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Tuija Vasara

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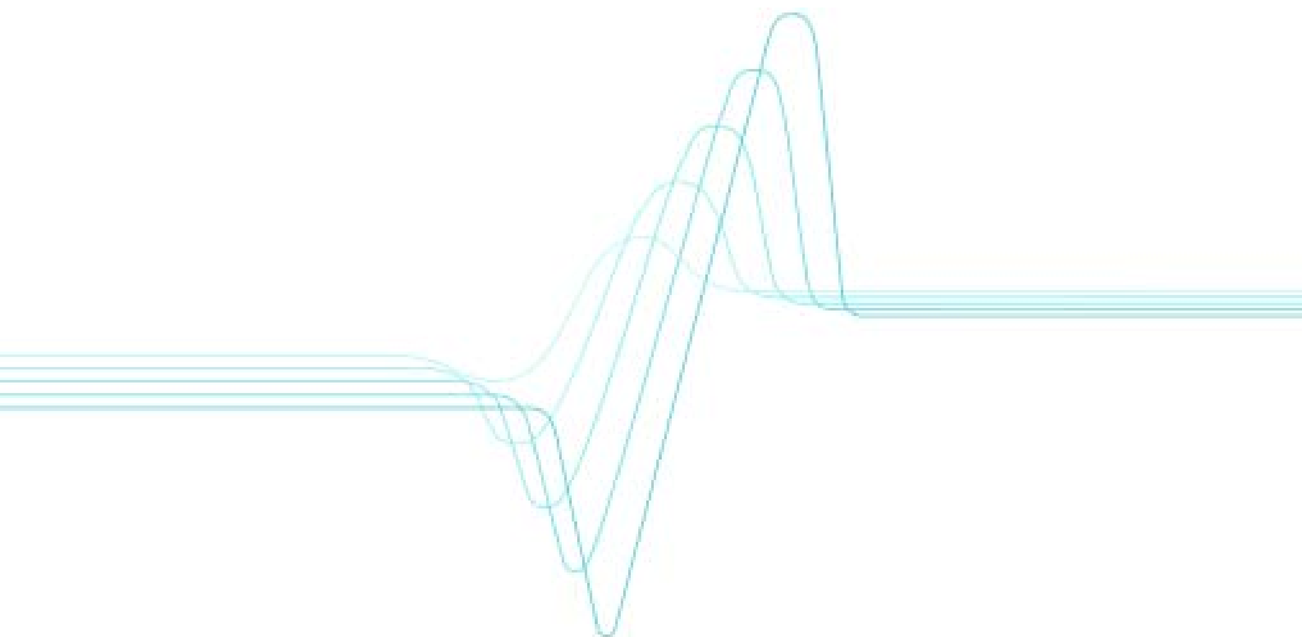

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Functional analysis of the RHOIII and 14-3-3 proteins of *Trichoderma reesei*

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Abstract

The filamentous fungus *Trichoderma reesei* is a biotechnologically important organism having an excellent capability to secrete hydrolytic enzymes e.g. cellulases. Allthough the protein production technology is well established, the protein secretion apparatus of *T. reesei* is still insufficiently known.

To isolate and characterise genes involved in secretion in *T. reesei*, a complementation screening in a *sec15-1 Saccharomyces cerevisiae* strain with a *T. reesei* expression cDNA library was performed. Sec15p is a component of the exocyst complex that is essential for the orientation of the secretion pathway and thus for polarised growth and secretion. Two signal transduction genes encoding the RHOIII and a truncated 14-3-3 protein (FTT1ΔC) were obtained as suppressors of the yeast *sec15-1* mutation. Two full-length FTT genes (FTTI and FTTII) were also cloned from *T. reesei* but they did not suppress this mutation.

Several other genetic interactions between late-acting secretory genes of *S. cerevisiae* and *T. reesei rho3* or *ftt1ΔC* were also detected and this further implies the involvement of these genes in regulation of protein secretion. The expression of *rho3* gene was shown to rescue the growth of mutations in genes encoding other exocyst components and Sec1p that is a regulator of secretory vesicle docking to the plasma membrane. *Ftt1ΔC* appeared to interact with genes involved in vesicle targeting along actin cables in yeast.

These *T. reesei* genes were also able to suppress the secretion defects of *sec15-1* or *sec2-41* strains of *S. cerevisiae*. The full-length *ftt1* enhanced the secretion of invertase in a wild type yeast although it could not suppress the growth of the yeast secretion mutations. The *ftt2* did not have any clear effect on secretion in yeast. Moreover, expression of *ftt1* slightly retarded the growth of wild type yeast cells while expression of *ftt2* enhanced it.

The *rho3* gene was disrupted in the *T. reesei* genome. The disruptants were able to grow as well as the parental strain in media with either glucose or cellobiose as the carbon source. However, in a medium with cellulose as the carbon source both growth and secretion were reduced in the disruptants. The morphology or actin organisation was not affected by the disruption. Therefore, the *rho3* gene would appear to be more involved in the regulation of secretion than morphogenesis in *T. reesei*.

Preface

The present study is carried out at VTT Biotechnology (formerly VTT Biotechnology and Food Research) during the years 1995–2001. The work was financially supported by the Viikki Graduate School in Biosciences and by grants from the University of Helsinki and Finnish Cultural Foundation, which are acknowledged.

I wish to express my gratitude to Research Director, Professor Juha Ahvenainen and Professor Hans Söderlund for excellent working facilities and the encouraging atmosphere at the VTT. Professor Timo Korhonen, head of the Division of General Microbiology, and Dr. Ritva Virkola, study secretary, are acknowledged for their ready cooperation during the final stage of my studies. Docents Vesa Olkkonen and Pekka Lappalainen are warmly thanked for their fast and thorough reviewing of the thesis manuscript and their useful suggestions to improve it.

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Lappeenranta, April 2002.

Tuija Vasara

List of publications

This thesis is based on the following original articles which are referred to as I–III in the text.

I Vasara, T., Saloheimo, M., Keränen, S. and Penttilä, M. 2001. *Trichoderma reesei rho3*, a homologue of yeast *RHO3*, suppresses the growth defect of yeast *sec15-1* mutation. Curr. Genetics **40**, 119–127.

II Vasara, T., Salusjärvi, L., Raudaskoski, M., Keränen, S., Penttilä, M. and Saloheimo, M. 2001. Interactions of the *Trichoderma reesei rho3* with the secretory pathway in yeast and *T. reesei*. Mol. Microb. **42**, 1349–1361.

III Vasara, T., Keränen, S., Penttilä, M. and Saloheimo, M. 2002. Characterisation of two 14-3-3 genes from *Trichoderma reesei*: interactions with yeast secretory components. Biochim. Biophys. Acta, in press.

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Abbreviations

AP	adaptor protein[1]
ATP	adenosine triphosphate
bip	gene encoding binding protein[1]
BHK	baby hamster kidney
Bni	forming homology protein[2]
Bnr	Bni1 related protein
bp	base pair
Bud	budding protein
Ca	calcium
CAP	adenylyl cyclase-associated protein
CBHI	cellobiohydrolase I
CHC1	a gene encoding clathrin heavy chain
COP	coatomer complex proteins
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
ERGIC	ER-Golgi intermediate compartment
GAP	GTPase activating protein
GDI	GDP dissociation inhibitor protein
GDP	guanosine diphosphate
GEF/GEP	guanine nucleotide exchange factor/protein
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
Hof	homolog of cdc15, protein involved in cytokinesis
hsp	heat shock protein

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[1] Note the nomenclature:

Filamentous fungi/mammals	*gene*		PROTEIN

[2] Note the nomenclature:

Yeast	*GENE*	*gene with mutation*	Protein

kb	kilobase
kDa	kilodalton
KIF	kinesin family
LPA	lysophosphatidic acid
MUL	methylumbelliferyl-β-D-lactoside
Myo	myosin
NSF	*N*-ethylmaleimide sensitive fusion protein
ORF	open reading frame
pdi	gene encoding protein disulphide isomerase, a chaperone
Pkc	protein kinase C
PM	plasma membrane
PP1	protein phosphatase 1
RNA	ribonucleic acid
SEC	secretory gene[2]
sec	mutant *SEC* gene[2]
Sec	secretory protein
SNAP	soluble NSF-attachment protein
SNARE	soluble NSF-attachment protein receptor
Snc	suppressor of the null allele of CAP, a vesicle-SNARE
SRP	signal recognition particle
Sso	suppressor of *sec one*, a target-SNARE
TGN	*trans*-Golgi network
TRAPP	transport protein particle
Vsm	vesicle SNARE master protein

1. Introduction

Filamentous fungi have a central role in carbon recycling in nature. They characteristically secrete large amounts of extracellular enzymes in order to degrade complex polymers derived from plants (e.g. lignin and cellulose) into mono- and oligomeric compounds which they use for their nutrition. The growth mode of filamentous fungi is well adapted for decomposition of plant residues. They grow by means of branching hyphae extending exclusively at apices which allows them to explore and penetrate various substrata. It has been suggested that hydrolytic enzymes are predominantly released from the tips (Wösten *et al*., 1991; Wessels, 1993) and therefore the growth and secretion of filamentous fungi are thought be intimately connected.

The ability of some filamentous fungal species (e.g. *Trichoderma reesei*, *Aspergillus niger*) to produce gram-quantities of protein per litre of culture medium (e.g. Durand *et al*., 1988; Verdoes *et al*., 1995) has been exploited by enzyme industry. In spite of well established protein production technology, the knowledge of basic cell biology and molecular mechanisms of protein secretion in filamentous fungi is still rather limited. In contrast, the secretion apparatus of the fungus *Saccharomyces cerevisiae* is extensively studied and has been found to be well conserved with that of higher eukaryotes (reviewed by Ferro-Novick and Jahn, 1994; Rothman, 1994). On the basis of this conservation, similar mechanisms are thought to prevail also in vesicle trafficking in filamentous fungi. However, the distinct features of filamentous fungi: high secretion capacity and filamentous growth, may bring about modifications in the classical secretion pathway, which is originally described by Palade (1975) and further reinforced by morphological and biochemical studies and yeast genetics.

1.1 Overview of the secretory pathway

The eukaryotic cell is a highly organised structure with distinct subcellular compartments. To maintain this compartmentalisation with specific protein and lipid compositions, mechanisms to certify that specific proteins are delivered to specific organelles or specific domains of plasma membrane have been generated.

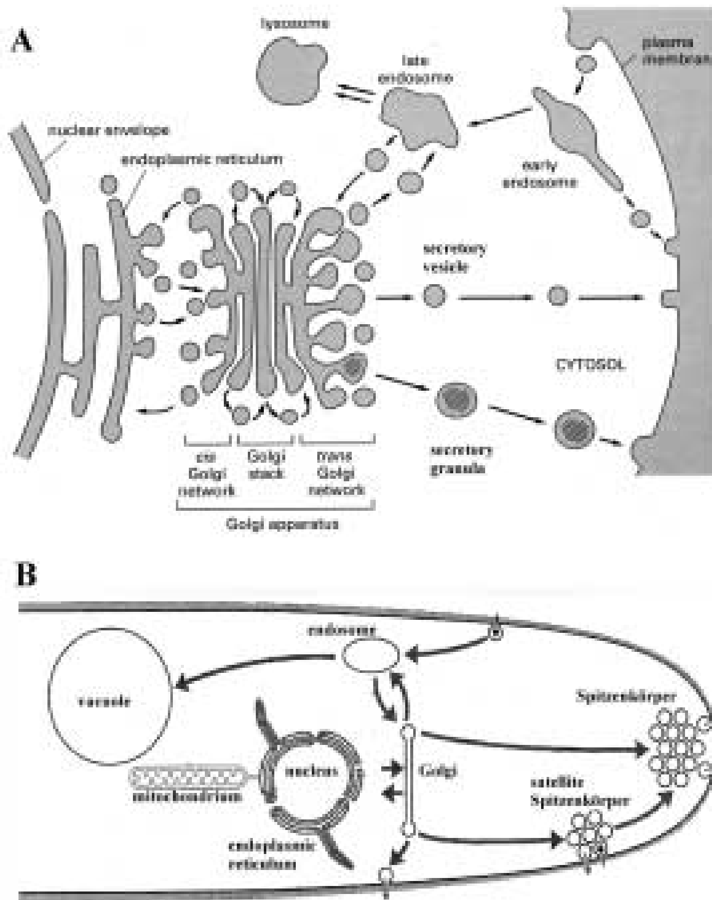

Figure 1. Schematic representation of the secretory pathways of mammalian cells (A) and filamentous fungi (B).

(A). Mammalian cells have a well-organised stack of Golgi apparatus cisternae. The ER-to-Golgi intermediate compartment (ERGIC) is not represented in this figure. In addition, the mechanism of transport through the cisternal stack is under debate. This figure represents the vesicular transport model for intra-Golgi transport. The alternative is cisternal maturation model (Pelham, 1998). Modified from Alberts et al.*, (1989)*

(B). A characterictic organelle in tip of hyphae, Spitzenkörper, consists of small accumulated secretory vesicles. It acts as a vesicle supply center, having a role in apical extension. The endoplasmic reticulum membrane has been observed to be in contact with mitochondrial membranes in the hyphae of filamentous fungi (Bourett et al.*, 1998; Fischer-Parton* et al.*, 2000) and in yeast (Prinz* et al.*, 2000a). Modified from Fisher-Parton* et al. *(2000).*

Proteins that are destined to the membranous bodies, which in fungi comprise endoplasmic reticulum, Golgi complex, endosomes and vacuole, to plasma membrane or to cell exterior have a signal sequence which ensures their entry into the secretory pathway. The entry station for newly synthesised proteins is the endoplasmic reticulum (ER). From the ER proteins are transferred through the Golgi complex unless having retention/retrieval signals, and in *trans*-Golgi network (TGN) the proteins are sorted to their final destination according to their localisation signals (Figure 1). Proteins are transported between the organelles in coated carrier vesicles that bud from one compartment and fuse, after being uncoated, selectively with an other compartment (Kuehn and Schekman, 1997).

