Characterisation of Rab-Effector Complexes at the Golgi Apparatus

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"The three little sentences that will get you through life.

Number one, 'Cover for me'.

Number two, 'Oh, good idea, boss'.

Number three, 'It was like that when I got here'."

Homer J. Simpson

Summary

The rab family of small, ras-like GTPases regulate membrane trafficking events in the secretory and endocytic pathways. They appear to be involved in all stages of vesicular transport, from vesicle budding and cargo selection to motility, docking and membrane fusion. Through a cycle of GTP binding and hydrolysis, rab-effector proteins are recruited to membrane sub-domains in a temporally and spatially specific manner. Several rab proteins localise to the Golgi apparatus, the organelle consisting of stacked, flattened, membrane-bound cisternae through which newly-synthesised proteins are transited and modified, and where proteins and lipids are sorted and packaged for transport to other subcellular destinations. The structure of the Golgi is maintained by a matrix of proteins, many of which have now been shown to be rab-effector proteins. This thesis focuses on Golgi-localised rab proteins and their effector proteins. The *cis*-Golgi-localised rab protein, rab1, is shown to interact with the Golgi matrix/golgins GM130 and p115 while rab2 binds GM130 as well as a novel tethering factor named golgin-45. siRNA-mediated depletion of these rabs and golgins revealed them to be important for the maintenance of Golgi structure and suggested that p115 is primarily recruited to Golgi membranes by its interaction with rab1 rather than its association with GM130. A search for additional rab1 effectors revealed potential interactions between rab1 and the phosphoinositide-binding/metabolising proteins centaurinβ2 and MTMR6. A search for novel effectors of the *trans*-Golgi-localised rab protein, rab6, was also made, revealing specific interactions with the dynactin subunit $p150^{glued}$ and the dynactin/dynein accessory proteins BicD1 and BicD2. These interactions are proposed to mediate the recruitment of dynactin/dynein to membranous cargo and control minus-end directed, microtubule-dependent vesicle motility. An additional rab6 effector, GOPC, was also identified, which may be responsible for cargo recognition and sorting. Finally, the *cis*-Golgi rab-effector and matrix protein, GM130, is shown to have a hitherto unsuspected role in the activation of a family of Golgi-localised Ste20 kinases. The implications of all these interactions for Golgi structure and function is discussed.

Chapter Overview

Contents

List of Figures

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

List of Tables

CHAPTER 4

CHAPTER 5

Abbreviations

ADP	Adenosine 5'-diphosphate
ARF	ADP-ribosylation Factor
ARL	Arf-like
ATP	Adenosine 5'-triphosphate
BFA	Brefeldin A
CGN	Cis-Golgi Network
COP	Coatomer Protein
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
EEA1	Early Endosome Antigen 1
EGF	Epidermal Growth Factor
EM	Electron Microscopy
ER	Endoplasmic Reticulum
ERGIC	Endoplasmic Reticulum-Golgi Intermediate Compartment
ERK	Extracellular signal Regulated Kinase
FYVE	Fab1, YOTB/ZK632.12, Vac1, EEA1
GAP	GTPase Activating Protein
GDI	GDP Dissociation Inhibitor
GDP	Guanosine 5'-diphosphate
GEF	Guanine Nucleotide Exchange Factor
GFP	Green Fluorescent Protein
GGA	Golgi-localising, γ-adaptin ear homology domain, ARF binding protein
GMP-PNP	Guanosine 5'-[β,γ-imido]triphosphate

GRASP	Golgi Reassembly Stacking Protein
GRIP	Golgin-97, RanBP2α, Imh1p, p230/golgin-245
GST	Glutathione-S-Transferase
GTP	Guanosine 5'-triphosphate
GTPγS	Guanosine 5'-[γ-thio]triphosphate
HeLa	Henrietta Lacks
HRP	Horseradish peroxidase
IGEPAL	(Octylphenoxy)polyethoxyethanol
IMAC	Immobilised Metal Affinity Chromatography
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
kb	kilobase
kDa	kiloDalton
LB	Luria-Bertani
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
MAP Kinase	Mitogen Activated Protein Kinase
MBP	Myelin Basic Protein
MDCK	Madin-Darby Canine Kidney
mRNA	messenger RNA
NAGT-I	n-acetylglucosaminyltransferase-I
NEM	N-ethyl Maleimide
Ni-NTA	Nickel-nitriloacetic acid
NRK	Normal rat kidney
NSF	NEM-sensitive Factor
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PFA	Paraformaldehyde
PH	Pleckstrin-homology
PLC	Phospholipase C
QDO	Quaduple Dropout
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase-PCR

SC	Synthetic complete
SDS	Sodium Dodecylsulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
siRNA	Small, interfering ribonucleic acid
SM protein	Sec1/Munc18 –related protein
SNAP	Soluble NSF Attachment Protein
SNARE	SNAP Receptor
TCA	Trichloroacetic acid
TGN	Trans-Golgi Network
UV	Ultra-violet
VSV-G	Vesicular Stomatitis Virus G protein
VTCs	Vesicular Tubular Clusters
YPDA	Yeast extract, Peptone, Dextrose, Adenine

Chapter 1: Introduction

1.1 The Secretory Pathway

The secretory pathway in eukaryotic cells is the pathway by which new proteins and lipids synthesized at the endoplasmic reticulum are delivered to different sub-compartments within the cell or secreted from the cell. The vectorial transport of this material through the endomembrane system is a subject, which has fascinated cell biologists for decades (Mellman and Warren 2000).

The pathway begins with the translocation of newly synthesized proteins into the endoplasmic reticulum (ER). In animal cells, this usually occurs co-translationally, as signal-peptides in the protein sequence direct the attachment of the ribosome to the ER membrane and the translocation of the protein through a pore (the translocon) into the internal lumen of the ER. For soluble secretory proteins, the signal sequence is at the amino terminus of the protein and is cleaved following the translocation of the protein into the ER lumen (Rapoport *et al.* 1996). Type I integral membrane proteins also have a cleavable N-terminal signal peptide as well as a stop-transfer signal later in the protein sequence, which prevents further translocation of the polypeptide through the translocon and becomes the transmembrane domain of the protein (Goder and Spiess 2001). Type II and type III membrane proteins have internal signal sequences that direct the insertion of the nascent polypeptide into the translocon (either the C- or N- termini of the protein respectively) but also act as transmembrane anchors so that integral membrane proteins of the correct topology are formed. Multispanning transmembrane proteins are formed by a combination of start- and stop-transfer sequences (Goder and Spiess 2001). For all integral membrane proteins, the transmembrane domains partition into the ER membrane during their passage through the translocon.

Following translocation, proteins are acted upon by numerous ER proteins responsible for ensuring their correct folding and processing. The ER quality control system utilises a specific glucosyltransferase and lectins to ensure that only correctly folded proteins are able to exit the ER (Ellgaard and Helenius 2003). The glucosyltransferase recognises misfolded proteins and adds glucose residues to the ends of N-linked sugar chains on the protein (Trombetta and Helenius 2000). This modified glycan is then recognised by the lectins calnexin and calreticulin and thereby retained in the ER. A glucosidase then acts to release the protein and allow it to be re-sampled by the glucosyltransferase. The cyclical action of these enzymes retain the protein in the ER until it has folded correctly (Ellgaard and Helenius 2001).

Once the protein is correctly folded, it must be transported from the ER to the Golgi apparatus and then onwards to other compartments of the secretory pathway. The pioneering work of Jamieson and Palade used electron microscopy autoradiography to first demonstrate this linear transport pathway in pancreatic acinar cells and to show the existence of vesicular carriers responsible for transferring material between the different organelles (Palade 1975). The mechanisms of vesicle transport will be discussed below but, put simply, vesicles form at a donor membrane, budding off to contain transportable cargo, before fusing with an acceptor membrane and delivering their contents. Thus, cargo is transported in vesicles from the ER to the Golgi apparatus, the key organelle of the secretory pathway.

1.2 The Golgi Apparatus

The Golgi was first identified over 100 years ago by the Italian histologist Camillo Golgi using a particular silver nitrate staining technique which revealed a distinctive reticular network in Purkinje cells (Golgi 1898). It wasn't until the advent of electron microscopy (EM), however, that real insights into the structure and function of the Golgi began to be made. Dalton and Felix were the first to observe the Golgi as a stack of flattened, membrane-bound, cisternae (Dalton and Felix 1954). EM autoradiography was used to show a role for the Golgi in sulphation and glycosylation (Godman and Lane 1964; Neutra and Leblond 1966) while, as mentioned above, Palade and co-workers were able to show the passage of secretory material through the Golgi stack (Palade 1975).

A complementary technique was that of cellular sub-fractionation, through which the glycosylation enzyme galactosyltransferase was found to be enriched in Golgi fractions and therefore identified as a resident Golgi marker protein (Fleischer *et al.* 1969; Morre *et al.* 1969). All of this pointed to a role for the Golgi apparatus in protein transport and modification. This concept was expanded on as the structure of the Golgi was further defined and found to consist of distinct sub-compartments containing different glycosyltransferase and other enzyme activities such as NADPase.

The Golgi cisternal stack has a defined polarity with a *cis* face exchanging material with the ER and a *trans* face communicating with the plasma membrane and endosomes. In between the *cis*- and *trans*- cisternae are the *medial*-cisternae. Albeit with some degree of overlap, the different cisternae contain a different complement of enzymes such that, as a secretory protein passes through the Golgi in a *cis* to *trans* fashion, it is acted upon sequentially by these enzymes to produce the correct glycosylation patterns and other modifications (Kornfeld and Kornfeld 1985).

In addition to the cisternae, two other structurally and functionally distinct Golgi sub-compartments have been recognised. The *trans*-Golgi network (TGN) is located at the *trans*-face of the Golgi stack but has a much more complex tubulo-vesicular structure than the *trans*-cisternae. The compartment again has a distinct subset of resident proteins and is involved in the sorting and packaging of cargo for delivery to the plasma membrane, lysosomes, or endosomes (Griffiths and Simons 1986).

At the *cis* face of the Golgi is another complex network of tubules and vesicles, variously defined as either the *cis*-Golgi network (CGN), the ER-Golgi intermediate compartment (ERGIC) or simply as vesicular-tubular clusters (VTCs). This network is thought to be formed by the fusion of ER-derived vesicles en route to the *cis*-Golgi (Schweizer *et al.* 1988). Whether these clusters fuse directly with a pre-existing *cis*-Golgi cisternae or whether they in fact develop themselves into the *cis*-Golgi is a matter of some debate, which reflects two differing views of the nature of the Golgi apparatus and the way in which biosynthetic material passes through it.

The first model to explain Golgi function was the cisternal maturation/progression model (Grasse 1957). This proposed that cisternae were formed at the *cis*-face of the Golgi before moving progressively through the Golgi stack, maturing as they did so into *medial*-cisternae followed by *trans*-cisternae. At the *trans*-face, the cisternae

would become fragmented as cargo was selected and packaged into vesicles for delivery to their final destinations.

As the existence of vesicular transport became accepted, an alternative model was proposed in which the Golgi cisternae were thought to be stable structures with transport between cisternae being mediated by vesicles (Farquhar and Palade 1981). Supporting this hypothesis was a series of *in vitro* assays by Rothman and colleagues which were able to recreate transport between two different populations of Golgi-derived membranes (Fries and Rothman 1980). Although there is good evidence to support the idea of anterograde transport through the Golgi by vesicles, many problems remain, not least the question of how large macromolecules such as procollagen fibrils, which are larger than the vesicles seen to be associated with the Golgi, are transported through the Golgi stack.

The cisternal maturation model may account for this by proposing that secretory material passes through the Golgi as cisternae mature, but that maturation occurs due to the consecutive recycling of enzymes from later Golgi compartments via transport vesicles (Pelham 1998).

To date, neither the cisternal maturation nor the stationary cisternae models have been proved conclusively. The reality may be that a combination of both processes occurs with some secretory material moving in anterograde transport vesicles while Golgi enzymes are recycled in retrograde vesicular carriers. Although it is not trivial to identify the contents of vesicles passing through the Golgi apparatus, secretory proteins as well as resident enzymes have been found in Golgi vesicles (Orci *et al.* 1997). Golgi enzymes in particular are clearly found in a type of Golgi vesicle known as COPI vesicles (see section 1.4). This reflects the general importance of retrieval and recycling in the secretory pathway.

1.3 Retention and Retrieval in the Secretory Pathway

Despite the high flux of material through the secretory pathway, the different compartments of the pathway are able to maintain separate identities with differing complements of resident proteins. This is probably due to a combination of the selective retention of resident proteins, the preferential incorporation of secretory cargo into anterograde vesicles and the retrieval of proteins which have 'escaped' to later compartments of the pathway.

The best understood retrieval mechanisms are those involved in the recycling of proteins back to the ER from the Golgi apparatus. Once ER-derived vesicles have fused together to form the CGN, a sorting process begins to ensure that resident ER proteins are returned. For type I ER membrane proteins, this involves an interaction between a 'retrieval motif' in their cytosolic domains and the γ subunit of the COPI protein coat of nascent retrograde vesicles (Harter *et al.* 1996; see section 1.4). This retrieval motif is also known as the dilysine motif due to its sequence of KKXX where X is any amino acid (Jackson *et al.* 1993).

Other types of integral membrane proteins may contain other retrieval motifs such as the N-terminal RKR motif found in the ATP-sensitive K^+ channel (Zerangue *et al.* 1999). Lumenal ER proteins contain a retrieval motif at the C-terminus, this time of the sequence KDEL (Munro and Pelham 1987). A membrane-spanning KDEL receptor, which recognises this motif, is responsible for recycling these proteins back to the ER (Lewis and Pelham 1992).

The mechanisms by which resident Golgi proteins remain localised to specific Golgi sub-compartments are less clear. Among the models proposed to explain this phenomenon are the self-association of 'like' enzymes to form large oligomeric structures, and the preferential partitioning of different proteins into membranes of different composition and thickness (Bretscher and Munro 1993; Nilsson and Warren 1994).

No matter how it is achieved, however, the different organelles of the secretory pathway maintain separate identities and transport between these compartments in either an anterograde or retrograde direction, occurs via the formation of transport vesicles and their specific fusion with their target membrane.

1.4 Mechanisms of Vesicle Budding

The first step in vesicle formation is the recruitment of proteins from the cytosol to the membrane surface to form a coat. Cargo must be selected into the nascent vesicle as the donor membrane invaginates. The vesicle must then detach from the membrane by scission of the bud neck. Finally, the vesicle must shed its protein coat before it is ready to fuse with its target membrane.

There are several different sets of coat proteins, defining different classes of vesicle within the secretory pathway. The best studied are the COPII vesicles mediating ER

to Golgi transport, COPI vesicles involved in Golgi to ER and intra-Golgi transport, and clathrin vesicles which transport material between the *trans*-Golgi and the endosomes (and also from the plasma membrane to the endosomes in the endocytic pathway). There are many similarities between these different types of vesicle but there are important differences also.

COPII (Coat Protein II) vesicles form at transitional ER sites – regions of the ER devoid of ribosomes and specialised for the export of material destined for the Golgi. The accumulation of COPII components at the transitional ER can be visualised in live cells using green fluorescent protein (GFP) tagged proteins (Hammond and Glick 2000; Stephens *et al.* 2000). The proteins themselves were originally identified in the budding yeast *Saccharomyces cerevisiae* using a combination of yeast genetics and an *in vitro* assay for ER to Golgi transport (Novick *et al.* 1980; Baker *et al.* 1988). Most of the mammalian homologues of the proteins involved have now been identified.

The initiator of COPII coat formation is the small GTPase Sar1p. The GDP-bound form of Sar1p is predominantly cytosolic but is recruited to the ER membrane by its guanine exchange factor (GEF) Sec12p (Barlowe and Schekman 1993). Once activated at the membrane, Sar1p-GTP is able to recruit additional components of the COPII coat, beginning with the Sec23p-Sec24p complex (Barlowe *et al.* 1994). The function of this complex appears to be both cargo selection (Sec23p can bind to a diphenylalanine motif in the cytosolic domains of the p24 family of transmembrane proteins required for the efficient ER to Golgi transport of some cargo molecules (Dominguez *et al.* 1998)) and the recruitment of SNARE proteins essential for the fusion of the COPII vesicle with its target membrane (Peng *et al.* 1999; Miller *et al.* 2003; Mossessova *et al.* 2003; and see section 1.5.4). Selectivity seems to arise from multiple cargo binding sites (Miller *et al.* 2003; Mossessova *et al.* 2003). The Sec13p-Sec31p complex is also recruited to the ER membrane resulting in membrane deformation and vesicle budding (Barlowe *et al.* 1994). Once the vesicle has completely formed, Sec23p acts as a GTPase activating protein (GAP) for Sar1p (Yoshihisa *et al.* 1993). The release of Sar1p-GDP into the cytosol triggers the uncoating of the COPII vesicle prior to its fusion with the CGN.

The formation of COPI vesicles is based on similar principles but involves a different set of components. The precise role of these carriers remains controversial but it seems that they function mainly in retrograde transport within the Golgi and from the

Golgi to the ER. They may also play a role in anterograde transport through the Golgi (Orci *et al.* 1997).

As with COPII vesicles, COPI coat formation begins with the recruitment of a small GTPase to the donor membrane. In the case of COPI, this GTPase is ARF1 (ADP-ribosylation factor 1) which is activated at the appropriate membrane by ARF1 GEF (Serafini *et al.* 1991; Franco *et al.* 1998). Once associated with the membrane, ARF1 is able to recruit the COPI complex, a heptameric complex normally consisting of the proteins α, β, β', γ, δ, ε, and ζ-COP (Waters *et al.* 1991). Different isoforms of these subunits can substitute for them in the COPI coat but the purpose of this *in vivo* is unclear. As discussed in section 1.3, the COPI complex (specifically the γ subunit) is responsible for cargo recognition by an interaction with dilysine motifs. p24 proteins are also recognised by the COPI complex during cargo recognition (Dominguez *et al.* 1998). In addition, recruitment of the COPI complex causes membrane deformation and vesicle budding. Interestingly, the kinetics of COPI coat recruitment appears to be regulated by the presence of suitable cargo. The GTPase activity of ARF1 is stimulated by ARFGAP and the COPI complex. However, this stimulation is reduced in the presence of a synthetic peptide corresponding to the sorting signal of a p24 protein (Goldberg 2000). This suggests a model in which the presence of suitable cargo for COPI transport inhibits ARF1 GTPase activity thereby allowing more time for coat assembly, vesicle budding, and scission before ARF1 does hydrolyse GTP, dissociates from the membrane and triggers uncoating.

ARF1 is also involved in the initiation of clathrin coat formation. In the case of TGN to endosome transport, ARF1 recruits the clathrin adaptor protein AP-1 (Stamnes and Rothman 1993). Different adaptor proteins act at other sites of clathrin coat formation and the mechanism of their recruitment is not so clear although it is nucleotide dependent.

The adaptor proteins are responsible for binding to many of the proteins involved in clathrin coat formation and function, including clathrin itself, as well as to cargo proteins via various sorting signals found in the cytosolic tails of many membrane proteins. The recruitment to the membrane of clathrin, with its distinctive triskelion structure, followed by its polymerisation into a basket-like network of hexagons and polygons, results in the deformation of the membrane and vesicle budding. Unlike COPI and COPII vesicles, scission requires an additional accessory factor – the

GTPase dynamin. Dynamin forms a ring at the neck of the bud which somehow results in vesicle fission. Clathrin coat formation appears to be much more regulated than COPI or COPII vesicle budding, as reflected in the existence of a variety of regulatory factors such as amphyphysin, epsin, and synaptojanin (Kirchhausen 2000). Many of these proteins are lipid-modifying enzymes that alter the lipid composition or distribution of the forming vesicle, enabling additional proteins to be recruited and also affecting membrane curvature. A further difference between clathrin vesicles and COPI/COPII vesicles is the mechanism of uncoating. While COPI and COPII vesicles uncoat following the release of the small GTPases ARF1 and Sar1p respectively, clathrin uncoating requires the additional activity of the heat-shock proteins Hsc70 and auxilin (Kirchhausen 2000).

Nevertheless, although important differences exist between the different types of vesicle coats, the basic principles of coat recruitment, cargo selection, membrane deformation, scission and uncoating remain the same. Similarly, the fundamental mechanisms by which vesicles dock and fuse with their target membranes are also conserved across all vesicle transport steps.

1.5 Mechanisms of Vesicle Targeting

A wide variety of proteins are involved in ensuring that a vesicle docks and fuses specifically with its target membrane. These proteins fall into different families, individual members of which act at individual vesicle transport steps in the secretory and endocytic pathways, reflecting the conserved nature of vesicle targeting. This conservation also allowed a number of different experimental approaches towards studying vesicle targeting to converge and reveal a general model for docking and fusion. The different families of proteins will be discussed below but first we will consider the various approaches used to reveal their function.

1.5.1 *In vitro* reconstitution

The first approach to successfully identify factors essential for membrane transport was the reconstitution *in vitro* of intra-Golgi transport by James Rothman and co-workers. The approach made use of a membrane protein from the vesicular stomatitis virus, called VSV-G, which passes through the secretory pathway of infected cells. Early Golgi or ER-derived vesicles from a viral-infected cell line unable to process

VSV-G oligosaccharides properly were isolated, and then mixed with Golgi membranes from wild type cells in the presence of ATP and cytosol. Under these conditions, VSV-G protein oligosaccharides were processed properly, indicating that fusion had occurred between the vesicles and Golgi membranes (Fries and Rothman 1980). Using this assay, it was possible to identify a protein essential for membrane fusion, which was sensitive to the alkylating agent N-ethyl-maleimide (NEM) (Block *et al.* 1988; Wilson *et al.* 1989). This NEM-sensitive factor (NSF) was found to be a AAA-type ATPase. Co-factors for NSF called SNAPs (Soluble NSF Attachment Proteins) were also identified in this way (Clary *et al.* 1990). NSF and the SNAPs were later found to be essential for the function of the SNARE (SNAP Receptor) proteins (Sollner *et al.* 1993), a family of proteins already implicated in vesicle fusion by biochemical studies of synaptic vesicles.

1.5.2 Synaptic vesicle characterisation

Studies on purified synaptic vesicles had identified a membrane protein called VAMP/synaptobrevin which was able to form a complex with a related protein located at the pre-synaptic membrane – syntaxin (Trimble *et al.* 1988; Bennett *et al.* 1992). When these two proteins were found to form a complex with one another and with NSF and SNAP, the proteins were reclassified as SNAP Receptors (SNAREs), with VAMP and related proteins being designated vesicle- or v-SNAREs and syntaxin and its relatives being known as target membrane- or t-SNAREs (Sollner *et al.* 1993). Support for a role for the SNAREs in membrane fusion came from the observation that they could be cleaved by certain neurotoxins such as botulinum toxin which inhibit synaptic vesicle exocytosis (Montecucco and Schiavo 1995). The identification of proteins related to VAMP and syntaxin in other organelles of the secretory and endocytic pathways suggested they were involved in a general mechanism of membrane fusion (Sollner *et al.* 1993). The SNARE proteins were also found to be present in yeast and, through a genetic approach, it had already been shown that NSF and SNAPs were important for secretion.

1.5.3 Yeast genetic screens

Another approach key to uncovering the basic mechanisms of membrane targeting and fusion was the application of genetic screens in *S. cerevisiae*. This approach was

begun by Randy Schekman and Peter Novick and resulted in the isolation of many conditional mutants deficient in secretion (Novick *et al.* 1980). Two of the genes thereby identified were *SEC18* and *SEC17* which encode the yeast homologues of NSF and α-SNAP respectively. When these proteins were identified in Rothmans *in vitro* transport assay, it became clear that the mechanisms of membrane targeting were conserved and the different experimental approaches began to converge. Thus, when the yeast genetic screen revealed the importance of Sec4p for secretion (Salminen and Novick 1987), it soon became clear that this protein represented the founding member of a family of small ras-related GTPases, the rabs, different members of which existed on different organelles of the secretory and endocytic pathways in both yeast and mammalian cells (Chavrier *et al.* 1990).

Rab proteins are one of a number of protein families involved in vesicle transport. These different families act together to ensure correct and efficient vesicle targeting, with individual family members acting at specific steps in the secretory and endocytic pathways. We will now consider these protein families in turn and discuss their general roles in vesicle docking and fusion.

1.5.4 SNARE proteins

As discussed above, SNARE proteins were implicated in membrane fusion by a number of different observations. The original 'SNARE hypothesis' proposed the formation of a four-helical bundle between SNAREs on the vesicle membrane and SNAREs on the target membrane (Sollner *et al.* 1993; Rothman 1994). This complex would drive membrane fusion as well as providing the necessary specificity to ensure only the correct membranes fused.

Most SNARE proteins are short type II membrane proteins with a characteristic cytoplasmic α-helical region. An atypical class of SNAREs however is exemplified by SNAP-25 (a protein unrelated to the SNAP proteins discussed in section 1.5.1), which is required for fusion of synaptic vesicles with the pre-synaptic membrane. SNAP-25 is a peripheral membrane protein that associates with the plasma membrane by a palmitoyl group. It has two separate α-helices, both of which are involved in SNARE complex formation. Thus, the core SNARE complex formed between a synaptic vesicle and the plasma membrane consists of one helix contributed by the v-SNARE VAMP on the vesicle, and three helices from the t-SNAREs on the target

membrane – one from syntaxin and two from SNAP-25 (Fasshauer *et al.* 1998; Sutton *et al.* 1998). Other SNARE complexes consist of helices contributed by four separate SNARE proteins but the pattern of one helix from the vesicle membrane and three from the target membrane remains the same (Parlati *et al.* 2000).

The SNARE complex is a highly stable, SDS-resistant complex and it has been proposed that the energy of its formation is able to drive membrane fusion (Lin and Scheller 1997). While some have proposed that additional molecules are required to catalyse fusion, *in vitro* reconstitution experiments measuring liposome fusion suggest that the SNAREs alone can suffice to drive fusion (Weber *et al.* 1998; Nickel *et al.* 1999).

The specificity of SNARE associations has also been called into question. Immunoprecipitation of SNARE complexes from yeast cell extracts had suggested that SNARE proteins were more promiscuous in their interactions than originally thought (von Mollard *et al.* 1997). Liposome reconstitution experiments have again appeared to answer this criticism. SNARE-mediated liposome fusion *in vitro* only occurs with a limited number of SNARE combinations, all of which correspond to a genetically or biochemically inferred *in vivo* interaction (McNew *et al.* 2000).

Some questions over SNARE function remain. Once membrane fusion has occurred, the members of the SNARE complex end up on the same membrane. The complex must first be disassembled – a process catalysed by NSF and its co-factor α-SNAP. It is here that the energy input required for membrane fusion is made in the form of ATP hydrolysed by NSF. This disassembly of *cis* complexes by NSF and α-SNAP may represent the NSF-dependent 'priming' step required immediately prior to membrane fusion (Mayer and Wickner 1997). Once the *cis* complexes have been disassembled, the v-SNAREs must be sorted and recycled to their original compartments. Since the majority of SNAREs are integral membrane proteins, this recycling cannot occur simply via the cytosol and must occur through vesicular transport instead. This is a potential problem because, if SNARE proteins were continuously active, a v-SNARE recycling in a vesicle might direct that vesicle to simply fuse back with the membrane it was supposed to be recycling from. This implies that SNARE activity must be regulated in some way and/or that, *in vivo*, SNARE proteins alone are not responsible for determining the directionality and specificity of membrane fusion.

1.5.5 SM proteins

A potential family of SNARE regulators is the Sec1/Munc18 (SM) family. There are 7 members of this family in the human genome (Bock *et al.* 2001), which appear to tightly bind to various SNARE proteins and regulate SNARE function in some way (Toonen and Verhage 2003). Whether this regulation is positive or negative is unclear. Overexpression of many family members inhibits vesicle fusion (Thurmond *et al.* 1998) but, on the other hand, loss-of-function mutants also inhibit fusion suggesting that the activity of these proteins is essential in some way. To make matters more confusing, different results are obtained with different members of the family (Toonen and Verhage 2003). This may reflect a specific adaptation of individual family members to fulfil different roles at different transport steps. In support of this is the fact that, even though the structure of the SM family proteins is conserved, their mode of binding to SNAREs is not (Gallwitz and Jahn 2003; Toonen and Verhage 2003). Some SM proteins bind to monomeric SNAREs while others specifically recognise SNARE complexes. SM proteins also show interactions with other proteins involved in vesicular transport and may provide a link between these proteins and SNARE-mediated membrane fusion (Toonen and Verhage 2003).

1.5.6 Rab proteins

The Rab proteins are a family of small ras-related GTPases, directing particular vesicle transport steps in the secretory and endocytic pathways (Novick and Zerial 1997). The precise ways in which they direct traffic are not entirely clear and may differ from Rab to Rab but it is certain that they play an important role in promoting the specificity and efficiency of vesicle docking and fusion. The first family members to be identified were both in yeast: Sec4p, required for exocytosis, and Ypt1p, required for ER to Golgi transport (Gallwitz *et al.* 1983; Salminen and Novick 1987; Segev *et al.* 1988).

As with all small GTPases, the Rabs cycle between the GTP (active) and GDP (inactive) bound states. In addition, the Rabs undergo a change in their localisation in response to their nucleotide state; a prenyl group at the C-terminus allows Rabs to become membrane associated in the GTP-bound form. The C-terminal 'hypervariable' region of the Rabs determines which organelle the active Rab is recruited to (Chavrier *et al.* 1991).

The GTP cycle is influenced by a number of other proteins. Membrane-bound Guanine nucleotide Exchange Factors (GEFs) catalyse the exchange of GTP for GDP, thereby activating the Rab and stabilising its membrane association. GTPase Activating Proteins (GAPs) stimulate the GTPase activity of Rabs, promoting their conversion to the inactive, GDP-bound state. GDP-GTP Dissociation Inhibitors (GDIs) are able to extract inactive Rabs from membranes by binding to the prenyl group and masking it in the cytosol. This interaction is only disrupted by the appropriate GEF (possibly in conjunction with a GDI Displacement Factor, (Dirac-Svejstrup *et al.* 1997)) and the Rab GTP cycle begins again. The YIP1 family of proteins may also be involved in rab targeting through interactions with the prenyl groups at the rabs C-terminus (Calero *et al.* 2003).

The purpose of this cycle is to regulate the membrane recruitment of Rab proteins and, in turn, the membrane recruitment/membrane organisation of various Rab effector proteins. The Rab may act as a timer, the longer it is 'on', the more its effectors become organised and the more efficiently is specific membrane docking and fusion promoted (Rybin *et al.* 1996). Certainly, for homotypic membrane fusion such as Rab5 mediated endosome fusion, this makes sense with Rab5 acting as a master controller, determining the overall rate at which membrane fusion proceeds. For heterotypic membrane fusion however, this timer function makes less sense as it would be wasteful for a transport vesicle to form and then not be able to fuse with its target membrane due to inactivation of the Rab. Active Rabs are therefore recruited to vesicles, either during cargo selection or following budding. The Rab protein would then recruit its effectors and remain active until after docking or fusion (Goud *et al.* 1988). Alternatively, some Rab proteins appear to localise to target membranes and recruit effector proteins there (Siniossoglou *et al.* 2000; Wang *et al.* 2000). In this case, regulation of Rab activity would regulate the ability of incoming vesicles to dock and fuse with the target membrane. Rab activity on both the vesicle and target membranes may therefore produce a two-way signal mediating a specific interaction between the two membranes.

The functions of the Rab effector proteins are many and varied and will be discussed elsewhere in this thesis. In general, however, they facilitate the specific and efficient fusion of membranes by acting at many stages of vesicle transport from vesicle formation to motility to attachment to the target membrane. The Rabs themselves

organise these multiprotein complexes. This is not just important in vesicular transport but also in maintaining ordinary organelle identity and function. The presence of Rabs on specific organelles (and specific sub-domains of these organelles) may allow a specific network of protein-protein (and protein-lipid) interactions to be built up which define that compartment. Unlike SNARE proteins which, as discussed in section 1.5.4, must recycle via transport vesicles, Rab proteins recycle via the cytosol and can therefore be restricted to a single membrane compartment and give that compartment its specific identity. The fact that more than 60 Rab proteins have been identified in mammalian cells (Bock *et al.* 2001), supports the idea that Rab proteins are capable of providing such specificity.

1.5.7 Tethering proteins

The third family of proteins involved in vesicle targeting is much more diverse in structure and function than either the SNARE or Rab proteins.

The idea of a group of proteins mediating the initial tethering together of membranes and providing an extra layer of specificity for membrane recognition events arose from several lines of evidence. Not only were there doubts as to the specificity of SNAREs pairing, but there were also doubts over the spatial specificity of SNAREs. For example, it was observed that exocytosis in budding yeast is restricted to the bud tip even though the t-SNAREs involved in exocytosis, Sso1p and Sso2p, are localised all over the plasma membrane (Brennwald *et al.* 1994). This implied that other factors were involved in specific membrane recognition. The existence of such factors was also suggested by experiments in which giant-squid axons injected with tetanus toxin, thus cleaving and inhibiting SNARE proteins and membrane fusion, were actually found to accumulate synaptic vesicles docked at the pre-synaptic membrane (Hunt *et al.* 1994). Also, SNARE-mediated liposome fusion proceeds at a much slower rate than *in vivo* vesicle fusion suggesting that additional factors are required for maximum efficiency (Fasshauer *et al.* 2002). Finally, the existence of 'tethering factors' was also indicated for docking and fusion events at membranes other than the plasma membrane by freeze fracture electron micrographs of the Golgi apparatus. These showed the existence of fibrous elements linking vesicles to Golgi membranes, leading to the idea of 'vesicles on strings' (Orci *et al.* 1998).

The identity of these additional factors has been found in a variety of proteins, many of which were discovered as autoantigens in patients suffering from autoimmune diseases. Although diverse in structure, they can be broadly classified into two groups – those which form large, relatively stable multimeric complexes, and those which are long coiled-coil proteins (Whyte and Munro 2002; Gillingham and Munro 2003).

The multimeric complexes involved in membrane tethering may be sub-divided on the basis of certain structural similarities. A subgroup of 'quatrefoil' tethering complexes which assemble in multiples of four subunits, some of which have a conserved N-terminal domain, has recently been proposed (Whyte and Munro 2002). The remaining multimeric tethering complexes seem to bear no similarity either to the quatrefoil complexes or to each other.

The quatrefoil complexes are the exocyst, an octameric complex required for the delivery of Golgi-derived vesicles to the plasma membrane (TerBush *et al.* 1996), the VFT/GARP (Vps52/53/54 or Golgi Associated Retrograde Protein) a tetrameric complex implicated in retrograde transport from endosomes to the TGN (Conibear and Stevens 2000), and the octameric COG (Conserved Oligomeric Golgi) complex involved in recycling through the Golgi (Whyte and Munro 2001). Many of the subunits of these complexes have a short conserved coiled-coil domain near the N-terminus but the function of this domain has yet to be resolved.

The non-quatrefoil complexes are TRAPP (Transport Protein Particle) involved in ER to Golgi transport which can act as a GEF for the yeast Rab Ypt1p (Sacher *et al.* 2001), the Class C Vps complex involved with docking and fusion to the yeast vacuole and mammalian endosomes (also a GEF for the Rab Ypt7p) (Rieder and Emr 1997), and the Dsl1p complex which may tether retrograde COPI vesicles to the ER membrane (Andag *et al.* 2001; Reilly *et al.* 2001).

The evidence for all of these complexes involvement in membrane tethering is varied and ranges from genetic studies in yeast to *in vitro* transport assays which suggest a function in vesicle targeting upstream of the SNAREs. The precise functions of all these complexes remain unclear, as does the significance of their multimerisation compared to the second group of tethering factors – the long, coiled-coil proteins.

Many proteins fall into this second class, although they appear to have little similarity to one another apart from the presence of long regions of coiled-coil. Commonly, these coiled-coil domains are in the centre of the protein with separate domains at the

N- and C- termini. The coiled-coil domain consists of heptad repeats of amino acids where residues 1 and 4 in the sequence are hydrophobic. This results in a helix that coils together with other helices to form an extended, rod-like structure (Burkhard *et al.* 2001). For tethering factors, this coiled-coil is normally a homodimer. Breaks in the coiled-coil sequence may produce hinges, allowing flexibility in the protein which may be important functionally, for example by bringing two tethered membranes closer together for downstream fusion to occur.

So far, proteins of this type have only been identified at the Golgi and on endosomes. The Golgi tethering proteins (also known as Golgins) will be discussed later in section 1.6. The endosomal tethering proteins are best exemplified by EEA1 (Early Endosomal Antigen 1;(Christoforidis *et al.* 1999)). A central coiled-coil domain is flanked at the N- and C- termini by domains responsible for localising EEA1 to the early endosome. A FYVE domain at the C-terminus binds to phosphatidylinositol (3) phosphate (PtdIns(3)P) while both the N- and C-termini contain domains able to bind to the endosome-localised Rab protein Rab5. Both Rab5 binding and PtdIns(3)P binding appear to be necessary for correct EEA1 localisation (Simonsen *et al.* 1998). The presence of such binding sites at both ends of the protein may suggest a simple mechanism for EEA1 function in membrane recognition. The protein is certainly important for such events since anti-EEA1 antibodies greatly inhibit endosome fusion in an *in vitro* assay and EEA1-depleted cytosol can only support the assay if recombinant EEA1 is re-added (Simonsen *et al.* 1998). The precise role of EEA1 may be more complicated however, since it binds directly to the SNARE proteins syntaxin 6 and syntaxin 13, and exists on endosomes in large oligomeric complexes also containing NSF and the Rab5 effectors Rabaptin-5 and Rabex-5 (McBride *et al.* 1999; Simonsen *et al.* 1999).

It is therefore apparent that a variety of proteins, including SNAREs, Rabs, and tethering factors, act together to ensure the specific and efficient targeting of vesicles to their target membranes as well as giving identity to membrane sub-compartments. This is true throughout the secretory and endocytic pathways, including the Golgi apparatus.

1.6 The Golgi Matrix

It is estimated that, in the exocrine pancreas, more secretory material enters the Golgi apparatus from the ER every 5 minutes, than there is protein in the Golgi (Barr and Warren 1996). How does the Golgi apparatus maintain its identity and structural integrity in the face of such a high flux of material?

One suggestion is that the Golgi apparatus is not, in fact, an independent, stable organelle, rather that it arises *de novo* from the ER. Indeed, under experimental conditions in which ER to Golgi transport is blocked (for example, by treatment with the fungal metabolite brefeldin A or with dominant-negative forms of Sar1p) many Golgi resident proteins relocalise to the ER (Ward *et al.* 2001).

However, even under these conditions, some Golgi-like structures remain distinct from the ER (Seemann *et al.* 2000). These remnants contain proteins believed to be important for the structure of the Golgi. Under certain conditions, Golgi membranes could be extracted with detergent leaving a proteinaceous skeleton that retained the organisation of Golgi cisternae (Slusarewicz *et al.* 1994). The existence of such a Golgi matrix was also supported by electron microscopy studies of the Golgi apparatus showing proteinaceous links between the cisternae (Cluett and Brown 1992).

Important components of this matrix are a family of proteins identified using a functional assay for the post-mitotic reassembly of Golgi stacks which can be inhibited by the alkylation agent NEM. This family, known as the GRASPs (for Golgi Reassembly Stacking Proteins) consists of two proteins of molecular weight 55 and 65 KDa (Barr *et al.* 1997; Shorter *et al.* 1999). GRASP65 is localised to the *cis*-Golgi while GRASP55 is predominantly at the medial Golgi. Both proteins are peripheral membrane proteins on the cytosolic face of Golgi cisternae, and are associated with the membrane by N-terminal myristoylation (although how the proteins are targeted specifically to Golgi membranes remains unclear). Both proteins are phosphorylated during mitosis which may be significant for the mitotic disassembly of the Golgi apparatus. In addition, several Golgi-localised coiled-coil autoantigens (or Golgins) were found to be part of the Golgi matrix, suggesting an importance not only in vesicle tethering, but also in maintaining Golgi structure.

The golgins were originally defined as a family of distantly-related coiled-coil proteins localised to the Golgi and found as antigens in autoimmune patients (Chan and Fritzler 1998). Additional Golgi coiled-coil proteins have since been discovered and classified as golgins even though they are not known to be autoantigens. Although little is known about the precise functions of many of these proteins, a number of them have had their roles better defined.

1.6.1 *Cis*-Golgi golgins

Perhaps the best-characterised golgins to date are those at the *cis*-Golgi. p115 was originally identified using Rothman's transport assay as being required for transport through the Golgi (Waters *et al.* 1992). It is a peripheral membrane protein mainly localised to the CGN and the *cis*-Golgi (Waters *et al.* 1992; Nelson *et al.* 1998) and is shown by rotary shadowing to be a homodimer with a N-terminal globular head domain and a C-terminal coiled-coil domain, and a length of 45nm (Sapperstein *et al.* 1995). A short acidic patch is located at the extreme C-terminus of the protein. The budding yeast homologue of p115, Uso1p, is similar in structure but is much larger in size (approximately 150nm; Yamakawa *et al.* 1996).

Studies on Uso1p provided insight into its function as a tethering factor. Along with Ypt1p (yeast Rab1), it is required for the docking of ER-derived COPII vesicles with Golgi membranes in an *in vitro* assay (Barlowe 1997; Cao *et al.* 1998). This would seem to occur upstream of SNARE function, since yeast carrying temperature-sensitive mutations in ER-to-Golgi SNARE proteins are still able to dock ER-derived vesicles with the *cis*-Golgi, and overexpression of the wild type SNAREs can compensate for mutations in USO1 and YPT1 (Sapperstein *et al.* 1996).

Recent studies on mammalian p115 have suggested a direct interaction with SNAREs, thereby linking tethering to fusion. Not only could p115 bind to certain early Golgi SNARE proteins but an *in vitro* assay showed that it could actually stimulate SNARE complex assembly (Shorter *et al.* 2002). Additionally, it has been shown that p115 is important for COPI, as well as COPII, vesicle docking, and that it is involved in the stacking of Golgi cisternae following mitosis (Sonnichsen *et al.* 1998; Shorter and Warren 1999). To accomplish these functions, p115 interacts with two additional golgins at the *cis*-Golgi: GM130 and giantin.

GM130 was originally identified (as golgin-95) using antisera from an autoimmune disease patient and, subsequently, by raising antisera against solubilised Golgi matrix

(Fritzler *et al.* 1993; Nakamura *et al.* 1995). Localised to the *cis*-Golgi, GM130 is predicted to contain extensive regions of coiled-coil forming a homodimer, and has a basic domain of 75 amino acids at its N-terminus, which binds to the C-terminal acidic patch of p115 (Nakamura *et al.* 1997). This interaction is disrupted at the onset of mitosis by the phosphorylation of GM130 on serine25 by Cdk1 (Lowe *et al.* 1998). This is thought to be crucial for the mitotic breakdown of the Golgi apparatus due to the continued budding of transport vesicles while p115-GM130 mediated vesicle tethering is inhibited. GM130 is targeted to *cis*-Golgi membranes by its tight binding to GRASP65, an interaction which requires the extreme C-terminus of GM130 (Barr *et al.* 1998).

Giantin, on the other hand, is an integral membrane protein of 400KDa, with a C-terminal transmembrane domain and a very large cytoplasmic domain containing extensive regions predicted to form coiled-coil (Linstedt and Hauri 1993). It is localised to the edges of the Golgi stack and on COPI vesicles and also binds p115 via its N-terminus (Sonnichsen *et al.* 1998). Two other integral membrane golgins, golgin-84 and CASP, have recently been identified with transmembrane domains closely related to that of giantin but with little sequence homology outside of this region (Bascom *et al.* 1999; Gillingham *et al.* 2002). The functional significance of some golgins having transmembrane domains while others (such as p115 and GM130) are peripheral membrane proteins associated either weakly or strongly with the membrane, is unclear. However, the yeast homologue of CASP, Coy1p, shows a genetic interaction with the SNARE protein Gos1p, an interaction which is lost if the conserved transmembrane domain is mutated (Gillingham *et al.* 2002). This implies that CASP and, by extension, golgin-84 and giantin, could be involved in transmitting membrane tethering events to SNARE-mediated fusion events.

How do p115, GM130, and giantin act together to mediate docking events at the *cis*-Golgi? The presence of giantin in COPI vesicles and GM130 on Golgi membranes, and the ability of both proteins to bind p115, suggests a model in which COPI vesicles are linked to their target membranes by a giantin-p115-GM130 ternary complex (Sonnichsen *et al.* 1998) - although this wouldn't apply to ER-targeted COPI vesicles. Such a model is supported by an *in vitro* assay in which the docking of COPI vesicles with Golgi membranes is inhibited if the vesicles are pre-incubated with anti-giantin antibody but is unaffected if the Golgi membranes are pre-incubated with anti-giantin.

Anti-GM130 antibodies, on the other hand, only inhibit the assay if they are pre-incubated with Golgi membranes but have no effect when pre-incubated with COPI vesicles. The same assay shows a dependence on p115 since addition of increasing amounts of the protein increases the rate of vesicle docking (Sonnichsen *et al.* 1998). The idea of a giantin-p115-GM130 ternary complex is contradicted however by evidence that GM130 and giantin compete for the same binding site on p115 (Linstedt *et al.* 2000). Also, studies on trafficking between the ER and the Golgi suggest that the three proteins act at kinetically distinct stages since anti-p115 antibodies inhibit transport at the CGN stage while anti-GM130 and anti-giantin inhibition occurs at the Golgi - with giantin inhibition occurring later than GM130 inhibition (Alvarez *et al.* 2001).

Since p115, GM130, and giantin are implicated in such a variety of different processes (including both retrograde and anterograde transport steps as well as cisternal stacking), it is possible that they act in different ways during different events. The effects of antibody inhibition may vary depending on precisely which process is being measured in a particular assay. In any case, the interactions of these golgins, both with each other and with other proteins, is clearly important for membrane tethering events at the *cis*-Golgi.

1.6.2 Late Golgi golgins

Far less is known about tethering events at later Golgi compartments. With the exception of GRASP55, no medial-Golgi components of the Golgi matrix have been characterised (Shorter *et al.* 1999). Several coiled-coil proteins localising to the *trans*-Golgi and TGN have been identified although their precise roles remain unclear. These *trans*-Golgi proteins include golgin-245 and golgin-97 (both identified as antigens in autoimmune patients), and the yeast protein Imh1p (Kooy *et al.* 1992; Fritzler *et al.* 1995; Griffith *et al.* 1997; Tsukada *et al.* 1999). A key feature of these proteins is the presence of a 50 amino acid sequence known as the GRIP domain (for golgin-97, Ran-binding protein 2α, Imh1, and p230/golgin-245) (Barr 1999; Kjer-Nielsen *et al.* 1999; Munro and Nichols 1999). The GRIP domain alone is sufficient for targeting to the *trans*-Golgi for which an invariant tyrosine residue is essential (McConville *et al.* 2002). The domain was originally suggested to have rab6 binding properties although, more recently, it has been implicated in binding to ARL1, a

Golgi-localised member of the ARF-like GTPase family (Van Valkenburgh *et al.* 2001). Little is known about the function of ARLs, but the interaction of ARL1 with GRIP domain proteins is dependent upon the conserved tyrosine residue essential for the Golgi targeting of these proteins (Van Valkenburgh *et al.* 2001).

IMH1 shows a genetic interaction with YPT6, the yeast homologue of rab6. Overexpression of Imh1p suppresses the temperature sensitive growth of yeast strains lacking Ypt6p and deletion of both IMH1 and YPT6 leads to a severe growth defect (Tsukada *et al.* 1999). As yet, however, no physical interaction has been found between Imh1p and Ypt6p. Nevertheless, it is clear that there is some sort of interaction between golgins and rab family members, as indicated by the IMH1/YPT6 and USO1/YPT1 data discussed above. Rab proteins also show an interaction with multimeric membrane tethering complexes since *RIC1* (the GEF for *YPT6*) shows synthetic lethality with *DOR1*, a component of the yeast COG complex (Whyte and Munro 2001). We shall now consider, therefore, those rab family members that are known to localise to the Golgi apparatus.

