells into mitosis via the sequential phosphorylation of GRASP55.

The depletion of GM130 and GRASP65 with specific siRNAs also results in the loss of such lateral connections without affecting the stacking of Golgi cisternae (Puthenveedu et al., 2006). In *Drosophila* S2 cells, when the single GRASP homolog, dGRASP, is depleted in combination with GM130, the Golgi stacks are fragmented (Kondylis et al., 2005). Interestingly, in *Drosophila* S2 cells, inhibition of Golgi separation during G2 prevents entry into mitosis (Kondylis et al., 2007). This furthers

the idea that Golgi inheritance is linked to mitotic entry but the role of the *Drosophila* GRASP homolog during this process is not yet understood.

In mammalian cells, the presence of either GRASP65 or GRASP55 is not required for Golgi structure, mitotic entry or mitotic Golgi fragmentation, although GRASP65 is required for mitotic exit via proper spindle formation. How then, can proteins which regulate Golgi fragmentation not be required for this process? This suggests that the GRASP proteins act as negative regulators of Golgi fragmentation and mitotic entry, while also serving additional functions. Therefore, the phosphorylations of these proteins during the G2/M transition, abrogates this negative regulation, allowing for mitotic Golgi fragmentation and entry of cells into mitosis.

But do these proteins have additional functions during the cell cycle? GM130 has been proposed to act as a scaffold for the mammalian Ste20 kinases, YSK1 and MST4, whose activity at the Golgi have been proposed to play a role in protein transport, cell migration and cell adhesion (Preisinger et al., 2004). Human GRASP55 has been suggested to play a role in the proper surface expression of TGF-α (Kuo et al., 2000). Both GRASP55 and GRASP65 interact with and help retain specific members of the p24 family of cargo receptors in the Golgi apparatus (Barr et al., 2001). Although general protein secretion is not affected in the absence of either GRASP65 or GRASP55, could these additional functions be a factor in regulating Golgi inheritance, spindle formation and hence mitotic entry?

We have recently published that the GRASP homolog in *Dictyostelium* is required for the unconventional secretion of a protein required for the development of spore cells (Kinseth et al., 2007). Do the mammalian GRASP proteins have a similar

function in unconventional secretion? Interestingly, similar to GRASP55 depletion, the disruption of GRASP in *Dictyostelium* shows no phenotype in the organization of Golgi membranes, conventional protein secretion or cell growth. Unconventional routes of secretion can use both non-vesicular and vesicular mechanisms. Could the control of unconventional secretion by GRASP be linked with its regulation of mitotic Golgi fragmentation and mitotic entry? These newly discovered functions of the GRASP proteins offers new insight on how the Golgi membranes may serve important and diverse functions. As we continue to study the physiological function of the GRASP proteins, their role in unconventional secretion and regulation of mitotic Golgi fragmentation, we will discover how the dynamics of Golgi membranes serve as a scaffold to regulate these many functions.

Materials and Methods

Expression and Purification of Recombinant Proteins

GRASP55 constructs were generated by PCR using a C6-glioma cDNA library (generated by Yusuke Maeda). The PCR fragments, full length GRASP55 (upstream primer 1: 5'-ggctggatccggctcctcgcagagcgtcgag-3', downstream primer 1: 5'-ccacaagcttttaagaagccccagaagcatttgcatcc-3'), C254 (amino acids 201-454) (upstream primer 2: 5'-ggctggatcccgaatacctacgcgtccctttg-3', downstream primer 1: 5'-ccacaagcttttaagaagccccagaagcatttgcatcc-3'); C204 (amino acids 251-454), (upstream primer 3: 5'-cctcggatccggagttgagcagagtctgtctgga-3', downstream primer 1: 5'-ccacaagcttttaagaagccccagaagcatttgcatcc-3'); C150 (amino acids 301-454), (upstream primer 4: 5'-ttatggatccctgatgcctttgtcagcagggc-3', downstream primer 1: 5'-ccacaagcttttaagaagccccagaagcatttgcatcc-3'); C100 (351-454 amino acids), (upstream primer 5: 5'-ttatggatccaacttacctggcattgcacctctc-3', downstream primer 1: 5'-ccacaagcttttaagaagccccagaagcatttgcatcc-3'); [201-304] (amino acids 201-304), (upstream primer 2: 5'-ggctggatcccgaatacctacgcgtccctttg-3', downstream primer 2: 5'-tattaagcttttaactggtaatgtggcagc-3'); [251-354] (amino acids 251-354) (upstream primer 3: 5'-cctcggatccggagttgagcagagtctgtctgga-3', downstream primer 3: 5'-tattaagcttttatcggggaggcaaggacggaa) were subcloned into the BamHI and HindIII restriction sites of the pQE30 bacterial expression vector (Qiagen, Chatsworth, CA). Recombinant proteins were induced to express with 0.5mM IPTG in either 71-18 (gift from Mike Yaffe) or XL-1-Blue (Stratagene) competent bacterial cells, lysed in lysis buffer (50mM Sodium Phosphate buffer pH 8.0, 300mM NaCl, 10% glycerol) and