To counterbalance the continuous withdrawal of membrane proteins and lipids from ER and Golgi complex during this anterograde transport, lipids and the proteins having retrieval signals have to be recycled by retrograde transport. Two major recycling pathways can be defined: recycling from the Golgi cisternae to the ER and recycling from PM or endosomes to the TGN (Goda and Pfeffer, 1988; Bos *et al*., 1993; Cosson and Letourneur, 1994).

1.1.1 Endoplasmic reticulum and Golgi(-like) complex

Proteins are transferred into the endomembrane structures by the translocation machinery. The hydrophobic signal sequences of polypeptides that are translated on ribosomes are recognised by signal recognition particle (SRP) which guides the nascent polypeptide-ribosome complexes to the cytosolic face of the ER membrane. After a release from SRP the signal sequence interacts with Sec61p complex in a second signal recognition step which then opens a protein-conducting channel formed by the Sec61p complex towards ER lumen. Soluble proteins can also be translocated across the ER membrane post-translationally. In that case polypeptides are targeted to the ER membrane by a single recognition step by the Sec61p complex (reviewed by e.g. Rapoport *et al*., 1996). Since ribosomal RNAs, signal sequences and SRPs have been demonstrated to be interchangeable between eukaryotes and prokaryotes (Powers and Walter, 1997; Prinz *et al*., 2000a) and since SRP have been also described in filamentous fungi (Thompson *et al*., 1995), it is likely that targeting and translocation of the nascent polypeptides in the ER utilise similar basic mechanisms in filamentous fungi as well.

Table 1. Secretion genes cloned from filamentous fungi.

Gene	Site of protein action	Function	Species	Reference
srpA	cytosol / ER membrane	Protein translocation	*Aspergillus niger*	Thompson *et al*., 1995
dpm1	cytosolic face of ER	Protein core glycosylation	*Trichoderma reesei*	Kruszewska *et al*., 2000
mpg1	cytosolic	Protein core glycosylation	*T. reesei*	Kruszewska *et al*., 1998
bip	ER lumen	Protein folding	*A. niger var awamori*	Hijarrubia *et al*., 1997
bipA	ER lumen	Protein folding	*A. niger*	Van Gemeren *et al*., 1997
pdi	ER lumen	Disulphide bond oxidation and isomerisation, protein folding	*Humicola insolens*	Kajino *et al*., 1994
pdi	ER lumen	Disulphide bond oxidation and isomerisation, protein folding	*A. niger*	Malpricht *et al*., 1996 Ngiam *et al*., 1997
pdi	ER lumen	Disulphide bond oxidation and isomerisation, protein folding	*A. oryzae*	Lee *et al*., 1996
pdi1	ER lumen	Disulphide bond oxidation and isomerisation, protein folding	*T. reesei*	Saloheimo *et al*., 1999
pdi	ER lumen	Disulphide bond oxidation and isomerisation, protein folding	*A. fumigatus*	Nigam *et al*., 2001
tigA	ER lumen	Disulphide bond oxidation and isomerisation, protein folding	*A. niger*	Jeenes *et al*., 1997
prpA	ER lumen	Disulphide bond oxidation and isomerisation, protein folding	*A. niger var awamori*	Wang and Ward, 2000
hac1	nuclear	Transcription factor of unfolded protein response	*T. reesei*	Saloheimo *et al*., submitted
ire1	ER membrane	Positive regulator of HACI mRNA splicing	*T. reesei*	Valkonen *et al*., unpublished

ptc2	cytosolic	Negative regulator of unfolded protein response	*T. reesei*	Valkonen *et al*., unpublished
sarA	cytosolic face of ER	GTPase involved in vesicle budding from ER	*A. niger*	Veldhuisen *et al*., 1997
sar1	cytosolic face of ER	GTPase involved in vesicle budding from ER	*T. reesei*	Veldhuisen *et al*., 1997
ypt1	cis-Golgi membrane	GTPase involved in vesicle fusion at Golgi	*Neurospora crassa*	Heintz *et al*., 1992
ypt1	cis-Golgi membrane	GTPase involved in vesicle fusion at Golgi	*T. reesei*	Saloheimo *et al*., submitted
yptA	cis-Golgi membrane	GTPase involved in vesicle fusion at Golgi	*A. niger*	Punt *et al*., 2001
nsf	cis-Golgi membrane / plasma membrane	ATPase involved in disassembly of SNARE complexes	*T. reesei*	Saloheimo *et al*., submitted
nsfA	cis-Golgi membrane / plasma membrane	ATPase involved in disassembly of SNARE complexes	*A. niger var awamori*	Saloheimo *et al*., submitted
kexB	trans-Golgi network	Proteolytic maturation of secreted proteins	*A. niger*	Jalving *et al*., 2000
srgA	Golgi- plasma membrane	GTPase involved in vesicle fusion at PM	*A. niger*	Punt *et al*., 2001
snc1	Golgi- plasma membrane	T-SNARE in vesicle fusion at PM	*T. reesei*	Valkonen *et al*., in prep.

In the lumen of the ER, the post-translational processing of polypeptides is initiated. The signal sequence is cleaved off by the signal peptidase complex, polypeptide chain core glycosylation takes place, disulfide bonds are formed, and the polypeptide is folded and, in some cases, oligomerised (reviewed by Hammond and Helenius, 1995). Each of these events requires a set of ER resident proteins that assist the process or function as catalysts. These proteins have been extensively characterised for animal cells and *S. cerevisiae* (see Ellgaard *et al*., 1999). Genes encoding proteins involved in protein folding have also been cloned in filamentous fungi, such as the chaperone protein *bip* belonging to the hsp70 family and protein disulphide isomerase (*pdi*) (see Table 1 and references therein). The primary quality control process ensures efficient folding of newly synthesized proteins. Proteins having hydrophobic patches exposed on the surface, incompletely trimmed core glycans or exposed free cysteines are transiently associated with the foldases and chaperones mentioned above or with the lectins calnexin and calreticulin until correctly folded (reviewed by Ellgaard *et al*., 1999). The prolonged association of a polypeptide with the ER-resident chaperones is a signal of protein mal-folding. Aberrant proteins are transported to the cytosol through the retro-translocation machinery and degraded there by the 20S proteasome (reviewed by Brodsky and McCracken, 1997). The proteins with correct tertiary structures are concentrated, packed into vesicles and transported further towards ER-Golgi intermediate compartment (ERGIC) (Saraste and Kuismanen, 1984) and *cis*-Golgi.

A true Golgi system of polarised cisternae has not been described in filamentous fungi. Nevertheless, they are thought to have equivalent structures based on studies with electron microscopy (Howard, 1981) and confocal microscopy (Bourett *et al*., 1998). Enzyme activities or genes encoding enzymes localised in Golgi complex/TGN in other eukaryotes have also been documented in filamentous fungi (Goller *et al*., 1998; Jalving *et al*., 2000; van Petegem *et al*., 2001). These Golgi-resident enzymes are responsible for sequential maturation of oligosaccharide side chains and activation of proproteins by proteolytic cleavage. The post-translationally modified proteins are distributed either to endosome/vacuole route(s) or to plasma membrane (PM) route(s) from TGN (reviewed by Keller and Simons, 1997; Bryant and Stevens, 1998).

1.1.3 Vesicle transport machinery

Proteins are enclosed in membrane-bound vesicles during their transport to various cellular destinations or to the cell exterior. The processes of vesicular membrane budding from the donor membrane and docking and fusion with the acceptor membrane require numerous protein factors which confirm that the appropriate proteins are packed in particular vesicles and delivered to particular target sites. The molecular details of these events in *S. cerevisiae* have been largely defined by secretory mutants originally isolated by Peter Novick and Randy Schekman (Novick *et al.* 1980). The membrane budding and fusion machineries are conserved in early and late secretion pathways but still specific for each secretory stage (Harter and Reinhard, 2000).

Coat proteins have two major roles on the donor membrane: they mechanistically force the vesicle formation and are also involved in protein sorting and concentration during vesicle budding (reviewed by Kirchhausen *et al.*, 1997; Kuehn and Schekman, 1997). Three distinct classes of coat proteins have been characterised in detail: COPII and COPI mediate anterograde and retrograde transport, respectively, in the early secretory pathway, and clathrin is involved in budding from TGN and plasma membrane. In addition, four adaptor protein (AP) complexes have been described. AP1 and AP2 appear to act together clathrin while the role of AP3 and AP4 is not thoroughly known (Bryant and Stevens, 1998; Harter and Reinhard, 2000; Lundmark and Carlsson, 2002). Specific recruitment of coat complexes as well as uncoating of vesicles require activity of small GTPases (reviewed by e.g. Aridor and Balch, 1996).

The vesicles are transported towards their target membrane along cell cytoskeleton. The transfer requires assistance of vesicle-associated motor proteins for microtubules and/or actin (Smith and Simmons, 2001).

Selective directing and docking of transport vesicles to their target membranes is achieved by 1) action of specific targeting complexes, e.g. TRAPP/Sec34p-Sec35p complex in early secretory pathway and exocyst in late secretory pathway (reviewed by Guo *et al.*, 2000), 2) by specific Rab GTPases which may recruit specific tethering/docking factors to membranes (Schimmöller *et al.*, 1998), 3) by specifically localised receptors on vesicle (vesicle-SNARE, v-

SNARE) and target membranes (target-SNARE, t-SNARE) (Söllner *et al.*, 1993; Advani *et al.*, 1998), and 4) by regulators of SNARE proteins (Gerst, 1999).

A number of genes encoding small GTPases involved in coat assembly (Veldhuisen *et al.*, 1997) and targeting (Punt *et al.*, 2001) have been cloned in filamentous fungi which suggests that similar molecular mechanisms in vesicle formation and targeting may exist in those organisms as well.

1.2 Polarised exocytosis

Exocytosis is a general term indicating the last stage of secretion, fusion of secretory vesicles with plasma membrane and liberation of their content to the cell exterior. Constitutive exocytosis, which is typical for all cells, was previously thought to arise by a simple bulk flow process (Rothman, 1987). However, proteins do have sorting signals which e.g. have been shown to direct certain proteins to specific domains of plasma membrane in polarised cells. In epithelial cells, proteins reach apical or basolateral surface domains by distinct pathways (reviewed by Keller and Simons, 1997). In addition, membrane and secretory proteins may undergo an additional sorting step in the basolateral route (Boll *et al.*, 1991; Saucan and Palade, 1994). *S. cerevisiae* has been suggested to possess three distinct secretory vesicle populations (Harsay and Bretscher, 1995; Roberg *et al.*, 1997; David *et al.*, 1998), and the transport of one population is regulated by extracellular stimuli (Roberg *et al.*, 1997). However, in yeast these different vesicle populations are directed to the same region of plasma membrane, the growing bud (Field and Schekman, 1980). In the same way, filamentous fungi are thought to direct the secretory vesicles to the growing apex of hyphae (Wösten *et al.*, 1991; Wessels, 1993) whereas no segregation of different cargoes into distinct vesicle populations has been reported so far. However, the existence of two separate secretory pathways in *Aspergillus niger*, constitutive and induced, has been proposed by Punt *et al.* (2001).

1.2.1 Vesicle transport

According to a common opinion the vectorial transport of secretory vesicles to the plasma membrane is executed by the cytoskeleton. The nucleation sites of

cytoskeletal microtubules and actin filaments determine the direction of organelle movement along these tracks. Microtubules, nucleated at the centrosome, point their plus ends at the cell periphery while actin filaments, nucleated at the membrane, attach by their barbed ends, and therefore plus-end directed motors move towards the membrane (DePina and Langford, 1999). The kinesin and myosin motor proteins are thus obvious candidates for delivering secretory vesicles to the plasma membrane.

It has been proposed that in animal cells the secretory vesicles are delivered by kinesin-dependent transport along microtubules to cell periphery and actomyosin-dependent transport near the final delivery site (Bi *et al*., 1997; Bridgman, 1999; Brown, 1999). Evidence has also been presented for the involvement of both microtubules and filamentous actin in the secretory vesicle transport of filamentous fungi. Deletion of conventional kinesin genes leads to reduced or hardly visible Spitzenkörper, the highly dynamic apical vesicle structure thought to contain secretory vesicles (Seiler *et al*., 1997, 1999; Wu, Q. *et al*., 1998), in concert with inhibition of secretion and hyphal extension (Seiler *et al*., 1997, 1999; Lehmler *et al*., 1997). Accordingly, Seiler *et al*. (1999) have proposed that the apical movement of secretory vesicles to the Spitzenkörper was defective in a kinesin mutant of *Neurospora crassa*. On the other hand, inhibition of actin polymerisation by cytochalasin A also decreased the secretion and disturbed hyphal growth (Torralba *et al*., 1998b; Khalaj *et al*., 2001) while microtubule inhibitor treatment increased the number of apical vesicles (Torralba *et al*., 1998a) in *Aspergillus*. Furthermore, since secretory vesicles have not been found to morphologically associate with microtubules in filamentous fungi (Heath, 1995) it has been suggested that microtubules may rather support actin organisation in the tip of hyphae and thus indirectly promote vesicle transport (Torralba and Heath, 2001; Khalaj *et al*., 2001). Nevertheless, it has been established that yeast and plants use exclusively actin filaments for vesicle transport (Jacobs *et al*., 1988; Cai *et al*., 1997; Pruyne *et al*., 1998; Karpova *et al*., 2000).

