An investigation of the *ypt* genes 1, 2 and 5 from *Schizosaccharomyces pombe*

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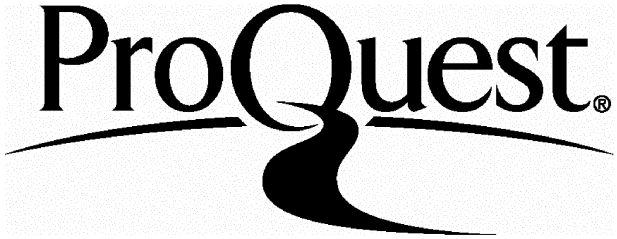

Abstract

The *ypt* genes from *Schizosaccharomyces pombe* encode low MW GTP binding proteins. These proteins are members of a large family each of which is thought to regulate a specific stage of cellular transport. The aim of this work was to analyse three ypt proteins in an attempt to elucidate their function *in vivo*. The genes examined were: *ypt5*, *ypt1* and *ypt2*. The endogenous gene was replaced with a recombinant version linked to a selectable marker.

Ypt5p is essential for growth on minimal media and the protein normally receives two geranylgeranyl groups on conserved cysteine residues at its C terminus. The *ypt5* gene was replaced with recombinant versions altered at their C termini. Strains containing the mutant ypt5 proteins which only received a single group were viable, however, a doubly mutated protein, which received no geranylgeranyl groups was not capable of sustaining cell growth. Western blotting revealed that both of the singly modified proteins showed less membrane association than the WT protein.

The *ypt1* gene was randomly mutated prior to recombination and a temperature sensitive strain defective in *ypt1* was produced. At restrictive temperatures the Golgi structure was severely disrupted, indicating that the ypt1 protein acts at an early stage in secretion. The strain does not accumulate the secretory protein acid phosphatase although a much reduced rate of secretion was observed. Sequencing revealed that the mutant allele was altered in a residue common to almost all known ypt proteins.

The *ypt2* gene was specifically mutated so as to encode a protein with the analogous mutation to that found in the ypt1 mutant protein. A strain was created which contained the mutant *ypt2* allele and it was found to be temperature sensitive for growth and to accumulate apparently fully glycosylated acid phosphatase, demonstrating that a late stage of secretion was blocked. The defect could be alleviated by expression of both WT ypt2p and mammalian rab8p, strongly suggesting that the two proteins are functionally homologous.

To those I love most

Sharon, Frances,

Tommy and Paul.

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Main abbreviations

Ab	antibody
ARF	ADP ribosylation factor
ATP	Adenosine Tri-Phosphate
BiP	Immunoglobulin heavy chain binding protein
CS	cold sensitive
CGN	*cis*Golgi Network
COP	coat protein
DNA	deoxyribonucleic acid
EM	electron microscopy
ER	endoplasmic reticulum
GDP	Guanine Di Phosphate
GTP	Guanine Tri Phosphate
GDS	GDP Dissociation Stimulator
GDI	GDP Dissociation Inhibitor
GAP	GTPase Activating Protein
Gal T	galactosyltransferase
GeGe	geranylgeranyl
IPTG	isopropyl β-D-thiogalatopyranoside
Kb	kilobases
kDa	kilo Daltons
NEM	N-ethylmaleimide
NSF	NEM sensitive factor
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
PMSF	phenylmethylsulphonyl fluoride
RNA	ribonucleic acid
SDS	sodium dodecylsulphate
S.cerevisiae	*Saccharomyces cerevisiae*
SNAP	soluble NSF attachment proteins
SNARE	SNAP receptor
S.pombe	*Schizosaccharomyces pombe*
TBE	tris borate electrophoresis buffer
TGN	*trans*Golgi Network
Tris	tris (hydroxymethyl) aminomethane
TS	temperature sensitive
VSV-G	Vesicular Stomatitis Virus surface glycoprotein
X-gal	halogenated indolyl-β-D-galactoside

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Chapter 1

Introduction

1.0 INTRODUCTION

1.1 Overview

In this introduction I shall begin by briefly considering some of the major transport pathways in both mammalian and yeast cells. I will go on to consider the process of vesicle trafficking: how vesicles form, how they fuse with membranes and how they target to the correct acceptor membrane. I will then concentrate on vesicular targeting and in particular the ypt/rab gene family. I will give a review of the function of ypt and rab proteins in their respective systems and I shall consider the modifications they receive and the proteins they interact with. Finally I shall introduce the system I have been using to investigate various aspects of ypt proteins.

1.1.1 Mammalian transport pathways

It is now known that specific proteins will pass through a series of specific organelles. The routes followed are termed transport pathways, examples of which are given in Fig. 1.1. Secretory proteins, for example, are transported from the ER through the Golgi apparatus and finally secreted from the plasma membrane (Rothman and Orci, 1992). Specific proteins are found to reside in particular organelles such as galactosyltransferase (Gal.T.) which is found in the Golgi complex (Roth and Berger, 1982). Gal.T. is transported along the secretory pathway until it reaches the correct Golgi compartment, the protein is then retained by a mechanism which has not yet been completely elucidated but is known to involve the membrane spanning domain of the protein (Nilsson et al., 1991).

Fig. 1.1 Transport Pathways

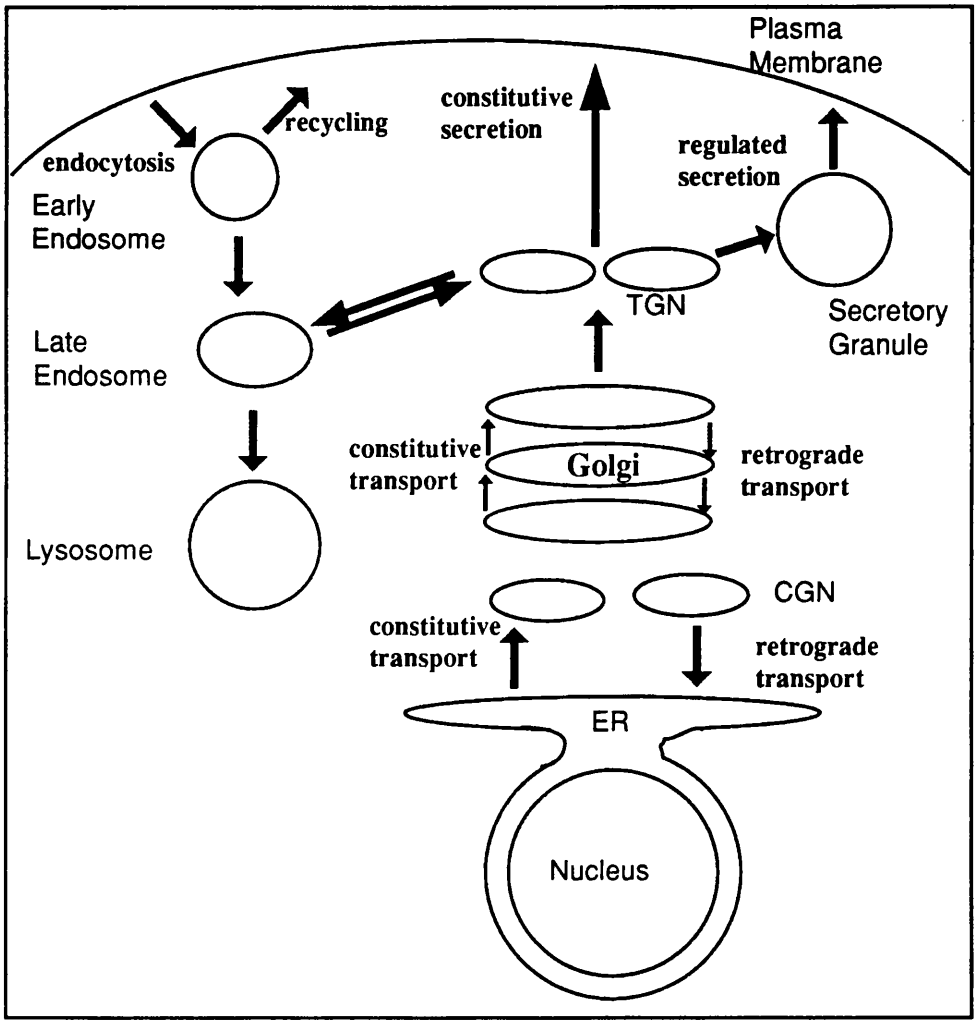


Fig. 1.1
The above gives a very simplified model of some of the major transport pathways within the mammalian cell. With each arrow representing a transport stage.

The endocytic pathway is typified by the transferrin receptor, which normally resides in coated pits on the plasma membrane (Hopkins, 1985). Transferrin binds to its receptor and is then endocytosed into clathrin coated vesicles (for review see Pearse and Crowther, 1987). The receptor and ligand complex then follow the endocytic pathway to the early endosomes. In the early endosomes the transferrin separates from its receptor and is transported, via vesicles to late endosomes and finally to the lysosome. The receptor is recycled from the early endosomes back to the plasma membrane (Klausner et al., 1983).

The endocytic and secretory pathways are also connected by transport vesicles. The hydrolytic enzymes which are found in the lysosome are transported through the secretory pathway until they reach the Trans Golgi Network (TGN), here they are sequestered by a mannose-6-phosphate receptor, sorted into clathrin coated vesicles and transported to late endosomes (Brown, et al., 1986). Another set of proteins such as TGN 38 are primarily localised to the TGN but it has been shown that they travel form the TGN to the plasma membrane and that they are then endocytosed and finally transported from late endosomes back to the TGN (Reaves et al., 1993).

1.1.2 Transport in yeast

A great deal is now known about transport in yeast and in some respects the pathways followed are very similar to those seen in mammalian cells (Fig. 1.2). Although morphologically the Golgi in *S.cerevisiae* consists of a series of separate cisternae, except in strains defective in certain yeast genes eg sec7 (Franzusoff and Schekman, 1989), it has recently been shown that the cisternae are biochemically distinct. Graham and Emr used a strain deficient in the sec18 gene to show that there are at least three separate compartments (Graham and Emr, 1991). They argued that the final compartment, that which contains the Kex2 enzyme, is involved in sorting of proteins which are to be sent to the yeast vacuole (Johnson, et al., 1987). In this way the final compartment of the yeast is behaving in an analogous manner to the TGN of mammalian cells.

Fig. 1.2 Transport pathways in yeast.

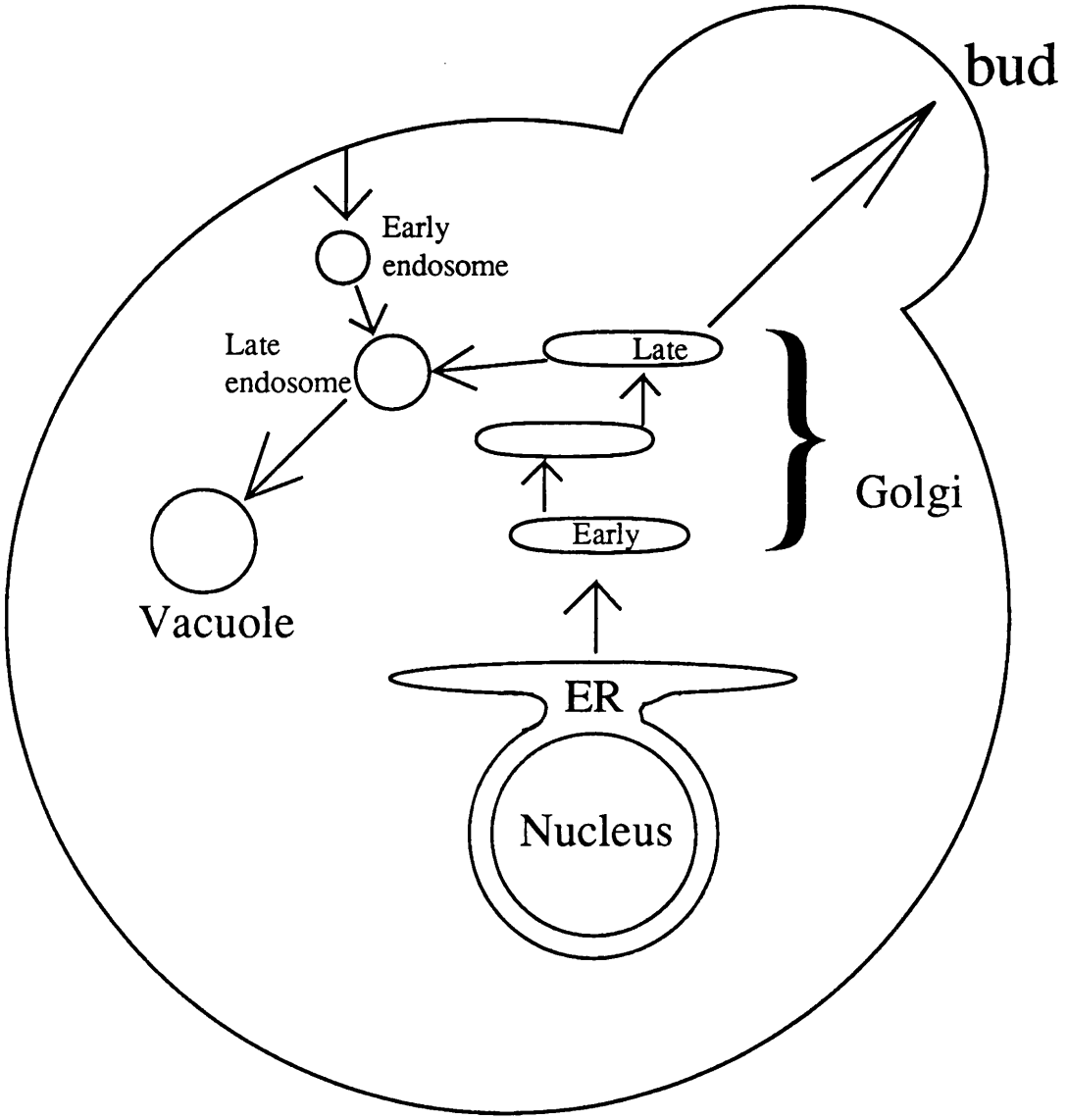


Fig. 1.2
This gives a highly stylised view of transport pathways in budding yeast with each arrow representing a separate transport stage. Secretory proteins travel from the ER through different Golgi cisternae and are secreted from the bud membrane.
Vacuolar proteins are sorted in a late Golgi compartment and are transported to late endosomes.
Proteins which are ebdocytosed travel through early and late endosomes prior to reaching the vacuole.

Not only is secretion very similar to that observed in higher cells, there is also evidence that yeast have an endocytic pathway. Singer and Riezman studied the uptake of α-factor and demonstrated that the protein passed through a separate compartment en route from the plasma membrane to the vacuole (Singer and Riezman, 1990). Recently the same group have been able to purify two separate endocytic compartments, which they call early and late endosomes (Singer-Kruger, et al., 1993). The group have been studying the degradation of α-factor in a cell line lacking the ypt7p (Wichmann, et al., 1992) and they have found that transport from the late endosomes to the vacuole is severely disrupted in this cell line (Schimmoller and Riezman, 1993). However, the α-factor is partially degraded and this led the workers to suggest that the late endosomal compartment represents the intersection of the endocytic and the vacuolar transport pathways.

1.1.3 Vesicle Trafficking

It would not be possible to have physically distinct organelles were it not for the process of vesicular transport which was first postulated by (Palade, 1975). By communicating via discrete lipid vesicles it is possible for organelles to maintain a biochemically distinct makeup. In order for vesicular traffic to occur several criterion must be met (Fig. 1.3). The target membrane must be able to form buds which in some cases would have a different composition from that of the donor organelle i.e. sorting would be occurring. The bud must then form a vesicle i.e. scission must occur. The vesicle would then have to targeted to a specific membrane and then be able to fuse to that membrane. I am now going to discuss what we know about these various events and then I shall concentrate on vesicular targeting.

Fig. 1.3 Vesicle trafficking

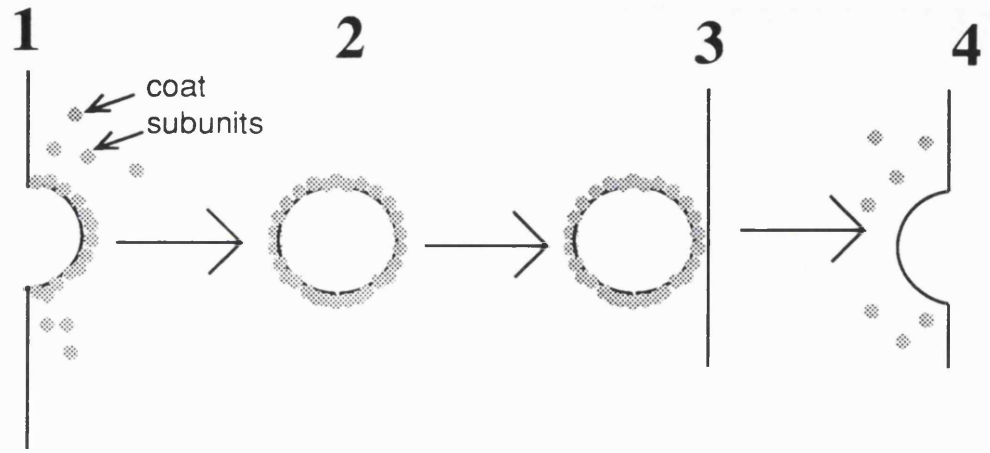


Fig. 1.3
This shows some of the criterion that must be met to facilitate vesicular transport.

1. The donor membrane must be able to become distorted to form a bud, perhaps with the aid of a coat as shown, and in some cases specifically sort specific components into the bud.

2. The bud must be able to pinch off to form a discrete transport vesicle.

3. The vesicle must be able to target specifically to the correct membrane.

4. The vesicle must next be able to fuse to the acceptor and must therefore lose its coat if it possesses one.

1.1.4 Vesicle formation and sorting

The process of vesicle budding is becoming well understood. It is now known that when vesicles are initially formed they are covered with a protein coat and it is the binding of the coat to the target membrane which distorts the membrane to form a bud (Osterman, et al., 1993). There are now known to be two kinds of coat protein, clathrin and the COPs.

Clathrin and adaptor complexes are found on endocytic and regulated secretory vesicles (Pearse and Bretscher, 1981). Constitutive secretory vesicles have a coat formed by Coatomer proteins (COPs) (Orci, et al., 1986). Another component of COP coated vesicles is ARF (ADP-ribosylation factor) which is a 20 kilo-dalton (kDa) GTP (Guanosine Tri-phosphate) binding protein which is myristylated on its N terminus (Serafini, et al., 1991). It has recently been shown that ARF is also required for the formation of the clathrin coated vesicles which bud from the TGN (Stamnes and Rothman, 1993).

A model for the formation of constitutive secretory vesicles is given in Fig. 1.4. ARF is recruited to membranes by an as yet unidentified exchange protein, which can be inhibited by Brefeldin A (Helms and Rothman, 1992). The exchange protein catalyses the exchange of GDP for GTP on the ARF protein. The ARF protein in its GTP bound state is then capable of membrane association. In the membrane the ARF associates with a receptor (Helms et al., 1993) and recruits the coatomer to the membrane (Palmer, et al., 1993). The recruitment of coatomer subunits is drives the formation of the coated bud (Osterman, et al., 1993).

Fig. 1.4 COP coated Vesicle Budding

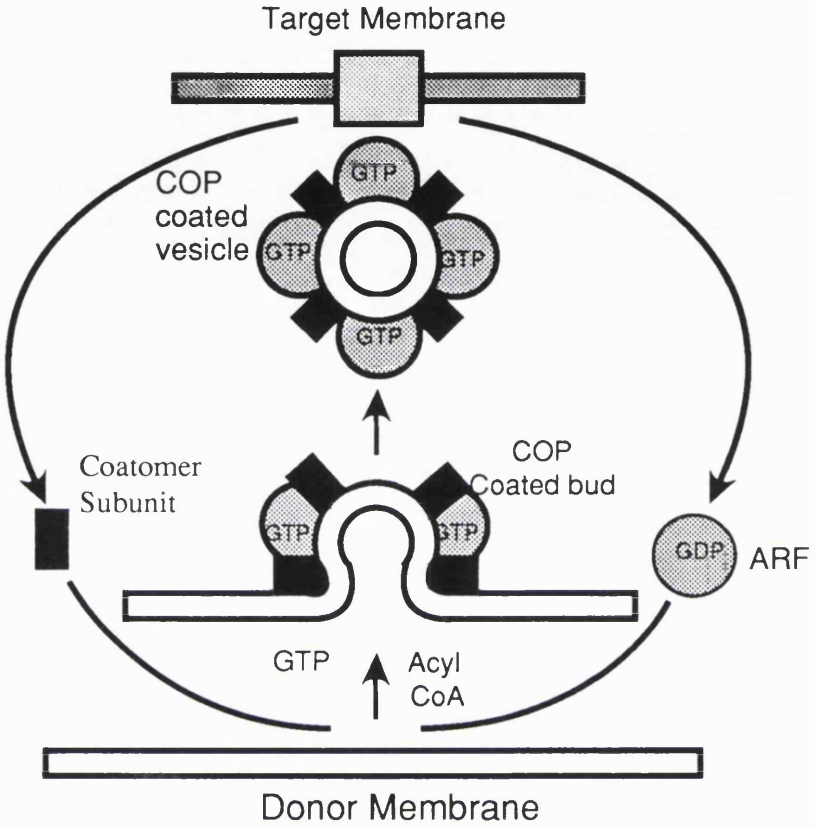


Fig. 1.4 COP coated vesicle budding
ARF is recruited to the donor membrane by a Guanosine exchange protein. The coatomer subunits are then recruited to the donor membrane after the ARF binds a membrane receptor. COP coated vesicle formation requires AcylCoA in addition to GTP. After vesicle targeting the Coatomer and ARF-GDP are released into the cytosol.

Clathrin coated vesicle formation is slightly more complex than that of COP coated vesicles (for review see Pley and Parham, 1993) as they are recruited by adaptor complexes. There are two kinds of adaptor complexes (Pearse and Robinson, 1984), AP1 which associates with TGN derived vesicles and AP2 which is associated with endocytotic vesicles (Ahle et al., 1988). Clathrin heavy and light chains then associate with adaptor complexes (for review see Pearse and Crowther, 1987). The clathrin forms a triskelion and polymerisation of the clathrin causes the formation of coated buds (Lin et al., 1991) which then pinch off to form coated vesicles in a process which requires ATP (Smythe et al., 1989).

The major difference between the two types of vesicle formed is that the COP coated vesicles are involved in constitutive transport and they are not thought to be any sorting of proteins into these vesicles i.e. they are involved in the "bulk flow" of proteins (Weiland, et al 1987). Whereas clathrin coated vesicles are involved in sorting, as adaptor complexes bind to the cytoplasmic tails of specific proteins (Bretscher et al., 1980; Pearse, 1988) thus ensuring selective transport of the correct membrane proteins and the exclusion of the other proteins that reside in the donor organelle.

As mentioned above the pinching off of clathrin coated vesicles is an energy dependant process (Smythe et al., 1989). It has also recently been shown that the formation of COP coated vesicles only requires palmitoyl-coenzyme A (Ostermann, et al., 1993). However, nothing at all is known about the actual molecular mechanism of the fission event itself.

1.1.5 Vesicle uncoating

As was discussed above when vesicles form they have a protein coat which has two functions firstly it binding to the membrane helps drive the formation of the vesicle and secondly its presence prevents the vesicle immediately fusing to its donor membrane. However, the vesicle must be capable of fusing with the correct membrane and it follows therefore that the vesicle must lose its coat prior to fusion. In the case of clathrin coated endocytic vesicles it appears that uncoating is catalysed by an ATPase (Schlossman, et al., 1984). The ATPase causes the clathrin coated vesicles to lose their coat some time after budding. In the case of COP coated vesicles it appears that the coat is lost after association with the target membrane (Melancon, et al., 1987). It was found that membranes contain an ARFGTPase activating protein (ARFGAP) and this protein stimulates ARF to hydrolyse its GTP and this causes uncoating (Ostermann, et al., 1993).

1.1.6 Vesicle Fusion

Vesicle fusion has also been studied in detail (for review see Rothman and Orci, 1992). Rothman's group has used an *in vitro* system to purify molecules involved in transport (Balch, et al., 1984). Molecules purified include the NEM-sensitive fusion protein (NSF) and soluble NSF attachment proteins (SNAPs). Interestingly both NSF and α-SNAP have yeast homologues, sec18p and sec17p respectively, which were both identified as being involved in secretion (Novick, et al., 1980). The proteins carry out analogous roles and the sec18p can functionally replace mammalian NSF in an *in vitro* assay (Wilson and Rothman, 1989). Others have shown that NSF is also involved in ER to Golgi transport (Beckers, et al., 1989) and early endosome fusion (Diaz et al., 1989) in mammalian cell. Whilst the sec18p has been shown to be involved in multiple stages of transport in yeast (Graham and Emr, 1991). It therefore appears that the underlying features of vesicular fusion are conserved throughout all eukaryotic cells and that vesicles share a common mechanism of fusion which involves NSF, SNAPs and various other proteins (Wilson, et al., 1992a).

1.1.7 Vesicle targeting

I have not yet discussed the mechanism by which vesicles target to their destination. Whilst both vesicle formation and fusion are beginning to be understood at the molecular level, it is much less clear how a transport vesicle is targeted. That is, what mechanism ensures that a vesicle which buds from the TGN fuses with the plasma membrane and not the ER. As said above membranes do contain an ARFGAP and as their a number of different ARF genes (Kahn, et al. 1991) it could be argued that they may be involved in mediating the specificity of membrane fusion. However, it is not clear whether the protein products of those ARF genes carry out distinct functions *in vivo*. There is a high degree of homology between all of the ARF protein sequences. The most divergent being ARF1 and ARF4 who's protein products have 80% identity at the amino acid level and both of which are capable of substituting for yeast ARF proteins (Kahn, et al. 1991).

Recently another family of low molecular weight GTP binding proteins have been identified in both mammalian and yeast cells. The proteins are now believed to be involved in the regulation of vesicular fusion. I shall now go on to review this family of proteins in depth and to consider their role in vesicular trafficking.

1.2 THE YPT/RAB GENE FAMILY

In yeast, where the proteins were first identified, they are termed ypt proteins which stands for Yeast Protein Transport. In mammalian cells the proteins are termed rabs as the first members were isolated from a RAt Brain cDNA library (Touchot, et al., 1987). As said the ypt proteins were initially identified in yeast and both ypt1p and sec4p were found to be involved in protein secretion within the cell (Segev, et al., 1988) (Novick, et al., 1980). As I shall discuss later further work on the yeast proteins revealed that they were associated with transport vesicles (Goud, et al., 1988) and transport vesicles were seen to accumulate in strains which contained mutant alleles of either the YPT1 or SEC4 genes (Segev, et al., 1988) (Novick, et al., 1980)

In the remainder of this introduction I shall briefly give an overview of the known ypt proteins, their general features and their specific roles *in vivo*. I shall go on to consider examples of the known mammalian rab proteins which are involved in the various forms of intracellular transport, for example constitutive and regulated exocytosis as well as endocytosis. Later I shall review the accessory proteins which interact with the ypt/rab proteins before presenting a unifying model depicting the role of the ypt/rab protein *in vivo*.

1.2.1 Nomenclature

The nomenclature used in this study to represent the various genes and proteins is as follows: In *Saccharomyces cerevisiae* (*S.cerevisiae*) genes are represented by capitals eg. YPT1, unless it is a recessive mutant in which case lower case is used eg. ypt1-1. In *Schizosaccharomyces pombe (S.pombe),* all genes, whether dominant or recessive, are presented in lower case italics eg. *ypt1*. Mammalian genes are all in lower case eg. rab5. Finally all proteins, from all species, are given in lower case and designated as proteins with a "p" suffix eg. ypt1p and rab6p.

1.2.2 Homologues

The ypt/rab proteins are often conserved between species and I shall discuss functional homology at greater length elsewhere. Homologues from different species are not always assigned the same number and in yeast not all ypt proteins are called ypt at all i.e. whilst the ypt1p from *S.cerevisiae* is the homologue of mammalian rab1p, the sec4p is homologous to ypt2p from *S.pombe* and rab8p from mammalian cells. The known *ypt* genes are listed in Table 1.1 along with their mammalian sequence homologues.

TABLE 1.1 SEQUENCE HOMOLOGUES

S.pombe	Mammalian	S.cerevisiae
YPT1	RAB1	YPT1
YPT2	RAB8	SEC4
YPT3	RAB11	--------
YPT4	-------	--------
YPT5	RAB5	--------
RYH1	RAB6	YPT6
-------	RAB7	YPT7

Table 1.1
This is a list of the known ypt genes from both *S.cerevisiae* and *S.pombe*. Also given are the known mammalian sequence homologues of the *S.pombe* and *S.cerevisiae* genes.

1.2.3 General Features

In this section I shall give a brief overview of some of the general features and properties of the known ypt/rab proteins. A generalised ypt/rab protein is shown in Fig. 1.5. All of the proteins have MW's ranging between 21 and 25kDa. As I shall discuss later the proteins are localised to specific endomembranes and believed to be involved in specific stages of transport. The proteins are all predicted to be hydrophilic and to have no membrane spanning domains, but *in vivo* they act as peripheral membrane proteins and membrane association is essential for the protein to function (Walworth, et al., 1989). Gallwitz's group discovered that ypt1p mutants which were unable to associate with membranes were also incapable of supporting growth *in vivo* (Molenaar, et al., 1988). Attachment to membranes is now known to be mediated by isoprenoid groups which are post-translationally added to conserved cysteine residues (Fig. 1.5) at the extreme C terminus of the rab protein (Khosravi, et al., 1991; Kinsella and Maltese, 1991).

(i) GTP binding

The ypt/rab proteins bind GTP and as such they contain structural features typical of ras-like GTP binding proteins. The regions involved in binding GTP have been most thoroughly characterised with respect to the ras oncogene (Pai, et al., 1989). The four domains, shown in Fig. 1.5, are (i) the GXXXXGKS/T which is the phosphate binding loop L1; (ii) the DTAG which interacts with the γ phosphate of GTP; (iii) NKXD which has been termed the guanine specificity region and (iv) the SAK/L which interacts with the D residue of (iii) (where X represents any amino acid). Although the ypt/rab proteins are technically GTPases they all have low rates of GTP hydrolysis which can be stimulated by GTPase Activating Proteins (GAPs) (Strom, et al., 1993; Walworth, et al., 1992). The GAP is believed to interact with effector domain on the ypt/rab protein (Fig. 1.5) (Becker, et al., 1991)

Fig. 1.5 ypt general structure

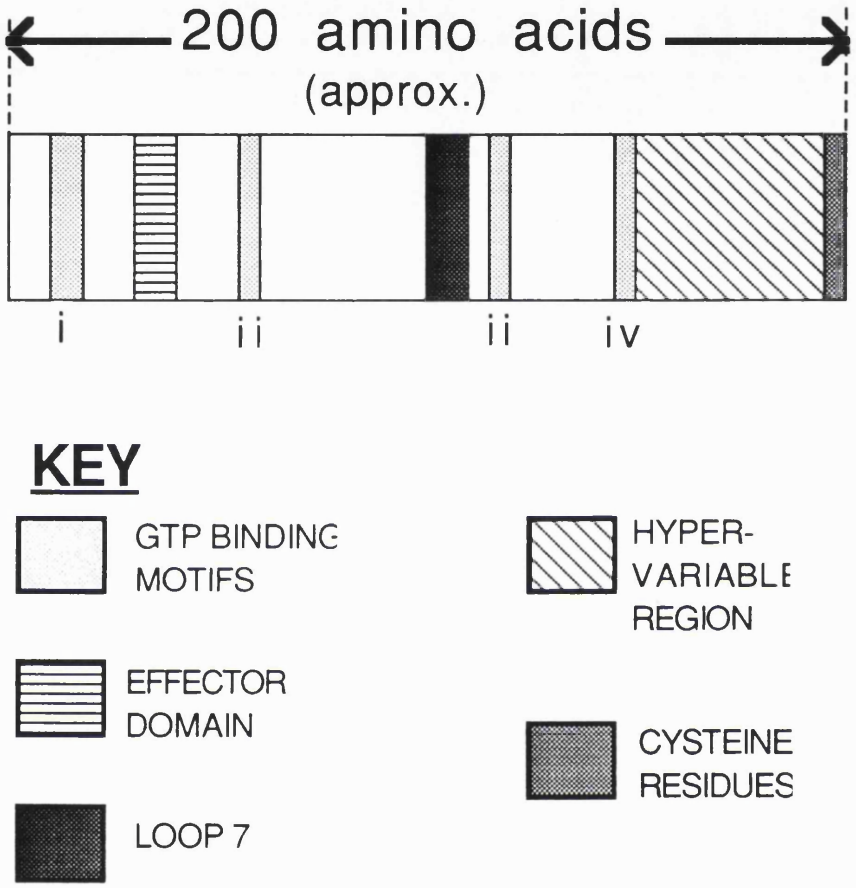


Figure 1.5
The domains present on a typical ypt/rab protein are shown. The four GTP binding domains are marked i- iv see main text for details.

(ii) Homology

The ypt/rab proteins are a family of proteins within the group of ras-related proteins and, as such, they all have approximately 30% amino acid identity with the mammalian ras protein, mainly in the GTP binding regions discussed in the previous section (Chavrier, et al., 1990). The proteins tend to be very highly conserved between species which indicates, but does not prove, that the role they play is the same in highly divergent species. Within the same species the proteins generally have around 50% identity to one another, although this does vary considerably (Chavrier, et al., 1990; Zahraoui, et al., 1989). Homologues between different species have much higher sequence identities, for example canine rab1 and ypt1p are 78% identical at the amino acid level. Homologues are not assigned solely on the basis of overall identity but on the conservation of the effector domain. The effector domain of ras interacts with a GTPase Activating Protein (GAP) and work done by Gallwitz's group indicates that ypt1p also interacts with a GAP via its effector domain (Becker, et al., 1991). It has been found that functional homologues, i.e. those which can be used to replace one another, have virtually identical effector domains (Haubruck, et al., 1990; Miyake and Yamamoto, 1990). The role of the effector domain and the characterisation of ypt GAPs will be discussed in more detail later .

(iii) Hypervariable domain and Localisation

Sequence comparisons both between homologues, and between proteins from the same species, show that the degree of identity varies along the length of the proteins. The most divergent region occurs downstream of the final GTP binding motif and is termed the hypervariable C terminal domain (Fig. 1.5). Chavrier and co-workers (Chavrier, et al., 1991) argued that the C terminal region was a good candidate for controlling the localisation of the rab protein *in vivo* as it was so highly divergent, also due to its proposed proximity to membranes and as it was known that the C terminal were required for membrane association (Molenaar, et al., 1988 ; Walworth, et al., 1989)

To test their hypothesis the group constructed a series of chimeras which they overexpressed in Baby Hamster Kidney (BHK) cells (Chavrier, et al., 1991). Both wild type (WT) rab5p and rab7p targeted to early and late endosomes respectively, when overexpressed, indicating that saturation of the localisation machinery did not occur. Addition of the C terminal 34 amino acids from rab7p to

the N terminal region of the rab5p resulted in a protein which localised to late endosomes. If the C terminal region of the rab7p was added to the N terminal region of the rab2p, a protein which normally localises to the intermediate compartment (Chavrier, et al., 1990b), the chimera again targeted to late endosomes.

The authors concluded that the hypervariable C terminal domain was indeed responsible for the localisation of rab proteins *in vivo*. However, the authors do not comment on the fact that the C terminal region is also the most divergent between species homologues eg. rab1p and *S.cerevisiae* ypt1p (Haubruck, et al., 1987). The divergence in primary sequence between homologues in different species would indicate that the targeting information is presumably determined by the secondary structure of the C terminus rather than its primary sequence.

(iv) Specificity

Although the C terminal domain is apparently involved in the localisation of ypt/rab proteins recent work by two groups (Brennwald and Novick, 1993; Dunn, et al., 1993) indicates that it does not control the functional specificity of the protein. Both Novick's and Botstein's groups studied ypt1p and sec4p from *S.cerevisiae* by constructing chimeras between the two proteins. Both groups reported very similar results.

Brennwald and Novick discovered that swapping the hypervariable C terminal domains (Fig. 1.5) of the two proteins resulted in chimeras which functioned according to the N terminal domain they possessed (Brennwald and Novick, 1993). That is the ypt1p, with the C terminal hypervariable region of sec4p (ypt1-Csec4p) would complement a temperature sensitive (TS) defect in a ypt1p deficient strain but it would not rescue a sec4p deficient strain. However, immunofluorescence studies indicate that the C terminal hypervariable domains were controlling the localisation of the proteins e.g. the ypt1-Csec4p was localised to the plasma membrane not the Golgi complex. These results confirm that the C terminal region controls localisation, as had been concluded by Chavrier and colleagues (Chavrier, et al., 1990b), but indicate that the specificity of the ypt proteins is not determined by the C terminal hypervariable domain. The authors explain the apparent anomaly by arguing that a small proportion of the ypt1-Csec4p associates with the correct membrane (i.e. the Golgi) allowing it to function as a wild type ypt1 protein.

Brennwald and Novick also exchanged the effector domains of the yptlp and sec4p. The effector domain (Fig. 1.5) is highly conserved between homologues in different species and is thought to interact with a GAP (Becker, et al., 1991). It was found that a yptlp containing the sec4p effector domain (ypt1-EFsec4p) would again rescue a strain deficient in the yptlp but not sec4p. It must be noted, however, that the reciprocal protein, sec4p containing the effector domain of yptlp, would not function as either yptlp nor sec4p. In fact it was never possible to convert yptlp to a functional sec4p which may indicate that it is easier to replace yptlp or alternatively there may be a more trivial reason, such as misfolding of the yptlp chimeras.

Botstein and co-workers (Dunn, et al., 1993) discovered that as few as nine amino acids from yptlp, corresponding to Loop 7 from ras (Fig 1.5), when engineered into sec4p allowed it to partially rescue a TS strain which contained a defective yptlp, although the protein was also capable of rescuing a sec4 TS strain. Presumably the Loop 7 region interacts with an accessory protein. In fact both groups engineered chimeras that were capable of simultaneously rescuing strains defective in both the yptlp and the sec4p. These results strongly indicated that the ypt proteins themselves were not solely responsible for the recognition of the target organelle but rather that they played a more regulatory role. Much more work remains to be done in identifying exactly which regions of the ypt proteins interact with other proteins *in vivo*.

1.2.4 YPT genes

(i) YPT1

The first YPT genes to be identified were in the budding yeast *S.cerevisiae*. The YPT1 gene was cloned and its protein product was shown to be essential for growth by Gallwitz and colleagues (Schmitt, et al., 1986). Originally it was thought that yptlp was involved in calcium regulation or in the organisation of microtubules within the cell (Schmitt, et al., 1986) (Schmitt, et al., 1988). Further work using conditional mutants of the yptlp revealed an accumulation of transport vesicles under non-permissive conditions (Segev, et al., 1988). The authors concluded that the protein was involved in vesicular transport. It is still not clear at exactly which stage yptlp acts. It is either between the ER and the cisGolgi, or in an early stage of intra-Golgi transport (Bacon, et al., 1989; Schmitt, et al., 1988; Segev, et al., 1988).