purified with Ni-agarose beads. The elutions were dialyzed in a low-salt buffer (50mM Sodium Phosphate buffer pH8.0, 50mM NaCl, 10% glycerol) using a Slide-A-Lyzer slide (Pierce). The dialyzed protein was then passed over a DEAE-Sephacel column (Pharmingen) and the GRASP55-containing flow-through was collected. Purified proteins were dialyzed against KHM plus 10% glycerol and concentrated with Microcon spin columns (Millipore). It is important to note that all recombinant proteins were purified in the complete absence of detergent.

Generation of Anti-GRASP55 Antibody and Antibody Affinity Purification

After the purification of 6His-tagged GRASP55 using Ni-agarose (Qiagen), the protein fractions were loaded onto a large preparative gel for additional purification from co purifying contaminants. Pure GRASP55 was then cut out of the SDS-polyacrylamide gel and eluted from the gel slices by electro-elution and used to inject rabbits. Each bleed was tested for reactivity with recombinant protein and total NRK lysate by Western blot analysis and by immunofluorescence. The anti-GRASP55 antibody was affinity-purified. In brief, an antigen column was generated by coupling approximately 4mg of recombinant full-length GRASP55 to activated CNBR beads (Sigma); the column was washed with PBS and incubated with the crude antiserum. Immediately after elution of the antibody with 100mM glycine (pH 2.5), the eluted sample was brought to neutral pH and dialyzed against PBS containing 10% glycerol in a Slide-A-Lyzer slide (Pierce). The affinity-purified antibody was then concentrated using Microcon spin columns (Millipore) to a final concentration of 10mg/ml. Pre-immune IgG was purified by binding the crude pre-immune serum to a Protein A-

Sepharose (Amersham Pharmacia Biotech) column. IgG was eluted with 100mM glycine (pH 2.5), dialyzed, and concentrated as described for the anti-GRASP55 antibody.

Additional Antibodies and Reagents

Antibodies used in this study were obtained from the following sources: GRASP65, GRASP55, Giantin, β-COP (Malhotra lab), GM130 (BD Biosciences), phospo-histone-H3 (Upstate Biotechnology). The siRNA oligos used in this study were purchased from Dharmacon and are: control siRNA (non-targeting siRNA #1, Dharmacon), GR55-1 (target seq: nngaagaucuauucagccuua) and GR55-2 (target seq: nncuauucagccuuaucgaaa). In order to create the SS-HRP-Flag plasmid (pSS-HRP-Flag), the SS-HRP cDNA sequence was amplified by PCR from a previously described plasmid encoding SS-HRP-KDEL (Connolly et al., 1994) and cloned into pEGFP-N1 (BD Biosciences) using SmaI-AgeI targets. The EGFP cDNA in the vector was removed by AgeI-NotI digestion and the resulting digested plasmid was ligated with pre-annealed complementary primers encoding both the Flag epitope and the AgeI-NotI restriction sites.

Cell Culture and transfections

HeLa cells were grown in complete medium consisting of DMEM (Gibco BRL) containing 10% FCS and L-glutamine at 37ºC in 6% CO_2 incubator. Knockdown transfections were performed on ~50-70% confluent HeLa cells using either Oligofectamine (Invitrogen) or HiPerFect (Qiagen), according to manufacturer's

instructions. Transfection reagents were washed away 6 hours after transfection to prevent cell death. Once transfected, cells were passaged to other plates or onto coverslips at the desired confluency, depending on the needs of each experiment. Unless otherwise stated, experiments were performed 3-4 days after siRNA transfection, when GRASP55 knockdown was found to be optimal.