Unconventional class V myosins have been associated with the polarised transport of Golgi-derived secretory vesicles in *S. cerevisiae* (Johnston *et al*., 1991; Govindan *et al*., 1995; Karpova *et al*., 2000) and with synaptic vesicles and pigment granules in animal cells (Wu *et al*., 1997; Evans *et al*., 1998; Bridgman, 1999). The C-terminal globular tail of this myosin has been shown to

interact with the vesicles in yeast (Reck-Peterson *et al.*, 1999; Schott *et al.*, 1999). A receptor for a mammalian myosin-Va on the vesicle membrane has been recently established to consist of a Rab protein and its effector protein (Wu *et al.*, 2002). In yeast, a small Rab GTPase Sec4p and its guanine nucleotide exchange factor (GEF), Sec2p, are likely to be involved in Myo2p-vesicle contact as defects in these three proteins are synthetically lethal (Govindan *et al.*, 1995; Schott *et al.*, 1999). In addition, cells with a *sec2* mutation accumulate post-Golgi vesicles (Walch-Solimena *et al.*, 1997) which have been suggested to be uncoupled from Myo2p because of the mutation (Schott *et al.*, 1999). Although Rab proteins including Sec4p have been suggested to act primarily as regulators of the exocyst complex and of the SNARE complex assembly (Søgaard *et al.*, 1994; Johannes *et al.*, 1996; Guo *et al.*, 1999b), there is also evidence for their involvement in vesicle budding and transport (Zerial and McBride, 2001) as well as in regulation of actin organisation (Kato *et al.*, 1996; Peränen *et al.*, 1996) in higher eukaryotes.

1.2.2 Vesicle targeting and tethering

Targeting of secretory vesicles to the appropriate sites of plasma membrane is carried out by a large protein complex located in sites of polarised growth in *S. cerevisiae* (TerBush and Novick, 1995) and in the area around tight junction (Grindstaff *et al.*, 1998) and developing axons (Hazuka *et al.*, 1999) in mammalian cells. In yeast, this complex has been denoted the exocyst complex, and it contains the Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84 proteins (TerBush *et al.*, 1996; Guo *et al.*, 1999a) (Figure 2). A homologous protein complex, the sec6/8 complex, has been isolated from rat brain (Kee *et al.*, 1997; Hsu *et al.*, 1998).

Sec3p is a subunit competent to reach the site of polarised exocytosis at each stage of cell cycle independent of the other exocyst subunits, of the early secretory pathway, and of the actin and septin cytoskeleton (Finger *et al.*, 1998) but dependent on small GTPase Rho1p (Guo *et al.*, 2001). Sec3p has thus been suggested to spatially mark the site of exocytosis at the plasma membrane (Finger *et al.*, 1998) in response to polarity guidance. Sec15p colocalises with the Rab GTPase Sec4p on secretory vesicles. If Sec4p is unable to bind GTP, as in *sec4-8* mutant cells, it does not interact with Sec15p, and Sec3p fails to

assemble with other components of the exocyst (Guo *et al*., 1999b). The network of molecular interactions between exocyst subunits, as studied by immunoprecipitation and the yeast two-hybrid method, has thus been suggested to link secretory vesicles to the plasma membrane (Figure 2) (Terbush and Novick, 1995; Guo *et al*., 1999a, b), and this assembly is controlled by a Rab GTPase, Sec4p (Guo *et al*., 1999b). The Rab-controlled reversible attachment of vesicles to plasma membrane is also called tethering.

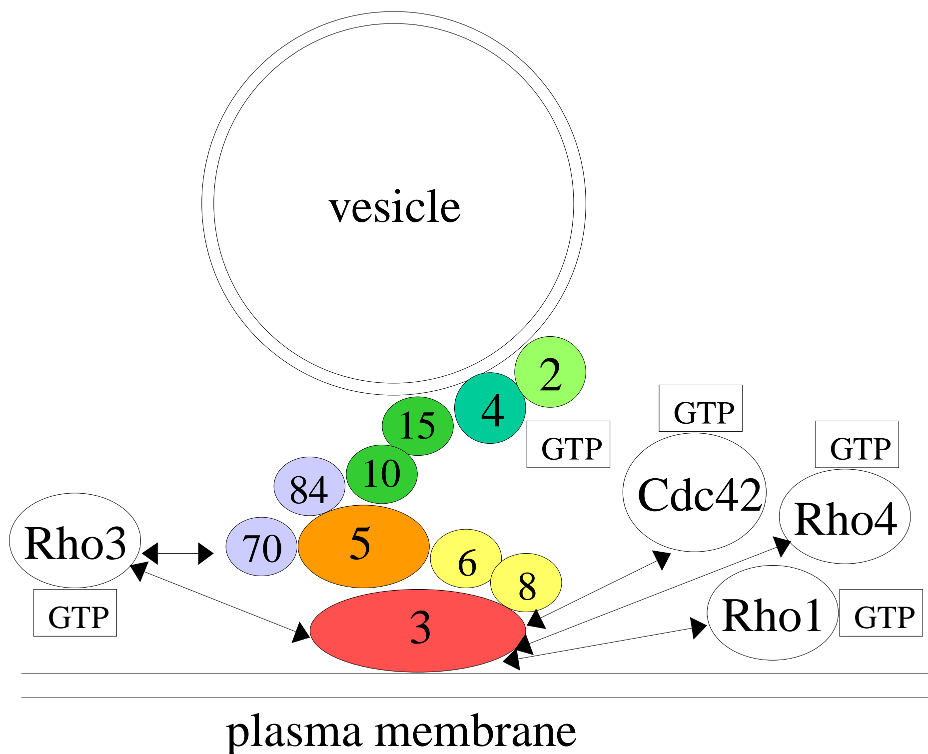

Figure 2. Schematic representation of the molecular structure of the exocyst complex in yeast. GTP-bound Sec4p, Sec2p and Sec15p interact on the surface of secretory vesicle while Sec3p marks the site of exocytosis on PM. The rest of the exocyst components are thought to link these sites by assembling according to a model presented by Guo et al. *(1999b). Several GTP-binding proteins have been found to interact with exocyst components (Adamo* et al.*, 1999; Robinson* et al.*, 1999; Guo* et al.*, 2001; Zhang* et al.*, 2001).*

The large size (>700 kDa) of the exocyst complex and the large number of its individual subunits could facilitate its interactions with lipids or with numerous proteins. Obvious candidates would be the proteins involved in cell polarisation through regulation of the cytoskeleton organisation such as Rho-type GTPases. Quite recently, such protein interactions have been found. Sec3p has been shown to interact with Rho1p (Guo *et al*., 2001) and Cdc42p (Zhang *et al*., 2001), and Exo70p with Rho3p in yeast (Adamo *et al*., 1999; Robinson *et al*., 1999) while Sec5 interacts with RalA in mammals (Moskalento *et al*., 2002; Sugihara *et al*., 2002). These GTPases are involved in formation or growth of yeast bud (Adams *et al*., 1990; Matsui and Toh-E, 1992b; Yamochi *et al*., 1994) or cytoskeletal remodelling in mammalian cells (Sugihara *et al*., 2002). Furthermore, it has been shown in yeast that *SEC3* and *SEC8* mutations are synthetically lethal with a mutant profilin gene encoding an actin-binding protein (Haarer *et al*., 1996). In mammalian cells, the Sec8 protein immunoprecipitates with actin (Shin *et al*., 2000).

1.2.3 Vesicle docking and fusion

In contrast to tethering, vesicle docking to the plasma membrane is an irreversible event. This docking process has been described in the SNARE hypothesis (Söllner *et al*., 1993). According to this hypothesis, vesicle docking is mediated by specific pairing of receptors on the vesicle membranes (v-SNAREs) and on the target membrane (t-SNAREs). In *S. cerevisiae*, Snc1p and Snc2p (Gerst *et al*., 1992; Protopopov *et al*., 1993) operate as the v-SNAREs, and Sso1p and Sso2p (Aalto *et al*., 1993) as well as Sec9p (Brennwald *et al*., 1994) as the t-SNAREs in exocytosis. In mammals there are several sorting alternatives with regulated/constitutive exocytosis to basolateral/apical membrane and, therefore, the number of vesicle- and target-SNAREs acting in exocytosis is larger (see Gerst, 1999). The plants also appear to have multiple forms of SNARE protein orthologs (Sanderfoot and Raikhel, 1999). In *Neurospora crassa*, one putative t-SNARE was identified in tips of the hyphae with antibodies against yeast Sso2p (Gupta and Heath, 2000), and a v-SNARE (Snc1) from *T. reesei* has been characterized (Valkonen *et al*., in prep.). The tip-high gradient of the *N. crassa* t-SNARE is a unique feature since both yeast and mammalian t-SNAREs are suggested to be distributed over the whole plasma membrane area (Brennwald *et al*., 1994; Garcia *et al*., 1995).

According to a current model (see Gerst, 1999), remnants of earlier docking and fusion events, that is the stable complex of v- and t-SNAREs on the same membrane (*cis*), must be disassembled before new docking. This is catalysed by *N*-ethylmaleimide sensitive fusion protein (NSF/Sec18p) and soluble NSF-attachment protein (α-SNAP/Sec17p) that associate with the ternary SNARE complex. ATP hydrolysis by NSF results in resolving of the complex. This is thought to allow SNAREs to bind stabilising factors which are removed before the next productive SNARE pairing. The nature of these stabilising factors is unclear, but Vsm1p in yeast (Lustgarten and Gerst, 1999) and synaptophysin in neuronal cells (Edelmann *et al*., 1995) have been suggested to mask v-SNAREs and inhibit unspecific binding to t-SNAREs (Gerst, 1999). Sec1 is suggested to act similarly on t-SNAREs since its binding on syntaxin inhibits formation of ternary complex in mammals (Zhang *et al*., 2000). However, in yeast it has been established that Sec1p binds to complexed SNAREs (Carr *et al*., 1999). Sec1 has probably other tasks in exocytosis in addition to acting as a SNARE regulator. In animal cells, different Sec1 mutants have different effects on transport (Wu, M. N. *et al*., 1998; Riento *et al*., 2000), which indicates that Sec1 can function in other complexes than that of syntaxin during exocytosis. Furthermore, expression of a mutant neuronal Sec1 having decreased affinity for syntaxin speeds up fusion pore broadening, thus implicating a role in fusion process itself (Fisher *et al*., 2001).

It is under debate whether SNARE proteins are functioning only before membrane fusion or also during bilayer mixing. Weber *et al*. (1998) have shown that liposome-anchored recombinant v- and t-SNARE proteins assemble into SNARE complexes, which leads to spontaneous fusion. This is consistent with the model of Fasshauer *et al*. (1997) where the energy provided for the formation of the very stable SNARE complex would be sufficient to drive fusion as well. However, Tahara *et al*. (1998) established that disruption of SNARE complex does not block fusion. Moreover, *trans*-SNARE complexes (v- and t-SNAREs on the different membranes) can be formed even if fusion is blocked (Ungermann *et al*., 1998). According to another model (Mayer *et al*., 1999), the transient *trans*-SNARE pairing glues a signal that docking is complete and fusion process can be proceeded by other factors. Pairing of SNAREs leads to Ca(2+) influx which activates the formation of complex between calmodulin and protein phosphatase 1 (PP1) in addition to other factors. These factors then

support the final bilayer mixing. The role of PP1 in bilayer mixing, in multiple steps of vesicle trafficking, has been established in yeast by Peters *et al*. (1999).

1.3 Small GTP-binding protein subfamily Rho

Small GTP-binding proteins are monomeric signalling molecules forming the Ras superfamily. This superfamily is divided into five families: Ras, Rho, Rab, Sar/Arf and Ran (reviewed by Takai *et al*., 2001). The Rho family is further composed of Rho, Rac, Rop and Cdc42 subfamilies (Valster *et al*., 2000; Takai *et al*., 2001). The Rho subfamily has five members, *RHO1-RHO5*, in fungal organisms (Table 2). The most thoroughly investigated fungal Rho protein is *S. cerevisiae* Rho1.

Table 2. Rho genes cloned from fungal organisms.

Gene	Accession number	Species	Reference
RHO1	M15189	*Saccharomyces cerevisiae*	Madaule *et al*., 1987
	D38180	*Schitzosaccharomyces pombe*	Nakano and Mabuchi, 1995
	D86430	*Candida albicans*	Kondoh *et al*., 1997
	AF210628	*Ashbya gossypii*	Wendland and Philippsen, 2001
	AB017639	*Filobasidiella neoformans*	Tanaka *et al*., 1999
	AF242351	*F. neoformans*	Chang and Penoyer, 2000
	AF279915	*Yarrowia lipolytica*	Leon *et al*., unpublished
	AAG12155	*Aspergillus fumigatus*	Beauvais *et al*., 2001
	AF338871	*Aspergillus nidulans*	Guest and Momany, unpublished
RHO2	M15190	*S. cerevisiae*	Madaule *et al*., 1987
	D38181	*S. pombe*	Nakano and Mabuchi, 1995
RHO3	D10006	*S. cerevisiae*	Matsui and Toh-E, 1992a
	AF210629	*A. gossypii*	Wendland and Philippsen, 2001
	AF242543	*Schitzophyllum commune*	Gorfer *et al*., unpublished
	AJ297909	*Trichoderma reesei*	Vasara *et al*., 2001 (I)
RHO4	D10007	*S. cerevisiae*	Matsui and Toh-E, 1992a
	AF195008	*A. gossypii*	Wendland *et al*., 2000
RHO5	Z71456	*S. cerevisiae*	Obermaier *et al*., unpublished

1.3.1 Structure and mechanism of function

The Rho GTPases are 20–30 kDa proteins possessing conserved amino acid sequences for interaction with GDP and GTP and for GTPase activity (G-1–G-4, Figure 3). The region primarily responsible for interaction with downstream effector proteins overlaps with the G-2 region (Bourne *et al.*, 1991; Takai *et al.*, 2001). The extreme carboxyl terminus of Rho proteins (CaaX, where a is any aliphatic amino acid, Figure 3) is a target for posttranslational modification by geranylgeranylation (CaaL) or farnesylation (CaaM/S) (Kinsella *et al.*, 1991). Lipid modification has been shown to be necessary for membrane binding (Seabra, 1998), and it is presumed that specific lipid modification may target GTPases to membrane microdomains (Gulli and Peter, 2001).