1.6.3 Golgi-localised rab proteins

Many of the 60-plus rab proteins in the human genome are reported to localise to the Golgi apparatus. They are believed to regulate a variety of transport steps to, through, and from the Golgi in both anterograde and retrograde directions.

One of the best-characterised Golgi-localised rabs is rab1 and its budding yeast homologue Ypt1p. Rab1 (of which there are 2 isoforms, a and b) is ubiquitously expressed in mammalian cells and is localised to the CGN and the *cis*- most cisternae of the Golgi stack (Plutner *et al.* 1991). Overexpression of a dominant-negative form of rab1, locked in its GDP-bound form was able to inhibit ER to Golgi transport and also early intra-Golgi transport (Tisdale *et al.* 1992; Nuoffer *et al.* 1994). Ypt1p is also important for the same transport steps in yeast, as evidenced by its requirement for the *in vitro* COPII vesicle docking assay described in section 1.6.1 and its genetic interactions with a variety of other proteins involved in ER to Golgi transport – deletion of Ypt1p is suppressed by overexpression of the SNARE proteins Sed5p, Bet1p, Bos1p, and a mutant form of the SM protein Sly1p (Dascher *et al.* 1991; Lian *et al.* 1994). Although Ypt1p is localised to both vesicles and target Golgi membranes, its activity seems to be required at the target membrane, since a Ypt1p

mutant which blocks ER to Golgi traffic *in vivo*, only affects an *in vitro* assay when present on acceptor membranes instead of wild type protein. Vesicles bearing the mutant Ypt1p are still competent for docking and fusion (Cao and Barlowe 2000). Ypt1p has been implicated in both the recruitment of Uso1p to membranes and in the activation of t-SNAREs prior to membrane fusion (Lupashin and Waters 1997; Cao *et al.* 1998).

A second rab protein, rab2, is also required for ER to Golgi transport in metazoan cells (Tisdale *et al.* 1992; Tisdale and Balch 1996). There does not appear to be a homologue in yeast, indicating that metazoan transport pathways are more complicated and differently regulated. This would partially explain the large evolutionary expansion of rab proteins between yeast and mammals (from 12 to over 60 members) since there is not a corresponding increase in the number of secretory/endocytic organelles in mammals (Bock *et al.* 2001).

The Golgi-localised rab protein rab6, on the other hand, does have a homologue in budding yeast, Ypt6p. There are, however, 3 isoforms of rab6 in mammalian cells – rab6a, a', and b. Rab6a and a' differ by only 3 amino acids, are generated by alternative splicing, and are ubiquitously expressed (Echard *et al.* 2000). Rab6b is more divergent and is primarily expressed in neurons (Opdam *et al.* 2000). All 3 proteins are localised to the Golgi apparatus and the TGN although it has been suggested they may play different roles *in vivo*.

Rab6a appears to be involved in retrograde Golgi-to-ER transport since overexpression of wild type or GTP-restricted rab6a stimulates the redistribution of Golgi-localised glycosylases to the ER (Martinez *et al.* 1994; Martinez *et al.* 1997) while a GDP-restricted mutant blocks this recycling pathway and also inhibits the transport of Shiga toxin from the Golgi to the ER (Girod *et al.* 1999). Recycling of other proteins, such as the KDEL receptor, is not affected by rab6a. This pathway would appear to be COPI-independent, since anti-COPI antibodies and ARF1 mutants do not affect the retrograde transport of Shiga toxin or glycosylases while they do inhibit the recycling of the KDEL receptor (Girod *et al.* 1999).

Rab6a', on the other hand, appears to be involved in endosomes to TGN transport, as assessed by the inhibitory effects of GDP-locked rab6a' on the transport of Shiga toxin to the TGN - although GDP-locked rab6a partially inhibits this assay as well (Mallard *et al.* 2002). Thus, the retrograde transport of proteins from endosomes,

through the Golgi, to the ER may be mediated by the sequential activity of rab6a' and rab6a. To date, however, no specific effectors of either rab6 isoform have been identified with the exception of rabkinesin-6, a motor protein proposed to interact specifically with rab6a in Golgi to ER transport (Echard *et al.* 1998). More recently, however, the veracity of this interaction and the involvement of rabkinesin-6 in transport events has been called into question (Hill *et al.* 2000).

Rab6 proteins are, nevertheless, clearly involved in transport steps in and around the Golgi, along with rabs 1 and 2 and other Golgi-localised rabs. The question remains of how rabs regulate these processes and how they interact with other proteins involved in transport events at the Golgi apparatus.

1.7 Characterisation of Rab-Effector Complexes

As discussed above, many proteins are known to be involved in regulating vesicle transport events through the Golgi apparatus, and in maintaining Golgi structure. There is evidence to suggest that many of these proteins, such as the rabs and golgins, may interact with each other. For example, the vesicle tethering factor p115 and rab1 and their yeast homologues, Uso1p and Ypt1p, clearly act at the same stage of the secretory pathway. Not only do USO1 and YPT1 show a strong genetic interaction (not in itself an indicator of a physical interaction) but the recruitment of Uso1p to membranes in an *in vitro* COPII vesicle docking assay shows a clear dependence on the amount of Ypt1p added to the assay (Cao *et al.* 1998).

The interaction of rabs with coiled-coil tethering proteins has already been found in other cases. The endosomal rab, rab5, interacts with several effector molecules in a nucleotide-dependent manner, including EEA1 and rabaptin-5, which are required for the docking and fusion of endosomes (Christoforidis *et al.* 1999). These effectors in turn interact with other proteins such as the SNARE molecules and SM proteins involved in endosome fusion (McBride *et al.* 1999; Nielsen *et al.* 2000).

It therefore seems likely that Golgi-localised rab proteins act similarly to recruit members of the golgin family as effectors. These effectors are likely to include proteins such as p115 and GM130 but, given the large number of effectors found to interact with rab5, may also include as yet uncharacterised proteins.

I therefore set out to characterise rab-effector complexes located at the Golgi apparatus using a combination of approaches, including yeast two-hybrid and

pulldowns from cytosol. In this thesis, I present data identifying and characterising a number of effectors of rabs 1, 2, 6, and 33b. In addition, I characterise a family of serine/threonine kinases that interact with the *cis*-Golgi golgin GM130, itself a rab effector. Together, this data provides insight into the various ways in which rab proteins and their effectors control aspects of membrane trafficking events at the Golgi apparatus.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Reagents

Reagents were obtained from Sigma (Sigma-Aldrich, Poole, UK), BDH (BDH Laboratory Supplies, Poole, UK), Fisher (Fisher Scientific, Loughborough, UK), or Merck (Darmstadt, Germany) unless stated in the text.

2.1.2 Equipment

A list of commonly used equipment and its suppliers is given in the table below:

Equipment	Description and manufacturer
Benchtop centrifuge	Eppendorf Centrifuge 5417C
Benchtop refrigerated centrifuge	Eppendorf Centrifuge 5417R
Cell Culture Incubators	Heraeus HeraCell
Cell Culture Safety Hoods	Heraeus HeraSafe
Centrifuge	Heraeus Multifuge 3 L-R
Film Developer	Kodak X-OMAT 2000 Processor
Heating block/mixer	Eppendorf Thermomix Compact
Incubators	Heraeus Function Line
Shakers	Infors AG Multitron
Sonicator	Bandelin Sonopuls
SW28 rotor	Beckman
SW40 rotor	Beckman

Transilluminators	UVP 2UV Transilluminator
	UVP Mini Darkroom and UV transilluminator
	UVP Gel Documentation System
Ultracentrifuge	Beckman Optima™ LE-80K
UV/Visible Spectrophotometer	Amersham-Pharmacia Ultrospec 3000pro
Waterbath	Haake DC10

2.1.3 Solutions

The composition of many common solutions not given in the text is given in the table below. The solvent is water, unless otherwise stated. Many of these solutions were prepared by Marianne Siebert and other technical staff of the Cell Biology Department, MPI For Biochemstry.

Solution	Composition
DNA Loading Dye (6x)	0.25% (w/v) bromophenol blue
	40% (w/v) sucrose in TE
LB	10 g/L Bacto-tryptone
	5 g/L Bacto-yeast extract
	10 g/L NaCl
LB-Agar	LB plus 15 g/L Bacto-agar
Milk-PBS (Blocking Buffer)	4% milk powder in PBS plus 0.2% (w/v) Tween-20
PBS	8 g/L NaCl
	0.2 g/L KCl
	1.44 g/L Na_2HPO_4
	0.24 g/L KH_2PO_4
	(pH 7.4)
Ponceau	0.2% Ponceau red in 1% acetic acid
RIPA-buffer	10 mM Tris-HCl pH 7.5
	150 mM NaCl
	1% (w/v) Triton-X 100
	0.1% (w/v) SDS
	1% (w/v) sodium deoxycholate

SDS-PAGE Lower Buffer (4x)	181.72 g/L Tris base
	4 g/L Na.SDS
SDS-PAGE Running Buffer (10x)	30.2 g/L Tris base
	188 g/L glycine
	10 g/L Na.SDS
SDS-PAGE Sample Buffer (3x)	For 100 ml:
	2.3 g Tris base
	9.0 g SDS
	30 ml glycerol
	Adjust pH to 6.8 with HCl, then adjust volume to 90 ml with dH_2O
	50 mg bromophenol blue
	Add 10% ß-mercaptoethanol prior to use
SDS-PAGE Transfer Buffer	1x SDS-PAGE running buffer
	10% methanol
SDS-PAGE Upper Buffer (4x)	60.6 g/L Tris base
	4 g/L Na.SDS
TAE (50x)	242.4 g/L Tris base
	57.2 ml/L glacial acetic acid
	100 ml/L 0.5M EDTA, pH 8.0
TE	10 mM Tris-HCl pH 8.0
	1 mM EDTA
TNE	10 mM Tris-HCl pH 7.4
	150 mM NaCl
	1 mM EDTA
	1% (w/v) IGEPAL
TNTE	10 mM Tris-HCl pH 7.5
	150 mM NaCl
	0.3% (w/v) Triton-X 100
	5 mM EDTA

2.1.4 Plasmids

The plasmids used in this thesis are listed in the table below. Unless otherwise stated, they were generated by myself in the course of this work. CP indicates plasmids made by Christian Preisinger, EF by Evelyn Fuchs, FAB by Francis Barr, JS by Julia Schaletzky.

Plasmid Name	Vector	Insert and Notes
pBS21	pQE32	p115 full length
pBS24	pQE32	GM130 full length
pBS25	pQE32	GM130ΔN433
pBS74	pGBT9	p115 full length
pBS81	pACT2	p115 full length
pBS82	pACT2	p115 aa's 1-606
pBS83	pACT2	p115 aa's 772-959
pBS84	pACT2	GM130 full length
pBS85	pACT2	GM130ΔN433
pBS86	pACT2	GM130ΔC271
pBS102	pGAT2	Rab1Q70L
pBS103	pGAT2	Rab2Q65L
pBS113	pGBT9	Rab2 wt
pBS114	pGBT9	Rab2Q65L
pBS119	pGBT9	Rab1Q70L
pBS137	pACT2	p115 aa's 124-437
pBS147	pGAT2	Rab6aQ72L
pBS150	pGBT9	Rab5Q79L
pBS151	pGBT9	Rab6aQ72L
pBS154	pGBT9	Rab33bQ92L
pBS157	pACT2	p115 aa's 1-437
pBS158	pACT2	p115 aa's 124-606
pBS166	pACT2	p115 aa's 1-123
pBS167	pACT2	Golgin-45 aa's 123-282
pBS170	pACT2	Golgin-45 full length
pBS171	pEGFP-C2	Golgin-45 full length

pBS173	pACT2	Golgin-45 aa's 1-282
pBS174	pACT2	Golgin-45 aa's 123-403
pBS179	pACT2	Golgin-45 aa's 1-122
pBS180	pACT2	Golgin-45 aa's 283-403
pBS201	pACT2	p115 aa's 607-772
pBS216	pACT2	p115 aa's 1-772
pBS220	pACT2	GM130 aa's 433-679
pBS221	pACT2	GM130 aa's 679-986
pBS258	pQE32	YSK1 full length
pBS329	pEGFP-C2	YSK1 full length
pBS331	pGBT9	YSK1 full length
pBS375	pEGFP-C2	MTMR6
pBS378	pEGFP-N1	MTMR6
pBS383	pEGFP-C2	Centaurinß2
pBS384	pEGFP-N3	Centaurinß2
pBS394	pcDNA3.1mycA	GM130 full length
pBS407	pcDNA3.1mycA	GM130S25D
pBS417	pFBT9	YSK1 full length
pBS449	pFBT9	YSK1 aa's 1-302
pBS457	pACT2	GM130ΔN74
pBS460	pACT2	GM130 aa's 75-271
pBS469	pGEX5X1	GM130 aa's 75-271
pBS481	pGBT9	Rab6a'Q72L
pBS482	pGBT9	Rab6a'T27N
pBS484	pFAT2	Rab6a'Q72L
pBS485	pFAT2	Rab6a'T27N
pBS501	pFAT2	Rab6a'D129N
pBS502	pGAT2	Rab6aD129N
pBS506	pACT2	$p150^{glued}$ aa's 1-904
pBS507	pACT2	$p150^{glued}$ aa's 540-1278
pBS512	pFBT9	YSK1 aa's 1-270
pBS513	pFBT9	YSK1 aa's 20-302
pBS517	pcDNA3.1mycA	YSK1 aa's 303-426

pBS518	pFBT9	YSK1 aa's 303-426
pBS523	pACT2	$p150^{glued}$ aa's 1-539
pBS524	pACT2	$p150^{glued}$ aa's 540-904
pBS525	pACT2	$p150^{glued}$ aa's 905-1278
pBS529	pGBT9	Rab6a wt
pBS530	pGBT9	Rab6aQ72L
pBS531	pGBT9	Rab6aT27N
pBS535	pFAT2	Rab6aT27N
pBS539	pEGFP-C2	BicD1 aa's 1-703
pBS540	pEGFP-C2	BicD1 aa's 704-975
pBS541	pEGFP-C2	BicD2 aa's 1-705
pBS542	pEGFP-C2	BicD2 aa's 706-824
pBS543	pQE32	$p150^{glued}$ aa's 540-1278
pBS568	pEGFP-C2	BicD2 full length
pBS590	pcDNA3.1mycA	MTMR6
pBS591	pcDNA3.1mycA	Centaurinß2
pBS603	pQE32	GOPC
pBS606	pACT2	GOPC
pBS607	pEGFP-C2	GOPC
pBS608	pcDNA3.1mycA	GOPC
pBS613	pFAT2	Rab33b wt
pBS614	pFAT2	Rab33bQ92L
pBS624	pQE32	BicD2 aa's 706-824
pBS625	pcDNA3.1mycA	GM130ΔN74
pBS626	pACT2	BicD1 aa's 1-703
pBS627	pACT2	BicD1 aa's 704-975
pBS628	pACT2	BicD2 aa's 1-705
pBS629	pACT2	BicD2 aa's 706-824
pFB191	pGAT2	Rab1 wt, FAB
pFB193	pGAT2	Rab2 wt, FAB
pFB206	pGAT2	Rab6a wt, FAB
pFB208	pGAT2	Rab11 wt, FAB
pFB226	-	NAGT-I-GFP, FAB

pFB648	pFBT9	GRASP55, CP
pFB732	pGEX5X1	Golgin-45 aa's1-122, FAB
pFB1072	pVL1393N6H	YSK1 full length, CP
pFB1289	pACT2	$p150^{glued}$ full length, FAB
pFB1346	pFBT9	MST3, FAB
pFB1350	pEGFP-C2	MST3, FAB
pFB1351	pcDNA3.1mycA	MST3, FAB
pFB1360	pFBT9	MST4, FAB
pFB1364	pEGFP-C2	MST4
pFB1365	pcDNA3.1mycA	MST4, FAB
pFB1366	pAcHis	MST4, FAB
pFB1374	pEGFP-C2	BicD1 full length, FAB
pFB1376	pACT2	BicD1 full length, FAB
pFB1380	pACT2	BicD2 full length, FAB
pFB1381	pQE32	BicD2 full length, FAB
pFB1440	pGAT2	Rab11Q70L, JS
pFB1518	pcDNA3.1mycA	YSK1 full length, FAB
pFB1519	pcDNA3.1mycA	YSK1K49R, FAB
pFB1520	pcDNA3.1mycA	YSK1 aa's 1-270, FAB
pFB1521	pcDNA3.1mycA	YSK1 aa's 1-302, FAB
pFB1522	pcDNA3.1mycA	YSK1 aa's 20-302, FAB
pFB1523	pcDNA3.1mycA	YSK1 aa's 275-426, FAB
pFB1606	pcDNA3.1mycA	MTM1, FAB
pFB1648	pAcHis	Rabkinesin-6, FAB
pFB1803	pACT2	GOPC aa's 256-454, EF
pFB1809	pEGFP-C2	GOPC aa's 1-255, EF
pFB1810	pEGFP-C2	GOPC aa's 142-454, EF
pFB1811	pEGFP-C2	GOPC aa's 1-141, EF
pFB1812	pEGFP-C2	GOPC aa's 142-255, EF
pFB1813	pEGFP-C2	GOPC aa's 256-454, EF
pFB1814	pFBT9	GOPC aa's 1-255, EF
pFB1815	pFBT9	GOPC aa's 142-454, EF
pFB1816	pFBT9	GOPC aa's 1-141, EF

pFB1817	pFBT9	GOPC aa's 142-255, EF
pFB1880	pACT2	Rab6a'Q72L, EF
pFB1881	pACT2	Rab6aQ72L, EF

2.1.5 siRNA oligonucleotides

The RNA oligonucleotides used in the course of this work for siRNA-mediated depletion of specific mRNAs are listed in the table below along with the target sequence. Oligonucleotides were obtained from Dharmacon Inc. Oligos were generally designed to a target sequence of AA(N19)TT with the sense oligo sequence reading (N19)dTdT, and the antisense oligo reading (N19 reverse complemented)dTdT.

Target Gene	Target Sequence
Human GM130	AA*CCCTGAGACAACCACTTCT*TT
Human golgin-45	AA*TCCGAGGAGCAGGAGATGG*AA
Human lamin-A	AA*CTGGACTTCCAGAAGAACATC*
Human p115	AA*GCCGAGACGATTCAAAAGC*TT
Human rab1	AA*CAGCGAAGGAATTTGCTGA*TT
Human rab2	AA*ATCATGCTTATTGCTACAG*TT

2.1.6 Antibodies

The antibodies used in the course of this work are listed in the tables below, along with their typical dilutions used for western blotting and immunofluorescence.

2.1.6.1 Primary antibodies

Name	Species	Antigen	Dilution for western blots	Dilution for immuno-fluorescence	Source
1E7	Mouse	Rat rab1b C-terminal peptide	1:200 (culture supernatant)	1:250 (purified) 1:2 (culture supernatant)	Gift from Angela Barnekow, Münster, Germany
9E10	Mouse	c-myc	1:1000	1:1000	-
Biogenes #4256	Rabbit	His-human YSK1	1:1000	1:1000	-
FBA34	Sheep	His-rat GRASP55	1:2000	1:2000	-
FBA35	Sheep	His-GFP	1:2000	1:2000	-
FBA36	Sheep	Human golgin-97 aa's 589-769	1:500	1:500	-
FBA37	Sheep	His-rat p115 aa's 772-959	1:2000	1:2000	-
FBA40	Sheep	His-rat GM130	1:2000	1:2000	-
FBA44	Rabbit	His-GST-mouse golgin-45	1:1000	1:1000	-
MPI#1111	Rabbit	His-GST-human rab2	-	1:1000	-
NN5-1	Rabbit	Rat GM130	1:1000	1:1000	Nobohiro Nakamura, Kanazawa, Japan
α–c-myc (A-14)	Rabbit	Human c-myc C-terminus	1:150	1:150	Santa Cruz Biotech

α-p50/ dynamitin	Mouse	Human p50/dynamitin	1:100	1:100	BD Biosciences, Pharmingen
α–dynein (intermediate chain) (70.1)	Mouse	Chicken cytoplasmic dynein	1:100	-	Sigma
α–golgin-160	Rabbit	Human golgin-160	1:1000	1:1000	-
α–golgin-97	Mouse	Human golgin-97	-	1:100	Molecular Probes
α–Histidine	Mouse	Hexahistidine epitope tag	1:1000	-	Dianova
α–Lamin	Mouse	Human Lamin	1:1000	1:1000	-
α–p150^{glued}	Mouse	Rat p150^{glued}	1:100	1:100	BD Biosciences, Pharmingen
α–rab6 (C-19)	Rabbit	Human rab6 C-terminal peptide	-	1:100	Santa Cruz Biotech
α–tubulin	Mouse	Tubulin	1:1000	1:1000	Sigma
α–YSK1 (C-19)	Goat	Human YSK1 C-terminal peptide	1:100	1:100	Santa Cruz Biotech
α–YSK1 (N-19)	Goat	Human YSK1 N-terminal peptide	1:100	1:100	Santa Cruz Biotech

2.1.6.2 Secondary antibodies

Name	Species	Antigen	Dilution for western blots	Dilution for immuno-fluorescence	Source
Cy3 -α-mouse	Donkey	Mouse IgG	-	1:1000	Jackson Laboratories
Alexa-488-α-mouse	Donkey	Mouse IgG	-	1:1000	Jackson Laboratories
HRP-α-mouse	Sheep	Mouse IgG	1:2000	-	Amersham-Pharmacia
Cy3-α-rabbit	Donkey	Rabbit IgG	-	1:1000	Jackson Laboratories
Alexa-488-α-rabbit	Donkey	Rabbit IgG	-	1:1000	Jackson Laboratories
HRP-α-rabbit	Donkey	Rabbit IgG	1:2000	-	Jackson Laboratories
Cy3-α-sheep	Donkey	Sheep IgG	-	1:1000	Jackson Laboratories
Cy2-α-sheep	Donkey	Sheep IgG	-	1:1000	Jackson Laboratories
HRP-α-sheep	Donkey	Sheep IgG	1:10000	-	Jackson Laboratories

2.2 Bacterial Methods

2.2.1 Growth and maintenance of *E.coli*

Bacteria were grown in LB medium containing an appropriate antibiotic for selection (typically either 100µg/ml ampicillin, 50µg/ml kanamycin, or 34µg/ml chloramphenicol). Short-term storage was on LB-agar plates plus antibiotic at 4°C.

2.2.2 Bacterial strains

The following *E.coli* strains were used in the course of this work:

Strain	Genotype	Use
XL1-blue	F'::Tn10 proA$^+$B$^+$ lacI^q Δ(lacZ)M15/recA1 endA1 gyrA96 (Nal^r) thi hsdR17 (r_K^- m_K^+) glnV44 relA1 lac	General cloning applications
GM2163	F$^-$ ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 rpsL136 dam13::Tn9 xylA5 mtl-1 thi-1 mcrB1 hsdR2	Cloning of non-methylated DNA for digestion with Dam or Dcm-sensitive restriction enzymes
BL21(DE3)*	F$^-$ ompT gal [dcm] [lon] hsdS$_B$ (r_B^- m_B^-; an E.coli B strain) with DE3, a λ prophage carrying the T7 RNA polymerase gene	Recombinant protein expression
JM109*	F' traD36 proA$^+$B$^+$ lacI^q Δ(lacZ)M15/ Δ(lac-proAB) glnV44 e14$^-$ gyrA96 recA1 relA1 endA1 thi hsdR17	Recombinant protein expression
TOP10	F$^-$ mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 deoR araD139 Δ(ara-leu)7697 galU galK rpsL (Str^R) endA1 nupG	TA-cloning of PCR products

*In some cases, BL21(DE3) and JM109 strains carrying the pRIL plasmid (Stratagene) were used. This plasmid encodes the tRNA genes for rare arginine, isoleucine, and leucine codons and conveys chloramphenicol resistance.

2.2.3 Preparation and transformation of chemically competent bacteria

To prepare chemically competent *E.coli*, a single colony was picked from a fresh plate and used to inoculate 50 ml of LB (with selection, if necessary) and grown overnight at 37°C with shaking. 1 ml of overnight culture was then used to inoculate 100 ml of fresh LB and the cells were grown at 37°C until OD_{600}= 0.5. The culture was then chilled on ice for 15 minutes before being transferred to sterile centrifuge tubes and centrifuged at approximately 5000 x g for 10 minutes at 4°C. The supernatant was discarded and the cell pellet resuspended in 0.4 total culture volume of TfbI (30 mM KOAc, 100 mM $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% (v/v) glycerol, pH adjusted to 5.8 with dilute acetic acid). The cells were incubated on ice for 15 minutes and then pelleted as before. The cell pellet was then resuspended in 0.04 volume TfbII (10 mM MOPS, 75 mM $CaCl_2$, 10 mM $RbCl_2$, 15% (v/v) glycerol, pH adjusted to 6.5 with dilute NaOH). After storing on ice for 15 minutes, the cells were aliquoted into 50 µl aliquots in sterile Eppendorfs, frozen in dry ice or liquid nitrogen, and stored at –80°C until needed.

For transformation, aliquots were thawed on ice before adding the DNA to them. In general, 5 µl of a ligation reaction was used for a transformation, or 1 µl of plasmid DNA. The cells and DNA were mixed and left on ice for 15 minutes before being heat-shocked in a 42°C water bath for 45-90 seconds. Cells were placed back on ice for 1 minute before the addition of either 250 µl (ligation transformation) or 700 µl (plasmid transformation) LB. The cells were then allowed to recover for 30-60 minutes at 37°C with shaking before being plated on LB-agar plates containing an appropriate selection antibiotic with a glass spreader. All of a ligation transformation was plated while only 80 µl of a plasmid transformation was plated. Plates were then incubated at 37°C.

2.2.4 Preparation and transformation of electrocompetent bacteria

To prepare electrocompetent *E.coli*, a single colony was picked from a fresh plate and used to inoculate 5 ml of LB and grown overnight at 37°C with shaking. 2.5 ml of the

overnight culture was then used to inoculate 500 ml of fresh LB in a 2 L flask and grown at 37°C until an OD_{600} of between 0.5 and 0.6. The culture was then chilled on ice for 15 minutes before being transferred to sterile centrifuge tubes and centrifuged at approximately 5000 x g for 10 minutes at 4°C. The supernatant was removed and the cells resuspended in 5 ml of sterile, ice-cold water. Centrifuge tubes were then filled with ice-cold water and centrifuged as before. The supernatant was removed and the cell pellets resuspended in the residual water. The pellets were pooled and the volume adjusted to 50 ml with ice-cold 10% (v/v) glycerol before centrifuging as above. The cell pellet was resuspended in an equal volume of ice-cold 10% (v/v) glycerol and the cells were aliquoted into 40 µl aliquots in sterile Eppendorfs, frozen in dry ice or liquid nitrogen, and stored at –80°C until needed.

For electroporation, sterile 2 mm electroporation cuvettes were placed on ice to chill while cells were thawed on ice. 1-2 µl of yeast miniprep DNA or 1-10 ng plasmid DNA was added to the cuvette followed by 40 µl of electrocompetent cells. The cuvette was flicked to mix the DNA and cells and left on ice for 5 minutes. The electroporator was set to 2.5 kV, 25 µFarads. Cuvettes were dried with a tissue wipe and placed into the chamber and the cells electroporated (ideally, with a time constant of 4.7). 1 ml of LB medium was then added to the cuvette and the cells were transferred to a sterile Eppendorf tube and incubated at 37°C for 1 hour. Cells were then pelleted at 4000 rpm in a microfuge and resuspended in 100 µl LB before being plated on LB-agar plates containing an appropriate selective antibiotic and grown at 37°C.

2.2.5 Plasmid DNA preparation from bacteria

Plasmid DNA was prepared using either miniprep or maxiprep kits (Qiagen, Hilden, Germany) according to the manufacturers instructions. Typically, minipreps were prepared from 2 ml of overnight cultures grown in LB plus selective antibiotic at 37°C and maxipreps were prepared from 150 ml overnight cultures.

2.2.6 Purification of recombinant His-tagged proteins from bacteria

A single colony was picked from a fresh plate and used to inoculate 25 ml of LB plus selective antibiotics and grown overnight at 37°C with shaking. 10 ml of the overnight culture was then used to inoculate 1 L of fresh LB plus antibiotics and

grown at 37°C until an OD_{600} of 0.5-0.6 was reached. Depending upon the protein being expressed, cultures were either shifted to 18°C, 23°C, or 30°C for 30 minutes before being induced with 0.5 mM Isopropyl β-D-thiogalactoside (IPTG) from a 1 M stock or induced immediately at 37°C. Cultures were then left for either 3 hours (37°C), 5 hours (30°C), or overnight (23°C and 18°C). Bacteria were then harvested by centrifugation at 3000 x g for 15 min at 4°C and cell pellets were frozen on dry ice for 20 min.

For protein purification, cell pellets were thawed by the addition of 10 ml of lysis buffer per litre of original culture. Lysis buffer consisted of 0.5 mg/ml lysozyme added to IMAC5 (20 mM Tris-HCl pH 8.0, 300 mM NaCl, 5 mM imidazole). The cell pellet was resuspended and incubated at 37°C for 15 minutes or until the lysate became extremely viscous. The lysate was sonicated on ice using a Bandelin Sonopuls sonicator (Bandelin, Berlin, Germany) until the DNA was sheared and the lysate became much less viscous. The lysate was then centrifuged at 28000 rpm for 30 minutes at 4°C in an SW28 rotor in a Beckman Optima LE-80K Ultracentrifuge (Beckman, USA) to pellet the cell debris. The cleared lysate supernatant was removed into a clean falcon tube. 0.5 ml of Ni-NTA agarose (Qiagen) per litre of original culture, washed in IMAC5, was added to the cleared lysate and the tube was rotated for 2 hours at 4°C to allow protein binding to occur. The resin was then pelleted by centrifugation at 1000 x g for 5 minutes at 4°C and washed in 2 x 30 ml of IMAC20 (20 mM Tris-HCl pH 8.0, 300 mM NaCl, 20 mM imidazole). The resin was then transferred to a clean column and washed in a further 30 ml of IMAC20. Bound protein was then eluted by the addition of 10 ml IMAC200 (20 mM Tris-HCl pH 8.0, 300 mM NaCl, 200 mM imidazole). The eluate was collected in 1.5 ml fractions. After SDS-PAGE analysis of these fractions, peak fractions containing recombinant protein were pooled and dialysed overnight at 4°C into a suitable buffer (usually PBS). Protein concentration was then determined, before aliquoting and storage.

2.2.7 Purification of recombinant GST-tagged proteins from bacteria

A single colony was picked from a fresh plate and used to inoculate 25 ml of LB plus selective antibiotics and grown overnight at 37°C with shaking. 10 ml of the overnight culture was then used to inoculate 1 L of fresh LB plus antibiotics and grown at 37°C until an OD_{600} of 0.5-0.6 was reached. Depending upon the protein

being expressed, cultures were either shifted to 18°C, 23°C, or 30°C for 30 minutes before being induced with 0.5 mM Isopropyl β-D-thiogalactoside (IPTG) from a 1 M stock or induced immediately at 37°C. Cultures were then left for either 3 hours (37°C), 5 hours (30°C), or overnight (23°C and 18°C). Bacteria were then harvested by centrifugation at 3000 x g for 15 min at 4°C and cell pellets were frozen on dry ice for 20 min.

For protein purification, cell pellets were thawed by the addition of 10 ml of lysis buffer (PBS plus 0.5 mg/ml lysozyme) per litre of original culture. The cell pellet was resuspended and incubated at 37°C for 15 minutes or until the lysate became extremely viscous. The lysate was sonicated on ice using a Bandelin Sonopuls sonicator (Bandelin, Berlin, Germany) until the DNA was sheared and the lysate became much less viscous. The lysate was then centrifuged at 28000 rpm for 30 minutes at 4°C in an SW28 rotor in a Beckman Optima LE-80K Ultracentrifuge (Beckman, USA) to pellet the cell debris. The cleared lysate supernatant was removed into a clean falcon tube. 0.5 ml of glutathione-sepharose (Amersham-Pharmacia, USA) per litre of original culture, washed in PBS, was added to the cleared lysate and the tube was rotated for 2 hours at 4°C to allow protein binding to occur. The resin was then pelleted by centrifugation at 1000 x g for 5 minutes at 4°C and washed in 2 x 30 ml of PBS. The resin was then transferred to a clean column and washed in a further 30 ml of PBS. Bound protein was then eluted by the addition of 10 ml Elution Buffer (100 mM Tris-HCl pH 7.4, 150 mM NaCl, plus 15 mM reduced gluitathione added immediately prior to use). The eluate was collected in 1.5 ml fractions. After SDS-PAGE analysis of these fractions, peak fractions containing recombinant protein were pooled and dialysed overnight at 4°C into a suitable buffer (usually PBS). Protein concentration was then determined, before aliquoting and storage.

2.3 DNA Methods

Standard DNA manipulations were performed essentially as described (Sambrook *et al.* 1989). Enzymes for DNA modification were obtained from New England Biolabs (Beverly, MA, USA), Promega (Madison, WI, USA), or Invitrogen (Leek, Netherlands) and used according to the suppliers instructions.

2.3.1 General cloning strategies

As far as possible, a standard cloning strategy, referred to as the "Shortway" system, was used when generating constructs, in order to make all inserts compatible with a wide range of different vectors. This involved generating (by PCR) inserts with a BamHI, BglII, or BclI site at the 5' end and a SalI or XhoI site at the 3' end. The ATC of the BamHI/BglII/BclI site was designed to be in frame with the start codon of the insert i.e.

GGG ATC CCC *ATG* (BamHI)

GAG ATC TCC *ATG* (BglII)

GTG ATC ACC *ATG* (BclI, *dam* sensitive)

where the restriction site is underlined and the start codon is in italics.

These restriction sites were added by PCR and the insert was TA-TOPO cloned into pCRIITOPO. This construct was then sequenced. The insert DNA could then be sub-cloned, in frame, into the following vectors:

Yeast 2-hybrid vectors (pGBT9, pFBT9, pACT2)

N-terminal His-tagging vectors (pQE32, pTrcHisC)

N-terminal GST-tagging vectors (pFAT2, pGEX-5X-1)

N-terminal GFP-tagging vectors (pEGFP-C2)

C-terminal GFP-tagging vectors (without STOP codon) (pEGFP-N1, 2, or 3)

N-terminal tagged mammalian expression vectors (pcDNA3.1/HisA, or /MycA)

Non-tagged mammalian expression vectors (pcDNA3.1+)

If a sequence of interest was not clonable using this approach, alternative restriction sites were added to the 5' and 3' ends.

2.3.2 Restriction digests and agarose gel electrophoresis of DNA

Analytical restriction digests were carried out in a total volume of 20 µl using 1 µl of each enzyme in an appropriate buffer to digest approximately 500 ng of DNA. Digests were carried out for 2 hours at an appropriate temperature before being mixed with DNA loading buffer and loaded for electrophoresis onto a 1% agarose gel (1% (w/v) agarose dissolved in 1 x TAE containing 0.8 µg/ml ethidium bromide). The gel

was run in 1 x TAE at a constant voltage of 80 V. DNA was visualized on a UV transilluminator.

Preparative restriction digests were performed similarly except that 2-3 μg DNA was used. Vector DNA was usually treated with alkaline phosphatase by the addition of 1 μl enzyme to the digestion mix for 15 minutes immediately prior to loading on the gel. Following electrophoresis, appropriate bands were excised using a clean scalpel blade and purified using a gel extraction kit (Qiagen) according to the manufacturers instructions.

2.3.3 Cloning digested DNA fragments

Ligations were performed in a total volume of 20 μl using 1 μl of T4 DNA ligase in its buffer. 100 ng of vector DNA was used along with enough insert DNA to make a 3:1 molar ratio of insert:vector DNA. Ligations were incubated for 2 hours at room temperature before transformation of 5 μl into bacteria for cloning.

2.3.4 Polymerase Chain Reaction (PCR)

Oligonucleotides for PCR reactions were obtained either in-house (MPI for Biochemistry or Beatson Institute), or from one of the following companies: MWG-Biotech (Ebersberg, Germany), Metabion (Martinsried, Germany), or Thermohybaid (Ulm, Germany). 100 μM stock solutions were prepared by dissolving the oligonucleotides in 1 x TE.

For all PCRs a GeneAmp PCR System 2400 thermocycler (Perkin-Elmer) was used. Reactions were carried out in a total volume of 50 μl. Reactions contained 1 x polymerase reaction buffer, 1.25 μM forward and reverse primers, 0.25 mM dNTPs, and 1 μl (2.5 U) *Pfu* turbo polymerase (Stratagene, Amsterdam, Netherlands) (often added after the mix had been pre-heated to 94°C). As a template, either 10 ng plasmid DNA or 5 μl marathon-ready cDNA library (Clontech, Palo Alto, CA, USA) was used. Typically, 25 cycles of denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 68°C for 2 minutes per kb to be amplified, were used with an initial denaturation step of 2 minutes and a final extension step of 2 minutes per kb. PCR products were then analysed by agarose gel electrophoresis and, if required, bands were excised, gel extracted, and used for cloning. In the case of PCR cloning from cDNA libraries, a Nested approach was generally used. A first

round of PCR was performed as described above. The PCR product was then directly purified using a PCR nucleotide removal kit (Qiagen) according to the manufacturers instructions. 5 µl of the purified PCR product was then used in a second round of PCR using a set of primers designed internally to the primers used in the first round. This PCR product was then purified by agarose gel electrophoresis and used for cloning.

2.3.5 Cloning of PCR products

Following gel extraction of PCR products, cloning was performed into the pCRIITOPO vector using the TA-TOPO cloning system (Invitrogen). Adenosine overhangs were added to the 3' ends of the PCR product by incubation of the product with 1 µl *Taq* polymerase (Promega) at 72°C for 30 minutes in the presence of polymerase buffer and 0.25 mM dNTPs in a total volume of 50 µl.

2 µl of this mix was then added to a sterile Eppendorf along with 0.5 µl TA-TOPO cloning mix and incubated for 5 minutes at room temperature. This mix was then transformed into TOP10 bacteria.

2.3.6 Site-directed mutagenesis

Point mutagenesis was carried out using the Quickchange method. Forward and reverse mutagenic primers were designed to be 33 bases long with the codon to be altered in the middle, with 15 unchanged bases on either side. The quickchange reaction was then set up in a total volume of 50 µl with 1 x *pfu* polymerase buffer, 1.25 µM forward and reverse primers, 0.25 µM dNTPs, 25 ng DNA template, and 1 µl (2.5 U) *pfu* turbo polymerase. The following cycling parameters were then used: 95°C for 30 seconds followed by 18 cycles of 95°C for 30 seconds, 53-55°C for 30 seconds, and 68°C for 2 minutes per kb of plasmid. After cooling to 4°C, 1 µl of the restriction enzyme DpnI was then added to the reaction mix for 1 hour at 37°C to digest methylated (parental) DNA. 5 µl of the reaction mix was then transformed into XL1-blue bacteria.

2.3.7 DNA sequencing

All insert DNAs generated by PCR or mutagenesis, were sequenced commercially by either Medigenomix (Martinsried, Germany), or in house at the Beatson Institute.

2.4 Protein Methods

2.4.1 SDS-PAGE (polyacrylamide gel electrophoresis)

Small (8 x 6.5 cm) SDS polyacrylamide gels were prepared as described (Sambrook *et al.* 1989). Samples were prepared in SDS-PAGE sample buffer and boiled for 5 minutes. Gels were run in a BioRad Mini-PROTEAN 3 gel chamber (BioRad, Hemel Hempstead, UK) in SDS-PAGE running buffer at 180 V, 50 mA.

2.4.2 Coomassie staining of SDS-PAGE gels

Protein gels were Coomassie stained by immersion in 0.01% Coomassie Brilliant Blue R-250 in 50% methanol, 10% acetic acid for 20 minutes, with shaking. Gels were then destained in 20% isopropanol, 20% acetic acid.

2.4.3 Mass-spectrometry

Sample preparation and analysis was carried out by Christian Preisinger (MPI For Biochemistry, Martinsried) according to a protocol adapted from Shevchenko *et al.* 1996). Briefly, Coomassie stained bands from protein gels were excised with a clean scalpel and cut into 1 mm cubes. The gel pieces were washed with 100 μl 50 mM NH_4HCO_3 for 10 minutes at 22°C, with shaking at 1000 rpm then shrunk in 100 μl acetonitrile for 10 min at 22°C. This was repeated once. Gel pieces were then dried in a speed vac for 5 min at 35°C. 10-15 μl of 10 mM DTT in 50 mM NH_4HCO_3 was added to the dried gel pieces and the mixture was incubated at 56°C for 45 min. The gel pieces were returned to room temperature and overlaid with 10-15 μl 55 mM iodoacetamide in 50 mM NH_4HCO_3. The reaction was left for 30 min at room temperature in the dark. The supernatant was removed and replaced with 100 μl 50 mM NH_4HCO_3 and washed as above. The gel pieces were dried again for 5 minutes. 10-15 μl trypsin solution (12.5 ng/μl trypsin in 50 mM NH_4HCO_3; Sequencing-grade modified trypsin, Promega) was added to the dried gel cubes and left on ice for 30 minutes. Additional 50 mM NH_4HCO_3 (5-15 μl) was added to the mixture and the digest reaction was performed for 16 hours at 37°C.

2 μl 10% TFA was added to the digest. 1 μl of acidified sample was spotted onto the sample matrix (1-Cyano-4-hydroxycinnamic acid, Bruker Daltonik GmbH, Germany). The spot was washed once with 0.2% TFA.

Mass spectrometry of the samples was performed on a ReflexIII instrument (Bruker) MALDI TOF mass spectrometer, equipped with a Scout 384 Ion source. The spectra were processed with Xmass 5.1.1 software (Bruker). Proteins were identified using the database programs MASCOT (http://www.matrixscience.com) and ProFound (http://129.85.19.192/profound_bin/WebProFound.exe).

2.4.4 Western blotting

Proteins run on SDS-PAGE gels were transferred to 45 µm Hybond-C Extra Nitrocellulose (Amersham Pharmacia Biotech, UK) by semi-dry blotting in transfer buffer (1 x SDS-PAGE running buffer plus 10% methanol) using a Trans-Blot SD Transfer Cell (BioRad, UK) at 15 V, 200 mA, for 45 minutes. Blots were then blocked in milk solution (4% milk powder in PBS, plus 0.1% Tween-20) for at least 1 hour before addition of the primary antibody.

Blots were usually incubated with the primary antibody in milk solution for 1 hour at room temperature before being washed 3 x 10 minutes in milk solution. Secondary antibodies linked to horseradish peroxidase were then also incubated with the blot for 1 hour before the blots were washed for a further 3 x 10 minutes in milk. Bound antibodies were then detected by chemiluminescence using ECL Western Blot Detection Reagents (Amersham) according to the manufacturers instructions, and exposed to Kodak X-Omat XAR-5 film for an appropriate length of time.

2.4.5 Determination of protein concentration

Protein concentrations were determined using the BioRad Protein Assay kit (BioRad), a modified version of the Bradford Assay. The dye reagent was diluted 1:5 in water and 1 ml 1 x reagent was used per assay point. Different concentrations of bovine serum albumin (BSA) were used to construct a standard curve by addition to 1 ml aliquots of the dye reagent, vortexing, and transferal to disposable cuvettes for measurement of the OD_{595} in an Ultrospec 3000 Pro spectrophotometer (Amersham Pharmacia). Protein samples to be measured were then analysed in the spectrophotometer and the concentration determined.

2.4.6 Protein precipitation

Proteins were sometimes precipitated, using trichloroacetic acid (TCA), for loading on SDS-PAGE gels. Typically, protein samples were adjusted to 1 ml with water. 7.5 µl 10% sodium deoxycholate was then added and the sample vortexed. 300 µl 55% TCA was then added, the sample vortexed, and left on ice for 45 minutes. Precipitated proteins were then recovered by centrifugation at 20000 x g for 20 minutes at 4°C. The supernatant was discarded and the pellet washed in 1 ml –20°C acetone before being centrifuged for a further 10 minutes. The supernatant was discarded and the pellet resuspended in an appropriate amount of 1.5 x SDS-PAGE sample buffer with a small amount of 1 M Tris-HCl pH 8.0 added, as necessary, to adjust the pH of the sample.

2.4.7 Rab-effector pulldowns

Pulldowns from rat liver cytosol and rat liver golgi using GST-tagged rab proteins were performed throughout the course of this work and the protocol was modified over time. The final and most-commonly used protocol is presented below and is an adaptation of protocols presented in, for example, (Christoforidis and Zerial 2000). Deviations from this standard protocol will be noted in the experimental chapters of this thesis.

Rat liver cytosol was prepared according to a published method (Levine *et al.* 1996) and dialysed into HNTM (50 mM HEPES-KOH pH 7.5, 100 mM NaCl, 0.5% Triton X-100, 5 mM $MgCl_2$). 2 mg (an excess) of recombinant GST-tagged rab protein was bound to 50 µl glutathione-sepharose beads (Amersham Pharmacia) for 1 hour at 4°C in PBS. The beads were then recovered and washed 3 x 1ml HNTM. The beads were then incubated in the presence of 12 mg cytosol plus either 100 µM GDP (wild type or GDP-locked rabs) or 100 µM GTP-γ-S or GMP-PNP (GTP-locked rabs) in a total volume of 1.8 ml overnight at 4°C. Beads were then washed in 3 x 1ml HNTM and bound proteins were eluted in 1 ml NE500 buffer (20 mM HEPES-KOH pH 7.5, 500 mM NaCl, 0.1% Triton X-100, 20 mM EDTA) for 30 minutes at room temperature. An excess of glutathione-sepharose beads was then added to the eluate for 2 x 30 minutes to rebind as much rab protein from the eluate as possible. The eluate was

then TCA precipitated and analysed by western blotting or coomassie brilliant blue staining as required.

2.4.8 Rab-effector binding assays

Direct binding between recombinant GST-tagged rab proteins and his-tagged effector proteins was assessed by immobilizing 4 μg rab protein on 15 μl glutathione-sepharose and then incubating the beads for 1 hour at 4°C with 10 μg effector protein in the presence of 100 μM GDP, GTP-γ-S or GMP-PNP in a total volume of 400 μl HNTM. Beads were washed in 3 x 1ml HNTM and then eluted directly in 1.5 x SDS-PAGE sample buffer for analysis either by western blotting or coomassie brilliant blue staining.

2.5 Yeast Methods

2.5.1 Strains, growth, and media

The *S.cerevisiae* strain PJ69-4A (James *et al.* 1996) was used for all two-hybrid experiments, the genotype of which is:

MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1$_{UAS}$-*GAL1*$_{TATA}$-*HIS3, GAL2*$_{UAS}$-*GAL2*$_{TATA}$-*ADE2, MEL1 met2= GAL7-lacZ*

Yeast were grown in either YPDA or SC Dropout media selecting for appropriate plasmids at 30°C. Short-term storage was on plates at 4°C.

YPDA media consisted of 20 g/l peptone (Difco/Becton-Dickinson), 10 g/l yeast extract (Difco), and 20 g/l glucose (plus 20 g/l Bacto-agar for YPDA-agar). The media was then sterilised in an autoclave and, after cooling to approximately 55°C, 6 ml 0.2% sterile filtered adenine hemisulphate was added.