(ii) SEC4

Sec4p was one of a large number of proteins, involved in secretion, to be identified by Schekman and co-workers (Novick, et al., 1980). Work done later by Novick's group demonstrated that the sec4p has an essential role in transport, in this case between the Golgi complex and the bud membrane (Goud, et al., 1988; Salminen and Novick, 1987). The use of mutant sec4 proteins allowed Walworth and co-workers to demonstrate that hydrolysis of GTP by sec4p is linked to fusion of transport vesicles with the bud membrane (Walworth, et al., 1992).

Both the ypt1 and sec4 proteins have been characterised in great detail and much of our current understanding of the function of ypt/rab proteins is based on them. As I shall discuss later both Novick's and Gallwitz's groups have utilised the power of yeast genetics to identify accessory proteins that interact with the ypt proteins *in vivo*

1.2.5 YPT cycle

As mentioned above all ypt and rab proteins are believed to undergo a cycle of GTP binding and hydrolysis. This information led Bourne to propose a model to explain the ypt protein's function (Bourne, 1988). Bourne argued that GTP hydrolysis was being coupled to unidirectional transport within the cell in a manner analogous to the action of GTP binding proteins involved in protein synthesis. The ypt/rab protein would associate with a vesicle budding from a donor membrane. The vesicle would then dock to the correct acceptor membrane with proteins in the vesicle recognising specific proteins in the acceptor. The ypt protein would hydrolyse its bound GTP and the energy would be used to lock the vesicle in a fusion competent state. If there was no GTP hydrolysis there could be no fusion, which explained why mutants incapable of hydrolysing GTP acted as dominant inhibitors of transport (Schmitt, et al., 1986; Walworth, et al., 1989 ; Tisdale, et al., 1992; Bucci, et al., 1992). He also predicted that each stage of membrane transport would be regulated by a distinct ypt protein.

1.3 MAMMALIAN RAB GENES

The study of YPT genes in *S.cerevisiae* yielded large amounts of vital information on the function of the proteins. Several obvious questions remained unanswered: was transport in higher eukaryotes also regulated by ypt proteins? And would the mammalian homologues function in exactly the same manner as in yeast cells? The study of mammalian rab proteins has given the answer to these questions and gone on to reveal much more.

The rab genes were initially isolated from a RAt Brain cDNA library (Touchot, et al., 1987). Rabs have also been isolated from mouse (Haubruck, et al., 1987) and canine libraries (Chavrier, et al., 1990) and show very high homology, at the amino acid level, to those identified in the initial screen.

As I stated earlier ypt/rab proteins have been localised to particular endomembranes and usually to specific stages of vesicular transport (Fig. 1.6). Rather than go through all of the known rab proteins, which at this time number at least thirty, I shall discuss a selection of the rab proteins which are involved in the various forms of intracellular trafficking. This will allow me to demonstrate the depth of our current knowledge of rab function. In general the results obtained from these studies are thought to be applicable to all of the known rab proteins.

Fig. 1.6 Rab Localisation

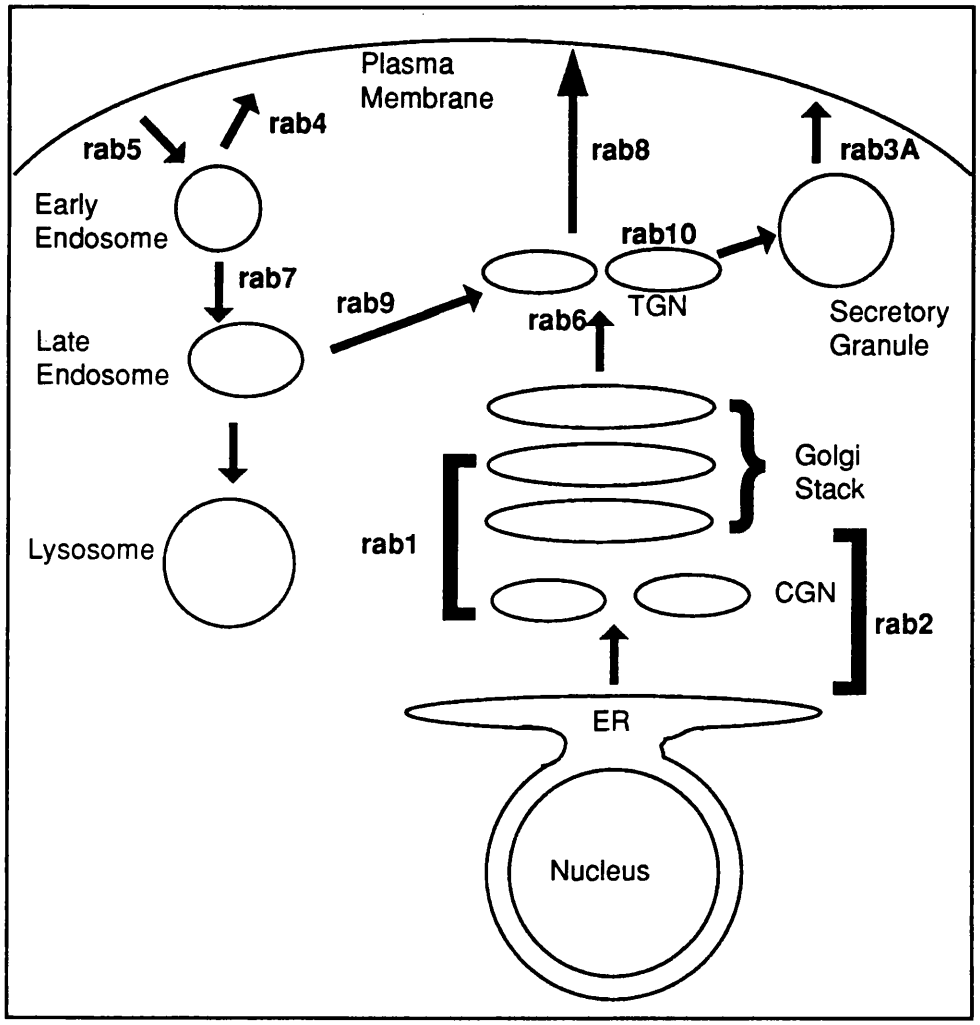


Fig. 1.6
The localisation of the best characterised rab proteins is shown above, with each tansport stage being represented with an arrow. In general the rab proteins have been localised to particular stages of membrane traffic. Except for rab6 which has been localised to the Golgi complex and rab10 which is thought to reside in the TGN.

1.3.1 Rab1

Rab1p is the homologue of the ypt1p. The rab1 gene was independently cloned by two groups. Gallwitz and colleagues screened a mouse cDNA library using *S.cerevisiae* YPT1 gene sequence as probes (Haubruck, et al., 1987). The predicted protein showed 71% identity to they ypt1p and was found to be expressed in all tissues examined. The group then demonstrated that rab1p was the functional homologue of the ypt1 by replacing the endogenous YPT1 gene with the murine rab1 gene (Haubruck, et al., 1989).

Touchot and co-workers also cloned rab 1 along with a number of other rab genes (Touchot, et al., 1987). Later the group identified a second ypt1p homologue which they termed rab1Bp (Vielh, et al., 1989). The rab1Bp is predicted to be 92% identical to the rab1Ap and the amino acid changes between them are all conservative. Touchot and co-workers compared the biochemical properties of the two proteins (Touchot, et al., 1989) and found them to be very similar, although rab1Ap does have a higher intrinsic rate of GTP hydrolysis and, as shown by northern blot analyses, the rab1A mRNA is always expressed at higher levels in the various tissue and cell lines studied. At this time it appears that the two proteins are functionally identical.

The rab1p action has been studied in great detail by Balch and colleagues. The group used semi-intact 15B Chinese Hamster Ovary (CHO) cells to investigate the role of rab1p in transport (Plutner, et al., 1991). Cells were infected with Vesicular Stomatitis Virus (VSV) and the glycosylation of the viral surface glycoprotein (VSV-G) was monitored. In the Golgi complex N-linked carbohydrate side chains are trimmed from a 9 mannose ER form to a 5 mannose form by mannosidase I (Mann I). Mann I was thought to be a *cis*Golgi marker, but recent work by Farquhar and colleagues has cast some doubt upon the exact localisation of Mann I (Velasco, et al., 1993) therefore Mann I can at this time only be used as a marker for the Golgi stack. Balch and co-workers discovered that the addition of a mAB against rab1Bp would completely block the trimming of VSV-G oligosaccharide indicating that the rab1Bp plays an important role either in ER to Golgi transport or in an early stage of intraGolgi transport (Fig. 1.6).

The group went on to study the rab protein's function *in vivo* by expressing both wild type and a dominant mutant form of the rab1Bp in HeLa cells (Tisdale, et al., 1992). The mutant rab1B$_{121I}$p is analogous to an oncogenic ras mutant (Barbacid, 1987). The ras mutant fails to bind both GTP and GDP, and acts as an activated form of the protein. Overexpression of wild type rab1Bp had no effect on the processing of VSV-G whereas tenfold overexpression of rab1B$_{121I}$p completely blocked processing of VSV-G.

The group then examined the localisation of VSV-G by immunofluorescence using a mutant form of the protein which accumulates in the ER at 39.5°C. By incubating the cells at 39.5°C and then shifting them to 32°C a wave of VSV-G could be sent through the secretory pathway. In the presence of overexpressed rab1B$_{121I}$p the VSV-G moved from the ER to punctate structures reminiscent of the CGN, but the protein did not enter the Golgi stack itself (Tisdale, et al., 1992). This would indicate that the rab1Bp is not involved in transport from the ER to the CGN.

The question still remained as to whether the rab1p acts at one or more stages of transport. This was resolved quite elegantly by Davidson and Balch (Davidson and Balch, 1993) who demonstrated that VSV-G goes through multiple stages of transport which could be defined by different oligosaccharide modifications. The protein first reaches a partial Endoglycosidase D sensitive stage termed G_{d1}, next a fully sensitive form G_{d2} and finally a neuraminidase sensitive form G_{ht}. All of the transport stages were inhibited by the addition of GTPγS to an *in vitro* assay, whereas only the earlier two stages could be inhibited by the dominant mutant rab1A$_{124I}$p. Therefore the rab1p certainly acts at more than one stage of vesicular transport.

Apparently one rab protein is regulating more than one stage of membrane transport. This contradicts Bourne's theory that each stage in membrane traffic will be regulated by a distinct rab protein. I believe that there is a possible explanation of the result. I think that the two rab1p variants could act at two sequential stages *in vivo*. It could well be that the two stages of transport are only distinct in higher eukaryotes and that rab1p has evolved to fulfil both stages. This would explain the lack of two ypt1p variants in *S.cerevisiae*. It would be interesting to engineer a cell line lacking one of the two rab1p variants and to examine carbohydrate processing to ascertain whether both rab1 proteins are essential. This hypothesis explains the need for both proteins and the apparent contradiction that arises from a protein supposedly involved in regulating the

fidelity of transport being involved in two separate transport stages.

1.3.2 Rab2

A second rab protein has also been implicated in the very early stages of exocytotic transport. The rab2 gene was identified by Touchot and co-workers (Touchot, et al., 1987). Antibodies raised against the protein product of the gene stained a perinuclear region in BHK cells and partially overlapped with the Golgi resident enzyme Mann II (Chavrier, et al., 1990b). However, nocodazole treatment, which disrupts the Golgi apparatus, caused segregation of the two markers and in HeLa cells electron microscopy revealed that rab2 co-localised with the CGN marker p53 (Chavrier, et al., 1990b). The localisation of rab2 to the CGN led researchers to argue that it must regulate a very early stage of the secretory pathway.

The functional involvement of rab2p in early transport was demonstrated by Tisdale and colleagues (Tisdale, et al., 1992). By expressing a dominant mutant of the rab2p- $rab2_{119I}$p- the researchers could completely block the acquisition of endoglycosidase H (Endo. H.) resistance by VSV-G in HeLa cells which had been transfected with the mutant rab protein. That the rab2p operates at an early stage of transport is clear (Fig. 1.6). What is not clear, however, is exactly which step the rab2p is involved in.

1.3.3 Rab3A

Both the rab1 and rab2 proteins are involved in constitutive transport, and have been identified in many different tissue types (Touchot, et al., 1989). There are, however, a growing number of tissue specific rab proteins, such as the recently identified rab17p (Lutcke, et al., 1993) which is specifically expressed in epithelial cells. The best characterised example of tissue specific distribution is that of the rab3Ap which is involved in regulated secretion.

Takai and co-workers purified small GTP binding proteins from bovine brain (Yamamoto, et al., 1988). They then went on to identify the corresponding cDNA clones (Matsui, et al., 1988). The group identified three closely related genes which they termed smg-25A, B and C (small molecular weight guanine binding proteins). Previously smg-25A had been partially cloned by Touchot and co-workers (Touchot, et al., 1987) they had termed the gene rab3A. To avoid confusion I shall henceforth refer to the gene as rab3A.

Takai and co-workers used antibodies raised against rab3Ap to examine its

distribution (Mizoguchi, et al., 1989). The protein was only found in the cerebellum, pancreas and adrenal medulla and not in other tissues such as spleen, liver and thymus. Closer investigation revealed that the protein localised to the synaptic regions of the cerebellum. The group had previously shown that expression of rab3A mRNA was induced when PC12 cells differentiated into sympathetic neurone-like cells (Sano, et al., 1989). These results led to the conclusion that rab3Ap is involved in regulated secretion. This idea was given greater credence when a separate group demonstrated that rab3Ap was associated with chromaffin granules in bovine adrenal cells (Darchen, et al., 1990).

Takai's group have gone on to study the function of rab3Ap in great detail. In particular they have identified a GDP Dissociation Inhibitor (GDI). This accessory protein has an important role in recycling rab3A from the plasma membrane to secretory vesicles. The identification and characterisation of GDI will be discussed later.

1.3.4 Rab5

The rab proteins discussed in the preceding sections are involved in either constitutive or regulated exocytosis in this section I am going to discuss the rab5 protein which has been shown to be involved in endocytosis. Several rab proteins have in fact been localised to the endocytic pathway, for example the rab4p was shown to be associated with early endosomes (Van der Sluijs, et al., 1991) and it is thought to be involved in the recycling from early endosomes to the plasma membrane (Van der Sluijs, et al., 1992B) (Fig. 1.6). Whilst the rab7 protein has been localised to late endosomes (Chavrier, et al., 1990b).

The rab5 gene was originally cloned by Chavrier and colleagues (Chavrier, et al., 1990). The group raised antibodies to the C terminal region of the protein and carried out localisation studies on both BHK and Madin-Derby Canine Kidney (MDCK) cells (Chavrier, et al., 1990b). The group found that the protein targeted to both the plasma membrane and to early endosomes but not with late endosomes.

The group went on to demonstrate that the rab5 protein was involved in transport between the plasma membrane and early endosomes. The workers constructed a point mutant of rab5 analogous to the oncogenic ras mutant discussed in section 1.3.1. The mutant rab5$_{I133}$p and the WT protein were both transfected into BHK cells and the cytosols from the cell lines were used in an *in vitro* endosome-endosome fusion assay (Gorvel, et al., 1991). It was found that the cytosol from

the cells containing the mutant rab5$_{I133}$p would inhibit fusion whereas cytosol from cells transfected with WT rab5 stimulated fusion when compared to cytosol from untransfected cells or from cells which had been transfected with WT rab2 as a control.

The group next went on to study the effects of overexpression of both WT rab5p and rab5$_{I133}$p *in vivo* (Bucci, et al., 1992). BHK cells were infected with recombinant T7 RNA polymerase vaccinia virus expressing either WT rab5p or rab5$_{I133}$p at approximately 15 fold above endogenous levels. The group found that expression of the mutant rab5$_{I133}$p decreased both Fluid Phase and Receptor-Mediated endocytosis whilst WT rab5p stimulated both processes. Microscopic analysis revealed that the early endosomes were enlarged in cells expressing the WT rab5p whilst in cells expressing the mutant rab5$_{I133}$p the early endosomes appeared to be fragmented (Bucci, et al., 1992). Whilst Western blotting revealed that the rab5 protein was present in purified Clathrin coated vesicles.

The group concluded that the rab5p is involved in the regulation of transport from the plasma membrane to early endosomes and as overexpression of WT rab5p increased the rate of endocytosis, that the protein must be involved in a rate limiting step. As overexpression resulted in an increase in the size of early endosomes the group concluded that the protein would be involved in a fusion event rather than at a budding stage.

The results discussed above along with many other studies have indicated that the rab protein products are involved in many stages of transport. Presumably they are carrying out analogous roles in the different stages of transport. In the following sections I am going to discuss the modifications that the proteins receive and review the proteins with which they interact. Finally I am going to present a model of ypt/rab protein function.

1.4 POST-TRANSLATIONAL MODIFICATIONS

The ypt/rab proteins are now known to undergo a variety of post-translational modifications. These modifications include isoprenylation and phosphorylation. In general these modifications alter the proteins membrane avidity. For example isoprenylation increases the hydrophobicity of the ypt/rab proteins. It is apparent that not all of the ypt/rab proteins are modified equally, for example whilst all of the proteins studied do receive geranylgeranyl groups some may receive two such groups and others only one. In the case of phosphorylation it appears that only a subset of the proteins is modified in this way and even then the effects of the

modification differ between different members of the family.

1.4.1 Geranylgeranylation.

All of the ypt/rab proteins thus far identified have two cysteine (C) residues at, or near, their extreme C terminus. The yptlp from *S.cerevisiae* is no exception ending C-C. Gallwitz and co-workers demonstrated that the C terminal cysteine residues of yptlp were necessary for the protein to function (Molenaar, et al., 1988). In a gene replacement experiment a diploid strain, which has two copies of YPT1, had one of its YPT1 genes replaced. The endogenous gene was replaced with mutants which had had either one or both of their C-terminal cysteine residues substituted with serine.

The diploid strain was now germinated to give haploid progeny. Half of the progeny would therefore contain the modified version of the yptlp. Progeny which contained the yptlp lacking both cysteine residues were not viable. A yptlp lacking both cysteine residues was found to be completely cytosolic (Molenaar, et al., 1988). The researchers concluded that a modification at the C-terminus was necessary for the protein to become membrane associated and that membrane association was essential for protein function.

Several groups demonstrated that rab proteins were being modified by the addition of a 20 carbon Geranylgeranyl (GeGe) group. Der and co-workers, and Kinsella and Maltese separately showed that various mammalian rab proteins received GeGe groups both *in vitro* and *in vivo* (Khosravi, et al., 1991; Kinsella and Maltese, 1991). The former demonstrated that both proteins ending CC (eg. the rab1Bp) and those terminating CXC (where X is any amino acid) (eg. the rab3Ap) received GeGe groups.

Kinsella and Maltese studied the incorporation of radiolabelled tritiated mevalonate into rab1Bp. They compared uptake of label into wild type and mutant forms of the protein. The rab1Bp terminates CC and mutants were used that had one of the cysteines replaced with a serine. In both cases incorporation of radiolabel was reduced to 10% of WT levels. It was unclear whether one or both of the residues was being modified in the WT protein.

In the case of the rab3Ap, which terminates CSC, it appears that both of the cysteine residues receive GeGe groups. Farnsworth and colleagues studied the modification of rab3A *in vivo* by adding a radiolabelled GeGe precursor to growing cells (Farnsworth, et al., 1991). Proteolytically digested rab3Ap from the cells was then examined by HPLC and compared to a modified peptide, this

revealed that both cysteine residues were receiving GeGe groups.

Thus far all of the ypt and rab proteins examined have been found to be geranylgeranylated *in vivo*. It therefore appears that all ypt/rab proteins receive this modification which allows them to associate with membranes. What is not clear is how many groups does each member of the family receive. Do they all receive two 20 carbon groups as the rab3Ap does? If they do receive different numbers of GeGe groups then why does this occur?

One of the aims of my work has been to study the effect of GeGe modifications on the ypt5p from *S.pombe*. In particular we wished to discover how many GeGe groups the ypt5p received and if the groups were essential for the function of the protein *in vivo*.

1.4.2 Phosphorylation

Phosphorylation is a very common form of modification. Proteins can either be modified on serine, threonine or tyrosine residues. As phosphorylation is a reversible modification it is often used to regulate the function of a protein. The most obvious examples of phosphorylation come during mitosis. During mitosis protein transport ceases, for review see (Warren, 1993b). The kinase that is thought to initiate transport inhibition is $p34^{cdc2}$ (Tuomikoski, et al., 1989). What remains unclear are the exact targets of phosphorylation and the mechanism by which phosphorylation leads to a block in transport.

The rab proteins are good candidates to be target proteins and their phosphorylation has recently came under scrutiny. Bailly and co-workers (Bailly, et al., 1991) studied four members of the rab family. They found that two of the proteins rab4p and rab1Ap, received phosphate groups during mitosis whilst two, rab2p and rab6p, did not. A motif recognised by the mitotic kinase $p34^{cdc2}$ was discovered very close to the C termini in both the rab4p and rab1Ap, and indeed it was shown that both proteins were directly phosphorylated by $p34^{cdc2}$. The proteins were only phosphorylated during mitosis and mitotic extracts depleted of $p34^{cdc2}$ could no longer modify recombinant protein. Rab4p was shifted to the cytosol during mitosis, whilst rab1Ap actually became more membrane associated (Bailly, et al., 1991).

Modification of the rab4p was studied in more detail by Mellman and colleagues, who had previously shown that the rab4p was associated with early endosomes (Van der Sluijs, et al., 1991). The group demonstrated very elegantly that the rab4p was involved in recycling from early endosomes to the plasma membrane

by overexpressing WT rab4p in CHO cells. Overexpression of rab4p caused a steady state redistribution of transferrin receptor to the plasma membrane from an internal locale (Van der Sluijs, et al., 1992b). However, a recombinant rab4p which had had its putative phosphorylation site, serine 196, abolished (rab4 ^{Q196}p) was not capable of stimulating recycling even though the protein was overexpressed 80 fold and was found to target correctly to the early endosomes.

The group demonstrated that the rab4p was modified at position 196 by blocking transfected cells in mitosis (Van der Sluijs, et al., 1992a) and then adding radiolabelled phosphate. Wild type rab4p was very quickly modified whereas rab4 ^{Q196}p was not. During interphase greater than 90% of the WT rab4p was membrane associated, whilst during mitosis 75% of WT rab4p became cytoplasmic. Mutant rab4 ^{Q196}p, however, did not change its distribution during mitosis, demonstrating quite clearly that phosphorylation is required for the change in distribution. A rab4p which lacked its C terminus, and was therefore exclusively cytoplasmic, was efficiently modified in mitotic cells indicating that phosphorylation takes place in the cytoplasm and presumably blocks the reassociation of the protein with membranes rather than causing dissociation directly.

Serine 196 is presumably a critical residue in a site recognised when the protein is removed from the membrane. It could be argued (Fig. 1.7) that during mitosis the cytoplasmic rab4p is phosphorylated causing it to dissociate from GDI protein. A consequence of this would be that rab4p would not be recycled to early endosomes and therefore transport would be inhibited. At the end of mitosis the rab4p would lose the phosphate and be able to reassociate with GDI prior to being to binding stimulated to bind membranes by GDP Dissociation Stimulator (GDS), a protein which catalyses the release of Guanosine nucleotides allowing GDP to be replaced with GTP, both GDI and GDS with be discussed in greater detail in the following section. Van der Sluijs and colleagues did observe a distinct lag between dephosphorylation of the rab4p and its subsequent reassociation with the membrane, which is consistent with the proposed model.

Fig. 1.7 Mitotic Phosphorylation

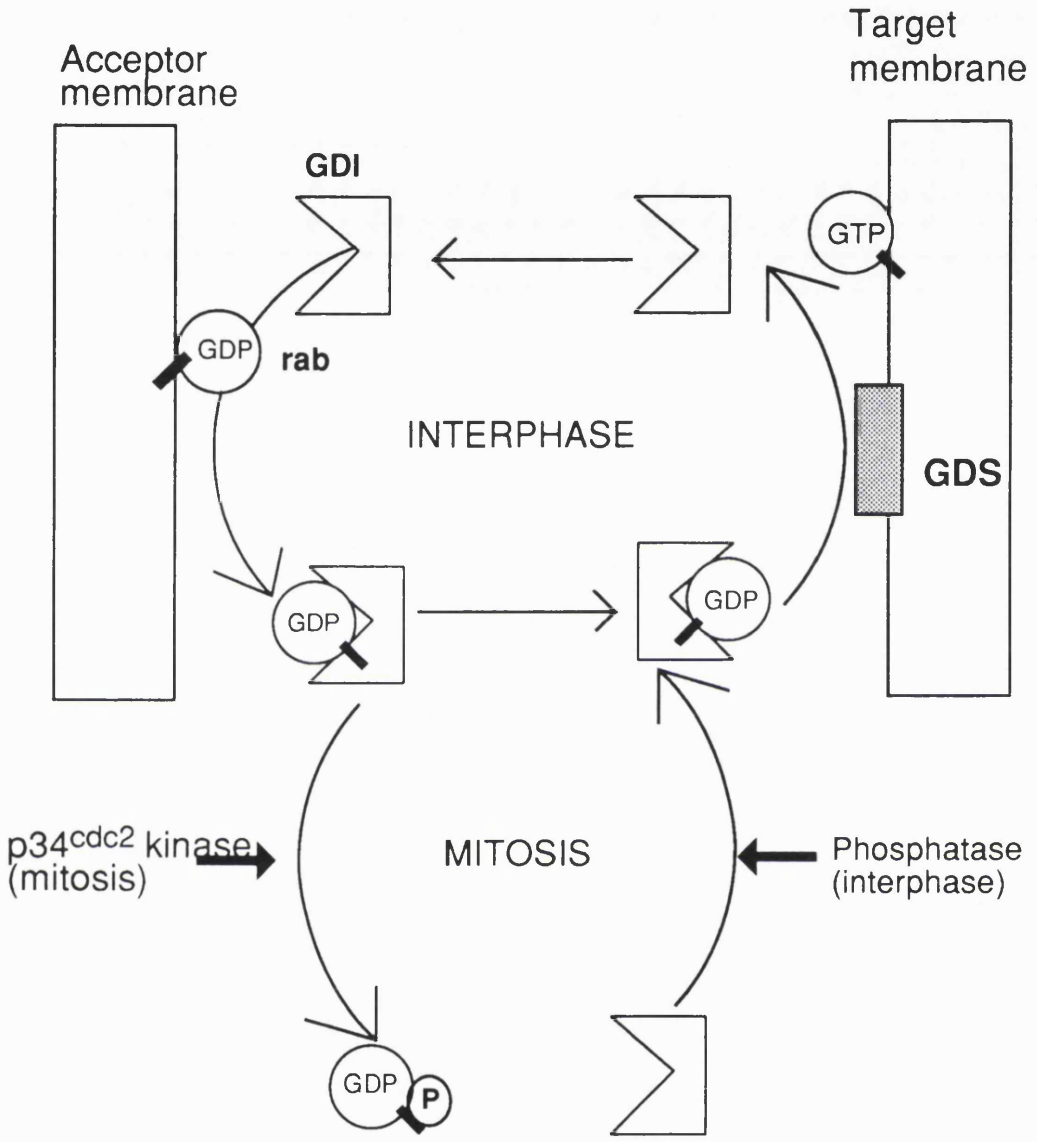


Fig. 1.7
During interphase GDI acts to cycle rab proteins from acceptor to target membranes. It is possible that during mitosis phosphorylation by $p34^{cdc2}$ causes dissociation of GDI from the rab protein. The rab protein would not be able to associate with its donor membrane resulting in transport inhibition. The phosphate group would be removed from the rab protein during interphase allowing reassociation with GDI and a resumption of transport.

A prediction one can make from the above hypothesis is that vesicles which accumulate during mitosis should not be associated with rab proteins. If this was found to be the case it would indicate an absolute necessity for rab proteins during vesicle targeting. It remains to be determined whether or not phosphorylation, directly or indirectly, is a general mechanism of regulating the localisation of rab proteins during mitosis. Interestingly the rab1Ap is also phosphorylated during mitosis (Bailly, et al., 1991) but it becomes more membrane associated, rather than less. It would be interesting to discover if phosphorylated rab1Ap is capable of supporting ER to Golgi transport in the *in vitro* system developed by Balch and colleagues (Tisdale, et al., 1992). If it were shown that a number of rab proteins did not associate with transport vesicles during mitosis then this would be a major step forward in our understanding of the molecular basis of transport inhibition during mitosis.

1.5 ACCESSORY PROTEINS

It has been proposed that rab proteins regulate specific stages of intracellular transport, but they cannot do this in isolation. The rab proteins must physically interact with other proteins *in vivo*. These other proteins are termed accessory proteins and a number of them have now been characterised in detail. In this section I want to consider a few of these proteins and in particular how they interact with ypt/rab proteins. At the end of this section I will present an updated version of the Bourne model which takes into account the roles played by the accessory proteins in controlling vesicle specificity.

1.5.1 Geranylgeranyl Transferase

The first protein I wish to discuss is rab Geranylgeranyl Transferase (GGTase). This protein differs from the other accessory proteins in a number of ways. GGTase modifies the rab protein directly and is only thought to interact with an individual rab protein once, shortly after it is translated. The other accessory proteins are not thought to modify the rab protein and can interact with an individual rab protein on a number of occasions.

Goldstein and co-workers identified the enzyme responsible for the attachment of GeGe to rab proteins. In a series of papers they report the purification and characterisation of rab GGTase. The enzyme was found to be composed of two components A and B (Seabra, et al., 1992b). Component B is a heterodimer of two subunits with MWs of 60 and 38 kDa. Both components were required to

modify rab proteins and it was shown that the reconstituted enzyme could modify rab proteins terminating either CC eg. rab1Ap, or CXC eg. rab3Ap (Seabra, et al., 1992b).

Seabra and co-workers then demonstrated that rab GGTase differs fundamentally from the other known prenyl transferases, CAAX farnesyltransferase and CAAX GG Transferase. The GGTase recognises conserved sequences in the rab proteins (Seabra, et al., 1992a) whilst the other transferases recognise only the extreme C terminus of their target proteins and in fact will recognise and modify peptides only four amino acids in length. Cloning of the GGTase demonstrated that the two subunits of component B are analogous to the two subunits which make up the other prenyl transferases (Armstrong, et al., 1993).

Component A of rab GGTase is, however, unique to the rab GGTase. Component A is a 95kDa protein (Andres, et al., 1993). Sequence comparisons revealed it to be the rat homologue of the human Chloroderemia gene. Andres and co-workers demonstrated that component A would bind rab proteins, whether the rab protein was prenylated or not. Component A would only release the rab protein in the presence of detergent. The authors argued that component A, which they renamed Rab Escort Protein (REP), interacts with translated rab protein and presents it to component B which acts as the catalytic component and modifies the rab protein by the addition of GeGe groups. The authors propose that REP is necessary because rab proteins receive two GeGe groups and that it stabilises the singly modified form of the rab allowing the second GeGe group to be added. The REP would then take the fully modified rab to the correct endomembrane.

1.5.2 GDP Dissociation Inhibitor (GDI)

One of the first accessory proteins identified was a GDP Dissociation Inhibitor (GDI) which was purified from bovine brain cytosol by Takai's group (Sasaki, et al., 1990) and shown to block the dissociation of GDP from rab3Ap. The same group went on to show that GDI could remove GDP-bound but not GTPγS-bound rab3Ap from synaptic vesicle membranes (Araki, et al., 1990), and that GDI and rab3Ap form a 1:1 complex in the cytosol.

GDI was cloned and found to encode a hydrophilic protein composed of 447 amino acids (Matsui, et al., 1990). The predicted protein was homologous to cdc25 from *S.cerevisiae*, a protein which interacts with the GDP-bound form of the ras protein. Bacterially expressed rab3Ap could not form a complex with GDI nor could purified rab3Ap which had had its C terminus removed by proteolytic

digestion (Araki, et al., 1991). This indicated that GDI binding to the rab3Ap required the presence of a geranylgeranyl moiety.

Studies revealed that GDI would also block the release of GDP from the sec4p. GDI was found to be capable of removing the GDP-bound form of the sec4p from purified yeast membranes (Sasaki, et al., 1991). Recent work by Pfeffer's group has shown that GDI will interact with the mammalian rab9 protein (Soldati, et al., 1993). Thus GDI, which was purified on the basis of its interaction with the rab3Ap, can bind to a range of ypt and rab proteins. GDI will not, however, interact with either the ras or rhoB proteins (Araki, et al., 1990). The conclusion is that GDI interacts with all of the ypt/rab proteins *in vivo*.

Takai and colleagues have proposed a model depicting the function of GDI (Fig. 1.8). They argue that GDI acts to recycle rab proteins from the acceptor compartment to the target compartment. After a transport vesicle has fused to the correct organelle the rab protein will presumably be in its GDP-bound state. It is argued that the GDI will associate with the GDP-bound form of the protein and remove it from the membrane. The interaction between GDI and the GDP-bound rab protein presumably involves binding of the geranylgeranyl moieties, therefore helping to mask the hydrophobic lipids from the cytoplasm. Interestingly GDI is homologous to REP which is also capable of binding the geranylgeranylated form of rab proteins. The GDI forms a 1:1 complex with the rab protein. The complex will remain intact until the rab protein binds GTP. It is possible that donor compartment, or the transport vesicles themselves, could contain specific GDP Dissociation Stimulator (GDS). The GDS would catalyse the exchange of GTP for GDP on the rab protein. The GDI would then dissociate from the GTP-bound rab protein allowing it to associate with the donor membrane/transport vesicle.

Fig. 1.8 Action of GDI

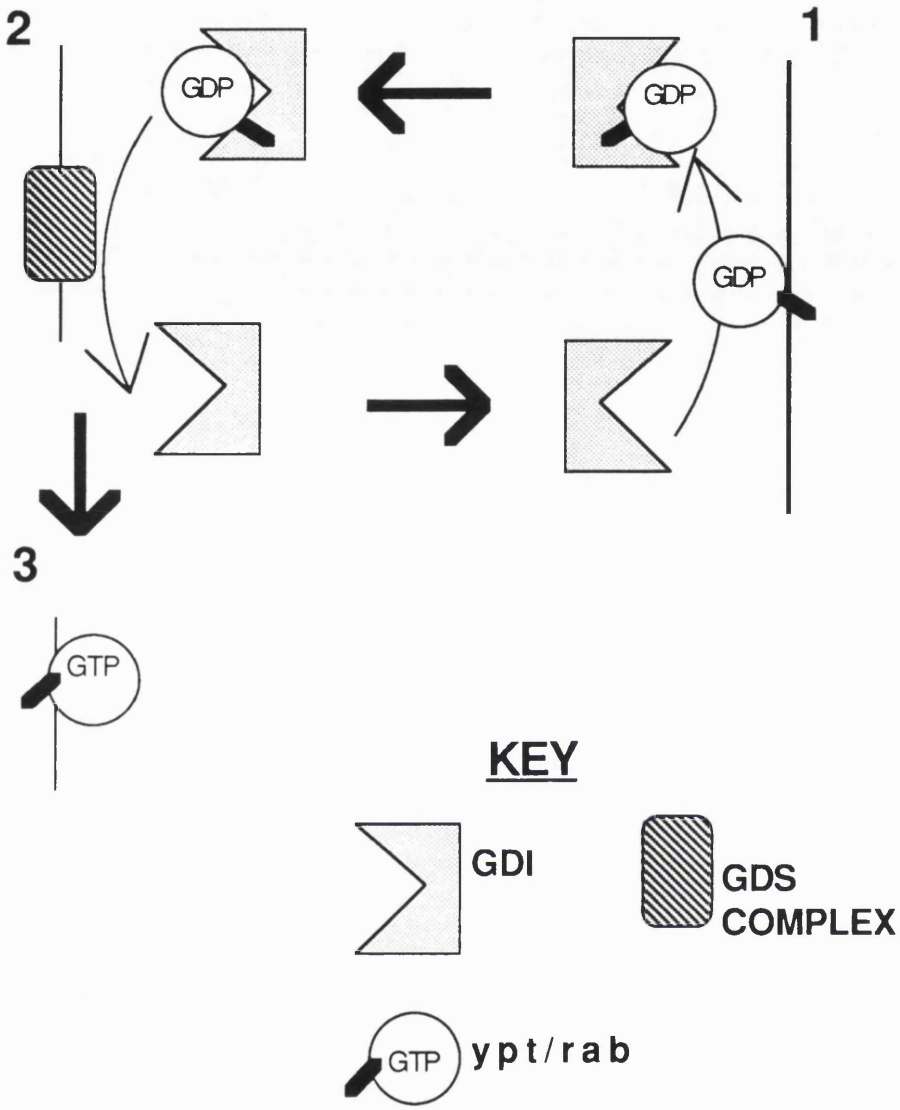


Figure 1.8

1. The ypt/rab protein in its GDP-bound state is complexed by GDI and removed from the acceptor membrane.
2. The ypt/rab-GDI complex travels through the cytosol until it encounters a GDS complex on the donor membrane/transport vesicle which stimulate the exchange of GDP for GTP.
3. The GDI releases the GTP-bound ypt/rab protein which inserts itself into the donor membrane/transport vesicle.

1.5.3 GDP Dissociation Stimulator (GDS)

Recent work by two groups appears to confirm the model presented above. Novick's group attempted to identify proteins that interacted with sec4p *in vivo*. To this end they used a strain of yeast containing a mutant form of the sec4p, termed sec4-8, which will not grow at 37°C. The strain was transformed with plasmids expressing yeast genes and the group selected transformants which would grow at 37°C. In this manner the researchers identified a gene they termed DSS4-1 (Dominant Suppresser of Sec4) (Moya, et al., 1993). Cloning of the chromosomal copy of the gene revealed that the protein product of the DSS4-1 gene has a single point mutation of aspartate to glycine which is precisely complementary to the mutation found in sec4-8p, of glycine to aspartate. The group concluded that the dss4p functionally interacts with sec4p *in vivo*.

Sequencing indicated that the dss4p was hydrophilic. However, Western blotting revealed that it associates with the 100,000g pellet (Moya, et al., 1993). During fractionation membranes are separated from the cytosol by a one hour 100,000g spin with the cytosolic components remaining in the supernatant. The presence of the dss4p in the pellet indicated that the protein was membrane associated *in vivo*. Studies demonstrated that both dss4-1p and dss4p stimulated the rate of GDP dissociation from sec4p by 6-8 fold. The dss4p did not, however, have any effect on either the ras protein or the rab3Ap and only slightly stimulated GDP dissociation from the ypt1p. These results indicate that the dss4p acts as a Guanosine Dissociation Stimulator specific for the sec4p. If the dss4p is present in the Golgi complex and/ or on secretory vesicles this would be compelling evidence that it plays a major role in recruiting the sec4p *in vivo* and could represent the exchange factor that causes the dissociation of the ypt/rab proteins from GDI (Fig. 1.8).