The small GTP-binding proteins cycle between an active GTP-bound, and an inactive GDP-bound state. In the active form they are able to interact with multiple downstream effector proteins. The GTPase activity hydrolyses GTP into GDP and phosphate, returning the protein into an inactive state. The factors regulating the nucleotide state of GTP-binding proteins are of particular importance for this cycling. They comprise the GTPase activating protein (GAP) which enhances the otherwise modest intrinsic GTPase activity (Bourne *et al.*, 1991), GDP dissociation inhibitor (GDI) which prevents GDP release and masks the lipid moiety thus keeping the GTPase cytosolic and inactive (Olofsson, 1999), and the guanine nucleotide exchange protein (GEP) which catalyses the replacement of GDP with GTP (Bourne *et al.*, 1991).

It is thought that upstream activation signals enhance the recruitment of GEP and GTPase/GDI complex to the plasma membrane where GDI is inactivated and the released GTPase is activated by the aid of GEP. Binding of GTP then induces a conformational change in the GTPase which allows the effector domain to interact with the target protein (Symons and Settleman, 2000; Gulli and Peter, 2001). However, according to a current model, the Rho proteins cycle constantly between GDP- and GTP-bound forms even in resting cells. In non-stimulated cells the majority of proteins are in GDP-bound state while cell stimulation causes a shift in equilibrium towards activated GTP-bound form. Probably the comprehensive activation of Rho-GTPases requires activation of GEPs as well as inactivation of GAPs and/or GDIs (Symons and Settleman, 2000; Gulli and Peter, 2001).

```
                                        G-1                             G-2
S. cerevisiae Rho1p    SIRR---------KLVIVGDGACGKTCLLIVFSKGQFPEVYVPTVFENYVADVEV-----  53
S. cerevisiae Rho5p    SI-----------KCVIIGDGAVGKTSLLISYTTNSFPTDYVPTVFDNYSTTIAIPNGTA  51

S. cerevisiae Rho3p    PIER---------KIVILGDGACGKTSLLNVFTRGYFPEVYEPTVFENYIHDIFV-----  59
E. gossypii Rho3p      PIER---------KIVILGDGACGKTSLLNVFTRGYFPKVYEPTVFENYIHDIFV-----  58
T. reesei RHOIII       TVQR---------KLVLLGDGASGKTSLLNVFTRGYFPTVYEPTVFENYVHDIFV-----  54
S. commune RHOIII      PIQR---------KVVVCGDGACGKTSLLNVFTRGFFTQVYEPTVFENYVHDLYI-----  60
                        ::*         *:*: ****.************:*. **********:**:::
S. cerevisiae Rho2p    AVRR---------KLVIIGDGACGKTSLLYVFTLGKFPEQYHPTVFENYVTDCRV-----  50
S. cerevisiae Rho4p    QMKRTNKLPDYHLKIVVVGDGAVGKTCLLISYVQGTFPTDYIPTIFENYVTNIEGP---- 116


                                                              G-3
S. cerevisiae Rho1p    -------------------------DGRRVELALWDTAGQEDYDRLRPLSYPDSNVVLIC  88
S. cerevisiae Rho5p    SSPLELDNGNDKRGSLSSASSSPSTDRKLYKINLWDTAGQEDYDRLRPLCYPQTDIFLIC 111

S. cerevisiae Rho3p    -------------------------DSKHITLSLWDTAGQEEFDRLRSLSYSDTQCIMLC  94
E. gossypii Rho3p      -------------------------DNQHITLSLWDTAGQEEFDRLRSLSYSDTHTIMLC  93
T. reesei RHOIII       -------------------------DNVHIELSLWDTAGQEEFDRLRSLSYDDTDLIVLC  89
S. commune RHOIII      -------------------------DDQLVELSLWDTAGQEEFDRLRSLSYAETHVIMIC  95
                                                 *.  : ******************** :*. *::*
S. cerevisiae Rho2p    -------------------------DGIKVSLTLWDTAGQEEYERLRPFSYSKADIILIG  85
S. cerevisiae Rho4p    -------------------------NGQIIELALWDTAGQEEYSRLRPLSYTNADVLMVC 151


                                                                      G-4
S. cerevisiae Rho1p    FSIDLPDSLENVQEKWIAEVLHFC--QG---------VPIILVGCKVDLRND-------- 129
S. cerevisiae Rho5p    FSVSEHASFANVTEKWLPELKQTSNIEGTSLYTKLGKYPILLVGTKSDLRDD-------- 163

S. cerevisiae Rho3p    FSIDSRDSLENVQNKWVGEITDHC--EG---------VKLVLVALKCDLRNNEN-ESN-- 140
E. gossypii Rho3p      FSVDSRDSLENVKNKWVSEIADHC--EG---------VKLVLVALKCDLRSSDE-YGNES 141
T. reesei RHOIII       YSVDSKDSLENVESKWVGEIADNC--PG---------VKLVLVALKCDLRQQEDDEPEDQ 138
S. commune RHOIII      FSVDNPTSLENVESKWLDEILEYC--PG---------VKLVLVDSKCDLRDD-------- 136
                       :*:*. *****:.**:.** : *    *         ****** *****..
S. cerevisiae Rho2p    FAVDNFESLINARTKWADEALRYC--PD---------APIVLVGLKKDLRQEAHFKEN-- 132
S. cerevisiae Rho4p    YSVGSKTSLKNVEDLWFPEVKHFC--PS---------TPIMLVGLKSDLY-EADN----- 194


S. cerevisiae Rho1p    PQTIEQLRQEGQQP----------------VTSQEGQSVADQIGATGYYECSAKTGYGVR 173
S. cerevisiae Rho5p    PATQKKLQEANSDY----------------VSQEEIDELVQRCGFMGYTECSAATQAGVR 207

S. cerevisiae Rho3p    AITPNNIQQDNSVSNDNGNNINSTSNGKNLISYEEGLAMAKKIGALRYLECSAKLNKGVN 200
E. gossypii Rho3p      AITPGSIQ--NQKYNGGG--------GNGLIPYDEGLAMAKQIGALRYLECSAKMNRGVN 191
T. reesei RHOIII       AAADGNAQREKPPT----------------ISYDEGLEVAKRIGASRYLECSAMKNRGVN 182
S. commune RHOIII      PAVLDRLQRYGTHTD----------------QYEEGLGVARRIRASRYLECSSKHNRGVN 180
                       . . .  *                       *:*** :*::* * ******   *:***
S. cerevisiae Rho2p    -ATDE------------------------MVPIEDAKQVARAIGAKKYMECSALTGEGVD 167
S. cerevisiae Rho4p    -LSD-------------------------LVEPSSAESLAKRLGAFAHIQCSARLKENID 228


S. cerevisiae Rho1p    EVFEAA----TRASLMG------------------------------------------- 186
S. cerevisiae Rho5p    EVFEQA----VRYAIYEPESPNQKSANHTLTDELTTATTNTNGDKNIREQKQQPHHNNST 263

S. cerevisiae Rho3p    EAFTEA----ARVALTAGPVAT-------------------------------------- 218
E. gossypii Rho3p      EAFTEA----ARCALTATPKGAR------------------------------------- 210
T. reesei RHOIII       EAFTEA----ARVALSVK------------------------------------------ 196
S. commune RHOIII      EVFLRGR---ARVTVHS------------------------------------------- 194
                       *.* ..    ** ::
S. cerevisiae Rho2p    DVFEVA----TRTSLLMK------------------------------------------ 181
S. cerevisiae Rho4p    EVFETAIHTLLSDSLYAPREPTHTIKN--------------------------------- 255


S. cerevisiae Rho1p    -------------------------------KSKTNG-----------KAK---KNTTEK 201
S. cerevisiae Rho5p    DSTLPKGSLQQEKEALNIKPTKKGQKDKIHEQSKSKGSKIASNNHHNKQAKPKTRNDKKK 323
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S. cerevisiae Rho3p   -------------------------------------------------------EVKSD 223
E. gossypii Rho3p     ------------------------------------------------------DSAPEA 216
T. reesei RHOIII      ---------------------------------------------------------KER 199
S. commune RHOIII     --------------------------------------------------------IRQG 198
                                                                               . .
S. cerevisiae Rho2p   ---------------------------------------------------------KEP 184
S. cerevisiae Rho4p   -------------------------------PFKRNTTRSDIDSSTGDTSVSIS-GTKRL 283


                          CaaX
S. cerevisiae Rho1p   KKKKCVLL 209
S. cerevisiae Rho5p   KKSKCVIL 331

S. cerevisiae Rho3p   SGSSCTIM 231
E. gossypii Rho3p     ESSSCTIM 224
T. reesei RHOIII      EDNKCTIM 207
S. commune RHOIII     SAGSCCVM 206
                        ..* :*
S. cerevisiae Rho2p   GANCCIIL 192
S. cerevisiae Rho4p   RKNKCIIM 291
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Figure 3. A ClustalW (1.81) multiple sequence alignment of fungal Rho3 proteins and other selected Rho proteins. Asterisks indicate conserved residues and dots indicate residues which share similar biochemical properties. The conserved amino acids of Rho3 proteins are also highlighted in red. The consensus amino acid sequences involved in GDP/GTP binding and GTP hydrolysis (G-1, G-2, G-3, G-4) and the prenylation sequences (CaaX) are in bold, and the putative effector loop is underlined. The Rho3p-specific amino acids are marked by arrows.

1.3.2 Regulation of the actin cytoskeleton organisation

Rho GTPases are primarily involved in regulation of the actin cytoskeletal organisation. This was first indicated in mammals by Paterson *et al*. (1990) who microinjected recombinant Rho[1] proteins into a variety of cell lines and discovered rapid induction of stress fiber formation. The stress fiber formation together with assembly of focal adhesions has been found to be induced in response to lysophosphatidic acid (LPA) (Ridley and Hall, 1992), known to act through a G-protein coupled receptor. Since stress fiber formation and assembly of focal adhesions were observed to be separate events (Nobes and Hall, 1995) it was suggested that multiple downstream effectors for Rho exist. Now, several Rho target proteins mediating actin-coupled functions are known in mammals and *S. cerevisiae* (Table 3).

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[1] In many cases Rho refers to RhoA but it could also mean the combined or partly overlapping functions of RhoA, RhoB and RhoC in mammalian cells (Aspenström, 1999).

Table 3. The effectors and functions of Rho proteins in animals and yeast. For references see Machesky and Hall, 1996; Tanaka and Takai, 1998; Adamo et al.*, 1999; Aspenström, 1999; Robinson* et al.*, 1999; Settleman, 1999; Bishop and Hall, 2000; Sah* et al.*, 2000; Guo* et al.*, 2001; Ozaki-Kuroda* et al.*, 2001; Takai* et al.*, 2001 and text. Abbreviations:* ***DAG****, diacylglycerol;* ***Dia****, homolog of diaphanous protein;* ***EF****, elongation factor;* ***ERM****, ezrin/radixin/moesin;* ***FA****, focal adhesion;* ***GFAP****, glial fibrillary acidic protein;* ***MAPK****, mitogen activated protein kinase;* ***PA****, phosphatidic acid;* ***PI4P-5K****, phosphatidylinositol-4-phosphate-5-kinase;* ***PKC****, protein kinase C;* ***PKN****, protein kinase N;* ***PLD****, phospholipase D;* ***Smy****, suppressor of myosin;* ***Spa****, spindle pole antigen;* ***Rok****, Rho kinase.*

GTPase	Target proteins	Effects	Cellular pathway
ANIMAL Rho	Rok	Phosphorylates and inactivates myosin phosphatase which results in increased regulatory light chain phosphorylation and actomyosin contractility.	Stress fiber formation
		Phosphorylates and activates LIM kinase which phosphorylates and inactivates actin depolymerising protein, cofilin.	
		Phosphorylates ERM proteins	Assembly of FA
			Cell migration
	Dia1, Dia2	Regulation of actin polymerization through interaction with profilin	Stress fiber formation
	PKN/ PRK1, PRK2	Phosphorylates actin crosslinking protein, α-adducin	Assembly of FA
			Cell migration
		Activates PLD which regulates PA levels	Secretion?
		?	Embryogenesis
		Activates MAPK pathway	Transcriptional activation
	PKC	Activates MAPK pathway	Transcriptional activation
	Citron	Phosphorylates GFAP?	Cytokinesis
	PI4P-5K	Regulation of actin-binding regulatory proteins,	Actin cytoskeleton organization?
		phosphoinositide metabolism	Secretion?
	DAG kinase	Regulates PA levels	Secretion?
	Kinectin	Kinesin binding	Vesicle trafficking?

YEAST	Bni1p	Regulation of actin polymerisation through profilin, Bud6p, Spa2p and EF1α	Actin polymerisation
Rho1p		?	Nuclear migration
	Pkc1p	Activates MAPK pathway	Cell wall synthesis
	Fks1p, Fks2p	Synthesizes β-1,3-glucan	Cell wall synthesis
	Sec3p	Plays part in vesicle targeting/tethering	Vesicle targeting
Rho3p	Myo2p	Transports secretory vesicles along actin filaments	Vesicle trafficking?
	Exo70p	Plays part in vesicle targeting/tethering	Vesicle targeting
Rho4p	Bnr1p	Interacts with Hof1p, Smy1p, profilin and Bud6p	Cytokinesis

Yeast Rho proteins, Rho1p-Rho4p, have been implicated in control of bud formation (Yamochi *et al.*, 1994; Matsui and Toh-E, 1992b) by controlling the actin cytoskeleton organisation and cell wall synthesis. Rho1p directs cell wall synthesis through two separate pathways. 1) It acts as a regulatory subunit of β-1,3-glucan synthetase (Drgonavá *et al.*, 1996; Arellano *et al.*, 1996; Kondoh *et al.*, 1997), an enzyme which is responsible for the formation of a major structural component of fungal cell wall (Sentandreu *et al.*, 1975). 2) The other downstream factor, Protein kinase C (Pkc1p) (Nonaka *et al.*, 1995), controls the expression of genes encoding cell wall components including the catalytic subunit of 1,3-β-glucan synthetase (see Banuett, 1998). Pkc1p is also involved in the control of the actin cytoskeleton organisation (Helliwell *et al.*, 1998) together with the formin homology protein Bni1p (Kohno *et al.*, 1996). This protein acts through direct interactions with actin, the actin-binding proteins profilin and Bud6p (Evangelista *et al.*, 1997), and elongation factor 1α (Umikawa *et al.*, 1998). The other formin homology protein, Bnr1p, is a specific target protein of Rho4p (Imamura *et al.*, 1997). It interacts in addition to profilin (Imamura *et al.*, 1997) and Bud6p (Kikyo *et al.*, 1999), also with Hof1p (Kamei *et al.*, 1998) which is involved in cytokinesis.