Synthetic complete (SC) dropout media was prepared as follows:

Amino acid base (-His/-Trp/-Leu/-Ura) was prepared by mixing 20 g alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, inositol, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine with 5 g adenine and 2 g para-aminobenzoic acid. To prepare dropout mixes, 36.7 g of amino acid base was mixed with either 2 g histidine, 4 g leucine, 2 g tryptophan, or 2 g uracil, as appropriate to form the correct dropout mix. SC dropout media consisted of 6.7 g/l nitrogen base (without amino acids, Difco), 2

g/l appropriate dropout mix, 20 g/l glucose and pH adjusted to between 5.5 and 6.5 (plus 20 g/l Bacto-agar for SC dropout agar). After autoclaving and cooling to approximately 55°C, 6 ml 0.2% sterile filtered adenine hemisulphate was added, unless dropout media was also to be –adenine.

2.5.2 Yeast transformation (frozen cell method)

To prepare frozen competent yeast cells, several colonies were picked from a freshly grown plate and grown overnight in YPDA at 30°C with shaking. The following morning, the overnight culture was diluted to an OD_{600} of 0.15 in fresh medium and grown at 30°C to an OD_{600} of 0.5-0.6 (1.2-1.5 x 10^7 cells). The cells were then harvested at 3000 rpm for 2 minutes at room temperature in a Heraeus centrifuge. The cells were then washed in one half culture volume sterile water and spun as before. The cells were then resuspended in 1/8th culture volume of LiSorb (100 mM LiOAc, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA pH 8.0, 1 M sorbitol (molecular biology quality, filter sterilized). The resuspended pellet was incubated for 5 minutes at room temperature before being spun as before. An additional spin was made to remove residual supernatant. The cell pellet was again resuspended in LiSorb (600 µl per 100 ml original culture). Carrier DNA was then added (10 µl per 100 µl yeast of 10 mg/ml salmon sperm DNA (Gibco), heat-treated at 95°C for 5 minutes). Cells were then aliquoted and frozen directly in a –80°C freezer where they were stored until use.

To transform frozen yeast cells, the cells were thawed at room temperature. 25 µl cells were used per transformation. 0.5 µl plasmid DNA for transformation was added to the cells, followed by 150 µl LiPEG (100 mM LiOAc, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 40% PEG3350, filter sterilized). The cells were then vortexed and incubated for 20 minutes at room temperature. 17.5 µl DMSO was then added and the cells were heat-shocked for 15 minutes in a 42°C waterbath. The cells were then pelleted at low speed in a microfuge, the supernatant removed, and the cells resuspended in 200 µl sterile PBS. 80 µl were the plated onto appropriate selective plates and grown at 30°C.

In the case of directed yeast two-hybrid experiments, transformed cells were originally grown on SC-Leu/-Trp plates to select for both the bait and prey plasmids. After 2-3 days growth, colonies were picked and restreaked onto SC-Leu/-Trp/-His

and/or SC-Leu/-Trp/-His/-Ade plates to assess the strength of the two-hybrid interaction.

2.5.3 DNA minipreps from yeast cells

To prepare plasmid DNA from yeast cells, either 1 ml of an overnight culture or a matchhead-sized ball of cells scraped from a fresh plate into 1 ml water, was pelleted at low-speed in a microfuge. The cell pellet was then resuspended in 500 µl buffer S (10 mM K_2HPO_4, pH 7.2, 10 mM EDTA, 50 mM β-mercaptoethanol (i.e. 100 µl per 25 ml added immediately prior to use), and 50 µg/ml zymolyase). The cells were then incubated at 37°C for at least 1 hour. 100 µl lysis buffer (25 mM Tris-Hcl, pH 7.5, 25 mM EDTA, 2.5% (w/v) SDS) was then added, vortexed, and the lysate incubated at 65°C for 30 minutes. 166 µl 3 M KOAc, pH 5.5 was then added to stop the lysis reaction, tubes were inverted to mix, and incubated on ice for 10 minutes. They were then centrifuged at 20000 x g in a microfuge at 4°C for 15 minutes and the supernatant was transferred to a clean Eppendorf tube. 800 µl cold ethanol was added, tubes were inverted to mix, and incubated on ice for 10 minutes before centrifugation as before for a further 10 minutes. The supernatant was removed and the pellet washed in 1 ml 70% ethanol. The pellet was allowed to air dry before resuspension in 40 µl sterile water.

2.6 Cultured Mammalian Cell Methods

2.6.1 Cell culture

Hela L cells were cultured at 37°C and 5% CO_2 atmosphere in Dulbeccos Modified Eagle Medium (DMEM) containing 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin (all Life Technologies).

2.6.2 Immunofluorescence

For immunofluorescence, cells were normally cultured on ethanol-flamed 15 mm coverslips in 6-well plates. Cells were fixed in either 3% paraformaldehyde or methanol, as indicated in the text.

For paraformaldehyde fixation, 3% paraformaldehyde was prepared by dissolving 3 g paraformaldehyde in 100 ml PBS at 80°C in a fume hood. 10 µl 1 M $CaCl_2$ and 10 µl

1 M $MgCl_2$ were added while stirring before the solution was allowed to cool to room temperature and pH adjusted to 7.4. The solution was then vacuum-filtered through a 0.45 μm filter and 15 ml aliquots were stored at –20°C. Coverslips were washed twice in 2 ml PBS and then fixed in 3% paraformaldehyde for 20 minutes. Coverslips were then washed once in 2 ml quench solution (50 mM NH_4Cl in PBS made immediately prior to use) before incubation in a further 2 ml quench solution for 10 minutes. The coverslips were then washed in 3 x 2ml PBS. If the cells were to be permeabilised, the coverslips were incubated for 5 minutes in permeabilisation solution (0.2% Triton X-100 in PBS) before washing in 3 x 2ml PBS.

For methanol fixation, coverslips were washed twice in 2 ml PBS before adding 2 ml –20°C methanol and placing them in a –20°C freezer for 4 minutes. The coverslips were then removed from the freezer and washed in 3 x 2ml PBS.

For antibody labeling, primary antibodies were diluted appropriately in PBS. A strip of parafilm was placed on a flat surface and 50 μl drops of the antibody solution were placed on the strip. Coverslips with fixed cells were then transferred, cell face down, onto the antibody drops. The coverslips were then covered with a moist, dark chamber and left for 1 hour at room temperature. After this incubation, the coverslips were floated by pipetting 200 μl under each one, before transferring them back, cell face up, to the 6-well plate. They were then washed in 3 x 2ml PBS. The same procedure was then followed for incubation with secondary antibodies conjugated to appropriate fluorophores. After washing in PBS for the final time, coverslips were mounted onto clean microscope slides by placing them, cell face down, onto a 10 μl drop of Moviol mounting medium. (Prepared as follows: 2.4 g Moviol 4-88 added to 6 g analytical grade glycerol while stirring. 6 ml of dH_2O then added and the solution was left for 2 hours at room temperature. 12 ml of 0.2 M Tris-HCl pH 8.5 was then added while stirring and the solution was mixed for 10 minutes at 50°C. The Moviol solution was then clarified by centrifugation at 3000 x g for 15 minutes and stored at –20°C aliquoted into glass vials.) The mounting medium used usually contained 1 μg/ml DAPI stain. The coverslips were then left for at least 30 minutes at room temperature to allow the Moviol to dry.

Images were collected using a ZEISS Axioskop-2 with a 63x Plan Apochromat oil immersion objective with a 1.4 NA, a 1300 by 1030 pixel cooled CCD camera (Princeton Instruments) and Metaview software (Universal Imaging Corp.).

2.6.3 Transient transfection of mammalian cells

DNA constructs were transiently transfected into mammalian cells using lipid-based transfection reagents. Specifically, either FuGENE 6 (Roche Diagnostics) or Effectene (Qiagen) were used according to the manufacturers instructions.

2.6.4 RNA interference

In order to selectively knockdown the expression levels of particular proteins, small interfering RNA duplexes (siRNAs) were transfected into cultured Hela L cells. The protocol used was essentially that of Elbashir *et al.* 2001. The open reading frames of target cDNAs were analysed for regions at least 100 bases downstream of the start codon containing the sequence AA(N19)TT. This sequence was then used to design an siRNA duplex consisting of 21 nucleotide sense and antisense RNA oligonucleotides paired to have a 2 nucleotide 3' overhang. RNA oligonucleotides were synthesized by Dharmacon Inc. To anneal the oligonucleotides, 20 μM single-stranded RNAs were incubated in annealing buffer (100 mM KOAc, 30 mM HEPES-KOH, pH 7.4, 2 mM MgOAc) in a total volume of 100 μl for 1 minute at 90°C followed by 1 hour at 37°C. This solution could then be stored at –20°C until needed. Hela L cells were usually passaged and allowed to grow and attach 24 hours before transfection with the siRNA duplex. For transfection, the lipid-based transfection reagent Oligofectamine (Invitrogen) was used. For the transfection of a single well of a six-well plate, the following amounts were used: 3 μl of 20 μM siRNA duplex and 3 μl Oligofectamine were added to 200 μl OptiMEM medium (Invitrogen) in a clean, RNAse-free Eppendorf tube. The mixture was pipetted up and down to mix and left for 25 minutes at room temperature. The entire mixture was then added, drop-wise, to the cells which were then incubated at 37°C, 5% CO_2 in DMEM until being processed for western blotting or immunofluorescence.

Chapter 3: Characterisation Of Rab-Effector Complexes At The Early Golgi

3.1 Introduction

Secretory material exits the ER in COPII vesicles, that fuse together to form vesicular tubular clusters, which then act as transport vehicles to the *cis*-Golgi (Presley *et al.* 1997). The ER-Golgi Intermediate Compartment (ERGIC) was originally thought to be a separate stable compartment from which material was transported to the Golgi by small budded vesicles (Lotti *et al.* 1992). It was later demonstrated, however, by visualizing GFP-tagged VSV-G protein in live cells, that fluorescent protein accumulated in the intermediate compartment, which then moved to the *cis*-Golgi (Presley *et al.* 1997). This movement is directed to the minus-end of microtubules and was dependent on the dynactin/dynein motor (Presley *et al.* 1997). Proteins are also recycled back to the ER from the intermediate compartment (Hauri and Schweizer 1992; Tang *et al.* 1995; Klumperman *et al.* 1998).

Once at the *cis*-Golgi, cargo is then moved on to the medial Golgi and later compartments either by anterograde transport vesicles or by the successive recycling of Golgi components in retrograde vesicles (see section 1.2).

Many of the proteins mediating targeting events at the early Golgi have been identified. Rabs 1 and 2 are involved in ER to Golgi transport and rab1 is also required for *cis*- to medial-Golgi transport in mammalian cells (Plutner *et al.* 1991; Tisdale *et al.* 1992). Accordingly, rab1 has been localised to the ER, ERGIC, and early Golgi (Plutner *et al.* 1991), while rab2 has been detected at the ERGIC (Chavrier *et al.* 1990). Another rab protein of potential relevance to transport events at the early Golgi is rab33b, which localizes to the medial Golgi and is implicated in retrograde transport (Zheng *et al.* 1998; Valsdottir *et al.* 2001).

The early Golgi coiled-coil proteins p115, GM130, and giantin are also implicated in ER to Golgi and intra-Golgi transport. p115 was originally identified as a protein required for an *in vitro* intra-Golgi transport assay (Waters *et al.* 1992), and is required *in vitro* for COPI vesicle docking (Sonnichsen *et al.* 1998). Uso1p, the budding yeast homologue of p115, is also essential for the docking of ER-derived COPII vesicles with the *cis*-Golgi (Barlowe 1997). The *cis*-Golgi matrix proteins GM130 and giantin both bind p115 (Nakamura *et al.* 1997; Sonnichsen *et al.* 1998), are both required for COPI vesicle docking *in vitro* (Sonnichsen *et al.* 1998), and are both involved in ER-to-Golgi transport *in vivo* (Alvarez *et al.* 2001).

Two other important proteins in the early Golgi are GRASP65 and GRASP55 (Barr *et al.* 1997; Shorter *et al.* 1999). GRASP65 is a peripheral membrane protein associated with the *cis*-Golgi, required for the post-mitotic reassembly of Golgi stacks (Barr *et al.* 1997). It is tightly bound to GM130, an interaction required for the targeting of both proteins to Golgi membranes (Barr *et al.* 1998).

The related protein, GRASP55, on the other hand, is localised to the medial Golgi and, though also required for the post-mitotic reassembly of the Golgi, it does not appear to bind GM130 (Shorter *et al.* 1999). GRASP55 and GRASP65 show a high degree of homology in their N-termini, with the first 212 amino acids of GRASP65 and 213 amino acids of GRASP55 showing 66% identity and 14% conservation. The C-termini are more divergent (Shorter *et al.* 1999). The conserved region includes the site on GRASP65 responsible for GM130 binding (Barr *et al.* 1998) suggesting that, although GM130 does not appear to bind GRASP55, a related protein may do so and fulfil a role at the medial Golgi similar to that of GM130 at the *cis*-Golgi.

A two-pronged approach was used to identify potential binding partners for GRASP55: a yeast two hybrid screen using GRASP55 as bait, and the immunoprecipitation of GRASP55 complexes from Golgi membranes. As shown in Figure 3.1, both approaches led to the identification of golgin-45 as an interactor of GRASP55. This protein, previously known as JEM-1, had been identified as a putative nuclear-localised transcription factor (Duprez *et al.* 1997), but was also identified as a resident of the Golgi apparatus in a mass spectrometric proteomic screen of purified Golgi membranes (Taylor *et al.* 2000).

The yeast two hybrid screen identified clones encoding amino acids 12-402 of human JEM-1/golgin-45, the only obvious sequence feature of which was a predicted coiled-

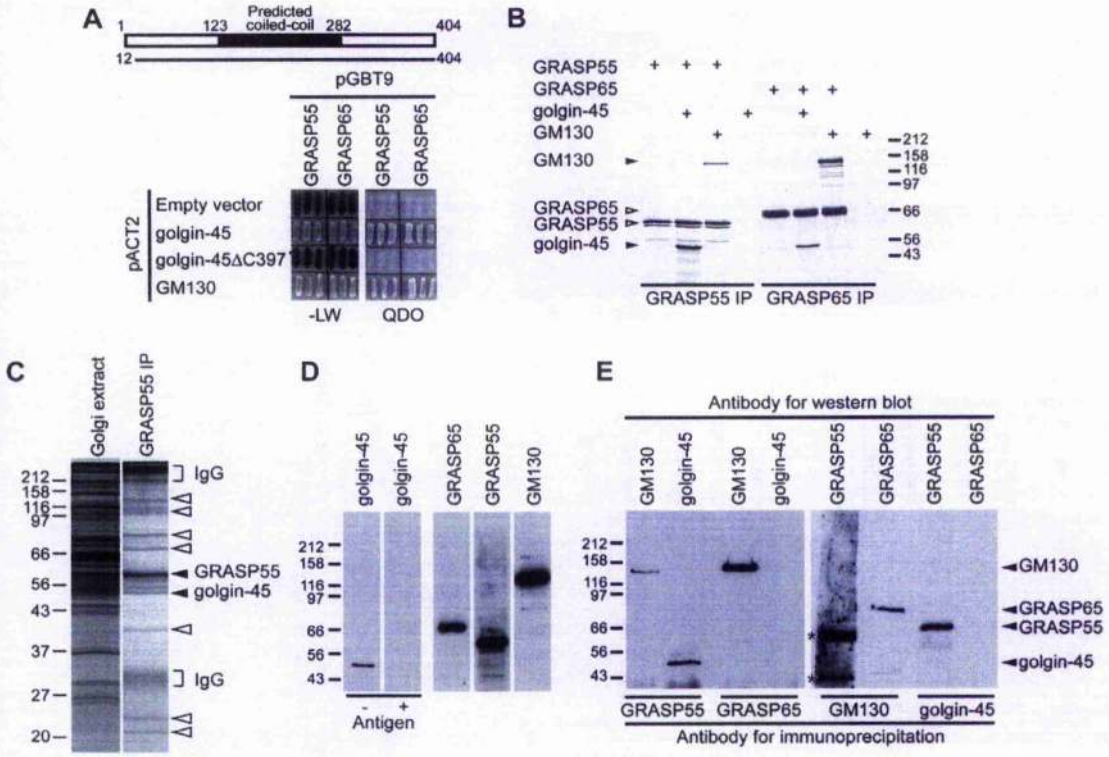


Figure 3.1 Golgin-45 is a novel GRASP55 interacting protein
(A) Amino acids 12-404 of golgin-45 were identified using the yeast two-hybrid system by screening GRASP55 against a human testis cDNA library. Golgin-45 and a C-terminal deletion mutant were tested for interaction with GRASP55 and GRASP65 in the yeast two hybrid system compared with GM130 and empty vector as positive and negative controls respectively. (B) Co-immunoprecipitations of *in vitro*-translated GRASP55 and GRASP65 with golgin-45 and GM130. (C) GRASP55 complexes were affinity purified from detergent extracts of Golgi membranes. Specifically interacting proteins were excised (arrowheads) and tryptic digests of the proteins contained therein analysed by mass spectrometry. (D) Golgi membranes (10 μg) were western blotted with antibodies to golgin-45 in the presence (+) or absence (-) of 10 μg/ml of recombinant golgin-45. Control blots are also shown for the antibodies to GRASP65, GRASP55, and GM130. (E) Immunoprecipitations were performed from 20 μg Golgi membranes with either sheep anti-GRASP55, mouse anti-GRASP65, sheep anti-GM130, or rabbit anti-golgin-45. GRASP55 and GRASP65 IPs were blotted with rabbit anti-GM130 and rabbit anti-golgin-45, GM130 IPs were blotted with rabbit anti-GRASP55 and mouse anti-GRASP65, and golgin-45 IPs were blotted with sheep anti-GRASP55 and mouse anti-GRASP65. Asterisks indicate a non-specific cross-reactivity of the rabbit anti-GRASP55 antibody to the sheep anti-GM130 antibody used for the IP.
The data in this figure was kindly provided by Francis Barr.

coil region between amino acids 123 and 282 (Figure 3.1 A). The GRASP55-golgin-45 interaction was dependent upon the last 7 amino acids of golgin-45, since deletion of these amino acids abolished the interaction. Golgin-45 also showed an interaction with GRASP65, (Figure 3.1 A), just as GM130 is able to interact with both GRASP proteins by yeast two hybrid (Shorter *et al.* 1999). These interactions were confirmed by immunoprecipitation of *in vitro*-translated proteins (Figure 3.1 B), with golgin-45 binding more GRASP55 than GRASP65 and GM130 binding more GRASP65 than

GRASP55 *in vitro* (Figure 3.1 B). Golgin-45 could also be co-immunoprecipitated with GRASP55 from Golgi membranes and identified by mass spectrometry (Figure 3.1 C) confirming the interaction *in vivo*. A polyclonal antibody was raised against golgin-45, which recognised a protein of the correct size in Golgi membranes by western blot (Figure 3.1 D). Immunoprecipitations from Golgi membranes with GRASP55, GRASP65, GM130, and golgin-45 antibodies, further confirmed the *in vivo* interaction and suggested that, *in vivo*, the main GRASP complexes are GRASP65-GM130 and GRASP55-golgin-45 (Figure 3.1 E).

This suggested that JEM-1/golgin-45 might, in fact, be a golgi-localised coiled-coil protein functioning in vesicle transport and maintenance of Golgi structure in a similar manner to GM130. I therefore set out to characterise the role of golgin-45 in transport events at the early Golgi, and to determine whether it and other early Golgi coiled-coil proteins were able to interact with the rab proteins thought to be involved in these steps.

3.2 Experimental Methods

3.2.1 Rab pulldowns from Golgi extract

0.5 mg recombinant GST-tagged rab proteins were bound to 50 µl glutathione-sepharose beads (Amersham Pharmacia Biotech). Wild type proteins in the presence of 10 µM GDP were used for rab-GDP samples, and proteins bearing activating point mutations (rab1Q70L, rab2Q65L, and rab6aQ72L) in the presence of 10 µM GTPγS were used for rab-GTP samples. The beads were incubated with 200 µg Golgi extract in HNTM (50 mM HEPES-KOH, pH7.2, 200 mM NaCl, 0.5% TX-100, 5 mM $MgCl_2$) in a total volume of 400 µl for 1 hour at 4°C. Beads were then washed three times in 500 µl HNTM containing 10 µM nucleotide before bound proteins were eluted in HNT containing 20 mM EDTA. Bound proteins were then precipitated with 12% (w/v) TCA and analysed by western blotting.

3.3 Experimental Results

3.3.1 Golgin-45 localises to the Golgi apparatus and behaves as a Golgi matrix protein

In accordance with its detection in Golgi membrane extract (Figure 3.1 D), golgin-45 was also localised to the Golgi apparatus by immunofluorescence (Figure 3.2). Staining of normal rat kidney (NRK) cells with anti-golgin-45 and anti-GRASP55 antibodies showed extensive co-localisation of the two proteins at the Golgi, consistent with their existence together in a complex similar to GRASP65/GM130 (Figure 3.2 A). Golgi matrix proteins such as GM130 remain in Golgi remnant structures when cells are treated with the drug brefeldin A (BFA), in contrast to resident Golgi enzymes, which redistribute back to the ER (Seemann *et al.* 2000). Staining for golgin-45 following BFA treatment reveals that golgin-45 behaves as expected for a matrix protein: its localisation becomes vesiculated and dispersed throughout the cytoplasm but the protein does not re-localise to the ER (Figure 3.2 B). Similarly, treatment of cells with the microtubule depolymerising drug, nocodazole, a treatment that disrupts the Golgi into 'mini-stacks' (Rogalski *et al.* 1984), also causes golgin-45 staining to become dispersed throughout the cytoplasm (Figure 3.2 B). Dispersal of golgin-45 and GRASP55 can also be induced by the overexpression of full length golgin-45 (Figure 3.2 C). Golgin-45 therefore displays the characteristics of a Golgi matrix protein and may be important for maintaining Golgi structure.

3.3.2 Golgin-45 is a rab2 effector

The Golgi matrix proteins p115 and GM130 have recently been identified as rab1 effector proteins, which specifically bind to the active, GTP-bound form of rab1 (Allan *et al.* 2000; Moyer *et al.* 2001; Weide *et al.* 2001). Since golgin-45 has several features in common with GM130, I tested the possibility that it, too, would specifically interact with a Golgi-localised rab protein. The GTP-restricted mutants of rabs 1, 2, 5, 6a, and 33b were cloned into the yeast two hybrid bait vector pGBT9 and screened against full length p115, GM130, and golgin-45 cloned into the prey vector pACT2 (Figure 3.3 A). Golgin-45 displayed a specific interaction with the GTP-restricted mutant of rab2 as assessed by growth on selective media. No interaction was seen with the other rab proteins tested. p115 showed an interaction with the

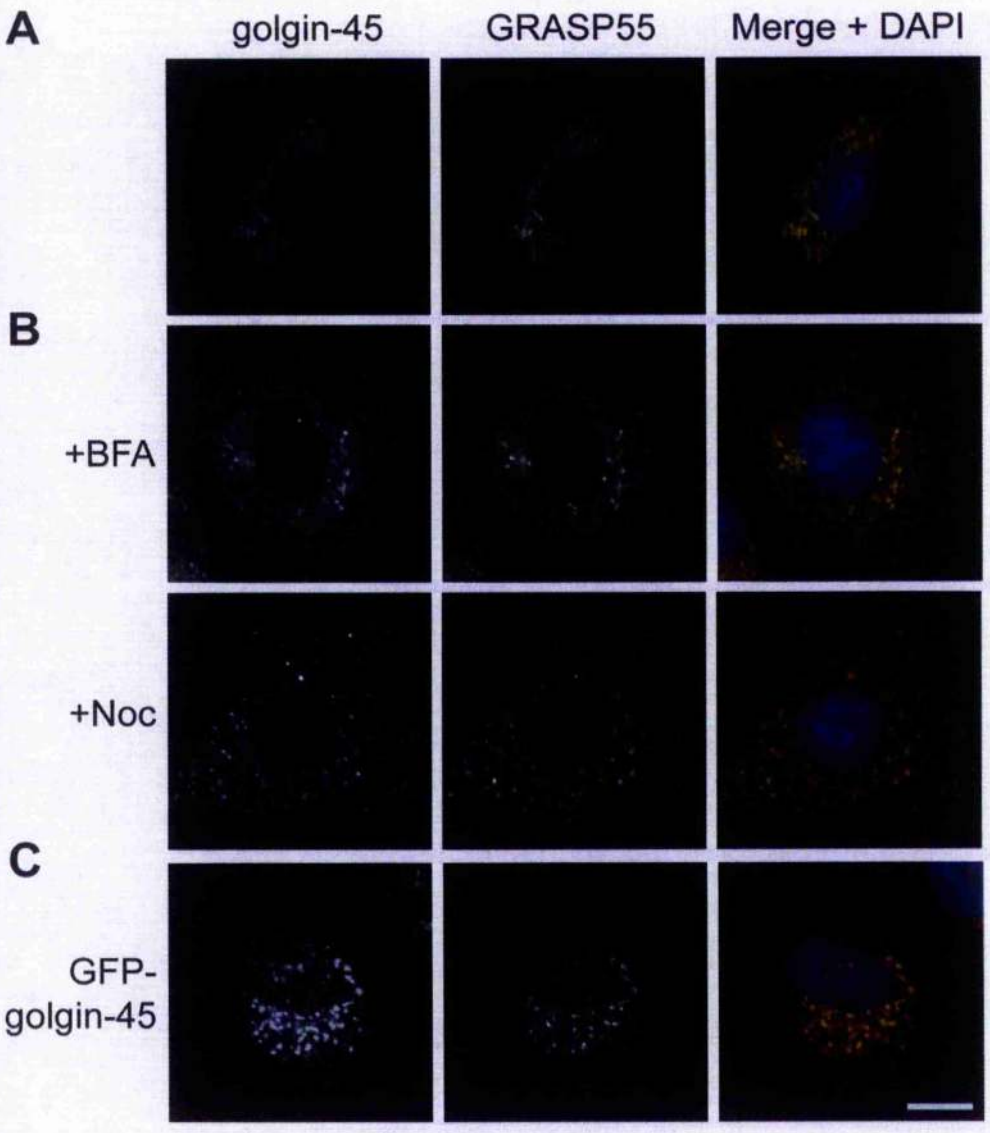
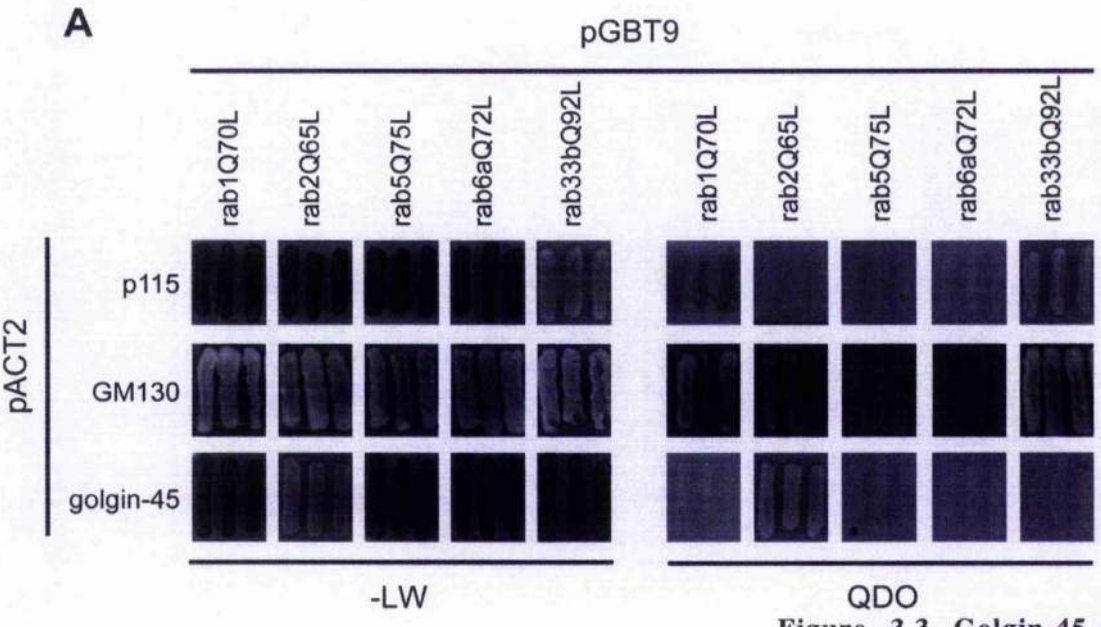


Figure 3.2 Golgin-45 behaves like a Golgi matrix protein
(A) NRK cells were fixed with 3% PFA and stained with rabbit anti-golgin-45 and sheep anti-GRASP55. (B) NRK cells were treated with either 5 μg/ml BFA for 30 minutes or 200 ng/ml nocodazole for 60 minutes prior to fixation and staining as in (A). The merged images in (A) and (B) show DNA (DAPI staining) in blue, golgin-45 in red, GRASP55 in green, and areas of overlap between golgin-45 and GRASP55 in yellow. (C) NRK cells were transfected with GFP-golgin-45 for 12 hours then fixed and stained with anti-GRASP55 (red in merged image).
Bar, 10μm.

activated mutant of rab1, confirming the interaction reported by Allan *et al.*, (2000). We also observed an interaction with the GTP-form of rab33b (Figure 3.3 A). GM130 interacted with rab1 in the yeast two hybrid system, consistent with the reports of Moyer *et al.*, and Weide *et al.*, (both 2001) but we also observed an interaction with rab2 and rab33b, suggesting that GM130 is capable of interacting with multiple rab proteins (Figure 3.3 A).



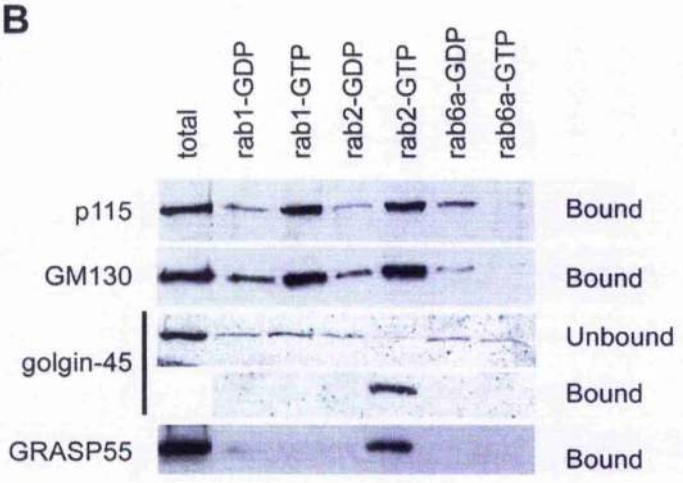


Figure 3.3 Golgin-45 is a specific binding partner of rab2

(A) Full length golgin-45, GM130, and p115 were tested in the yeast two hybrid system for interactions with the activated rab mutants rab1Q70L, rab2Q65L, rab5Q75L, rab6aQ72L, and rab33bQ92L. Interactions were assessed by growth on quadruple dropout media. (B) Rab1, 2, and 6 beads loaded with either GDP or GTPγS were incubated with Golgi extract and the specifically eluted proteins were analysed by western blotting.

To confirm the specific interaction of golgin-45 with rab2-GTP, pulldowns were performed from Golgi membranes using GST-tagged rab proteins in either the GDP- or GTP-bound forms (Figure 3.3 B). Golgin-45 was specifically bound by activated rab2 but not rab2-GDP or GTP-loaded rab1 and rab6a. The protein was correspondingly depleted in the unbound fractions (Figure 3.3 B). GRASP55 was also pulled down by rab2-GTP (Figure 3.3 B), consistent with its interaction with golgin-45 since no interaction was detected between GRASP55 and rab2 in the yeast two hybrid system (data not shown). p115 and GM130 were pulled down by activated rab1 and rab2 (Figure 3.3 B). This supports the finding by yeast two hybrid (Figure 3.3 A) that GM130 interacts with both of these rab proteins. Since full length

p115 does not interact with activated rab2 by yeast two hybrid, its presence in the rab2-GTP pulldown is presumably due to binding to GM130.

3.3.3 p115 binds rab1 directly

Since neither the yeast two hybrid system nor pulldowns from Golgi extract are proof of a direct interaction between a rab protein and a putative effector, I performed a direct binding assay between rabs 1, 2, 6a and p115 using recombinantly expressed proteins (Figure 3.4). p115 could be specifically and quantitatively bound to rab1-GTP (rab1Q70L GTP-restricted mutant in the presence of GMP-PNP) but not to rab1-GDP (wild type rab1 in the presence of GDP) nor to either mutant or wild type rab2 and rab6a proteins (Figure 3.4).

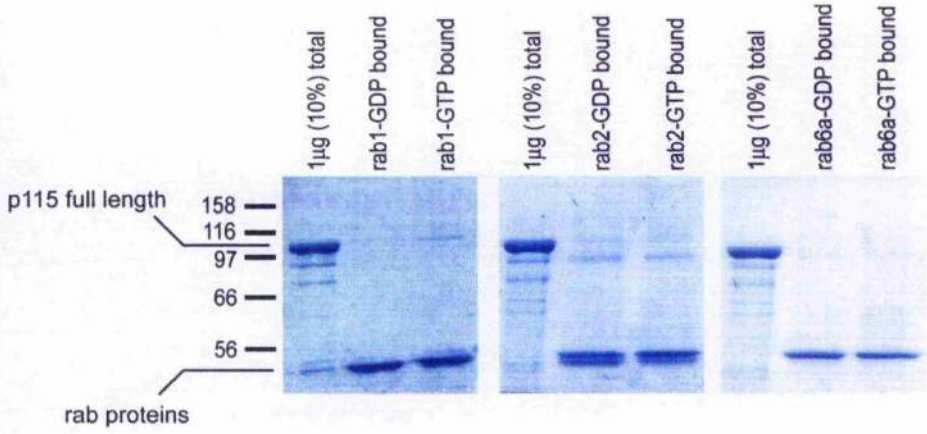


Figure 3.4 p115 binds directly to rab1
4 µg recombinantly expressed GST-tagged rab proteins were incubated with 10 µg His-tagged p115 and 15 µl glutathione-sepharose beads in the presence of either 10 µM GDP or GMP-PNP in a total volume of 400 µl HNTM for 1 hour at 4°C. Bound proteins were eluted in SDS-PAGE sample buffer and analysed by SDS-PAGE. p115 was specifically bound by activated rab1 only. Gels have been scaled to match.

3.3.4 p115 contains distinct rab binding sites

Due to problems with the expression and purification of functional rab33b protein from *E.coli*, we were unable to assess direct binding between p115 and rab33b to confirm the yeast two hybrid interaction seen in Figure 3.3 A. However, I used the yeast two hybrid system to further investigate this potential interaction and map the rab33b binding site on p115 as well as the rab1 binding site (Figure 3.5).

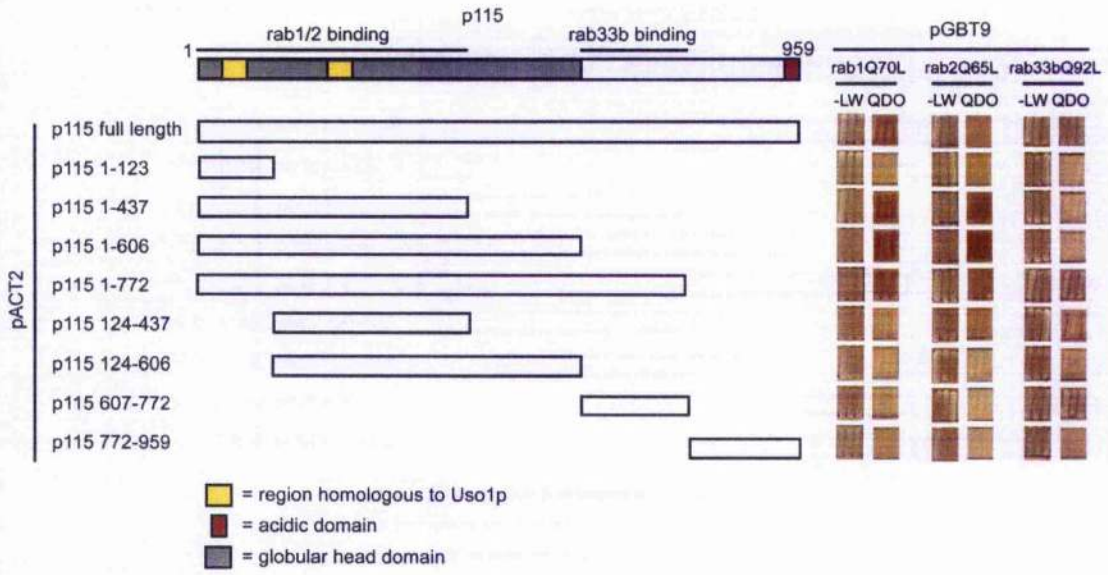


Figure 3.5 Mapping of rab binding sites on p115 by yeast two hybrid
Deletion mutants of p115 were cloned into the yeast two hybrid prey vector pACT2 and were screened against the GTP-restricted point mutants rab1Q70L, rab2Q65L, and rab33bQ92L cloned into the bait vector pGBT9. Interactions were assessed by growth on quadruple dropout media.

p115 consists of a globular head domain at the N-terminus, a coiled-coil tail, and an acidic C-terminus. The head domain contains two regions particularly homologous to Uso1p, the yeast homologue of p115 (Sapperstein *et al.* 1995).

Yeast two hybrid analysis suggests that the rab1 binding site lies in this region of p115 (Figure 3.5). The smallest fragment of p115 able to interact with rab1 by yeast two hybrid encompasses amino acids 1-437. This contains both of the regions highly conserved between p115 and Uso1p. Deletion of either of these two regions abolishes the interaction (e.g. neither p115 1-123 nor p115 124-437 demonstrates an interaction with activated rab1). Surprisingly, even though full length p115 shows no interaction with activated rab2 by yeast two hybrid, the same p115 fragments that interact with rab1 are also able to interact with rab2 (Figure 3.5). The significance of this is unclear and may simply be an artefact of expressing deletion mutants of the protein.

These fragments do not, however, interact with GTP-restricted rab33b. The rab33b binding site instead maps to amino acids 607-772 of p115, corresponding to the neck region of the protein linking the head and tail domains (Figure 3.5). p115 therefore appears to have two distinct rab binding sites in its sequence: a rab1 binding site (also capable of interacting with rab2 in the yeast two hybrid system) in the head domain of the protein, and a rab33b binding site in the neck region.

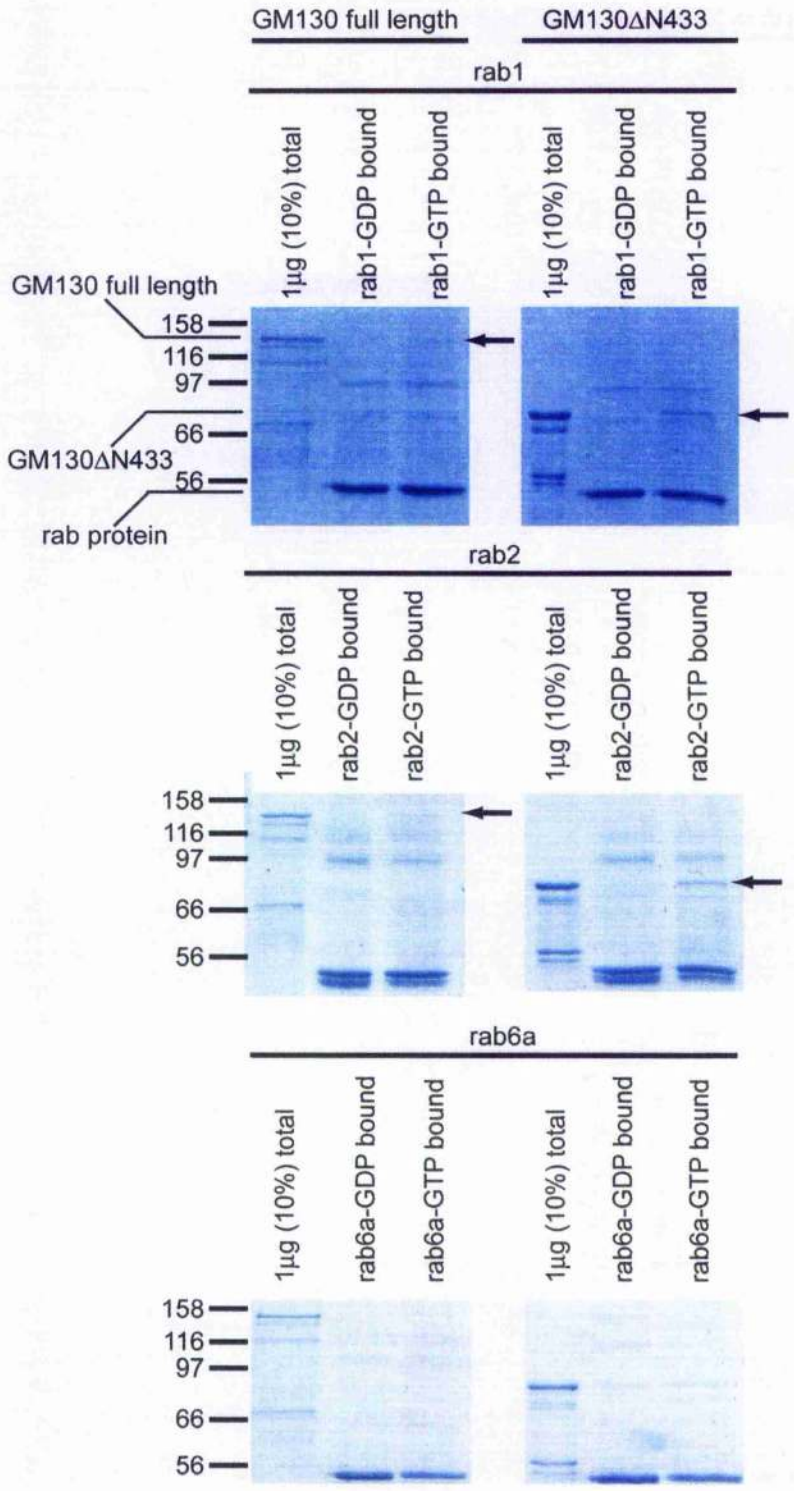


Figure 3.6 GM130 binds directly to rab1 and rab2
4 µg recombinantly expressed GST-tagged rab proteins were incubated with either 10 µg His-tagged GM130 or GM130ΔN433 and 15 µl glutathione-sepharose beads in the presence of either 10 µM GDP or GMP-PNP in a total volume of 400 µl HNTM for 1 hour at 4°C. Bound proteins were eluted in SDS-PAGE sample buffer and analysed by SDS-PAGE. Both GM130 and GM130ΔN433 were specifically bound by activated rab1 and rab2 as indicated by the arrows. Gels have been scaled to match.

3.3.5 GM130 binds rabs 1 and 2 directly

Direct binding assays were also performed between recombinantly expressed His-tagged GM130 protein and GST-tagged rab proteins (Figure 3.6). The C-terminal half of GM130 (GM130ΔN433) was also expressed and used in binding assays. As shown in Figure 3.6, both full length GM130 and GM130ΔN433 were able to bind to activated rab1 and rab2 but not to rab1-GDP or rab2-GDP. Neither protein was able to bind rab6a (Figure 3.6). This supports the data from Figure 3.3 in which GM130 demonstrated interactions with rabs 1 and 2 by yeast two hybrid and by pulldowns from Golgi membranes. Additionally, the binding of GM130ΔN433 suggests that the binding sites for rabs 1 and 2 lie in the C-terminus of GM130.

3.3.6 GM130 contains distinct rab binding sites

To further define the binding site for rabs 1 and 2 on GM130 as well as to map the potential binding site for GM130, deletion mutants of GM130 were cloned into pACT2 and screened in the yeast two hybrid system against rab GTP-restricted point mutants cloned into pGBT9 (Figure 3.7). In agreement with the binding data from Figure 3.6, GM130ΔN433 interacts with activated rab1 and rab2. This region also interacts with activated rab33b. The N-terminus of GM130 (GM130ΔC271) showed no interaction with any of the rabs tested.

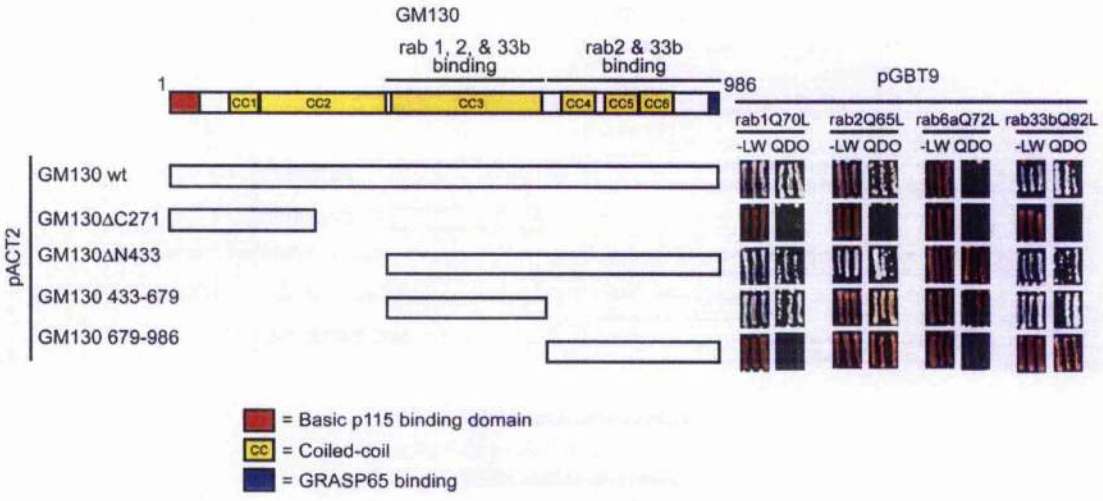


Figure 3.7 Mapping of rab binding sites on GM130 by yeast two hybrid
Deletion mutants of GM130 were cloned into the yeast two hybrid prey vector pACT2 and were screened against the GTP-restricted point mutants rab1Q70L, rab2Q65L, rab6aQ72L, and rab33bQ92L cloned into the bait vector pGBT9. Interactions were assessed by growth on quadruple dropout media. Rab6aQ72L shows a slight interaction with GM130ΔN433 and GM130 433-679 but this is most likely background activation since levels of growth are much less than that seen for the same GM130 fragments with the other 3 rabs.

The C-terminus of GM130 is predicted to contain 4 coiled-coil regions as shown in Figure 3.7. Analysis of further deletion mutants of GM130 suggested that rabs 1, 2, and 33b were all able to bind to amino acids 433-679 of GM130, a region predicted to form one long coiled-coil. Additionally, GM130 amino acids 679-986, encompassing three short stretches of coiled-coil, was able to interact with activated rab2 and rab33b, but not with activated rab1. This suggests the presence of at least two distinct rab binding sites in the C-terminus of GM130, both of which consist extensively of coiled-coil. These binding sites appear to be able to interact with more than one rab protein but the two sites have slightly different specificities with amino acids 433-679 able to bind rabs 1, 2, and 33b and amino acids 679-986 only binding rabs 2 and 33b.