A mammalian homologue of the dss4 protein, termed mss4p, has also been reported (Burton, et al., 1993). The cDNA was cloned from a rat cDNA library, in exactly the same way as for DSS4-1, due to the ability of its protein product to rescue the Temperature Sensitive (TS) defect in the sec4-8 strain. Sequencing revealed that mss4 encoded a 14kDa protein which has 27% identity to the dss4 protein. Purified mss4p was capable of stimulating release of GDP from sec4p and, to a lesser extent, from both ypt1p and rab3Ap.

The identification of dss4 and mss4 proteins raises many interesting questions. Firstly is there a family of related Guanosine Dissociation Stimulators each specific for a different ypt/rab protein? One way of addressing this question would be to investigate the effects of the mss4p on various rab proteins in particular on the rab8p, as it is the sequence homologue of the sec4p. Another question which must be addressed is how dss4p is associated with the membrane? One intriguing possibility is that these proteins form part of a large complex responsible for recruiting the rab proteins to the correct endo-membrane (Fig. 1.8).

1.5.4 (i) GTPase Activating Proteins (GAPs)

Purified ypt/rab proteins have low intrinsic rates of GTP hydrolysis (Kabcennel, et al., 1990) which led researchers to postulate the existence of GTPase Activating Proteins (GAPs) as had been identified for the ras protein (Trahey and McCormick, 1987). Tan and co-workers partially purified a GAP activity from porcine liver which would stimulate the rate at which purified ypt1p hydrolysed GTP (Tan, et al., 1991). The GAP is apparently specific as it also activated the rab1p, the mammalian homologue of ypt1p, but did not activate either rab2p or ryh1p.

A GAP has recently been identified that activates ras by interacting with a region of the ras protein termed the effector domain (Adari, et al., 1988). To discover if the rab1GAP also interacts with rab protein's effector domain Becker and colleagues produced a series of mutant ypt1 proteins which had been altered in the effector domain (Becker, et al., 1991). The endogenous gene was replaced by each of the mutant genes to produce a series of mutant strains. It was found that one of the strains, containing the mutant protein ypt1M41p, was not viable, and that another strain, with the mutant ypt1N44p, would not grow at 37^{o}C.

The two mutant ypt1 proteins were purified and found to be capable of binding GTP and of nucleotide exchange (Becker, et al., 1991). However, the mutant protein ypt1M41 was not stimulated by the rab1GAP activity, whilst ypt1N44 protein was only activated 33% compared to WT ypt1p activation. The group went on to partially purify a GAP activity from yeast cytosol (Tan, et al., 1991). Again the ypt1GAP activated ypt1p but not ryh1p. When the ypt1GAP was assayed on the mutant proteins the non-functional protein, ypt1M41 was not stimulated at all whilst the temperature sensitive form ypt1N44p was only partially activated. These results indicated that the ypt1p interacts with a specific GAP *in vivo* via its effector domain, and that this interaction is necessary for the

function of the ypt1p.

Novick and co-workers have provided further evidence for specific GAP activities *in vivo*. They constructed a mutant form of the sec4p, sec4-Leu79p which has an unmeasurably low rate of GTP hydrolysis and is incapable of sustaining growth at low temperatures when present as the sole copy of the sec4p (Walworth, et al., 1992). The workers partially purified a GAP activity which only activated the sec4-Leu79p to 30% of activated WT sec4p. The activation of sec4p could be competitively inhibited by GTPγS-bound sec4p but not by GTPγS-bound ypt1p, indicating that the purified GAP activity is specific for sec4p.

The specificity of GAP proteins was investigated further by Jamieson and co-workers (Jena, et al., 1992). They purified separate GAP activities for sec4p and ypt1p from rat pancreas. The two GAPs partitioned differently between the membrane and cytosol, with ypt1GAP being 95% membrane associated whereas sec4GAP was only 65% membrane associated. Both putative GAPs were tightly membrane associated as salt washing did not remove them from the membrane.

Recently Gallwitz's group succeeded in cloning the ypt6GAP (Strom, et al., 1993). The GAP was not essential for growth, although it should be borne in mind that ypt6p itself is not essential. The purified GAP did not activate any of the other ypt proteins from *S.cerevisiae* or mammalian rab2p to any great extent compared to its activation of the ypt6p. They also report the purification of another GAP which activates ypt7p. It will be of great interest to learn whether the ypt6GAP is membrane associated and if the GAP has activity on ypt6p homologues from other species such as the rab6p and *S.pombe ryh1* (Hengst, et al., 1990)

At the moment the exact role of the GAPs remains unclear. The GAPs do appear to play a vital role *in vivo* and they also appear to act specifically. The GAP may well reside on the target organelle, for example in the case of sec4GAP the predicted localisation would be on the cytoplasmic side of the plasma membrane. After vesicle docking the GAP could activate the ypt protein to hydrolyse its bound GTP and the energy released would be used to lock the vesicle to the target membrane.

(ii) rab3A GAPs

Rab3Ap, as discussed earlier, differs from the majority of the known rab proteins in that it is involved in regulated exocytosis rather than constitutive transport. Burstein and colleagues reported the purification of two rab3AGAP activities from rat brain samples (Burstein, et al., 1991). One of the GAP activities was membrane associated and is apparently an integral membrane protein. The second GAP activity was purified from the cytoplasm and gel filtration studies indicate that it has a MW of approximately 400kDa (Burstein, et al., 1991). The most interesting observation of all was that the GAP activities were found in all rat tissues examined which sharply contrasts with the distribution of rab3Ap. It would be interesting to discover whether the GAP activities were specific for rab3Ap, as would be predicted if GAPs are involved in regulating the fusion of vesicles to the correct membrane.

How can one explain the fact that rab3Ap has two, apparently separate, GAPs. What one might suggest is that rab3Ap is involved in a regulated process in which the granules do not fuse with the plasma membrane until after the correct signal, which may well be a rise in intracellular calcium levels. Prior to fusion the cytosolic GAP would continually interact with rab3Ap causing it to hydrolyse bound GTP to GDP. The GDP-bound rab3Ap would then be removed from the vesicle by GDI and recycled via its exchange protein. The rab3Ap would continue in this futile cycle and the vesicle would never be capable of fusing. Upon the appropriate signal the cytosolic GAP would be inactivated. The vesicle would now be able dock with the plasma membrane and the rab3A protein would interact with the membrane associated GAP. This system would therefore allow the rapid response observed in regulated exocytosis.

However, it should be noted that rab3A interacts with at least one additional protein *in vivo* .This protein has been termed rabphilin (Shirataki, et al., 1993) and it may also help to regulate the function of rab3Ap. It also must be borne in mind that a number of other proteins have also been implicated in the regulation of regulated exocytosis, such as synaptotagmin which binds phospholipids in a calcium dependant manner (Brose, et al., 1992). Recently its been shown that antibodies against synaptotagmin will inhibit regulated exocytosis if they are injected into PC12 cells (Elferink, et al., 1993). It is therefore clear that the rab3Ap is not solely responsible for the control of regulated exocytosis and probably acts as one of a number of fusion regulators.

(iii) Effector domain and GAP interaction

It has been observed by a number of groups that peptides analogous to the effector domain of the rab3Ap are potent inhibitors of transport *in vitro*. Balch and co-workers demonstrated that the a peptide identical to the effector domain of the rab3Ap would inhibit early stages of transport *in vitro* (Plutner, et al., 1990) and that a modified form of the peptide termed rab3AL was an even stronger inhibitor of transport. The rab3AL peptide was analogous to a ras mutant which fails to interact with rasGAP (Adari, et al., 1988). The group concluded that the peptides blocked the interaction of the endogenous rab proteins with their GAPs which in turn leads to inhibition of transport. It should be noted that peptides identical to the effector domains of rab1p and rab2p were insoluble and could therefore not be used (Plutner, et al., 1990).

Fernandez and co-workers used the same peptides to study regulated exocytosis of secretory granules in mast cells (Oberhauser, et al., 1992). The group found that both the rab3 and the rab3AL peptides stimulated complete degranulation of mast cells, this is in striking contrast with the peptides effect on early transport. The group went on to show that the peptides were competing with the endogenous rab3A protein. It could be that in this case the peptide is interacting with the cytosolic rab3AGAP and therefore allowing the secretory granule to dock with the plasma membrane.

1.5.5 SUPPRESSORS of LOSS of YPT1 (SLY) GENES

In the above examples both biochemical and genetic means have been used to identify accessory proteins. On occasion both methods used independently can identify homologous proteins in different species. Gallwitz and colleagues devised a system to isolate genes whose expression could compensate for loss of ypt1p *in vivo* (Dascher, et al., 1991). The group identified three genes whose overexpression could allow loss of ypt1p. The group also identified a fourth gene, termed SLY1, whose expression could compensate for the absence of ypt1p even at the single copy level.

(i) SLY1

The group cloned and sequenced both the plasmid borne and the WT genomic copies of SLY1 gene (Dascher, et al., 1991). They found that the protein encoded by the plasmid borne form of SLY1 actually contained a point mutation and the allele was therefore renamed SLY1-20. The WT copy of the sly1p could not replace the ypt1p even when expressed at very high levels. Next the group wanted to identify which stage of transport sly1p is involved in (Ossig, et al., 1991). Ossig and co-workers found that cells depleted of sly1p accumulated ER and an under-glycosylated form of invertase, indicating that sly1p acts at an early stage of transport. Sly1p was overexpressed in the sec4-8 strain but it did not rescue the TS phenotype indicating that sly1p only interacts with ypt1p and does not interact with ypt proteins in general.

Sequence analysis of the sly1p revealed that it has high homology with sec1p (Aalto, et al., 1992) which has been shown to be involved in transport between the Golgi complex and plasma membrane, and to interact with sec4p in *S.cerevisiae* (Aalto, et al., 1991). Both the sly1p and the sec1p are related to a third protein from *S.cerevisiae* called slp1p. Slp1p is required for transport from the Golgi to the vacuole (Wada, et al., 1990). Aalto and colleagues argue that these three proteins are all members of a large family. Each member of the family would interact with a different ypt protein in a specific stage of membrane transport. It will be of interest to identify homologues of these proteins in higher eukaryotic cells as well as to search for more members of the family in *S.cerevisiae*. The exact role of these proteins is not at all clear. Sly1p apparently interacts with ypt1p *in vivo*, although this has not be shown directly, but a mutated version of the protein can allow transport to continue in the absence of the ypt1p. These results suggests that sly1p acts downstream of ypt1p perhaps as part of a large fusion complex either on the transport vesicle or on the target organelle itself.

(ii) SLY 2, 12 and 41

The three multicopy suppressers were named SLY2, 12 and 41. Sequencing of the genes revealed that the sly2 and sly12 proteins are homologous. SLY2 encodes a 214 amino acid protein whereas sly12p is predicted to be only 142 amino acids in length (Dascher, et al., 1991). Both proteins have a hydrophobic stretch of amino acids, near their C terminus, which is indicative of a membrane spanning domain. Sly41p is larger, predicted to have 452 amino acids, and is highly hydrophobic. Sequence comparison reveals sly41p is homologous to a phosphate translocator found in chloroplasts (Dascher, et al., 1991).

Depletion experiments reveal that of the three only sly12p is an essential protein. Cells lacking sly2p are both TS and cold sensitive (CS). The lack of sly2p could be completely compensated by the overexpression of sly12p and the TS, but not the CS, phenotype could be rescued by expression of sly1-20p (Ossig, et al., 1991). These results suggest that sly12 and sly2 proteins interact directly with both sly1p and ypt1p *in vivo*. The proteins are presumably all part of a large protein complex involved in membrane fusion.

(iii) V and T SNAREs

Sequencing of SLY2 and 12 revealed that both genes had been cloned previously. SLY2 is identical to SEC22, which was found in the same screen that located SEC4 (Novick, et al., 1980). SLY12 is BET1 a gene whose protein product had been shown to be involved in an early transport stage (Newman and Ferro-Novick, 1987).

Recent work by Rothman's group has given us an insight into the role of the two sly proteins *in vivo*. This group have been studying intra-Golgi transport using an *in vitro* system (Balch, et al., 1984). This system has allowed them to identify and characterise a number of proteins involved in the transport machinery. N-ethylmaleimide-sensitive fusion protein (NSF) is 76Kd protein which acts as a tetramer (Glick and Rothman, 1987). The protein was originally shown to be involved in transport within the Golgi but is now believed to operate in all transport stages both in higher eukaryotes and in yeast (Beckers, et al., 1989; Diaz, et al., 1989; Graham and Emr, 1991). NSF was shown to attach to Golgi membranes via soluble NSF attachment proteins (SNAPs) (Weidman, et al., 1989). The SNAPs were also shown to be involved in general intracellular membrane fusion (Kaiser and Schekman, 1990). NSF could only bind to SNAPs which were themselves attached to membranes. This led to the idea of SNAP receptors (SNAREs) which would be membrane bound proteins which would in turn bind SNAPs.

It was shown by Wilson and colleagues that NSF bound SNAPs in the presence of an integral membrane protein (Wilson, et al., 1992a). Sollner and colleagues purified SNAREs from bovine brain (Sollner, et al., 1993) using myc-tagged NSF and recombinant SNAPs. Sequencing of the SNAREs revealed that they had all been previously identified. They were: SNAP-25 (Oyler, et al., 1989) which is found in pre-synaptic terminals of neurones, but has not been precisely localised; syntaxins A and B (Bennet, et al., 1992) both of which are localised to the active zone of presynaptic membranes and VAMP-2 (Sudhof, et al., 1989) which is found on synaptic vesicles. VAMP-2 is inserted into vesicles via a hydrophobic C-terminal segment. Both the sly2 and sly12 proteins are predicted to have a similar segment at their C termini and in fact sequence comparison reveals that the sly proteins are homologous to VAMP-2.

Fig 1.9 V and T SNAREs

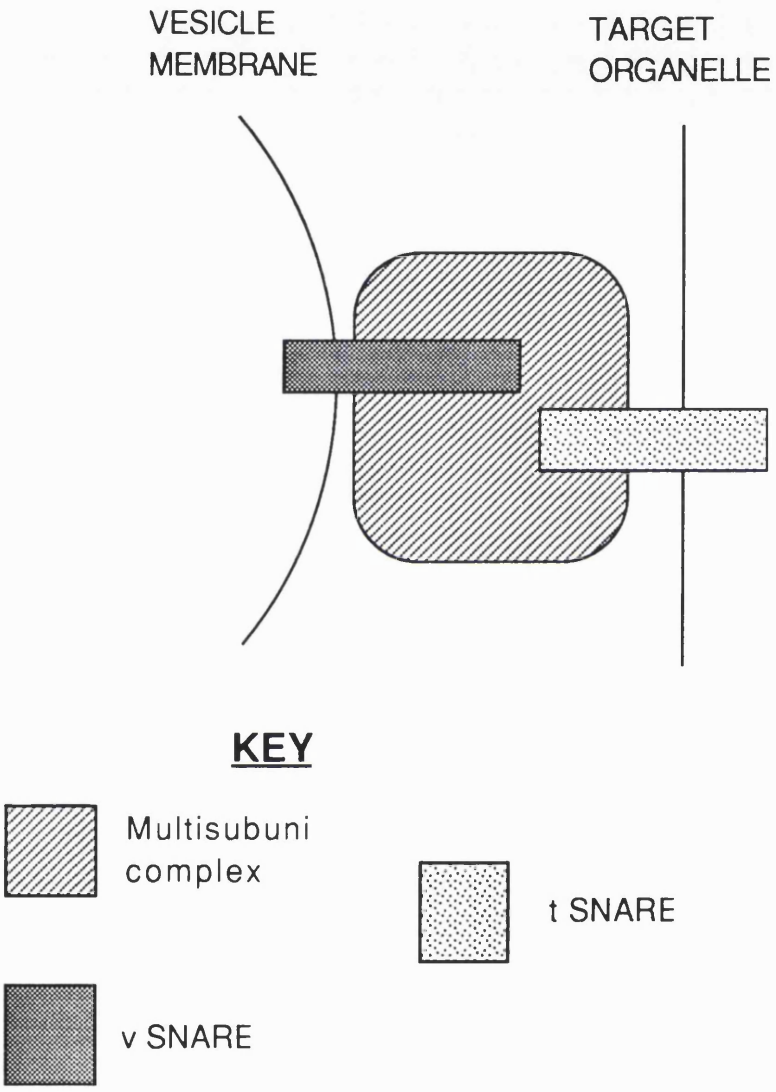


Figure 1.9 gives a highly stylised view of recognition between a v and t SNARE pair. The multisubunit complex will contain SNAPs and NSF.

Rothman and colleagues proposed that the purified SNAREs act to control the specificity of membrane transport (Fig. 1.9). They propose that the SNAREs come in two types: those found on vesicles called v-SNAREs and those found on the target membrane called t-SNAREs (Sollner, et al., 1993) . A v and t-SNARE pair would recognise each other and control each stage of vesicular transport. The sly2 and 12 proteins would therefore be yeast v-SNAREs. Other v and t-SNARE homologues have also been identified in yeast (for review see Warren, 1993a) . For example the recently cloned SSO genes (Aalto, et al., 1993) were found to encode proteins homologous to syntaxins A and B, as shown by the FASTA search.

The idea of v and t-SNARE pairs specifying each stage of transport is, in my opinion, only partially correct. The model does not consider the recycling of SNAREs or the transport of v-SNAREs to their donor compartments. That is a v-SNARE which targets a vesicle from the TGN to the late endosomes must pass through a number of vesicles en route to the TGN. During the transport the v-SNARE cannot be operable ie. it cannot target the vesicle it travels in to the late endosomes. What I believe is happening is that the v and t-SNAREs work in parallel with ypt/rabs with both sets of proteins contributing to vesicular targeting. A model for this "dual recognition" is presented in the next section.

1.6 YPT/RAB CYCLE

The study of ypt/rab accessory proteins has revealed that the docking of vesicles to their target membrane and the subsequent fusion events are more complex than was originally surmised. Fig. 1.10 presents a model based on our knowledge to date. The ypt/rab protein in its GDP-bound state travels through the cytosol complexed to GDI. At the donor membrane, or on the vesicle itself, the ypt/rab is stimulated to exchange GDP for GTP by a membrane associated GDS protein. The rab protein will be inserted into the membrane. It will then bind to a complex of proteins which include the v-SNARE.

Fig. 1.10 ypt/rab cycle

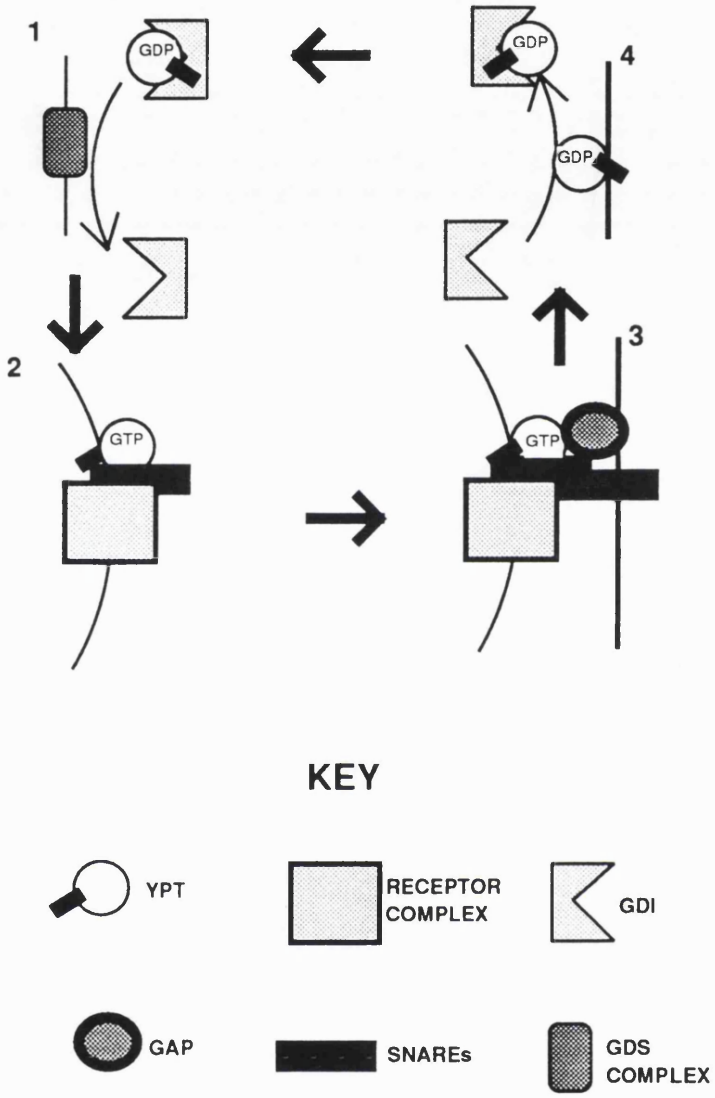


Fig. 1.10
1. The GDP-bound rabp is complexed to GDI in the cytoplasm. The GDI-rabp complex interacts with a GDS on the donor compartment/ transport vesicle, causing a GDP- GTP exchange.
2. The GTP-bound rabp associates with a complex on the transport vesicle. The complex may include a v-SNARE.
3. At the acceptor a v and t SNARE pair interact. A GAP on the membrane stimulates the rabp to hydrolyse its GTP. The energy released being used to lock the vesicle in a fusion competent state.
4. GDI removes the GDP-bound rabp from the membrane and recycles it to the donor membrane.

The vesicle would then bud from the donor and travel to the target membrane. The complex containing the ypt/rab protein would then be involved in docking the vesicle to the correct acceptor compartment. Presumably the v-SNARE on the vesicle will bind the t-SNARE on the target membrane. The ypt/rab protein would act as a second level of control on specificity with it being activated by a GAP activity on the membrane to hydrolyse its bound GTP to GDP. This allows for a two-step recognition process: the v and t-SNARE pair, and the rab protein with its specific GAP. The dual recognition model explains the need for specific rab proteins, SNAREs and GAPs. The energy of hydrolysis would lock the vesicle in a fusion competent state. Fusion would continue with NSF etc all playing a role. After hydrolysis the rab would be in a GDP-bound state and could therefore be removed from the membrane by GDI and the cycle would continue.

1.7 *Schizosaccharomyces pombe*

The fission yeast *S.pombe* was first isolated from beer in Africa. *S.pombe* differs quite markedly from the primitive budding yeast *S.cerevisiae*. One of the most notable differences is in the growth cycle of the two yeasts. *S.cerevisiae* grows by forming a bud which grows in size to eventually form a daughter cell which separates from the parental cell. *S.pombe* is a fission yeast and multiplies in a manner which is more similar to higher eukaryotes. The *S.pombe* cell is cylindrical, about 3.5μm in diameter and 7μm in length. Growth occurs throughout the cell cycle with extension occurring at one end of the cell at any particular time, for a review of *S.pombe* cell growth see (Johnson, et al., 1989). When the cell has doubled in length a septum starts to form at the centre of the cell which increases in size eventually separating the two daughter cells.

1.7.1 The Golgi complex

S.pombe not only differs from *S.cervisiae* in its growth cycle but also in its morphological and biochemical makeup, both of which are closer to that seen in higher eukaryotes. As stated earlier the Golgi in *S.cerevisiae* consists of separate cisternae whereas in mammalian cells stacks are observed. The Golgi complex in *S.pombe* is readily observable. The number of cisternae in the stack varies but on average there are three per stack with twenty such stacks per cell (Ayscough, 1993). The number of stacks increases during the cell cycle. There is no morphological evidence of any asymmetric distribution of enzymes within the stacks, with electron microscopy indicating that galactosyltransferase is

distributed throughout each stack (Ayscough, 1993). Analysis of secretory proteins has revealed that, unlike *S.cerevisiae*, which only adds mannose to carbohydrate side chains, both galactose and mannose are added to the sugar groups of secretory proteins as they pass through the Golgi complex (Chappell and Warren, 1989).

1.7.2 *S.pombe* genetics

Although *S.pombe* is only distantly related to the budding yeast *S.cerevisiae* it does have the same features that makes *S.cerevisiae* such a genetically tractable organism. *S.pombe* exists normally as a haploid organism but nutrient deprivation will result in the formation of diploid zygotes which will normally sporulate to form haploid progeny (Egel, 1989). Strains can be forced to remain diploid by depriving them of specific nutrients the haploids cannot synthesise. The availability of both haploid and diploid strains makes *S.pombe* a very useful organism in terms of allowing gene disruptions and in the production of conditional mutants.

Recently a large number of genetic techniques, including transformation and the construction of expression vectors have successfully been applied to *S.pombe* (Moreno., et al., 1990). *S.pombe* has now proved to be an excellent model for higher eukaryotes, for example the cell cycle in *S.pombe* has been studied in great detail (Fantes, 1989).

1.7.3 YPT genes from *S.pombe*

Until recently only two YPT genes had been identified in *S.cerevisiae* whereas in mammalian cells a large number of rabs had been identified. A number of groups, including our own, attempted to locate *ypt* genes in *S.pombe*. It was reasoned that the proteins identified could well be models for those already identified in mammalian cells. A total of six genes were rapidly identified by various genetic screening methods (Fawell, et al., 1989; Fawell, et al., 1990; Haubruck, et al., 1990; Hengst, et al., 1990; Miyake and Yamamoto, 1990). Sequencing revealed all of the genes discovered, except for *ypt4*, were homologues of previously identified mammalian genes (Table 1.1).

1.8 AIM OF PROJECT

At the beginning of this work our group had recently identified a number of *ypt* genes from *S.pombe* which we now wished to characterise. My initial aim was to establish a system which would allow the endogenous *ypt* genes to be replaced by recombinant copies linked to a selectable marker. The system would then be used to generate conditional mutants of the *ypt* genes by either specifically or randomly mutagenising the recombinant gene prior to replacement. The resultant strains would then be examined both biochemically, by examining the carbohydrate modifications of secretory proteins, and morphologically by microscopic analysis.

By this route we hoped to identify which stage of transport each ypt protein was operating at. We also wished to use mutant strains to discover if they could be rescued by the expression of the mammalian homologue i.e. would overexpression of rab8 protein rescue a strain with a defective *ypt2* gene.

As said we wished to produce stains of yeast containing *ypt* genes with specific point mutations. Doing this would allow us to correlate *in vitro* results with results *in vivo*. In particular we wished to investigate the post-translational modifications received by the ypt proteins and the effect of these modifications, or lack of them, on the protein *in vivo*.

Chapter 2

Materials and Methods

2.1 MATERIALS

2.1.1 Media

S.pombe were grown in either YPD or in minimal media PM supplemented with the appropriate nutrients, vitamins and minerals. The cultures were grown either on plates or in liquid with mild agitation at the temperatures indicated. The plates were sealed with thin strips of Parafilm to stop dehydration. Bacteria were grown using LB containing 40μg/ml Ampicillin, unless otherwise stated, they were grown at 37°C either in liquid whilst shaking or on plates.

2.1.2 Chemicals

All chemicals used were purchased from Sigma or BDH and were of analytical grade unless specified otherwise. All restriction enzymes used were from GibCo-BRL, UK.

2.1.3 General Solutions

All of the following are autoclaved unless stated otherwise. The volumes stated were required to make 1 litre. At all times distilled water was used to bring up to volume. Distilled water was used throughout the course of this project.

TE	10ml	1M Tris-HCl pH 7.5
	1ml	1M Na-EDTA
PBS	10g	NaCl
	0.25g	KCl
	1.43	Na_2HPO_4
	0.25g	KH_2PO_4
LISB	5ml	1M Tris-HCl pH7.5
	1ml	1M $MgCl_2$
	1ml	1M EGTA
	0.1ml	1M EDTA
SEQ mix		
SP1	218.6g	Sorbitol
	14.7g	NaCitrate

	7.1g	Na_2HPO_4
	80ml	1M EDTA
	pH 5.6	(use orthophosphoric acid)
Phenol/Chloroform	500ml	Buffered Phenol
	480ml	Chloroform
	20ml	Isoamyl Alcohol
	Not autoclaved- store at 4°C	
PEM	30.24g	PIPES
	1ml	1M $MgSO_4$
	1ml	1M EGTA
PEMS	PEM +	218.6g Sorbitol
PEMF	PEM +	5ml Fish Skin Gelatin
TAE	4.84g	Tris base
	1.142ml	Glacial acetic acid
	1ml	1M EDTA pH8
TBE	10.8g	Tris base
	5.5g	Boric acid
	1ml	1M EDTA pH8
TB1	20ml	1M Tris-HCl pH7.5
	2ml	1M EDTA pH8
	200ml	1M LiCl
TB2	400ml	100% PEG 4000
	100ml	1M LiCl
	500ml	TE
	Filter sterilise no autoclave	
LAB1	100ml	1M LiAc pH4.9
LAB2	500ml	100% PEG 4000
	Filter sterilise no autoclave	

LAB3	2.5g	Bacto-yeast extract
	75ml	20% Glucose
	50ml	Adenine/ Leucine/ Uracil as required 1.5mg/ml.
	Add Glucose after autoclaving.	
2 x SB	100ml	20% SDS
	50ml	1M Tris-HCl pH6.8
	2ml	1M EDTA pH8
	100ml	100% Glycerol
	0.3g	Bromophenol Blue
	20ml	β-mercaptoethanol
	Not autoclaved	
Protease Inhibitors	1g	Aprotinin
	1g	Leupeptin
	1g	Pepstatin
	1g	Antipain
	1ml	1M Benzamidine
	Not autoclaved	
HE	11.915g	HEPES
	5ml	1M EDTA pH8
	100ml	1M NaCl
	pH to 7.9 with NaOH	
HK	47.66g	HEPES
	10ml	1M EDTA pH8
	200ml	1M NaCl
	100ml	20% SDS
	pH to 7.9 with NaOH	
	Add SDS after autoclaving.	
TFB1	6ml	5M KAc
	12g	$RbCl_2$
	10ml	1M $CaCl_2.2H_2O$
	9.9g	$MnCl_2$
	150ml	100% Glycerol
	pH to 5.8 with acetic acid.	

	Filter sterilise and store at 4°C	
TFB2	3g	PIPES
	75ml	1M $CaCl_2.2H_2O$
	1.2g	$RbCl_2$
	150ml	100% Glycerol
	pH to 6.5 with KOH	
	Filter sterilise and store at 4°C	
Solution 1	2.5ml	20% Glucose
	10ml	1M EDTA pH8
	25ml	1M Tris-HCl pH8
	Add Glucose after autoclaving	
Solution 2	50ml	20% SDS
	200ml	1M NaOH
	no autoclave store in plastic container	
Solution 3	250g	KAc
	150g	HAc
SEQ1	100ml	1M NaCl
	900ml	TE pH8
5 x Ficoll	20ml	1M Tris-HCl pH7.5
	100ml	1M EDTA
	0.1g	Bromophenol Blue
	1.75ml	Ficoll 400
	Not autoclaved	
Depurinating solution	250ml	1M HCl
Denaturing solution	500ml	1M NaOH
	500ml	3M NaCl
Church solution	500ml	1M $NaPO_4$
	350ml	20% SDS
	1ml	1M EDTA

	1g	BSA
	Not autoclaved	
LS solution	25 : 25 : 7 ratio	
	1M Hepes pH8: DTM : OL	
	Not autoclaved	
DTM	250ml	1M Tris-HCl pH8
	25ml	1M MgCl2
	25ml	1M DTT
	0.1ml	1M dCTP/dGTP/dTTP
	Not autoclaved	
OL	90 OD	units/ml hexamers
	1ml	1M Tris-HCl pH8
	1ml	1M EDTA pH8
	Not autoclaved	
Blocking buffer	50g	Dried milk powder
	10ml	20% Tween-20
	Made up in PBS	
	Not autoclaved	
5 x RB	30g	Tris base
	144g	Glycine
	5g	SDS
	Not autoclaved	

Composition of Media

	Per Litre	
LB	10g	Bactopeptone
	5g	Yeast Extract
	10g	NaCl
YPD	20g	Bactopeptone
	10g	Yeast Extract
	100ml	20% Glucose
	20g	Agar (for plates)
YES	500ml	2 x Glucose

	500ml	2 x YES
	20g	Bacto agar (Difco)
	(For plates)	
	Glucose added after autoclaving	
2 x Glucose	60g	Glucose
	Not autoclaved	
2 x YES	10g	YE
	500mg	adenine, histidine leucine, uracil and lysine hydrochloride
PM	50ml	20 x PPN
	20ml	50 x Salts
	100ml	20% Glucose
	50ml	Adenine/ Leucine/ Uracil as required 1.5mg/ml.
	1ml	1000 x Vitamins
	0.1ml	10,000 x Minerals
	20g	Agar - for plates.
	Glucose, vitamins and minerals added after autoclaving	
20 x PPN	60g	KH phthallate
	36g	Na_2HPO_4 (anhydrous)
	100g	NH_4Cl (absent from nitrogen free medium.)
50 x Salts	53.3g	$MgCl_2.6H_2O$
	50g	KCl
	2g	Na_2SO_4
1000 x Vitamins	1g	Pantothenic Acid
	10g	Nicotinic Acid
	10g	Inositol
	10mg	Biotin
10000 x Minerals	5g	Boric Acid
	4g	$MnSO_4$

4g	$ZnSO_4$
2g	$FeCl_2.6H_20$
1.6g	Molybdic Acid
1g	KI
0.4g	$CuSO_4.5H_2O$
10g	Citric Acid

Vitamins and Minerals were both filter sterilised not autoclaved.

2.2 *S.pombe* METHODS

2.2.1 Centrifugation and incubation

Harvesting and washing of cells was carried out using a Jouan CR422 Benchtop centrifuge at 4000 rpm, which is equivalent to 3080g, all spins were at 4°C. An MSE eppendorf centrifuge was also used at either at 13000 rpm, unless otherwise stated this was done at room temperature. *S.pombe* were grown at 30°C and bacteria were grown at 37°C (again unless otherwise stated) in Gallenkamp Cooled incubators. Eppendorfs were incubated in Anderman Thermostat 5320 eppendorf incubator.

2.2.2 Cell Counting

Cell numbers, for growth curves, were ascertained using an improved Neubauer Haemocytometer (Weber U.K.). 10µl of cells were added to the haemocytometer and the number of cells present in the central 25 squares counted, multiplying this figure by 10^4 gave the number of cells per ml in the original culture.

2.2.3 Reversion assessment (Boeke, et al., 1984)

Strains made ura^+ by transformation were checked for stability using 5-Fluoroorotic acid (FOA). Cells were grown under non-selective conditions, in YPD media. Aliquots of approximately 1 x 10^6 cells were then plated onto 3cm diameter minimal plates supplemented with uracil and containing 1mg/ml of FOA. The plates were then placed at 30°C for 4-5 days, only cells which lacked *ura4* gene were capable of growth.

2.2.4 Lithium Chloride (LiCl) Transformation (Broker, 1987)

Linear DNA was introduced using this method. Early log phase cells were harvested by centrifugation for 5 minutes at 4000rpm. The cells were washed in distilled water, resuspended in 0.6ml of TB1 and incubated at 30°C, with

mild agitation, for 1 hour. The cells were split into 200µl aliquots to which between 0.2 - 2µg of DNA, in 10µl of TE, was then added. The cells were incubated for a further 30 minutes at 30°C with no agitation prior to the addition of 0.7ml TB2. After another 30 minute incubation at 30°C the cells were heat shocked at 46°C for 25 minutes. The cells were then plated onto selective plates and incubated at the appropriate temperature.

2.2.5 Lithium Acetate (LiAc) Transformation (Okazaki, et al., 1990)
40ml of logarithmic phase cells were harvested at 4000rpm for 5 minutes, washed twice in distilled water and resuspended in 0.4ml of LAB1. 100µl aliquots of the cells were transferred to 1.5ml eppendorf tubes and incubated at 30°C for 1 hour. 3-5µg of DNA, in 15µl of TE, was added and the incubation was continued for a further hour at 30°C. Next 0.29ml of LAB2 was added to each eppendorf which were again incubated at 30°C for 1 hour. The cells were heat shocked at 43°C for 15 minutes, pelleted at 6500 rpm for 30 seconds and transferred to a flask containing 4ml of LAB3. The cells were allowed to recover for 3-4 hours at 30°C and then 1ml aliquots were spread onto a selective plates. The plates were then incubated at the appropriate temperature.

2.2.6 Random spore analysis
This technique was used to examine the effect of structural changes on a protein's ability to sustain vegetative growth. WT diploid cells had one copy of a gene replaced with an altered version linked to the selectable marker *ura4*. The transformed diploid strains were then induced to sporulate by growth on nitrogen free media for 2-3 days at 25°C. The diploid yeast formed spore containing asci under these conditions, the spores were released by incubating a loopful of cells in 1ml of water containing 2µl of helicase (Dupont) at 37°C overnight. The spores were washed 3 times in water and plated onto YES plates at a density of 500 spores per plate. The spores germinated and were allowed to grow at 25°C until colonies appeared. The colonies were then replicated onto minimal plates with or without uracil.

2.2.7 Fractionation
40ml of log phase cells, approximately 2 x10^8, were harvested at 4000rpm for 5 minutes, washed in distilled water, resuspended in 0.4ml of SP1 and transferred to 1.5ml eppendorfs. Cells were spheroplasted by pelleting at 6500rpm for 15 seconds, and resuspended in 1ml of SP1, containing 0.4mg/ml Zymolyase 20T (Seikagaku), and incubated at 37°C for 1 hour. The

spheroplasts were pelletted, washed twice in SP1 and resuspended in 0.3ml of LISB. The spheroplasts were vortexed vigorously and placed at 4°C for 30 minutes. Spheroplasts were now passed through a 21 gauge needle 20 times and again vortexed vigorously. Unlysed cells and nuclei were removed by a 15 second spin at 6500rpm. The supernatant was now spun using a TL100.2 rotor, in a Beckman TL-100 ultracentrifuge at 4°C, at 50,000rpm (equivalent to 100,000g) for 1 hour, yielding a supernatant (S100) and a pellet (P100). The P100 was resuspended in 100µl of TE. Protein was precipitated from both the S100 and P100 and resuspended in 80µl of 2 x SB.

2.2.8 Sucrose Gradient Fractionation

The procedure was exactly as described above. The spheroplasts were lysed by passage through a 21 gauge needle, and then the 0.3ml homogenate was laid onto a 12ml continuous sucrose gradient, from 75% W/W - 15% W/W. The gradients were poured, at 4°C, from the base using a Buchler Auto-densi flow peristaltic pump. The gradients contained 0.1M TE pH 7.4, 1.25mM DTT, 0.25mM PMSF and 1ml/L of protease inhibitors. The gradients were centrifuged in a SW40 rotor at 40,000rpm for 16 hours at 4°C, using a Beckman L8-M ultracentrifuge. The gradients were then fractionated into 1ml aliquots. The density of sucrose in each fraction was calculated using a Brix 0-50% refractometer. The fractions were then stored at -20°C.