The rearrangements of the actin cytoskeleton have key roles in cellular processes such as morphogenesis and cytokinesis, cell adhesion to extracellular matrix, cell motility and phagocytosis in mammals (van Aelst and D'Souza-Schorey, 1997). Parts of the activities of Rho in exocytosis and cellular polarity are also mediated through the actin cytoskeleton organisation whilst actin-independent pathways downstream of Rho also exist in these processes (see below).

1.3.3 Regulation of membrane traffic

Mammalian cells display frequently a physical barrier for exocytosis formed by the cortical network of actin filaments (Aunis and Bader, 1988). Removal of this barrier has been suggested to trigger exocytosis (Muallem *et al.*, 1995). On the other hand, efficient exocytosis also needs the actin cytoskeleton since secretory vesicles have been reported to be transported along actin filament tracks (see 1.2.2). Furthermore, endocytosis appears to require the actin cytoskeleton (Lamaze *et al.*, 1997; Ayscough, 2000). Therefore Rho proteins have been

anticipated to have a role in membrane traffic through their effect on the actin cytoskeleton (Caron and Hall, 1998).

However, Rho proteins have been found to regulate secretion also independently of actin. This has been demonstrated by application of toxins which disturb actin polymerisation or stabilise actin filaments together with activated forms of Rho. In this way the signalling pathways controlling secretion and cytoskeletal rearrangements have been found to be divergent and act in parallel in mast cells (Norman *et al*., 1996; Sullivan *et al*., 1999). In *S. cerevisiae*, interaction between Rho3p and proteins involved in vesicle trafficking and targeting (Myo2p and Exo70p) has been demonstrated (Adamo *et al*., 1999; Robinson *et al*., 1999). This interaction was abolished by a mutation in the effector region of Rho3p (Robinson *et al*., 1999). Cells expressing mutated *RHO3* also accumulated secretory vesicles but did not exhibit defects in the actin cytoskeleton (Adamo *et al*., 1999). Similarly, the regulation of both clathrin-dependent (Lamaze *et al*., 1996) and clathrin-independent endocytosis (Schmalzing *et al*., 1995) by Rho was unaffected by changes in the actin cytoskeleton, indicating independence of the regulatory pathways of actin organisation and membrane traffic.

1.4 14-3-3 proteins

The 14-3-3 proteins were first described by Moore and Perez (1967) as abundant acidic mammalian brain proteins with a molecular weight of approximately 30 kDa. The name is due to a particular migration pattern in two-dimensional gel chromatography. Later it was found that these proteins are widely expressed in other mammalian tissues and other eukaryotes (see Aitken *et al*., 1992). Furthermore, the 14-3-3 protein sequences are remarkably well conserved between species (Wang and Shakes, 1996) and a broad range of target proteins whose fate is regulated by the 14-3-3 proteins have been described (Table 4) implying an essential role of 14-3-3 proteins in many signalling pathways. These target proteins include e.g. various receptors, kinases, GTPases, enzymes, cytoskeletal proteins, and proteins involved in cell cycle, in transcription or in control of apoptosis.

Table 4. Interaction partners of 14-3-3 proteins. The partners for some fungal 14-3-3 proteins (Bmh1p and Bmh2p in S. cerevisiae*, Rad24 and Rad25 in* Schitzosaccharomyces pombe *and Cip3 in* Lentinus edodes*) are shown in red, green and blue, respectively. For references see the supplementary table of Fu* et al.*, 1999 at* http://pharmtox.annualreviews.org/ *and references therein, and Roberts* et al.*, 1997; Toroser* et al.*, 1998; Beck and Hall, 1999; Dorner* et al.*, 1999; Benzing* et al.*, 2000; Kanai* et al.*, 2000; Laronga* et al.*, 2000; Pierrat* et al.*, 2000; Tanaka* et al.*, 2000; Wang* et al.*, 2000; Zhou* et al.*, 2000; Bunney* et al.*, 2001; Cahill* et al.*, 2001; van Hemert* et al.*, 2001a; supplementary table of van Hemert* et al.*, 2001b at* [http://www.interscience.wiley.com/jpages /0265-9247/Suppmat/](http://www.interscience.wiley.com/jpages/0265-9247/Suppmat/)*; Sehnke* et al.*, 2001; Zhai* et al.*, 2001. Abbreviations:* ***A20****, tumor necrosis factor α-inducible zinc finger protein;* ***APX****, ascorbate peroxidase;* ***ASK****, apoptosis signal-regulating kinase;* ***Bcr,*** *breakpoint cluster region protein,* ***Bop****, bypass of* PAM1*;* ***Cas****, cellular apoptosis susceptibility;* ***CAP****, adenylyl cyclase-associated protein;* ***Cdc****, cell division cycle protein;* ***CDK****, cyclin dependent kinase;* ***CDPK****, calcium-dependent kinase;* ***Chk****, cheakpoint kinase;* ***CRM****, chromosomal maintenance protein;* ***EmBP****, Em promoter binding protein;* ***FKHR****, forkhead in rhabdomyosarcoma,* ***FRAP****, FKBP12 translational control factor-rapamycin-associated protein;* ***GABA****, gamma-aminobutyric acid;* ***GM-CSF****, granulocyte/macrophage colony stimulation factor;* ***Gplb-IX****, platelet adhesion factor;* ***GR****, glucocorticoid receptor;* ***GS****, glutamine synthase;* ***HAT****, histone acetyl transferase;* ***HDAC****, histone deacetylase;* ***HTERT****, telomerase;* ***IGF1R****, insulin-like growth factor 1 receptor;* ***Ii****, invariant chain;* ***IL****, interleukin;* ***Ip****, inositol polyphosphate;* ***IRS****, insulin receptor substrate;* ***KIF****, kinesin family;* ***Ksc****, Inositol phosphate 6-kinase;* ***KSR****, kinase suppressor of ras;* ***MEKK****, mitogen-activated protein kinase kinase kinase;* ***MLK****, mixed lineage kinase;* ***Msn****, multicopy suppressor of* snf1 *mutation;* ***NFAT****, nuclear factor of activated T cells;* ***NHE****, Na(+)/H(+) exchanger;* ***NS****, non-structural protein;* ***OMT****, caffeic acid O-methyltransferase;* ***Pcl****, PI-specific phospholipase;* ***PCTAIRE1****, cdc2-related serine/threonine kinase;* ***PHD****, plant homeodomain;* ***PI****, phosphatidylinositol;* ***PKC****, protein kinase C;* ***PKU****, protein kinase U;* ***PTPH****, protein tyrosine phosphatase;* ***Rad****, Ras associated with diabetes;* ***Raf****, Ras factor;* ***Rem****, Rad and Gem-related;* ***RGS****, regulator of G protein signalling;* ***RIN****, Ras interacting protein;* ***Slob****, slowpoke calcium-dependent potassium channel binding protein;* ***SPS****, sucrose phosphate synthase;* ***ST-IV****, CMP-NeuAC:GM1 α2,3 sialyl transferase;* ***TAF****, TBP-associated factor;* ***TAZ****, transcriptional co-activator with PDZ-binding motif;* ***TBP****, TATA-binding protein;* ***TF****, transcription factor;* ***TPS****, trehalose-6-phosphate-synthase;* ***UCP****, uncoupling protein;* ***VP****, viviparous;* ***WPK****, wheat SNF1-related protein kinase.*

Kinases	Phosphatases	Other enzymes	Transcription factors	G-proteins and factors	Receptors	Docking and adaptor proteins	Cytoskeletal proteins	Miscellaneous proteins
ASK1	Cdc25	APX3	CRM1	Rad	α2adrenergic receptor	A20	Integrin β	BAD
Bcr	43 kDa Ip 5-phosphatase	ATP synthase	DAF16	Rem	GABA receptor	Cbl	Keratin 8/18	Bop3
CDK2	PTPH1	GS	DNA topo-isomerase IIα	RGS	GM-CSF, IL-3, IL-5 β chain	IRS1	KIF1C	Calmodulin
CDPK1,2		H(+)-ATPase	EmBP1, VP1	P190Rho GEF	Gplb-IX	p130(Cas)	vimentin	CAP
Chk1		13-lipoxygenase	FKHRL1		GR	RIN1		Ecm13
Ksc1		Nitrate reductase	Gcr2		IGF1R			Exoenzyme S
KSR		OMT1	HAT1		IL-9Rα			Fin1
MEKK1,2,3		Pcl1	HDAC4,5					FRAP
MLK2		Serotonin N-acetyltransferase	hTAF(II)32					Hepatitis C core protein
PCTAIRE1		SPS	HTERT					Iip35
PI 3-kinase		ST-IV	Msn2,4					Mei2
PKC		Starch synthase	NFAT					Middle T antigen
PKUα		TPS	p53					NHE1
Raf-1		Tryptophan hydroxylase	PHD finger TF					NS2 proteins
Ste20		Tyrosine hydroxylase	Rtg3					Slob
Wee1			TAZ					UCP2,3
WPK4			TBP					
			TFIIB					
			TLX-2					

1.4.1 Structure and mechanism of function

14-3-3 proteins exist as several isoforms in animal and plant cells and predominantly as two isoforms in fungal cells (van Hemert *et al.*, 2001a). The crystal structures of human 14-3-3 tau (τ) (Xiao *et al.*, 1995) and zeta (ζ) (Liu *et al.*, 1995) isoforms have been determined. 14-3-3 proteins function as dimers, and each subunit of the dimer is composed of nine antiparallel α-helices organised in two structural domains. The four N-terminal helices take part in the dimer formation The invariant helices $\alpha3$, $\alpha5$, $\alpha7$, and $\alpha9$ form an amphipathic groove in the inner surface of a cup-like structure. A cluster of charged and polar residues of helices 3 and 5 outline one side of the groove, and a cluster of hydrophobic residues of helices 7 and 9 form the other side of the groove. The variant helices form the outer surface of the structure.

14-3-3 proteins have been suggested to bind their target proteins by the amphipathic groove (Liu *et al.*, 1995). Many of the target proteins contain a special binding motif R(S/Ar)XpSXP or RX(Ar/S)XpSXP (pS denotes phosphorylated serine or threonine and Ar denotes aromatic residue) recognised by 14-3-3 proteins (Muslin *et al.*, 1996; Yaffe *et al.*, 1997). However, 14-3-3 proteins also interact, presumably using a similar ligand binding site, with ligands having divergent phosphoserine motifs or unphosphorylated motifs (see Fu *et al.*, 2000 and references therein). Stable association of most target proteins with 14-3-3 requires a 14-3-3 dimer (Tzivion *et al.*, 2000).

According to a current model, the 14-3-3 dimer could control the localisation of its target molecule through interacting with it by one subunit and simultaneously masking the putative targeting information nearby with the other subunit (Muslin and Xing, 2000). Alternatively, the dimer could also bind to two target motifs, that are present either in a single molecule or in two different proteins. In this way, the 14-3-3 dimer could regulate either protein conformation and activity or facilitate the interaction between its target proteins (Fu *et al.*, 2000; Tzivion and Avruch, 2002).

1.4.2 14-3-3 proteins regulate the conformation of their target proteins

14-3-3 proteins have been found to regulate important enzymes in e.g. nutrient-sensing pathways in plants. Nitrate reductase catalyses the first step in nitrate assimilation. It is induced during active photosynthesis and inactivated in darkness. The inactivation is mediated by serine phosphorylation and subsequent binding of 14-3-3 proteins to the phosphorylated site. In contrast, binding of 14-3-3 dimer to the phosphorylated motif in the autoinhibitory C-terminal domain of plasma membrane H^+-ATPase results in the activation of the enzyme and subsequent stomatal opening (see Finnie *et al.*, 1999; Roberts, 2000, and references therein).

In mammalian Raf-1 activation, the 14-3-3 proteins appear to have a more subtle role. Raf-1 is a Ser/Thr kinase acting between the small GTP-binding protein Ras and kinase MEK in a signal transduction cascade induced by growth factors. 14-3-3 proteins have been suggested to take part in Raf-1 activation by two different ways. In the absence of activation signals the 14-3-3 proteins bind to the site in the N-terminal inhibitory portion of Raf-1 thus maintaining it inactive. In response to signals, activated Ras localises Raf-1 to the plasma membrane where phosphatidylserines replace 14-3-3 and abolish the inhibitory effect. 14-3-3 may subsequently bind to another site in the kinase domain of Raf-1 to stabilise the active conformation (Morrison and Cutler, 1997; Yip-Schneider *et al.*, 2000).

1.4.3 Regulation of subcellular localisation of target proteins by 14-3-3 proteins

The localisation of a given protein is often equally important for its function as its activation state. 14-3-3 proteins have been found to promote the cytoplasmic localisation of many of their target proteins. Tethering of e.g. transcription factors and cell cycle regulators within the cytoplasm by interaction with 14-3-3 prevents their access to the nucleus and thereby their action. The 14-3-3 proteins are frequently masking the nuclear import signal in these target proteins (Muslin and Xing, 2000 and references therein; Wang *et al.*, 2000; Kanai *et al.*, 2000). In *S. cerevisiae*, the 14-3-3 homologue Bmh2p has been found to retain Msn2p and

Msn4p in the cytoplasm in response to glucose. Removal of glucose caused a release of these transcription factors from Bmh2p (Beck and Hall, 1999). Similarly with the control of nuclear localisation, an interaction between the phosphorylated motif of pro-apoptotic protein BAD and 14-3-3 blocks the entry of BAD into mitochondria, presumably due to masking of the site through which BAD interacts with the protein Bcl-X_L that has a mitochondrial targeting signal (Muslin and Xing, 2000 and references therein).

On the other hand, the 14-3-3 proteins have been found to stimulate import of precursor proteins into both mitochondria and chloroplasts (Komiya *et al*., 1997; May and Soll, 2000).