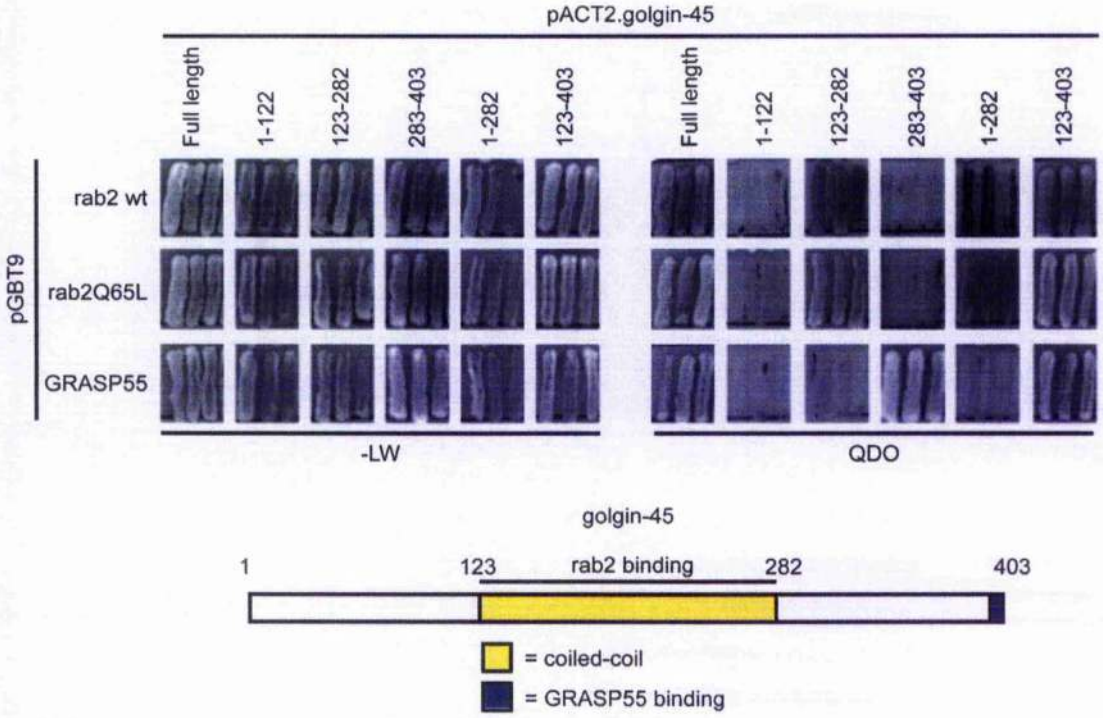


Figure 3.8 Rab2 binds to the coiled-coil region of golgin-45
Deletion mutants of golgin-45 were cloned into the yeast two hybrid prey vector pACT2 and were screened against rab2 wild type, the GTP-restricted point mutant rab2Q65L, and GRASP55 cloned into the bait vector pGBT9. Interactions were assessed by growth on quadruple dropout media.

3.3.7 Rab2 binds the coiled-coil region of golgin-45

It was not possible to express and purify soluble golgin-45 in *E.coli*, and so we were unable to perform a direct binding assay between golgin-45 and rab2. It was possible, however, to map the rab2 binding site to the central, coiled-coil domain of golgin-45 using the yeast two hybrid system (Figure 3.8). Deletion mutants of golgin-45 were

cloned into pACT2 and screened against rab2. The region encompassing amino acids 123-282 was necessary and sufficient for the interaction with activated rab2 (Figure 3.8). Only mutants containing the C-terminus of golgin-45 were able to interact with GRASP55, agreeing with the finding that the last 7 amino acids of golgin-45 are essential for this interaction (Figure 3.1 A).

3.3.8 Depletion of rabs and golgins by RNA interference

The data presented above suggests that a network of interactions exists at the early Golgi between rabs and coiled-coil proteins. To investigate the function of these proteins in maintaining Golgi structure, rab1, rab2, p115, GM130, and golgin-45 were specifically depleted in cells using the technique of RNA interference (Elbashir *et al.* 2001). This technique uses 21 base RNA duplexes to target particular mRNAs for degradation, thereby reducing protein levels in the cell. Duplexes were therefore designed to target early Golgi proteins and the effects on Golgi structure were monitored.

3.3.8.1 Depletion of rab1 disrupts the Golgi apparatus

Treatment of HeLa cells with rab1 small interfering RNA (siRNA) duplexes resulted in the depletion of rab1 and the disruption of Golgi structure (Figure 3.9). After 72 hours, rab1 was no longer detectable by immunofluorescence compared to control cells treated with siRNA targeting lamin-A (Figure 3.9 A-C). At this time point, GM130 was found in small, punctate structures diffuse throughout the cytosol while control cells displayed a normal, reticular, perinuclear, staining pattern (Figure 3.9 A). p115 staining was also diffused throughout the cell in the absence of detectable rab1 (Figure 3.9 B). The medial Golgi-localised enzyme *n*-acetylglucosaminyltransferase-I (NAGT-I), on the other hand, redistributes to give a nuclear envelope and ER-like staining pattern when rab1 is depleted (Figure 3.9 C). Thus, depletion of rab1 gives rise to a phenotype similar to the treatment of cells with BFA or dominant negative mutants of Sar1p, in which forward transport from the ER to Golgi is blocked. Golgi resident enzymes recycle back to the ER, while Golgi matrix proteins remain in small Golgi remnant structures.

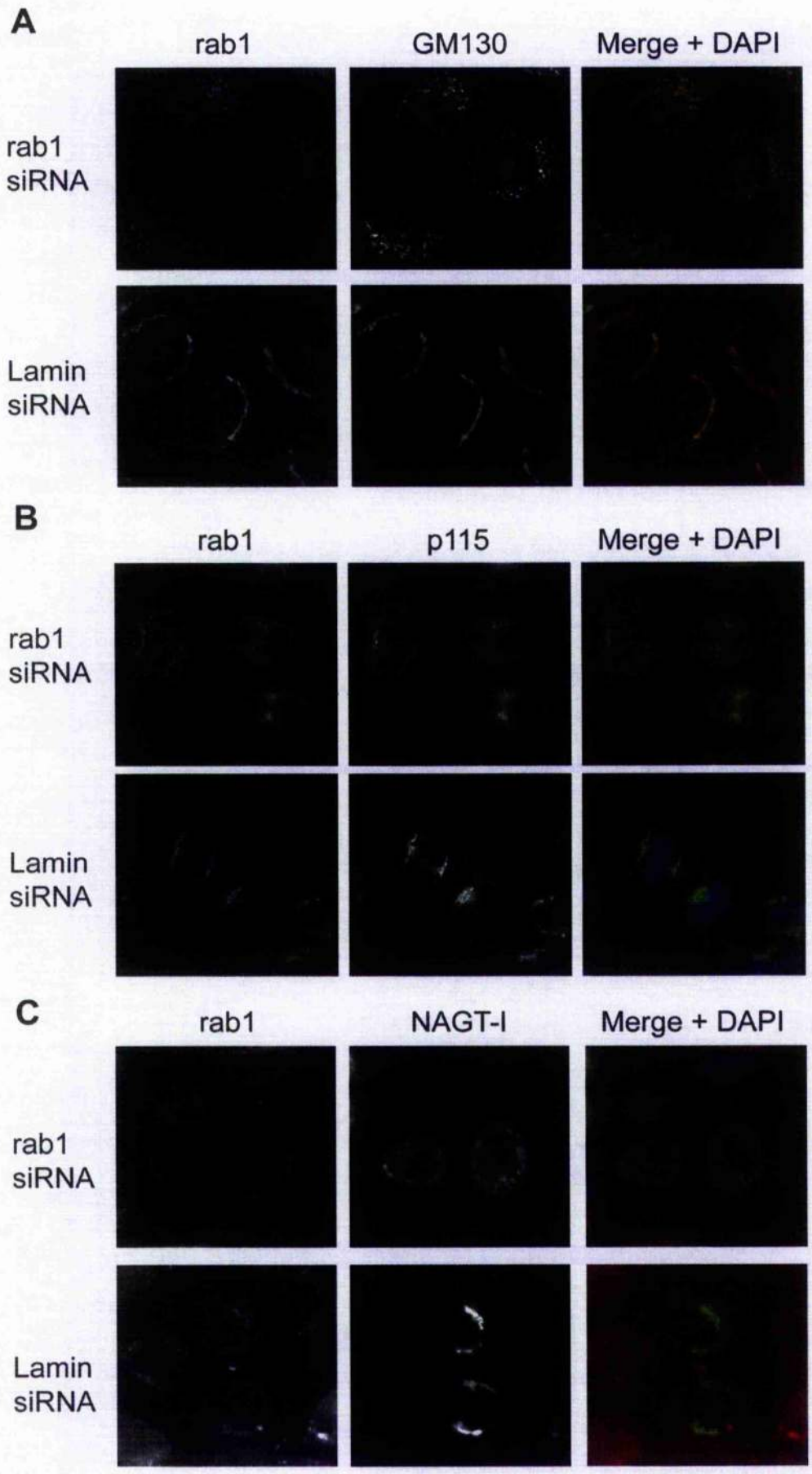


Figure 3.9 Depletion of rab1 disrupts the Golgi apparatus
(A) HeLa cells were treated with siRNA duplexes targeting either rab1 or lamin-A for 72 hours prior to fixation with 3% PFA and staining with mouse anti-rab1 and sheep anti-GM130. In the merged images, DNA (DAPI staining) is in blue, rab1 is in red, and GM130 is in green. (B) HeLa cells were treated with siRNA duplexes targeting either rab1 or lamin-A for 54 hours and then transfected with a plasmid encoding GFP-tagged NAGT-I for a further 18 hours. Cells were then fixed with 3% PFA and stained with mouse anti-rab1. In the merged images, DNA (DAPI staining) is in blue, rab1 is in red, and GFP-NAGT-I is in green.

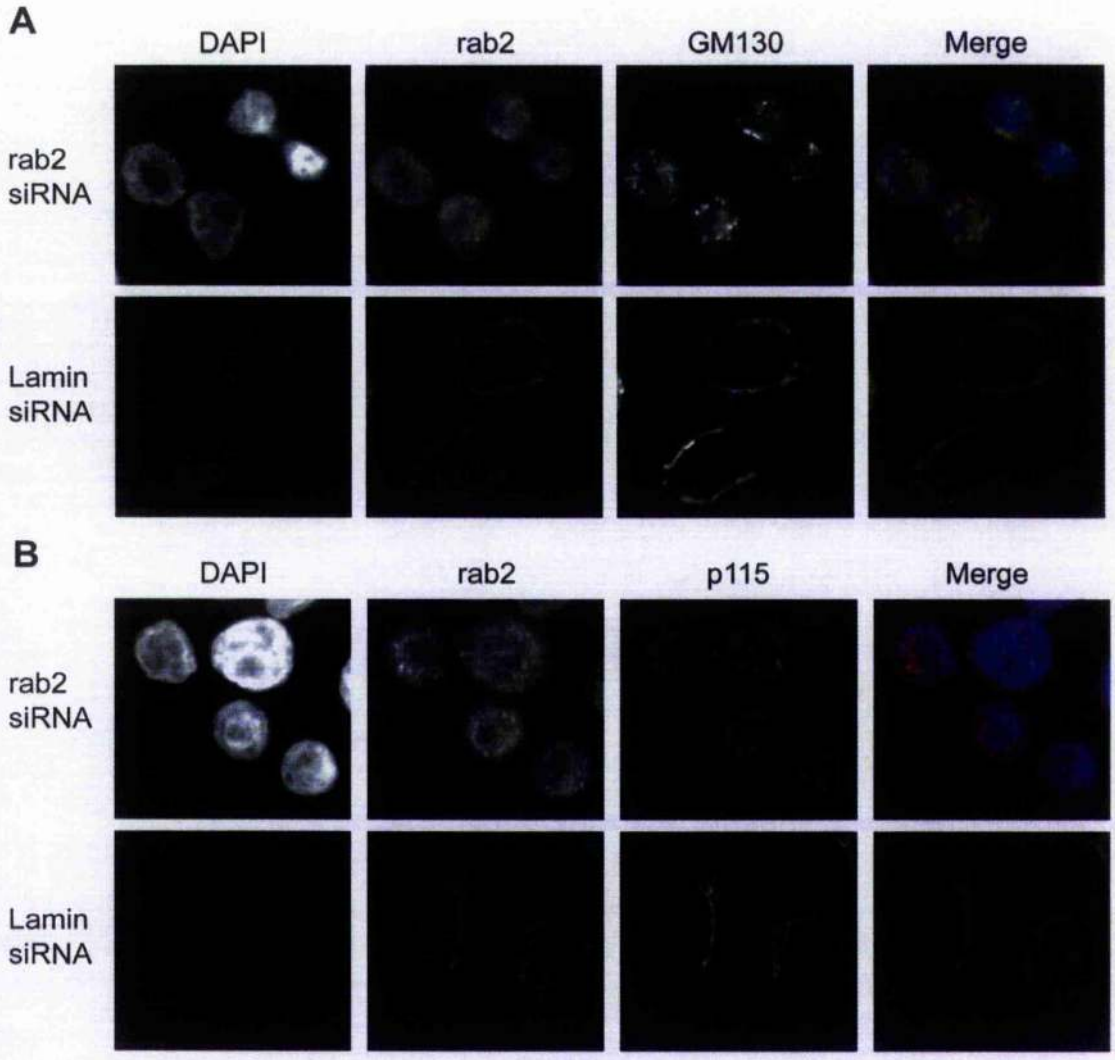


Figure 3.10 Depletion of rab2 disrupts the Golgi and causes cell death
HeLa cells were treated with siRNA duplexes targeting either rab2 or lamin-A for 72 hours prior to fixation with 3% PFA and staining with rabbit anti-rab2 and either sheep anti-GM130 (A) or sheep anti-p115 (B). In the merged images, DNA (DAPI staining) is in blue, rab2 is in red, and GM130/p115 is in green.

3.3.8.2 Depletion of rab2 disrupts the Golgi apparatus and causes cell death

Treatment of HeLa cells with rab2 siRNA duplexes resulted in the depletion of rab2, the disruption of Golgi structure, and an adverse effect on cell viability (Figure 3.10). After 72 hours, rab2 was no longer detectable by immunofluorescence compared to control cells treated with lamin-A siRNA (Figure 3.10 A and B). This corresponded to an increase in cell death and a marked change in cellular morphology: rab2-depleted cells became very small and rounded with compact nuclei taking up almost the entire cell and little cytoplasm remaining. GM130 and p115 staining was still present but was disrupted into punctate structures compared to control cells (Figure 3.10 A and B).

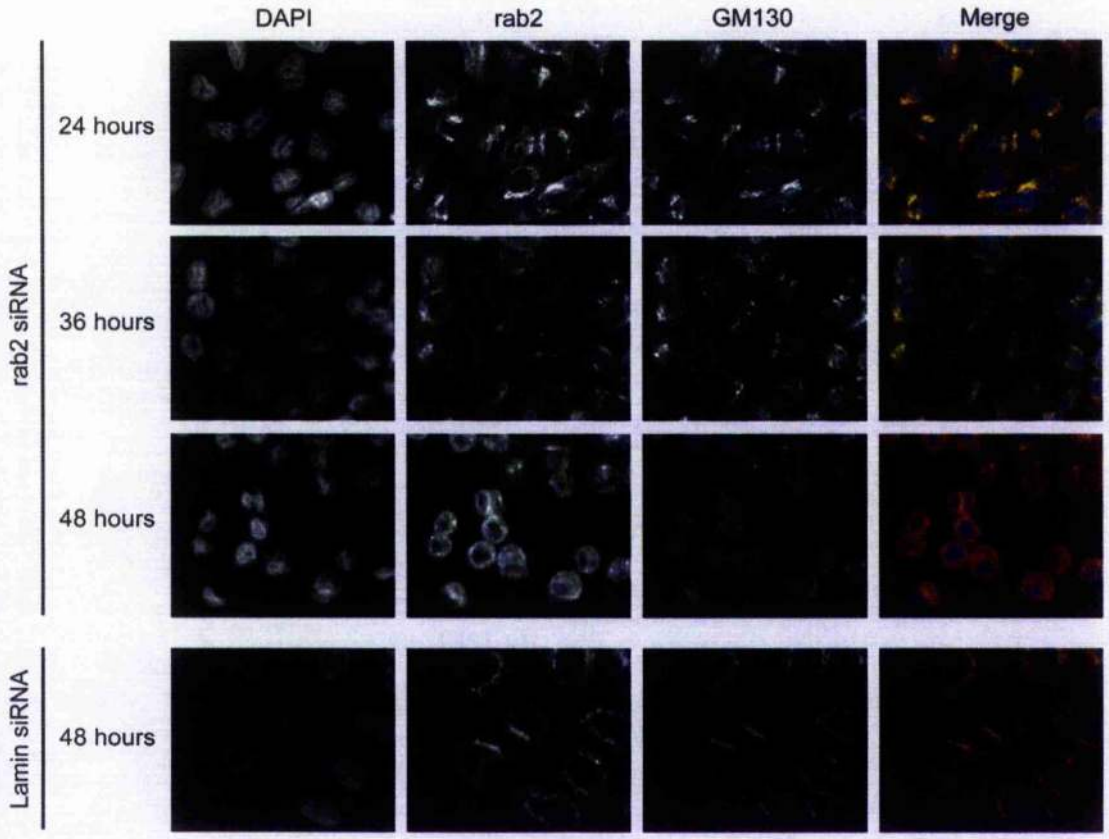


Figure 3.11 Depletion of rab2 correlates with changes in cell morphology
HeLa cells treated with siRNA duplexes targeting rab2 for 24, 36, or 48 hours were fixed with cold methanol and stained with rabbit anti-rab2 and sheep anti-GM130. In the merged images, DNA (DAPI staining) is in blue, rab2 is in red, and GM130 is in green. Control cells treated with siRNA duplexes targeting lamin-A for 48 hours were fixed and stained identically.

In order to better follow the changes associated with depletion of rab2, siRNA-treated cells were fixed at earlier time points and stained for rab2 and GM130 (Figure 3.11). This analysis showed a decrease in rab2 staining between 24 and 48 hours of treatment concomitant with an increase in the number of dying cells displaying an rounded morphology and reduced cytoplasmic volume. After 24 hours, the cells still appear normal with rab2 staining still present and GM130 staining appearing regular. At 36 hours, an intermediate phenotype is seen, in which rab2 levels are partially, but not completely, depleted and GM130 staining shows that the Golgi has become disrupted into smaller punctate and reticular structures. Most cells however are still flat and normally shaped. By 48 hours, the majority of cells have lost rab2 staining, as well as normal GM130 staining, and have become condensed and rounded up (Figure 3.11). Lamin-A siRNA-treated cells at the same time point are unaltered in

terms of Golgi structure and cell shape. The difference in size between rab2-depleted and control cells is readily apparent.

Depletion of rab2 therefore results in the loss of Golgi structure with a deleterious effect on the cell as a whole.

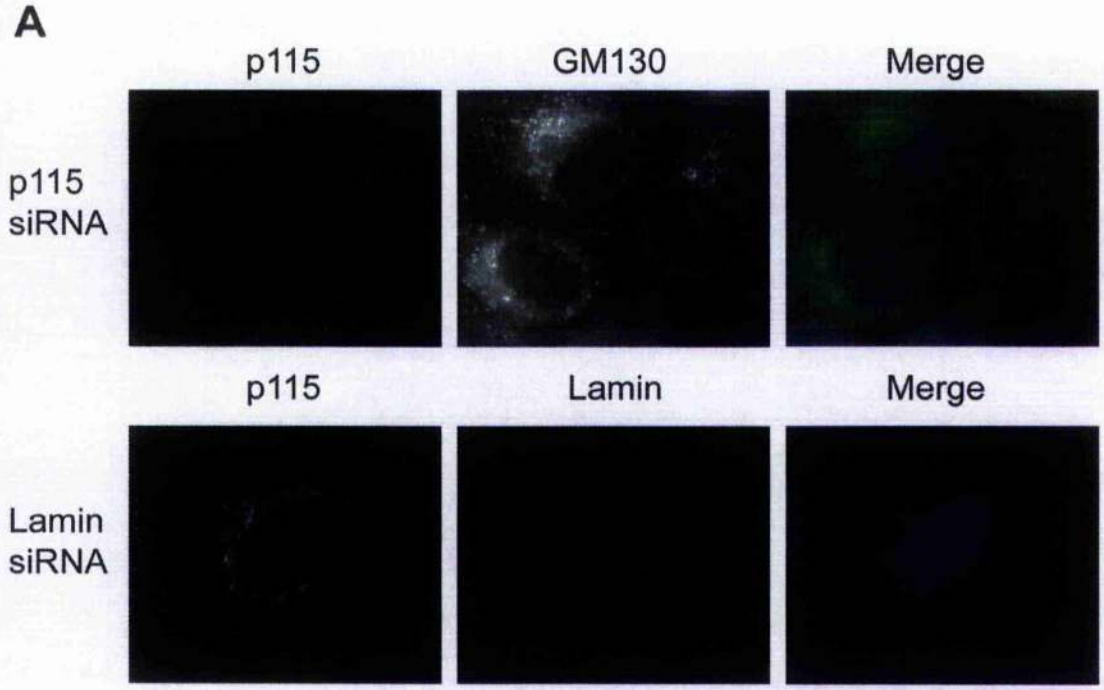


Figure 3.12 Depletion of p115 disrupts the Golgi apparatus

(A) HeLa cells were treated with siRNA duplexes targeting either p115 or lamin-A for 120 hours prior to fixation with cold methanol. 1.6μg/ml aphidicolin was added to the cells after 48 hours in order to prevent cells from overgrowing. p115 siRNA-treated cells were stained with sheep anti-p115 and rabbit anti-GM130. Lamin-A siRNA-treated cells were stained with sheep anti-p115 and mouse anti-lamin. In the merged images, DNA (DAPI staining) is in blue, p115 is in red and GM130/lamin is in green. (B) HeLa cells treated as in (A) were extracted in TNTE buffer after 120 hours. 25μg extract was western blotted and probed with sheep anti-p115 and, as a loading control, mouse anti-tubulin.

3.3.8.3 Depletion of p115 disrupts the Golgi apparatus

Treatment of cells with siRNA duplexes targeting p115 resulted in the depletion of p115 and the disruption of the Golgi apparatus (Figure 3.12). Depletion of p115 protein levels took a longer time than that required to deplete rab1 or rab2 but, after 120 hours, p115 levels were undetectable by either immunofluorescence (Figure 3.12

A) or western blotting (Figure 3.12 B) compared to lamin-A siRNA-treated cells. At this time, normal Golgi structure was disrupted, as assessed by staining for GM130 (Figure 3.12 A) which was redistributed into small, punctate structures diffuse throughout the cytoplasm reminiscent of the staining pattern seen following rab1-depletion (Figure 3.9 A). Control cells showed normal levels of p115 and a normal p115 staining pattern. p115 is therefore also essential for the maintenance of normal Golgi structure.

3.3.8.4 Depletion of GM130 disrupts the Golgi apparatus and changes cell morphology

Treatment of cells with siRNA duplexes targeting GM130 resulted in the depletion of GM130, disruption of the Golgi apparatus, and an alteration in cellular morphology (Figure 3.13). After 72 hours, GM130 was no longer detectable by immunofluorescence (Figure 3.13 A-C) or by western blotting (Figure 3.13 D) compared to control cells treated with lamin-A siRNA. p115 levels remained unaffected (Figure 3.13 D) but its staining pattern was altered such that smaller, punctate structures throughout the cytoplasm and, in some cells, a reduced perinuclear staining compared to the extended reticular network seen in control cells (Figure 3.13 A). The staining patterns of other Golgi markers were also affected with golgin-97 (*trans*-Golgi) staining becoming dispersed into smaller, punctate structures (Figure 3.13 B) and GRASP55 (medial Golgi) staining almost entirely disappearing (Figure 3.13 C).

Strikingly, cell morphology was also altered by treatment with GM130 siRNA duplexes. DAPI staining revealed the nuclei to be misshapen while long processes formed from the cytoplasm projecting outwards from the centre of the cell (Figure 3.13 A-C).

p115 is proposed to be recruited to *cis*-Golgi membranes by binding to the N-terminus of GM130 (Nakamura *et al.* 1997). Phosphorylation of GM130 serine25 by Cdk1 at the onset of mitosis is thought to inhibit this interaction, thereby preventing p115-GM130 mediated vesicle docking and initiating Golgi mitotic fragmentation (Lowe *et al.* 1998). We therefore reasoned that transfection of cells with a siRNA-resistant form of GM130 should be able to restore normal p115 staining to GM130-depleted cells. GM130 siRNA-treated cells were transiently transfected after 24 hours with a

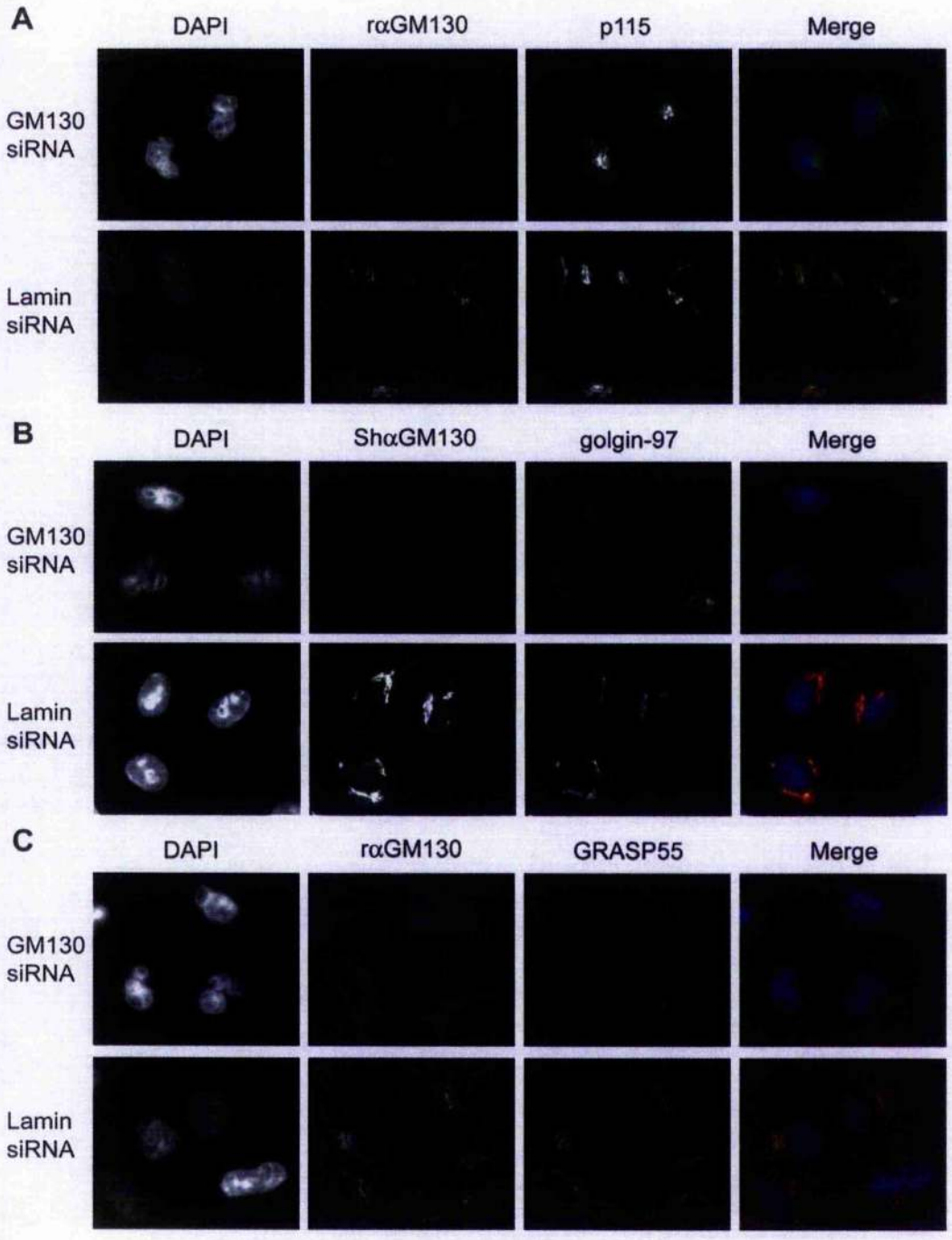


Figure 3.13 Depletion of GM130 disrupts the Golgi apparatus and alters cell shape

(A-C) HeLa cells were treated with siRNA duplexes targeting either GM130 or lamin-A for 72 hours prior to fixation with either 3% PFA (A) or cold methanol (B and C). Cells were then stained with either rabbit anti-GM130 (A and C) or sheep anti-GM130 (B) as well as either sheep anti-p115 (A), mouse anti-golgin-97 (B), or sheep anti-GRASP55 (C). In the merged images, DNA (DAPI staining) is in blue, GM130 is in red, and p115/golgin-97/GRASP55 is in green. (D) HeLa cells treated as in (A-C) were extracted in TNTE and 25μg extract was western blotted and probed with sheep anti-GM130, sheep anti-p115, and, as a loading control, mouse anti-tubulin.

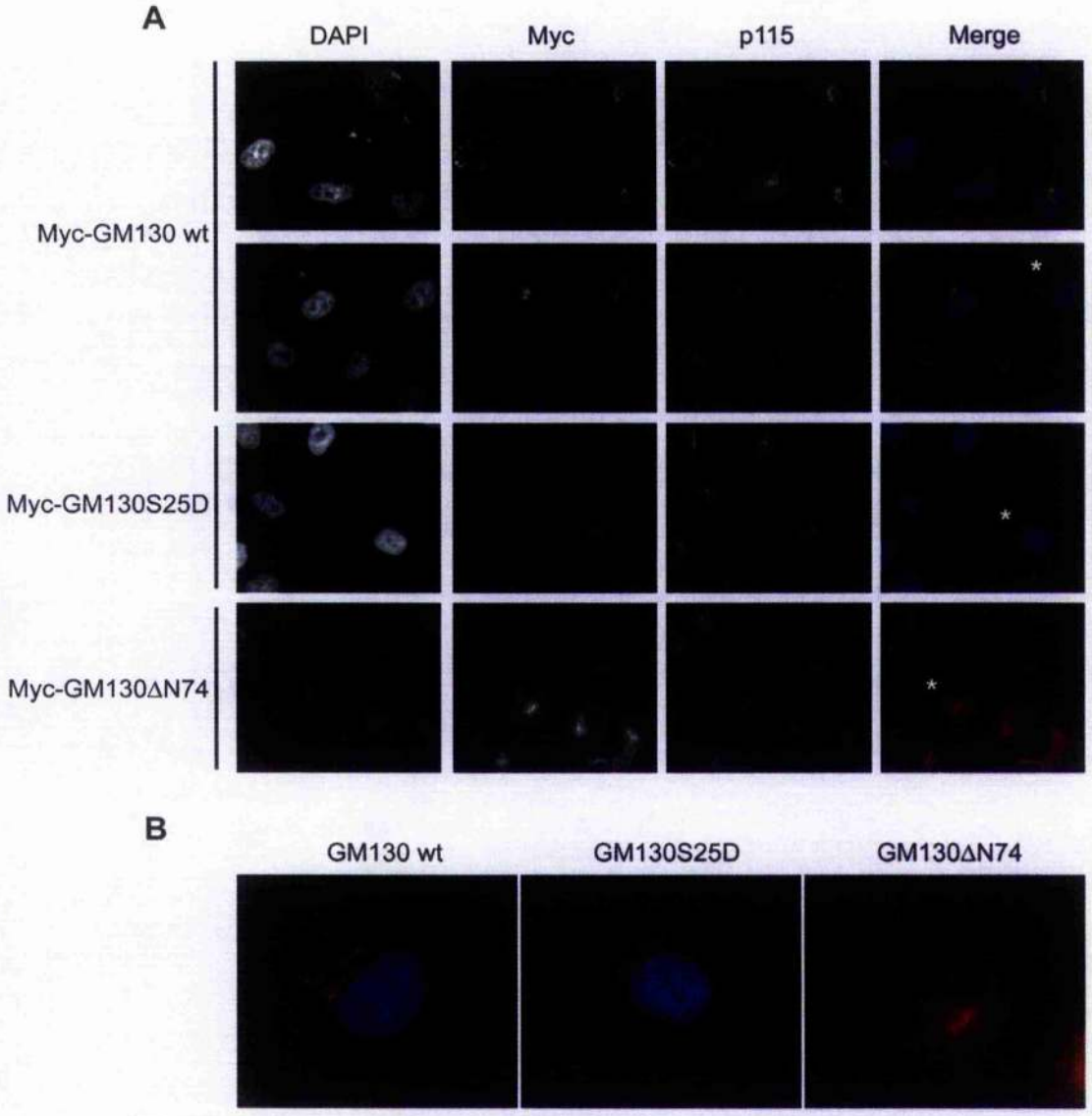


Figure 3.14 Transient transfection of wild type, but not mutant, rat GM130 restores p115 localisation in GM130-depleted cells

(A) HeLa cells were treated with siRNA duplexes targeting GM130 for 24 hours before transfection with myc-tagged rat GM130 constructs for a further 48 hours prior to fixation with 3% PFA and staining with mouse anti-myc and sheep anti-p115. Note that p115 localisation is restored in cells transfected with wild type GM130 or GM130S25D but not in neighbouring, non-transfected cells nor in cells transfected with GM130ΔN74. (B) Cells marked with an asterisk in (A) are shown enlarged. In the merged images in (A) and (B), DNA (DAPI staining) is in blue, myc-tagged GM130 is in red, and p115 is in green.

plasmid encoding myc-tagged rat GM130 and fixed with 3% PFA after a further 48 hours (Figure 3.14). The nucleotide sequence of rat GM130 differs from human GM130 in the region targeted by the GM130 siRNA duplex and should therefore be resistant to siRNA-mediated degradation.

Staining of cells with anti-myc revealed that myc-tagged GM130 was expressed in siRNA-treated cells and localised to perinuclear structures reminiscent of the Golgi

apparatus in untreated cells (Figure 3.14). p115 co-localised to these structures while it was absent in neighbouring, non-transfected cells, suggesting that rat GM130 was able to rescue p115 localisation in GM130-depleted cells. Transfection of cells with a mutant rat GM130 (GM130S25D) thought to mimic the Cdk1-phosphorylated form of GM130 was also able to restore p115 localisation (Figure 3.14). However, a deletion mutant of rat GM130 lacking the first 74 amino acids (GM130ΔN74) was unable to restore normal p115 localisation, even though the GM130 mutant itself appeared to localise to a Golgi-like structure (Figure 3.14). This suggests that the N-terminus of GM130 is required for the correct localisation of p115 to the *cis*-Golgi and that either binding is not regulated by phosphorylation or that the mutation of serine25 to aspartate does not accurately mimic the phosphorylation of serine25 by Cdk1 since this mutant, along with wild type GM130, successfully rescues the siRNA phenotype of GM130-depleted cells.

3.3.8.5 Rab1 is the major membrane receptor for p115

GM130 is not, however, the only proposed membrane-associated receptor for p115, since rab1 is also able to recruit p115 (Allan *et al.* 2000). To assess the contributions of rab1 and GM130 to the membrane-association of p115, I compared the localisation of p115 in rab1- and GM130-depleted cells and in cells simultaneously treated with rab1 and GM130 siRNAs (Figure 3.15). In control cells, treated only with lamin-A siRNA, p115 displays a normal, perinuclear, reticular staining pattern. When rab1 alone is depleted from the cells, p115 is diffused throughout the cell with some small, punctate structures remaining (Figure 3.15). In GM130-depleted cells, p115 staining appears to become more concentrated in a compacted, perinuclear region, as seen previously in Figures 3.13 and 3.14. This structure also stains positive for rab1 (Figure 3.15). Some cells treated with GM130 siRNA also show a diffuse, cytoplasmic staining pattern for p115, but these cells also no longer stain for rab1. This is also demonstrated in cells simultaneously depleted of both GM130 and rab1 (Figure 3.15). All cells depleted of GM130 (as assessed by the change in cellular morphology described in section 3.3.8.4) show a change in p115 localisation and membrane-association. However, it is only in cells also depleted of rab1 that p115 staining is completely lost from membrane structures (compare the two cells indicated by white arrows in Figure 3.15 with the two neighbouring cells). This suggests that,

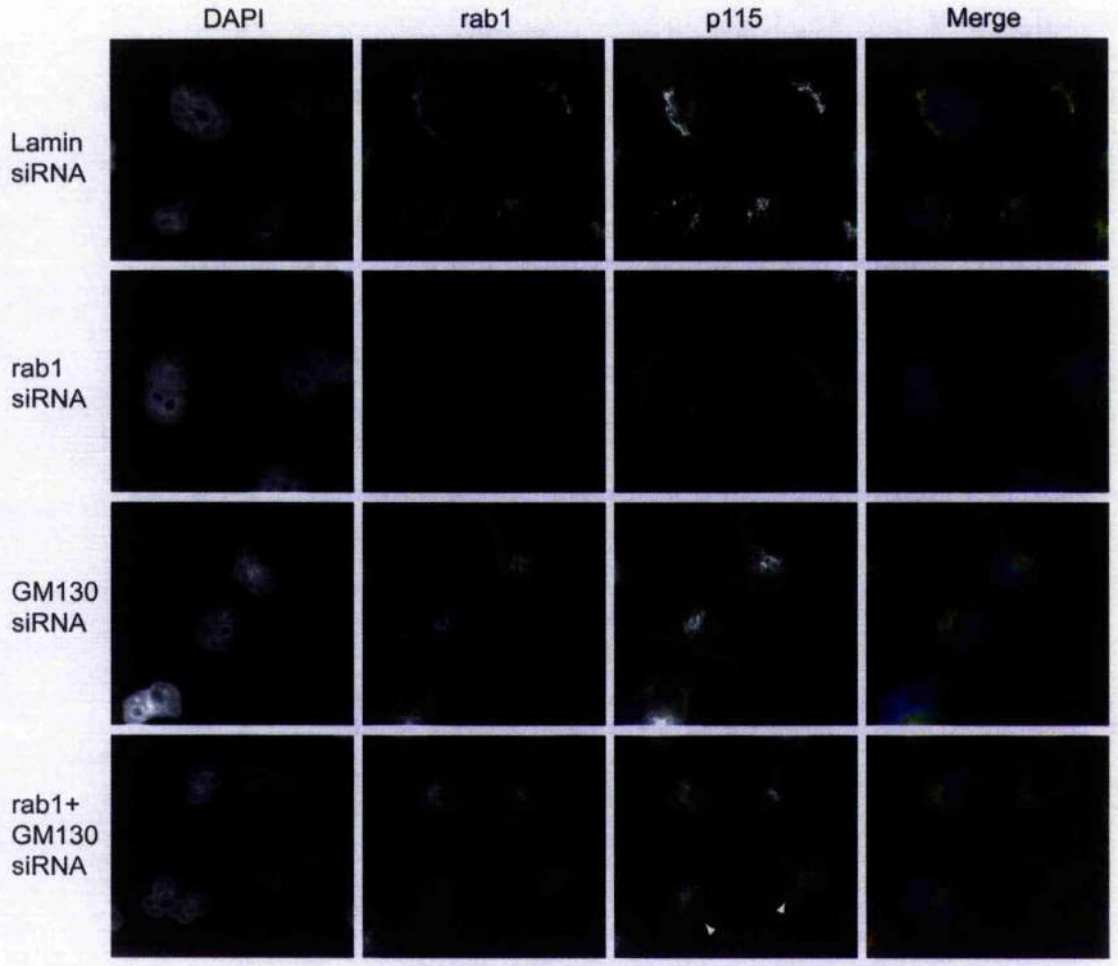


Figure 3.15 Depletion of rab1 and GM130 affects p115 localisation and membrane-association

HeLa cells were treated with siRNA duplexes targeting either lamin-A alone, rab1 and lamin-A, GM130 and lamin-A, or rab1 and GM130. Cells were fixed with 3% PFA after 72 hours and stained with mouse anti-rab1 and sheep anti-p115. The white arrows indicate two cells in the GM130/rab1 double RNAi that are completely depleted of rab1. Note, that in these two cells, p115 staining is completely lost when compared to the two neighboring cells, which still display some rab1 staining. In the merged images, DNA (DAPI staining) is in blue, rab1 is in red, and p115 is in green.

although both rab1 and GM130 are important for p115 localisation, the major protein responsible for the membrane-association of p115 is rab1.

3.3.8.6 Depletion of golgin-45 disrupts the Golgi apparatus

Treatment of cells with siRNA duplexes targeting golgin-45 resulted in the depletion of golgin-45 and disruption of the Golgi apparatus (Figure 3.16). After 48 hours, golgin-45 was undetectable by immunofluorescence compared to lamin-A siRNA-treated cells (Figure 3.16 A-C). At this time point, normal Golgi structure was disrupted, as assessed by staining for GRASP55 (Figure 3.16 A) and GM130 (Figure

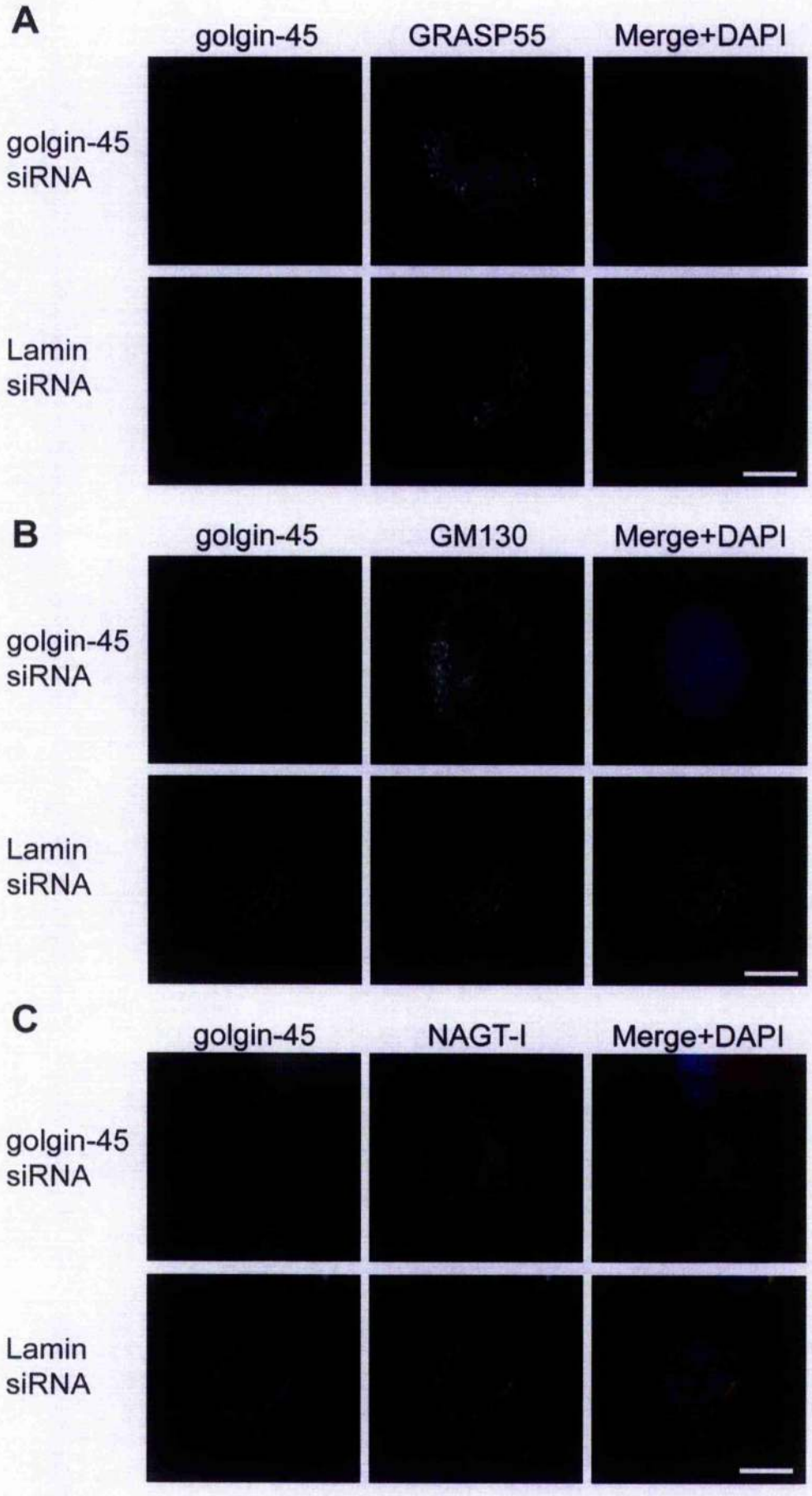


Figure 3.16 Depletion of golgin-45 disrupts the Golgi apparatus
HeLa cells were treated with siRNA duplexes targeting either golgin-45 or lamin-A for 48 hours (A-B) or 54 hours (C) prior to fixation with 3% PFA. Cells in (C) were transfected with a plasmid encoding GFP-tagged NAGT-I after 36 hours. Cells were stained with rabbit anti-golgin-45 and either sheep anti-GRASP55 (A) or sheep anti-GM130 (B). In the merged images, DNA (DAPI staining) is in blue, golgin-45 is in red, and GRASP55/GM130 /NAGT-I is in green.
Bars, 10μM.

3.16 B). These two matrix proteins, medial and *cis*-localised respectively, redistribute to small, punctate structures diffused throughout the cytoplasm. The medial Golgi enzyme, NAGT-I, on the other hand, redistributes to give a nuclear envelope and ER-like staining pattern in golgin-45-depleted cells compared to lamin-A-depleted cells (Figure 3.16 C). Golgin-45 therefore appears to be essential for the maintenance of Golgi structure.

3.4 Discussion

3.4.1 Golgin-45 is a novel Golgi matrix protein localised to the medial Golgi

Golgin-45 was identified as an interactor of the medial Golgi matrix protein GRASP55 by a yeast two hybrid screen and purification of endogenous GRASP55 complexes (Figure 3.1). It has several features in common with other components of the Golgi matrix, particularly with GM130. It contains a region predicted to form a coiled-coil structure and, like GM130, it interacts via its C-terminus with a GRASP protein (Figure 3.1 A; (Barr *et al.* 1998)). The protein behaves as expected for a Golgi matrix protein *in vivo*, in that it redistributes from the Golgi stack to smaller, punctate, Golgi remnants upon treatment with BFA or nocodazole (Figure 3.2 A and B) rather than to the ER as would be expected for a Golgi resident enzyme (Seemann *et al.* 2000).

Golgin-45 is clearly important for the maintenance of Golgi structure since both overexpression of the protein (Figure 3.2 C) and its depletion by siRNA (Figure 3.16) disrupts the Golgi. This is not as paradoxical as it may first appear. The Golgi apparatus faces a constant flux of material through its structure and, in order to maintain its normal structure and function this flux must be carefully controlled (Warren and Malhotra 1998). Overexpression or depletion of a component important for regulating transport through the Golgi might therefore be expected to disrupt normal traffic flow and hence Golgi structure.

3.4.2 Golgins are rab effectors

If Golgi-localised coiled-coil proteins such as golgin-45 are key regulators of membrane traffic through the Golgi apparatus, they may do this via interactions with the rab family of small GTPases known to be regulators of vesicle transport. Using

the yeast two hybrid system, golgin-45 was found to specifically interact with the activated form of rab2, GM130 was able to interact with rab1, rab2 and rab33b, while p115 displayed interactions with rab1 and rab33b (Figure 3.3 A). The interactions between rab1 and p115, rab1 and GM130, and rab33b and GM130 agree with published data (Allan *et al.* 2000; Moyer *et al.* 2001; Valsdottir *et al.* 2001; Weide *et al.* 2001). Binding assays were also used to confirm the interactions of rabs 1 and 2. Both p115 and GM130 are pulled down from Golgi membranes by rab1 (Figure 3.3 B) and the recombinant proteins are able to bind directly (Figures 3.4 and 3.6). Golgin-45 and GM130 are pulled down from Golgi membranes by rab2 (Figure 3.3 B) and recombinant GM130 binds directly (Figure 3.6). p115 is also pulled down by rab2 (Figure 3.3 B) but full length p115 does not bind directly (Figure 3.4) nor does it interact by yeast two hybrid (Figure 3.3 A). It is most likely therefore that p115 is pulled down by rab2 as a result of its interaction with GM130.