2.2.9 Enzymatic assays

(i) Galactosyltransferase (Chappell and Warren, 1989)

14.75µl of each sample, or of distilled water as a control, was added to 35.25µl of Gal.T. assay mix in an 1.5ml eppendorf this gave a final concentration of 0.1M Hepes pH 7.0, 1mM $MnCl_2$, 25nmols UDP-[^{3}H] Galactose(specific activity of 1-2mCi/mmol), 200mM methyl mannose, 1% TX-100, 500µM UDP-Galactose and 10mM $MgCl_2$. The samples were incubated at 37°C for 1 hour. Dowex 1 chloride columns were used to remove excess UDP-Gal. The Dowex was washed twice with distilled water, and then 0.8ml of the Dowex added to a Pasteur pipette sealed at the base with 1cm of glass wool. The columns were washed with 2ml of 5M NaCl, 2ml 0.1M HCl and finally with 5ml distilled water. The samples were added to the column and the columns were washed with 1.5ml of water. The wash through and the eluted materials were combined. 5ml of scintillation fluid was then added to each sample and the amount of tritium determined using a Beckman LS 6500IC Scintillation counter.

(ii) Acid phosphatase (Schweingruber, et al., 1986)

Assays on both gradient fractions and on secretion from spheroplasts were carried out in essentially the same manner. 50µl aliquots of material, or water as a control, were added to 0.5ml of 2mM Nitrophenylphosphate in 0.1M NaAc pH 4.0. The samples were incubated at 30°C for 40 minutes. The reaction was stopped by the addition of 0.5ml 1m NaOH. The OD at 405nm was then read.

2.2.10 Electron Microscopy

1 x 10^8 cells were harvested at 4000rpm by 5 minutes, resuspended in 1ml of distilled water and transferred to a 1.5ml eppendorf. The cells were then washed three times in distilled water, resuspended in 1ml of 2% $KMnO_4$ and incubated at room temperature for 45 minutes. The cells were again washed three times in distilled water and the pellet was covered by 1ml of 70% ethanol. The cells were incubated at 4°C for 1-2 days. Next the cells were dehydrated by being washed and incubated in increasing concentrations of ethanol. The samples were incubated twice at 70 and 90% and three times in 100%, in each case for 15 minutes. The pellets were then placed in 1ml of propylene oxide for ten minutes, and then in 50% propylene oxide: 50% Epon resin for 1 hour. Finally the pellets were incubated twice in neat Epon (Taab Laboratories Equipment Ltd.) for 2 hours. The pellets were embedded in resin in separate moulds and baked at 65°C overnight. The samples were sectioned and mounted on carbon/formvar coated grids. Staining was carried out using 3% uranyl acetate for 10 minutes and 4 minutes in lead citrate (Reynolds, 1963). The stained sections were viewed in a Philips CM10 electron microscope.

2.2.11 Indirect Immunofluorescence

(i) Paraformaldehyde fixation

20ml of logarithmic phase cells were pelleted at 4000rpm for 5 minutes, resuspended in 1ml PBS and transferred to an eppendorf. Cells were pelleted, at 6500rpm for 15 seconds, resuspended in 0.5ml of 3% paraformaldehyde in PBS and incubated at room temperature for 1 hour with occasional inversion. Next the cells were pelleted, washed twice in PEM and once in PEMS. Samples were spheroplasted by resuspending in 1ml of PEMS containing 0.5mg of Zymolyase and 2.5mg of Novozyme (NovoBioLabs Ltd.) and incubated at 37°C for 1 hour. The spheroplasts were pelleted and washed three times in PEMS. The spheroplasts were permeabilised by resuspending in

1% TX-100, in PEMS for 30 seconds. The samples were again pelleted, washed once in PEMS and twice in PEM. Non-specific binding sites were blocked by incubating samples in 1ml of PEMF for 1 hour. The cells were pelleted and resuspended in 0.4ml of PEMF containing the first antibody, typically 1/100 dilution for a polyclonal antibody. The spheroplasts were put on a rotating wheel at 4°C overnight. The samples were then washed three times in PEMF and resuspended in 100-200ml PEMF containing fluorophore conjugated second antibody, again diluted 1/100, and rotated at 4°C for 4 hours. Finally the samples were washed three times in PEMF and resuspended in as small a volume as possible, approximately 10µl depending on the amount of sample. The samples were dried onto poly-lysine coated coverslips and mounted in Citifluor containing 1mg/ml DAPI. The cells were viewed using a Zeiss Axiophot microscope and photographed using Ilford HP5 film.

(ii) Methanol fixation

1 x 10^8 of logarithmic phase cells were filtered directly onto a piece of GFC Whatman in a filter which had been pre-cooled at -20°C. The filter papers were transferred to a 15ml Falcon containing 10ml methanol which had also been cooled at -20°C. The samples were now placed at -20°C for 10 minutes. The cells were then shaken off the filter paper by vortexing and the filter paper was removed and discarded. The cells were spun down at 4000rpm for 5 minutes. The procedure now followed that outlined above from the spheroplasting stage onwards.

2.2.12 *S.pombe* RNA preparation

Whilst carrying out this procedure gloves were worn and all tips and glassware were autoclaved. Falcon tubes used were from freshly opened packs. These precautions were necessary due to the danger of contamination by RNAse. 200ml cultures of logarithmic phase cells were spun down at 4000rpm for 5 minutes, washed once in distilled water and transferred to a 15ml Falcon tube. The pellet was resuspended in 500µl HE buffer, cooled on ice, to which 2ml of baked acid washed glass beads (425-600mm) were added. The tube was vortexed six times for 30 seconds, between each the tube was left on ice. 1ml of cooled HE was now added, the tube was vortexed and spun at 4000rpm for 30 seconds and the supernatant was removed to a fresh 15ml Falcon. This stage was repeated twice more. The pooled supernatants were now incubated at 37°C for 1 hour with 1.5ml of HK buffer containing 200µg of proteinase K. The samples were now treated with 3ml of phenol/chloroform, the samples were vortexed and spun at 4000rpm for 5

minutes. The upper aqueous phase was transferred to a fresh Falcon tube. The RNA in the aqueous phase was precipitated by addition of 0.3ml 3M NaAc (pH 5.6), 10ml of 100% ethanol and a 1 hour incubation on dry ice. The precipitated RNA was pelleted by spinning at 4000rpm for 10 minutes and washed in 70% ethanol before being thoroughly dried and resuspended in 0.5ml of distilled water. The amount of RNA was calculated by measuring the OD at 260nm, an absorbance of 1 being equivalent to 40mg/ml.

2.2.13 *S.pombe* DNA preparation

5ml of logarithmic phase cells were harvested by centrifugation at 4000rpm for 5 minutes. The cells were washed, resuspended in 1ml of distilled water and transferred to a 1.5ml eppendorf. The cells were spun down at 6,500 rpm for 30 seconds and resuspended in 0.25ml of SP1 containing 0.4mg/ml Zymolyase 20T. The cells were spheroplasted by incubation at 37°C for 1 hour. The spheroplasts were pelleted and washed twice in fresh SP1. The spheroplasts were next resuspended in 0.5ml TE and 50µl of 10% SDS, and vortexed vigorously. Next 165µl of 5M KAc was added and the samples were incubated on ice for 30 minutes. Cell debris was removed by centrifugation at 13,000 rpm for 10 minutes, the supernatant was transferred to a fresh eppendorf containing 750µl cold isopropanol. The samples were then placed on dry ice for 5 minutes to precipitate nucleic acid. The precipitate was pelleted at 13000 rpm for10 minutes and resuspended in 300µl TE and 1µl of RNase (10mg/ml). The samples were incubated at 37°C for 30 minutes. After digestion of RNA the samples were extracted by addition of 300µl phenol/chloroform, the samples were vortexed and spun at 13000 rpm for 5 minutes. The upper aqueous phase was removed to a fresh eppendorf and the DNA precipitated by the addition of 30µl 3M NaAc and 0.8ml 100% ethanol. The DNA was pelleted, washed in 70% ethanol and allowed to air dry. The DNA was now resuspended in 20µl TE.

2.2.14 Protein Precipitation (Wessel and Flugge, 1984)

Samples were resuspended in 0.1ml of TE, or distilled water, in an 1.5ml eppendorf. 0.4ml of methanol was added to each to each sample. The samples were then vortexed and spun at 13000 rpm for 10 seconds. Next 0.1ml of chloroform was added and the samples were again vortexed and spun briefly. Then 0.1ml of distilled water was added to each sample, the samples were again vortexed and spun for 1 minute. The precipitated protein accumulated at interphase between aqueous and organic layers. The upper aqueous layer was removed and discarded, with care being taken not to remove the protein,.

0.3ml of methanol was added to the remaining material and each sample was again vortexed and spun for 2 minutes. The supernatant was removed and the precipitated protein was allowed to dry before being resuspended in the appropriate buffer.

2.2.15 Endoglycosidase H (Endo.H.) digestion

S.pombe secretory proteins receive N linked carbohydrate modifications which include polymannose and galactose residues. Due to the heterologous nature of the modifications fully glycosylated proteins run as a smear on SDS gels. To resolve the smears Endo.H. digestion can be carried out as this will remove all *S.pombe* N linked carbohydrates. 10µl of protein extracts in 2 x SB were added to 1µl 3M NaAc pH5.6, 1µl 100mM PMSF in ethanol, and 0.05µl Protease Inhibitor mix in the presence or absence of 1µl Endo.H. (Boehringer) in a total volume of 50µl. The digestions were incubated overnight at 37°C. 40µl of 2 x SB was then added to each sample which were then heated to 95°C for 5 minutes prior to being separated by SDS-PAGE.

2.2.16 Western Blotting

Protein samples were separated by SDS-PAGE in 1 x RB, at a constant current of 37mA whilst being water cooled. The gels had a 5% acrylamide stacking phase which contained 0.125M Tris-HCl pH6.8, 0.1% SDS and were polymerised using 10% APS (5µl/ml gel) and TEMED (1µl/ml gel). The main phase of the gel had a variable acrylamide content 8-15% depending on the size of the proteins being investigated. This phase contained 0.38M Tris-HCl pH8.8, 0.1% SDS and was also polymerised using 10% APS (1µl/ml gel) and TEMED (0.4µl/ml gel). Pre-stained rainbow markers (Amersham) were used to allow determination of molecular weight.

The proteins were transferred from the gel to Hybond-C super membranes (Amersham) using a Hoeffer semi-dry blotter according to the manufactures instructions. The membranes were stained with Ponceau S, to confirm protein transfer, and then washed four times in PBS. The membranes were then blocked overnight in 100ml blocking buffer. The first antibody was then added in 10ml blocking buffer, the dilution depended on the antibody being used generally around 1 in 10000 for polyclonal antibodies. The membrane was sealed in a hybridisation bag, along with the antibody solution, and then incubated at room temperature for 1 hour. The membrane was washed 4 times in 0.2% Tween 20 (in PBS) and incubated with HRP conjugated second antibody which was specific for the first antibody (again diluted in blocking buffer). After incubation with the second antibody the membrane was again

washed 4 times and the antibodies visualised using Amersham's ECL reagents according to the manufacturers instructions.

2.2.17 Surface density measurements (Griffiths, et al., 1989)

The analysis of *S.pombe* cells was carried out in the following manner. Random photographs of cells were taken and printed at a magnification of 22,000. The photographs were overlaid with a 15mm square grid. The points of membranes lying on grid lines were scored (Σi). Golgi cisternae were defined as being between 0.4 and 0.1μm in length with dilated rims at either end of the cisterna.

The total area of the cells was calculated by counting all grid intersections on the cells (Σp). The surface density of each organelle therefore equalled:

$$\text{Surface density} = \Sigma i / d\Sigma p$$ (where d= distance between grid lines in microns)

This was done for at least ten micrographs and mean and standard deviation values were calculated

2.2.18 cDNA production

cDNA was produced using *S.pombe* RNA. The RNA was first incubated with DNase. 40μl of RNA was added to 40μl of RQ buffer, which contained 40mM Tris pH 7.9, 10mM NaCl, 6mM $MgCl_2$ and 0.1mM $CaCl_2$, and with 5μl of RNase free DNase (Boehringer). The samples were incubated in a 1.5ml eppendorf for 30 minutes at 37°C. The samples were then heated at 95°C for 5 minutes to inactivate the DNase. The RNA was then extracted with phenol/chloroform and precipitated with ethanol. The RNA was resuspended in 40μl of distilled water. Next the following were added to the RNA: 10μl of 5 x reverse transcriptase buffer (Gibco-BRL), 8μg of poly dT (supplied by the ICRF oligonucleotide synthesis unit), 4μl RNasin (Boehringer), 8μl of 10mM dinucleotide triphosphates (Boehringer) and 1μl of reverse transcriptase (Boehringer). The sample was incubated at 85°C for 5 minutes and then immediately laced on ice for 5 minutes. The sample was then incubated at 42°C for 90 minutes, after this the cDNA was extracted with phenol/ chloroform and then precipitated with ethanol and finally resuspended in 20μl of distilled water. 5μl of cDNA solution was then amplified by PCR.

2.2.19 GTP binding (Lapetina and Reep, 1987)

Protein extracts were made as described and the proteins were separated by SDS-PAGE. The proteins were then transferred to nitrocellulose filters exactly as described except that the transfer buffer lacked SDS. The filters were then incubated briefly in binding buffer; 50mM Tris pH7.5, 0.3% Tween 20, 5mM $MgCl_2$ and 1mM EGTA. The filter was then incubated in binding buffer containing 40μCi of [α-^{32}P]GTP at room temperature for one hour. The filter was then washed in fresh binding four times for ten minutes. The filter was then allowed to air dry prior to being exposed to photographic film overnight.

2.3 Bacterial Methods

2.3.1 Preparation of competent cells.

All tips and plasticware used in this procedure were aseptic, care was taken to avoid contamination of cells eg all procedures were carried out in the presence of a flame. Bacteria were grown to an OD 550nm = 0.48 in 100ml of LB. The cells were chilled on ice for 5 minutes and then pelleted at 4000rpm for 5 minutes. The pellet was gently resuspended in 40ml of ice cold TFB1 and transferred to two 50ml Falcon tubes. The cells were left on ice for 30 minutes and then pelleted at 4000rpm for 10 minutes. The supernatants were decanted and the pellets gently resuspended in 4ml of ice cold TFB2. The cells were again left on ice for 30 minutes. The cells were then split into 0.6ml aliquots in 2ml Nunc cryo tubes and immediately frozen on dry ice. The cells were stored at -70°C until required for transformation.

2.3.2 Bacterial Transformation

An aliquot of competent cells was removed from the -70°C freezer and placed on ice to melt. 1ng of DNA in 10µl of TE was added to 100µl of competent cells. The cells were then placed on ice for 30 minutes. The cells were heat shocked at 37°C for 4 minutes and then 200µl of LB added to each tube. The samples were incubated at 37°C for 1 hour. The cells were then spread onto LB plates containing 40µg/ml of ampicillin to select transformants. All bacterial plasmids used contained the ampicillin resistance gene.

2.3.3 Small scale plasmid preparation

A 5ml bacterial culture was grown to mid log phase. in LB-AMP 1.5ml of culture was transferred to an eppendorf and cells pelleted at 13000 rpm for 15 seconds. The pellet was resuspended in 75µl of solution 1, 150µl of solution 2 and 90µl of solution 3. The samples were then vortexed vigorously and cell debris pelleted by spinning for 1 minute. The supernatants were removed to fresh eppendorfs containing 1ml of cold 100% ethanol and vortexed. The precipitated nucleic acids were pelleted and resuspended in 25µl of TE, 30µl of 5M LiAc was then added, to precipitate RNA, the samples were vortexed and placed on ice for 3 minutes. Precipitated RNA was removed by centrifugation and the supernatant transferred to an eppendorf containing 0.7ml 100% ethanol. The samples were vortexed , the precipitated DNA was pelleted and washed in 70% ethanol. The DNA was allowed to dry and resuspended in 20µl TE.

2.3.4 Large scale plasmid preparation

Bacterial strains were grown in 100ml of LB-AMP until mid log phase and then split into two 50ml Falcon tubes. The cells were pelleted at 4000rpm for 5 minutes, resuspended in 5ml solution 1 and combined into a single Falcon. To this was added 10ml of solution 2 and 5ml solution of 3. The samples were then vortexed, and cell debris was removed by a 4000rpm spin. The supernatant was removed to a fresh Falcon containing 20ml of isopropanol to precipitate nucleic acids. The precipitated nucleic acids were pelleted and resuspended in 1.5ml TE. 2ml of 5M LiCl was added to each sample, to precipitate RNA, the tubes were vortexed and placed on ice for 5 minutes. After spinning at 4000rpm for 5 minutes the supernatant was transferred to a 15ml Falcon containing 9ml of 100% ethanol and mixed thoroughly to precipitate DNA. The DNA was pelleted, washed in 70% ethanol, resuspended in 0.6ml TE and transferred to an eppendorf. RNase was added to a concentration of 40μg/ml, the samples were incubated at 37°C for 15 minutes and 0.3ml of 20% PEG 8000 in 2.5M NaCl was added to precipitate DNA. The samples were incubated on ice for 5 minutes and pelleted at 13000 rpm for 5 minutes. The pellets were dried and resuspended in 0.6ml TE. Each sample was then extracted twice with equal volumes of chloroform and then twice with phenol, finally the DNA was precipitated by the addition of 1ml of 100% ethanol. The DNA was pelleted, washed in 70% ethanol and resuspended in 0.2ml TE. The concentration of DNA was ascertained by the sample's OD at 260nm as 1 unit = 50μg/ml DNA.

2.3.5 Polymerase Chain Reaction (PCR)

PCR was used for various purposes during this project. One of the main uses was in engineering restriction enzyme sites, to aid in cloning, this was carried out in the following way. The DNA was prepared as described for large scale plasmid preparations. The reactions were carried out in 0.5ml eppendorf tubes. Approximately 0.5ng of plasmid template was used in each reaction. The DNA was resuspended in 100μl buffer containing 0.25mM of each dNTP, 10mM Tris-HCl pH 8.3, 50mM KCl, and 1.5mM $MgCl_2$ and 100pmols of each primer. Each sample then received 0.5μl of Cetus ampliTaq. Finally the reaction mixes were overlaid with mineral oil to prevent evaporation. PCR was carried out in a Techne Programmable Dri-Block PHC-1.

The reaction conditions varied depending on the primers used and the length of DNA being amplified, but they were generally as follows:-

Denature	92°C	1 minute
Anneal	40-60°C	1-2 minutes
Extension	72°C	1-5 minutes

Repeat cycle for 25-40 times. When finished 1 final 10 minute extension was carried out.

PCR was also used in sequencing genes directly from *S.pombe*. This was carried out using two primers one of which was biotinylated (Hultman, et al., 1989). *S.pombe* cells were taken from fresh plates and resuspended in 30µl TE containing 1% TX-100. The cells were lysed by heating at 95°C for 5 minutes and vortexed vigorously. 10µl samples were used in each 100µl reaction exactly as outlined above. In general, however, the extension times used were longer than for purified DNA and the maximum length of product was limited to around 2Kb.

2.3.6 Purification of DNA

During cloning DNA fragments often needed to be separated and purified. DNA amplified by PCR was also purified by this method. DNA was electrophoresed through agarose gels containing 1 x TAE and 1µg/ml ethidium bromide. The gels contained between 0.7 and 2.5% agarose depending on the size of fragments being separated. The gels were run in 1 x TAE buffer. at a constant voltage of 120V in LKB GNA-100 gel boxes. Lambda DNA digested with *Bst* X1 was used as molecular weight markers (Amersham). The DNA was visualised under UV and the section of gel containing the DNA was excised and placed in a 1.5ml eppendorf. The DNA was then purified using the Geneclean II kit (Bio 101, La Jolla California USA). This involved adding 0.5ml NaI to each gel slice , which were then incubated at 56°C for 10 minutes. The samples were allowed to cool for 3 minutes. The cooled samples were vortexed and 30µl Glassmilk was added to each sample. They were then incubated at room temperature for 10 minutes. Bound DNA was brought down in a 13000 rpm for 10 seconds, and the supernatant was removed. The pelleted DNA was then washed three times in 1ml NEW WASH and finally resuspended in 50µl of distilled water. The sample was incubated at 56°C for 10 minutes and spun for 20 seconds. The supernatant, containing the DNA, was transferred to a fresh 1.5ml eppendorf.

2.3.7 DNA Sequencing

Sequencing of PCR fragments was carried out as follows. The DNA was resuspended in 80µl of water after purification. 30µl of avidin coated magnetic beads (Dynabeads, Dynal UK) were prepared. The beads were pulled down using a magnetic block (Magnetic Particle Concentrator, Dynal UK), the beads were never vortexed at any stage, the supernatant was removed and the beads washed 3 times in 0.5ml in SEQ1. The beads were resuspended in 30µl of SEQ1 and added to the DNA. The DNA was incubated at 28°C for 10 minutes and then agitated at room temperature for 25 minutes. A further 100µl of SEQ1 was added, the beads were then pelleted using the magnetic block and the supernatant removed. The beads were washed again in 100µl SEQ1 and then 0.1ml of 0.15M NaOH added. The beads were incubated at 28°C for 10 minutes, pelleted as before and the supernatant removed. The NaOH treatment was repeated and then the beads were washed 4 times in 0.1ml of water. Finally the beads were resuspended in 14µl of water. Sequencing was carried out using Sequenase 2.0 kit (USB). 7µl was used in each sequencing reaction. To each sample 2µl of 5 x SEQ MIX ((USB): 200mM Tris pH7.5, 100mM $MgCl_2$ and 250mM NaCl)was added. and 2µl of sequencing primer (0.5pmols/µl.). The samples were heated at 70°C for 2 minutes and allowed to cool slowly to less than 35°C. Next 1µl of 0.1M DTT was added , 2µl of G mix, 2µl of Sequenase (diluted 1:5 from stock) and 0.5µl of S^{35} dATP was added to each sample. The samples were then incubated at room temperature for 5 minutes and 3.5µl aliquots of each were added to 4 tubes containing 2.5µl of each termination mix. The termination mixes each contained all 4 dNTP's and one dideoxynucleotide. The samples were incubated at 37°C for 10 minutes. The beads were then brought down using the magnetic block. The beads were then resuspended in 4µl stop solution and incubated at 37°C for a further 15 minutes, finally 3µl of water was added to each sample.

The samples were now ready for running through acrylamide gel. The gel was prepared by mixing 40ml of 6% acrylamide with 175µl 10% ammonium persulphate and 20µl TEMED. The gel was set using wedge spacers and run using 1 x TBE buffer at a constant power of 40 Watts using a Pharmacia LKB 2197 power supply. The gel was pre run for 30 minutes prior to loading of samples. The samples were heated at 75°C for 2 minutes just before loading and then immediately placed on ice. Samples were then loaded, either 2.5µl or 4µl depending on well size. The gel was run between 2 and 4 hours and then fixed in a litre of 10% methanol and 10% glacial acetic acid for 15 minutes. The gel was then transferred to Whatman 3MM paper and dried for 1 hour

before being exposed to photographic film overnight.
Plasmid DNA was also sequenced. 5µl of plasmid DNA was incubated with 4µl 1M NaOH, 0.8µl 5mM EDTA and 10.2µl water. The samples were vortexed and incubated at room temperature for 10 minutes. 2µl of 2M NH_4Ac was added to each sample, which were then vortexed. Next 60µl of 100% ethanol was added and again the samples were vortexed. The samples were incubated on dry ice for 5 minutes and the precipitated DNA pelleted by spinning at 13000rpm for 10 minutes, the pellet was washed in 70% ethanol and dried thoroughly. The sample was resuspended in 7µl of water and the procedure, from this stage, was exactly as described above. Except at the final stage were stop solution was added directly to the samples and no additional water was required.

2.3.8 Southern Blotting

This technique was used both when identifying gene fragments from an *S.pombe* library and also when examining strains to identify whether or not they were homologous recombinants. DNA was digested with the appropriate restriction enzyme at 37°C, The digested DNA then had 5 x Ficoll added to give a final of concentration of 1 x Ficoll. The DNA was run through agarose gels as previously described, fresh 1 x TBE buffer was always employed. The gel was then photographed under UV light against a ruler, to allow sizing of fragments. The gel was depurinated, to allow DNA transfer, in 400ml depurinating solution for 10 minutes. The gel was rinsed in water and denatured twice in 200ml denaturing solution for 15 minutes. The DNA was then transferred as follows. Two pieces of 3MM, the same size as gel, were soaked in denaturing solution and placed on a clean glass plate, above this was a piece of Genescreen filter (Amersham, UK) which had been soaked first in water then in denaturing solution, the gel was placed on top of the filter, then two more pieces of 3MM and finally a stack of dry tissues. A glass plate was placed on top and after 30 minutes a weight was put on the plate, the DNA was transferred overnight. The Genescreen was then marked to indicate the position of wells to allow oriented. The filter was washed in 50mM PO_4^- for 10 minutes and placed on a dry piece of 3MM. Next the filter was baked at 80°C for 2 hours and then the DNA crosslinked by exposure to UV light.
The probe for the filter was prepared thus: 60ng of DNA was resuspended in 11.6µl of water in an 0.5ml eppendorf and incubated at 95°C for 5 minutes, before being transferred to ice. The tube was now placed behind radioactive screens and 11.4µl of LS solution added along with 2µl 5mg/ml BSA, 0.5µl of Klenow fragment and 5µl ^{32}P dATP. The sample was incubated at 37°C for 2

hours, whilst covered with a lead pot. The labelling was stopped by the addition of 1µl 0.5M EDTA, 1µl tRNA 5mg/ml, 5µl 3M NaAc pH 5.6 and 50µl isopropanol. The sample was incubated on ice for 5 minutes and precipitated DNA spun down for 10 minutes at 13000rpm. The supernatant was removed and the pellet dried and resuspended in 100µl of water.
The filter was now probed, it was prehybridised for 1 hour , in 10ml church buffer, at 65°C in a sealed bag. 30µl of the radioactive probe was heated at 95°C for 5 minutes and then added to 5ml church buffer in a 15ml Falcon. The probe was added to the filter and the bag resealed. The bag was then placed inside a second bag, for safety, and then in a box and incubated in a 65°C waterbath overnight. The filter was washed in SB1 buffer twice at room temperature and twice at 65°C for 15 minutes. The filter was then air dried, transferred to a piece of 3MM and exposed to photographic film at -70°C.

2.3.9 Cloning from a Lambda library (Sambrook, et al., 1989)
Ypt genes were cloned from a lambda phage DNA library. The appropriate restriction enzyme fragments were identified by Southern blotting and cloned into plasmids. 10µl of lambda DNA was incubated with 10µl BSA (1mg/ml), 10µl React buffer (GibCo BRL), 10µl spermidine(10mM) and 10µl of restriction enzyme (GibCo BRL) 10 units/µl, in a total volume of 0.1ml. This was incubated at 37°C for 2 hours. The DNA fragment was then purified on agarose gels as described previously.

2.3.10 Cloning (Sambrook, et al., 1989)
The plasmid was prepared by restriction enzyme digest, approximately 1µg of DNA was digested with 10 units of enzyme, in a total volume of 20µl, at 37°C for 1 hour. For single enzyme digests the plasmid was dephosphorylated by the addition of 5µl alkaline phosphatase 1unit/µl (Boehringer Mannhiem, UK), 5µl buffer and 20µl water. The DNA was incubated for 1 hour at 37°C. Dephosphorylation was stopped by the addition of 0.5µl 1M EDTA, the sample was then heated to 70°C for 10 minutes. The samples were extracted with 60µl of phenol/chloroform and the aqueous phase transferred to a fresh eppendorf. The DNA was precipitated by the addition of 5µl 3M NaAc and 0.2ml of 100% ethanol. The DNA was pelleted and resuspended in 0.1ml of water to give a final concentration of approximately 10ng/µ l. If the plasmid was cut with more than one restriction enzyme then there was no need to dephosphorylate, however, the plasmid must be gel purified. The plasmids were now ready for ligation.

2.3.11 Ligation

In general 3 fold molar excess of linear insert was used over the plasmid vector. 10ng of vector (1µl) was added to 3µl of insert, 2µl of ligase buffer and 1µl T4 DNA ligase (GibCo-BRL) in a total volume of 10µl. The ligations were incubated at room temperature for 1-2 hours. The ligation mix was then used to transform competent cells. If the vector used contained the Lac Z gene then, prior to plating, 25µl 100mM IPTG and 25µl X-Gal, in dimethylformamide, were added to allow selection of recombinant clones.

2.3.12 Hydroxylamine Mutagenesis

Random mutagenesis of linear DNA was executed using hydroxylamine, a compound which induces C - T transitions. 0.35g of hydroxylamine and 0.09g of NaOH were diluted in 5ml water, this was done at start of each experiment. 200ng aliquots of linear DNA were incubated with 0.5ml of hydroxylamine solution at 67°C for varying lengths of time between 20 and 60 minutes. The DNA was then precipitated by the addition of 10µl BSA (5mg/ml), 10µl 5M NaCl and 1ml 100% ethanol. The precipitated DNA was then spun down and washed twice in 70% ethanol. The DNA was resuspended in 0.3ml of a 3M NaAc/ ethanol mixture (1/10). The DNA was once again spun down and washed twice in 70% ethanol. The DNA was then resuspended in 10µl of TE and used to transform yeast.

2.4 *S.pombe* strains

Strain	Genotype	Reference
556	Haploid (WT) ade6-216 ura4-D18 leu1-32	P. Nurse
611	Diploid (WT) ura4-D18/ ura4-D18 ade6-216/ ade6-210 leu1-32/ leu1-32	P.Nurse

The following strains are all as 556 and were created in this study.

Strain	Phenotype
CSCypt5	ade$^-$ leu$^-$ ura$^+$ - contains WT *ypt5* gene linked to *ura4*
CSSypt5	ade$^-$ leu$^-$ ura$^+$ - contains a modified *ypt5* gene
SSCypt5	ade$^-$ leu$^-$ ura$^+$ - contains a modified *ypt5* gene

CSSypt5::ypt5	ade$^-$ leu$^+$ ura$^+$-as CSSypt5 but with WT *ypt5* cDNA on plasmid
SSCypt5::ypt5	ade$^-$ leu$^+$ ura$^+$-as SSCypt5 but with WT *ypt5* cDNA on plasmid
ypt1-1	ade$^-$ leu$^-$ura$^+$- TS, modified *ypt1* gene linked to *ura4*
ypt2-1	ade$^-$ leu$^-$ ura$^+$-TS, modified *ypt2* gene linked to *ura4*

Various strains were created from both *ypt1-1* and *ypt2-1*. These strains were ade$^-$ leu$^+$ ura$^+$ and had various growth phenotypes as described in the text.

2.5 Oligonucleotides used in study

Y5X2	5'-GGATCCGGATCCACAAGAGAAAGCCTATTCCC-3'
UOUT	5'-GGATCCGGATCCAAAAAGTTTCGTCAATATCAX-3'
Y2-3'	5'-GTGATTGTGAGGATCAGCGTC-3'
Y2-EXT	5'-GGATCCGGATCCTGTACATTAAGTCCAACCTGA-3'
Y2SPE	5'-GGATCCACTAGTACCATTTATACAATA-3'

Y2VNDOWN

5'-AAGACATGATTTTCCATTACCTGAATCTCCTATGAGAA-3'

Y2CC

5'-GGATCCGGTACCCTAACAACACCTCTTCACCGTACGATCG-3'

Y2VNUP

5'-ATAGGAGATTCAGGTAATGGAAAATCATGTCTTCTTCT-3'

YPT1 5' Bam

5'-GGATCCGGATCCGCATATACGATGAATCCAGAG-3'

YPT1 3' Bam

5'-GGATCCGGATCCGCGCATTCAGAGGTATAACC-3'

Chapter 3

Ypt5

3.1 INTRODUCTION

ypt5 was identified by screening an *S.pombe* genomic library with degenerate oligonucleotides (Armstrong, et al., 1993). The cloned gene was sequenced by both Sally Bowden and Deborah Gurr, and they found that the protein encoded by the gene had 63% identity to the recently identified mammalian rab5p (Zahraoui, et al., 1989). The predicted protein did not show a high degree of homology to either of the known ypt proteins from *S.cerevisiae*. As I discussed in the introduction the rab5p had been localised to early endosomes by Chavrier and co-workers (Chavrier, et al., 1990b). This led to the hypothesis that the *S.pombe* ypt5 protein would also operate in endocytosis. Again as discussed in the introduction Zerial and colleagues went on to demonstrate that the rab5p was involved in transport from the plasma membrane to the early endosomes (Bucci, et al., 1992; Gorvel, et al., 1991).

To determine if the ypt5p was essential a knock out experiment was carried out, by Sally Bowden and John Armstrong, in which a diploid strain of *S.pombe* had one of its copies of *ypt5* disrupted. The strain was then sporulated and examined by tetrad dissection. This revealed that although the *ypt5*$^-$ progeny failed to grow on minimal media they would grow, albeit very slowly, on rich YE media. Examination of cells lacking ypt5p by electron microscopy (carried out by Rose Watson), revealed that the cells had grossly altered morphology, in particular they accumulated membranous structures (Armstrong, et al., 1993). These results along with the strong homology to the rab5p led to the hypothesis that ypt5p was involved in endocytosis in *S.pombe* and that under most growth conditions endocytosis is an essential process.

Ypt5p was expressed in HeLa cells (by John Armstrong) and found to co-localise with the transferrin receptor by indirect-immunofluorescence (Armstrong, et al., 1993). This indicated that the ypt5p was being targeted to the early endosomes. This was further evidence that the ypt5p was the functional homologue of the rab5p. Interestingly the homology between rab5p and ypt5p is not consistent throughout the length of the two proteins, with the extreme N and C termini showing the lowest degrees of conservation. As I discussed in the introduction Chavrier and colleagues argue that the localisation of a rab protein is determined by its C terminus (Chavrier, et al., 1991). The lack of homology between the rab5p and ypt5p at their C termini again indicates that localisation is not determined by the primary sequence of the C termini but presumably by a secondary structural motif.

The aim of my work on the ypt5p was to investigate the function of the protein in more detail, in the hope that this would allow us to test our hypothesis that the ypt5p was indeed involved in endocytosis. The initial intention was to develop a system which would allow the replacement of the endogenous *ypt5* gene with a recombinant copy linked to a selectable marker. The replacement was to be carried on a haploid strain to allow one step gene replacement. Once the system was in place it could be utilised to produce strains containing randomly mutated copies of the *ypt5* gene. Alternatively the system could be used to examine the effects of specific point mutations on the function of the ypt5 protein *in vivo*. It was hoped that initially this would lead to a better understanding of the role of ypt5p, and in the longer term allow the study of endocytosis in *S.pombe*, if indeed that is the stage of transport at which ypt5p operates.

RESULTS

3.2 GENETIC MANIPULATION

3.2.1 Gene replacement

The coding sequence and the 5' upstream region of *ypt5* were amplified by polymerase chain reaction (PCR) from the plasmid pYPT5 (Armstrong, et al., 1993) and cloned into pBSM13$^-$, which had had its *NdeI* site removed by *NdeI* digestion followed by blunt ending and re-ligation, to form pY5M13. The 3' non-coding region of *ypt5* and the *S.pombe ura4* gene (Grimm, et al., 1988) were then both amplified and joined by PCR, and finally cloned into pY5M13 to give pY5U4 (Fig. 3.1). The pY5U4 plasmid therefore had a 5.1kilobase (kb) insert containing the *ypt5* gene linked to the *ura4* gene.

The insert was excised by digestion with the restriction enzymes *SphI* and *SstI*. After digestion the insert was purified, to remove any uncut plasmid, and used to transform 556, a haploid strain of *S.pombe*. 556 lacks the *ura4* gene and is in fact ade$^-$ leu$^-$ ura$^-$. A complete deletion of the endogenous *ura4* gene was required to ensure that the linear insert did not recombine with the *ura4* gene. If for example a strain of yeast which had only a partial deletion of the *ura4* gene was used, then recombination with the *ura4* gene on the linear insert would result in yeast which were ura$^+$ but still contained the endogenous *ypt5* gene. Transformants were plated on minimal media supplemented with adenine and leucine but lacking uracil in order to select for recombinants.

In order to produce random mutants a high rate of homologous recombination was thought to be required as only a fraction of all the homologous recombinants would have been mutated and of course any non-homologous mutants would not demonstrate a phenotype due to the presence of the endogenous WT gene, unless the mutated gene was a dominant conditional mutant as has been seen for the YPT1-1 allele in *S.cerevisiae* (Schmitt, et al., 1988). The rate of homologous recombination was initially ascertained by PCR. Twenty transformants were selected at random and streaked onto fresh minimal plates, after 2 days an aliquot of cells were examined by PCR, using one oligonucleotide which recognised a region close to the C terminus of *ura4* and a second which bound downstream of the site of recombination (Fig. 3.2). The results of a typical screening are shown in Fig. 3.3. The results obtained by PCR were independently checked by Southern blot analysis using a probe which binds downstream of the site of recombination (Fig. 3.1)

Fig. 3.1 pY5U4 Insert and Replacement

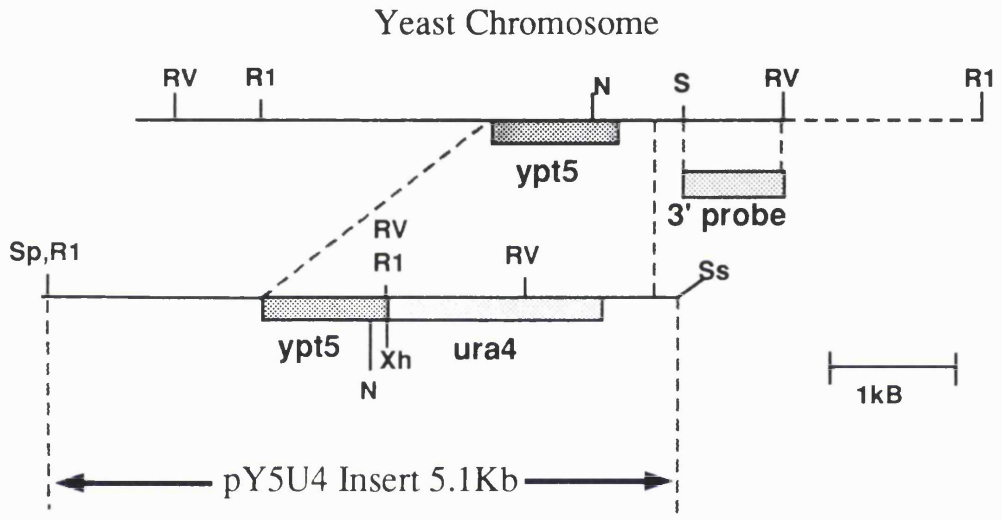


Fig. 3.1
The pY5U4 insert has the *ura4* gene cloned approximately 40bp downstream of the *ypt5* coding sequence.
The region of homologous recombination between the endogenous gene and the linear insert is shown.
Important restriction sites are marked. The enzymes are as follows: N- Nde1, R1- Eco RI, RV- Eco RV, S- Sal I Sp- Sph I, Ss- Sst I and Xh-Xho I

KEY

ypt5 coding sequnce

ura4 gene

hybridisation probe

Fig. 3.2 Homologous recombination

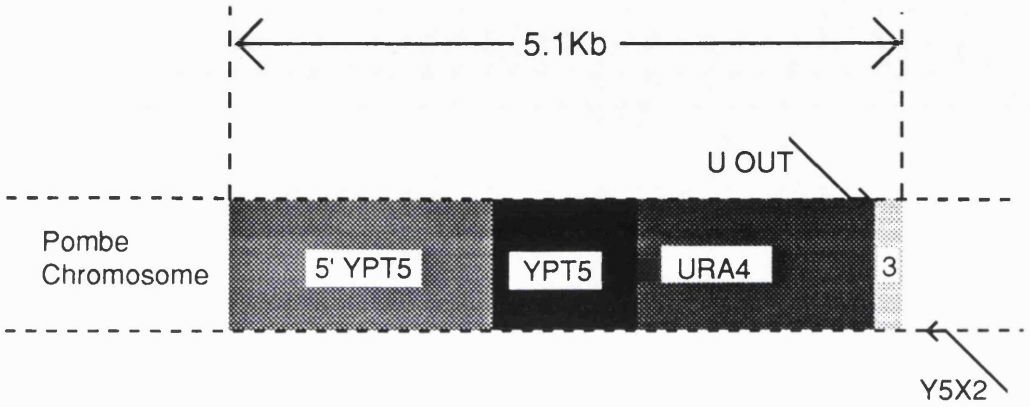


Fig. 3.2
Recombination was checked by PCR using U OUT, which recognises a region at the 3' end of the ura4 gene, and Y5X2, which binds outwith the region of recombination. Homologous recombinants will therefore give a 650bp product by PCR, whilst WT and non-homologous recombinants will give no product.