1.4.4 14-3-3 proteins are involved in membrane traffic

In mammals, the first indication of the involvement of 14-3-3 proteins in vesicle trafficking was the finding that a mixture of 14-3-3 isoforms from brain cytosol reactivated the Ca^{2+}-dependent exocytosis in permeabilised adrenal chromaffin cells (Morgan and Burgoyne, 1992). This stimulation was suggested to be due to reorganisation of the cortical actin barrier (Roth and Burgoyne, 1995) and could be related to increasing secretory vesicle availability during the ATP-dependent priming stage of exocytosis (Chamberlain *et al*., 1995). Similarly, the *Drosophila* 14-3-3 protein called Leonardo has been proposed to regulate the releasable pool of synaptic vesicles. In addition, Leonardo was found to colocalise with synaptic vesicles in *Drosophila* (Broadie *et al*., 1997). Similarly, certain isoforms of mammalian 14-3-3 proteins have been reported to colocalise with *trans*-Golgi network-derived vesicles in epithelial cells (Fiedler *et al*., 1997) or bind to synaptic membranes (Martin *et al*., 1994). In another experimental system, 14-3-3 proteins were found to associate with a kinesin-like motor protein KIF1C which is involved in the retrograde trafficking between the Golgi complex and ER (Dorner *et al*., 1999).

Of the two 14-3-3 proteins of *S. cerevisiae*, Bmh2p is associated with membrane trafficking. Expression of the *BMH2* is able to rescue the lethality of the clathrin heavy chain gene (*CHC1*) deletion. In addition, the slow growth of viable strains of *chc1*Δ yeast is further impaired by single *bmh* mutations (Gelperin *et al*., 1995). Roth *et al*. (1999) showed that expression of dominant-negative alleles,

consisting of either amino- or carboxy-terminal region of Bmh2p, disturbs polarised exocytosis. The 14-3-3 proteins thus appear to be involved in multiple steps of vesicular trafficking. The possible interactions with phospholipids or actin cytoskeletal components have been proposed to mediate their effects on membrane flow (Dorner *et al*., 1999; Roth *et al*., 1999).

1.5 Aims of the study

The original aim of this work was to clone genes involved in secretion from the filamentous fungus *Trichoderma reesei*, by complementation in yeast. One of the yeast genes chosen to be complemented was *SEC15*. Sec15p is a component of the exocyst complex, and it mediates the interaction between the Rab-protein Sec4p, secretory vesicle and the rest of the exocyst complex. Two signal transduction genes encoding the RHOIII and a 14-3-3 protein were obtained as suppressors of the yeast *sec15-1* mutation. These genes provided putative links between exocytosis and other cellular processes such as e.g. regulation of the actin cytoskeleton. More specific aim of this study was to characterise the genes further by studying their genetic interactions with secretory components, and by examining their effects on growth and secretion in yeast and/or in *T. reesei*.

2. Materials and methods

The detailed descriptions of the materials and methods are presented in the original publications I–III.

Library	Source	Used in
T. reesei λZAP-cDNA library	Stålbrand *et al*., 1995	I, III
T. reesei yeast expression cDNA library	Margolles-Clark *et al*., 1996	I, III
T. reesei cosmid library	Mäntylä *et al*., 1992	I, III

Strain	Source	Used in
BHY44	B.K. Haarer	II, III
BY55	P. Brennwald	II, III
DH5α	Life Technologies	I–III
GG583	G.P.H van Heusden	III
H1152	S. Keränen	II, III
HS33-1	S. Keränen	I–III
MBY12-6D	R. Schekman	II, III
NY3	P. Novick	II, III
NY13	P. Novick	I–III
NY24	P. Novick	II, III
NY770	P. Novick	II, III
NY772	P. Novick	II, III
NY774	P. Novick	II, III
NY776	P. Novick	II, III
NY778	P. Novick	II, III
NY780	P. Novick	II, III
NY782	P. Novick	II, III
NY784	P. Novick	II, III
NY786	P. Novick	I–III
NY1213	P. Novick	II, III
QM9414	Mandels *et al*., 1971	I, II
Rut C30	Montecourt and Eveleigh, 1979	III
Sf821-8A	R. Schekman	II, III
TOP10F'	Invitrogen	I, II
XL1-Blue MRF'	Stratagene	III
YMR505	Y. Matsui	I
203A	This work	II
343B	This work	II

Plasmid	Description	Used in
pLA4	*rho3* disruption plasmid in Bluescript SK-	II
pMS48	*rho3* expression plasmid in pAJ401	I, II
pMS49	*ftt1ΔC* expression plasmid in pAJ401	III
pTV*rho3*	*rho3* cDNA in Bluescript SK-	I
pTV*PGK-rho3*M20	$rho3^{Gly20Val}$ expression plasmid in pAJ401	I
pTV*PGK-rho3*M2	$rho3^{Thr25Asn}$ expression plasmid in pAJ401	I
pTV*PGK-rho3*M126	$rho3^{Asp126Ala}$ expression plasmid in pAJ401	I
pTV*GAL1-rho3*	*rho3* expression plasmid in pYES2	I
pTV*GAL1-rho3*M20	$rho3^{Gly20Val}$ expression plasmid in pYES2	I
pTV*GAL1-rho3*M25	$rho3^{Thr25Asn}$ expression plasmid in pYES2	I
pTV*GAL1-rho3*M126	$rho3^{Asp126Ala}$ expression plasmid in pYES2	I
pTV*PGK-RHO3*	*RHO3* expression plasmid in pAJ401	II
pTV*GAL-RHO3*	*RHO3* expression plasmid in pYES2	II
pTV*ftt1*	*ftt1* cDNA in Bluescript SK-	III
pTV*ftt2*	*ftt2* cDNA in Bluescript SK-	III
pTV*PGK-ftt1*	*ftt1* expression plasmid in pAJ401	III
pTV*PGK-ftt2*	*ftt2* expression plasmid in pAJ401	III
pTV*ADH-ftt1*	*ftt1* expression plasmid in pYcDE-2	III
pTV*ADH-ftt2*	*ftt2* expression plasmid in pYcDE-2	III
pTV*PGK-ftt1ΔC(TRP1)*	*ftt1ΔC* expression plasmid in pAJ401 (*TRP1* selection marker)	III

Cosmid	Description	Used in
F.1	A cosmid harbouring *rho3* and *ftt1* genes	I, III
H.2	A cosmid harbouring *ftt1* and *ftt2* genes	III

Method	Used in
Complementation of yeast disruption	I, III
Determination of activity against MUL	II
DNA methods and Southern hybridisation	I–III
Dry weight measurement	II
Gene disruption from fungal genome	II
Immunofluorescence microscopy of yeast cells	I, II
Immunofluorescence microscopy of fungal cells	II
Light microscopy of fungal cells	II
Log phase growth assay	III
Measurement of α-amylase activity	III
Measurement of invertase activity	II, III
Measurement of plasma membrane H^+-ATPase activity	II
Polymerase chain reaction	I, II
Protease activity measurement	II
RNA extraction and Northern hybridisation	III
Screening of a cDNA library	I, III
Screening of a cosmid library	I, III
Suppression of yeast ts mutations	I–III
Total protein determination	II
T. reesei cultivation	II
T. reesei transformation	II
Western blotting	III
Yeast cultivation	I–III
Yeast transformation	I–III

3. Results

3.1 Isolation of suppressors for yeast *sec15-1* mutation from *T. reesei* genome (I, III)

The organisms which grow in a polarized way target their secretory vesicles to limited areas of plasma membrane. For example, budding yeast directs them towards daughter cell and filamentous fungi towards growing apex (Field and Schekman, 1980; Wösten *et al*., 1991; Wessels, 1993). In yeast, Sec15p has been found to play an important role in secretory vesicle targeting as a component of the exocyst complex and as a protein which interacts with the secretory vesicles and with Sec4p, the central regulator of exocyst assembly (Guo *et al*., 1999b). Since the general molecular mechanisms of exocytosis are assumed to be well conserved between different organisms, a complementation screening in a *sec15-1* yeast strain with a *T. reesei* expression cDNA library was performed.

For this screening, the *T. reesei* expression library (Margolles-Clark *et al*., 1996) constructed under the control of strong *PGK1* (3-phosphoglycerate kinase) promoter in a multicopy vector (Saloheimo *et al*., 1994) was transformed into the *sec15-1* mutant yeast strain. The transformants were shifted to the restrictive temperature (+37°) where the mutant cells were not able to grow. In multicopy suppression numerous copies of a gene are able to revert the mutant phenotype which in this case was temperature-sensitivity to normal. Approximately one cDNA clone out of 10000 was able to support growth of the yeast strain at +37°. The cDNA library plasmids from these colonies were isolated for retransformation and partial sequencing.

The *T. reesei* equivalent of the yeast *SEC15* gene was not among the isolated clones but they included two genes encoding signal transduction molecules which belong to Rho proteins and 14-3-3 proteins. At that time, there was very little information on the involvement of these two groups of proteins in secretion. However, these two genes were sequenced and chosen for further analysis. During the sequencing it was found that the 14-3-3 cDNA that was able to suppress the *sec15-1* mutation was not a full-length one but encoded a protein lacking 115 amino acids from its carboxyl-terminus (III, Figure 1A). The same 14-3-3 cDNA was found in two independent cDNA clones, truncated exactly at the same position (unpublished).

3.2 Characterisation of the *T. reesei* genes *rho3*, *ftt1*, and *ftt2* (I, III)

3.2.1 Sequence analysis

A comparison of the amino acid sequences of the isolated suppressors with the SWISSPROTEIN data bank revealed that the closest homologues for the two chosen clones were *Saccharomyces cerevisiae* Rho3p (I) and *Trichoderma harzianum* 1433 (III). The cDNAs encoding these proteins were also isolated from the *T. reesei* λZAP cDNA library of the strain Rut-C30 (Stålbrand *et al.*, 1995) by using the suppressor cDNAs as probes. During this screening two *14-3-3* cDNAs were obtained which were different from the one isolated as a suppressor. The first was the full-length clone of the *14-3-3* cDNA found to suppress *sec15-1* mutation and the second was also a full-length cDNA but different in sequence. These genes were denoted *ftt1* and *ftt2* (**f**ourteen **t**hree **t**hree), respectively. The C-terminally truncated *14-3-3* cDNA was named *ftt1ΔC* (III). One cDNA was isolated with the *rho* probe, and the gene was consequently designated *rho3* (I).

The *T. reesei* genomic fragments carrying these genes were isolated from the cosmid library (Mäntylä *et al.*, 1992). Two types of cosmid clones were picked up in the screening. One of them carried *rho3* and *ftt1* genes while the other harboured *ftt1* and *ftt2* genes. Based on the overlapping nature of these cosmids it was suggested that the *ftt1*, *ftt2* and *rho3* genes were located relatively adjacent to each other on the same chromosome (unpublished data). On the basis of Southern analysis data, the distance between the *ftt1* and *ftt2* genes was estimated to be approximately 11 kb (III), and the distance between *ftt1* and *rho3* chromosomal genes about 21 kb (unpublished data).

The short promoter area of the *rho3* gene which was sequenced contained a putative TATA-element (TATAAA) but no other known regulatory regions. The gene contained an open reading frame (ORF) of 634 bp which is interrupted by four introns located in the 5’ region of the coding sequence. According to Southern analysis in non-stringent conditions, no other gene closely related to *rho3* exists in the *T. reesei* genome (I, Figure 3). Presumably *T. reesei* has also other *rho* genes similarly as other fungi (see Introduction, Table 2). However,

their sequences are probably so different that they are not detected in Southern analysis performed with low stringency.

The *14-3-3* genes are extremely well conserved across isoforms and through species as diverse as yeast and human (Wang and Shakes, 1996). Genomic Southern analysis suggested that the *T. reesei* genome contains two genes encoding 14-3-3-related proteins (III) like most other fungal species (van Hemert *et al*., 2001a), except *Lentinus edodes* and *Candida albicans* which, according to recent reports, would have only one *14-3-3* gene (Zhou *et al*., 2000; Cognetti *et al*., 2002). Both of the *T. reesei ftt* genes had four introns in their coding regions and, interestingly, the site of only one of the introns was conserved. The *ftt1* had one additional exceptionally long intron at the 5' flanking region (III). The genomic sequences of *T. reesei rho3*, *ftt1* and *ftt2* have been submitted to the EMBL data bank under the accession numbers AJ297909, AJ297910 and AJ297911, respectively.

The *rho3* gene encodes a 207 amino-acid protein with a calculated molecular weight of 23.1 kDa. The closest homologues found in a database search were, in addition to *Saccharomyces cerevisiae* Rho3p (61% identity) mentioned above, *Ashbya gossypii* Rho3p (65% identity), and *Schizophyllum commune* RHOIII (58% identity) (I, Figure 2B). As anticipated, the regions involved in GTP binding and hydrolysis have the strongest conservation (Figure 3). The G-2 and G-3 regions were identical in all the known fungal Rho3 proteins. Interestingly, the glutamic acid in G-2 region and the serine residue in G-3 region appear characteristic of Rho3 proteins since in other fungal Rho proteins the corresponding amino acids are valine and alanine, respectively. The sequence stretches following the G-1–G-3 regions and the regions close to the C-terminus are also highly conserved, as they are among all fungal Rho proteins. However, some amino acids turned out again to be typical of Rho3 proteins. In contrast, soon after G-4 region there is a stretch of poor homology. The *T. reesei* RHOIII and the other Rho3 proteins have a farnesylation signal in the carboxy-terminus (CaaM; a is any aliphatic amino acid) (Kinsella *et al*., 1991) although a geranyl-geranylation signal is characteristic of other Rho proteins (Boguski and McCormick, 1993).