Golgin-45 and GM130 are the first known effectors of rab2, a protein previously implicated in anterograde transport (Tisdale *et al.* 1992). This suggests that rab2-mediated vesicle transport occurs at the level of both the *cis*- and medial Golgi. The interaction of GM130 with rab33b, a protein implicated in retrograde transport (Valsdottir *et al.* 2001), suggests that GM130 is also involved in retrograde trafficking, fitting with its involvement in COPI vesicle docking (Sonnichsen *et al.* 1998).

The ability of GM130 and, potentially, p115 to bind multiple rab proteins would therefore fit with the multiple roles assigned to these proteins. Interestingly, mapping of the rab binding sites on these proteins revealed that these sites were distinct, raising the possibility that these golgins may be able to bind multiple rab proteins simultaneously. The globular head domain of p115 binds rab1 (and rab2, although not in the context of the full length protein) while the neck domain binds rab33b (Figure 3.5). Amino acids 433-679 of GM130 bind rab1, rab2, and rab33b while amino acids 679-986 bind rab2 and rab33b (Figure 3.7). Both of these sites contain extensive regions of coiled-coil, a motif that appears to be a common feature of rab-binding proteins. Similarly, the rab2 binding site on golgin-45 maps to the central coiled-coil domain of the protein (Figure 3.8).

3.4.3 Rabs and golgins are essential for maintaining Golgi structure

As discussed in section 3.4.1, golgin-45 is essential for maintaining Golgi structure since its depletion by siRNA disrupts the Golgi (Figure 3.16). siRNA analysis of other early Golgi proteins suggests they are also essential for normal Golgi structure and function.

Depletion of rab1 causes Golgi matrix proteins to redistribute into punctate Golgi remnants while Golgi enzymes relocalise to the ER (Figure 3.9). This is similar to the effects seen upon BFA treatment or addition of dominant negative Sar1p mutants which block ER to Golgi transport (Seemann *et al.* 2000). Since rab1 itself is implicated in ER to Golgi transport (Tisdale *et al.* 1992), depletion of the protein might be expected to block this transport step and produce such similar effects.

Rab2 is also important for ER to Golgi transport (Tisdale *et al.* 1992), but its depletion by siRNA produces a markedly different effect (Figure 3.10 and 3.11). Normal Golgi structure is disrupted, but the rest of the cell is also strongly affected as they shrink in size, round up, and eventually die. This corresponds in time with the loss of rab2 and disruption of the Golgi (Figure 3.11) but it is certainly possible that the siRNA duplex designed to target rab2 has additional effects on other mRNA(s) or is toxic in some other way. The design of a second oligonucleotide pair targeting rab2 would help to determine the specificity of the rab2-siRNA phenotype but the ultimate test of specificity would come from the ability of an siRNA-resistant rab2 to rescue the phenotype. Unfortunately, technical difficulties with transiently transfecting rab2 siRNA-treated cells mean that rescue experiments must await the generation of a stable cell line expressing a resistant form of rab2. If this phenotype is a direct result of rab2-depletion, it is possible to envisage several explanations depending upon subtle differences between rab1 and rab2 function. Since golgin-45 depletion does not display a similar phenotype, the explanation is not likely to involve the disruption of rab2-golgin-45 function. Rab2 has been reported to be essential for the maturation of pre-Golgi intermediates (Tisdale and Balch 1996). Prevention of this step might cause an accumulation of proteins in the ER and subsequent activation of the unfolded protein response (Kaufman *et al.* 2002), leading to cell death. Alternatively, loss of rab2 might result in too much material exiting the ER due to an ER retention defect, transiting through the Golgi, and being secreted, resulting in a cell stress response.

This possibility could be tested for by examining the localisation of various ER resident proteins.

Depletion of p115 results in a more expected phenotype – similarly to rab1 depletion, Golgi matrix proteins are redistributed into punctate remnant structures (Figure 3.12). Depletion of GM130, also results in the disruption of the Golgi apparatus (Figure 3.13). GM130 function has been closely linked to Golgi structure, given that its phosphorylation by Cdk1 has been proposed to cause Golgi fragmentation at the onset of mitosis (Nakamura *et al.* 1997; Lowe *et al.* 1998). This model was recently contradicted however by a study characterising the conditional lethal mutant cell line ldlG, which is deficient in expression of the low-density lipoprotein receptor at the plasma membrane (Vasile *et al.* 2003). Although ldlG cells contain no detectable GM130 at 34°C, the temperature permissive for growth, they nevertheless have an organised and functional Golgi apparatus (Vasile *et al.* 2003). This is not to say these cells are entirely normal, as they exhibit reduced levels of protein secretion at the non-permissive temperature of 39.5°C and eventually die. This temperature-sensitive phenotype corresponds to a reversible disassembly of the Golgi at 39.5°C. Importantly, the growth and secretion phenotypes of ldlG cells can be rescued by transfection to restore expression of GM130 (Vasile *et al.* 2003).

In contrast to these results, depletion of GM130 in HeLa cells by siRNA does result in disruption of the Golgi apparatus (Figure 3.13). This discrepancy may simply be due to cell-type differences in the way in which Golgi structure is maintained or may be due to the higher temperature (37°C) at which siRNA treated HeLa cells were incubated. The efficiency of siRNA-treatment was greatly reduced at lower temperatures so that this latter possibility could not be tested (data not shown). Alternatively, a low level of GM130 may still exist in ldlG cells at 34°C which, although undetectable by immunofluorescence, is still sufficient to maintain Golgi structure. This critical threshold may be crossed in siRNA-treated HeLa cells. In any case, the data in Figure 3.13 suggests that, like p115, GM130 is essential for maintaining Golgi structure in HeLa cells at 37°C.

Depletion of GM130 also results in general changes in cell morphology as the cell nuclei become more irregularly shaped and cytoplasmic projections are formed (Figure 3.13). Again, it is possible that these effects are not related to GM130 depletion and are secondary effects of the GM130 siRNA duplex, although it is

tempting to speculate that depletion of a factor important for anterograde and retrograde transport through the Golgi might have effects on the morphology of the nuclear envelope and the plasma membrane. Transfection with siRNA-resistant rat GM130 was not able to rescue the cell morphology phenotypes (Figure 3.14), although this may be because the expression levels of rat GM130 are not sufficient when transiently transfected, or that rat GM130 only partially rescues the lack of human GM130. Generation of stable cell lines expressing rat GM130 would better address this question. Transient transfection of rat GM130 was able to restore the localisation of p115 to the Golgi however (Figure 3.14). This rescue depended on the N-terminus of GM130 since a deletion mutant did not restore p115 localisation although a serine to aspartate mutant purported to mimic the mitotic phosphorylation of GM130 was still able to rescue p115 staining (Figure 3.14). Nevertheless, this suggests that GM130 is important for the correct localisation of p115 to the *cis*-Golgi and proves the potential of rescue experiments in the further analysis of siRNA-induced phenotypes.

Further experiments, however, suggest that rab1, rather than GM130, is the principle determinant of p115 localisation (Figure 3.15). Direct comparison of the effects of depleting rab1 and GM130, either individually or simultaneously, revealed that the loss of p115 staining on membranous structures correlated with the presence or absence of rab1 in the cells. GM130 depletion also had an effect on p115 localisation but the effect was not as marked as seen for rab1. This broadly agrees with the findings of Nelson *et al*, (1998) who found that p115 constructs lacking the C-terminal GM130-binding site were still able to localise to the Golgi. Constructs lacking the first 283 amino acids from the N-terminus, however, failed to localise to the Golgi (Nelson *et al.* 1998). This N-terminal domain, containing the two regions most homologous to Uso1p, corresponds to the region found to interact with rab1 in the yeast two hybrid system (Figure 3.5).

Taken together, these results suggest that the recruitment of p115 to Golgi membranes is primarily dependent upon rab1 while GM130 binding is responsible for the correct organisation of these membranes. Since rab1 binds GM130 and, in rab1-depleted cells, GM130 is mislocalised (Figure 3.9), p115 is presumably neither efficiently recruited nor organised.

3.4.4 Summary

A number of rab proteins, golgins, and GRASP proteins exist at the early Golgi, which are essential for maintaining the normal structure and function of the Golgi. These proteins interact with one another – golgins with GRASPs, golgins with other golgins, and, perhaps most importantly in terms of regulatory events, golgins with rab proteins. A network of protein-protein interactions therefore establishes and maintains the early compartments of the Golgi apparatus.

Chapter 4: Identification Of Novel Rab-Effector Complexes At The Early Golgi

4.1 Introduction

As discussed in Chapter 3, rab proteins of the early Golgi are able to bind effector proteins in a nucleotide-dependent manner. Rab1 binds p115 and GM130 (Figure 3.3; (Allan *et al.* 2000; Moyer *et al.* 2001; Weide *et al.* 2001)); rab2 binds GM130 and golgin-45 (Figure 3.3); and rab33b binds p115 and GM130 (Figure 3.3; (Valsdottir *et al.* 2001)).

More recently, additional rab1 effectors have been identified in the form of golgin-84 and MICAL-1 (Diao *et al.* 2003; Satoh *et al.* 2003; Weide *et al.* 2003). The possibility exists that yet more effector proteins of rab1 and other early Golgi rabs remain to be found. Support for this idea comes from the fact that several proteins in addition to p115 were retained on GST-rab1-GTP beads in a pulldown from rat liver cytosol (Allan *et al.* 2000), and that the endosomal rab protein, rab5, binds 22 interacting proteins from bovine brain upon large-scale affinity chromatography (Christoforidis and Zerial 2000).

This latter approach involved the incubation of 1 g GST-rab5 with cytosol prepared from 14 bovine brains (Christoforidis and Zerial 2000). For logistical reasons, this approach was not considered desirable for the identification of new Golgi rab effectors. We therefore considered alternative methods for identifying interactors of small GTPases.

The ras-related small GTPase ran controls a variety of cellular processes, including nuclear transport and mitotic spindle assembly. It accomplishes these different functions through interactions with a variety of effector proteins (Dasso 2002). Affinity chromatography has also been used to identify some of these effectors (see,

for example, (Kutay *et al.* 2000)). This experiment is performed on a much smaller scale than the rab5-binding experiments, using only a few mg of GTPase and less than 1 ml of HeLa cell extract. The key difference between the two methods appeared to be that bovine brain cytosol was bound to rab5 over 2 hours (Christoforidis and Zerial 2000), while HeLa extract was bound to ran overnight (Kutay *et al.* 2000). Overnight binding might therefore allow maximal binding to occur despite working with less starting material.

I therefore incorporated the approach of Kutay *et al.*, into the rab binding protocol used in Chapter 3. The resulting method, detailed in section 2.4.7, was then used to try to identify additional rab1- and rab2- binding proteins from rat liver cytosol.

4.2 Experimental Methods

4.2.1 Rab binding assay from transfected HeLa cells

HeLa cells were transfected for 20 hours with plasmids encoding myc-tagged proteins using the standard protocol. One 6 cm dish was sufficient for each binding reaction to be performed. Each dish of cells was harvested in 300 µl lysis/binding buffer (10 mM Tri-HCl, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2% TX-100, COMPLETE™ Protease Inhibitor Tablets (Roche)) for 45 minutes on ice before centrifugation for 10 minutes at 20000 x g at 4°C. Cell lysates transfected with the same construct were pooled before being re-aliquoted into each binding reaction.

For each reaction, 10 µg GST-rab protein (either rab1 wild type, rab1Q70L, rab2 wild type, or rab2Q65L) was bound to 15 µl glutathione-sepharose beads and then incubated for 3 hours at room temperature with 300 µl transfected HeLa lysate in the presence of 1 mM GDP (wild type rabs) or GMP-PNP (GTP-restricted mutants). Beads were then washed in 3 x 1ml lysis/binding buffer and bound proteins eluted in SDS-PAGE sample buffer. Bound proteins were then analysed by SDS-PAGE, western blotted, and probed with rabbit anti-myc antibody.

4.3 Experimental Results

4.3.1 Identification of MTMR6 and centaurinβ2 as putative rab1 effectors

Overnight incubation of the GTP-restricted point mutant of rab1, rab1Q70L, immobilised on glutathione sepharose, with rat liver cytosol, resulted in the specific binding of a number of proteins compared to rab1-GDP and rab2 (Figure 4.1). Two protein bands of approximately 90KDa and 70KDa in size, clearly visualised by Coomassie staining, were excised from the gel, subjected to tryptic digest, and then analysed by MALDI-TOF (Table 4.1). This analysis identified the 90KDa band (band A in Figure 4.1) as being Centaurinβ2, a member of the centaurin family of PtdIns(3,4,5)P_3-binding proteins (Jackson *et al.* 2000). The 70KDa band (band B in Figure 4.1) was identified as MTMR6, a member of the myotubularin family of inositol lipid phosphatases (Laporte *et al.* 1998).

Protein	Score	Sequence Coverage (%)	Number of peptides matched
Mouse Centaurinβ2 (RIKEN cDNA 9530039J15)	92	16	10
Mouse MTMR6 (RIKEN cDNA 4022440C11)	74	20	13

Table 4.1 Identification of putative rab1-interacting proteins from rat liver cytosol
Allowing for partial oxidation of methionine residues, complete modification of cysteine by iodoacetamide during sample preparation, and single missed trypsin cleavages, searches were performed of monoisotopic peptide masses in the NCBInr database using the search engine at http://www.matrixscience.com/cgi/index.pl?page=../home.html. (An explanation of the scoring system is provided at this site; a score >67 is considered significant). Tolerance was set to 70-100 parts per million to allow for errors in peptide mass determination.

Little is known of the cellular localisation or function of these two proteins. Phosphoinositides are known to play key roles in the regulation of membrane trafficking events (Odorizzi *et al.* 2000; De Matteis *et al.* 2002). The involvement of both proteins in phosphoinositide metabolism and signalling, together with the

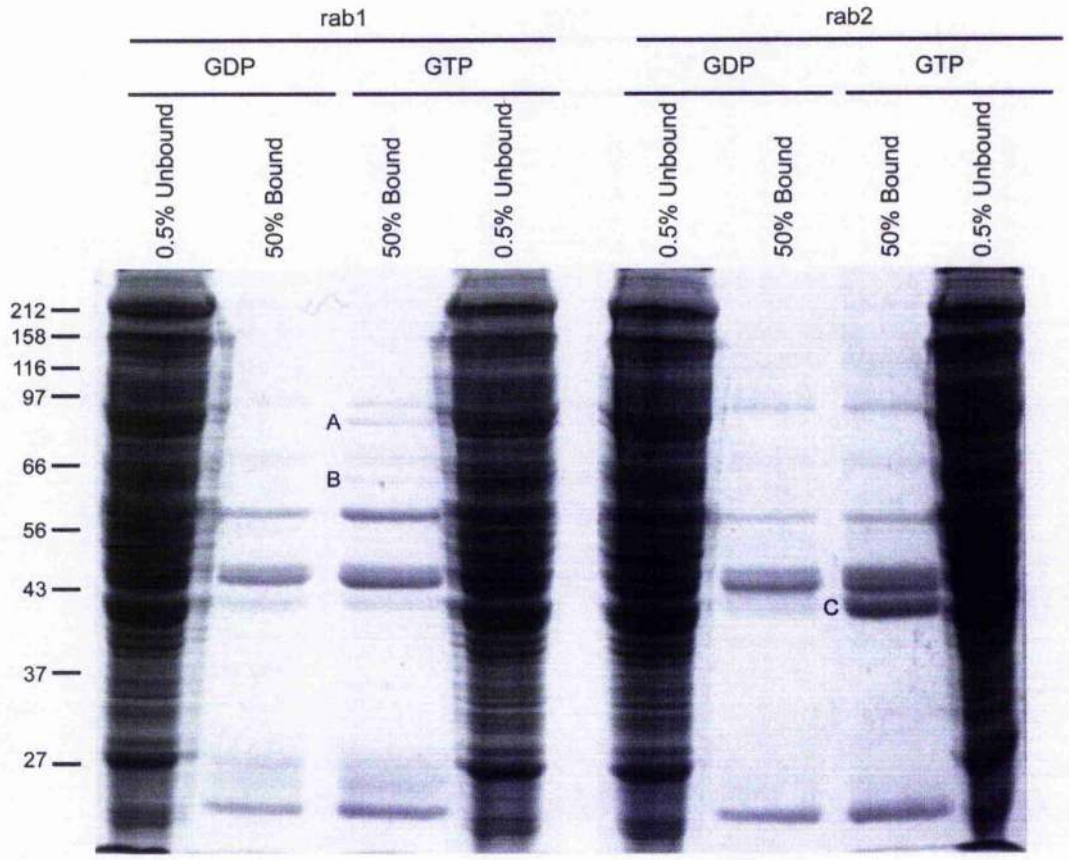


Figure 4.1 Centaurinβ2 and MTMR6 are specifically bound from rat liver cytosol by rab1-GTP

12 mg rat liver cytosol was bound overnight to either rab1 wild type, rab1Q70L, rab2 wild type, or rab2Q65L protein immobilised on glutathione-sepharose. Bound proteins were specifically eluted, TCA precipitated, and analysed by SDS-PAGE and Coomassie brilliant blue staining. The bands denoted A, B, and C were excised from the gel due to their specific binding to rab1-GTP (bands A and B) or rab2-GTP (band C). They were then subjected to tryptic digest, and analysed by MALDI-TOF. Band A was identified as Centaurinβ2, band B was found to be MTMR6, and band C was identified as arginino-succinate synthetase. The positions of molecular standards (KDa) are indicated on the left hand side.

presence of a domain homologous to the Arf GAP domain of ArfGAP1 in centaurin proteins (Jackson *et al.* 2000), therefore suggested their possible involvement in vesicle transport and made them intriguing candidates for further investigation as potential rab1 effectors.

A pulldown was also performed with rab2, in which a protein of approximately 40KDa was strongly and specifically bound only to the GTP-restricted point mutant rab2Q65L (band C in Figure 4.1). This protein was identified by MALDI-TOF as arginino-succinate synthetase, a metabolic enzyme of the urea cycle. This protein has not been reported to have any connection with membrane transport events and could not be localised to the Golgi apparatus by either antibody staining or overexpression

of myc- and GFP-tagged constructs (data not shown). In addition, an interaction between rab2Q65L and arginino-succinate synthetase could not be seen in the yeast two hybrid system (data not shown) and so the potential interaction of this enzyme with rab2-GTP was not pursued any further.

4.3.2 Rab1 binds MTMR6 and centaurinβ2 from transfected HeLa cells

No interaction between rab1Q70L and MTMR6 and centaurinβ2 could be seen in the yeast two hybrid system (data not shown) and recombinant expression of sufficient protein to perform direct binding assays proved difficult. In order to provide some corroboration for an interaction between these proteins and rab1, plasmids encoding myc-tagged MTMR6 and centaurinβ2 were transfected into HeLa cells and the extracts were bound to immobilised rab proteins prior to analysis by western blotting (Figure 4.2).

Although poorly expressed, a small amount of myc-MTMR6 was specifically bound to activated rab1 but not to rab1-GDP or rab2. In contrast, the closely related protein myotubularin (MTM1) is not bound by either rab (Figure 4.2). Centaurinβ2 was bound significantly more by rab1-GTP than rab1-GDP. Smaller amounts were bound by both the active and inactive forms of rab2, but the nucleotide-specificity of rab1 binding, and the greater efficiency of binding to rab1-GTP, suggests that a specific interaction between rab1 and centaurinβ2 might also exist. Finally, as a positive control, both rab1 and rab2 were able to bind myc-tagged GM130 in a GTP-dependent manner (Figure 4.2).

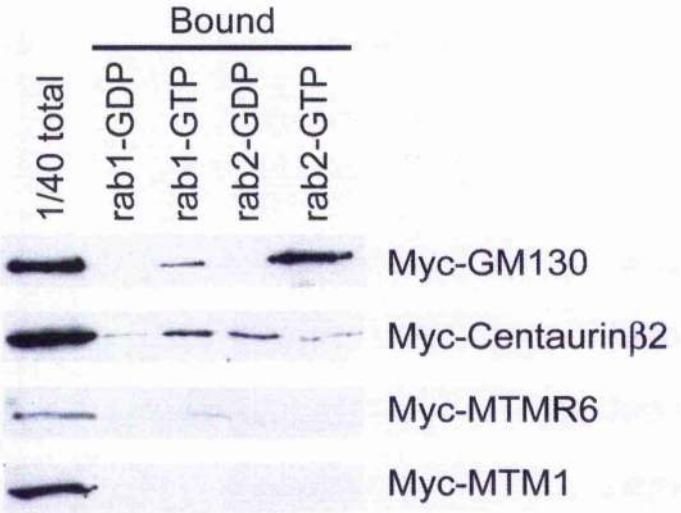


Figure 4.2 Centaurinβ2 and MTMR6 are specifically bound from transfected HeLa cells by rab1-GTP
Lysates from HeLa cells transfected with plasmids encoding myc-tagged GM130, centaurinβ2, MTMR6, and MTM1 were incubated for 3 hours at room temperature with 10 μg rab1 wild type, rab1Q70L, rab2 wild type, or rab2Q65L. Bound proteins were eluted, western blotted, and probed with rabbit anti-myc polyclonal antibody.

4.3.3 Overexpressed MTMR6 and centaurinβ2 have a predominantly cytosolic localisation

In the absence of specific antibodies to either MTMR6 or centaurinβ2, I attempted to determine the localisation of the two proteins by transfecting HeLa cells with plasmids encoding GFP-tagged constructs.

Both MTMR6 and centaurinβ2 contain putative pleckstrin homology (PH) domains and might, therefore, be expected to be partially membrane associated (Jackson *et al.* 2000; Wishart and Dixon 2002).

Upon overexpression, however, both proteins predominantly localise to the cytosol (Figures 4.3 and 4.4). MTMR6, tagged at either the N- or C-terminus with GFP, is mainly cytosolic with a slight concentration in a perinuclear region corresponding to the position of the Golgi apparatus (Figure 4.3). Similarly, GFP-tagged centaurinβ2 can be seen in many cells to have a slight accumulation in the region of the Golgi (Figure 4.4) but whether this indicates an actual association with the Golgi remains to be seen.

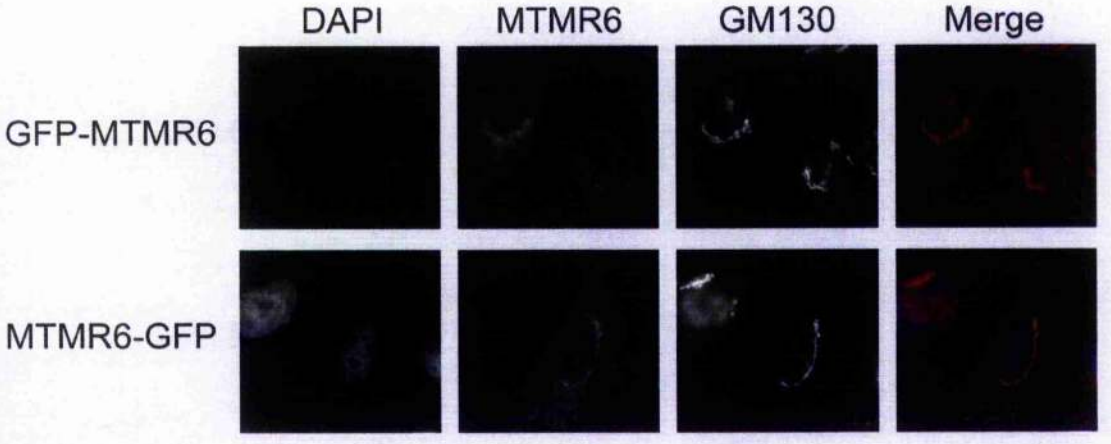


Figure 4.3 Overexpressed MTMR6 localises to the cytosol of HeLa cells
HeLa cells were transfected for 18 hours with plasmids encoding MTMR6 tagged at either the N- or C- terminus with GFP. Cells were fixed with 3% PFA and co-stained with sheep anti-GM130. In the merged images, DNA (DAPI staining) is in blue, MTMR6 is in green, and GM130 is in red.

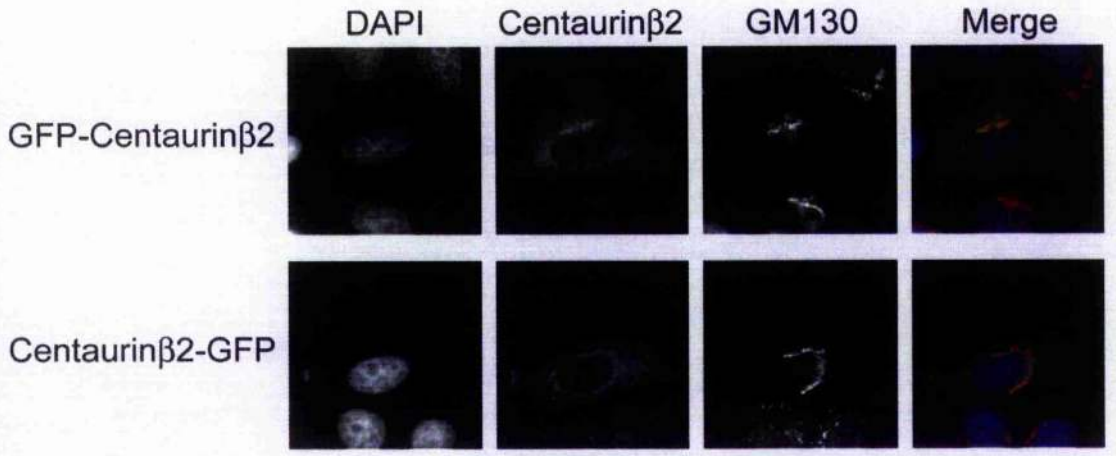


Figure 4.4 Overexpressed centaurinβ2 localises to the cytosol of HeLa cells
HeLa cells were transfected for 18 hours with plasmids encoding centaurinβ2 tagged at either the N- or C- terminus with GFP. Cells were fixed with 3% PFA and co-stained with sheep anti-GM130. In the merged images, DNA (DAPI staining) is in blue, centaurinβ2 is in green, and GM130 is in red.

4.4 Discussion

4.4.1 Overnight binding of rat liver cytosol to immobilised rab proteins allows the identification of putative rab effectors

In order to identify potential new rab effector proteins without using large amounts of starting material, immobilised rab1 and rab2 proteins were incubated overnight with rat liver cytosol. Bound proteins were analysed by SDS-PAGE and Coomassie staining, revealing a number of protein bands not corresponding to the known rab1/rab2 effectors p115, GM130, and golgin-45 (Figure 4.1).

The two proteins bound specifically to activated rab1 were identified by MALDI-TOF as MTMR6 and centaurinβ2, two proteins linked to phosphoinositide binding and metabolism (Table 4.1). Both proteins could also be bound by rab1-GTP when overexpressed in HeLa cells (Figure 4.2). Whether these represent genuine *in vivo* interactions is unclear. The interactions could not be confirmed in the yeast two hybrid system. This could be because the interactions are not direct or because both centaurinβ2 and MTMR6 form a tripartite complex with rab1. It is interesting to note that the two proteins are pulled down in approximately equal amounts by rab1 from rat liver cytosol (Figure 4.1, compare bands A and B).

Given that they were pulled down from rat liver cytosol rather than from Golgi extract, it will be important to determine whether either of these proteins co-localise

with rab1 at the Golgi. We were unable to raise antibodies capable of recognising endogenous MTMR6 by western blot or immunofluorescence. Since no good antibody exists for any myotubularin-related protein, this may reflect a general feature of this family. In the absence of specific antibodies, MTMR6 and centaurinβ2 localisation could only be determined by overexpression of tagged constructs. This showed that both proteins predominantly localise to the cytoplasm (Figures 4.3 and 4.4). Some perinuclear staining can be seen but this is by no means proof of localisation to the Golgi apparatus.

4.4.2 Phosphoinositide signalling in the regulation of membrane trafficking

Although it is unclear whether rab1 interacts with centaurinβ2 and MTMR6, and whether they localise to the Golgi apparatus *in vivo*, it is worth considering the possibility that these two proteins represent novel rab-effectors, given the role of phosphoinositide signalling in membrane trafficking events.

Many phosphoinositide kinases and phosphatases have been identified and linked to membrane trafficking at particular organelles (Odorizzi *et al.* 2000; De Matteis *et al.* 2002). For example, mutants of the yeast phosphoinositide 3-kinase, Vps34p, show defects in vacuolar protein sorting (Stack *et al.* 1995). The generation of particular phosphoinositides at specific membrane sub-domains may then help recruit specific protein factors via their phosphoinositide binding motifs. The rab5 effector EEA1 requires the presence of 3'-phosphorylated phosphoinositides in order to localise to endosomal membranes, which it binds via its FYVE domain (Stenmark *et al.* 1996; Patki *et al.* 1997).

The generation of 3'-phosphorylated phosphoinositides is therefore important for transport from the TGN to the endosomes/yeast vacuole. Based on the localisation of proteins which metabolise or bind PtdIns3P, PtdIns(3,5)P_2, and PtdIns(3,4,5)P_3, these three phosphoinositides are thought to be enriched in late Golgi compartments (De Matteis *et al.* 2002). PtdIns(3,4,5)P_3 is thought to play a key role in regulating Arf function at the TGN via its recruitment of various Arf-binding proteins, including the centaurin family (Jackson *et al.* 2000).

Earlier Golgi compartments are thought to be more enriched in 4'-phosphorylated phosphoinositides, which play a role in different trafficking events (De Matteis *et al.* 2002). PtdIns4P is important in controlling Golgi to plasma membrane trafficking

since this pathway is strongly inhibited in mutants of the yeast phosphoinositide 4-kinase, Pik1p (Audhya *et al.* 2000) and mutants of the yeast phosphoinositide 4-phosphatase, Sac1p, can rescue Sec14p mutants (a phosphoinositide transfer protein) deficient in protein transport to the cell membrane (Whitters *et al.* 1993). In mammalian cells, these phosphoinositide 4-kinases and phosphatases localise to the Golgi apparatus ((De Matteis *et al.* 2002), and references therein). Indeed, kinase-dead forms of mammalian PtdIns4Kβ affect Golgi organisation after BFA washout and effect Golgi to plasma membrane transport in MDCK cells (Godi *et al.* 1999; Bruns *et al.* 2002).

PtdIns(4,5)P_2, on the other hand, has been proposed to play a role in ER to Golgi transport by recruiting spectrin (Godi *et al.* 1998), as well as in the formation of post-Golgi vesicles and maintenance of Golgi structure (Sweeney *et al.* 2002).

Thus, 4'-phosphorylated phosphoinositides appear to be important earlier in the secretory pathway than 3'-phosphorylated phosphoinositides. If MTMR6 and centaurinβ2 are genuine rab1 effectors they might therefore be expected to be involved in 4'-phosphorylated phosphoinositide metabolism and binding.

4.4.3 MTMR6 is a phosphoinositide 3-phosphatase

Although centaurinβ2 itself has not been directly investigated, other centaurin family members are PtdIns(3,4,5)P_3 binding proteins able to act as Arf GAPs and/or effectors (Jackson *et al.* 2000). Centaurinβ2 might therefore be expected to localise to later Golgi compartments.

The myotubularins are a family of phosphoinositide 3-phosphatases (Laporte *et al.* 1998). Members of the family have been linked to disease: MTM1 to X-linked myotubular myopathy, and MTMR2 to Charcot-Marie-Tooth disease type 4B (Laporte *et al.* 1996; Bolino *et al.* 2000). Following on from the work presented here, we investigated MTMR6 and found that both it and MTM1 preferentially used PtdIns3P and PtdIns(3,5)P_2 as substrates (Schaletzky *et al.* 2003). MTMR6s involvement in 3'-phosphorylated phosphoinositide metabolism makes it unlikely to be a factor in transport events at the early Golgi.

The interactions of rab1 with MTMR6 and centaurinβ2 may not therefore represent genuine *in vivo* interactions despite the importance of both rab GTPase and phosphoinositide signalling in membrane trafficking.

4.4.4 Summary

Two proteins, MTMR6 and centaurinβ2, were identified as specific interactors of the activated form of rab1. We were unable to characterise this potential interaction further and the localisation of these two proteins remains unclear. If MTMR6 and centaurinβ2 are involved in the regulation of membrane trafficking events however, they are likely to be involved in later steps than those controlled by rab1.

Chapter 5: Identification And Characterisation Of Novel Rab-Effector Complexes At The Late Golgi

5.1 Introduction

The *trans*-Golgi network (TGN) is a complex network of tubules and vesicles located at the *trans* face of the Golgi (Griffiths and Simons 1986). There is a corresponding complexity in the membrane trafficking events with which the TGN is involved, since it is the major site of sorting events in the secretory pathway and an important interface between the secretory and endocytic pathways (Rohn *et al.* 2000). Secretory proteins and lipids are sorted for delivery to the plasma membrane (and targeted to either the apical or basolateral membranes in polarised cells), secretory granules, or the endosomal/lysosomal system. Retrograde transport also occurs from the endosomes to the TGN as well as transport from the TGN to earlier Golgi compartments and the ER.

Many of these transport steps involve clathrin-coated vesicles and a variety of adaptor proteins, which recognise specific sorting signals within the cytoplasmic tails of integral membrane cargo proteins (Robinson and Bonifacino 2001). Some of these cargo proteins are themselves receptors for luminal cargo. The classical example of this is the mannose 6-phosphate receptors, which are involved in the transport of newly-synthesised acid hydrolases from the TGN to the endosomal-lysosomal system (Ghosh *et al.* 2003). The cytoplasmic tail of the mannose 6-phosphate receptor contains several sorting signals recognised by both GGA (Golgi-localised, γ-ear-containing, ADP-ribosylation factor-binding) and AP-1 adaptor proteins for incorporation into clathrin-coated vesicles and transport to endosomes (Honing *et al.* 1997; Puertollano *et al.* 2001; Zhu *et al.* 2001).

The small GTPase rab6 has been implicated in several trafficking steps at the late Golgi and is localised to the medial and *trans* cisternae, as well as the TGN (Goud *et al.* 1990; Antony *et al.* 1992).

Analysis of mutants of the budding yeast rab6 homologue, Ypt6p, has suggested its involvement in endosome-to-Golgi transport and in retrograde intra-Golgi transport from the TGN to earlier Golgi compartments (Tsukada *et al.* 1999; Siniossoglou *et al.* 2000; Bensen *et al.* 2001). Ypt6p has alternatively been proposed to play a role in anterograde transport at the early Golgi (Li and Warner 1996), but kinetic analysis of conditional YPT6 mutants suggests that the transport defects observed are more likely due to inhibition of recycling pathways at the non-permissive temperature (Luo and Gallwitz 2003).

In mammalian cells, there are two ubiquitously expressed isoforms of rab6, a and a', which differ by only three amino acids (Echard *et al.* 2000). Rab6a has been implicated in retrograde intra-Golgi and Golgi to ER transport by reports that overexpression of wild type or GTP-restricted rab6a induces the redistribution of a *trans*-Golgi galactosyltransferase to the ER (Martinez *et al.* 1994; Martinez *et al.* 1997). The overexpression of GDP-restricted rab6a blocks the transport of Shiga toxin and glycosylation enzymes to the ER, but this transport is not affected by microinjection of anti-COPI antibodies, suggesting the pathway is COPI-independent (Girod *et al.* 1999). Rab6a', on the other hand, has been shown to be required for endosome-to-TGN transport in permeabilised cells although GDP-restricted rab6a also partially inhibited the same transport step (Mallard *et al.* 2002).

A number of rab6-interacting proteins have been identified. The kinesin-like protein rabkinesin-6 was found in a yeast two hybrid screen using rab6aQ72L as a bait. It was localised to the Golgi apparatus in interphase cells and found to affect Golgi membrane dynamics (Echard *et al.* 1998). Since kinesins control plus-end directed microtubule-based movement, the interaction between rab6a and rabkinesin-6 was proposed to control the rab6a/microtubule dependent Golgi-to-ER retrograde transport pathway (Martinez *et al.* 1997; White *et al.* 1999). Correspondingly, rab6a' was not found to interact with rabkinesin-6 (Echard *et al.* 2000). However, subsequent data showed that rabkinesin-6 is primarily expressed in mitotic cells and localises to the midbody of dividing cells. Microinjection of anti-rabkinesin-6 antibodies inhibited

cell division, suggesting the main role of this protein is in cytokinesis rather than in interphase membrane trafficking (Hill *et al.* 2000).

Additional rab6 interactors include GAPCenA, a centrosomal protein proposed to act as an activating protein for rab6, thereby controlling Golgi dynamics (Cuif *et al.* 1999), and rab6 interacting protein 2 A and B which may be involved in endosome-to-TGN transport (Monier *et al.* 2002). In yeast, the VFT quatrefoil tethering complex, required for the fusion of endosome-derived vesicles with the Golgi, has been found to interact with Ypt6p, as well as the late Golgi SNARE protein Tlg1p (Siniossoglou and Pelham 2001), while Ric1p and Rgp1p form an effector complex catalysing nucleotide exchange on Ypt6p (Siniossoglou *et al.* 2000). The GRIP domain-containing protein Imh1p has also been implicated in Ypt6p function (Tsukada *et al.* 1999).

Given the array of transport events controlled by rab6 isoforms, it is highly likely that further rab6 effectors remain to be found. In order to identify additional rab6 interacting proteins, I performed a series of binding experiments from rat liver cytosol, using the protocol developed in Chapter 4 and identified a number of putative rab6-effectors by MALDI-TOF.

5.2 Experimental Results

5.2.1 Components of the dynein/dynactin complex are bound from rat liver cytosol by rab6a-GTP

Overnight incubation of the GTP-restricted point mutant of rab6a, rab6aQ72L, immobilised on glutathione sepharose, with rat liver cytosol, resulted in the specific binding of a number of proteins (Figure 5.1). These proteins were bound to a lesser extent by rab6a-GDP and were not seen to bind rabs 1 and 2 (see, for example Figure 4.1). These protein bands were excised from the gel, subjected to tryptic digest, and then analysed by MALDI-TOF (Table 5.1). This analysis identified the $p150^{glued}$ subunit of dynactin and the TGN-localised dynactin interacting protein bicaudal D2 (BicD2; (Hoogenraad *et al.* 2001)) as being preferentially bound by activated rab6a. We were unable to identify additional dynactin subunits such as p50/dynamitin in the 50-60 KDa region of the gel, due to the presence of rab-guanine nucleotide dissociation inhibitor (rab-GDI) in this region (Figure 5.1). In addition, we were able

to identify rab6IP2 (Monier *et al.* 2002) and rab escort protein (REP-1), a protein essential for the delivery of newly synthesised rab proteins to membranes (Alexandrov *et al.* 1994) in the rab6a-GTP bound fraction.

Protein	Score	Sequence Coverage (%)	Number of peptides matched
Rat p150^{glued}	109	21	22
Mouse rab6IP2	80	19	20
Rat REP-1	79	22	10
Human BicD2	63	15	18
Mouse rab-GDI	126	37	17

Table 5.1 Identification of rab6-interacting proteins in rat liver cytosol
Allowing for partial oxidation of methionine residues, complete modification of cysteine by iodoacetamide during sample preparation, and single missed trypsin cleavages, searches were performed of monoisotopic peptide masses in the NCBInr database using the search engine at http://www.matrixscience.com/cgi/index.pl?page=../home.html. (An explanation of the scoring system is provided at this site; a score >67 is considered significant). Tolerance was set to 70-100 parts per million to allow for errors in peptide mass determination. REP-1 and BicD2 ran in the same doublet band on the gel and so were doubly identified in a single digest. This leads to a decreased identification score due to the increased amount of peptides disturbing and suppressing each other.

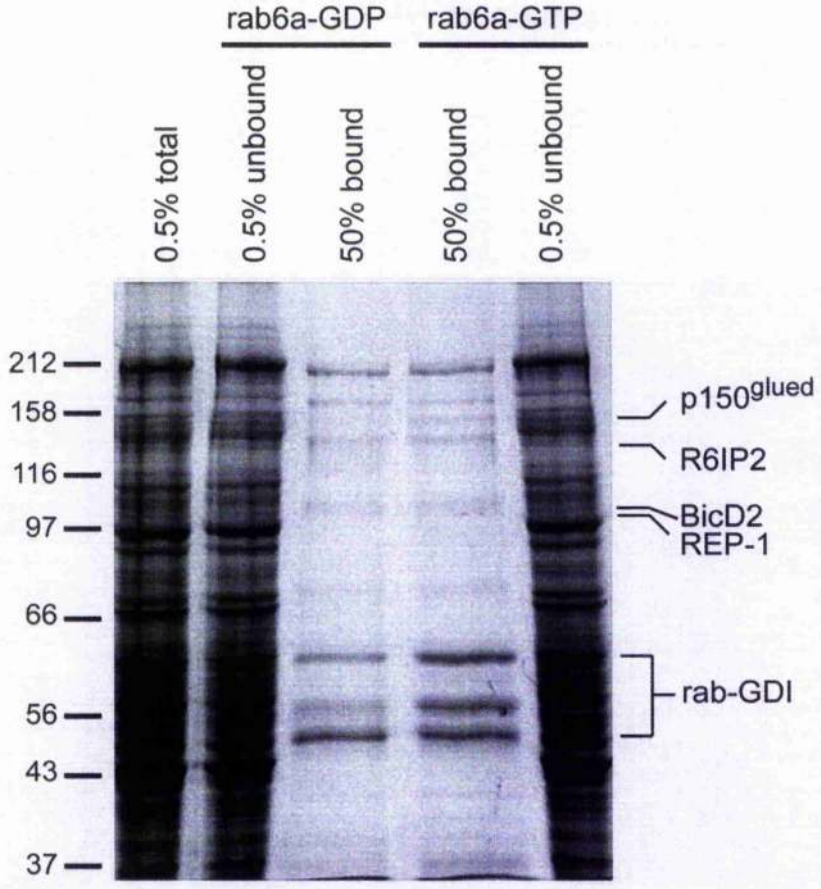


Figure 5.1 p150^{glued} and bicaudal D2 are bound from rat liver cytosol by rab6a-GTP
12 mg rat liver cytosol was bound overnight to either rab6a wild type or rab6aQ72L protein immobilised on glutathione-sepharose. Bound proteins were specifically eluted, TCA precipitated, and analysed by SDS-PAGE and Coomassie brilliant blue staining. Bands were excised from the gel, subjected to tryptic digest, and analysed by MALDI-TOF. The position of bands identified as p150^{glued}, rab6 interacting protein 2 (R6IP2), bicaudal D2 (BicD2), rab escort protein (REP-1), and rab-guanine nucleotide dissociation inhibitor (rab-GDI), are indicated. The positions of molecular standards (KDa) are indicated on the left hand side.

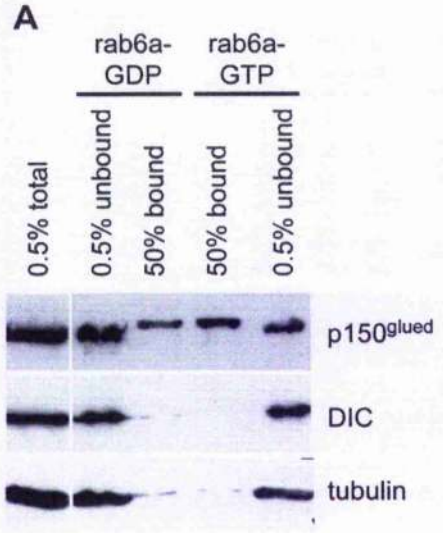
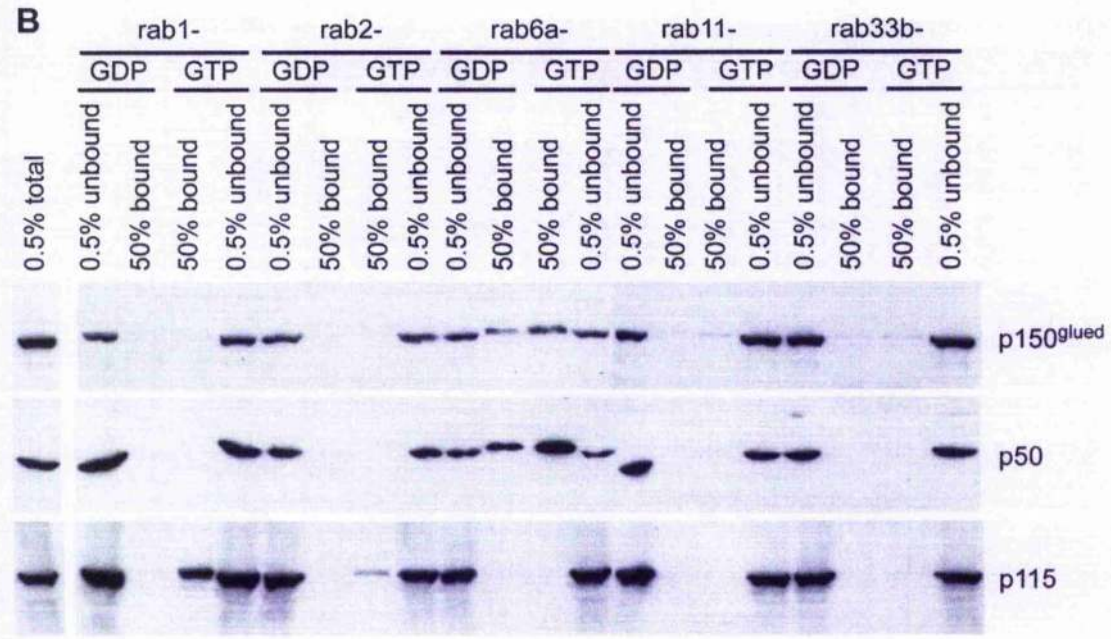


Figure 5.2 The dynactin complex is a specific binding partner for rab6

(A) Samples prepared as in Figure 5.1 were western blotted and probed with either mouse anti-p150^{glued}, mouse anti-dynein light intermediate chain, or mouse anti-tubulin. (B) Binding assays were performed as above using the wild type and GTP-restricted point mutants of rab1, rab2, rab6a, rab11, and rab33b. Specifically eluted proteins were analysed by western blotting with either mouse anti-p150^{glued}, mouse anti-p50 dynamitin, or sheep anti-p115.



5.2.2 Dynactin is a specific binding partner for rab6

To confirm the interaction between dynactin and rab6a, samples from the rat liver cytosol binding assay were western blotted and probed with specific antibodies (Figure 5.2). p150^{glued} was again found to bind rab6a-GTP more efficiently than rab6a-GDP while tubulin and the light intermediate chain of the dynactin-interacting motor protein dynein were not found in the bound fraction (Figure 5.2 A). A variety of rab proteins were tested and p150^{glued} binding was found to be specific to rab6

(Figure 5.2 B). In addition, a second subunit of dynactin, p50/dynamitin, was specifically bound to rab6, suggesting that the dynactin complex as a whole is a rab6 binding partner. Under the same conditions, p115 was bound specifically by rab1 but not by rab6 (Figure 5.2 B). We were unable to probe for BicD2, due to the lack of a specific antibody.

5.2.3 Rab6 interacts with p150^{glued} and BicD2 by yeast two hybrid

To further confirm the interaction between rab6 and components of the dynactin complex, p150^{glued} and BicD2 were cloned into the yeast two hybrid prey vector pACT2 and screened against rab6a cloned into the bait vector pGBT9 (Figure 5.3). Both p150^{glued} and BicD2 showed a weak interaction with rab6a wild type and a much stronger interaction with the GTP-restricted mutant rab6aQ72L as assessed by growth on quadruple dropout medium. No interaction was seen with the GDP-restricted mutant rab6aT27N or with the GTP-bound form of rab1 (Figure 5.3). BicD1, a BicD2-related protein, (Baens and Marynen 1997) also displayed a specific nucleotide-dependent interaction with rab6a. p115, on the other hand, only interacted with rab1Q70L and not with rab6 (Figure 5.3).