Fig. 3.3 PCR analysis of transformed yeast

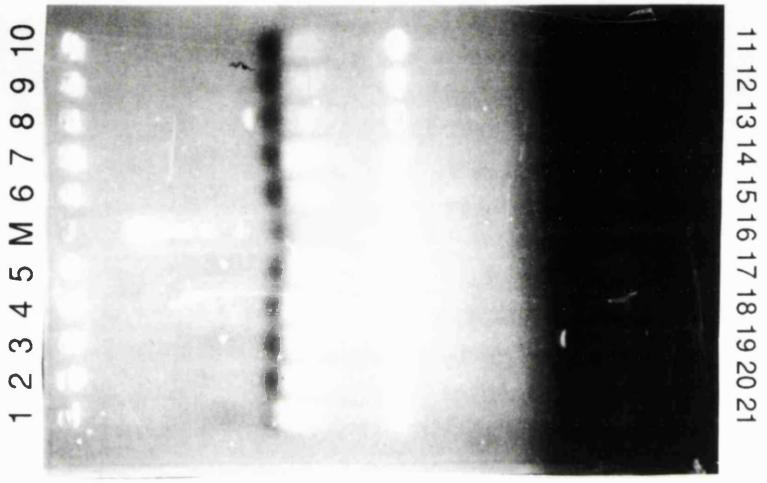


Fig. 3.3
Yeast were transformed with a linear DNA fragment containing the *S.pombe ura4* gene. Transformants were selected on media lacking uracil. In the above experiment 21 yeast colonies were randomly selected and streaked onto fresh plates. After two to three days single colonies were analysed by PCR, as described in materials and methods, using the Y5X2 and UOUT oligonucleotides (Fig. 3.2).The PCR products were then separated on a 1% agarose containing ethidium bromide. The DNA was visualised by U.V. light.
In the above two of the colonies in lanes 8 and 19 gave a positive band of approximately 650bp. The strains were later analysed by Southern blotting to confirm that they were homologous recombinants. The marker lane (M) contained λ phage DNA which had been digested with Bst X1 restriction enzyme (Amersham). The smallest marker visible is 702bp in length.

The rate of homologous recombination was found to be approximately 10% of the total number of viable transformants. This rate of recombination was felt to be too low to allow selection of conditional mutants after random mutagenesis. One problem with random mutagenesis is that the *ura4* gene will also be mutagenised and therefore a high percentage of any conditional mutants discovered would presumably be in the *ura4* rather than the *ypt5* gene. A higher rate of *ura4* conditional mutants was predicted due to the lack of the endogenous *ura4* gene. In the absence of an endogenous gene all of the conditional mutants of the *ura4* gene would express their phenotype, whereas in 90% of cases *ypt5* conditional mutants would have their phenotype masked by the expression of the endogenous *ypt5* gene (assuming a 10% rate of homologous recombination). In addition as the yeast are selected for the presence of the *ura4* gene, this selects for strains in which the gene has completely recombined, whereas the *ypt5* gene on the insert may have only partially recombined with the endogenous *ypt5* gene so the actual rate of complete *ypt5* recombinants would be much lower than 10%. We would predict that another consequence of our screening method would enrich for *ypt5* alleles altered towards the 3' region of the gene. The low rate of homologous recombination did not, however, preclude replacement of the endogenous gene with specifically point mutated copies of the *ypt5* gene.

The stability of the transformed strains had to be checked, that is how often the transformed strain spontaneously lost the *ura4* gene and regained a ura$^-$ phenotype. To check stability one homologous recombinant was selected at random and allowed to grow under non-selective conditions. Yeast cells were then spread out on plates containing 5-fluoroorotic acid (FOA), which is an analogue of a uracil precursor. Cells containing the *ura4* gene express the enzyme orotidine -5'-phosphate decarboxylase and they cease to grow in the presence of FOA (Boeke, et al., 1984). It is not clear why the cells cease to grow but it is thought that the FOA is converted into 5-fluoro-UMP and eventually into fluorinated ribonucleotides and deoxynucleotides. Out of 1 x 10^6 cells plated onto media supplemented with 1mg/ml of FOA all failed to grow which indicates that the homologous recombinants are stable and therefore could be used as the basis of future study.

3.2.2 Modification of C termini

As discussed in the introduction all ypt and rab proteins receive large isoprenoid groups as a post translational modification. The twenty carbon geranylgeranyl (GeGe) groups are added to highly conserved cysteine residues at the C terminus of the proteins. Our group had for some time been working in collaboration with Dr A. Magee's group studying the modifications of the *S.pombe* ypt proteins. It was

shown that ypt1, 3 and 5 proteins all received geranylgeranyl groups both *in vitro* and *in vivo* (Newman, et al., 1992). Further it was shown that ypt5p is methylated *in vivo*. What was not clear was whether ypt5p, which terminates in cysteine-serine-cysteine (CSC), is modified on one or both cysteine residues and whether mutants altered at the C terminus will be functional in *S.pombe*.

To begin to answer these questions it was necessary to produce a series of singly and doubly mutated *ypt5* cDNA's. This was carried out by Chris Newman (a Graduate student in Dr Magee's laboratory), who engineered *ypt5* mutants in which either one or both of the cysteine residues was replaced with a serine residue. The recombinant and WT *ypt5* cDNA's were termed pEXV-YPT5 CSC, CSS, SSC and SSS depending upon the C terminal sequence (Giannakouros, et al., 1993).

The plasmids which had been created all had the *ypt5* cDNA rather than the genomic copy of the gene. Therefore plasmids which contained a mutated copy of the *ypt5* gene linked to *ura4* had to be created. To do this the C terminal 225bp *Nde1-Xho1* fragment of pY5U4 (Fig. 3.1) was replaced by the corresponding *Nde1-Sal1* fragment from each of the pEXV-YPT5 plasmids. The plasmids created were termed pY5U4-CSC, CSS, SSC and SSS. The newly created plasmids were all sequenced to confirm the absence of any further mutations.

3.2.3 Haploid transformation

The pY5U4 plasmids containing the mutated *ypt5* genes were now used in gene replacement experiments as described above. A parental 556 strain was transformed with linear inserts from each of the plasmids. Transformants were selected on minimal media lacking uracil and screened by PCR. Of the transformants screened, approximately thirty for each of the four inserts, none had homologously recombined. This was an unexpected result especially for the wild type CSC control. From previous results I would have predicted two to four homologous recombinants per insert providing the protein encoded by the mutant gene was functional. It is possible that the homologous recombinants were growing much more slowly than the non homologous recombinants, or that the slightly different insert had a lower rate of homologous recombination and the location of homologous recombinants would require the screening of many more transformants.

3.2.4 Diploid transformation

To circumvent any possible problems in growth rate or viability, a diploid strain of *S.pombe*, 611, was transformed with the various *ypt5* constructs. Resultant recombinants contained one WT copy of the *ypt5* gene. The production of a double homologous recombinant was deemed unlikely due to the low rate of homologous transformation. The only circumstance which would have resulted in the failure to produce homologous transformants would have been if the mutant *ypt5* gene had displayed a dominant negative phenotype. A dominant gene is one that displays its phenotype in the presence of the WT gene. However, a dominant mutant which resulted in cell death would give no transformants whatsoever and would therefore have been easily detectable.

As mentioned earlier *S.pombe* are normally haploid but they can be forced to be diploid by mating two haploid strains with different defects in the adenine biosynthetic pathway. The resultant diploid will be capable of synthesising adenine and can therefore be kept diploid by growing on media lacking adenine. The 611 strain is ade^{+} ura^{-} leu^{-}

611 was transformed with each of the four inserts and transformants selected by growth on minimal media supplemented with leucine. The transformants were screened as before and in this case homologous recombinants were recovered (Table 3.1). The rate of homologous recombination varied tremendously between the four inserts. Whether the difference was significant was not clear and I would have had to screen many more transformants to allow this to be determined with any accuracy.

Table 3.1 Rate of Homologous Recombination

YPT5 Plasmid	No. of homologous recombinants	No. of non-homologous recombinants	Rate of homologous recombiation (%)
-CSC	2	48	4
-CSS	2	18	10
-SSC	7	22	24
-SSS	4	26	13.3

Table 3.1
A diploid strain (611) was transformed with linear inserts containing recombinant *ypt5* genes. The *ypt5* genes had been altered at their C termini and were named according to the final three amino acids they possessed. Transformants were screened by PCR to identify homologous recombinants.

3.2.5 Viability of mutants

The diploid homologous recombinants could now be examined to ascertain whether the mutant ypt5 proteins were capable of supporting growth of yeast cells or not. It must be noted that, strictly speaking *ypt5*, is not an essential gene. However, cells lacking the ypt5p will not grow on minimal media and under these conditions *ypt5* can be considered to be essential. The diploid strains were examined by a process termed Random Spore Analysis. The diploids were made to undergo meiosis and to produce haploid spores by growth on media lacking nitrogen. Of the four haploid spores produced by each diploid two should contain the WT *ypt5* gene and also be ura$^-$ whilst the other two progeny will contain the recombinant gene linked to *ura4* and therefore be ura$^+$.

Spores were released from their ascii, plated onto rich media and allowed to germinate. After the formation of colonies the transformants were replicated onto plates either lacking or supplemented with uracil. If the recombinant ypt5 protein was not capable of supporting growth the progeny would have been ura$^-$ in phenotype. However, if the recombinant ypt5p was functional the progeny would have been predicted to be 1: 1 ura$^+$: ura$^-$. The results of the Random Spore Analysis are given in Table 3.2. The diploids containing the control CSC and the two singly mutated copies of the ypt5 protein terminating CSS and SSC, all gave an approximately 1: 1 ratio indicating that the singly mutated copies of ypt5 protein were functional. However, the strain containing ypt5-SSSp had less than 1% ura$^+$ progeny indicating that a ypt5 protein lacking both C terminal cysteine residues was non-functional. Southern blotting of diploid strains confirmed that they contained one WT and homologously recombined copy of the *ypt5* gene (Fig. 3.4). Ura$^+$ haploid progeny of the diploid strains were also examined to confirm that they contained only a single copy of *ypt5* and that this copy was an homologous recombinant (Fig. 3.4).

Table 3.2 Random Spore Analysis

STRAIN	URA+ Progeny	URA- Progeny
-CSC	28	40
-CSS	35	36
-SSC	28	32
-SSS	1	157

Table 3.2
Diploid strains, which had had one of their endogenous *ypt5* genes replaced with a recombinant version, were examined by random spore analysis to determine the functional ability of the altered ypt5 protein. The strains were named depending on which recombinant *ypt5* gene they possessed. The haploid progeny were screened to determine whether they could grow in the absence of uracil= ura^+ which indicate that the recombinant ypt5 protein was functional.

Fig 3.4 Southern blot analysis of ypt5 replacement strains.

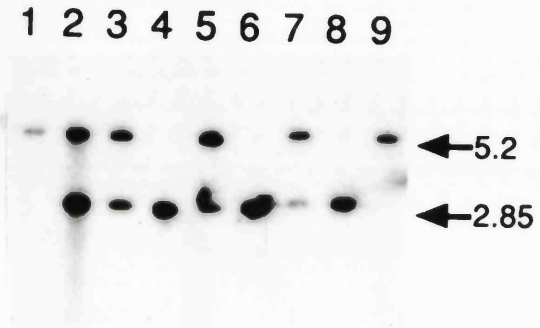


Fig. 3.4

Haploid and diploid strains were analysed by Southern blotting. Total DNA was digested with *EcoR I* and *Eco RV* . The digested DNA was probed as shown in Fig. 3.1. The WT gene gave a 5.2Kb product whilst the homologous recombinant gave only a 2.8Kb product. The results clearly show that the diploid strains contained two copies of *ypt5*, one of which is recombinant, whereas the ura$^+$ haploid strains analysed had only a single copy of *ypt5* which was recombinant.

Lanes. 1: 611 (parental diploid). 2: SSS diploid. 3: CSC diploid. 4: CSC haploid. 5: CSS diploid. 6: CSS haploid.

7: SSC diploid. 8: SSC haploid. 9: 556 (haploid WT)

3.3 Analysis of mutant strains

The results obtained by Random Spore Analysis indicated that the ypt5 protein was functional with only a single cysteine residue at or near its C terminus. These results are similar to those obtained in studies on both yptlp and sec4p from *S.cerevisiae* (Molenaar, et al., 1988; Walworth, et al., 1989). Work done by our collaborators confirmed that the doubly mutated ypt5 protein, which terminates SSS, was not modified *in vivo* or *in vitro* (Giannakouros, et al., 1993). It was also discovered that the singly mutated forms of ypt5p only received 50% of the GeGe groups compared to WT ypt5p, per cysteine residue. These results indicated that both of the cysteine residues in the WT protein were modified with GeGe moieties.

3.3.1 Localisation of ypt5 protein

(i) Mammalian Cells

As I stated in the introduction the function of isoprenoid groups is to increase the hydrophobicity of the protein being modified. The modified protein will therefore be capable of membrane association. We wished to investigate the effect of the various mutations on the partitioning of the ypt5 proteins between the membrane and the cytosol.

Our collaborators transfected COS cells with both mutant and WT *ypt5* cDNAs. The distribution of the ypt5p in the various transfectants was then examined by fractionation and Western blotting, using a polyclonal antibody raised to a C terminal peptide of the ypt5p (Newman, et al., 1992), the antibody was reported to specifically recognise the ypt5 protein. The Western blotting demonstrated that less than 10% of each singly mutated ypt5 protein was associated with membranes whereas approximately 50% of WT protein was membrane associated (Giannakouros, et al., 1993). This indicated that the singly mutated copies of the ypt5 protein were less tightly membrane associated *in vivo*.

(ii) *S.pombe*

The distribution of the ypt5 proteins in the various haploid strains was examined. The strains were named according to which ypt5 protein they possessed, for example, SSCypt5. A cell fractionation experiment allowed the distribution of the ypt5 protein to be ascertained. After removal of whole cells and nuclei from *S.pombe* cell extracts, the remainder of the extract was then divided into membrane and cytosol fractions by centrifugation. An 100,000g spin for one hour resulted in a pellet which contained membrane associated proteins (P100), with the supernatant containing the cytosolic proteins (S100). The protein from both samples was precipitated and resuspended in 2 x sample buffer (SB). The P100 and S100 protein fractions were run through a 12% acrylamide gel. The proteins were transferred to a nitrocellulose filter and Western blotting was carried out using an anti-ypt5 antibody (diluted 1: 5000) (Fig. 3.5). Ypt5p has an approximate MW of 23kDa although the main form of the ypt5 protein had a slightly greater MW on the gel (Fig. 3.5). Breakdown products of the ypt5 protein can be seen, especially in the cytoplasm. It has been reported that ypt/rab proteins are rapidly degraded by *S.pombe* cytosol (Giannakouros, et al., 1993).

Fig 3.5 Western Blot Analysis of ypt5 protein's distribution

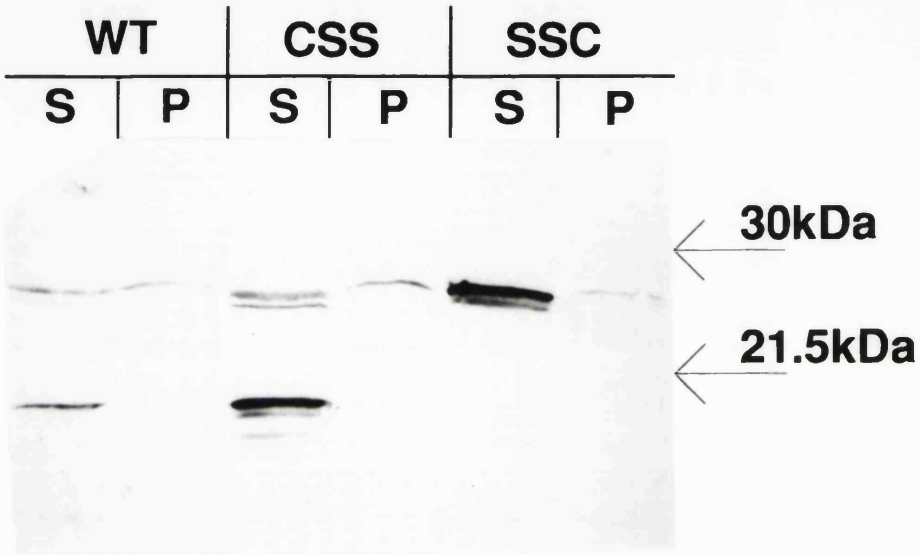


Fig. 3.5
Haploid strains were fractionated into pellet (P) and supernatant (S) by an 100,000g spin. The protein was precipitated and separated by SDS-PAGE. The proteins were transferred to nitrocellulose and blotted with an anti-ypt5 antibody. MW's shown represent standard proteins Carbonic anhydrase (30kDa) and Trypsin inhibitor (21.5kDa).

3.4 Growth properties of mutant strains

The two haploid strains, SSCypt5 and CSSypt5, were next examined to determine whether they displayed any growth defects. All of the following experiments were carried out in minimal media which had been supplemented with adenine and leucine and the growth properties were determined solely by visual inspection of the cultures.

The strains were grown at the permissive temperature of 30°C prior to be shifted to other temperatures. Both of the mutant strains were capable of growing at 37°C In addition both strains were capable of growing at 18°C both in liquid and on plates. It was observed that on a number of occasions the SSCypt5 strain failed to grow after storage on plates which had been incubated at 4°C.

The growth of the strains was next observed at 14°C. Initially observations indicated that the strains would not grow at this temperature. It was observed if the strains were transformed with a pSM^- plasmid containing the *ypt5* cDNA then they were capable of growth at 14°C. The cDNA was cloned by Vas Ponnambalam who sequenced it to ensure there were no point mutations (V. Ponnambalam pers. comm.).

Initially this experiment was carried out by growing the cells to stationary phase at the permissive temperature of 30°C. The cells were then diluted into fresh media and split, half being placed at 14°C and the rest remaining at 30°C. However, if the experimental procedure was changed so that after dilution the cells were allowed to grow at 30°C for a further four hours, prior to shifting to 14°C, a different picture emerges. Under these growth conditions both the CSSypt5 and SSCypt5 strains were seen to grow at 14°C.

3.5 Morphological Analysis of CSSypt5 strain

After observing that the stains containing the mutant ypt5 proteins were apparently not growing at 14°C the CSSypt5 strain was examined by electron microscopy. The CSSypt5 strain transformed with the pSM$^-$ plasmid containing the *ypt5* cDNA. The observation was only carried out on the CSSypt5 strain as initially the SSCypt5 strain failed to transform. The original transformation procedure used was the LiCl method (Broker, 1987) which involves a heat shock at 46°C for 25 minutes, the SSCypt5 strain failed to give any viable transformants using this method. Later a gentler method of transformation using LiAc (Okazaki, et al., 1990) was employed. In the LiAc method the heat shock is at 43°C for 15 minutes. This method of transformation was successful with the SSCypt5 strain.

The CSSypt5 strain and the same strain also containing the WT *ypt5* cDNA (CSSypt5:YPT5) were grown to stationary phase in minimal media at 30°C. The cells were then diluted with fresh media and split into two cultures. One culture was incubated at 30°C the second at 16°C for 12 hours. The cells were fixed using potassium permanganate and examined by electron microscopy (all electron microscopy was carried out by Rose Watson). Typical cells are shown in Fig. 3.6.

At 30°C the CSSypt5 (B) strain is very similar to the parental 556 strain (A) except that the vacuoles appear to be slightly more fragmented than is normally seen. However, the CSSypt5 strain which had been shifted to 16°C (C) were very different from control cells, as it now had a population of large membrane enclosed structures. The structures could easily be distinguished from mitochondria as their membranes were much more densely stained. The material, within the structures, stained more heavily than the nuclear material indicating that the structures were not part of the nucleus. In the control strain CSSypt5:YPT5 (D) these structures were not seen indicating that their presence was due, whether directly or indirectly, to the mutant copy of *ypt5*. The observations were based purely on visual inspection. Quantification of cell size or level of internal membranes would need to be carried out to precisely characterise the morphological alterations occurring in the mutant strain.

Fig. 3.6 Analysis of CSSypt5.

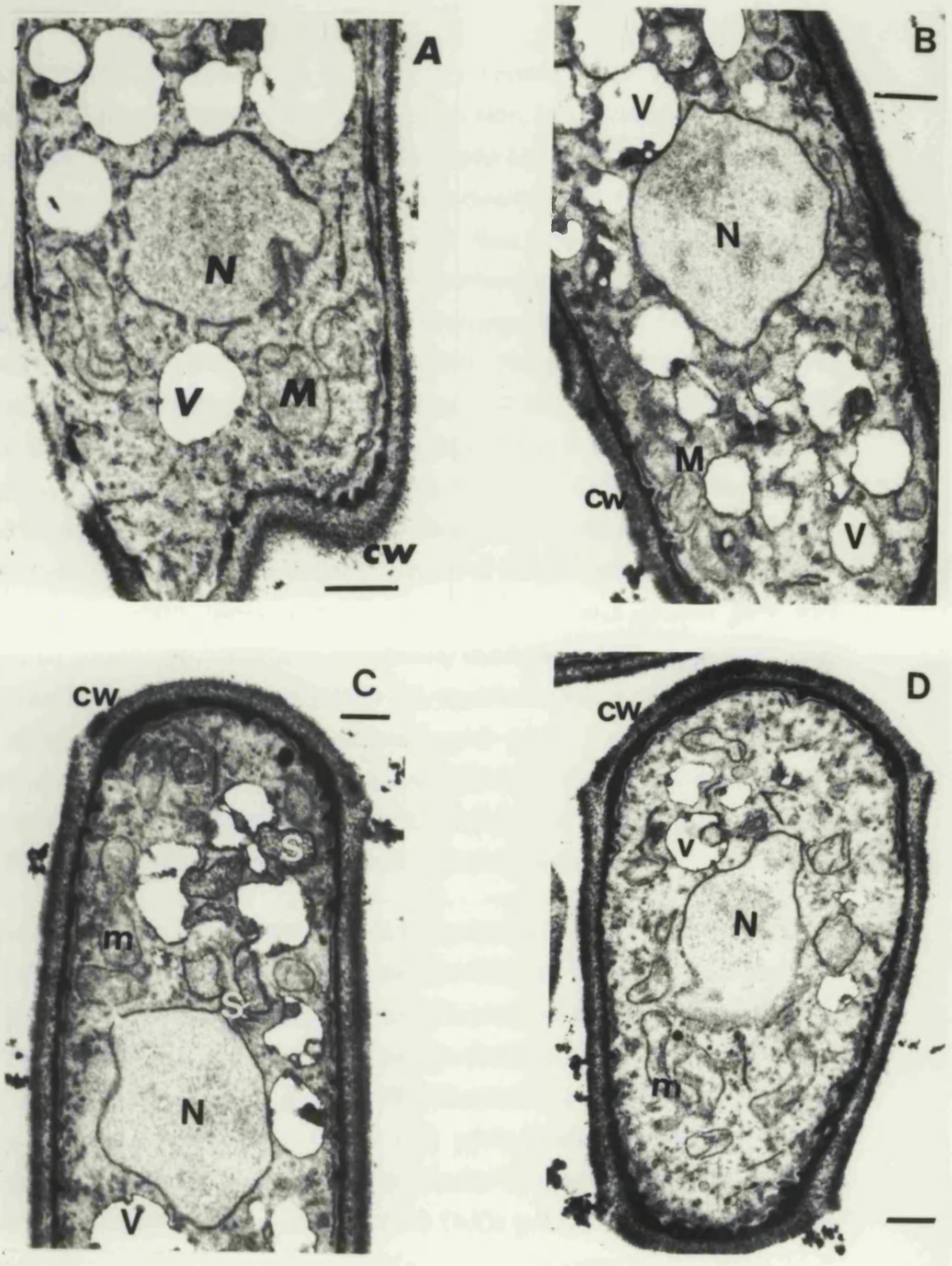


Fig. 3.6

Cells were grown in liquid at 30°C and either left at this temperature or shifted to 16°C for 12 hours. (A) 556 30°C (B) CSSypt5 30°C. (C) CSSypt5 16°C. (D) CSSypt5: YPT5 16°C. CW- cell wall. G- Golgi. M- mitochondria. N- nucleus. S- membrane structures. V- vacuole. Bar 0.5μ.m.

3.6 Discussion

The initial aim of this work was to develop a system which would allow the replacement of the endogenous *ypt5* gene in *S.pombe*. In an attempt to do this the *ypt5* gene and flanking sequences were cloned into a plasmid. The *S.pombe ura4* gene was also cloned into the plasmid 40bp downstream of the *ypt5* coding sequence. Purified insert from the plasmid was then used to transform a haploid strain of *S.pombe*. The rate of homologous recombination was then determined by PCR and later confirmed by Southern blotting. The rate of homologous recombination was found to be approximately 10%. The system was developed with the intention of producing conditional mutants of the *ypt5* gene. It had been intended to mutagenise the insert prior to transformation in the hope that we could subsequently select for conditional mutants. However, it was felt for reasons outlined in section 3.2.1 that the rate of homologous recombination was too low to allow the rapid production of conditional mutants of the *ypt5* gene. We believed that it would be potentially more productive to replace the endogenous gene with recombinant copies which had been specifically mutagenised, as a high percentage of the homologous recombinants recovered, approximately 1 in 10 of all viable transformants, would contain the altered *ypt5* gene. The exact fraction of recombinants which would contain the altered *ypt5* allele would vary depending on which part of the *ypt5* gene was modified i.e. those parts of the *ypt5* gene closest to the *ura4* gene would be selected for with the greatest efficiency.

As I discussed in the introduction all ypt/rab proteins have a conserved cysteine motif at their C terminus. It has been shown that the cysteine residues are modified with Geranylgeranyl (GeGe) moieties (Khosarvi, et al., 1991; Kinsella and Maltese, 1991). The ypt proteins from *S.pombe* were also found to have cysteine residues at their C termini (Miyake and Yamoto, 1990; Haubruck, et al., 1990). It had been previously shown that ypt1p, ypt3p and ypt5p from *S.pombe* received geranylgeranyl groups when they were expressed *in vitro* (Newman, et al., 1992). It had also been shown that the addition of the GeGe groups was necessary for the membrane association of the *S.pombe* proteins *in vivo* (Newman, et al., 1992).

In an attempt to further characterise the modifications that the ypt5p was receiving a number of specifically mutated *ypt5* cDNAs were created by Chris Newman in Dr A. Magee's laboratory. The *ypt5* alleles were altered at their extreme C terminus so as to encode either -CSC (control), -CSS, -SSC or -SSS. Dr Magee's group wished to study the modifications that the mutant proteins received. In parallel we were going to examine whether the mutant ypt5 proteins were functional in an *S.pombe* cell by specifically replacing the endogenous *ypt5* gene with the mutant alleles.

The mutant cDNAs were used to create mutant genes as described in section 3.2.2. The mutant genes linked to the selectable marker *ura4* were then used to transform a diploid *S.pombe* strain. The replacement experiment was initially attempted on the haploid 556 strain, in retrospect this was not the best strategy as only a positive result was possible i.e. a non-functional allele would give no homologous recombinants but of a lack of homologous recombinants would not prove that the protein encoded by the allele was non-functional. After transformation the diploid strains were screened and homologous recombinants identified both by PCR and by Southern blotting.

The diploid strains were next analysed by Random Spore Analysis which revealed that the two singly altered ypt5 proteins yptCSSp and ypt5SSCp were both functional i.e. strains containing the mutant alleles as the sole copy of *ypt5* were viable on minimal media. However, the doubly mutated protein ypt5SSSp was not functional. Work by our collaborators indicated that ypt5SSSp was not modified *in vitro* or *in vivo* whilst the singly mutated proteins ypt5CSSp and ypt5SSCp received only 50% GeGe groups per cysteine residue in comparison to the WT ypt5p. It was concluded therefore that the ypt5p receives two GeGe groups, as is seen for the rab3Ap (Farnsworth, et al., 1991), and that the GeGe groups were essential for the function of the ypt5p. The results obtained in *S.pombe* cells were similar to those reported for both yptlp and sec4p from *S.cerevisiae* (Molenaar, et al., 1988; Walworth et al., 1989). Both groups reported that removal of C terminal cysteine residues resulted in a non-functional protein which was completely cytoplasmic.

We then went on to analyse the localisation of the various ypt5 proteins both in transfected COS cells and in *S.pombe*. Our collaborators found that the WT ypt5p demonstrated 50% membrane association, both of the singly altered proteins were less than 10% membrane associated whilst the doubly mutated ypt5SSSp was completely cytoplasmic (Giannokouros, et al., 1993). Western blotting of *S.pombe* cell extracts confirmed that the point mutants ypt5SSCp and ypt5CSSp were less membrane associated than the WT ypt5p. In order to quantify the distribution of mutant proteins in *S.pombe* the experiment shown in Fig. 3.5 will have to be repeated in the presence of purified ypt5 protein to demonstrate that the observed bands are all specific. Also controls will have to be carried out to ensure that the ypt5 proteins are all being recovered to the same extent. For example the proteins were precipitated using the methanol/ chloroform method (Wessel and Flugge, 1984) and it would be desirable to repeat the experiment with a different precipitation procedure to ensure that the all of the various forms of the ypt5p were being recovered equally. Furthermore one could precipitate protein from total cell

extracts and compare the level of ypt5p with that found in the membrane and cytoplasmic fractions to ensure that the recovery levels are equal in the different strains.

I next went on to analyse the growth of the mutant strains at various temperatures. This analysis was done by visual inspection and therefore any conclusions on the growth properties will have to be confirmed by quantification. It was observed that the two mutant strains, termed SSCypt5 and CSSypt5, were capable of growing at 30°C, 37°C and 18°C. It was initially observed that the cells displayed a growth deficiency at 14°C in comparison to WT 556 cells, however, if the experimental procedure was altered such that the cells placed at 14°C were not in stationary phase then it was seen that the strains were capable of growing. Also it was observed that the SSCypt5 strain would no longer grow after storage at 4°C. In the future it would be desirable to repeat the growth analyses at different temperatures in a much more rigorous manner, with regular cell counts and with the experiment being repeated on several occasions. The results obtained seem to imply that the mutant cells may lose viability as they enter stationary phase. This can be analysed by plating out cells from cultures at different stages of growth eg when the cells are growing logarithmically and then as they enter stationary phase and after prolonged stationary phase. The cell numbers can be counted prior to plating onto fresh media, or dilution into fresh media, and viability can be determined by for example counting the number of colonies that appear on the plate.

Electron microscopy revealed the accumulation of large membrane enclosed structures in CSSypt5 cells which had been grown to stationary phase and then diluted into fresh media and incubated at 16°C. These structures were not seen in the same strain transformed with WT *ypt5* cDNA indicating that their presence was due to the defective ypt5 protein. If it is shown that these structures arise as a direct result of the recombinant ypt5 proteins then the most compelling explanation, for their production, is that they arise as a result of a defect in endocytosis. It will be interesting to examine the morphological effects in the SSCypt5 strain to determine whether this strain also forms these structures. It will also be interesting to analyse cells which have been incubated in stationary phase for varying lengths of time.

Assuming that the structures are formed due to a defect in endocytosis there are two possible explanations for their production (Fig. 3.7). It is possible that a non-functional ypt5 protein would somehow cause a block in budding from a compartment, presumably indirectly as a result of sequestering components which are required for budding. However, I believe that a more plausible explanation, bearing in mind that ypt proteins are thought to regulate fusion, is that the lack of functional ypt5 protein may allow aberrant fusion by not blocking the association of the wrong v and t SNARE pair.

At the moment work is going on to develop an endocytosis assay in *S.pombe* when there is such an assay we will then be able to ascertain whether the mutant ypt5 proteins are deficient in endocytosis or not. Using the gene replacement system it may be possible to engineer strains which are cold sensitive due to a mutation in the *ypt5* gene. If this could be achieved it would greatly enhance our ability to study the role of ypt5 *in vivo*.

Fig. 3.7 Effect of mutant ypt5p

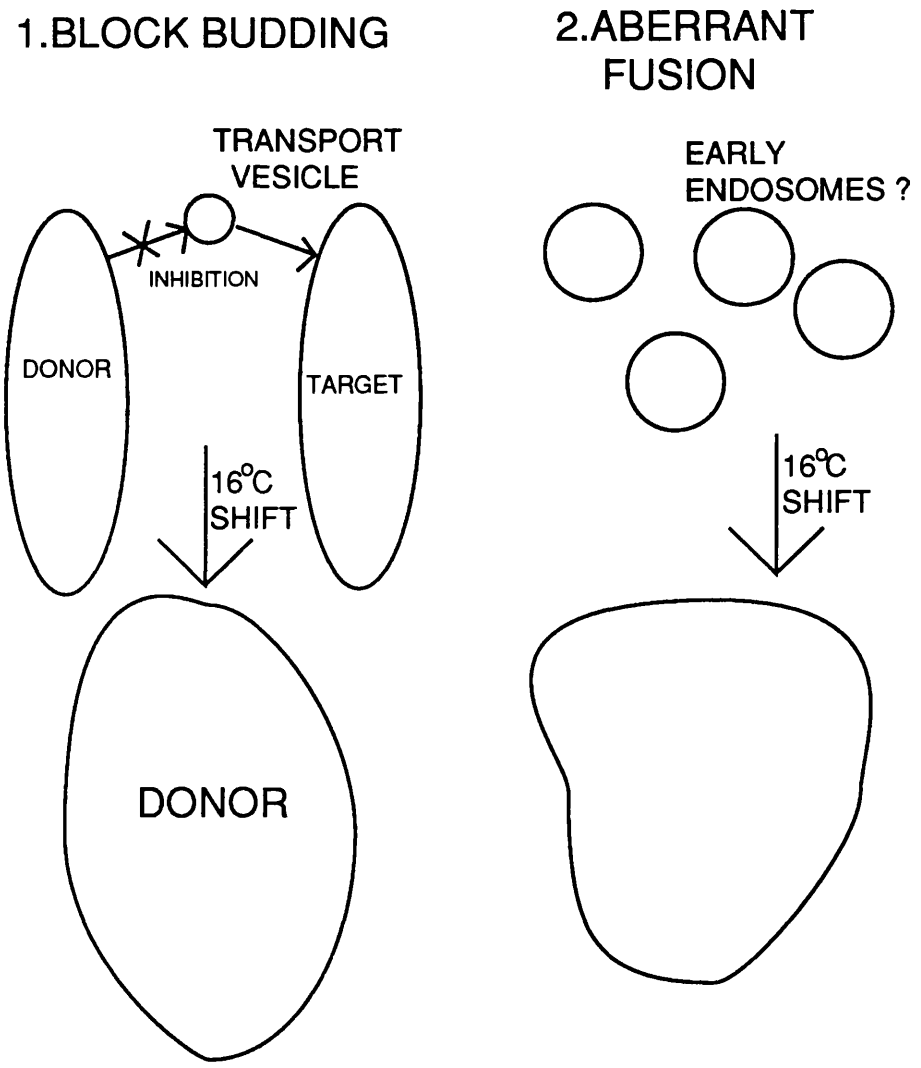


Fig. 3.7
The strain containing mutant ypt5 protein accumulates large membrane enclosed structures. Presumably ypt5p as rab5p is involved in endocytosis. The structures can arise by two mechanisms.

(1) Transport from a compartment is blocked but the compartment continues to receive vesicles.

(2) Pre-existing smaller structures fuse aberrantly to produce an enlarged organelle .

Chapter 4

ypt1

4.1 INTRODUCTION

As discussed in the introduction one of the first ypt/rab genes to be identified was YPT1 from *S.cerevisiae*. Studies demonstrated that the protein product of the YPT1 gene acts at an early stage of transport (Segev, et al., 1988). The mammalian homologue, rab1p, has been identified and it too acts at an early stage of secretion (Plutner, et al., 1991). Recently an *S.pombe* homologue of YPT1 was discovered by both ourselves and others (Fawell, et al., 1989; Miyake and Yamamoto, 1990). This gene was also termed *ypt1*.

Sequencing demonstrated that *ypt1* encoded a 203 amino acid protein which has 73% identity to *S.cerevisiae* ypt1p and 79% identity to murine rab1p. Miyake and Yamamoto demonstrated that the ypt1 protein was essential for cell growth and that the ypt1 protein could functionally replace *S.cerevisiae ypt1*p

The aim of my work was too characterise the *ypt1* gene in greater detail in order to ascertain the function of the ypt1 protein *in vivo*. It was hoped that this would allow the determination of whether or not the ypt1p is involved in multiple stages of transport, as has been claimed for rab1 (Plutner, et al., 1991). The strategy was to produce a strain of yeast which was TS for growth due to the presence of a mutated *ypt1* gene. In order to produce conditional mutants the endogenous gene would be replaced either with a specifically mutated *ypt1* gene or alternatively with randomly mutated copies. The gene replacement strategy to be employed was exactly that used for *ypt5*.

Any conditional mutants produced would then be examined both biochemically and morphologically in an attempt to deduce the role of the ypt1 protein. For example one would predict that strains containing a defective ypt1 protein would accumulate secretory proteins due to a disruption in transport. The analysis of accumulated secretory proteins by Western blotting would reveal the extent of the carbohydrate modifications they had received and would indicate which stage of transport was being inhibited. If the cessation of growth, after shifting to the restrictive temperature was swift this would allow multiple forms of accumulated protein to be visualised (assuming that the ypt1 protein is involved multiple stages of transport). This methodology has been used to show that the protein product of the SEC18 acts at multiple stages of transport in *S.cerevisiae* (Graham and Emr, 1991).