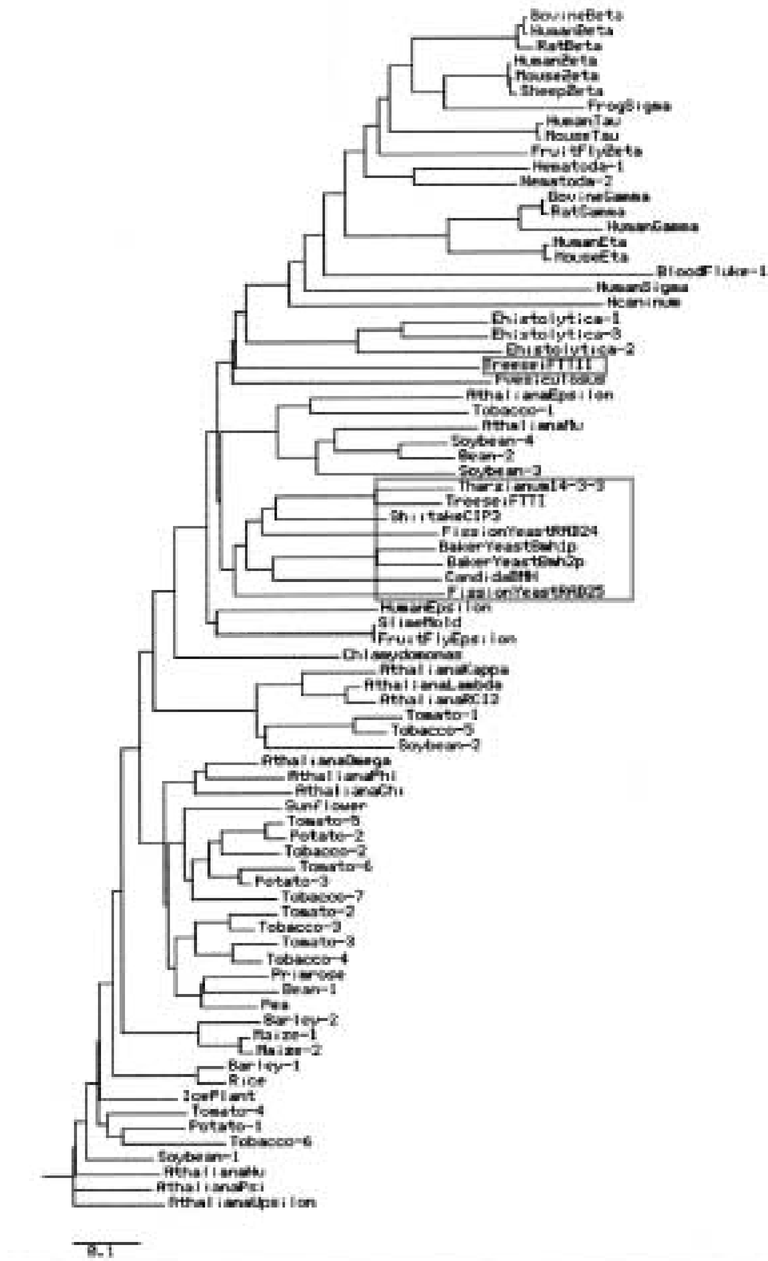

Figure 4. A phylogenetic tree of selected 14-3-3 isoforms from various species. The tree is drawn with Phylodendron (0.8d) by using a ClustalW (1.81) multiple sequence alignment data. The scale bar indicates 10% divergence. Note that the fungal 14-3-3 proteins form a distinct cluster not including T. reesei *FTTII.*

The FTTI and FTTII are small acidic proteins of 264 and 272 amino acids, with calculated molecular weights of 29.6 and 30.4 kDa, respectively. The comparison of amino acid sequences revealed that *T. reesei* FTTI is closely related to *Trichoderma harzianum* Th1433 protein (98% identity) while FTTII is relatively distant from those two proteins (III, Figure 1B). Notably, the 3' flanking regions of *ftt1* and *Th1433* were also highly identical (86%). Comparable high identities in 3' flanking regions have been found between human and rodent 14-3-3 ε isoforms, and between ζ isoforms of frog and human (Jin *et al.*, 1996; Miura *et al.*, 1997). The reason for this conservation is not known. The fungal 14-3-3 proteins form a cluster in the phylogenic tree based on the sequence alignment of currently known 14-3-3 proteins (Figure 4), except that FTTII is not located in this cluster. This further indicates its distinctiveness. The highest conservation between fungal 14-3-3 proteins is located in regions forming the putative α3, α5, α7 and α9 loops (Figure 5) which have been suggested to outline the amphipathic groove in the protein structure (Liu *et al.*, 1995).

3.2.2 Complementation of yeast gene disruptions by expression of the *T. reesei rho3, ftt1* or *ftt2* (I, III)

To study whether the *T. reesei* RHOIII is a functional homologue of the corresponding yeast protein we transformed the *rho3* cDNAs, cloned in expression plasmid pAJ401 (Saloheimo *et al.*, 1994), into yeast *rho3* disruption strain YMR505 (Matsui and Toh-E, 1992b). The cells of the *rho3* disruption strain expressed *RHO4*, a gene that has redundant function with *RHO3*, from the *GAL7* promoter (Matsui and Toh-E, 1992b). Therefore these cells survived on a galactose-containing medium but did not grow on a glucose-containing medium. Expression of *T. reesei rho3* from the *PGK1* promoter supported the growth of *rho3* disruptants also on a glucose-containing medium (I, Figure 4).

We studied further by immunofluorescence microscopy if overexpression of *T. reesei rho3* could also restore the actin organisation and chitin delocalisation defects, which have been reported to be caused by the lack of yeast Rho3p (Kagami *et al.*, 1997). We did not find severe defects in actin organisation in YMR505 cells grown on glucose. Instead, the chitin localisation defect was more evident. Overexpression of *T. reesei rho3* did rescue also these

morphological defects (I, Figure 5), indicating the functional resemblance of these proteins from the two fungal species.

The complementation abilities of *T. reesei ftt1*, *ftt2* or *ftt1ΔC* were examined by transformation of these cDNAs, cloned in yeast expression plasmids, into yeast *bmh1/bmh2* disruption strain, GG583 (van Heusden *et al*., 1995). Deletion of both *BMH* genes has been shown to be lethal while deletion of single *BMH* gene appeared to have no effect. Therefore, GG583 is a diploid strain having one active and one disrupted copy of *BMH1* and *BMH2* genes. Expression of either *ftt1* or *ftt2* from a *ADH1* promoter in GG583 cells gave four viable spores (III, Figure 3) while transformants with the empty vector or transformans expressing *ftt1ΔC* from *PGK1* promoter resulted in two or three viable spores in each tetrad after dissection. One of the spores expressing either *ftt1* or *ftt2* grew more slowly than the others giving rise to a smaller colony. The spores were analysed by cultivating on media lacking either leucine or uracil as *LEU2* or *URA3* were used for disruption of *BMH1* or *BMH2*. The analysis revealed that the smaller colony had cells which lack both *BMH1* and *BMH2*. Thus both *ftt1* and *ftt2* could functionally replace the native yeast genes, although not with full efficiency. Despite its sequence divergence from other fungal 14-3-3 proteins, FTTII appeared to be equally efficient as FTTI in complementing the lack of the yeast *BMH* genes. The truncated *ftt1* did not complement this deficiency as was anticipated (III).

3.2.3 Characterisation of *rho3* mutations (I)

To find a mutation which enables studies of RHOIII function in *Trichoderma* and to further compare the *T. reesei* RHOIII and yeast Rho3p, we introduced three mutations into the *T. reesei rho3* cDNA analogous to yeast *rho3* mutations reported (Imai *et al*., 1996). We studied the mutant phenotypes in wild type, *sec15-1* and *rho3* disruptant yeast strains by expressing these *T. reesei rho3* mutations. The mutated amino acids were positioned in the GTP binding and hydrolysis regions: Glycine-20 was replaced by valine or aspartic acid-126 by alanine to inhibit GTP hydrolysis and thus produce constitutively active proteins, and threonine-25 was replaced by asparagine to generate a dominant inhibitory phenotype. These amino acid substitutions have been widely used to generate

dominant mutations among *ras* and *rho* family GTPases (Boguski and McCormick, 1993; Bishop and Hall, 2000).

We found that overexpression from the constitutive *PGK1* promoter of *rho3*^{Gly20Val} and *rho3*^{Thr25Asn} did not produce viable cells while overexpression of *rho3*^{Asp126Ala} appeared to have no effect on growth. We then introduced these mutated cDNAs under the control of inducible *GAL1* promoter into yeast. In this way, transformants were obtained also with the expression plasmids of *rho3*^{Gly20Val} and *rho3*^{Thr25Asn}. Expression of *rho3*^{Gly20Val} appeared to suppress the *sec15-1* mutation while expression of other mutations did not. *rho3*^{Gly20Val} also seemed to slightly enhance the growth of this strain when compared to cells expressing wild type *rho3*. None of the mutations had negative effects on growth of wild type yeast in any temperature tested (I. Figure 6). Since *RHO4* is also expressed from a galactose-inducible promoter in the *rho3* disruption strain (YMR505) we could not examine whether expression of these mutations could complement the *rho3* disruption. Based on the results obtained, *rho3*^{Gly20Val} thus seemed to be an active mutation while the others appeared as loss-of-function mutations. The *rho3*^{Asp126Ala} mutation thus resulted in different phenotype from the corresponding yeast *rho3* mutation while the others produced similar phenotypes as the yeast mutations (I, Table 1).

3.3 Effects of *rho3*, *ftt1*, *ftt2* and *ftt1ΔC* expression on growth (III)

The mammalian and yeast 14-3-3 proteins have been implicated in cell cycle control and signal transduction pathways affecting growth (Baldin, 2000). To study whether expression of *T. reesei rho3, ftt1* or *ftt2* is regulated according to growth rate in *T. reesei*, lactose-limited chemostat cultivations were analysed by Northern hybridisation. The expression of all these genes was found to be independent of dilution rates (directly proportional to the growth rate) ranging from 0.02 to 0.07 1/h (unpublished; III, Figure 8). The *rho3* probe hybridised with a single mRNA of 1.5 kb (unpublished). The probe fragments from *ftt1* and *ftt2* were chosen from the 3' flanking region in order to avoid cross hybridisation of these highly conserved genes. Interestingly, three transcripts for *ftt2* (1.9, 1.65 and 1.43 kb) were found in Northern analysis while one (1.35 kb) hybridised

with the *ftt1* probe. The three transcripts of *ftt2* maintain their relative intensities similar in all chemostat cultures with different growth rates (III, Figure 8).

To study if expression of *T. reesei ftt* cDNAs has any dominant effect on growth of yeast, in the same way as expression of Bmh2p fragments had according to Roth *et al*. (1999), a wild type yeast strain was transformed with plasmids expressing *ftt1*, *ftt2*, *ftt1ΔC* or with the vector pAJ401. The cultures of different transformants were maintained in the logarithmic growth phase by repeated dilutions. The experiment showed that expression of both *ftt1* and *ftt1ΔC* retarded the growth of wild type yeast during log phase growth while expression of *ftt2* enhanced it (III, Figure 7).

3.4 Suppression of yeast late-acting secretory mutations by *T. reesei rho3* and C-terminally truncated *ftt1* (II, III)

3.4.1 Suppression of growth defects

The *T. reesei rho3* and *ftt1ΔC*, originally isolated as multicopy suppressors of yeast *sec15-1* mutation, were subjected to further experiments with other late-acting yeast secretory mutations to search for possible new interactions. Overexpression of *T. reesei rho3* from the *PGK1* promoter in a multicopy vector suppressed, in addition to *sec15-1*, also mutations *sec1-1*, *sec1-11*, *sec3-2*, *sec3-101*, *sec6-4*, and *sec8-9* (II, Figure 1A). Overexpression of full-length *ftt1* or *ftt2* from a similar construct did not suppress the growth defect of any mutations tested while overexpression of *ftt1ΔC* suppressed *sec2-41*, *sec3-101*, *sec7-1* and *sec15-1* (III, Figure 4). It also enhanced the growth of mutations *sec1-1*, *sec1-11*, *sec4-8*, *sec9-4* and *sec19-1* (Table 5).

Since the *T. reesei* RHOIII and yeast Rho3p are highly identical at the amino acid level and since they were found to be functionally similar we tested if overexpression of yeast *RHO3* could also suppress the growth defects of late-acting secretory mutations. For that reason the coding region of *RHO3* was produced by PCR and cloned into the same expression vector as *T. reesei rho3*.

Table 5. Suppression of yeast ts secretory mutations by overexpression of T. reesei rho3*,* S. cerevisiae RHO3 *or* T. reesei ftt1ΔC*. Plus indicates enhancement of growth at permissive temperature, two plusses indicates weak suppression and three plusses full supression. Minus indicates no effect on growth. The < denotes minor inhibition of growth, << indicates strong inhibition of growth and <<< denotes lethal effect. ND, not determined.*

Mutation	*rho3(PGK1)*	*RHO3(PGK1)*	*RHO3(GAL1)*	*ftt1ΔC(PGK1)*
sec7-1	-	-	-	+++
sec4-8	<	-	-	++
sec2-41	-	-	-	+++
sec19-1	-	-	-	++
sec15-1	+++	<<<	+	+++
sec10-2	-	<<<	-	-
sec5-24	-	<<<	+	-
sec6-4	++	<	+	-
sec8-9	++	<<<	+	-
sec3-2	+++	ND	ND	+++
sec3-101	+++	<<<	<<	+++
sec1-1	+++	-	+++	++
sec1-11	+++	-	+++	++
sec9-4	-	-	-	++
sso2-1	-	-	-	-
sec17-1	-	-	-	-
sec18-1	-	-	-	-

It was found that overexpression of yeast *RHO3* from the *PGK1* promoter is lethal in several secretory mutant strains: *sec3-101, sec5-24*, *sec8-9*, *sec10-2* and *sec15-1*. Furthermore, it did not suppress the mutations of *sec1-1*, *sec1-11* and *sec6-4* as overproduction of *T. reesei rho3* did but rather retarded the growth of these mutant cells at the permissive temperatures when compared to cells carrying the empty vector (II) implying thus synthetic dosage toxicity between excess *RHO3* and these *sec* mutations.

Since *PGK1* is a strong constitutive promoter we cloned the *RHO3* gene under inducible *GAL1* promoter to reveal possible dosage suppression effect. The

growth assay on a galactose-containing medium showed that only *sec1-1* and *sec1-11* mutations were suppressed (II, Figure 2). However, the growth of the *sec5-24*, *sec6-4*, *sec8*-9 and *sec15-1* mutant strains was improved at permissive temperatures while, interestingly, the growth of *sec3-101* was inhibited (II, Figure 2; Table 6).

3.4.2 Suppression of the secretion defect of yeast *sec15-1* and *sec2-41* mutants