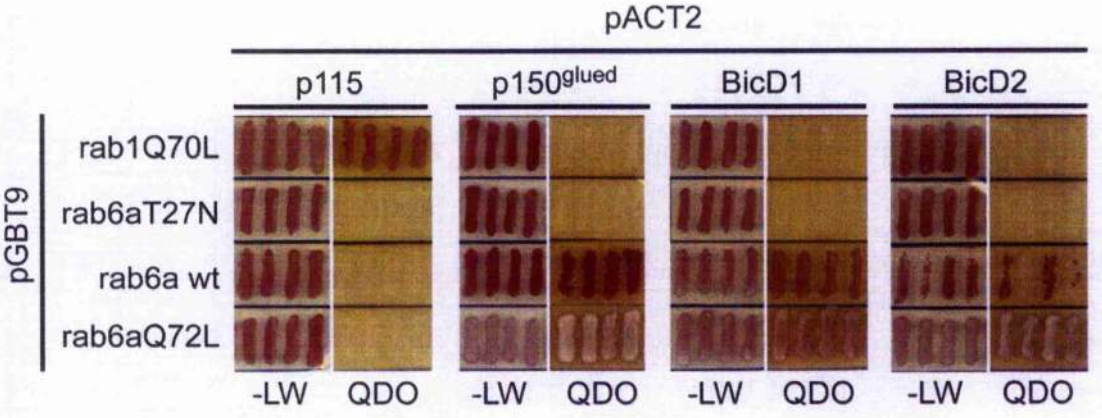


Figure 5.3 Rab6 interacts with p150^{glued}, BicD1, and BicD2 by yeast two hybrid
Full length p115, p150^{glued}, BicD1, and BicD2 were tested in the yeast two hybrid system for interactions with the activated rab mutants rab1Q70L and rab6aQ72L, as well as wild type rab6a and the inactive mutant rab6aT27N. Interactions were assessed by growth on quadruple dropout media.

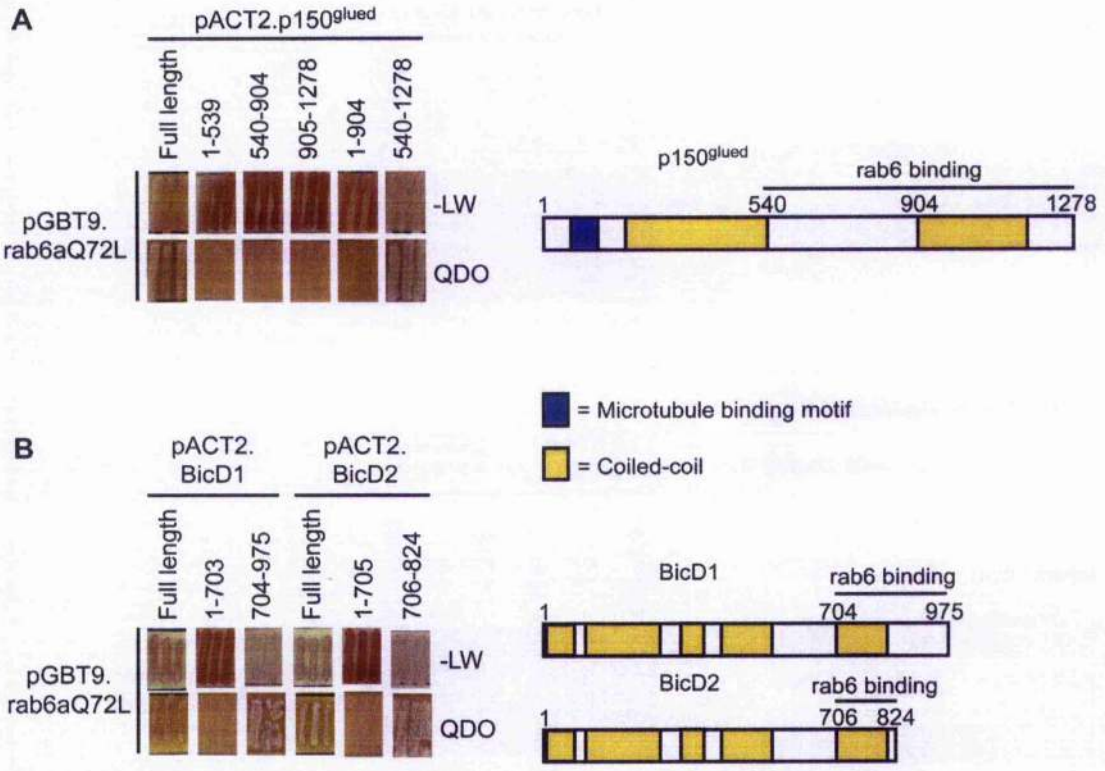


Figure 5.4 Identification of rab6 binding sites on p150^{glued}, BicD1, and BicD2
Deletion mutants of either p150^{glued} (A) or BicD1 and BicD2 (B) were cloned into the yeast two hybrid prey vector pACT2 and were screened against the GTP-restricted point mutant rab6aQ72L cloned into the bait vector pGBT9. Interactions were assessed by growth on quadruple dropout media.

5.2.4 Identification of rab6 binding sites on p150^{glued}, BicD1, and BicD2

The yeast two hybrid system was then used to map the rab6 binding site on these putative rab6 effectors (Figure 5.4). p150^{glued} has a microtubule binding motif near its N-terminus and has two regions predicted to form coiled-coil. The second, C-terminal-most, of these two coiled-coil regions appears to be important for the interaction with rab6 since deletion mutants of p150^{glued} lacking this region show no interaction with rab6aQ72L (Figure 5.4 A). This region alone, however, is not sufficient for the rab6-interaction, since p150^{glued} amino acids 905-1278 does not interact with rab6 either. The middle region of p150^{glued}, not predicted to form coiled-coil, is also necessary but not sufficient for the interaction with rab6, since p150^{glued} amino acids 540-1278 but not amino acids 540-904 interact with rab6aQ72L. Thus, p150^{glued} amino acids 540-1278 interact with rab6. p150^{glued} amino acids 828-1059 showed no interaction with rab6 (data not shown) suggesting that the rab6 binding site doesn't lie specifically at the junction between the central domain of p150^{glued} and the second coiled-coil. It is possible that amino acids 540-1278 are necessary for the

correct folding and presentation of the binding site. It is interesting to note that the second coiled-coil domain and the extreme C-terminus of p150^{glued} also regulates the interaction of dynactin with membranous cargo (Kumar *et al.* 2001).

BicD1 and BicD2 both contain five separate regions predicted to form coiled-coil. BicD1 contains an extended C-terminal region compared to BicD2. It is the C-terminus of both proteins that contains the binding site for rab6 (Figure 5.4 B). BicD2 amino acids 706-824, which consists extensively of coiled-coil, interacts with rab6aQ72L in the yeast two hybrid system while amino acids 1-705 show no interaction. Interestingly, this C-terminal region of BicD2 has previously been implicated in binding to p50/dynamitin and localising BicD2 to the Golgi apparatus (Hoogenraad *et al.* 2001). Similarly, BicD1 amino acids 704-975, consisting of a coiled-coil domain and the extended C-terminal region, interacts with rab6 while BicD1 amino acids 1-703 does not (Figure 5.4 B).

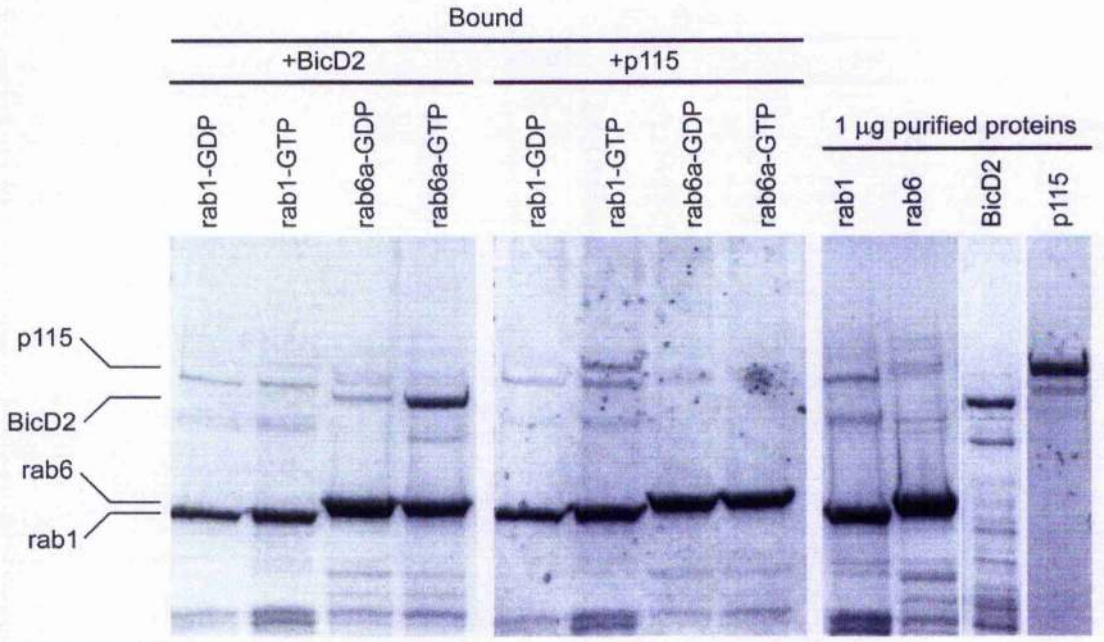


Figure 5.5 BicD2 binds directly to rab6a

4 µg recombinantly expressed GST-tagged rab proteins were incubated with 10 µg His-tagged BicD2 or p115 in the presence of 15 µl glutathione-sepharose beads and 10 µM either GDP or GMP-PNP in a total volume of 400 µl HNTM for 1 hour at 4°C. Bound proteins were eluted in SDS-PAGE sample buffer and analysed by SDS-PAGE. BicD2 was specifically bound by activated rab6a.

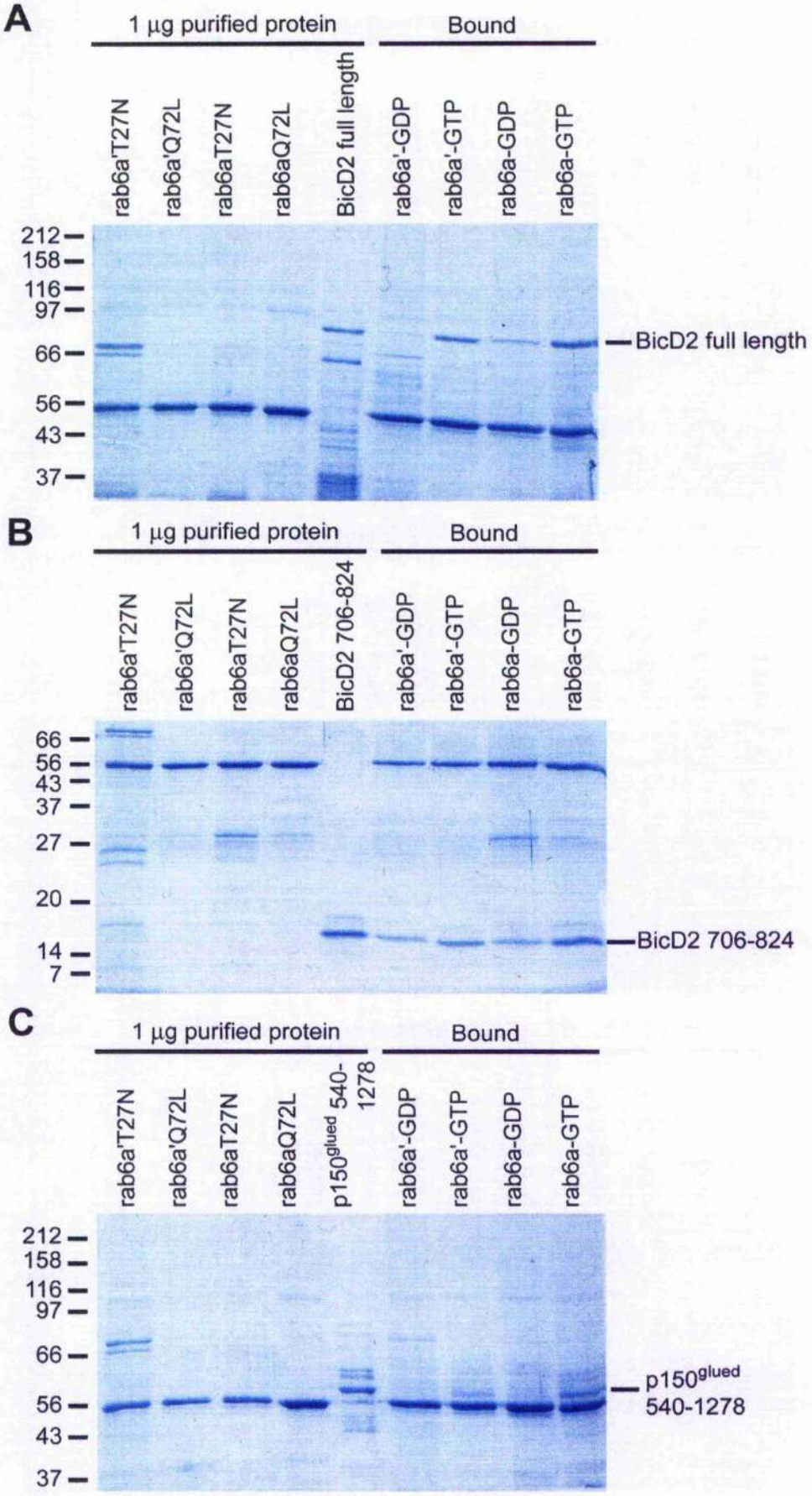


Figure 5.6 BicD2 and p150^{glued} directly bind to both rab6 isoforms
4 µg recombinantly expressed GST-tagged rab proteins were incubated with 10 µg His-tagged full length BicD2, BicD2 amino acids 706-824, or p150^{glued} amino acids 540-1278 in the presence of 15 µl glutathione-sepharose beads and 10 µM either GDP or GMP-PNP in a total volume of 400 µl HNTM for 1 hour at 4°C. Bound proteins were eluted in SDS-PAGE sample buffer and analysed by SDS-PAGE. The positions of molecular standards (KDa) are indicated on the left hand side.

5.2.5 BicD2 and p150^{glued} bind rab6 directly

To prove a direct interaction between rab6 and BicD2, I performed a direct binding assay using recombinantly expressed proteins (Figure 5.5). BicD2 specifically and quantitatively bound to rab6a-GTP (rab6aQ72L in the presence of GMP-PNP) compared to rab6a-GDP (wild type rab6a in the presence of GDP) and rab1. This is in contrast to p115, which bound rab1-GTP but not rab6a (Figure 5.5).

I was unable to express soluble, full-length recombinant p150^{glued} and so was unable to test wild type p150^{glued} for direct binding to rab6. I was, however, able to express recombinant p150^{glued} amino acids 540-1278, the region identified as binding rab6 by yeast two hybrid (Figure 5.4 A). p150^{glued} 540-1278 specifically and quantitatively bound rab6a-GTP compared to rab6a-GDP (here, the GDP-restricted point mutant rab6aT27N in the presence of GDP) (Figure 5.6 C).

To date, the only putative rab6 effector that has been shown to differentiate between rab6a and a' is rabkinesin-6 (Echard *et al.* 1998; Echard *et al.* 2000). Full length BicD2 and p150^{glued} 540-1278 were able to bind rab6a' as well as rab6a in a nucleotide-dependent manner (Figure 5.6). Additionally, BicD2 amino acids 706-824, was also able to bind both isoforms of rab6, confirming this region as the rab6 binding site (Figure 5.6 B).

5.2.6 BicD1 and BicD2 target to the Golgi apparatus by their C-termini

Full length BicD1 localises to the Golgi apparatus when overexpressed in HeLa cells and shows a high degree of co-localisation with rab6 (Figure 5.7). The region of the protein responsible for this appears to be the C-terminal region capable of interacting with rab6, since GFP-tagged BicD1 amino acids 704-975 also co-localises with rab6 at the Golgi while BicD1 1-703 displays a diffuse nuclear and cytoplasmic staining pattern (Figure 5.7).

Similarly, full length GFP-tagged BicD2 targets to the Golgi apparatus and co-localises with rab6 (Figure 5.8 A). As reported previously (Hoogenraad *et al.* 2001), this localisation is also mediated by the C-terminus of the protein. BicD2 amino acids 706-824 co-localises with rab6 while BicD2 1-705 is predominantly nuclear with a diffuse cytoplasmic staining pattern in higher expressing cells (Figure 5.8 A). BicD2 1-705 also has a tendency to form aggregates in both the nucleus and cytosol.

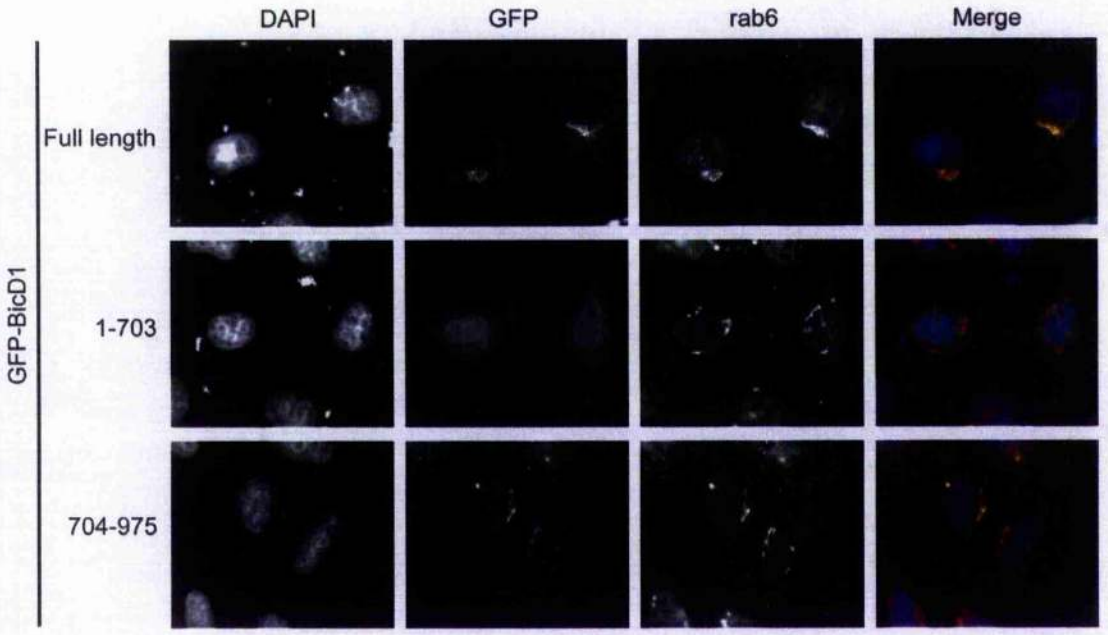


Figure 5.7 BicD1 targets to the Golgi apparatus by its C-terminus
Full length BicD1 and deletion mutants were cloned into the N-terminal GFP-tagging vector, pEGFP-C2 and transfected into HeLa cells for 18 hours. Cells were fixed in 3% PFA and co-stained with rabbit anti-rab6. In the merged images, DNA (DAPI staining) is in blue, GFP-BicD1 is in green, and rab6 is in red. Areas of overlap between BicD1 and rab6 are in yellow.

Full length BicD2 also forms aggregates in cells expressing high amounts of the protein. Interestingly, these aggregates are able to recruit rab6 (Figure 5.8 B, white arrows) while the aggregates of BicD2 1-705, which lack the rab6 binding site, do not recruit the rab protein (Figure 5.8 A, middle panels, white arrowheads).

5.2.7 Rabkinesin-6 does not bind directly to rab6

The interaction between rab6 and BicD1, BicD2, and $p150^{glued}$ implicates rab6 in the regulation of motor-based, microtubule-dependent transport. Rab6 has previously been proposed to be involved in such events by interacting with the kinesin family member rabkinesin-6 (Echard *et al.* 1998) although this protein's main role appears to be in cytokinesis (Hill *et al.* 2000). Using recombinantly expressed rabkinesin-6, I was unable to detect direct binding to either rab6a or rab6a' (Figure 5.9). In addition to the data of Hill *et al.*, this further suggests that rabkinesin-6 is not involved in interphase membrane trafficking events and that the interaction of rab6 with the BicD proteins and dynactin is key to the microtubule-dependent movement of rab6-positive structures.

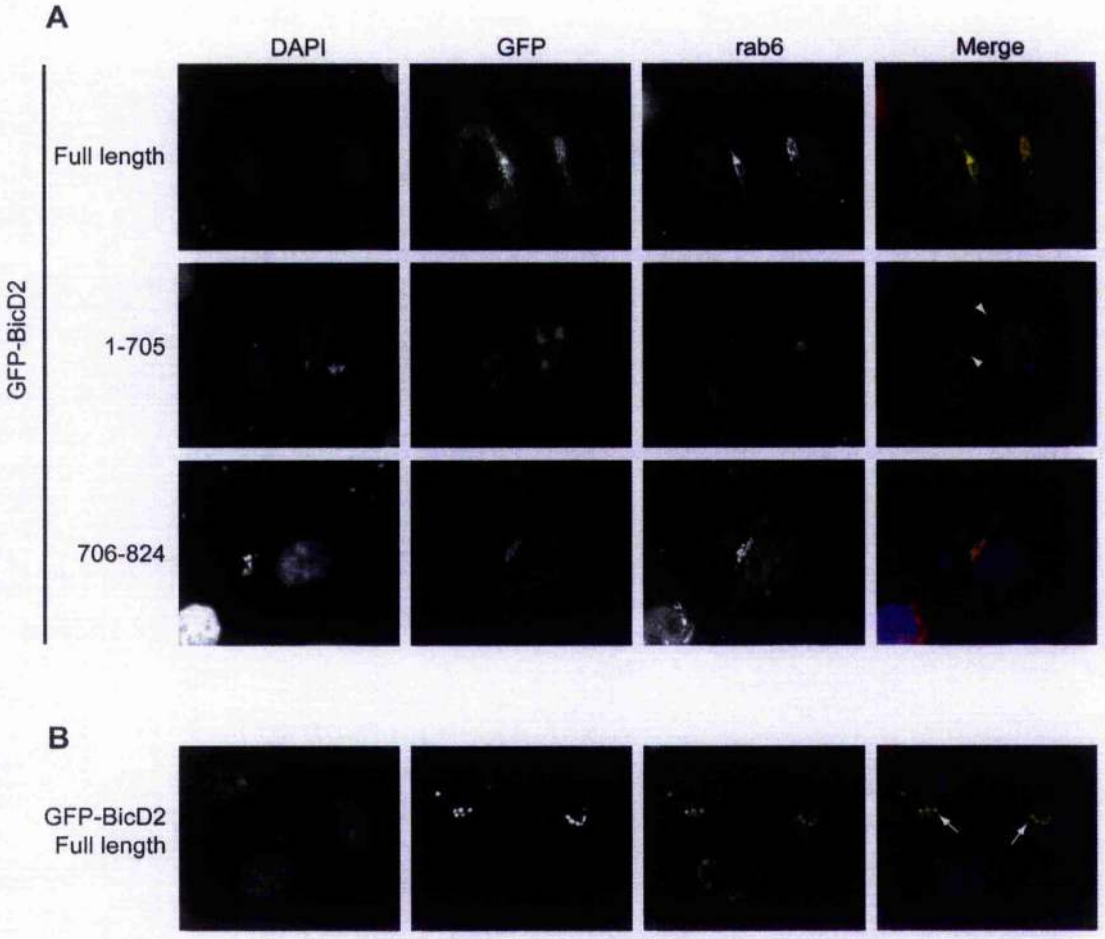


Figure 5.8 BicD2 targets to the Golgi apparatus by its C-terminus
(A) Full length BicD2 and deletion mutants were cloned into the N-terminal GFP-tagging vector, pEGFP-C2 and transfected into HeLa cells for 18 hours. Cells were fixed in 3% PFA and co-stained with rabbit anti-rab6. In the merged images, DNA (DAPI staining) is in blue, GFP-BicD2 is in green, and rab6 is in red. Areas of overlap between BicD2 and rab6 are in yellow. White arrowheads indicate examples of GFP-BicD2 1-705 aggregates that do not recruit rab6. (B) High overexpression of GFP-BicD2 full length results in numerous protein aggregates containing both BicD2 and rab6. White arrows indicate examples of GFP-BicD2 aggregates that recruit rab6. (Transfection and staining conditions as in A).

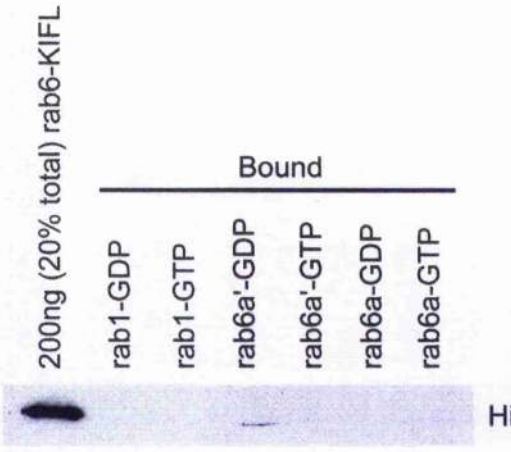


Figure 5.9 Rabkinesin-6 does not bind directly to rab6
4 μg recombinantly expressed GST-tagged rab proteins were incubated with 1 μg His-tagged full length rabkinesin-6 (rab6-KIFL) in the presence of 15 μl glutathione-sepharose beads and 10 μM either GDP or GMP-PNP in a total volume of 400 μl HNTM for 1 hour at 4°C. Bound proteins were eluted in SDS-PAGE sample buffer and analysed by western blotting with mouse anti-histidine tag.

5.2.8 Identification of GOPC as a putative rab6 effector

A further overnight binding experiment from rat liver cytosol, this time comparing proteins bound by GTP-restricted rab6aQ72L with proteins bound by point mutants mimicking the nucleotide free state (rab6aD129N and rab6a'D129N). Although no specific bands of interest could be seen to bind either of the two nucleotide free rabs, two bands of approximately 60KDa were seen to be present only in the fraction bound to rab6aQ72L (Figure 5.10). These protein bands were excised from the gel, subjected to tryptic digest, and analysed by MALDI-TOF (Table 5.2). The upper of these two bands (band A in Figure 5.10) was identified as rab-GDI, seen previously to bind rab6 from rat liver cytosol (Figure 5.1). Rab-GDI tryptic peptides were also found in the corresponding gel region of the fractions bound by rab6aD129N and rab6a'D129N. The lower band (band B in Figure 5.10) was identified as the Golgi-localised PDZ-domain-containing protein GOPC (Yao *et al.* 2001). Tryptic peptides derived from this protein were not found in the corresponding gel region of the fractions bound to rab6aD129N and rab6a'D129N, suggesting that GOPC may be a specific effector of rab6.

Protein	Score	Sequence Coverage (%)	Number of peptides matched
Mouse PIST/GOPC	1.19	22	8

Table 5.2 Identification of GOPC as a rab6-interacting protein from rat liver cytosol
Allowing for partial oxidation of methionine residues, complete modification of cysteine by iodoacetamide during sample preparation, and single missed trypsin cleavages, searches were performed of monoisotopic peptide masses in the NCBInr database using the search engine at http://prowl.rockefeller.edu/cgi-bin/ProFound. (An explanation of the scoring system is provided at this site). Tolerance was set to 70-100 parts per million to allow for errors in peptide mass determination.

5.2.9 GOPC is a specific binding partner of rab6

GOPC (Golgi associated PDZ and coiled-coil motif containing protein) was identified in a yeast two hybrid screen as an interaction partner of Frizzled, an integral membrane protein thought to act as a receptor for Wnt-signalling pathways (Yao *et al.* 2001). It has also been identified as PIST (PDZ domain protein interacting specifically with TC10), in a yeast two hybrid screen using the rho GTPase family member TC10 as bait (Neudauer *et al.* 2001) and FIG (Fused in glioblastoma), as a

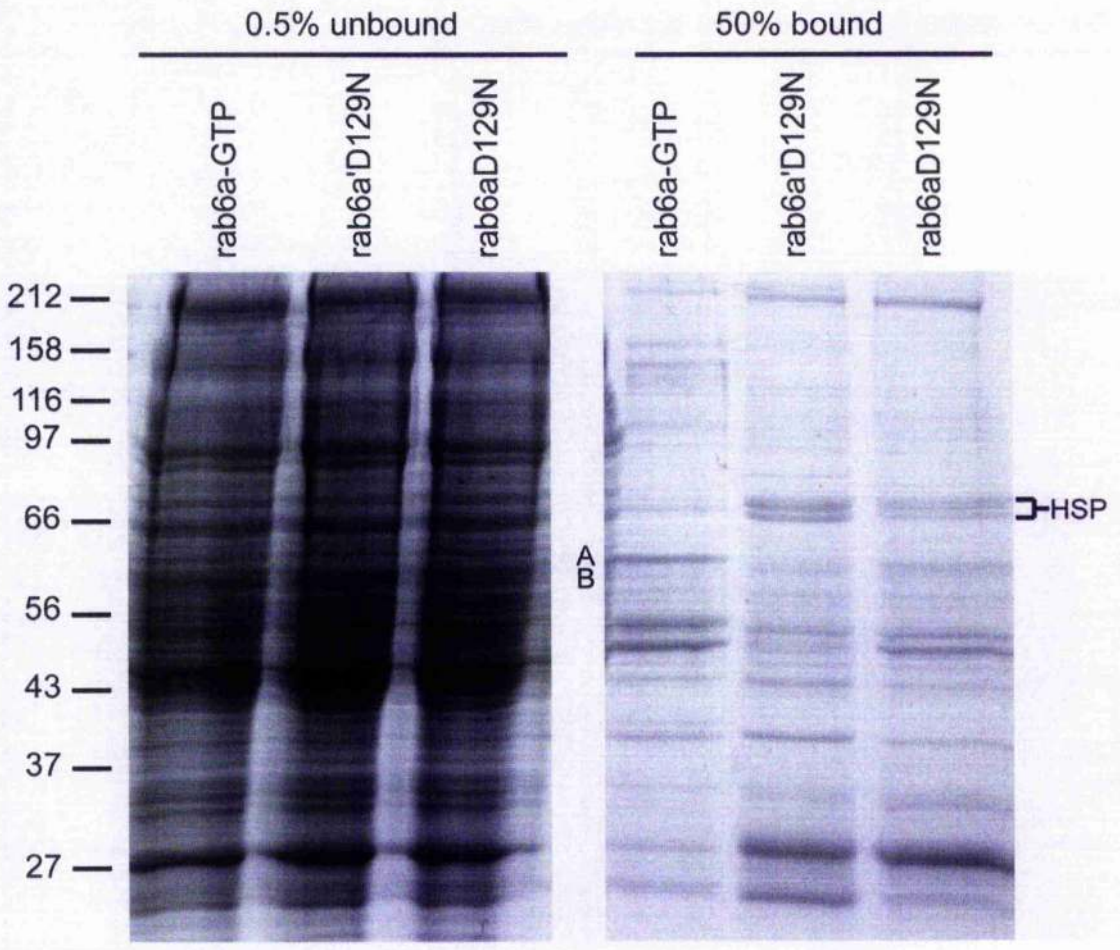


Figure 5.10 GOPC is bound from rat liver cytosol by rab6a-GTP
12 mg rat liver cytosol was bound overnight to either rab6aQ72L, rab6aD129N, or rab6a'D129N protein immobilised on glutathione-sepharose. Bound proteins were specifically eluted, TCA precipitated, and analysed by SDS-PAGE and Coomassie brilliant blue staining. The bands of approximately 70KDa are heat shock protein (HSP) derived from the original purification of rab6aD129N and rab6a'D129N. The bands denoted A and B were excised from the gel due to their specific binding to rab6a-GTP. They were then subjected to tryptic digest, and analysed by MALDI-TOF. Band A was identified as rab-GDI, and band B was found to be GOPC. The positions of molecular standards (KDa) are indicated on the left hand side.

Golgi-localised protein interacting with the SNARE protein syntaxin 6 (Charest *et al.* 2001). The protein contains a PDZ domain near its C-terminus and two regions predicted to form coiled-coil (see diagram in Figure 5.15). Two additional regions show a particularly high level of conservation between GOPC in mouse and the putative homologue in *C. elegans*, zk849.2 (Yao *et al.* 2001).

The interaction of GOPC with rab6 was confirmed using a binding assay from transfected HeLa and the yeast two hybrid system (Figure 5.11). Using the method described in section 4.2.1, lysates from HeLa cells transfected with plasmids encoding

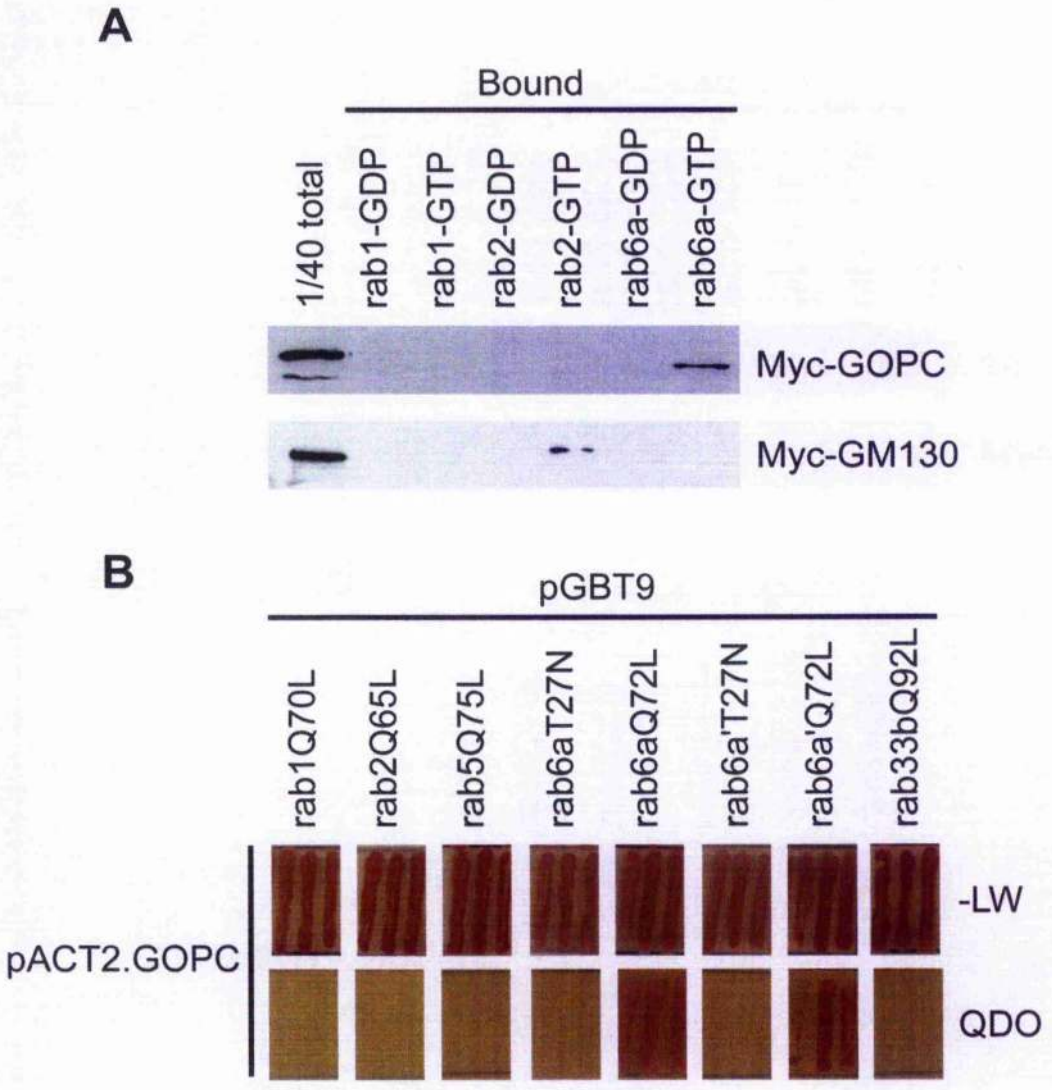


Figure 5.11 GOPC is a specific binding partner of rab6
(A) Lysates from HeLa cells transfected with plasmids encoding myc-tagged GOPC and GM130 were incubated for 3 hours at room temperature with 10 μg rab1 wild type, rab1Q70L, rab2 wild type, rab2Q65L, rab6a wild type, or rab6aQ72L. Bound proteins were eluted, western blotted, and probed with rabbit anti-myc polyclonal antibody. (B) Full length GOPC was tested in the yeast two hybrid system against the activated rab point mutants rab1Q70L, rab2Q65L, rab5Q75L, rab6aQ72L, rab6a'Q72L, and rab33bQ92L and against the GDP-restricted point mutants rab6aT27N and rab6a'T27N. Interactions were assessed by growth on quadruple dropout media.

either myc-tagged GOPC or myc-tagged GM130 were bound to immobilised rab proteins and analysed by western blotting. This analysis revealed that myc-GOPC was specifically bound by rab6a-GTP and not by rab6a-GDP or by rab1 or rab2 (Figure 5.11 A). Similarly, when GOPC was cloned into the yeast two hybrid prey vector pACT2 and screened against a number of activated rab mutants in the bait vector pGBT9, an interaction was only seen with the two isoforms of rab6, a and a' (Figure 5.11 B). This interaction was not seen with the GDP-locked mutants of rab6.

5.2.10 GOPC binds rab6 directly

To determine whether the interaction between GOPC and rab6 is direct, I performed a direct binding assay between recombinantly expressed His-tagged GOPC and point mutants of rab6a and rab6a'. Both isoforms of rab6 were able to bind GOPC in a nucleotide-dependent manner (Figure 5.12). Therefore, as with $p150^{glued}$, BicD1, and BicD2, GOPC appears to be a specific rab6 effector which does not distinguish between the two ubiquitously expressed rab6 isoforms.

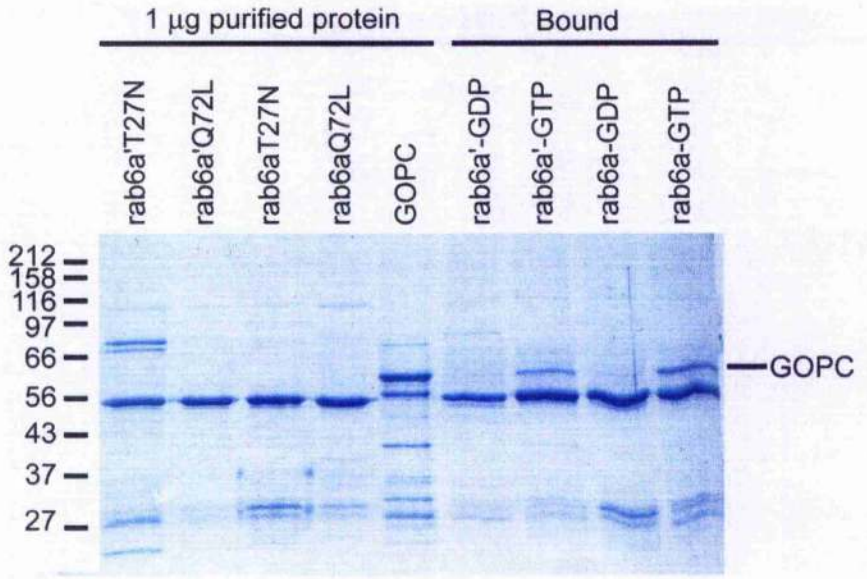


Figure 5.12 GOPC binds directly to both rab6 isoforms
4 µg recombinantly expressed GST-tagged rab proteins were incubated with 10 µg His-tagged full length GOPC in the presence of 15 µl glutathione-sepharose beads and 10 µM either GDP or GMP-PNP in a total volume of 400 µl HNTM for 1 hour at 4°C. Either GDP-restricted or GTP-restricted point mutants of the rab proteins were used. Bound proteins were eluted in SDS-PAGE sample buffer and analysed by SDS-PAGE. The positions of molecular standards (KDa) are indicated on the left hand side.

5.2.11 GOPC and rab6 co-localise

GOPC has previously been localised to the Golgi apparatus (Charest *et al.* 2001; Yao *et al.* 2001) and immunoelectron microscopy has found the protein predominantly on the *trans*-cisternae and TGN in spermatids (Yao *et al.* 2002). To examine the localisation of GOPC in HeLa cells, cells were transfected with plasmids encoding GFP-tagged GOPC and counterstained for various Golgi markers (Figure 5.13). GFP-GOPC shows extensive co-localisation with rab6 (Figure 5.13 A), supporting an

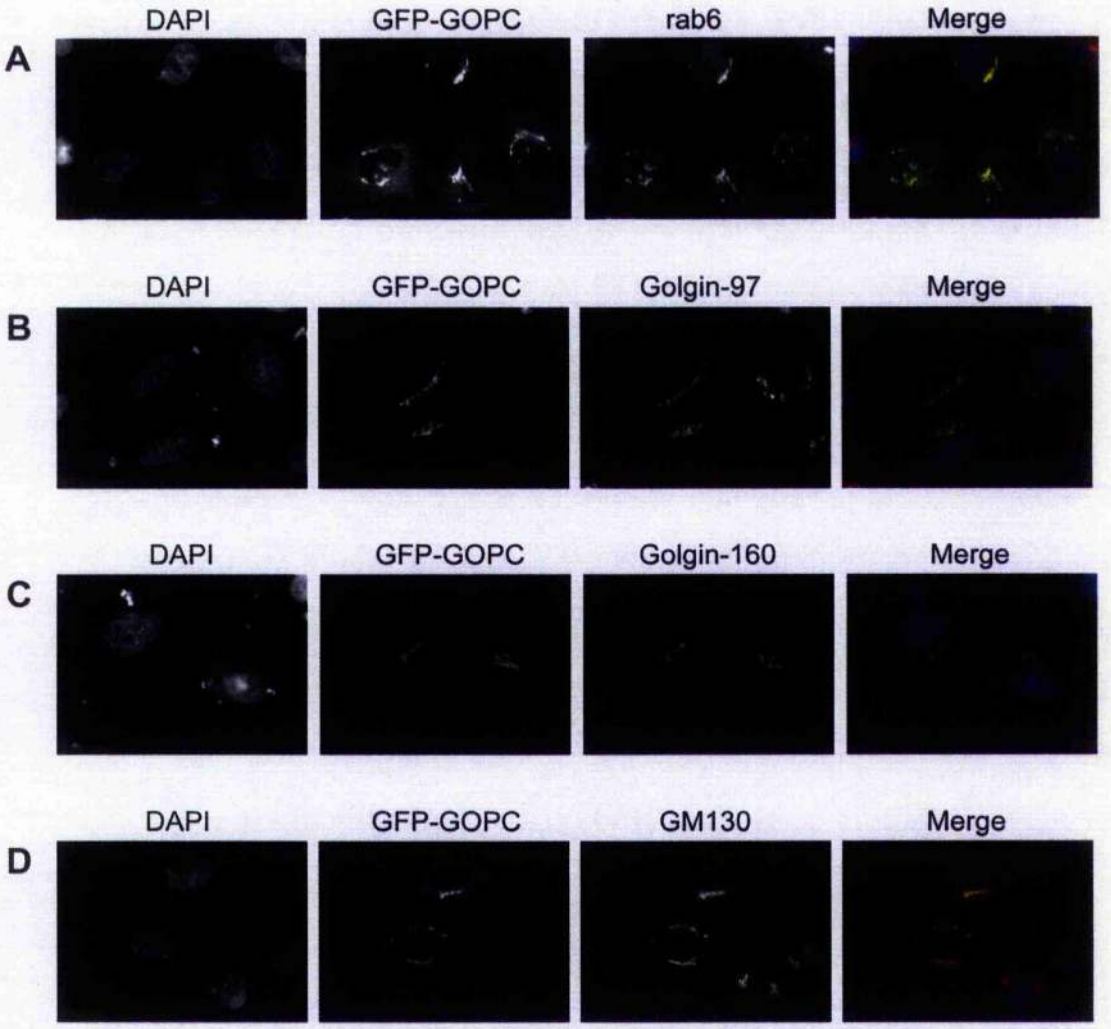


Figure 5.13 GFP-GOPC co-localises with rab6 at the Golgi apparatus
HeLa cells were transfected for 18 hours with a plasmid encoding GOPC tagged at its N-terminus with GFP. Cells were fixed in 3% PFA and co-stained with either rabbit anti-rab6 (A), sheep anti-golgin-97 (B), rabbit anti-golgin-160 (C), or sheep anti-GM130 (D). In the merged images, DNA (DAPI staining) is in blue, GFP-GOPC is in green, and rab6/golgin-97/golgin-160/GM130 is in red. Areas of overlap between GOPC and the Golgi markers are in yellow.

interaction between the two proteins *in vivo*. The distribution of GFP-GOPC partially overlaps with, but is distinct from, that of two other Golgi proteins, golgin-97 and golgin-160 (Figure 5.13 B and C). GFP-GOPC staining shows the least overlap with GM130, indicating that GOPC is not localised to the *cis*-Golgi (Figure 5.13 D).

Similarly, myc-tagged GOPC shows a high degree of co-localisation with rab6 at the Golgi apparatus (Figure 5.14 A) but only partial co-localisation with golgin-160 and GM130 (Figure 5.14 B and C).

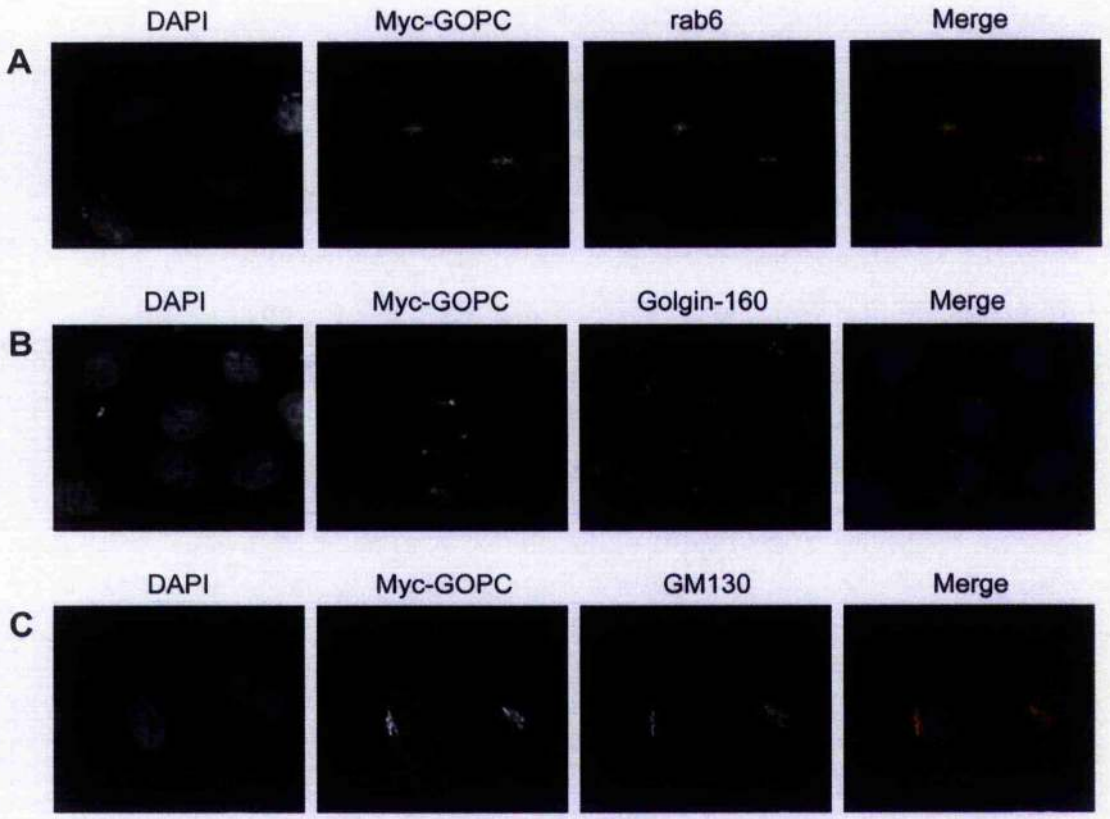


Figure 5.14 Myc-GOPC co-localises with rab6 at the Golgi apparatus
HeLa cells were transfected for 18 hours with a plasmid encoding GOPC tagged at its N-terminus with myc. Cells were fixed in 3% PFA and co-stained with either rabbit anti-rab6 (A), rabbit anti-golgin-160 (B), or sheep anti-GM130 (C). In the merged images, DNA (DAPI staining) is in blue, myc-GOPC is in red, and rab6/golgin-160/GM130 is in green. Areas of overlap between GOPC and the Golgi markers are in yellow.