RESULTS

4.2 GENETIC MANIPULATION

4.2.1 Gene replacement

The *ypt1* gene was cloned from a *S.pombe* genomic library, along with both its 5' and 3' non-coding regions, into the plasmid pBSM13$^-$. The *ura4* gene was inserted directly after the *ypt1* coding region to form the plasmid pY1U4. pY1U4 has a 5.1Kb insert which can be excised by digestion with *Pvu II* (Fig. 4.1). This digestion does leave some of the plasmid sequence at either end of the insert, but this did not prevent recombination. The insert does differ from that of pY5U4 in that there is approximately 1Kb of non-coding sequence at either side of the *ypt1/ura4* genes whereas in pY5U4 there is only 200bp of 3' non-coding sequence downstream of the *ura4* gene.

As described for *ypt5,* the insert was purified through an agarose gel prior to being used to transform WT 556 cells. Transformants were selected by their ability to grow on media lacking uracil. The transformants were stable, as they retained their ura$^+$ phenotype after growth in non-selective conditions. This was shown by plating cells onto FOA, no revertants were identified out of 1 x 10^6 cells plated. Five of the transformants were selected at random and screened by Southern blot analysis (Fig. 4.2). This showed that all five of the transformants were homologous recombinants (the high rate of homologous recombination was later confirmed by PCR which indicated > 98% homologous recombination). The reason for this very high rate is not known. It may be that the *ypt1* locus influences the rate of homologous recombination or, alternatively, it could be that having 1Kb of downstream sequence is important in directing the recombination event. This high rate of homologous recombination meant the production of conditional *ypt1* mutants by randomly mutating the *ypt1* gene prior to transformation, could be attempted.

Fig. 4.1 pY1U4 insert and *ypt1*

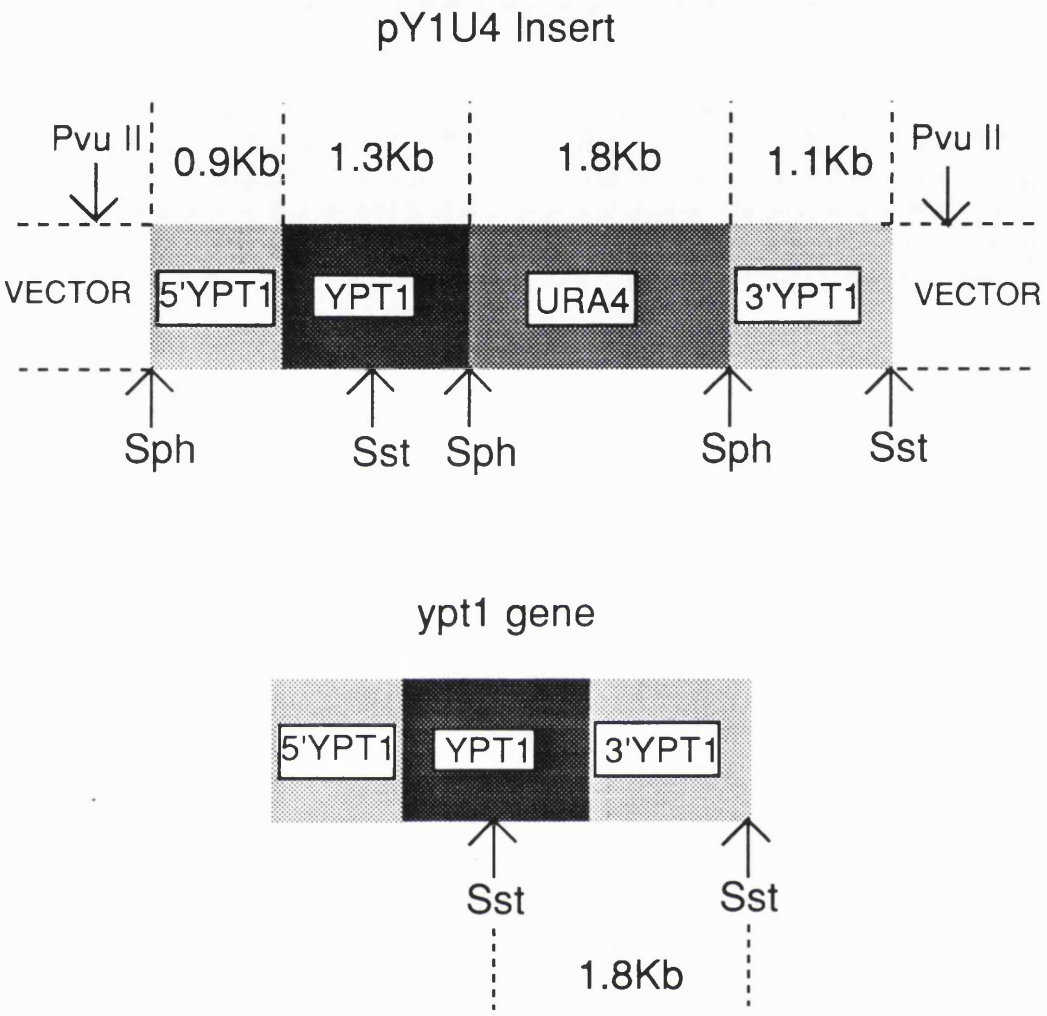


Fig. 4.1
The Y1U4 construct was formed by subcloning the ura4 gene into an Sph1 site downstream of the ypt1 gene. Recombinants were screened using the 1.8Kb Sst1 fragment shown.

Fig. 4.2 Southern blot analysis of ypt1 transformants

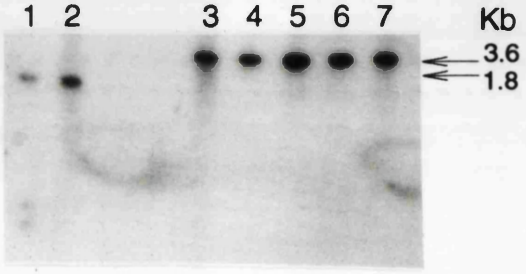


Fig. 4.2
Analysis of *ypt1* transformants. DNA was made from WT and five randomly selected transformants. The DNA was digested with *Sst 1* and run out on an agarose gel.
The DNA was transferred to a filter and probed using the *Sst 1* fragment from the *ypt1* gene.
Lanes: (1). Purified *Sst 1* fragment. (2) WT 556 DNA
(3-7) DNA from randomly selected transformants.

4.2.2 Random Mutagenesis

In an attempt to produce *ypt1* mutants, the linear insert from pY1U4 was mutagenised with Hydroxylamine (HAM). HAM causes C- T transitions as it deaminates Cytosine causing DNA polymerase to insert an Adenine in place of Guanine upon replication. Purified insert was incubated with HAM for increasing lengths of time prior to transformation. The transformants were plated onto media lacking uracil and grown at 25°C. As expected the increasing exposure to HAM caused a decrease in the number of viable transformants (Fig. 4.3). The viable transformants were replica plated onto fresh selective plates and incubated at 25°C, 30°C and 37°C. Of the 326 transformants screened three failed to grow at 37°C but would grow at the other temperatures. Since the whole insert had been mutagenised the TS strains identified were potentially mutant in the *ura 4* gene rather than the *ypt1* gene. The three mutants were therefore replicated onto plates supplemented with uracil. It was found that only one strain still failed to grow at 37°C and this strain was tentatively termed *ypt1-1*. It was assumed that the other two strains were both defective in the *ura4* gene and they were not studied any further. The assumption could have been proved conclusively by transformation with a plasmid containing the *ura4* gene followed by plating of the transformants at 37°C. If the transformants were viable at this temperature then this would indicate that the strain was indeed defective in the *ura4* gene.

To prove that the *ypt1-1* strain contained a defective *ypt1* gene, the strain was next transformed with a WT copy of the *ypt1* gene in the plasmid pIRT3. As a control the strain was also transformed with plasmid alone. Transformants were then analysed by growing them initially at 30°C and then shifting them to 37°C or leaving them at 30°C (Fig. 4.4). It was observed that cells which had been transformed with the plasmid alone rapidly ceased to divide at the 37°C whereas those transformed with the WT *ypt1* gene were capable of growth at 37°C, although the growth rate observed was less than at 30°C. These results indicated that the strain was defective in the *ypt1* gene.

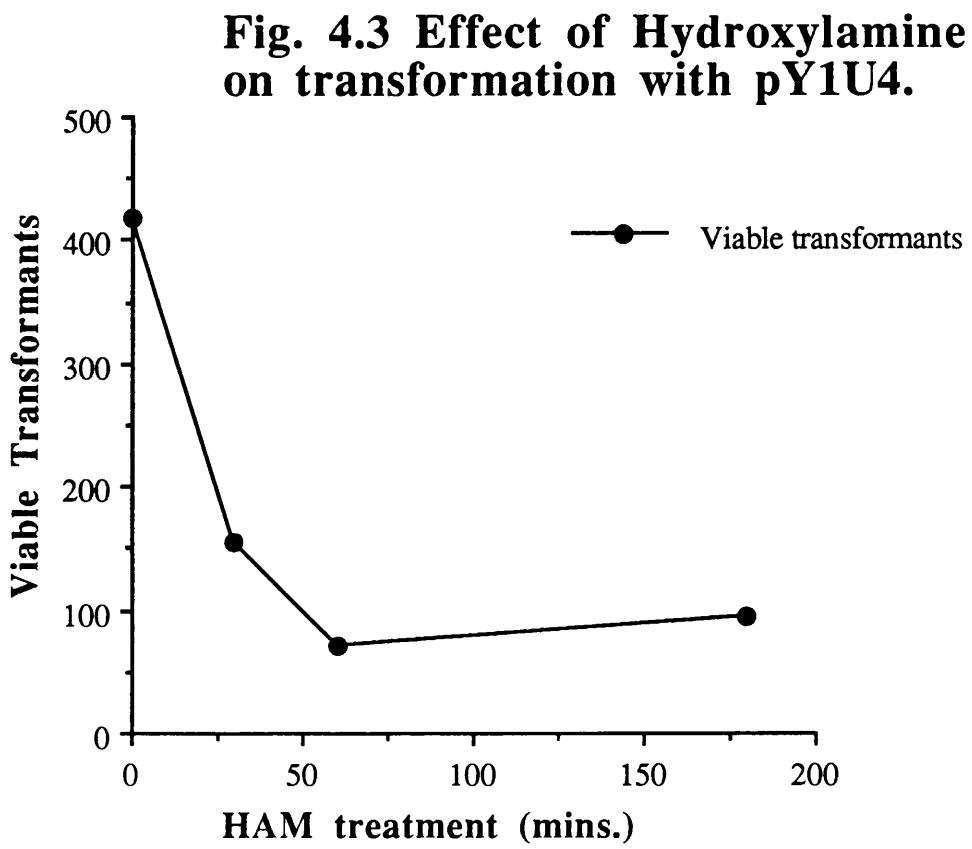


Fig. 4.3

The insert from pY1U4 was excised by *Pvu II* digestion and purified from an agarose gel. 200ng aliquots of the insert were treated with hydroxylamine (HAM) for varying lengths of time. The DNA was then separated from the HAM by phenol extraction followed by ethanol precipitation. The inserts were next used to transform the 556 cell line. Transformants were grown on selective plates at 25°C.

Fig. 4.4 Rescue of ypt1-1 strain with WT ypt1

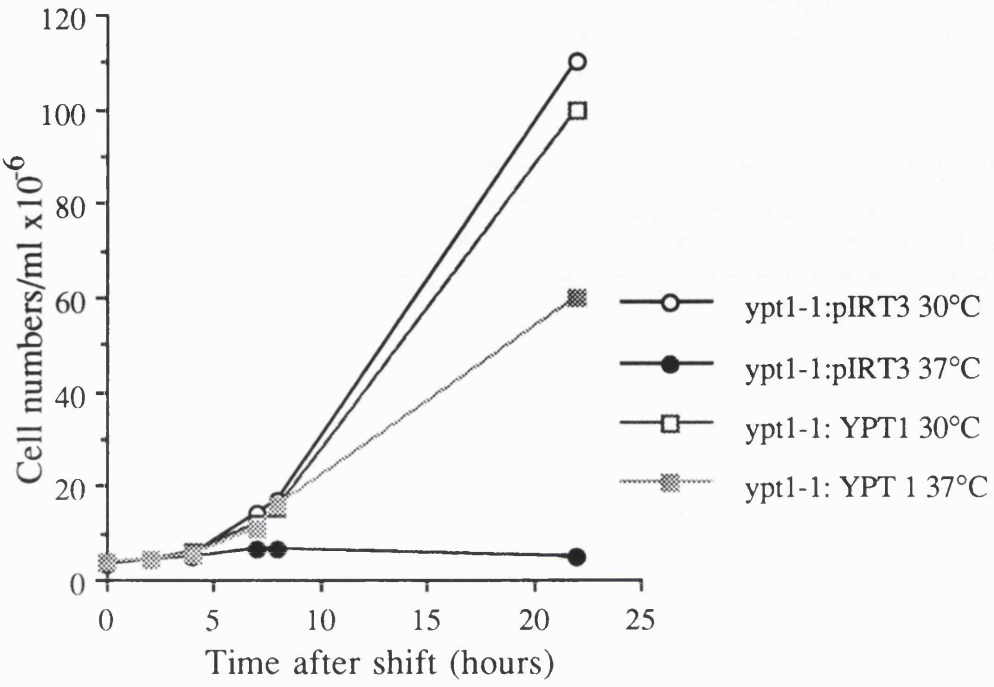


Fig. 4.4

The *ypt1-1* strain was transformed with control plasmid or plasmid containing the genomic copy of *ypt1* to give Y1:pIRT3 and Y1:pYPT1 respectively. The two strains were grown at 30°C in minimal media and then shifted to 37°C or left at the original temperature. Cell numbers per ml were calculated using a haemocytometer. The strain transformed with plasmid control continued to display a TS phenotype whilst the Y1: pYPT1 strain was able to grow at 37°C.

4.3 ANALYSIS OF *ypt1-1* STRAIN

4.3.1 Transport in mutant strain

(i) Protein Secretion

As we had produced a strain which was defective in the *ypt1* gene we now wished to analyse it in an attempt to ascertain at which stage of transport its protein product operates. The secretion of acid phosphatase was monitored to determine whether secretion was being disrupted in the mutant cell line. Secretion from the cells was compared to that from the parental 556 strain. The cells had their cell walls removed to form spheroplasts, therefore strictly speaking it was secretion from spheroplasts that was being monitored.

The cells were harvested during logarithmic growth at 30°C. The cells were then spheroplasted again at 30°C and finally resuspended in minimal media which had been supplemented with 1.2M Sorbitol. The spheroplasts were then incubated at either 30°C or 37°C and aliquots taken at various time points and assayed for the presence of acid phosphatase (Fig. 4.5). It was found that secretion from the *ypt1-1* strain was very much reduced in comparison to WT cells at both 30°C and 37°C. The reason for the reduction in secretion at both temperatures, was not known. We speculated that it may have been due to the fact that spheroplasts rather than whole cells were being examined. However, it was evident that the secretion of acid phosphatase was impaired in the *ypt1-1* as one would have predicted if the ypt1p is involved in constitutive secretion.

Fig. 4.5 Acid phosphatase secretion

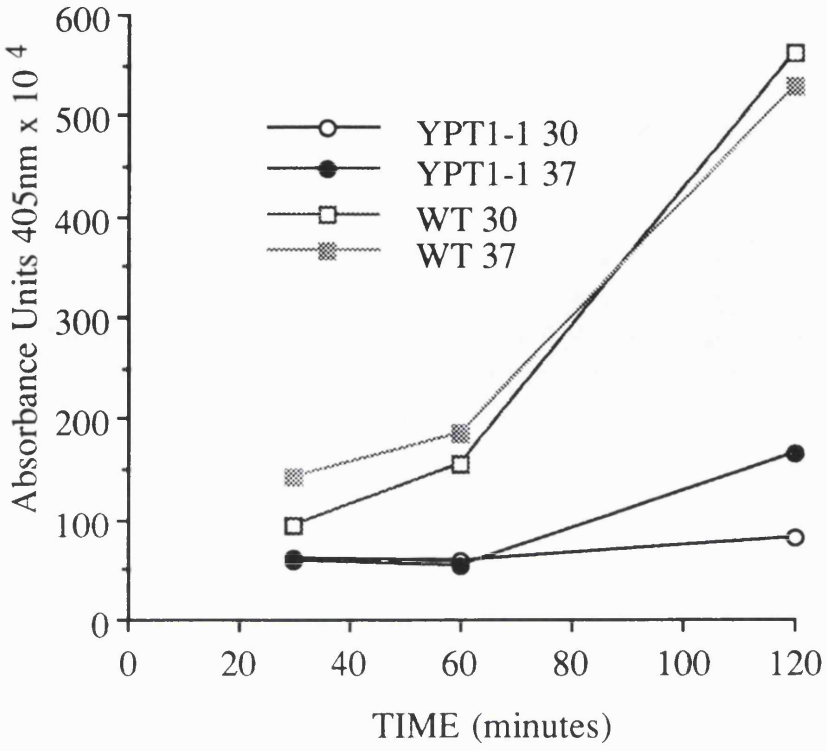


Fig. 4.5

WT and *ypt1-1* cells were harvested during logarithmic growth at 30°C in minimal media. The cells were spheroplasted and approximately 2 x 10^8 of spheroplasts were resuspended in 3ml of minimal media supplemented with 1.2M sorbitol. The spheroplasts were incubated at either 30°C or 37°C. At various time points 50µl aliquots were removed. The spheroplasts were spun down and the remaining supernatant was assayed for acid phosphatase as described in the methods section.

(ii) Protein accumulation

After discovering that transport apparently was being disrupted in the *ypt1-1* strain we wished to determine exactly which stage of transport was being disrupted. One way of identifying which stage of transport is being disrupted is to examine in which form a secretory protein is being accumulated. That is to say if transport was being disrupted between the ER and the Golgi, then one would predict that a secretory protein would accumulate in an ER modified form.

We decided to look for the accumulation of acid phosphatase within the cell, as we had been given a monoclonal antibody 7B4 (a kind gift from Ernst Schweingruber) which was reported to recognise all forms of acid phosphatase (Schweingruber, et al., 1986B). *S.pombe* have two forms of the secretory protein acid phosphatase expressed by the genes *pho1* and *pho4*, both of which have now been cloned (Elliott, et al., 1986) (Yang and Schweingruber, 1990). *Pho1* encodes which is phosphate repressible acid phosphatase (Maundrell, et al., 1985) which has an apparent MW of 54kDa (Schweingruber., et al., 1986). The thiamin repressible *pho4* gene encodes a protein with an apparent MW of 56kDa but is expressed at lower levels than the *pho1* gene.

Protein extracts were taken from both *ypt1-1* and 556 cells which were growing logarithmically. The cells had been grown in liquid minimal media at the permissive temperature of 30°C and either shifted to the restrictive temperature of 37°C for four hours or left at 30°C. The protein extracts were digested or mock treated with Endo. H. The Endo. H. digest was carried out to remove N-linked carbohydrates and hence would allow the visualisation of the protein if it had been heavily glycosylated, as glycosylation causes the protein to run as a heterogeneous smear (Schweingruber, et al., 1986B). The protein extract was then mixed with 2 x SB and electrophoresed on a 12% acrylamide gel. The proteins were transferred to nitrocellulose and probed with 7B4 at a 1 in 10 dilution. The protein bands were then visualised by chemiluminescence (Fig. 4.6). Surprisingly although a protein band was seen in the 556 strain both at 30°C and at 37°C, there was very little acid phosphatase visible in the *ypt1-1* protein extracts . It was thought that the strain may be expressing the protein at a lower level than WT perhaps as a consequence of the *ypt1-1* lesion.

Fig. 4.6 Western Blotting of *ypt1-1* and 556.

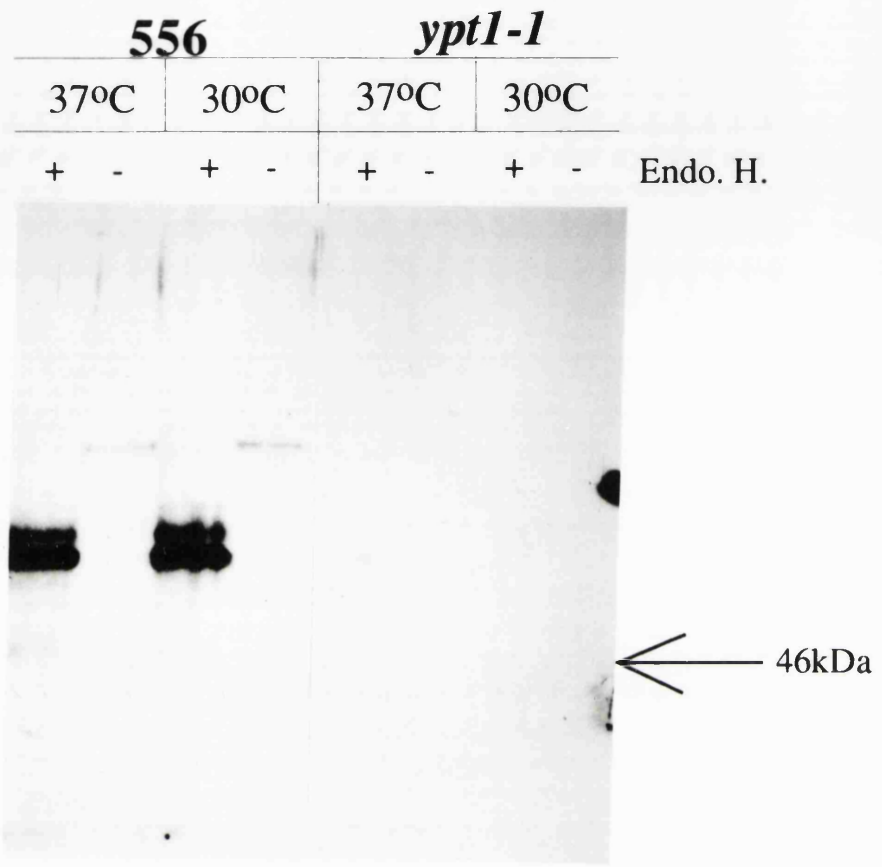


Fig. 4.6
Western analysis of acid phosphatase accumulation in 556 and *ypt1-1* cells. The cells were grown at 30°C and either shifted to 37°C for 4 hours or left at 30°C . Protein extracts were incubated in the presence or absence of Endo. H.
The MW marker was ovalbumin (46kDa).

In an attempt to overcome any potential problem of low expression levels the *ypt1-1* strain, and 556 as a control, were transformed with plasmids containing *pho4* cDNA. The cDNA was cloned into pEVP11 plasmid which gives a high expression as it contains the *S.pombe adh* promoter (Russell and Nurse, 1986). The strains were transformed with a plasmid containing a *pho4* cDNA which had been modified at its C termini to have the *S.pombe* ER retrieval signal ADEL (Pidoux and Armstrong, 1992). The reason for this was that the pho4-ADEL protein should be retrieved to the ER (Pidoux and Armstrong, 1992). The protein had, in addition, been modified, with the c-myc epitope (Munro and Pelham, 1987) constructs which is recognised by the monoclonal Ab 9E10 (kindly supplied by Gerard Evan).

Thus the strains created were WT: *pho4-adel* and *y1-1: pho4-adel.* The strains were selected by growth in minimal media supplemented with adenine and uracil, in the case of the WT strains, or simply adenine in the case of the *ypt1-1* strains. The cells were grown in liquid minimal media and harvested during logarithmic growth. Again the cells were grown at 30°C and then either shifted to 37°C or left at 30°C. The extracts were digested or mock treated with Endo. H. (Fig. 4.7). The proteins were electrophoresed through a 12% acrylamide gel, transferred to nitrocellulose and probed with 9E10 at 1 in 1,500.

It was found that this case the WT:*pho4-adel* strain did accumulate the 80kDa ER form of acid phosphatase, as has been previously seen (Pidoux and Armstrong, 1992). However, the *ypt1-1* strain did not accumulate acid phosphatase at a detectable level.

Fig. 4.7 Western of transformed *ypt1-1* and 556 strains.

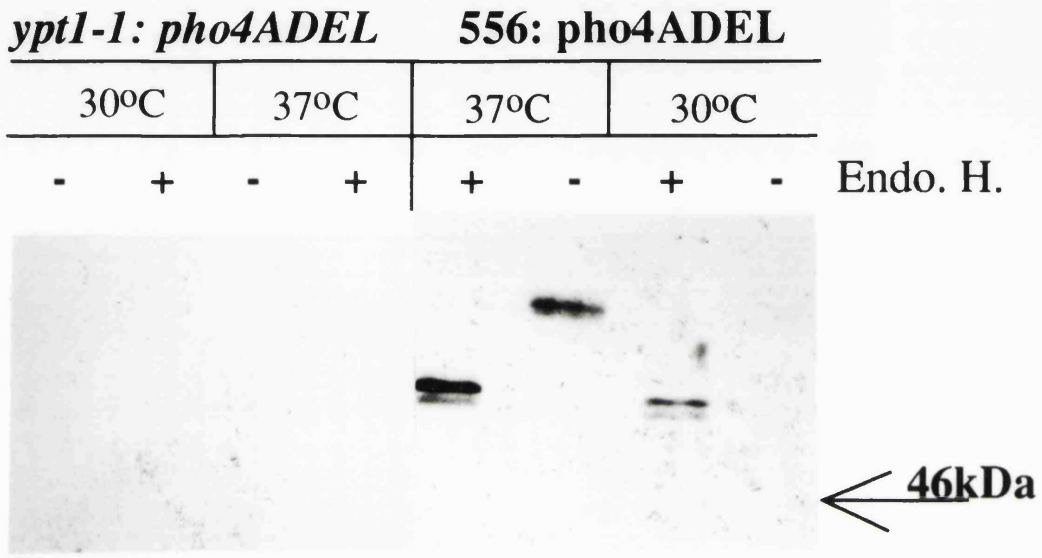


Fig. 4.7
The *ypt1-1* and 556 strains were both transformed with a plasmid encoding the pho4p tagged with ADEL. Cells were grown at 30°C and then shifted to 37°C for four hours. Protein extracts were digested or mock treated with Endo. H.
MW marker shown is that of ovalbumin.

4.3.2 Morphological analysis of strain

The ypt1 protein was predicted to be involved in an early stage of secretion due to its homology to both mammalian rab1p and *S.cerevisiae ypt1*p. The *ypt1-1* strain was therefore analysed morphologically to ascertain whether there was any gross disruption of the cellular architecture at either the permissive temperature or after shifting to the restrictive temperature.

(i) Light microscopy

Examination of cells by visual inspection revaled no gross morphological differences between the *ypt1-1* strain and the parental 556 strain. Neither were any gross differences observed after both strains had been shifted to 37°C for four hours. Cells which had been left at 37°C for 24 hours, however, did show gross morphological alterations from 556 cells. The *ypt1-1* cells were greatly enlarged and they appeared to contain a large amount of membranous material.

(ii) Fluorescence microscopy

There are now good markers for both ER and Golgi resident enzymes in *S.pombe*. The ER can be visualised with antibodies raised against BiP (Pidoux and Armstrong, 1993). By visual inspection there was no discernible change in the ER structure in the *ypt1-1* strain after it had been shifted to 37°C for four hours.

Recently a galactosyltransferase (Gal. T.) has been cloned from *S.pombe* (Chappell, et al., 1994). Antibodies have been raised against the protein which stain approximately ten to twenty punctate structures within each cell depending on cell length (Ayscough, 1993). The Golgi structure of the *ypt1-1* cells was examined by indirect-immunofluorescence (Fig. 4.8). This work was carried out in collaboration with Kathryn Ayscough. Initially the cells were grown, shifted to restrictive temperature and then harvested by myself, the rest of the procedure was then carried out by Kathryn Ayscough and the results have been previously reported (Ayscough, 1993).

Fig. 4.8 Galactosyltransferase distribution in *ypt1-1*.

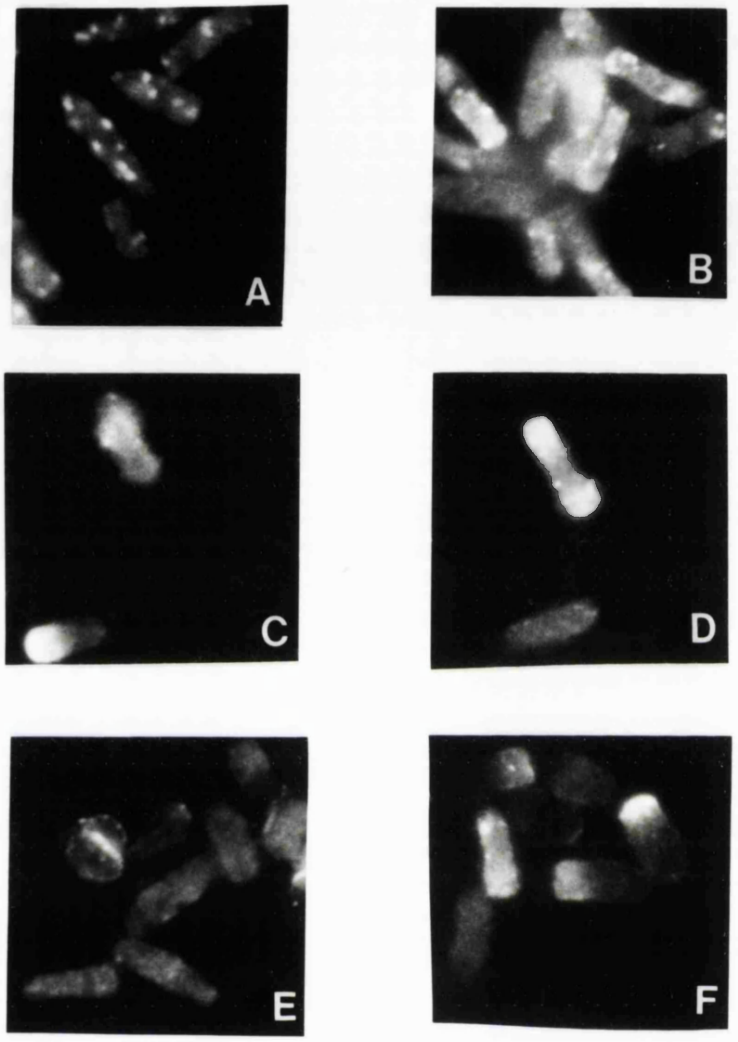


Fig. 4.8

The distribution of galactosyltransferase was examined in WT and *ypt1-1* cells by indirect immunofluorescence. The cells were grown at 30°C and then either left at this temperature or shifted to 37°C. (A) WT cells 37°C for four hours. (B) *ypt1-1* 30°C. (C, D) *ypt1-1* 37°C for 2 hours. (E, F) *ypt1-1* 37°C for four hours. In WT cells discrete dots can be observed, presumably Golgi stacks. In the *ypt1-1* strain at 30°C discrete spots were still observed but with a slightly higher background staining. However, in *ypt1-1* cells shifted to 37°C the punctate staining pattern is replaced with a much more diffuse staining pattern and in some cases the ends of the cell were heavily stained.

At 30°C a punctate pattern of spots was observed (Fig. 4.8 B) which is very similar to the staining pattern observed in WT cells, although the staining in the *ypt1-1* cells was somewhat more diffuse than is normally seen. The staining pattern changed dramatically when the cells were shifted to 37°C. The enzyme had apparently redistributed to one end of each cell (C, D) and at later time points the staining became much more diffuse with very few punctate structure visible (E, F). The cells had also swollen in size and many appeared to be unable to complete septation. This was not an effect of temperature as WT cells shifted to 37°C displayed a normal punctate staining (A).

(iii) Electron microscopy

The indirect-immunofluorescence results indicated that the Golgi apparatus may well be breaking down in the *ypt1-1* strain after shifting of the cells to 37°C. To investigate this further the strain was examined by electron microscopy. The cells were grown at the permissive temperature of 30°C and then during logarithmic phase growth they were shifted to 37°C for varying lengths of time, or left at 30°C as a control (Fig. 4.9). Electron microscopy revealed that after only four hours at the restrictive temperature the Golgi stacks were readily apparent in the *ypt-1* cells incubated at 30°C (B). At the restrictive temperature of 37°C the Golgi stacks had almost all completely vanished and this disappearance was apparently coupled to the appearance of novel vesicular structures which were 100nm-250nm in diameter (C, D). Golgi stacks were still visible the control WT cells grown at 37°C for four hours (A), which indicated that the effect was not due to the shift to 37°C.

The distribution of internal membrane in the cells was calculated, again in collaboration with Kathryn Ayscough (Ayscough, 1993). It was found that in cells incubated at 30°C 14% of the internal membrane was present in Golgi stacks whereas in cells incubated at 37°C for four hours this had dropped to 2% (Fig. 4.10). The amount of both the peripheral ER and the Nuclear Envelope remained unchanged whilst the level of internal ER, which includes unstacked Golgi cisternae and the newly formed vesicular structures, had risen by approximately 8%. Interestingly the overall amount of internal membrane had decreased by 9% which indicated that not all the membrane from the Golgi stacks had redistributed to other internal organelles.

Fig. 4.9 Analysis of *ypt1-1*

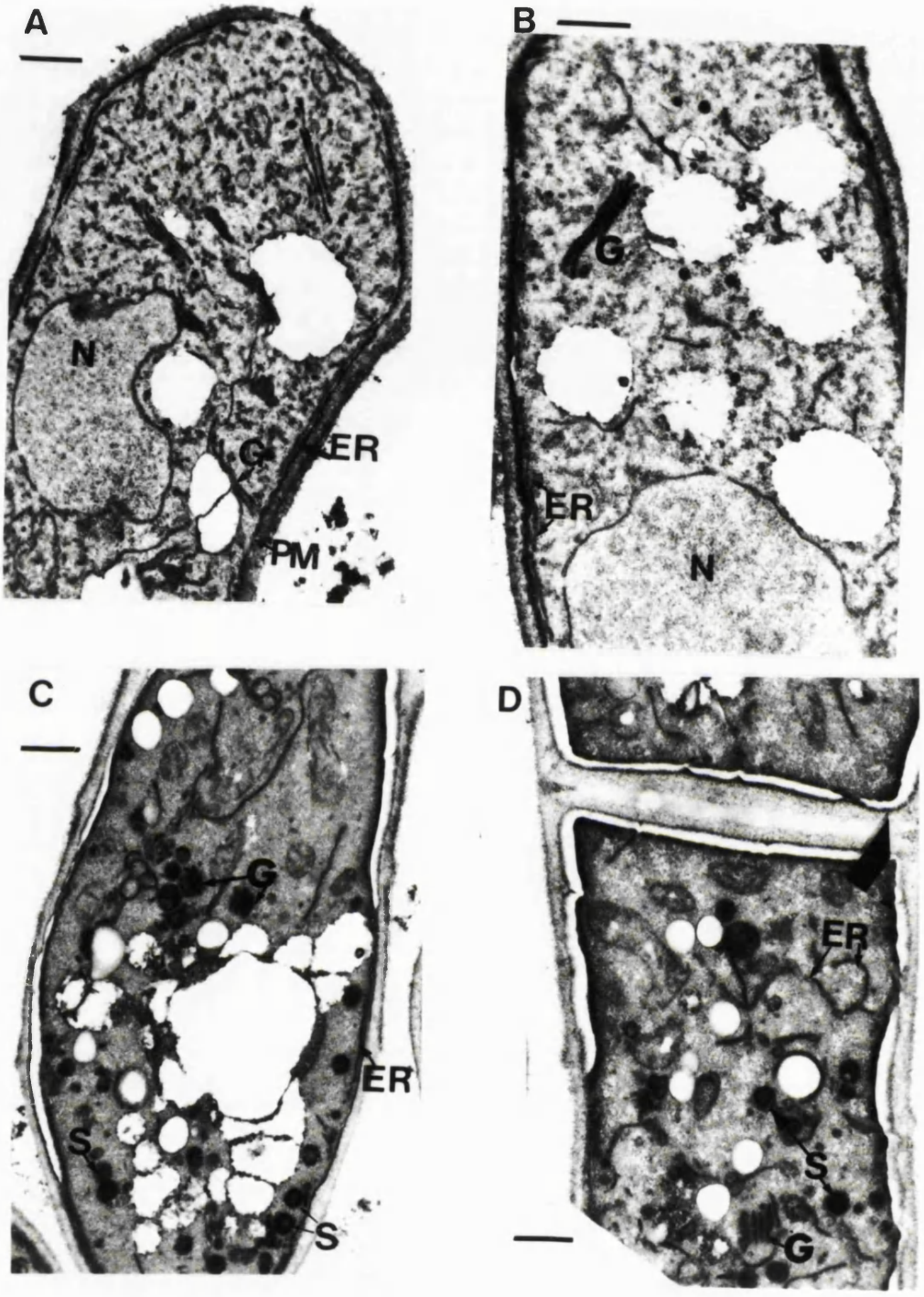


Fig. 4.9

WT and *ypt1-1* cells were examined by electron microscopy. Cells were grown in minimal media at 30°C and then shifted to 37°C for four hours, or left at 30°C. (A) WT 37°C for four hours. (B) *ypt1-1* 30°C. (C, D) *ypt1-1* 37°C for four hours. ER-endoplasmic reticulum. G- Golgi. N- Nucleus. PM-plasma membrane. S- vesicular structures. Bar 0.5μm.

Fig. 4.10 Surface density of secretory organelles.

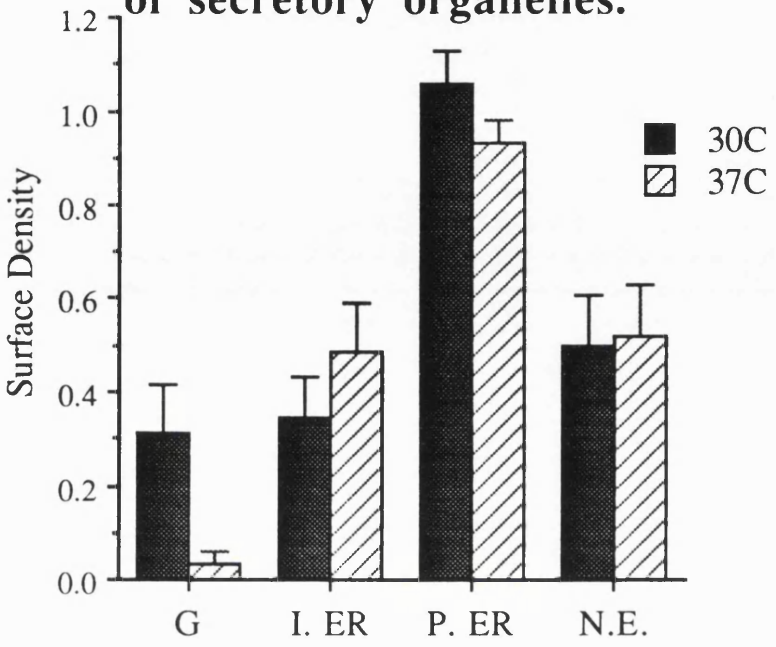


Fig. 4.10

The *ypt1-1* strain was examined by electron microscopy. Cells were grown at 30°C and then shifted to 37°C for four hours or left at 30°C. The amount of internal membrane was quantified from at least ten randomly selected micrographs. The membrane categories characterised were stacked Golgi (G), nuclear envelope (NE), peripheral ER (P. ER) and internal ER (I.ER). Internal ER includes all non-peripheral membrane and therefore would include any unstacked Golgi cisternae. Golgi cisternae are considered to be stacked when at least two cisternae are closely apposed.