Immunofluorescence Microscopy

For immunofluorescence studies, cells were fixed with 4% formaldehyde in PBS, blocked with blocking buffer (2.5% FCS, 0.1% Triton-X-100, 0.05% sodium azide in PBS). Cells were incubated with primary antibody (as mentioned above) followed by a PBS wash and 30 min incubation in secondary antibody (rabbit or mouse anti-goat-Alexa Fluor-488 or-594 from Molecular Probes) and Hoechst 33342 staining (Molecular Probes). Cells were mounted using Fluor Save Reagent (Calbiochem) and visualized by immunofluorescence microscopy. The Nikon Mirophot-FXA fluorescence microscope was used to visualize samples. Pictures were taken with an attached Olympus camera using MagnaFire computer software to record and save picture files. All pictures were opened in Adobe Photoshop and Illustrator.

Electron Microscopy

Control and silenced cells were fixed with 1% glutaraldehyde in Hepes buffer, embedded in Epon 812 and cut into thin sections. Analysis of thin sections was performed under Tecnai-12 electron microscope (FEI, Philips, Einhoven the Netherlands). Images were taken using a ULTRA VIEW CCD digital camera.

Preparation of Mitotic, Non-synchronous Cytosol and the Golgi Fragmentation Assay

NRK cells were grown in complete medium consisting of α-MEM (Gibco BRL) containing 10% FCS, L-glutamine, 10U/ml penicillin, and 100ug/ml streptomycin at 37ºC in 5% CO2 incubator. Cytosol from NRK cells arrested in mitosis or from a non-synchronous population, at a concentration of 12-14mg/ml, was prepared as described previously (Acharya et al., 1998). Permeabilization of cells grown on coverslips and the mitotic Golgi fragmentation assay was performed as described (Acharya et al., 1998). In brief, cells that have been treated with 2mM thymidine for 8-14hr were permeabilized on ice with 30µg/ml digitonin in KHM buffer (25mM HEPES-KOH [ph 7.4], 125mM potassium acetate, and 2.5mM magnesium acetate). Permeabilized cells were washed with 1 M KCl-containing KHM and incubated with non-synchronous or mitotic cytosol in the presence of 200µg/ml anti-GRASP55 antibody, pre-immune IgG, recombinant rat GRASP55, or its C-terminal fragments, and an ATP-regenerating system. Incubations were carried out in a volume of 50µl. After 60 minutes of incubation at 32ºC, cells were fixed and processed for immunofluorescence using either anti-giantin or anti-mannosidase II antibody to visualize the structure of the Golgi. For each experiment, 200-300 cells per coverslip were analyzed for their Golgi morphology.

Microinjections of GRASP55-specific reagents

Affinity-purified anti-GRASP55 antibody or pre-immune-IgG, all at a concentration of 5mg/ml, were injected into approximately 400 aphidicolin-arrested

NRK cells (aphidicolin was used at 2.5µg/ml) roughly 45 min after removal of the S-phase block. C100 microinjection was performed in 200 cells at a concentration of 3mg/ml. In both cases, cells were then incubated in complete medium for approximately 7.5 hrs prior to fixation. For BFA treatment, BFA was added 3 hours before fixation as previously determined. In all cases cells were stained with anti-phospho-Histone H3 and Hoechst to visualize the mitotic state and organization of the DNA.

ssHRP Secretion Assay

48 h after transfection with siRNA, the cells were transfected with the SS-HRP-Flag plasmid, using Lipofectamine 2000 (Invitrogen). 30 µl of extra-cellular media was harvested 72 h after the initial siRNA transfection. HRP activity was measured using enhanced chemiluminescence (ECL) as described previously (Bard et al., 2006).

VSV-G transport assay

48 h after transfection with siRNA the cells were transfected with *ts*045VSV-G-GFP construct and cultured at 40°C for 20 h. 100 υg/ml of cycloheximide was then added prior to 2 hr incubation at 20°C. After incubation at 32°C for 40 min. the cells were harvested with a cell dissociation buffer (Invitrogen) and fixed with 4% paraformaldehyde. After blocking with PBS containing 1.5% serum and 0.1% sodium azide, the labeling of surface VSV-G-GFP was performed for 30 min of incubation at

4°C with the anti-VSV-G mAb 8G5F11, which is specific for the extracellular domain of VSV-G. After washings with the blocking buffer, the cells were incubated with the secondary (APC)-labeled anti-mouse IgG antibody (Jackson ImmunoResearch) for 30 min at 4°C. After washing, the cells were analyzed on a FACScalibur flow cytometer (BD Biosciences). The amount of VSV-G present at the cell surface (APC positive) of cells transfected with both siRNA (cy3 positive) and VSV-G (GFP positive) after subtracting the background was normalized by the GFP intensity.