5.2.12 GOPC interacts with rab6 via its N-terminal coiled-coil domain

In order to define the rab6 binding site on GOPC, deletion mutants of GOPC were cloned into the yeast two hybrid bait vector pGBT9 and were screened against the activated point mutants of rab6a and rab6a' cloned into the prey vector pACT2 (Figure 5.15). One of these mutants, GOPC amino acids 256-454, containing the PDZ domain and C-terminus of GOPC, was self-activating when fused to the GAL4 binding domain in pGBT9 (data not shown). This fragment was therefore cloned into pACT2 and screened against pGBT9.rab6aQ72L. This analysis revealed that the N-terminus and first coiled-coil domain of GOPC (amino acids 1-141) was sufficient to mediate an interaction with rab6 and that deletion of this region abrogates the interaction (Figure 5.15).

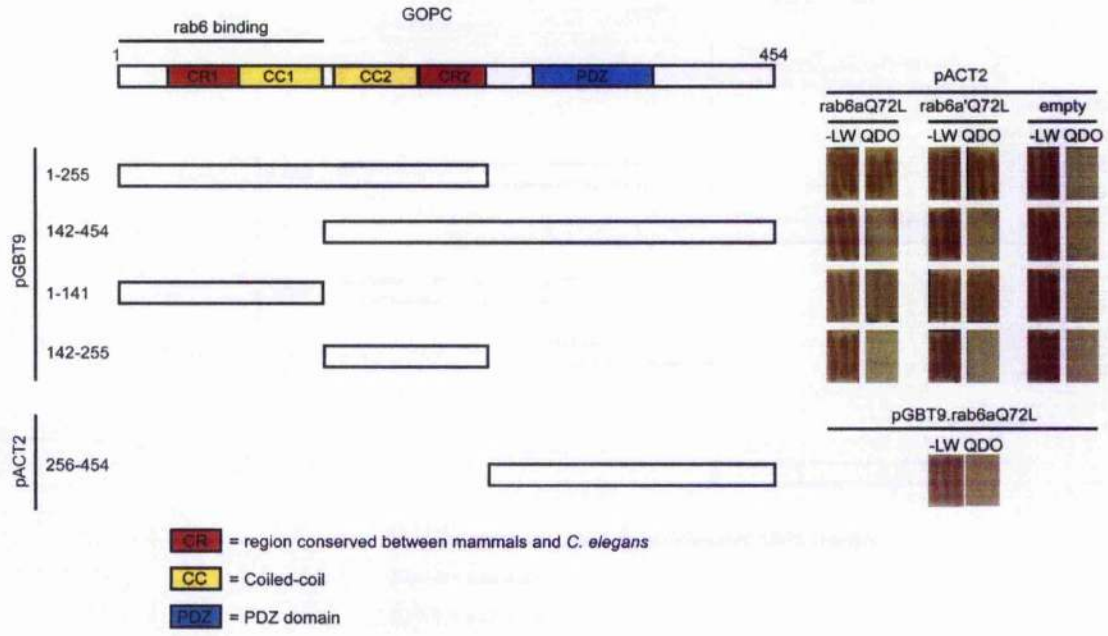


Figure 5.15 GOPC interacts with rab6 via its N-terminal coiled-coil domain
Deletion mutants of GOPC were cloned into the yeast two hybrid bait vector pGBT9 and screened against rab6aQ72L and rab6a'Q72L cloned into the prey vector pACT2 as well as empty pACT2 as a control. Due to problems with self-activation, GOPC 256-454 was cloned into pACT2 and screened against pGBT9.rab6aQ72L. Interactions were assessed by growth on quadruple dropout media.

5.2.13 GOPC targets to the Golgi apparatus via its second coiled-coild domain

The same deletion mutants used to map the rab6 binding site on GOPC, were cloned into a N-terminal GFP-tagging vector and transfected into HeLa cells (Figure 5.16). GOPC amino acids 1-141, while able to interact with rab6, was not able to target to the Golgi apparatus as seen for the full length protein, instead having a nuclear and cytoplasmic localisation. The C-terminus of GOPC (amino acids 256-454), containing the proteins PDZ domain, was also insufficient for correct Golgi localisation and was predominantly localised to the nucleus. GOPC 1-255 localised to the Golgi as well as the full length protein. While deletion of the first 141 amino acids of the protein resulted in a less efficient targeting to the Golgi, GOPC 142-255, containing the second coiled-coil domain and second conserved region, still displayed some Golgi localisation in addition to cytoplasmic staining (Figure 5.16). This region therefore appears to be essential for the Golgi targeting of GOPC, in agreement with previously published observations (Charest *et al.* 2001; Yao *et al.* 2001). It should be noted that the distribution of rab6 appears unaffected by the overexpression of any of the GOPC deletion mutants.

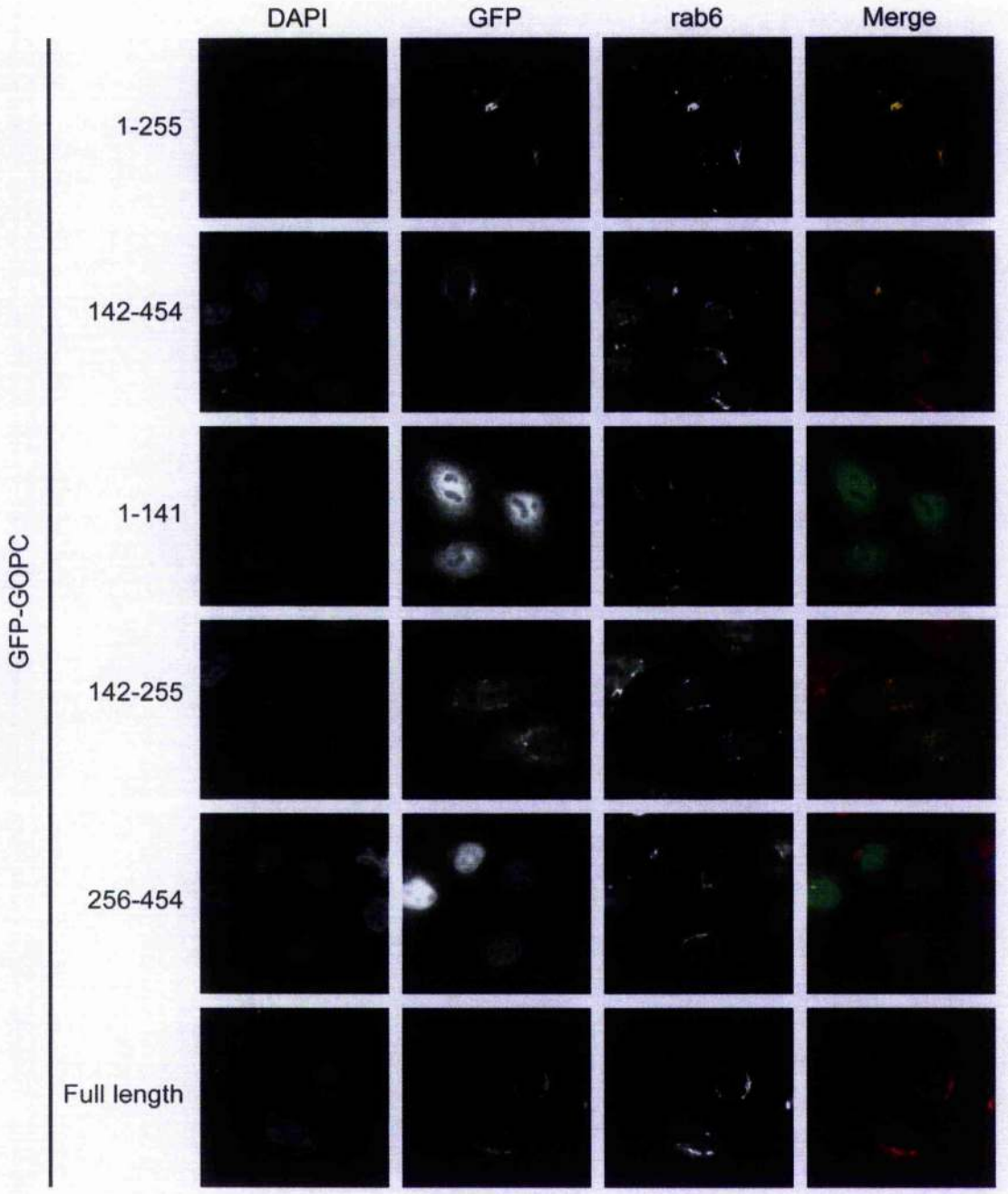


Figure 5.16 GOPC targets to the Golgi apparatus via its second coiled-coil domain
Deletion mutants of GOPC were cloned into the N-terminal GFP-tagging vector pEGFP-C2 and transfected into HeLa cells for 18 hours. Cells were fixed in 3% PFA and co-stained with rabbit anti-rab6. In the merged images, DNA (DAPI staining) is in blue, GFP-GOPC is in green, and rab6 is in red. Areas of overlap between GOPC and rab6 are in yellow.

Table 5.3 shows a summary of the rab6-binding and Golgi-targeting properties of the various GOPC deletion mutants.

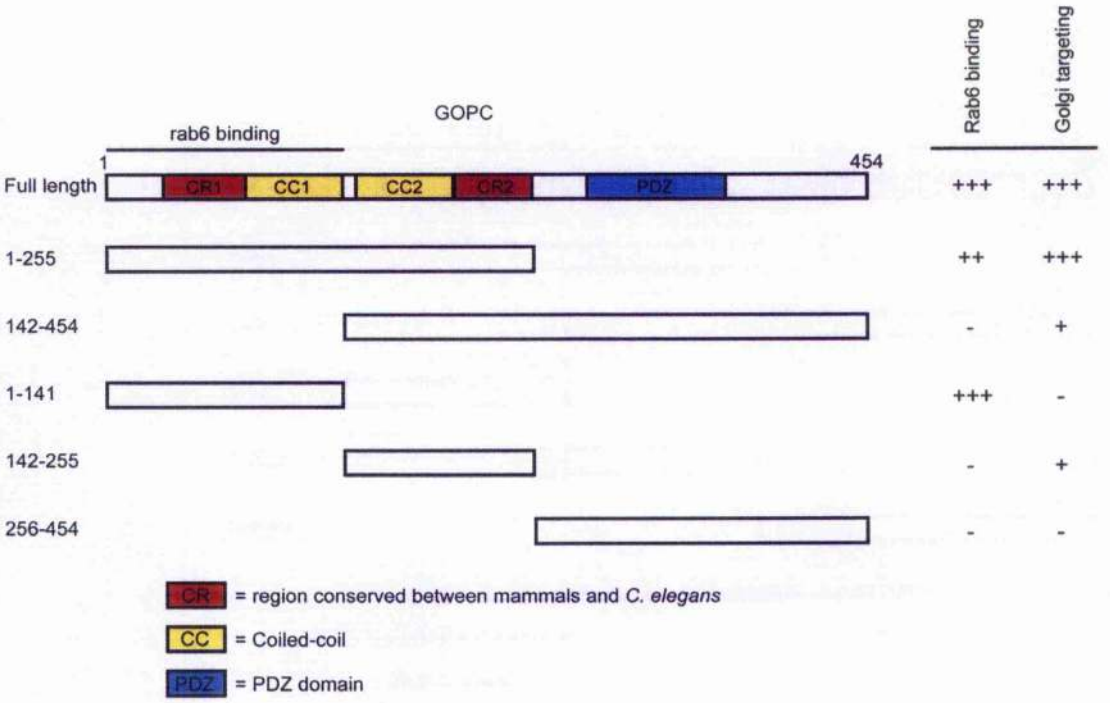


Table 5.3 Rab6 binding and Golgi targeting properties of GOPC deletion mutants

5.3 Discussion

5.3.1 Rab6 regulates the recruitment of the dynactin complex to Golgi membranes

The dynactin subunit p150^{glued} and the dynactin-interacting protein BicD2 were bound by activated rab6 from rat liver cytosol and identified by MALDI-TOF (Figure 5.1 and Table 5.1). Dynactin is a multisubunit protein complex essential for the activity of the microtubule-based motor protein dynein (Karki and Holzbaur 1999; King and Schroer 2000). Dynein is a minus-end directed motor that, together with dynactin, mediates a variety of intracellular motility processes including membrane trafficking events (Karki and Holzbaur 1999; Allan *et al.* 2002). The p150^{glued} subunit of dynactin is able to bind both microtubules and the intermediate chain of dynein (Vaughan and Vallee 1995; Waterman-Storer *et al.* 1995).

BicD2 is one of two mammalian homologues of the *Drosophila* protein Bicaudal-D, which is involved in the development of polarity in *Drosophila* oocytes and has been linked genetically to a transport pathway involving dynein and dynactin (Suter *et al.* 1989; Wharton and Struhl 1989; Swan *et al.* 1999). Mammalian BicD2 localises to the Golgi apparatus and directly binds to the p50/dynamitin subunit of dynactin. Overexpression of the N-terminal domain of BicD2 disrupts minus-end-directed organelle transport (Hoogenraad *et al.* 2001).

The interaction of dynactin with rab6 was found to be specific for this rab protein (Figure 5.2), and the interaction of both $p150^{glued}$ and BicD2 with rab6 was confirmed using the yeast two hybrid system (Figure 5.3). This analysis revealed that BicD1 also interacts with rab6. *In vitro* binding assays showed that the interactions between rab6 and $p150^{glued}$/BicD2 are direct and common to both ubiquitously expressed isoforms of rab6, a and a' (Figures 5.5 and 5.6).

The yeast two hybrid system was also used to map the rab6 binding sites on $p150^{glued}$ and the BicD proteins (Figure 5.4). The binding site on $p150^{glued}$ could not be defined very precisely, but was localised to the C-terminal two-thirds of the protein, while the binding site on BicD2 was mapped to the C-terminal coiled-coil domain known to target BicD2 to the Golgi ((Hoogenraad *et al.* 2001); Figure 5.8). Interestingly, a number of loss-of-function mutations in *Drosophila* BicD map to the C-terminal coiled-coil (Ran *et al.* 1994; Oh *et al.* 2000). The C-terminus of BicD1, which contains a homologous region of coiled-coil plus an extra domain of approximately 150 amino acids, also contained the rab6 binding site and targeted BicD1 to the Golgi (Figures 5.4 and 5.7).

This suggests that rab6 binding is crucial for the membrane targeting of dynactin. Further evidence for this was supplied by experiments that demonstrated the rab6-dependent recruitment of dynactin to Golgi membranes *in vitro* and *in vivo* (Figure 3 in (Short *et al.* 2002)). The C-terminus of $p150^{glued}$ has been proposed to regulate the interaction of dynactin with membranous cargo (Kumar *et al.* 2001). Dynein/dynactin can be recruited to protein-free liposomes containing phosphatidic acid in the presence of an acidic phospholipid-binding spectrin, and mediate their motility (Muresan *et al.* 2001). Spectrin and acidic phospholipids are unlikely to be the only regulators of dynactin-membrane association *in vivo* however, as they would not provide sufficient levels of specificity (Karcher *et al.* 2002).

The interactions of rab6-GTP with p150^{glued} and the BicD proteins would therefore be ideally placed to regulate the specific recruitment of dynactin to membrane compartments for transport. The C-terminus of p150^{glued} (including the C-terminal coiled-coil domain) may inhibit dynactins association with membranes since *in vitro* addition of this fragment inhibits dynactin membrane binding while dynactin constitutively binds to membranes in the presence of a p150^{glued} mutant lacking the C-terminus ((Kumar *et al.* 2001). It is possible that binding of activated rab6 to the C-terminus of p150^{glued} relieves this inhibition and allows dynactin to bind to membranes.

Associations between rab proteins and motor proteins controlling organelle motility have been reported previously. Rab27a regulates the peripheral distribution of melanosomes in melanocytes by binding its effector protein melanophilin, which, in turn, binds the actin-based motor protein myosin-Va (Hume *et al.* 2001; Wu *et al.* 2001; Wu *et al.* 2002). The late endosomal/lysosomal rab protein, rab7, binds RILP which then recruits dynein/dynactin to late endosomes and lysosomes, promoting their movement towards the minus-end of microtubules (Cantalupo *et al.* 2001; Jordens *et al.* 2001).

Rab6 itself has previously been suggested to bind the plus-end directed kinesin protein, rabkinesin-6 (Echard *et al.* 1998). I was unable to detect an interaction between these two proteins (Figure 5.9) which, together with the observations that rabkinesin-6 is primarily expressed in mitotic cells and plays a role in cytokinesis (Hill *et al.* 2000), suggests that a rab6-rabkinesin-6 interaction is not important for interphase membrane transport. Nevertheless, given the range of membrane trafficking events associated with rab6, one might predict rab6 to be involved in bi-directional transport and to be connected with plus-end-directed motors as well as the minus-end-directed dynein motor. Although dynactin has until now only been linked with dynein function, a recent report demonstrated that p150^{glued} was also able to bind kinesinII and was required for bi-directional organelle transport (Deacon *et al.* 2003). Interestingly, kinesinII may be involved in retrograde Golgi-to-ER transport (Le Bot *et al.* 1998), a microtubule-dependent transport step previously suggested to be regulated by rab6 (Martinez *et al.* 1997; White *et al.* 1999). The rab6-dependent membrane recruitment of dynactin might therefore result in the recruitment of either dynein or kinesinII, depending on the transport step concerned.

The bicaudal-D proteins have been implicated specifically in dynein/dynactin transport pathways (Swan *et al.* 1999; Hoogenraad *et al.* 2001) and, in particular, with Golgi-to-ER retrograde transport (Matanis *et al.* 2002). It is unclear, however, how a role in Golgi-to-ER trafficking would fit with a role in minus-end-directed transport since, in theory, transport from the Golgi to the ER should be a plus-end-directed activity. It is also unclear what functional differences exist (if any) between BicD1 and BicD2. Both proteins localise to the Golgi apparatus and both bind rab6. The function of the C-terminal extension in BicD1 compared to BicD2 should be of interest in answering this question.

5.3.2 GOPC is a rab6 effector

The Golgi-localised, PDZ domain-containing protein, GOPC/PIST/FIG was also bound from rat liver cytosol by activated rab6 (Figure 5.10 and Table 5.2). This interaction was confirmed as being specific to rab6 by binding assays from transfected HeLa extract and by the yeast two hybrid system (Figure 5.11). *In vitro* binding assays showed the interaction to be direct and common to both rab6a and rab6a' (Figure 5.12).

Rab6 and GOPC completely co-localise in cultured HeLa cells (Figures 5.13 A and 5.14 A). There is a partial overlap between GOPC, golgin-160, and golgin-97 distribution (Figures 5.13 B-C and 5.14 B) but little between GOPC and GM130 (Figures 5.13 D and 5.14 C). The targeting of GOPC to the Golgi apparatus appears to be mediated by the second coiled-coil motif and second highly conserved region since amino acids 142-255 are necessary and sufficient for the proteins localisation (Figure 5.16). This is in agreement with previous findings (Charest *et al.* 2001; Yao *et al.* 2001). This targeting domain, however, is distinct from the rab6 binding site, which maps to amino acids 1-141, containing the first coiled-coil motif and first conserved region (Figure 5.15). Rab6 would not, therefore, appear to be essential for localising GOPC to the Golgi although constructs lacking the rab6 binding site seem to target less well than constructs able to bind rab6 (compare, for example, the localisation of GFP-GOPC 1-255 with GFP-GOPC 142-454 in Figure 5.16). Rab6 binding is perhaps required for efficient Golgi localisation.

Charest *et al.*, (2001) found that the second coiled-coil domain and conserved region of GOPC binds syntaxin 6 and suggested this to be the mechanism of Golgi targeting.

Syntaxin 6 is a t-SNARE localised primarily to the TGN (Bock *et al.* 1996; Bock *et al.* 1997), which has been implicated in TGN-to-endosome and rab6a'-controlled endosome-to-TGN transport (Bock *et al.* 1997; Mallard *et al.* 2002). The interaction of GOPC with both a rab protein and a t-SNARE clearly suggests it is involved in membrane trafficking. This situation is not without precedent, since syntaxin 6 has previously been shown to bind the rab5 effector protein EEA1 (Simonsen *et al.* 1999).

GOPC was originally identified as an interactor of Frizzled, a plasma membrane receptor for Wnt signalling proteins (Yao *et al.* 2001). It has since been shown to interact with two other cell surface receptors: the δ2 glutamate receptor and the cystic fibrosis transmembrane conductance regulator (CFTR; (Cheng *et al.* 2002; Yue *et al.* 2002)). All of these interactions depend on the PDZ domain of GOPC, a domain also responsible for an interaction with the Golgi-localised chloride channel ClC-3B (Gentzsch *et al.* 2003).

Interestingly, overexpression of GOPC reduces the cell surface expression levels of CFTR by reducing the rate of its transport to the plasma membrane and decreasing the half-life of its cell surface localisation (Cheng *et al.* 2002). This suggests that GOPC may play a general role in controlling the cell surface expression of a variety of plasma membrane receptors either by negatively regulating anterograde transport to post-Golgi compartments or by promoting retrograde transport to the Golgi or from the Golgi to ER. The interaction of GOPC with rab6 indicates that a role in retrograde transport is more likely although it is possible that this interaction is simply required for the recycling of GOPC back to the *trans*-Golgi.

GOPC overexpression also results in a reduction in the overall levels of CFTR and ClC-3B (Gentzsch *et al.* 2003). GOPC has also been found to interact, via its second coiled-coil domain, with Beclin1, a protein known to promote the cellular degradative pathway of autophagy (Yue *et al.* 2002). GOPC might therefore also be involved in sorting processes at the TGN directing proteins for degradation at the lysosomes.

A final clue to the function of GOPC comes from an analysis of GOPC-deficient mice (Yao *et al.* 2002). Male mice lacking GOPC are infertile due to a failure in acrosome formation during spermatogenesis. The acrosome is formed by the fusion of vesicles derived from the *trans*-Golgi, again implicating GOPC in post-Golgi trafficking events. Although GOPC is ubiquitously expressed in all human and mice tissues

tested (Charest *et al.* 2001; Cheng *et al.* 2002), infertility was the only defect reported in GOPC-knockout mice (Yao *et al.* 2002).

GOPC therefore appears to be a molecule which regulates the trafficking of integral membrane proteins between the *trans*-Golgi and post-Golgi compartments possibly via a rab6-mediated retrograde transport step.

5.3.3 Rab6a and rab6a' show identical interactions

The two ubiquitously expressed rab6 proteins, rab6a and rab6a', differ by just three amino acids as a result of alternative splicing (Echard *et al.* 2000). Rab6a' has an isoleucine instead of valine at position 62 and two alanines instead of threonine and valine at positions 87 and 88. Nevertheless, despite this small difference in amino acid sequence, the two proteins have been reported to have different functions *in vivo,* with rab6a proposed to regulate retrograde intra-Golgi and Golgi to ER transport and rab6a' implicated in endosome to Golgi traffic (Martinez *et al.* 1994; Martinez *et al.* 1997; Echard *et al.* 2000; Mallard *et al.* 2002).

The most obvious explanation for the ability of these two rabs to control different transport steps would be an ability to bind different effector proteins. The double alanine/threonine-valine substitution at positions 87 and 88 lies at the end of the putative $\alpha 2$ helix corresponding to the switchII region predicted to change conformation depending on the nucleotide state of the rab protein, thereby mediating interactions with effector proteins (Echard *et al.* 2000). Small differences in this region might therefore have a large effect on protein interactions. To date, however, the only protein shown to differentiate between rab6a and rab6a' is rabkinesin-6 (Echard *et al.* 2000) which, as discussed above, may not in fact be a rab6 effector. All other rab6 effectors, including GOPC, $p150^{glued}$, and the BicD proteins, appear to interact with both isoforms of rab6 (see, for example, Figures 5.6 and 5.12). One possibility is that rab6a and rab6a' differ in the affinities and/or kinetics of their interactions resulting in differential effects when the proteins and their mutants are overexpressed in cells. Alternatively, small differences in the kinetics of nucleotide exchange and/or hydrolysis may result in slightly different functions.

Whatever the differences between the a and a' splice variants, rab6 appears to control retrograde transport steps from the endosomal system to the *trans*-Golgi /TGN and from the *trans*-Golgi to earlier compartments. It does this through the recruitment of

a variety of effectors including, as shown here, components of the dynactin complex involved in microtubule-based motility and a PDZ domain-containing protein able to bind integral membrane proteins and regulate their appearance at the cell surface. Nizak *et al.*, have recently reported the generation of a recombinant antibody specific for the GTP-bound form of rab6 *in vivo* (Nizak *et al.* 2003). Tools such as this will doubtless help the precise function and mechanism of rab6 activity to be defined more fully in future.

5.3.4 Summary

Four proteins were identified as novel effectors of rab6, a *trans*-Golgi localised regulator of retrograde transport steps. $p150^{glued}$ is a component of the dynactin complex required for dynein-dependent, microtubule-based motility of membranous cargo. BicD1 and BicD2 are dynein/dynactin-associated proteins also essential for a microtubule-based transport pathway. GOPC is a PDZ domain-containing protein which binds a variety of integral membrane proteins and influences their transport to the cell surface as well as potentially being involved in sorting to degradative pathways and in acrosome formation. Rab6 therefore regulates retrograde transport through interactions with a variety of different proteins.

Chapter 6: Identification Of A Golgin-Kinase Complex At The Golgi Apparatus

6.1 Introduction

Emerging evidence suggests that the Golgi apparatus may be the site of numerous signalling events important for such diverse cellular processes as growth, differentiation, motility, mitosis, and apoptosis (Ferri and Kroemer 2001; Bivona and Philips 2003; Rios and Bornens 2003).

Fragmentation of the Golgi has been found to be necessary for entry into mitosis, suggesting that the Golgi acts as a sensor controlling mitotic entry (Sutterlin *et al.* 2002). The pro-apoptotic cysteine protease caspase-2 partially localises to the Golgi, where it specifically cleaves golgin-160 in response to pro-apoptotic signals (Mancini *et al.* 2000). Other Golgi matrix proteins are also cleaved during mitosis: GRASP65 by caspase-3 (Lane *et al.* 2002) and p115 by caspases-3 and –8 (Chiu *et al.* 2002). In all cases, these cleavages result in a fragmentation of the Golgi apparatus during apoptosis, which can be slowed by expression of cleavage-resistant forms of the three Golgi proteins.

Many signalling molecules have been localised to the Golgi including members of the rho/rac/Cdc42 family of small GTPases (reviewed in Rios and Bornens 2003 and Bivona and Philips 2003). Ras has also recently been shown to localise to the Golgi from where it is able to signal to downstream targets with different kinetics from plasma membrane-associated ras (Chiu *et al.* 2002). Ras activation at the Golgi was shown to be dependent on the tyrosine kinase Src (Chiu *et al.* 2002). Another protein kinase associated with the Golgi apparatus is Protein Kinase A since one of its anchoring proteins, AKAP350, has been localised to the organelle (Shanks *et al.*

2002). Thus, the Golgi apparatus may act as a signalling platform, sensing and integrating signals and regulating downstream events.

The Ste20 (sterile 20) family of serine/threonine protein kinases is implicated in a variety of signalling pathways (Kyriakis 1999; Dan *et al.* 2001; Bokoch 2003). The founding member of the family, budding yeast Ste20p, was originally identified as a signalling component of the yeast mating pathway (Leberer *et al.* 1992), but is also important for other events, including the regulation of actin organisation and cell polarity (Holly and Blumer 1999). Ste20p acts as an upstream activator of MAP kinase cascades in response to a variety of extracellular signals(Wu *et al.* 1995; Drogen *et al.* 2000) and is regulated by binding to $G_{\beta\gamma}$ subunits of heterotrimeric G proteins and by the small GTPase Cdc42 (Leeuw *et al.* 1998; Lamson *et al.* 2002).

Approximately 30 Ste20 kinases exist in mammals, which can be classified into two sub-groups: p21-activated kinase (PAK)-family kinases and germinal centre (GC)-kinases (Dan *et al.* 2001). The main difference between these two groups is the position of the kinase domain. PAK kinases have a C-terminal kinase domain while GC kinases possess a N-terminal kinase domain and a C-terminal regulatory domain (Kyriakis, 1999; Dan et al., 2001a).

The PAK kinases are regulated by rac and Cdc42 via a Cdc42 and Rac interactive binding (CRIB) motif in their N-terminal regulatory domains (Bokoch 2003). Like Ste20p, PAK-kinases affect a wide range of downstream processes. PAK1 controls actin remodelling and affects cell motility (Sells *et al.* 1997; Sells *et al.* 1999). PAK-kinases have also been implicated in MAP kinase signalling pathways (Bokoch 2003), by, for example, the phosphorylation of Raf-1 by PAK1 in response to integrin activation (Chaudhary *et al.* 2000). Apoptosis is also regulated by PAK-kinases (Bokoch 2003). PAK2 is cleaved by caspases, resulting in a constitutively active form of the kinase, which promotes cell death (Lee *et al.* 1997; Rudel and Bokoch 1997). PAK1, on the other hand, promotes cell survival by phosphorylating and inhibiting the pro-apoptotic protein Bad (Schurmann *et al.* 2000).

Relatively little is known, however, about the activation, regulation, and physiological functions of GC kinases, although some members of the family are thought to be involved in stress-activated MAP kinase pathways (Kyriakis 1999).

The GC kinases can be further sub-divided into 8 groups based on sequence homologies (Dan *et al.* 2001). One of these sub-groups, group III, contains the

kinases MST3 (Schinkmann and Blenis 1997), MST4 (Lin *et al.* 2001; Qian *et al.* 2001; Dan *et al.* 2002), and YSK1 (Pombo *et al.* 1996; Osada *et al.* 1997).

YSK1 (for Yeast Sps1/Ste20-related kinase 1), also known as SOK1 (Ste20/oxidant stress response kinase 1), was found to be activated by reactive oxygen intermediates but not by any other environmental stresses, nor by growth factors (Pombo *et al.* 1996). The kinase was unable, however, to activate any of the known MAPK pathways (Pombo *et al.* 1996; Osada *et al.* 1997) and a physiological function for YSK1 remains unknown.

Serendipitously, having isolated YSK1 in a yeast two hybrid screen using rabkinesin-6 as a bait protein, we discovered that GFP-tagged YSK1 localised to the Golgi apparatus. This was interesting, given the wide range of processes regulated by Ste20 kinases, and the increasing importance of the Golgi apparatus as a signalling platform. Therefore, although the interaction with rabkinesin-6 was not investigated further, I performed a series of experiments designed to characterise the localisation of YSK1 and determine the mechanism by which the protein targets to the Golgi.

6.2 Experimental Methods

6.2.1 Yeast two hybrid screen using YSK1 as bait

Full length, wild type, YSK1 was cloned into the modified yeast two hybrid bait vector pFBT9 which, in contrast to pGBT9, carries a kanamycin resistance cassette rather than ampicillin resistance. This plasmid was transformed into the yeast strain PJ69-4A using the standard frozen yeast cell transformation protocol described in section 2.5.2. This bait strain was then screened against a human testis Matchmaker™ cDNA library (Clontech), using the 'TRAFO' yeast transformation protocol available at http://www.umanitoba.ca/faculties/medicine/biochem/gietz/, and performed at the 30X scale as follows:

50 ml SC-Trp was inoculated with the YSK1 bait strain and grown overnight at 30°C with shaking. The following morning, this culture was diluted in 150 ml YPDA medium to an OD_{600} of 0.2-0.3 (equivalent to approximately 7.5×10^8 cells). This culture was then grown at 30°C for 3-4 hours until an OD_{600} of 0.4-0.6 (approximately 2×10^7 cells/ml) was reached. Cells were then harvested by centrifugation at room temperature at 3000 x g for 5 minutes. The cell pellet was washed in half the culture

volume sterile water and centrifuged as before. The pellet was then resuspended in 3 ml 100 mM LiOAc and incubated for 15 minutes at 30°C before being centrifuged once again. A transformation mix was prepared by mixing, in order, the following components: 7.2 ml 50% PEG, 1.08 ml 1 M LiOAc, 1.5 ml 2 mg/ml single-stranded herring sperm DNA, and 1.02 ml sterile water. This mixture was added to the cell pellet and the pellet resuspended by vortexing. 2 μg library cDNA was then added to the mix and vortexed. The transformation mix was incubated for 30 minutes at 30°C and then heat shocked for 40 minutes at 42°C with occasional mixing. The cells were centrifuged as before, resuspended in 40 ml sterile water and plated onto 15 cm dishes of SC-Leu/-Trp/-His/-Ade (QDO) agar. The plates were incubated for 4 days at 30°C after which, large colonies were re-streaked onto fresh QDO plates. Library plasmids were recovered by performing minipreps from yeast cells (Section 2.5.3), and electroporation of the plasmid DNA into XL1b *E.coli* (Section 2.2.4) followed by selection on LB-Ampicillin plates. Recovered plasmids were then re-transformed into PJ69-4A yeast by the frozen cell method along with either pFBT9.YSK1 or empty vector. Plasmids allowing growth on QDO in the presence of YSK1 but not in the presence of empty vector were selected for sequencing.

6.2.2 Direct binding of YSK1 and MST4 to GM130

1 μg recombinant, His-tagged, YSK1 or MST4, expressed in baculovirus-infected Sf9 cells was incubated with 5 μg either GST-tagged GM130 amino acids 75-271, golgin-45 amino acids 1-122, or GST alone for 1 hour at 4°C in HNTM buffer in the presence of 15 μl glutathione-sepharose and 100 μM ATP in a total volume of 300 μl. Beads were then washed in 3 x 1ml HNTM and bound protein was eluted directly in SDS-PAGE sample buffer. Samples were then western blotted and probed with either goat anti-YSK1 N19 (Santa Cruz Biotech, USA) or affinity purified rabbit anti-YSK1/MST4. Loading controls were analysed by SDS-PAGE and Coomassie brilliant blue staining.

6.3 Experimental Results

6.3.1 YSK1 is a Golgi-localised kinase

Rabbit polyclonal antisera were raised against his-tagged full length YSK1 recombinantly expressed in *E.coli*. When the antisera was tested by immunofluorescence on cultured HeLa cells, it gave a Golgi-like, perinuclear staining pattern (Figure 6.1 A). Co-staining with anti-GM130 antibodies showed a partial co-localisation of YSK1 with the cis-Golgi matrix protein. A similar staining pattern was seen in the human melanoma cell line MelJuso with anti-YSK1 sera strongly staining the Golgi and co-localising with GM130 (Figure 6.1 B).

These results suggest that endogenous YSK1 localises to the Golgi apparatus. This corresponded to previous observations that both GFP- and myc-tagged YSK1 localised to the Golgi upon transfection into HeLa cells (Figure 6.1 C and D).

6.3.2 Characterisation of rabbit anti-YSK1

The polyclonal antiserum was affinity purified and, to further characterise the antibody, recombinant YSK1 expressed in baculovirus-infected Sf9 cells was western blotted and probed. The affinity-purified antibody recognised a band of approximately 60 KDa but recognition was blocked by pre-incubation of the antiserum with the bacterially expressed antigen (Figure 6.2 A). Additionally, the rabbit antiserum recognised proteins of the correct size in HeLa extract, rat liver cytosol, and rat liver Golgi membranes (Figure 6.2 A). The slight difference in apparent molecular weight between recombinant YSK1 and endogenous protein is due to the his-tag on the recombinant protein.

Next, I examined the specificity of the antibody with respect to the two kinases most closely related to YSK1, namely MST3 and MST4 (Dan *et al.* 2001). These kinases have, respectively, 72.3% and 68.1% identity with YSK1 over the full length of the protein. HeLa cells were transfected for 18 hours with plasmids encoding GFP-tagged kinases before cell extracts were prepared in TNTE buffer. 20μg of each extract was western blotted and probed with the affinity-purified rabbit antibody (Figure 6.2 B). The antibody strongly recognised GFP-YSK1 but was also able to recognise GFP-MST4. The antibody did not recognise GFP-MST3, however, even though equal amounts of all three kinases were present, as revealed by probing the

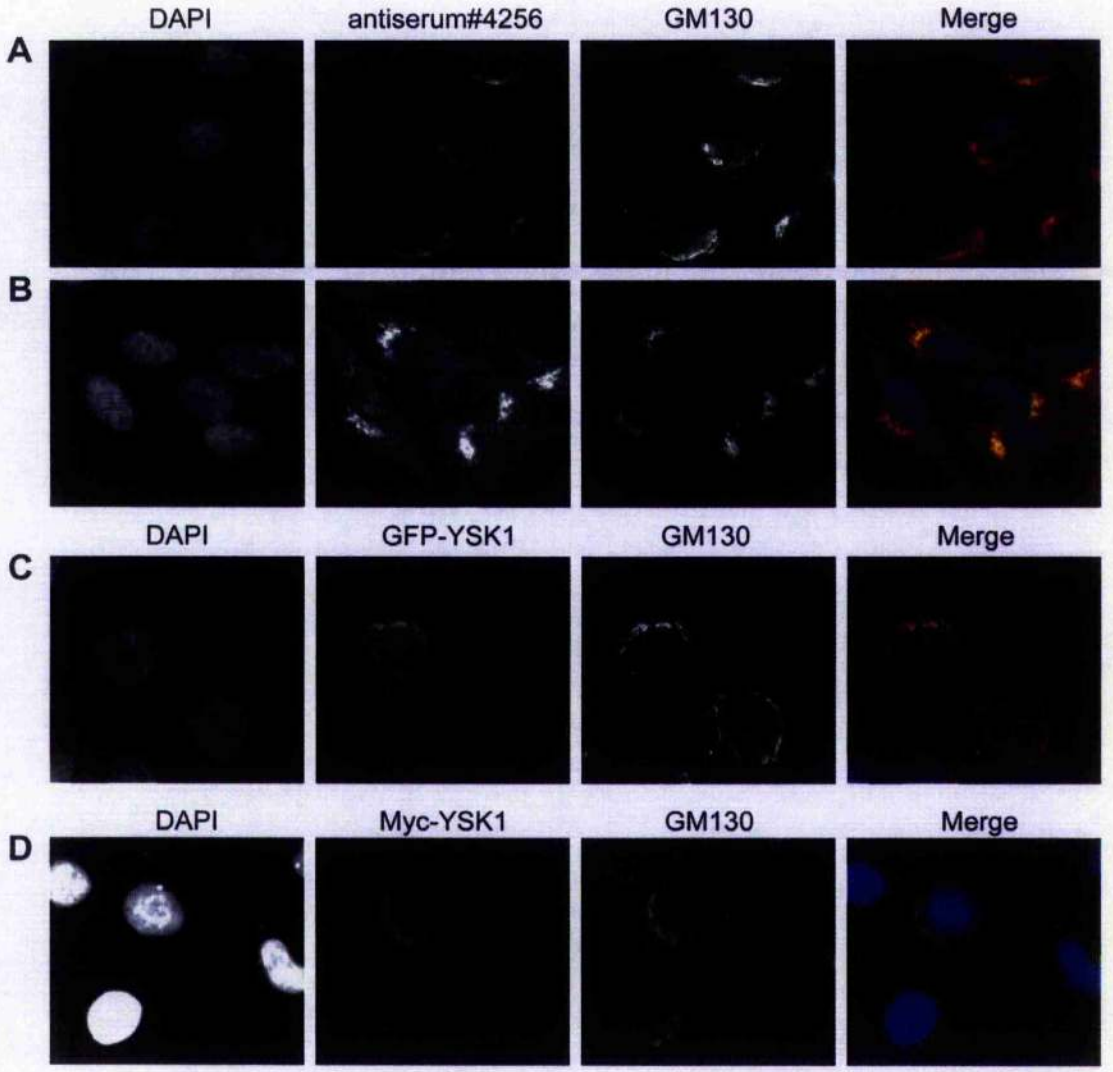


Figure 6.1 YSK1 is a Golgi-localised kinase
(A and B) HeLa cells (A) and MelJuso cells (B) were fixed with 3% PFA and co-stained with rabbit polyclonal antiserum #4256, raised against recombinant YSK1, and sheep anti-GM130. (C and D) HeLa cells were transfected for 18 hours with plasmids encoding either GFP-tagged YSK1 (C) or Myc-tagged YSK1 (D) prior to fixation with 3% PFA. Cells were then stained with sheep anti-GM130 and, in (D) with mouse anti-myc. In the merged images, DNA (DAPI staining) is in blue, YSK1 is in either green (A and C) or red (B and D), and GM130 is in either red (A and C) or green (B and D). Areas of overlap between YSK1 and GM130 are in yellow.

extracts with sheep anti-GFP (Figure 6.2 B). Thus, the rabbit 'anti-YSK1' polyclonal antibody is not entirely specific for YSK1 and also recognises MST4. This is not too surprising, given that full length YSK1 was used as the antigen for immunisation and the high level of identity between the two proteins.

Two different anti-YSK1 antibodies are available commercially from Santa Cruz Biotechnology, California, USA. These antibodies were raised in goats against peptides corresponding to either the N-terminal or C-terminal amino acids of YSK1

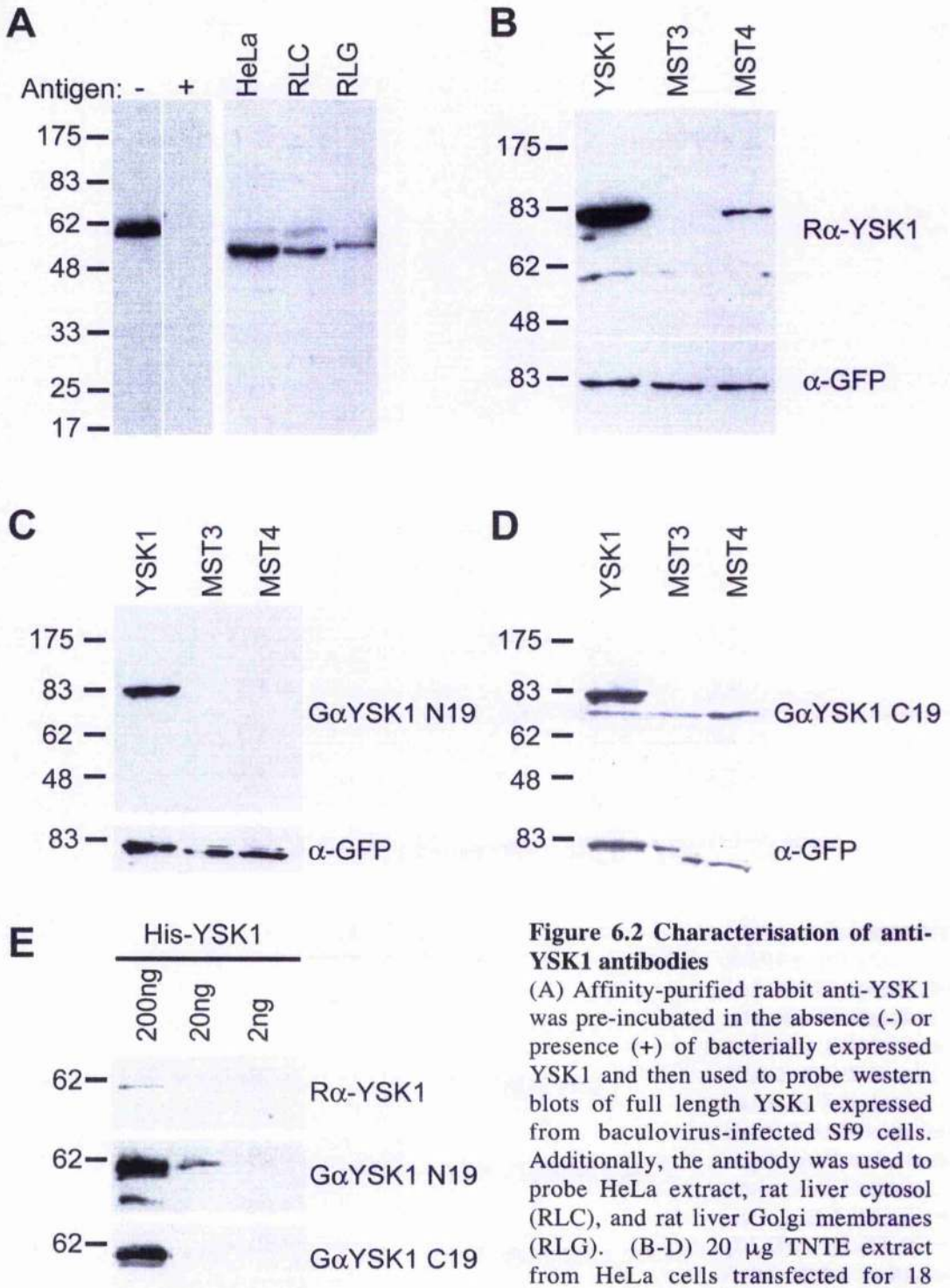


Figure 6.2 Characterisation of anti-YSK1 antibodies

(A) Affinity-purified rabbit anti-YSK1 was pre-incubated in the absence (-) or presence (+) of bacterially expressed YSK1 and then used to probe western blots of full length YSK1 expressed from baculovirus-infected Sf9 cells. Additionally, the antibody was used to probe HeLa extract, rat liver cytosol (RLC), and rat liver Golgi membranes (RLG). (B-D) 20 μg TNTE extract from HeLa cells transfected for 18 hours with plasmids encoding GFP-tagged YSK1, MST3, or MST4 were western blotted and probed with either rabbit anti-YSK1 (B), goat anti-YSK1 N-terminus (C), or goat anti-YSK1 C-terminus (D). All three blots were probed with sheep anti-GFP as a loading control. (E) The three anti-YSK1 antibodies were used to probe decreasing amounts of recombinant YSK1 expressed in Sf9 cells. The positions of molecular standards (KDa) are indicated on the left hand side of each blot.

and might therefore be expected to be completely specific for YSK1. The specificity of these two antibodies was tested as before. Both antibodies recognised GFP-tagged YSK1 in HeLa cell extracts but not GFP-MST3 or GFP-MST4 (Figure 6.2 C and D). The antibody raised against the C-terminal 19 amino acids of YSK1 appeared to recognise an unknown additional protein in HeLa extract too large to be the endogenous kinase.

Finally, the relative affinities of the three antibodies for YSK1 was tested by western blotting serial dilutions of Sf9-expressed YSK1 (Figure 6.2 E). This suggested that the two goat antibodies (at the concentrations used for both western blotting and immunofluorescence) have a higher affinity for YSK1 than the rabbit antibody. This is not a direct quantitative test however, since different secondary antibodies are used for either the goat or rabbit antibodies.