4.4 Sequencing of *ypt1-1*

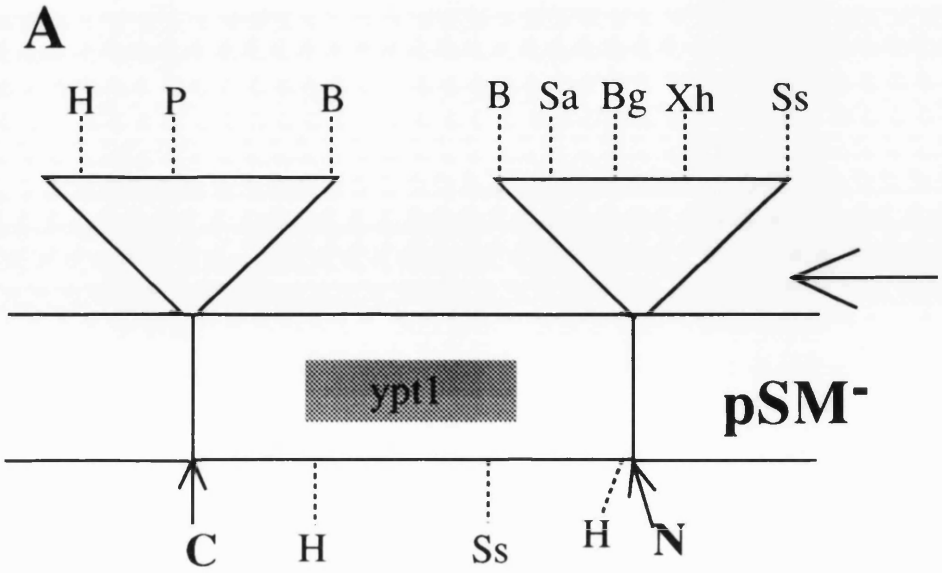
Having established that the *ypt1-1* strain was defective in the *ypt1* gene I wished to investigate the nature of the lesion in *ypt1*. The *ypt1* gene from the *ypt1-1* strain was sequenced directly from the chromosome using PCR. In this method one biotinylated primer is used in the PCR reaction (Hultman, et al., 1989). After gel purification, of the DNA, one strand is recovered by binding to magnetic beads coated with avidin. The purified single stranded DNA can then easily be sequenced. Sequencing revealed that only one codon had been altered in the *ypt1-1* allele. Codon 19 had been changed from GTC, which encodes valine, to AAC, which encodes asparagine. The residue is thought to reside in the first GTP binding motif of the protein (Fig. 1.5). The residue is thought to be equivalent to valine 14 (Val 14) in H-ras. Crystalographic studies have shown that Val 14 is involved in binding the guanine ring of both GTP and GDP (Pai, et al., 1989).

4.5 GTP binding of the ypt1-1 protein

In an attempt to analyse the effect of the lesion in greater detail the *ypt1* cDNA was cloned from both the 556 strain and the *ypt1-1* strain into pBSM13$^-$. The cDNA was recovered from total *S.pombe* cDNA by PCR using oligonucleotides which recognised the extreme 5' and 3' ends of the cDNA(Fig. 4.10A) and had *BamH1* restriction sites. The cDNAs were cloned into the *BamH1* site on the plasmid and sequenced. The WT cDNA contained no mutations whilst the *ypt1-1* cDNA was only altered in codon 19. Both cDNAs were now cloned into the *S.pombe* expression vector pSM$^-$. The orientation was checked by digestion with *Sst1* and separately with *Hind III* (Fig. 4.11A).

Both plasmids were now used to transform the WT 556 strain and transformants were selected by growth on minimal media which had been supplemented with both adenine and uracil. A GTP binding blot was carried out (Lapetina and Reep, 1987). Protein extracts were made from the 556 strain, *ypt1-1* strain and from both newly created strains 566:ypt1 and 556:ypt1-1. The proteins were separated on a acrylamide gel containing SDS and then transferred to nitrocellulose in the absence of SDS. The filter was then incubated in the presence of [α-^{32}P]GTP, washed and exposed to photographic film (Fig. 4.11 B). In the 556:ypt1 i.e. the strain containing overexpressed WT ypt1p, there was an intense GTP binding band of approximately 25kDa which was not seen in the 556:ypt1-1 strain, which contained the mutant ypt1-1p. Also the 556 strain appeared to have a slightly more intense band in this region in comparison to the *ypt1-1* strain.

Fig. 4.11 *ypt1* cDNA cloning and expression



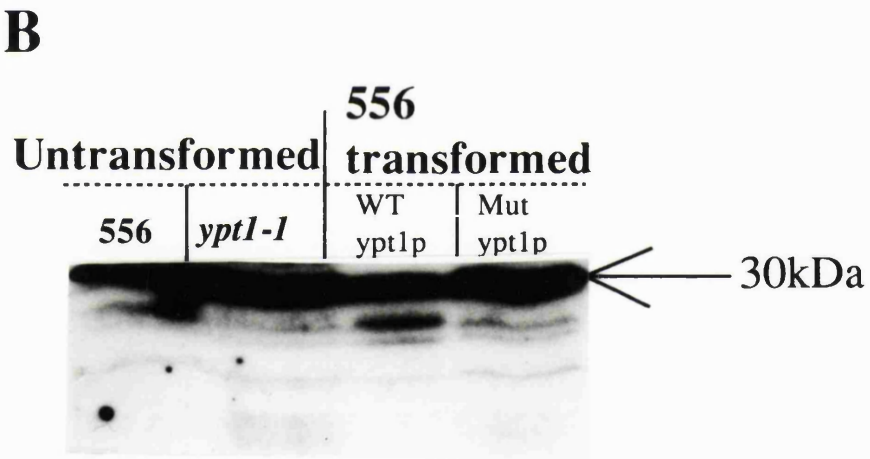


Fig.4.11
Cloning and expression of *ypt1* cDNA.

(A) The *ypt1* cDNA was cloned into pSM$^-$. The N and C termini ofthe *ypt1* cDNA are shown in bold. The large arrow indicates direction of transcription. Restriction sites shown are: B- Bam HI; Bg- Bgl II; H- Hind III; Sa- Sal I; Ss- SstI and Xh- Xho I.

(B) Protein extracts were separated by SDS-PAGE, transferred to nitrocellulose and incubated with radiolabelled GTP. The filter was washed and exposed to photographic film. The MW marker is that of carbonic anhydrase.

4.6 DISCUSSION

The initial aim of this work was to engineer a system that would allow the replacement of the endogenous *ypt1* gene, this was done in almost exactly the same manner as had been used for the replacement of the *ypt5* gene described in chapter 3. The *ypt1* gene was cloned into a plasmid along with the selectable marker *ura4* and the upstream and downstream non-coding regions of the *ypt1* gene. An insert was excised from the plasmid, purified and used to transform the 556 strain of *S.pombe* again as described for *ypt5*. Screening of transformants, initially by Southern blotting and subsequently by PCR, revealed that the transformants produced were homologous recombinants. This differed from the results seen with the *ypt5* replacement system which had yielded a large number of non-homologous recombinants. The most obvious explanation of this is that the *ypt1* construct contained over 1Kb of downstream sequence whilst the *ypt5* construct had only 200bp of downstream sequence.

In an attempt to produce a conditional mutant the purified insert was mutagenised prior to transformation. Transformants were initially grown at 25°C and then replicated onto plates incubated at either 25°C, 30°C or 37°C. Potential TS strains were then replicated a second time onto media which contained uracil, to ensure that the strain did not contain a defective *ura4* gene. One strain was isolated which grew at both 25°C and 30°C but not at 37°C. This strain was termed *ypt1-1*.

The *ypt1-1* strain was then transformed with a plasmid containing a genomic copy of *ypt1*. The resultant strain would now grow at 37°C, however, the growth rate observed was lower than at 30°C. The growth of the strain must be analysed in detail to ascertain whether its growth does differ significantly from that of the parental 556 strain, and if so to determine what those differences are. If indeed the strain does not display WT growth characteristics, then there are several possible explanations which could all be checked.

It may be that the strain contains a second mutation in a separate gene, this could easily be investigated by crossing the strain with a WT strain. The resultant progeny, which have the TS phenotype, can then be analysed by transformation with the WT *ypt1* gene. If the progeny still display the same

phenotype this would indicate that the strain does not have a second separate mutant gene. Unless the second gene is very closely linked to the *ypt1* locus in which case it may well continue to cosegregate with the *ypt1-1* allele.

A second possibility is that the genomic copy of the *ypt1* gene is not being expressed at high enough levels to compensate for the mutant ypt1p. One could therefore transform the strain with the *ypt1* cDNA in a plasmid which has a high expression level, for example pEVP11 (Russel and Nurse, 1986). If the higher expression of the WT ypt1p, which could be analysed by Western blotting alleviated the TS phenotype then that would indicate that the phenotype of the *ypt1-1* strain was due to a mutant ypt1p.

Another possibility is that the mutant ypt1p inhibits transport to a certain extent even in the presence of WT ypt1p. This could be investigate by transforming a WT strain with a plasmid which expresses the mutant ypt1p. The growth rate of the resultant strain could then be analysed.

I next attempted to characterise the stage of transport being disrupted in the *ypt1-1* strain. Analysis of acid phosphatase secretion from spheroplasts indicated that secretion was very much reduced in the *ypt1-1* strain in comparison to WT cells. However, Western blotting revealed no accumulation of acid phosphatase intracellularly. This was a somewhat surprising result as I had expected to see protein accumulation as had been seen previously in cells which contain a mutant *S.cerevisiae ypt1*p (Segev, et al., 1988).

It was thought that the strain was in some way down regulating protein synthesis in response to the *ypt1-1* lesion, therefore the strain was transformed with a plasmid containing the *pho4* gene (Pidoux and Armstrong, 1992). The expressed protein contained the c-myc epitope to allow identification of the protein with the 9E10 monoclonal antibody (Munro and Pelham, 1987). The *pho4* construct expressed a protein which terminated ADEL. It had been shown previously that this protein was retrieved back to the ER in transformed cells (Pidoux and Armstrong, 1992). Again no protein was seen to accumulate in the *ypt1-1* strain expressing the pho4 protein, whereas in WT cells an 80kDa form of the pho4p was observed.

It was concluded that either proteins were not being translated at the same rate as in WT cells or proteins which accumulated in the *ypt1-1* strain were rapidly degraded. At the moment the results do not distinguish between these two possibilities. It may be possible to carry out a pulse chase experiment using

^{35}S to label newly translated protein followed by immunoprecipitation after varying lengths of chase. This would allow us to demonstrate whether the *pho4* mRNA is being translated and to deduce the rate of degradation of the protein.

In my opinion it is more likely that the protein is being translated correctly and that accumulated protein is being rapidly degraded. The main reason for preferring this possibility is that at 30°C the *ypt1-1* strain has a growth rate which is very similar to that of WT cells, indicating that the cell must be transporting proteins to its surface and continuing to produce new organelles. If the general level of protein production had been decreased then one might expect to see a commensurate decrease in growth rate. Of course this pre-supposes that other secretory and membrane proteins are not accumulating in the *ypt1-1* strain and that what has been observed is not unique to acid phosphatase.

Morphological analyses of the *ypt1-1* strain has proved very informative. Immunofluorescence studies indicated that at 37°C the Golgi marker galactosyltransferase was redistributed from discrete dots to a much more diffuse pattern which, in some cases, was concentrated at one end of the cell. This suggested that the Golgi complex was being disrupted in the *ypt1-1* strain at the restrictive temperature. The disruption of the Golgi complex was confirmed by electron microscopic analysis of the strain. This demonstrated that after four hours at 37°C the Golgi stacks, which are normally readily apparent in *S.pombe* cells, had almost completely vanished and those that were present were greatly reduced in size, and novel, densely stained organelles had been formed. The nature of these novel organelles is not known at this time. They presumably are formed due to the breakdown of the Golgi stacks and it is interesting to note that both Golgi stacks and the novel organelle stain heavily under the conditions used. One possible explanation is that they represent remnants of the Golgi stacks.

Both the WT and the mutant *ypt1* cDNAs were cloned into pSM^{-}. The cDNAs were then transformed into the 556 strain and protein extracts from the cell lines were analysed for GTP binding proteins, along with extracts from both the 556 strain and the *ypt1-1* strain. The 556 strain which was expressing the WT ypt1p possessed an intense GTP binding protein with an approximate MW of 25kDa which was not seen in the 556 strain expressing the ypt1-1p. The simplest explanation of this result is that the mutant ypt1-1p is defective in GTP binding. This is consistent with sequencing data which revealed that

the *ypt1-1* gene encodes a protein which is mutated in its first GTP binding domain. However, these results do not prove that the ypt1-1p is defective. This can only be shown conclusively by studies on both the purified WT and mutant proteins. Studies on the purified protein would allow us to investigate not only the GTP and GDP binding capabilities of the protein but also its rate of GTP hydrolysis, GDP/GTP exchange rates etc., as has been done for other mutant ypt and rab proteins (Walworth, et al., 1989; Bucci, et al., 1992).

The observed results indicate that *ypt1* gene encodes a protein which is involved in the secretory pathway as a mutant strain containing an altered ypt1p secretes acid phosphatase at much lower levels than is seen in the WT strain. However, at this time we cannot be certain which stage of transport is being disrupted, although there is evidence that the ypt1p operates at an early stage of secretion. Firstly by analogy with its homologues *S.cerevisiae* ypt1p and rab1p which have both been shown to operate early in secretion (Segev, et al., 1988; Plutner, et al., 1991). Secondly in the mutant strain a shift to the restrictive temperature leads to the rapid breakdown of the Golgi complex indicating that a block in ypt1p function in turn leads to a block in transport into the Golgi complex.

It is not clear why the Golgi complex breaks down in the *ypt1-1* strain but it may be that the Golgi continue to form transport vesicles and therefore would theoretically bud itself out of existence (Fig. 4.12). This would explain why galactosyltransferase is apparently relocated from the Golgi complex to the plasma membrane and why the overall level of internal membrane falls by 9% after the strain is incubated at 37^{o}C. Interestingly it has recently been reported that inhibition of protein syntheis causes Golgi breakdown (Ayscough and Warren, 1993). However. under these conditions although by immunofuorescence the Golgi does break down there is no redistribution of stain to the plasma membrane. It may well be that inhibition of protein synthesis blocks all vesicular fusion steps and hence causes Golgi breakdown but does not allow Golgi enzymes to redistribute to the cell surface.

Fig. 4.12 Role of ypt1p

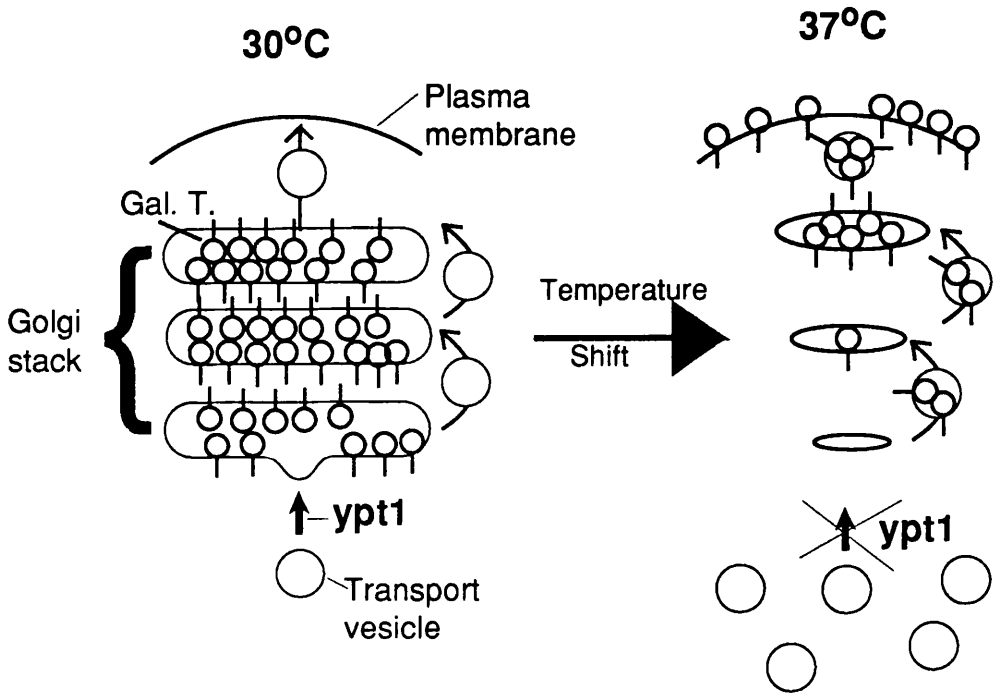


Fig. 4.12
Under normal conditions the ypt1p is involved in a very early stage of transport. After shifting to the non-permissive temperature transport is inhibited and therefore the Golgi complex will no longer receive any vesicles. However, as subsequent stages of transport are not inhibited the Golgi will continue to form vesicles. Eventually this will result in a loss of Golgi structure and the movement of the marker enzyme Gal. T. from the Golgi to the plasma membrane.

Chapter 5

ypt2

5.1 INTRODUCTION

The two original ypt/rab proteins identified were *S.cerevisiae* ypt1p and sec4p. In the previous chapter I discussed work on the characterisation of ypt1p, the *S.pombe* homologue of *S.cerevisiae* ypt1p. In this chapter I am going to discuss ypt2p, the *S.pombe* homologue of the sec4p. The *S.pombe ypt2* gene was cloned by both our group and others (Fawell, et al., 1990; Haubruck, et al., 1990). Sequence comparison revealed that the predicted ypt2 protein is 55% identical to the sec4 protein at the amino acid level, and that there is complete identity between the two proteins' effector domains (Haubruck, et al., 1990).

The homology between ypt2p and the sec4p led Haubruck and colleagues to speculate that the ypt2p was the functional homologue of the sec4p (Haubruck, et al., 1990). To investigate their hypothesis the workers functionally replaced endogenous sec4p with ypt2 protein. The yeast strain created grew as WT indicating that the ypt2p was indeed the functional homologue of the sec4p. Haubruck and colleagues also demonstrated that the ypt2 protein was essential for the growth of *S.pombe* cells.

The ypt2p is the functional homologue of the sec4p, but it is closer in sequence to both sas1p from *Dictostelium* (Saxe and Kimmel, 1988) and to the plant protein ara3 from *Arabidopsis thaliana* (Anai, et al., 1991). Interestingly the sec4p is less closely related to the two higher eukaryotic proteins than is the ypt2p. Recently a mammalian homologue of ypt2p and sec4p has also been identified and has been termed rab8p (Chavrier, et al., 1990). Sequence comparison reveals that again ypt2p is more closely related to the mammalian protein, 63% identity at the amino acid level, than it is to the sec4p.

The ypt2p can substitute for the sec4p but it appears to be much closer in sequence, and presumably in function, to proteins found in higher eukaryotic cells. Work by Novick and colleagues over a number of years has demonstrated quite clearly that the sec4 protein is involved in targeting of vesicles from the Golgi complex to the plasma membrane of the bud (Salminen and Novick, 1987; Walworth, et al., 1989). This stage of transport has no exact equivalent in either *S.pombe* or higher eukaryotic cells, which grow by fission rather than budding. An attractive hypothesis is that as organisms have developed, their ypt/rab genes have evolved also, and that *ypt2* represents a stage in development between the primitive SEC4 gene and the equivalent genes found in higher eukaryotes.

The primary aim of this work was to develop a system which would allow the replacement of the endogenous *ypt2* gene with a recombinant copy and then go on to use this system in an attempt to produce conditional mutant strains defective in the *ypt2* gene. Subsequently the strain could be used to identify at which stage of transport the ypt2 protein acts. A conditional strain could also be used to ascertain whether the rab8p is functionally homologous to the ypt2p, as its sequence indicates. The system we intended to use was exactly as described for both *ypt1* and *ypt5*, with the *ypt2* gene being linked to the selectable marker *ura4*.

The work I shall describe in this chapter was carried out by myself and my colleague Sally Bowden. Specifically the work described in sections 5.2 and 5.3.1 was carried out by Sally Bowden, with the rest of the work being done by myself. Electron microscopy was again done by Rose Watson. The results of most of this work have previously been reported (Craighead, et al., 1993).

RESULTS

5.2 GENETIC MANIPULATION

5.2.1 Gene Replacement

The strategy of gene replacement employed was identical to that used for both *ypt5* and *ypt1*. The *ypt2* gene and accompanying 3' and 5' untranslated regions were cloned from an *S.pombe* genomic library into pBSM13$^-$. The *ura4* gene was then cloned downstream of the *ypt2* coding sequence to form the plasmid pY2U4 (Fig. 5.1). The 4.8Kb insert could be excised from the plasmid by *Eco R1* digestion. The insert was purified and used to transform the WT 556 strain. As before transformants were selected by growth on minimal plates lacking uracil. The recombinants were then screened by PCR (Fig. 5.1) to determine the rate of homologous recombination. It was found that the insert would direct homologous recombination in approximately 15% of all recombinants investigated.

5.2.2 Point Mutation

The relatively low rate of homologous recombination obtained with the Y2U4 insert precluded the use of random mutagenesis in the production of a mutant strain. However, the sequence of the mutant ypt1-1p was known and it was found that the valine residue which had been modified was highly conserved in the ypt/rab family (Fig. 5.2) (Chavrier, et al., 1990) (Pai, et al., 1989). The ypt2p also possesses a valine residue, at position 20, analogous to Val 19 in ypt1p. It was thought that the introduction of an asparagine residue at position 20 in ypt2p would result in a protein which was a conditional mutant.

The *ypt2* gene was specifically modified so as to encode asparagine in place of valine at codon 20 (Ho, et al., 1989) (Fig. 5.3). The resultant plasmid pY2U4-VN was then used in a gene replacement experiment as described above. Transformants were again screened by PCR and an homologously recombined strain selected. The *ypt2* gene from the strain, tentatively termed *ypt2-1*, was then sequenced directly from the genome using PCR and biotinylated primers (Hultman, et al., 1989), as described in chapter 4. Sequencing revealed that codon 20 had been altered, from encoding Val to coding for Asn, and that there were no further mutations present.

Fig. 5.1 pY2U4

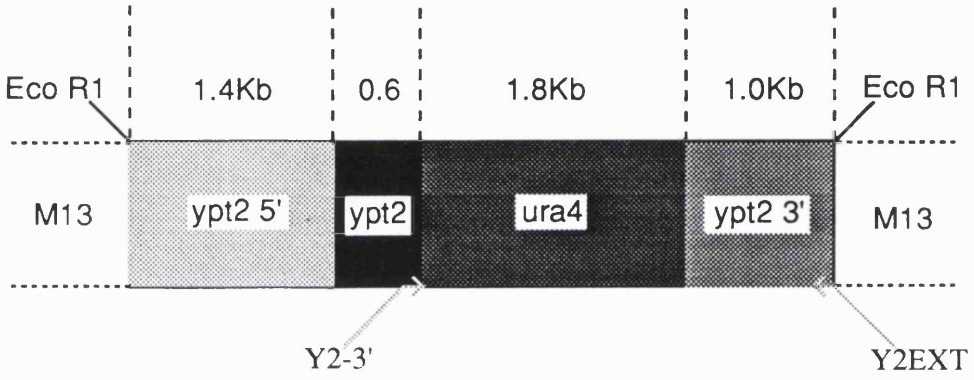


Fig. 5.1
The pY2U4 insert is shown. The 4.8Kb insert can be excised by *EcoR1* digestion. Transformants can be screened by PCR using the oligonucleotides Y2-3' and Y2EXT which yield a 1Kb product with WT and non-homologous recombinants. Homologous recombinants give a 2.8Kb product allowing them to be easily distinguished.

Fig. 5.2 Conservation of Val19

Protein						
sp ypt1	**ser**	**gly**	**val**	**gly**	**lys**	**ser**
sp ypt2	**ser**	**gly**	**val**	**gly**	**lys**	**ser**
sp ypt3	**ser**	**gly**	**val**	**gly**	**lys**	**ser**
Sc YPT1	**ser**	**gly**	**val**	**gly**	**lys**	**ser**
Sc SEC4	**ser**	**gly**	**val**	**gly**	**lys**	**ser**
rab1	**ser**	**gly**	**val**	**gly**	**lys**	**ser**
rab4	ala	**gly**	thr	**gly**	**lys**	**ser**
rab5	ala	**gly**	**val**	**gly**	**lys**	**ser**
rab8	**ser**	**gly**	**val**	**gly**	**lys**	thr
H-ras	gly	**gly**	**val**	**gly**	**lys**	**ser**

Fig. 5.2
The valine residue which was found to be mutated in the mutant protein ypt1-1, is part of a highly conserved motif which is involved in the binding of guanine nucleotides. The valine residue is underlined and conserved residues are shown in bold. The *S.pombe* ypt1p sequence was used as the basis for sequence comparisons.

Fig. 5.3 Mutation of *ypt2*

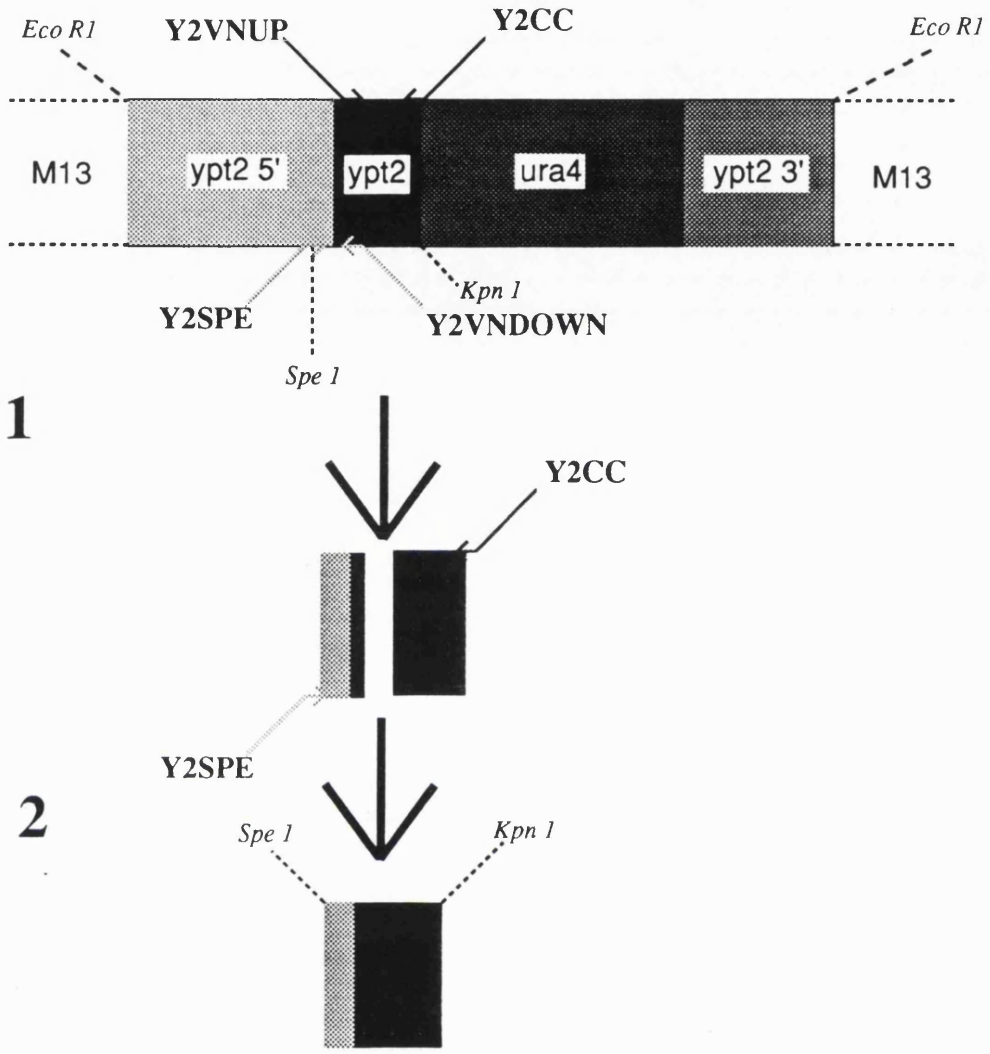


Fig 5.3
The ypt2 gene was specifically mutated by two rounds of PCR with the pY2U4 plasmid being used as the template.
(1) In two seperate reactions the plasmid was amplified with Y2SPE plus Y2VNDOWN, and Y2CC plus Y2VNUP. The two VN primers encoded the required mutation.
(2) The two products were gel purified and joined in a third round of PCR. The oligos were added after the first five cycles. The product was digested with *Spe 1* and *Kpn 1* and cloned into the original plasmid.

5.3 ANALYSIS OF *ypt2-1* STRAIN

5.3.1 Growth properties

The growth properties of the *ypt2-1* strain were now analysed. Initially it was observed that the *ypt2-1* strain would not grow on minimal plates incubated at 37°C. In an attempt to analyse the growth defect further the growth of the strain in minimal media was investigated. Cells were grown at 30°C and then shifted to 37°C. It was observed that *ypt2-1* cells would continue to grow at 30°C but would not grow at 37°C (Fig. 5.4). It was concluded that the *ypt2-1* strain had a TS defect and in an attempt to prove that the defect was due to the presence of an altered *ypt2* gene, the strain was transformed with pSM^+ (Jones, et al., 1988) containing the *ypt2* cDNA (Fig. 5.5), as the *ypt2* gene has no introns the gene is identical to the cDNA. The resultant strain was found to grow on minimal plates at both 30°C and at 37°C (Fig. 5.6). The strain was seen to grow in liquid media also at 37°C, however, this was carried out by visual inspection and the growth rate of the strain in liquid media has not been ascertained.

5.3.2 Complementation of TS defect

One of the original aims of this project was to determine whether the rab8p was the functional homologue of the ypt2p In an attempt to show complementation the rab8 cDNA was cloned into the expression vector pSM (Fig. 5.5) in order to allow it to be transformed into the *ypt2-1* strain. As a control other rab cDNAs were also cloned into the vector (Fig. 5.5). All of the rab cDNAs were supplied by Marino Zerial. The rab containing plasmids were now transformed into the *ypt2-1* strain. The *ypt5* containing plasmid (supplied by Vas Ponnambalam), the rab11 plasmid (supplied by J. Armstrong) and the *ypt1* containing plasmid (described in chapter 4) were also used to transform *ypt2-1*. The pSM plasmid contains the *S.cerevisiae* LEU2 gene, which is equivalent to *S.pombe leu1*, and transformants could therefore be selected by growth on media lacking leucine. All of the transformants were viable at 30°C, however, if the minimal plate was incubated at 37°C then only the *ypt2* and rab8 transformed strains were viable (Fig. 5.6). This provided strong evidence that the rab8p is the functional homologue of ypt2p. Further it demonstrated that the *ypt2* gene could not be substituted with other *S.pombe ypt* genes, even when overexpressed. This indicated that each ypt protein carries out a specific function *in vivo*.

Fig. 5.4 Growth of ypt2-1 versus WT at 30 and 37°C.

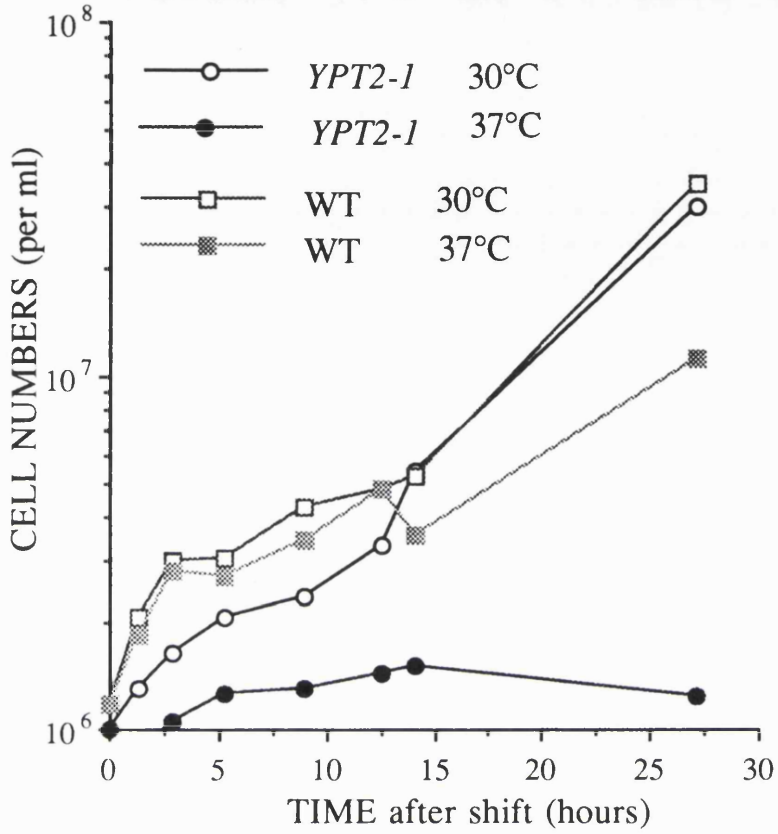


Fig. 5.4

The *ypt2-1* and 556 strains were grown in minimal media at 30°C. The cells were then shifted to 37°C or left at 30°C. Cell numbers were acertained using a haemocytometer.

Fig. 5.5 ypt and rab expression plasmids

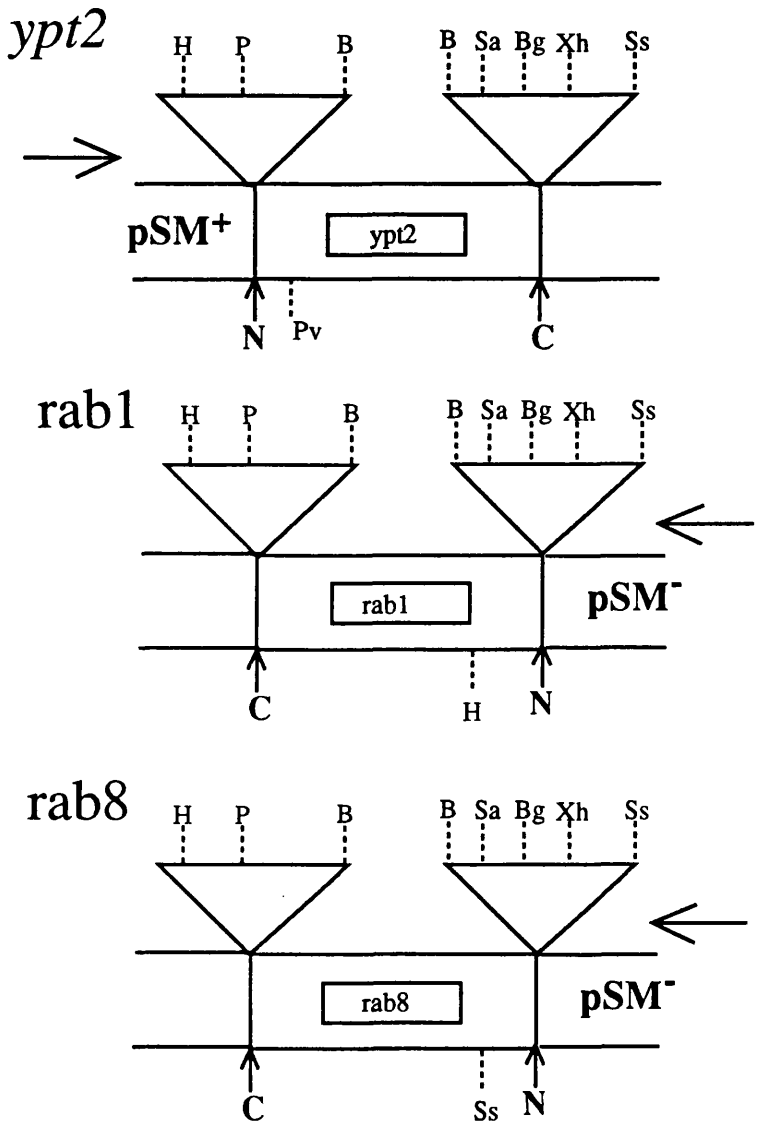


Fig. 5.5 This shows the cloning of various ypt and rab cDNAs into pSM. The restriction sites shown are:
B- Bam HI; Bg- Bgl II; H- Hind III; Pv- Pvu I
Sa- Sal I; Ss- SstI and Xh- Xho I.
Ypt1 was orientated with a Pvu I and Sst I digest.
Rab1 was orientated with a Hind III digest.
Rab8 was orientated with a Sst I digest.
The arrow indicates the direction of transcription and the N and C termini are shown in bold.

Fig. 5.6 Rescue of *ypt2-1*

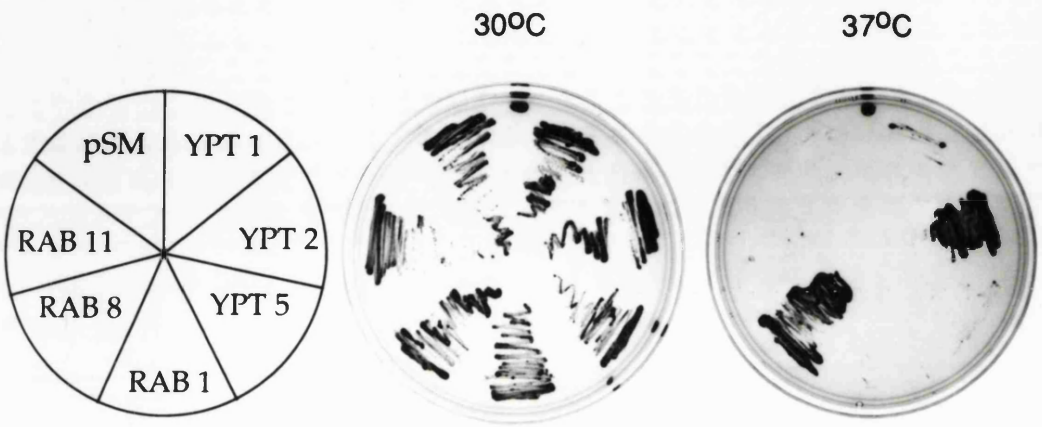


Fig. 5.5

The *ypt2-1* strain was transformed with a variety of *S.pombe* and mammalian ypt/rab genes, as described. The transformants were grown on minimal plates and then streaked onto fresh minimal plates which were incubated at either 30°C or 37°C. The TS defect could be alleviated by expression of either *ypt2* or rab8 but not by any of the other genes.