Phosphopeptide mapping

Recombinant GRASP55 was incubated for 1 hr at 32°C with cytosol purified from thymidine-arrested, non-synchronous, and nocodazole-arrested NRK cells. Recombinant GRASP55 was immunoprecipitated with Ni-Ag beads, washed in buffer and eluted with 100mM imidazole containing phosphate buffer. Samples were processed for tandem mass-spec analysis.

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Chapter 3, in part, will be submitted for publication of the material as it may appear. I, Matthew A. Kinseth, am the primary author of the paper

Discussion and Future Directions

In the 10yrs since the identification of the GRASP proteins, no true physiological function has been found. The majority of work done in cell culture (yeast, *Drosophila* and mammalian cells) along with a number of biochemical assays has produced controversial data. Gene disruption of the GRASP homolog in the developing organism, *Dictyostelium discoideum* has revealed the first physiological function for these proteins in regulating unconventional secretion.

The physiological link between the GRASP proteins and the mechanism of unconventional protein secretion should open a new chapter in understanding why these proteins are conserved. Unconventional protein secretion can use both non-vesicular and vesicular routes, both of which are not well understood. The discovery that Golgi associated proteins plays a role in this process may bridge the gap between the membrane dynamics of conventional and unconventional secretion as well as help discover new mechanisms for how the endomembrane system controls the trafficking of cargo outside of the cell.

Whether the GRASP proteins are conserved for unconventional secretion remains unknown. It may be that GRASP, and therefore the Golgi membranes, are indirectly involved in unconventional secretion, but rather are required for the trafficking of specific cargo needed for the release of proteins in an unconventional manner. Regardless of the mechanism directly used by the GRASP proteins, this opens up further questions to be explored.

It becomes imperative to continue the study of the GRASP proteins in other developing system, cellular networks as well as specialized cells where defects in unconventional secretion would be physiologically relevant. In addition, further work must be done to understand the functional homology of these proteins.

GRASP in Dictyostelium and unconventional secretion

1) Is GRASP in *Dictyostelium* phosphorylated during mitosis and does this control mitotic entry? It has been published that *Dictyostelium* Golgi membranes fragment during mitosis. It therefore may be likely that GrpA may serve similar functions as seen in mammalian cells.

2) Can either mammalian GRASP55 or GRASP65 rescue the *grpA*$^-$ spore viability phenotype? Understanding the functional homology of the GRASP proteins will be invaluable in understanding why the GRASPs are conserved.

3) Is the control of mitotic Golgi fragmentation and unconventional secretion linked via the GRASP proteins? Understanding whether the two functions of the GRASP proteins are somehow linked is crucial for discovering the physiological relevance of these proteins throughout eukaryotes.

GRASP in mitosis

1) What do the C-terminal peptides of the GRASP proteins sequester away from the Golgi to prevent mitotic fragmentation?

Although the C-terminal regions of both GRASP55 and GRASP65 are capable of inhibiting mitotic Golgi fragmentation, it remains unknown what factors these regions are sequestering. As mentioned previously, the C-termini could be acting as a scaffold for a variety of protein-protein interactions. Therefore it would be important to understand the interactions required for GRASP-mediated Golgi fragmentation.

2) What role do the conserved PDZ-like domains serve?

Interestingly, the N-terminus is not mitotically regulated but remains the only conserved region of these proteins. PDZ domains are known interactors with proteins and lipids and understanding how these domains function during mitosis will also reveal additional mechanisms. There is some evidence that polo-like kinase interacts with GRASP65 but no experiments to date have shown what function this serves. The link between Plk-1, GRASP65 and spindle formation brings forth obvious experiments, for instance is Plk-1 mislocalized in GRASP65-depleted cells? Such experiments will serve to better understand this mechanism. There is much less information on mammalian GRASP55, therefore any additional experiments will further our understanding.

Most importantly is the new link between GRASP function as a regulator of mitotic Golgi fragmentation and unconventional secretion. The endomembrane system may act as a scaffold for many functions and the GRASP proteins may have been conserved to help regulate the membrane dynamics of unconventional secretion, convention secretion, mitotic Golgi fragmentation, cytoskeletal-membrane interactions and hence the entry of cells into mitosis.

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