6.3.3 Immunofluorescence on HeLa cells with goat anti-YSK1 antibodies

Having shown both goat anti-YSK1 antibodies to be specific for YSK1 and to have an apparently higher affinity for YSK1 than the rabbit antibody by western blotting (Figure 6.2 C-E), I tested the antibodies by immunofluorescence. Somewhat surprisingly, neither of the two goat antibodies showed any staining of untransfected HeLa cells. This was not because the antibodies were unable to recognise YSK1 by

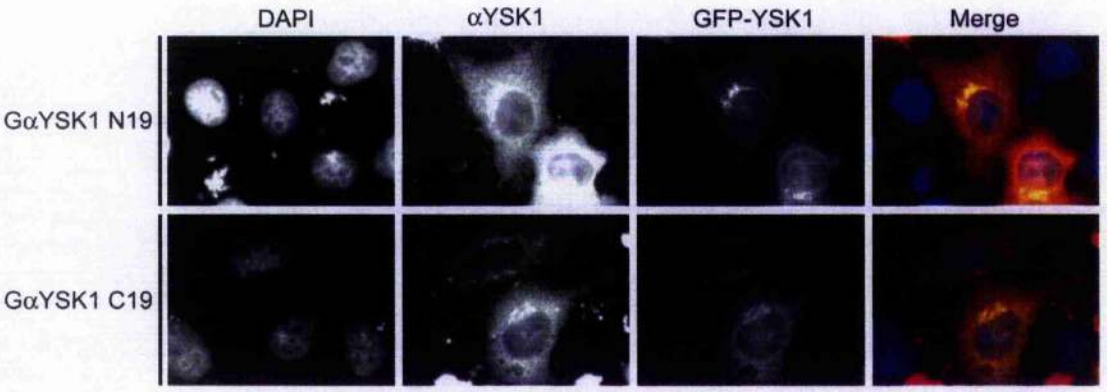


Figure 6.3 Goat anti-YSK1 antibodies only recognise exogenous YSK1 by immunofluorescence

HeLa cells were transfected for 18 hours with a plasmid encoding GFP-tagged YSK1 and fixed with 3% PFA. Cells were then stained with either goat anti-YSK1 N-terminus or goat anti-YSK1 C-terminus. In the merged images, DNA (DAPI staining) is in blue, YSK1 antibody is in red, and GFP-YSK1 is in green.

immunofluorescence, since, in HeLa cells transfected with GFP-YSK1, the antibodies stained YSK1 both at the Golgi and in the cytosol (Figure 6.3).

The rabbit anti-YSK1 was able to stain the Golgi of untransfected HeLa cells (Figure 6.1 A) even though its affinity for YSK1 is seemingly lower (Figure 6.2 E). This apparent discrepancy could be explained if MST4, which is also recognised by the rabbit antibody (Figure 6.2 B), also localises to the Golgi apparatus. In this case, the Golgi-localised protein recognised in immunofluorescence by rabbit anti-YSK1 could be MST4.

6.3.4 MST4, but not MST3, localises to the Golgi

MST3 and MST4 were cloned into a N-terminal myc-tagging vector (pcDNA3.1MycA) and were transfected into HeLa cells (Figure 6.4). Myc-MST3 is localised diffusely throughout the cytosol, in contrast to myc-MST4 which, like myc-YSK1, localises to the Golgi apparatus where its distribution partially overlaps with GM130. This suggests that MST4 is also a Golgi-localised kinase, and that the Golgi-staining detected by the rabbit anti-'YSK1' in HeLa cells by immunofluorescence may represent MST4 rather than YSK1.

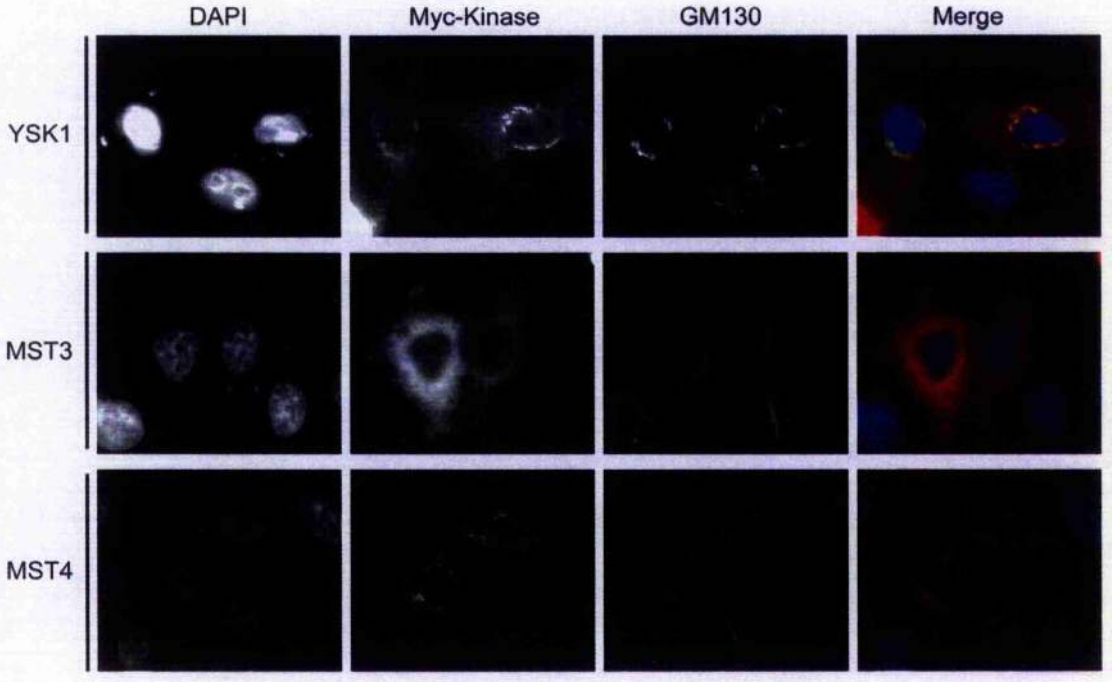


Figure 6.4 MST4 is a Golgi-localised kinase
HeLa cells were transfected for 18 hours with plasmids encoding either myc-YSK1, myc-MST3, or myc-MST4 prior to fixation with 3% PFA. Cells were co-stained with mouse anti-myc and sheep anti-GM130. In the merged images, DNA (DAPI staining) is in blue, myc-tagged kinases are in red, and GM130 is in green. Areas of overlap between the kinases and GM130 are in yellow.

6.3.5 YSK1 and MST4 bind to the *cis*-Golgi matrix protein GM130

Protein kinase A localises to the Golgi apparatus via an interaction with the adaptor protein AKAP350 (Shanks *et al.* 2002). To help identify the mechanism by which YSK1 targets to the Golgi, we performed a yeast two hybrid screen using YSK1 as bait in order to identify potential interacting proteins. A total of $2.2x10^6$ transformants from a human testis cDNA library were screened and two plasmids were found to specifically interact with YSK1 upon re-transformation. The two plasmids were sequenced and found to encode variants of full length human GM130 containing a 27 amino acid insertion at position 57. Despite this insertion in the p115 binding domain of GM130, the two clones were able to interact with p115 as well as YSK1 in the yeast two hybrid system (Figure 6.5). Full length rat GM130 also interacted with YSK1, suggesting that this interaction is not dependent upon the 27 amino acid insertion present in the positive yeast two hybrid screen clones.

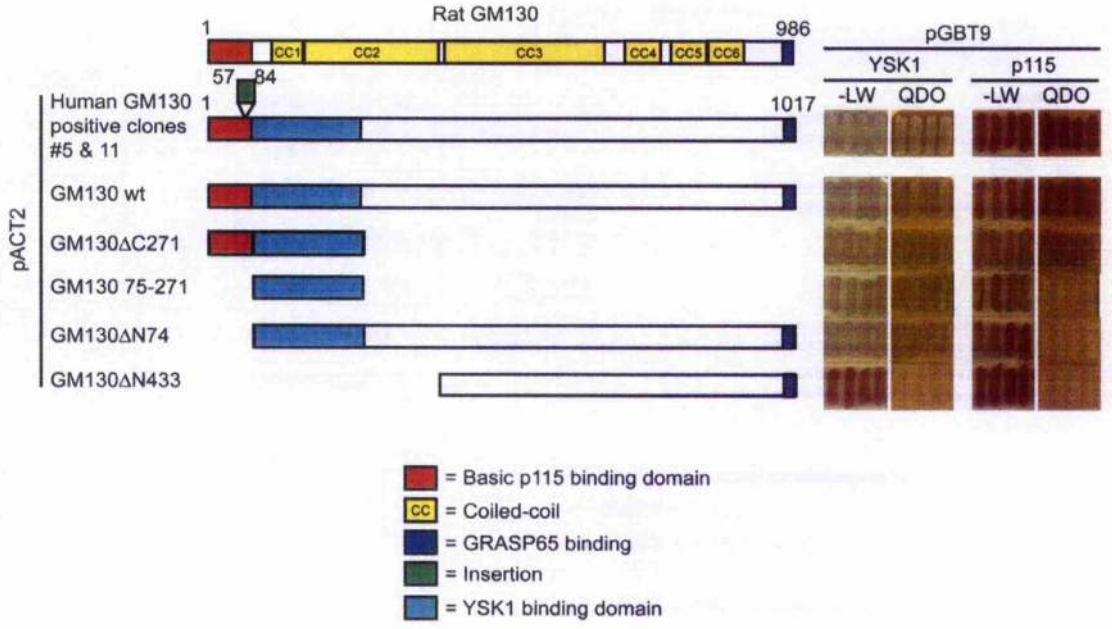


Figure 6.5 GM130 is identified in a yeast two hybrid screen as an interactor of YSK1
A yeast two hybrid screen was performed using a human testis cDNA library and YSK1 as bait. Two positive clones were identified corresponding to a variant of GM130 containing a 27 amino acid insertion near the N-terminus. Full length rat GM130 was also able to interact with YSK1. To map the YSK1 binding site for YSK1 on GM130, deletion mutants of GM130 were cloned into pACT2 and screened against YSK1 and p115 cloned into pGBT9. Interactions were assessed by growth on quadruple dropout media.

To map the binding site for YSK1 on GM130, a series of rat GM130 deletion mutants were cloned into pACT2 and screened against YSK1 in the yeast two hybrid system. A deletion mutant comprising the N-terminal 271 amino acids of GM130 (GM130ΔC271) was able to interact with YSK1 (and, as expected, with p115), while a construct containing the C-terminal half of the protein (GM130ΔN433) interacted with neither YSK1 nor p115 (Figure 6.5). This suggested that the YSK1 binding site lies close to the p115 binding site in the N-terminus of GM130. The two sites are distinct however, since GM130ΔN74, which lacks the first 74 amino acids of GM130, interacted with YSK1 but not with p115. A further mutant, GM130 amino acids 75-271, showed that these amino acids are sufficient to mediate the interaction with YSK1 (Figure 6.5).

We then tested whether MST3 or MST4 could interact with GM130. Like YSK1, MST4 could also interact with GM130 in the yeast two hybrid system, while MST3 showed no interaction (Figure 6.6). This corresponds to the localisation of YSK1 and MST4 at the Golgi while MST3 is apparently cytosolic (Figure 6.4). As with YSK1, MST4 interacts with the N-terminal 271 amino acids of GM130 (Figure 6.6). Neither of the three kinases interact with the medial-Golgi matrix protein golgin-45.

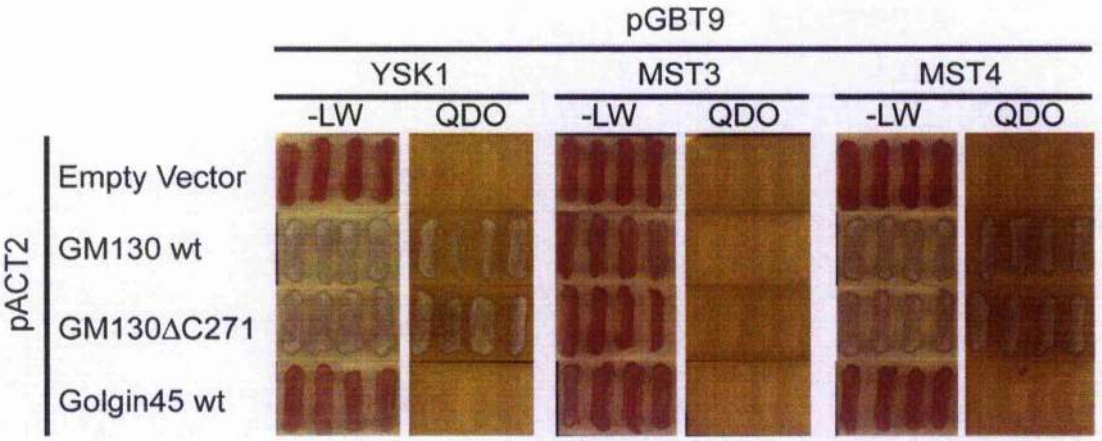


Figure 6.6 GM130 interacts with YSK1 and MST4, but not MST3

The related Ste20 kinases YSK1, MST3, and MST4 were cloned into the yeast two hybrid vector bait vector pGBT9 and screened against either full length GM130, GM130ΔC271, or golgin-45 cloned into the prey vector pACT2 or against empty vector. Interactions were assessed by growth on quadruple dropout media.

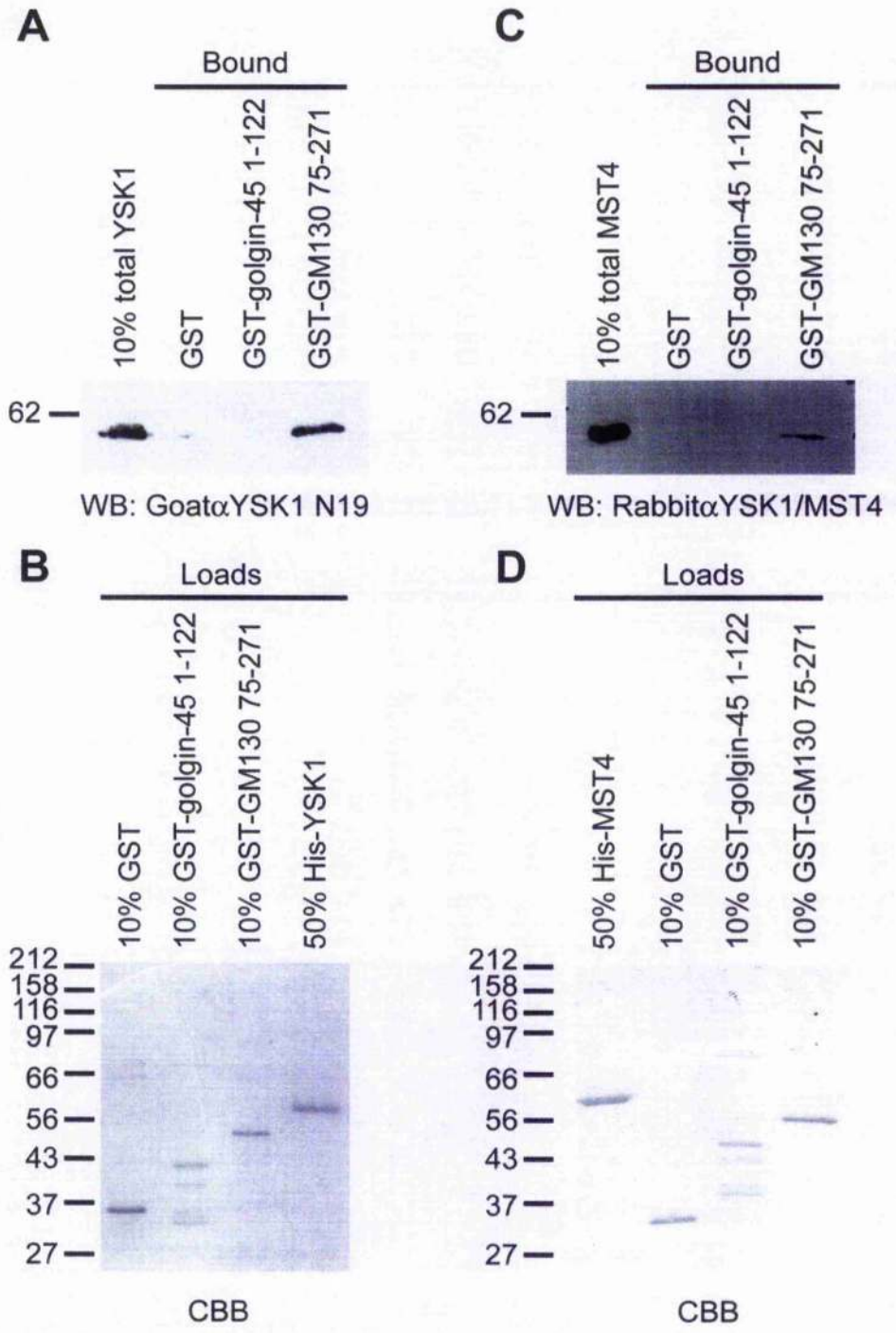


Figure 6.7 YSK1 and MST4 bind directly to GM130

(A and B) 1 μg recombinant YSK1 was incubated with immobilised GST-GM130 amino acids 75-271, GST-golgin-45 amino acids 1-122, or GST alone for 1 hour at 4°C. Bound proteins were western blotted and probed with goat anti-YSK1 N19 (A). 500 ng of each protein used in the experiment was also analysed by SDS-PAGE and Coomassie brilliant blue staining (B). (C and D) 1 μg recombinant MST4 was incubated with immobilised GST-GM130 amino acids 75-271, GST-golgin-45 amino acids 1-122, or GST alone for 1 hour at 4°C. Bound proteins were western blotted and probed with rabbit anti-YSK1/MST4 (C). 500 ng of each protein used in the experiment was also analysed by SDS-PAGE and Coomassie brilliant blue staining (D).

6.3.6 YSK1 and MST4 bind GM130 directly

Protein binding assays were performed to test whether the interactions between YSK1/MST4 and GM130 were direct (Figure 6.7). Recombinant YSK1 expressed in baculovirus-infected Sf9 cells was able to bind to immobilised GST-tagged GM130 amino acids 75-271 but not to GST alone or the N-terminus of golgin-45 (amino acids 1-122) (Figure 6.7 A and B). Similarly, Sf9-expressed MST4 only bound to GST-GM130 75-271 (Figure 6.7 C and D), suggesting that both kinases are able to bind GM130 directly.

6.3.7 Mapping of the GM130 binding site on YSK1

YSK1 and MST4, like other members of the GC-kinase family, possess a conserved N-terminal kinase domain and a C-terminal domain believed to be regulatory in function (Kyriakis 1999). In order to map the binding site for GM130 on YSK1, a number of YSK1 deletion mutants were cloned into the yeast two hybrid bait vector pGBT9 and screened against GM130 cloned into the prey vector pACT2. A YSK1 deletion mutant (YSK1 1-302) missing most of the C-terminus but containing the kinase domain was still able to interact with GM130 in the yeast two hybrid system (Figure 6.8). The minimal kinase domain encompasses amino acids 20-270 of YSK1. YSK1 20-302, containing the minimal kinase domain and an additional 32 amino acids at its C-terminus was also able to interact with GM130. Deletion of these 32 amino acids (YSK1 1-270) abolished the interaction however, suggesting that amino

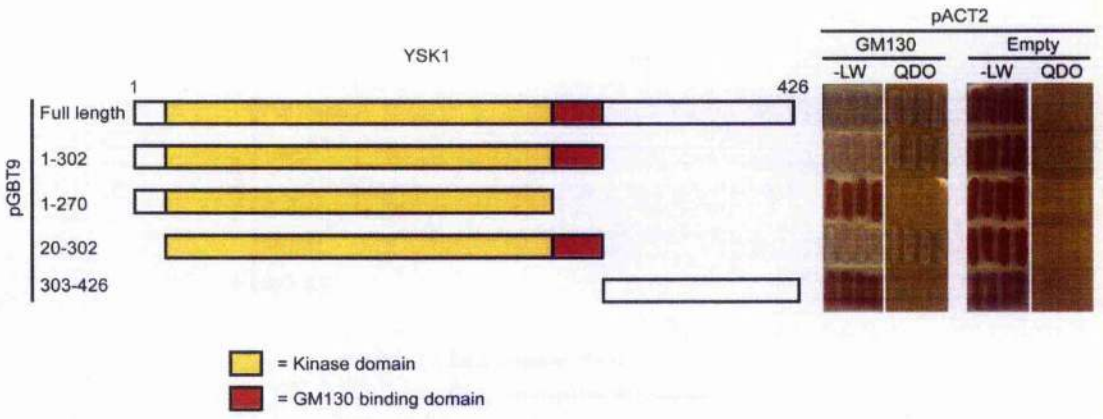


Figure 6.8 Mapping of the GM130 binding site on YSK1
Deletion mutants of YSK1 were cloned into the yeast two hybrid bait vector pGBT9 and screened against GM130 cloned into the prey vector pACT2 and against pACT2 alone. Interactions were assessed by growth on quadruple dropout media.

acids 271-302 of YSK1 are necessary for GM130 binding. A final deletion mutant, YSK1 303-426, was unable to interact with GM130 (Figure 6.8).

The short region between the kinase domain and the C-terminus is well conserved in MST4 suggesting that this region of MST4 is probably also crucial for GM130 binding. The region is also well conserved in MST3 however, suggesting that subtle differences in amino acid sequence may have a marked effect on the kinases ability to bind GM130.

6.3.8 Targeting of YSK1 to the Golgi apparatus

The same deletion mutants used to map the GM130 binding site on YSK1 were cloned into a N-terminal myc-tagging vector (pcDNA3.1mycA) and transfected into HeLa cells along with an additional deletion mutant (YSK1 275-426) and a kinase dead point mutant (YSK1K49R) (Figure 6.9). None of the deletion mutants tested were able to target to the Golgi, instead displaying a diffuse nuclear and cytoplasmic staining pattern. Only YSK1 amino acids 1-302 displayed a slight concentration in the vicinity of the Golgi but was not truly Golgi-localised as seen for myc-tagged full length kinase. YSK1 amino acids 20-302, although capable of interacting with GM130 in the yeast two hybrid system, did not show any Golgi localisation (Figure 6.9).

Interestingly, kinase dead YSK1 was also unable to target to the Golgi, suggesting that kinase activity may be required for Golgi localisation.

6.3.9 Depletion of GM130 disrupts YSK1/MST4 localisation

Treatment of cells with siRNA duplexes targeting GM130 results in the depletion of GM130, disruption of the Golgi apparatus, and an alteration in cellular morphology (section 3.3.8.4). It also results in the loss of YSK1 and MST4 staining (Figure 6.10). After 72 hours, YSK1/MST4 staining is dispersed throughout the cytosol and in a few Golgi remnant structures similar to the effect seen on p115 localisation when GM130 is depleted (Figure 3.13 A).

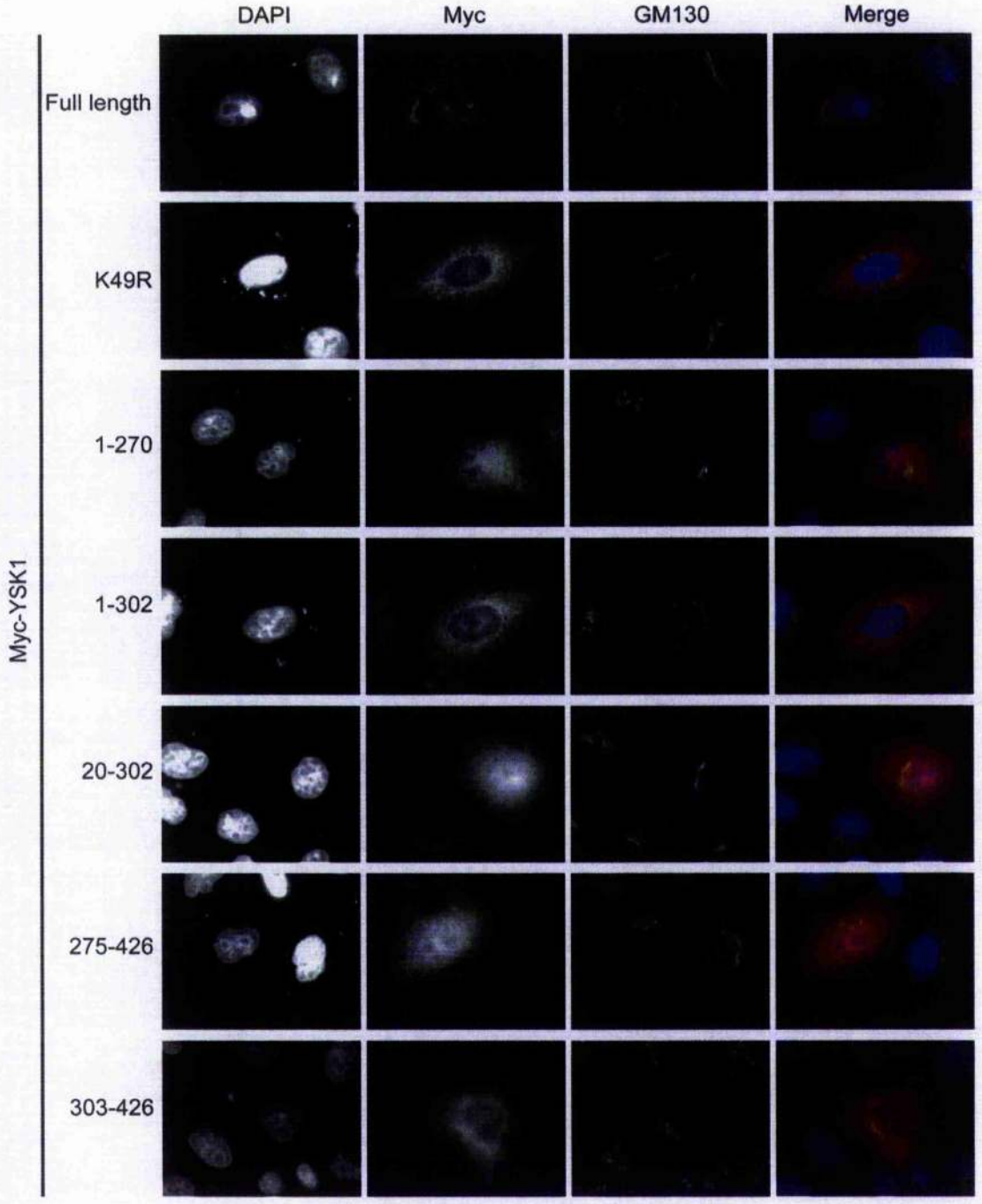


Figure 6.9 YSK1 mutants do not localise to the Golgi
Deletion mutants of YSK1 and a kinase dead point mutant (YSK1K49R) were cloned into the N-terminal myc-tagging vector pcDNA3.1mycA and transfected into HeLa cells for 18 hours. Cells were fixed in 3% PFA and co-stained with mouse anti-myc and sheep anti-GM130. In the merged images, DNA (DAPI staining) is in blue, myc-YSK1 is in red, and GM130 is in green. Areas of overlap between YSK1 and GM130 are in yellow.

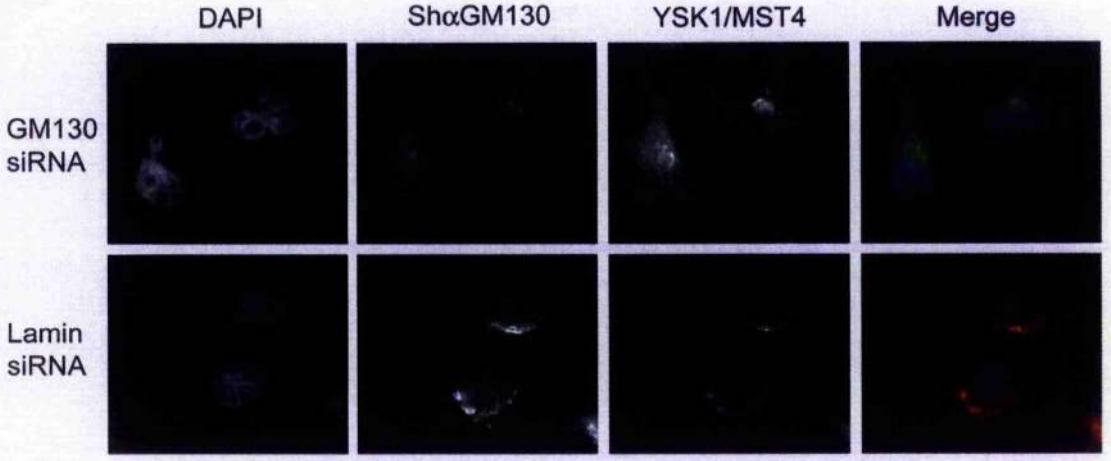


Figure 6.10 Depletion of GM130 disturbs YSK1/MST4 localisation
HeLa cells were treated with siRNA duplexes targeting either GM130 or lamin-A for 72 hours prior to fixation with 3% PFA. Cells were then stained with sheep anti-GM130 and rabbit anti-YSK1/MST4. In the merged images, DNA (DAPI staining) is in blue, GM130 is in red, and YSK1/MST4 is in green.

6.4 Discussion

6.4.1 YSK1 and MST4 are Golgi-localised Ste20 kinases

Having serendipitously identified YSK1, a member of the GC-kinase subfamily of Ste20 kinases, as localising to the Golgi (Figure 6.1) we were able to show that the closely related protein MST4 is also located at the Golgi (Figure 6.4). A third closely related protein, MST3, did not localise to the Golgi, at least in HeLa cells (Figure 6.4).

A rabbit polyclonal antibody was raised against bacterially expressed YSK1 which stained the Golgi in HeLa and MelJuso cells by immunofluorescence (Figure 6.1 A and B), and recognised a protein of the correct size in HeLa and rat liver extracts by western blotting (Figure 6.2 A). Testing of the antibody on transfected HeLa extracts revealed that the antibody recognised both YSK1 and MST4 but not MST3 (Figure 6.2 B). Thus, the antibody recognises both of the kinases, which localise to the Golgi. Since two commercially available antibodies specific for YSK1 do not stain the Golgi in untransfected HeLa cells, but are capable of staining overexpressed YSK1 (Figure 6.3), the rabbit antibody most likely recognises MST4 in HeLa cells by immunofluorescence. mRNA for YSK1, MST3, and MST4 could all be detected by RT-PCR in HeLa cells (data not shown), but, taken together, these findings suggest that MST4 is the major Golgi-localised Ste20 kinase in HeLa cells.

6.4.2 YSK1 and MST4 bind the *cis*-Golgi matrix protein GM130

A yeast two hybrid screen was performed using YSK1 as bait to screen a human testis cDNA library. Two positive clones were isolated, both of which were found to encode a novel variant of full length human GM130 containing a 27 amino acid insertion at position 57 (Figure 6.5). This insertion was not necessary for the interaction with YSK1 since rat GM130 was also able to interact. The yeast two hybrid system was also used to show that MST4, but not MST3, is able to bind GM130 (Figure 6.6). The interactions of YSK1 and MST4 with GM130 were shown to be direct in a binding assay using recombinantly expressed proteins (Figure 6.7). Thus, the two GC-kinases which localise to the Golgi are able to bind GM130 while MST3, which appears to be cytosolic, does not. This suggests that GM130-binding may be the mechanism by which YSK1 and MST4 localise to the Golgi.

The binding site for YSK1 on GM130 was mapped to amino acids 75-271 and is thus distinct from the p115 binding site which lies in the N-terminal 74 amino acids (Figure 6.5). MST4 also interacts with the first 271 amino acids of GM130 (Figure 6.6). Although the p115 and YSK1 binding sites are distinct, it will be important to determine whether p115 and YSK1 can bind GM130 simultaneously, or whether two separate pools of GM130 exist *in vivo*.

A 32 amino acid region lying C-terminal to the minimal kinase domain of YSK1 was found to be required for the interaction with GM130 (Figure 6.8). *In vivo*, kinase activity appears to be required for Golgi localisation, since two kinase dead point mutants, YSK1K49R and YSK1D158A, are distributed throughout the cytosol (Figure 6.9 and data not shown). The K49R mutant is still able to interact with GM130 in the yeast two hybrid system however (data not shown). None of the deletion mutants tested targeted properly to the Golgi (Figure 6.9), possibly because YSK1 folding is perturbed by these mutations and fails to adopt a native conformation, and thus lacks activity. The mutants do not aggregate, however, suggesting that the proteins are not completely misfolded.

Depletion of GM130 by siRNA perturbs YSK1/MST4 localisation in a very similar manner to the effect seen on the distribution of p115 (compare Figures 3.13 A and 6.10). Although GM130 depletion disrupts the overall structure of the Golgi apparatus, the similarity between p115 and YSK1/MST4 localisation upon siRNA

treatment supports the idea that YSK1 is at least partially localised to the Golgi by its interaction with GM130.

6.4.3 Function of the GM130-YSK1/MST4 interaction

To date, no substrates have been identified for either YSK1 or MST4. Having identified GM130 as a YSK1/MST4-interacting protein, we tested in *in vitro* kinase assays whether it was also a target for phosphorylation by these kinases. GM130 was not phosphorylated by YSK1 or MST4 and neither were a number of other Golgi-localised proteins, including p115, golgin-45, GRASP55, and GRASP65 (Christian Preisinger, unpublished observations). YSK1 is able to phosphorylate the model substrate myelin basic protein (MBP) *in vitro* however. We observed that this activity is greatly stimulated in the presence of the kinase-binding fragment of GM130 in a dose-dependent manner (Figure 6.11). Similar results are obtained with MST4. This indicates that GM130 is able to regulate the kinase activity of YSK1 and MST4. The increase in kinase activity upon addition of GM130 corresponds to the autophosphorylation of a threonine residue in the T-loop of the two kinases (Christian Preisinger, unpublished data). The 32 amino acids important for the interaction with GM130 are between the kinase domain and C-terminal domain (believed to be regulatory in GC-kinases). This would place GM130 binding in an excellent position to regulate kinase function.

It remains to be seen whether all Golgi-localised YSK1 and MST4 in the cell is bound to GM130 and whether all kinase bound to GM130 is constitutively active. The kinases may cycle on and off from GM130/the Golgi, thereby undergoing a rapid cycle of activation/deactivation. The activity of phosphatases on the T-loop phosphorylation could regulate such a cycle. A further question is whether active YSK1/MST4 remains at the Golgi (and functions there), or whether, once activated, the kinase translocates to a different site to fulfil its function. The development of phospho-specific antibodies, specific for the active forms of YSK1 and MST4, should help answer such questions.

Full activity of YSK1 and MST4 may therefore require extra signals in addition to GM130 binding, particularly if the physiological role of these kinases is in a specific signalling pathway rather than in a constitutive aspect of cellular function.

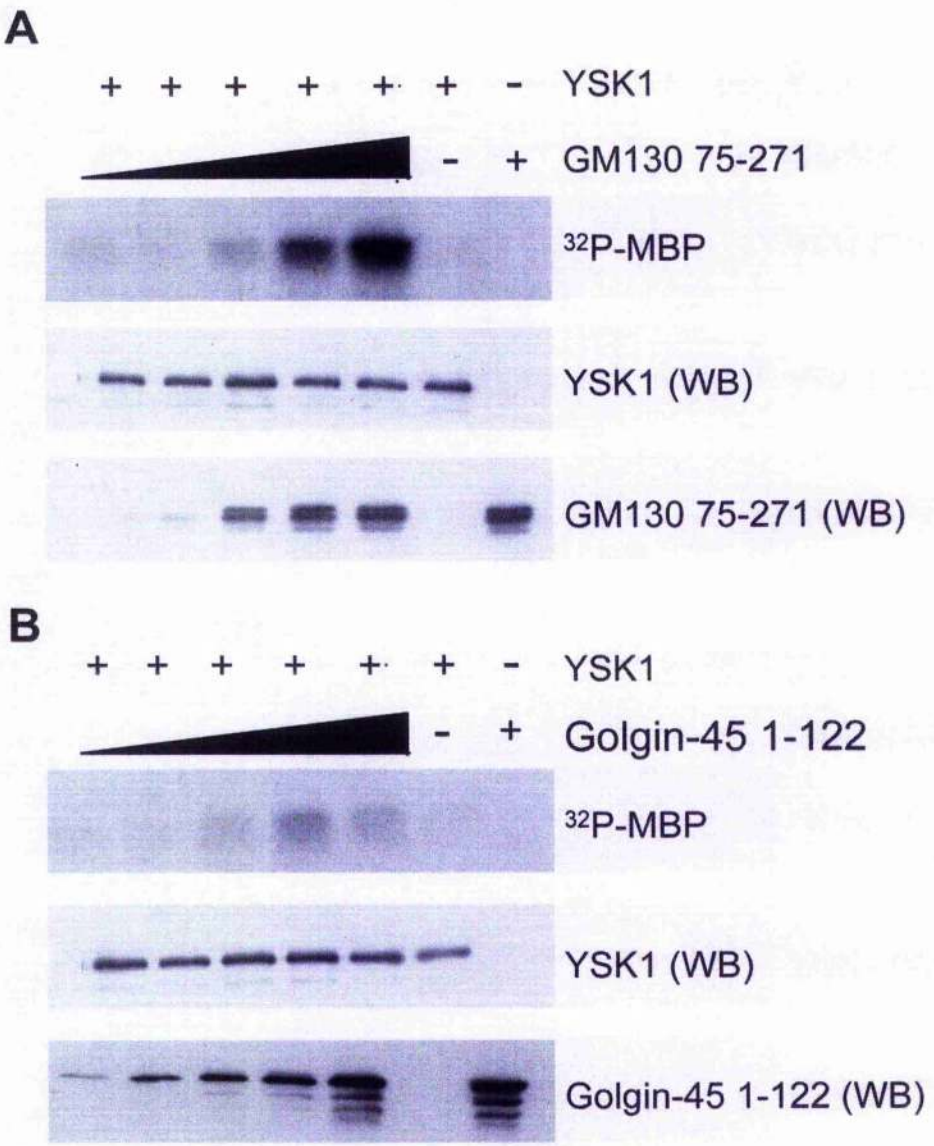


Figure 6.11 GM130 stimulates the kinase activity of YSK1
(A) Increasing amounts of GST-GM130 amino acids 75-271 were added to an *in vitro* kinase assay containing recombinant YSK1 and myelin basic protein (MBP). Phosphorylation of MBP was monitored by ^{32}P-labelling while YSK1 and GM130 levels were assessed by western blotting. Note that the amount of MBP-phosphorylation increases in proportion to the amount of GM130 75-271 added to the assay. (B) Same as in (A) but using GST-golgin-45 amino acids 1-122 in place of GST-GM130 75-271. The phosphorylation of MBP by YSK1 is not stimulated by the presence of golgin-45 1-122.
The data in this figure was kindly provided by Christian Preisinger.

6.4.4 Physiological function of YSK1 and MST4

No physiological function has so far been shown for YSK1, although it has been suggested to be stimulated by oxidative stress (Pombo *et al.* 1996). MST4 has been implicated in cell growth and transformation via the ERK pathway (Lin *et al.* 2001),

and has recently been found to be stimulated by EGF treatment of cells and to control anchorage-independent growth and proliferation (Sung *et al.* 2003).

The closely related kinase MST3 has been shown to activate p42/44 MAPK (Zhou *et al.* 2000) and has been proposed to have a pro-apoptotic function following caspase-mediated cleavage and nuclear translocation of the kinase domain (Huang *et al.* 2002). A *Drosophila* homologue of the related kinases MST1 and MST2 has recently also been shown to promote apoptosis as well as restricting cell growth and proliferation (Harvey *et al.* 2003; Wu *et al.* 2003).

A role for MST-related kinases in apoptosis is potentially intriguing since, in addition to YSK1 and MST4, GM130 also binds p115 and GRASP65, two proteins known to be cleaved by caspases resulting, in the case of p115, in a pro-apoptotic cleavage fragment (Chiu *et al.* 2002; Lane *et al.* 2002).

Alternatively, the localisation of YSK1 and MST4 to the Golgi could indicate a role in the regulation of cell polarity, as proposed for other Ste20 kinases (Holly and Blumer 1999; Sells *et al.* 1999; Dan *et al.* 2001). Changes in cell polarity must be accompanied by cytoskeletal rearrangements and the secretion of proteins and lipids to specific subdomains of the plasma membrane. Golgi-localised kinases would be in an ideal location to regulate these processes.

Other signalling events are also now known to occur at the Golgi apparatus. Ras has recently been shown to be activated at the Golgi apparatus following the Src-and PLCγ- dependent activation of the exchange factor RasGRP1 (Chiu *et al.* 2002; Bivona *et al.* 2003). Why should signalling events such as this, which often result in alterations in transcription activity, be localised to the Golgi? One possibility is that the Golgi matrix offers an ideal structure for the organisation and regulation of signalling events, either downregulating pathways by sequestration of molecules or promoting signalling by organising proteins in close proximity to one another as seen for signalling events at the plasma membrane. Another possibility is that kinases such as YSK1 and MST4 play a duel role. Firstly taking part in signal transduction pathways resulting in changes in transcriptional activity, but also then directly affecting certain aspects of Golgi structure and function. This could be important since any changes in cellular activity such as growth and proliferation rates or differentiation must be accompanied by changes in the rate and direction of membrane trafficking as well as cytoskeletal rearrangements. Co-ordination of the

nuclear and Golgi localised events required to effect these changes would therefore be desirable. Identification of the physiological roles of YSK1 and MST4, and their relationship to GM130, must await identification of *in vivo* substrates however.

6.4.5 Summary

Two related Ste20 kinases, YSK1 and MST4, were found to localise to the Golgi apparatus. A yeast two hybrid screen enabled the identification of the *cis*-Golgi matrix protein GM130 as an interacting protein of both kinases and the reciprocal binding sites on YSK1 and GM130 were mapped. Although the physiological function of YSK1 and MST4 is unclear, GM130 binding appears to regulate their kinase activity.

Chapter 7: Future Perspectives

The integrity of the secretory and endocytic pathways depends upon the efficient packaging of cargo molecules into vesicles and their specific delivery to target membranes. In this thesis, I have focussed on the roles of the rab family of small GTPases and their effector proteins in vesicular transport, in particular on rab proteins localised to the Golgi apparatus.

Through their cycle of GTP binding and hydrolysis, rab proteins cycle through active and inactive, membrane-bound and cytosolic, states, thereby regulating membrane trafficking events. They have been shown to be involved in almost all stages of vesicle transport, from vesicle budding and motility to tethering and fusion (Zerial and McBride 2001).

Rab proteins accomplish all of this through the nucleotide-dependent recruitment of a variety of effector proteins. It has become clear, both through work presented here and through the published work of numerous other groups, that many of these effectors are coiled-coil proteins important for tethering membranes together and docking vesicles to their target membrane. Those coiled-coil tethering factors that are localised to the Golgi are collectively referred to as golgins.

Other rab effector proteins have different functions however. In this thesis I have presented data showing that rab effector proteins include microtubule-motor accessory proteins (BicD and p150^{glued}), putative membrane protein-sorting proteins (GOPC), and kinase regulatory proteins (GM130). Rab effectors may also include proteins involved in phosphoinositide metabolism (MTMR6 and Centaurinβ2).

One interesting consideration is whether different rab proteins have similar complements of effector proteins. To date, rab1 effectors (e.g. p115, GM130, golgin-84) have tended to be of the coiled-coil, membrane tethering type, while known rab6 effectors (e.g. BicD, p150^{glued}, GOPC) are more diverse in function and do not include any conventional membrane docking proteins. Of course, more effector proteins of each rab probably remain to be identified and known effectors may prove to have

additional functions, such as GM130 activating the Ste20 kinases YSK1 and MST4, but it is likely that the overall complement of effector-types differs between rabs. This would represent specialisations of rab proteins for specific transport steps and reflect the role of rabs as organisers of protein complexes at membrane surfaces to accomplish particular tasks.

Nevertheless, it is likely that membrane tethering is important for all vesicle trafficking steps. It is possible, however, that many of the coiled-coil proteins labelled simply as 'tethering factors' are, in fact, much more active in their function than previously thought. Golgin-84, for example, has been proposed to have a specific role in the lateral organisation of Golgi stacks (Diao *et al.* 2003), while p115 may actively promote SNARE complex assembly for membrane fusion (Shorter *et al.* 2002). One can imagine other possible functions for coiled-coil 'tethering proteins' such as negative regulation of membrane fusion by keeping membranes apart and providing transient binding sites for vesicles in order to limit their diffusion. The stacking of Golgi cisternae, for example, would represent a case in which membranes are tethered together but do not undergo fusion.

If there are such functional differences between golgins, structural differences between the proteins may be significant. Is the length of a golgin important? Why is GM130 approximately three times the size of golgin-45 but three times smaller than giantin? Why are some golgins peripheral membrane proteins while others are integral membrane proteins? In some cases, the transmembrane domain of a golgin may be important for the proteins specific function. The transmembrane domain of Coy1p (the yeast homologue of CASP, a golgin related to golgin-84 and giantin) is clearly important for some aspect of Coy1p function, since mutation of conserved residues in the transmembrane domain abolishes a genetic interaction with the SNARE protein Gos1p (Gillingham *et al.* 2002). This suggests that integral membrane golgins may link membrane tethering events to SNARE-mediated fusion events.

Further functional differences are likely to exist between golgin-type tethering proteins and multimeric tethering complexes such as the COG and VFT complexes. In yeast, Ypt6p has been shown to interact with the VFT complex (Siniossoglou and Pelham 2001), suggesting that rab proteins also regulate this type of tethering complex.

Whatever the precise function of tethering factors such as p115 and GM130, the question remains of how this function is regulated by rab proteins. The rab activity could simply be to recruit tethering factors to a specific part of the membrane, thereby facilitating their assembly into a docking complex. Alternatively, rab proteins might induce small conformational changes in their effectors, thereby activating or inhibiting their function. For example, the rab9 effector, TIP47, undergoes a conformational change upon rab9 binding, resulting in an increased affinity for the cytoplasmic domain of the mannose-6-phosphate receptor, which it helps to recycle back to the Golgi (Carroll *et al.* 2001). Rab binding might similarly alter the affinity of a tethering factor for its binding partner. On the other hand, larger rab-induced conformational changes in a golgin might directly assist vesicle docking and fusion by 'collapsing' a tethered vesicle onto its target membrane. Breaks in the coiled-coil sequence of a golgin might act as hinges to facilitate such a process.

Another outstanding question is over the valency of rab-effector interactions. As demonstrated in chapter 3 of this thesis, GM130 is an effector of rab1, rab2, and rab33b, while p115 is possibly an effector of rab33b as well as rab1. This is similar to the situation with endosome-associated tethering proteins where the effectors rabenosyn and rabaptin-5 bind both rab4 and rab5 (Vitale *et al.* 1998; de Renzis *et al.* 2002). Does this reflect a function for GM130/p115 in multiple processes, each one of which is regulated by a different rab? This would imply the existence of sub-pools of effector bound to different rabs, possibly with distinct localisations and binding partners. Or do multiple rabs bind to a single golgin as part of the same process and act co-operatively to ensure specificity? It remains to be seen whether multiple rabs can actually bind to a single effector simultaneously but one possibility is that multiple rab-binding occurs to order sequential events so that, for example, a rab1-GM130-dependent process is followed by a rab2-GM130-directed event.

The binding of effector proteins by multiple rabs lends an extra layer of complexity to the organisation of membrane domains by rab proteins. Different members of the rab family are able to target to specific compartments within the cell. Precisely how this targeting is achieved is unclear but, once a rab protein is localised and activated, it is able to recruit its effectors either from the cytosol or from within the two-dimensional plane of the membrane. These effectors can then be organised and a series of protein-protein and protein-lipid interactions set up to establish a specific membrane sub-

domain. The participation of rab proteins at all stages of vesicle transport, from cargo selection to docking and fusion, suggests that vesicles may be looked on as rab-organised membrane sub-domains, which, during their lifetime, are able to physically separate from one membrane and join with another.

Essential to these events is the regulation of rab activity. An understanding of the function of specific nucleotide-exchange factors and GTPase activating proteins will be key to understanding how membrane trafficking events are regulated.

Thus, future work should focus on the regulation of rab activity as well as the regulation by rabs of their effector proteins and the specific functions of these effectors. A general view of the function of rab proteins is already in place; what remains is to develop a greater understanding of the specific ways in which individual transport steps are carried out.

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