5.3.3 Accumulation of acid phosphatase

(i) Density distribution

After developing the replacement system and the subsequent production of the conditional strain defective in the *ypt2* gene we now wished to analyse the strain in an attempt to deduce at which stage of transport the ypt2 protein was operating. It was thought that if the strain was defective in transport then this should cause an internal accumulation of acid phosphatase. Therefore we attempted to analyse the predicted accumulation by density centrifugation. This was done because it was thought that by comparing the distribution of the acid phosphatase to that of intracellular markers we could determine in which compartment the acid phosphatase was accumulating.

Both the WT and the *ypt2-1* strains were grown in minimal media at 30°C. Then during logarithmic growth the cells were either shifted to 37°C or left at 30°C for four hours. The cells were spheroplasted and fractionated as described in materials and methods. After centrifugation the gradients was fractionated and analysed for acid phosphatase activity. This indicated that there was a much increase level of acid phosphatase in the *ypt2-1* strain (Fig. 5.7).

The gradients were next assayed for galactosyltransferase (Gal. T.) activity and aliquots were separated by SDS-PAGE and probed with anti BiP antibody, the Western blot was then quantified by laser densitometry. The Gal. T. activity shows a very similar distribution to that of the accumulated acid phosphatase whilst the BiP is localised to a compartment with a quite different distribution (Fig. 5.8). This indicates that the acid phosphatase is present in a compartment which has a similar density to that of the Golgi complex.

Fig. 5.7 Acid phosphatase accumulation

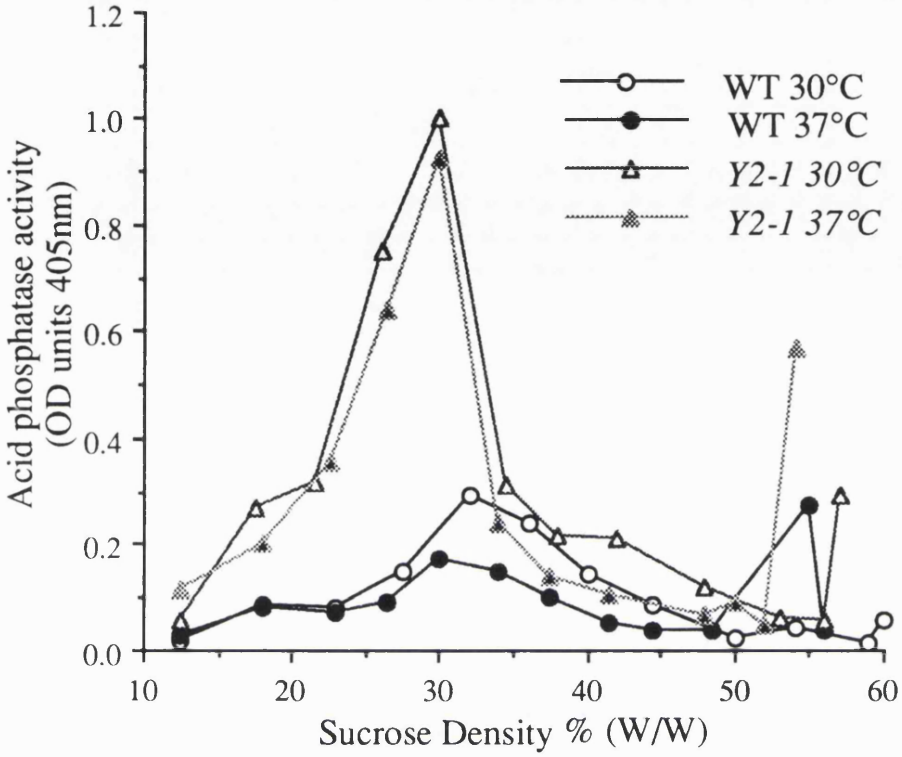


Fig 5.7

Extracts were taken from both WT and *ypt2-1* cells which had either been grown solely at 30°C or had been shifted to 37°C for four hours prior to fractionation. Aliquots were taken and assayed for enzyme activity. Acid phosphatase accumulated mainly at approximately 30% sucrose and at the bottom of the gradient, which presumably represented unlysed cells.

Fig. 5.8 Distribution of A.P. versus organelle markers.

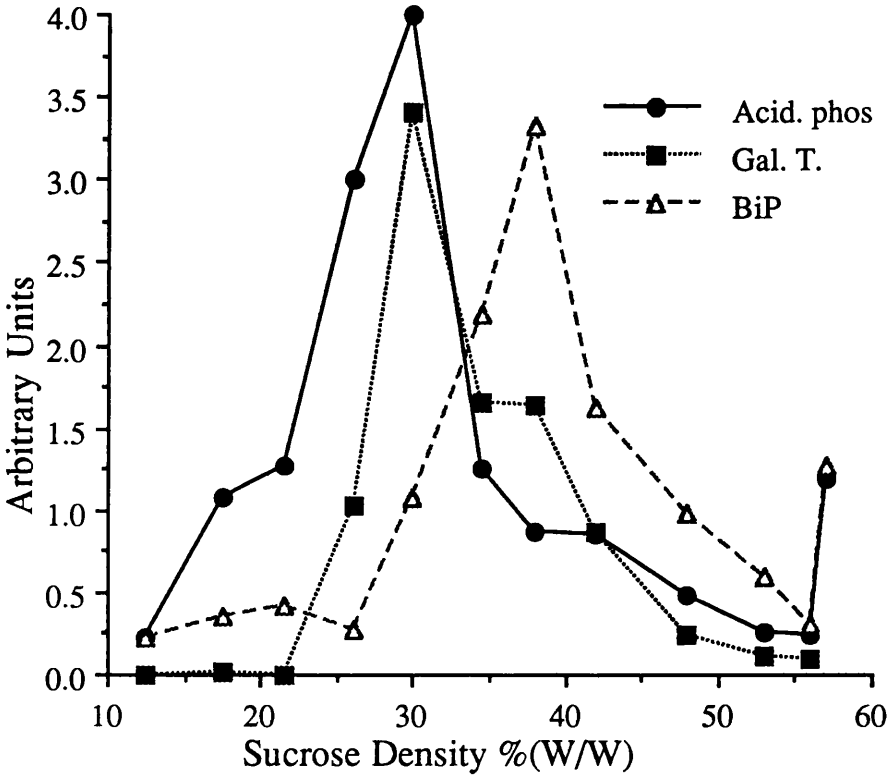


Fig. 5.8

The sucrose gradients described in Fig. 5.7 were assayed for the presence of the Golgi marker galactosyltransferase (Gal. T.) and aliquots were analysed by Western blotting for the presence of BiP. The distribution of the two markers was compared to that of acid phosphatase. The results shown above are for the *ypt2-1* strain grown at 30°C.

(ii) Western Blotting

The sucrose gradient fractionation indicated that the *ypt2-1* strain accumulated the secretory protein acid phosphatase. What it did not reveal was which stage of transport was being disrupted. In an attempt to deduce which stage of transport was being inhibited Western blotting experiments were carried out. WT and *ypt2-1* cells were grown in minimal media at 30°C and again either left at this temperature or shifted to 37°C for four hours. Two hours after shifting cycloheximide was added, to a final concentration of 100µg/ml, to block further protein production (Ayscough, 1993). Protein extracts were treated or mock digested with Endo. H. overnight and separated by SDS-PAGE prior to being transferred to nitrocellulose. The filter was probed with the 7B4 monoclonal Ab (Schweingruber, et al., 1986).

The Western blotting revealed that at 37°C the *ypt2-1* strain accumulated apparently fully modified acid phosphatase and there was also a slight accumulation of the 72kDa ER form (Fig. 5.9). There was some accumulation of acid phosphatase at 30°C also, as shown by Endo H digestion. In the *ypt2-1* there appear to be two forms of acid phosphatase visible after Endo. H. digestion whereas in the WT tracks there is only one major form of the protein. As discussed in chapter 4 there are two forms of acid phosphatase, pho1p (Elliot, et al., 1986) and pho4p (Yang and Schweingruber, 1990) and the monoclonal antibody recognises both proteins. At this time we cannot conclude which protein is being accumulated in the ypt2-*1* strain, also it must be noted that Schweingruber and co-workers have reported multiple forms of acid phosphatase visible after Endo. H. digestion (Schweingruber, et al., 1986).

5.3.4 Secretion

Although we could detect an accumulation of acid phosphatase within the cell we had not yet shown that secretion was defective in the *ypt2-1* strain. Secretion from spheroplasts was therefore assayed. The cells were grown in minimal media at 30°C and then spheroplasted, again at 30°C. The cells were resuspended in media supplemented with sorbitol as an osmotic support. The spheroplasts were then incubated at 30°C or 37°C and aliquots were assayed for acid phosphatase at various time points. Fig. 5.10 shows the results of a typical experiment, each point represents the average of two samples and the experiment was repeated three times. It was found that the rate of secretion from *ypt2-1* spheroplasts was very much lower than the rate from WT cells.

Fig. 5.9 Western Blotting

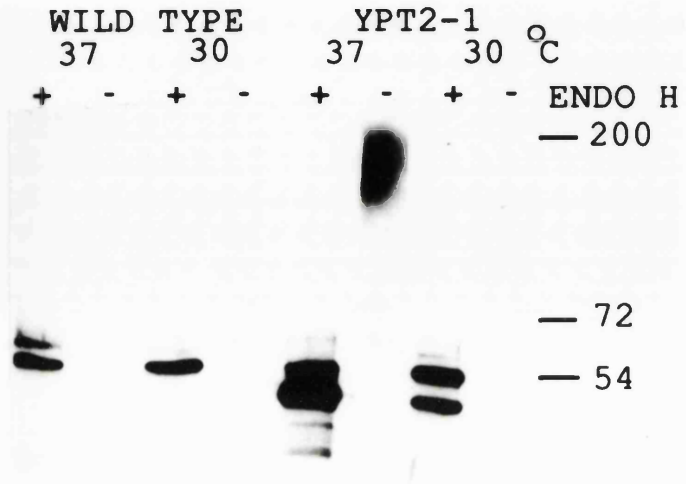


Fig. 5.9

Extracts were taken from *ypt2-1* and WT cells. The cells had been grown at 30°C and either left at this temperature or shifted to 37°C. Two hours after shifting the cells cycloheximide was added to growing cells, to a final concentration of 100μg/ml, to block further protein synthesis. The extracts were run separated by SDS-PAGE and transferred to nitrocellulose and blotted with the anti-acid phosphatase antibody 7B4.

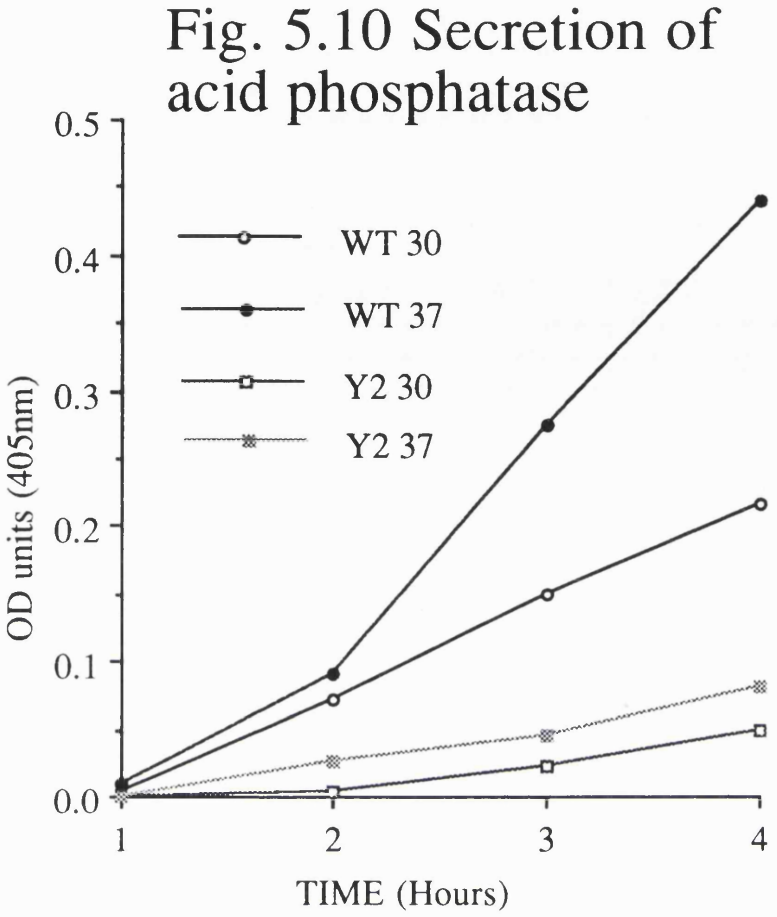


Fig. 5.10

The secretion of acid phosphatase from both *ypt2-1* and 556 spheroplasts was analysed. Cell were grown in minimal media at 30°C and then spheroplasted also at 30°C. Approximately 2 x 10^8 spheroplasts were resuspended in 3ml of minimal media supplemented with 1.2M sorbitol and incubated at either 30°C or 37°C. 50µl aliquots were removed at the time points indicated. The spheroplasts were spun down and the supernatant assayed for acid phosphatase.

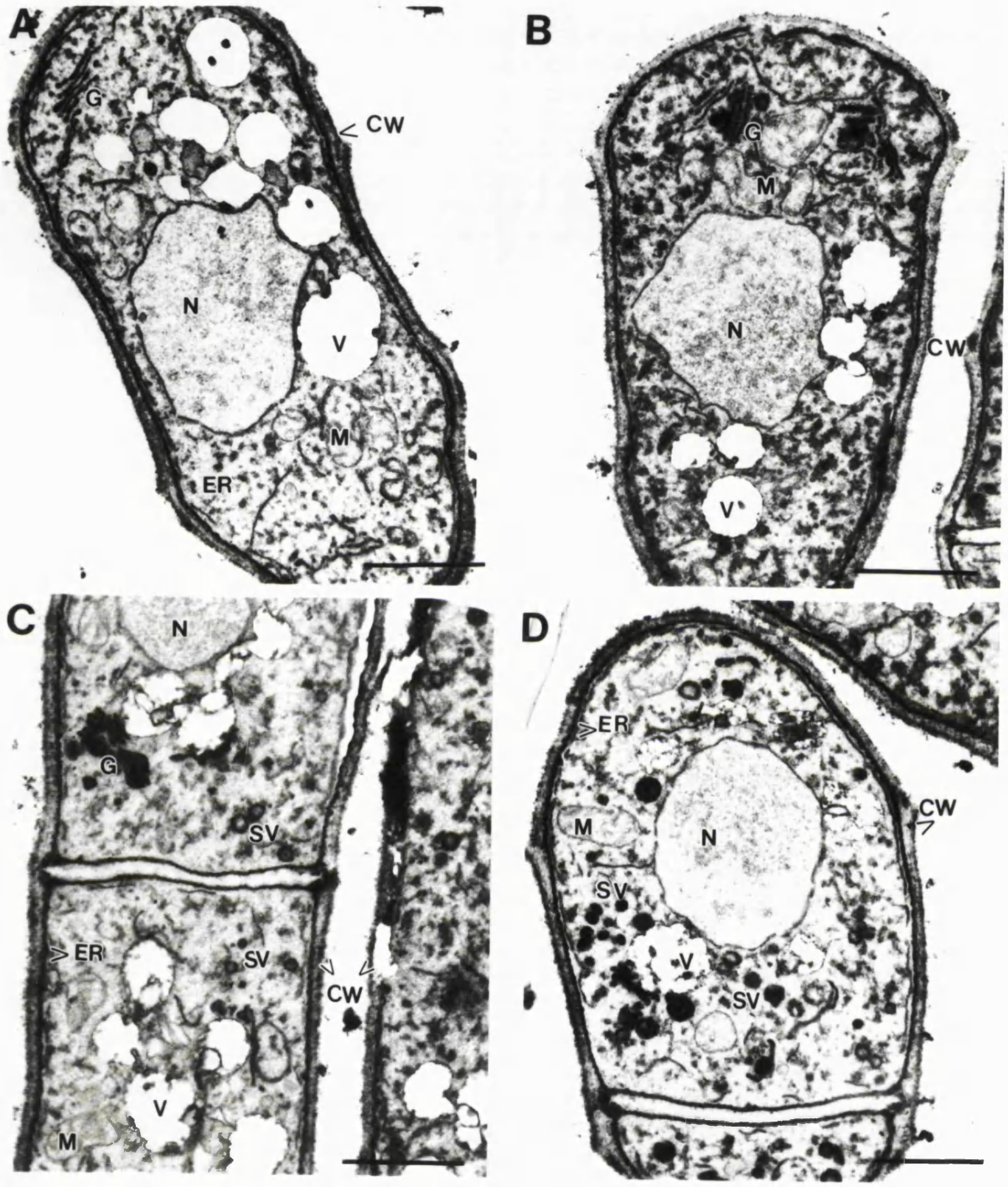
5.4 Electron microscopy

The *ypt2-1* strain was next examined in an attempt to correlate the proposed block in secretion with changes in the cellular architecture. The cells were grown in minimal media at 30°C prior to shifting to 37°C. After four hours the cells were harvested and fixed with potassium permanganate (Fig. 5.11). After incubation at 37°C the cells accumulated densely staining vesicles with diameters ranging between 130 and 170nm (C, D). These vesicles were not observed in *ypt2-1* cells which had remained at 30°C (B), or in WT cells which had also been shifted to 37°C for four hours (A). The organelles in the *ypt2-1* cells incubated at 37°C were not apparently disrupted by the block in transport, Golgi complexes, vacuoles and the ER were all readily apparent. The staining, sectioning and photographing of the electron micrographs were all carried out by Rose Watson.

Fig. 5.11

WT and *ypt2-1* cells were grown to mid-log phase at 30°C in minimal medium. The cells were then either shifted to 37°C or left at 30°C for four hours. The cells were then fixed with potassium permanganate and examined by electron microscopy. (A) WT cells shifted to 37°C for four hours. (B) *ypt2-1* cells grown at 30°C. (C, D) *ypt2-1* cells shifted to 37°C for four hours. CW: cell wall. ER: endoplasmic reticulum. G: Golgi complex. M: mitochondrion. N: nucleus. V: vacuole. SV: vesicles. Bar 1μm.

Fig. 5.11 Electron Microscopy



5.5 DISCUSSION

The aim of this work was the production of a strain of yeast defective in the *ypt2* gene. Subsequently the strain could be examined to identify at which stage the ypt2 protein is operating at, and to determine whether mammalian rab8p is indeed the functional homologue of ypt2p The first stage in the production of a conditional strain was the development of a system to allow the replacement of endogenous *ypt2*. This kind of replacement had already been developed, and successfully applied, to both *ypt1* and *ypt5*. Again a system was developed to allow the replacement of the endogenous *ypt2* gene with a recombinant copy linked to the *ura4* gene.

The scheme again proved successful and screening demonstrated that the endogenous gene was replaced in 15% of viable transformants. This rate is much lower than for *ypt1* but higher than that achieved with *ypt5*. It is not clear what determines the rate of homologous recombination although the most obvious explanation is that different genomic locations will have differing rates of recombination.

Due to the low rate of homologous recombination it was decided that the production of a conditional strain by random mutagenesis would be problematic, for reasons outlined in chapter 3. We had already produced a point mutant of *ypt1* which conferred a TS phenotype when present as the only copy of *ypt1*. As the mutated valine was conserved throughout the ypt/rab family (Fig. 5.2) including the ypt2p, it was decided to mutate the *ypt2* gene specifically in an attempt to produce an analogous mutant allele.

The *ypt2* gene, in pY2U4, was mutated giving pY2U4-VN, as the gene now encoded an asparagine in place of a valine residue at codon 20. The insert was then used to transform haploid 556 and an homologously recombined strain selected. The stain grew normally at 30°C but failed to grow when shifted to 37°C (several independent transformants displayed the same phenotype). The *ypt2-1* strain was transformed with WT *ypt2* and the resultant strain was capable of growth on plates at 37°C. The growth characteristics of the strain in liquid media have not been carefully examined and it would be interesting to do this to confirm that the strain is only defective in the *ypt2* gene.

The *ypt2-1* strain was next transformed with a variety of different *S.pombe ypt* and mammalian rab cDNAs. This was done in an attempt to prove that mammalian rab8p is the functional homologue of the ypt2p. It was found that the expression of WT ypt2p and mammalian rab8p would allow the *ypt2-1* strain to grow on plates at 37°C. This indicates that the rab8p is the functional homologue of ypt2p, however, this analysis was carried out on plates and it would be interesting to analyse the growth of the strains in liquid. If the transformed *ypt2-1* strain was capable of growing in liquid then this would be very strong evidence that the rab8p is functionally homologous to ypt2p. Interestingly recent work has shown that epitope tagged rab8p was incapable of rescuing a sec4 TS strain (Chen, et al., 1993). In the introduction to this chapter it was argued that the ypt2p may represent an evolutionary intermediate between sec4p from *S.cerevisiae,* and the rab8p found in higher eukaryotes. It has been shown that the rab8p will substitute for the ypt2p although it cannot replace the sec4p (Chen, et al., 1993). This is strong evidence that the ypt2p is an intermediate between the sec4p and the rab8p. In the future it is probable that the ypt2p will provide a better model for the late acting rab proteins than the sec4p.

A sucrose gradient fractionation indicated that the *ypt2-1* strain accumulated acid phosphatase at both 30°C and 37°C. Western blotting experiments confirmed that the strain accumulated the secretory protein and indicated that the accumulated protein was fully modified. Finally secretion studies demonstrated a reduction in secretion in the *ypt2-1* strain in comparison to the WT 556 strain. Therefore it can be concluded that the ypt2p does operate in the secretory pathway of *S.pombe* cells. Whilst blotting indicates that the accumulated protein is heavily glycosylated we cannot be certain which modifications the protein has received as the carbohydrates on the accumulated protein have not been analysed in any way.

Analysis of the sucrose gradient indicated that the acid phosphatase was present in a compartment whose density was similar to that of the Golgi complex, as defined by the presence of Gal. T. Whilst electron microscopy reveals the accumulation of electron dense vesicular structures in the *ypt2-1* strain after incubation at the restrictive temperature, these structures have a diameter which ranges between 130 and 170nm. The accumulated vesicles are therefore very similar in size to the post-Golgi vesicles purified by Novick and colleagues (Walworth and Novick, 1987). Presumably the shift to 37°C completely inhibits the transport between the Golgi and plasma membrane

leading to the accumulation of Golgi derived transport vesicles. It would be very interesting to discover whether the accumulated structures contained acid phosphatase. Unfortunately the monoclonal Ab 7B4 does not appear to work in immuno-localisation studies. What we may be able to do is analyse the sucrose gradient to ascertain whether the fraction which contain the acid phosphatase also contain the vesicular structures. If this were the case this would be strong evidence that the vesicular structures are accumulated transport vesicles.

In conclusion the results of this study indicate that the ypt2 protein is involved in transport from the Golgi complex to the plasma membrane (Fig. 5.12). This has been shown by the production of a conditional strain with a defective *ypt2* gene. The strain accumulates the secretory protein acid phosphatase in an apparently fully modified form, and at the restrictive temperature the strain also accumulates densely staining vesicular structures. The TS defect can be corrected by expression of both WT ypt2p rab8p. As the rab8p has been shown to operate in transport from the Golgi complex to the plasma membrane (Huber, et al., 1993) this again provides indirect evidence that the ypt2p is carrying out an analogous role in *S.pombe*.

What has not been proved conclusively is that the accumulated secretory protein is within the vesicles. It may be possible to purify the vesicles using the same protocol that was used to purify post-Golgi vesicles in *S.cerevisiae* (Walworth and Novick, 1987). This would allow the biochemical analysis of the structures: for example the purified structures could be examined by Western blotting for the presence of acid phosphatase and in the longer term sequencing of proteins which are specifically enriched in the vesicles could be carried out, perhaps allowing the identification of potential v-SNAREs.

Fig 5.12 The role of ypt2p

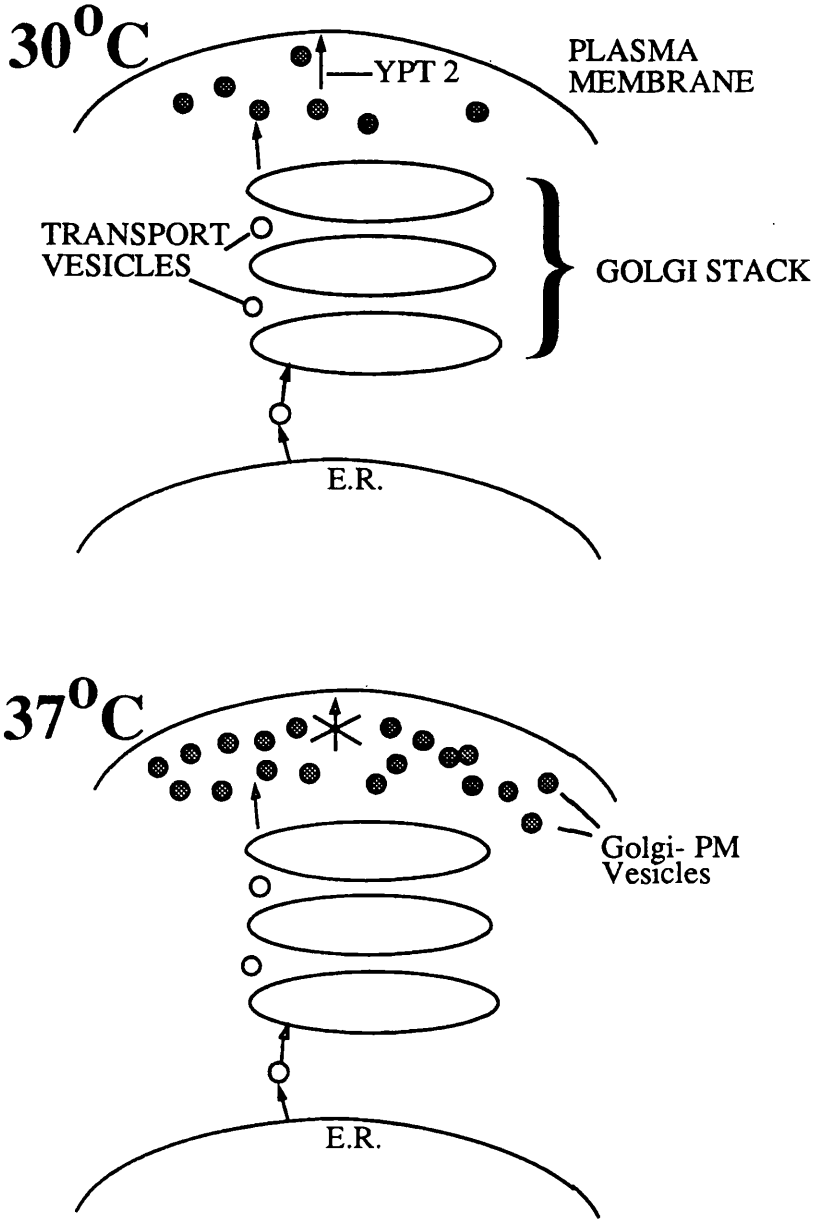


Fig. 5.12
In the *ypt2-1* strain at 30°C transport is normal except between the Golgi and plasma membrane which is reduced. When the cells are shifted to 37°C the ypt2 protein ceases to function resulting in a transport block and the accumulation of transport vesicles.

Chapter 6

Conclusion

6.1 Introduction

In this final chapter I am going to briefly go over the system used in this study. I shall then go on to consider the results obtained in the study of the various proteins. In particular I will discuss the protein's role *in vivo*. and the possible ways in which the studies presented in this work can be developed in the future. Finally I shall present depicting the predicted function of the various *S.pombe* ypt proteins.

6.2 Gene replacement

All three *ypt* genes studied in this work were replaced using a system whereby the endogenous gene was homologously replaced with either randomly or specifically altered copies of the gene linked to a selectable marker. In each case it was possible to replace the endogenous gene, however, the efficiency of homologous replacement versus non-homologous varied greatly between the three genes studied. The *ypt1* gene had a rate of homologous recombination which approached 100% whereas the *ypt5* and ypt2 genes had much lower rates of recombination. At the moment it is not possible to conclude why such a variability in replacement efficiency is seen. Quite possibly the locus of the gene will greatly effect the rate of homologous recombination, perhaps because the *ypt* gene is physically close to a second gene whose expression could well be disrupted by the introduction of the *ura4* gene. However, what is clear is that the system employed was successful in all cases and the system could be employed in the future to allow one step gene replacement in *S.pombe*.

6.3 Specific and random mutagenesis

Ypt genes were replaced with both randomly and specifically mutated genes. In the case of *ypt1* a conditional mutant strain was produced by random mutagenesis using HAM. In the future the same or different mutagenic agents could be used in an attempt to produce conditional mutants of both *ypt1* itself and other *ypt* genes. *Ypt2* and *ypt5* were both replaced with specifically altered copies of the gene. This protocol proved extremely efficient and allowed the *in vivo* study of mutant proteins whose properties had already been predicted. In general both methodologies will be useful in the future study of both *ypt* genes and possibly of other *S.pombe* genes.

6.4 *ypt5*

In chapter three I discussed the replacement of the *ypt5* gene with recombinant copies altered at the C terminus. It was shown that the ypt5p required the presence of at least one GeGe moiety in order to function correctly. It was found that the mutant protein which had only a single GeGe moiety were only poorly membrane associated in comparison to WT protein which has two GeGe moieties. It was therefore concluded that the GeGe moieties allow the protein to associate with lipid membranes *in vivo* and that membrane association was essential for the protein's function.

The studies presented here do not demonstrate at which stage of transport the ypt5p is operating although work done by others and sequence comparisons indicates that it is the functional homologue of rab5p and by extension that it functions at an early stage of endocytosis (Armstrong, et al., 1993; Bucci, et al., 1992) (Fig. 6.1). It was observed that the SSCypt5 strain, which contains a altered ypt5 protein, may well not survive entry into stationary phase. Much work will have to be done in the future to investigate the phenotype of the SSCypt5 strain in greater detail. Also in the future it may well be possible to use the replacement system developed to produce conditional mutant strains defective in the ypt5p. If the SSCypt5 strain does display specific and reproducible defects then it could be used to identify proteins which interact with the ypt5p *in vivo* as has been done with strains deficient in the sec4p (Burton, et al., 1993; Moya, et al., 1993) and completely lacking the *S.cerevisiae* ypt1p(Dascher, et al., 1991).

6.5 *ypt1*

The *ypt1* gene was replaced with a randomly mutagenised copy and a conditional strain was produced. Rescue studies indicated that the defect in the strain is due, at least in part, to the altered *ypt1* gene. Studies on the strain have allowed us to conclude that the ypt1p operates at an early stage of secretion (Fig. 6.1). This was concluded because spheroplasts show very low rates of secretion in comparison to WT spheroplasts and also as it was observed that at the restrictive temperature the stacked Golgi complexes observed in *S.pombe* very rapidly break down, indicating a defect in transport. Another reason for concluding that the ypt1p operates at an early secretion stage is by analogy to both *S.cerevisiae* ypt1p and rab1p, the homologues of ypt1p, which have both been shown to be involved in early stages of transport (Schmitt, et al., 1988; Segev, et al., 1988; Plutner, et al., 1991). GTP binding

studies indicated that the mutant ypt1p was deficient in binding GTP and sequencing of the gene indicated that the protein it encoded was altered in its first GTP binding domain (Pai, et al., 1989).

Although the ypt1-1 strain was thought to be defective in secretion no internal accumulation of either endogenous acid phosphatase or of acid phosphatase expressed from a plasmid was observed. In fact the level of protein was less than that in the WT strain. The reasons for this phenotype will have to be investigated in the future. The strain could also be used, as was discussed above, to look for surpressors of the TS defect again in the hope of identifying proteins which functionally interact with the ypt1p. It will also interesting to characterise the mutant protein in much greater detail and it might be possible to determine why the presence of the mutant protein results in a conditionally mutant strain.

6.6 *ypt2*

As described above the ypt1-1p was predicted to be altered in its first GTP binding domain. Sequence comparison revealed that the residue is conserved in a large number of both ypt and rab proteins. A *ypt2* gene was therefore specifically modified so as to encode a protein with the analogous mutation. It was thought that the introduction of this allele would yield a strain deficient in the *ypt2* gene. It has been shown previously that analogous mutants in different ypt/rab proteins have a similar phenotype. For example a dominant ras mutation (Barbacid, 1987) has been introduced into a number of rab and ypt proteins all of which have a dominant inhibitory effect on growth (Schmitt, et al., 1986; Walworth, et al., 1989; Plutner, et al., 1991; Gorvel, et al., 1991).

It was found that the strain produced by recombination was indeed TS for growth and that this defect could be alleviated by expression of WT ypt2 protein. The strain could also be rescued by WT rab8p expression but not by expression of other rab or ypt proteins. This indicates that the ypt proteins in *S.pombe* are carrying out functionally distinct roles *in vivo* which is necessary if the proteins are involved in the regulation of vesicle specificity. It also indicated that the ypt2 protein was carrying a functionally analogous role to the rab8p i.e. it was involved in a late stage of transport (Fig. 6.1). Secretion studies confirmed that transport was disrupted in the cell line and Western blotting experiments indicated that the transport block was at a late stage of transport.

Sucrose gradient fractionation demonstrated that the accumulated secretory protein was in a compartment with a similar density to that of the Golgi. This observation will need to be investigated further to determine whether the protein accumulates in post Golgi vesicles or in the Golgi itself. Now it has been shown that the rab8p can alleviate the defect in the strain it may be possible to use this as an assay for producing rab8p variants which are either completely non-functional or are conditional mutants.

It has been shown that two different *ypt* genes display a TS defect when they were altered in analogous valine residues. As the valine residue is conserved throughout almost all known ypt/rab proteins it is probable that both ourselves and others will engineer the same mutation into a variety of other family members. Presumably this will allow the production of various TS strains of yeast deficient in specific ypt proteins. In mammalian cells the altered rab proteins could be studied using the different *in vitro* assays (Bucci, et al., 1992; Plutner, et al., 1991) that are now available, and perhaps they could be used as the basis of new assays designed to determine the function of the various rab proteins.

Again as discussed above the replacement system could be used to produce more conditional mutants in the various *ypt* genes. If a number of conditional alleles of a particular gene exist, then the strains could be used to identify allelic suppressers. That is other genes whose mutation/ overproduction will rescue some of the mutant alleles but not all. The benefit of this is that in this way one can identify proteins which interact with the ypt protein i*n vivo* and do not simply allow the cell to by-pass the need for the ypt protein. Similarly it may be possible to identify suppressers which can alleviate the same defect in different *ypt* genes. For example a protein whose mutation/ overexpression can rescue both the *ypt1-1* and *ypt2-1* strains. Such a suppresser would presumably interact with a number of ypt proteins *in vivo* in the same way as GDI (Matsui, et al., 1990; Soldati, et al., 1993).

6.7 *S.pombe* ypt proteins in the secretory pathway

The results of the work presented here and of a number of other studies (Hengst, et al., 1990; Armstrong, et al., 1993; Miyake and Yamamoto, 1990; Haubruck, et al., 1990) have indicated that the *S.pombe* ypt proteins play specific roles in intracellular transport. Fig. 6.1 depicts at which stage in transport each of the ypt proteins is predicted to operate. As in both higher eukaryotes and in *S.cerevisiae* it appears that the ypt protein act to regulate specific stages of vesicular traffic within the *S.pombe* cell. Much work is sill required to precisely characterise the role of each of the known ypt proteins and also to identify and characterise any other ypt proteins which may be present in *S.pombe*.

Fig. 6.1 Role of *S.pombe* ypt proteins

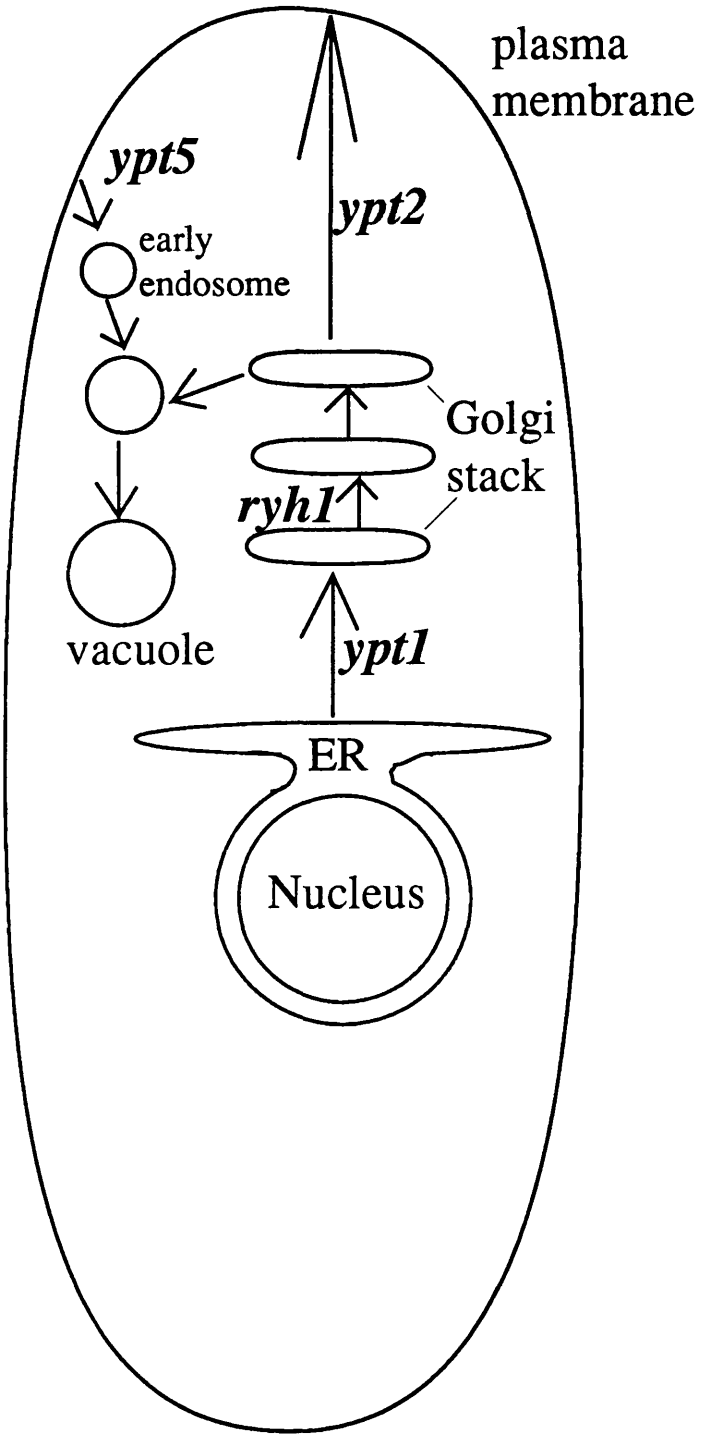


Fig. 6.1
Above gives a highly stylised view of transport within an *S.pombe* cell with each transport stage being represented with an arrow. The predicted localisation of various ypt proteins is shown. The ryh1p was studied by Gallwitz and colleagues (Hengst, et al., 1990).

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