In fungal cells the growth and secretion are believed to be intimately connected. We studied whether the secretion defect was suppressed in similar conditions as the growth defect in two of the secretory mutant strains, *sec15-1* and *sec2-41*. We used three assays: secretion of invertase, secretion of α-amylase, and glucose-induced medium acidification. Invertase is an endogenous enzyme of yeast which is liberated into the periplasmic space in the absence of glucose. α-amylase is a heterologous protein from *Bacillus amyloliquefaciens*, and its activity could be measured from the growth medium. The glucose-induced medium acidification method monitors plasma membrane H^+-ATPase activity. The latter analysis was chosen since it has been reported that invertase and plasma membrane H^+-ATPase are transported in different vesicle populations between the Golgi complex and plasma membrane in yeast (Harsay and Bretscher, 1995). We thus wanted to test whether both of these postulated secretion routes are affected by the suppression.

It was revealed that overexpression of *rho3* in a *sec15-1* strain could rescue the invertase secretion defect but not as efficiently as the growth defect (II, Figure 3). Expression of *T. reesei rho3* also supported the transport of plasma membrane H^+-ATPase to the plasma membrane (II, Figure 4). Presumably, *rho3* overexpression also enabled the delivery of glucose transporters to the plasma membrane, since glucose, added in the medium of starved cells, should be taken into the cell in order to supply energy for plasma membrane H^+-ATPase. Phosphate transporter were probably also transported to the PM since vanadate, used to inhibit plasma membrane H^+-ATPase, has been suggested to utilise phosphate transporters when moving into the cell (Bowman, 1983). An additional indication for the rescue of the yeast secretion defect by *T. reesei rho3* was that *sec15-1* cells expressing *rho3* and incubated at the restrictive

temperature had wild type actin network while the cells carrying empty vector had severely disrupted actin organisation (II, Figure 5). This improvement of actin structures could be observed at the same time point where the invertase secretion defect was partially rescued. Thus, the cells expressing *rho3* could also transport the patch assembly factors needed for actin nucleation.

Overexpression of *ftt1ΔC* also suppressed the invertase secretion defect of *sec15-1* (unpublished) and *sec2-41* (III, Figure 5A). Moreover, α-amylase activity reached the wild type level in growth medium of *sec15-1* (unpublished) and *sec2-41* (III, Figure 5C) cells expressing *ftt1ΔC* at the restrictive temperature. However, the latter response was clearly delayed compared to growth suppression (III, Figure 5D). To exclude the possibility that this would be caused by changes in the FTT1ΔC protein level during cultivation, Western analysis with an antiserum raised against yeast Bmh1p was performed. This antiserum could detect the expressed FTT1ΔC in approximately equal amounts at all time points (III, Figure 5B, E).

3.5 Enhancement of wild type yeast secretion by *ftt1* (III)

Since overexpression of *T. reesei ftt1ΔC* could suppress the secretion defect of two yeast secretory mutants, we wanted to test if overexpression of full length *ftt1* or *ftt2* would have any effect on secretion in wild type yeast. The invertase secretion assay showed that overexpression of *ftt1* in a wild type strain clearly enhanced secretion (III, Figure 6). The full-length *ftt2* and truncated *ftt1* did not have clear effects on invertase secretion (III, Figure 6).

3.6 Effects of *rho3* disruption on growth and secretion in *T. reesei* (II)

To find out the effect of RHOIII on growth and secretion in *T. reesei*, the gene encoding RHOIII was disrupted by replacing the coding region with hygromycin resistance gene. The Southern screening of transformants by a probe containing a fragment of *rho3* coding region revealed two putative disruptants not hybridising with this probe. It was further confirmed that these disruptants

contained additional DNA replacing the coding region of *rho3* and that no other copies of the replacement cassette were in the genome.

The effects of *rho3* disruption in these disruptants were examined by growing them in media supplemented with different carbon sources: glucose which supports efficient growth but not protein secretion, cellobiose which allows efficient growth and moderate secretion, or cellulose which is a powerful inducer of protein secretion (Ilmén *et al*., 1997; Margolles-Clark *et al*., 1997). In glucose and cellobiose-containing media no difference in growth, measured as dry weight accumulation and pH decrease, was obtained between the disruptants and the parental strain (II, Figure 7D). In contrast, in cellulose-containing medium both growth, according to pH decrease, and total protein secretion were retarded in the disruptants when compared to the parental strain (II, Figure 7A, B). Because cellulose is a solid carbon source, growth could not be monitored as dry weight accumulation in this medium. The amount of cellulase activity against methylumbelliferyl-β-D-lactoside (MUL) substrate and protease activity showed similar results as total protein secretion (II, Figure 7C, E).

Disruption of *rho3* from the filamentous fungus *Ashbya gossypii* has been reported to result in morphological abnormalities and impairment in actin organisation: The tips of growing hyphae in *rho3* disruptants were swollen but this was temporal, and eventually, a new hyphal tube emerged having the original direction of growth (Wendland and Philippsen, 2001). We studied by light microscopy whether *T. reesei rho3* disruptants grown in glucose or cellulose-containing medium were morphologically aberrant, focusing especially on tips and branching of hyphae, and found no differences between the parental strain and disruptants. The actin and tubulin organisation was studied by immunofluorescence microscopy and again, no differences between disruptants and the parental strain could be detected.

4. Discussion

4.1 Characterisation of *T. reesei rho3*, *ftt1* and *ftt2* genes

4.1.1 *T. reesei* RHOIII belongs to a distinctive group of Rho proteins

Five genes encoding Rho-proteins are present in the completely sequenced *S. cerevisiae* genome. Homologues for at least Rho1p, Rho3p and Rho4p have also been isolated from filamentous fungi (see Introduction, Table 2) mainly by PCR with oligonucleotide primers based on yeast sequences. In the present study, the homology between budding yeast *RHO3* and *T. reesei rho3* was established by sequence comparison and by complementation of the growth and morphology defects of yeast *rho3* disruption strain by *T. reesei rho3* expression. Furthermore, the experiments in which mutations were introduced into conserved amino acids involved in GTP binding and hydrolysis of *T. reesei rho3*, provided largely similar results as those of yeast *rho3* mutations except that the amino acid substitution of alanine-126 for aspartic acid produced a loss-of-function phenotype while in yeast the analogous mutation caused dominant cold-sensitivity (Imai *et al*., 1996). Similar discrepancy of phenotypes has also been found between the mutations of *A. niger sarA* and yeast *SAR1* genes encoding GTPases involved in formation and uncoating of ER-derived secretory vesicles (Veldhuisen *et al*., 1997).

Different Rho proteins have been suggested to carry out at least partially different functions via distinct target proteins (see Introduction, Table 3). The effector loop (underlined in Figure 3) is considered to be important for functional specificity of GTPases. The sole amino acid which distinguishes the effector loop of Rho3 proteins from the other fungal Rho proteins is glutamic acid-46 (yeast Rho3p numbering) (Figure 3). This glutamic acid was mutated in studies of Adamo *et al*. (1999). Amino acid substitution of serine was lethal while substitution of glycine produced cold-sensitive growth and severe defects in actin organisation. However, in the same study mutations of two other effector loop amino acids also produced cold-sensitive phenotype and defects in actin organisation or, interestingly, defects in post-Golgi secretion. Therefore, also other regions than the effector domain should be involved in binding of RHOIII to a special downstream factor regulating secretion. In agreement with

this, the crystal structure of the complex between PKN and RhoA revealed the regions required for interaction between these proteins (Maesaki *et al.*, 1999), and they cover residues both in the effector loop and outside this area. Although there is no information on the role of the corresponding Rho3p regions in effector binding, it is interesting to note that in many cases the amino acids conserved in Rho3 proteins but not in other Rho proteins are localised in these regions outside the effector loop and implicated in PKN binding by RhoA. Thus, e.g. arginine-38 could be a residue important for the specificity as the corresponding amino acid is lysine in Rho1 proteins, leucine in Rho2 proteins, arginine in Rho3 proteins, glutamine in Rho4 proteins and threonine in Rho5 protein (Figure 3).

On the basis of the prenylation signal the Rho3 proteins are farnesylated and not geranyl-geranylated which is typical of other Rho proteins. Prenylation is known to have an important role in the localisation of several small GTPases into membranes and their subsequent activation (Cox and Der, 1992). The significance of the difference between the two prenyl groups is, however, unclear, although they might direct proteins to specific membrane subdomains (Gulli and Peter, 2001). The Rho3 proteins could thus localise differently than Rho1 or Rho2 proteins. This could increase the specificity of protein function since the cellular subcontext has been suggested to affect the interaction between GTPases and their putative effectors (Bishop and Hall, 2000).

4.1.2 *T. reesei* FTTII sequence is distinct from other fungal 14-3-3 proteins

The 14-3-3 proteins are highly conserved and ubiquitously expressed. The identity of the *T. reesei* FTTI amino acid sequence to other 14-3-3 proteins ranges from 47% to 98%, while *T. reesei* FTTII is 47% to 72% identical to other proteins in this group. Most fungal organisms have two 14-3-3 isoforms that are more similar to each other than they are to proteins from any other species (Wang and Shakes, 1996). However, *T reesei* FTTI is more identical to *T. harzianum* 1433 than to *T. reesei* FTTII. FTTII is relatively distant from the other fungal 14-3-3 proteins, which is reflected by its segregation from the fungal cluster in the phylogenic tree (Figure 4). The amino acid differences mostly lie within the loop α2 while the amino acid sequence of α3, α5, α7 and

$\alpha 9$ loops, which are thought to interact with target proteins (Liu *et al*., 1995), are very conserved (Figure 5). This can explain why both *T. reesei* isoforms were equally capable in suppressing the lethality of yeast *bmh1*/*bmh2* disruption. However, they could not compensate for the Bmh protein functions to the full extent, since the cells expressing either *ftt1* or *ftt2* and lacking both *BMH1* and *BMH2* showed clearly slower growth than those harbouring an endogenous 14-3-3 gene.

Despite the high conservation of the putative effector binding region, the different 14-3-3 isoforms have been found to have partly different functions (Rittinger *et al*., 1999). The yeast isoforms Bmh1p and Bmh2p, for example, are highly identical (98%) with each other (van Heusden *et al*., 1995). Still, Bmh2p has numerous target proteins which do not interact with Bmh1p (van Hemert *et al*., 2001a). Such an isoform specificity has been proposed to be a consequence of either different subcellular localisation or transcriptional regulation of the isoforms (Rittinger *et al*., 1999). The exceptionally high conservation of the 3' flanking region between *T. reesei ftt1* and *T. harzianum 1433* raises the question whether this region has a role in *ftt1* mRNA targeting and thus subcellular localisation of the protein. The polarized distribution of certain mRNAs has been found to be determined by 3'-UTR elements in animals (Aronov *et al*., 2001; Simmonds *et al*., 2001). However, no indication of known targeting signals in the 3' flanking region of *ftt1* exists. Differential expression of different 14-3-3 isoforms, or even different mRNAs of a single isoform, have been observed during development or in different tissues (Swanson and Ganguly, 1992; Wang and Shakes, 1997). The *T. reesei ftt2* gene expresses three mRNAs whose comparative levels in chemostat conditions were equal at all growth rates. The expression of *ftt1* did not vary according to the growth rate either. Therefore, the expression study carried out did not illuminate the possible different functions of *T. reesei* FTTI and FTTII during *T. reesei* growth.

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Tharzianum14-3-3      --MGH---EDAVYLAKLAEQAERYEEHVENMKIVASEDRDLTVEERNLLSVAYKNVIGAR 55
TreeseiFTTI           --MGH---EDAVYLAKLAEQAERYEEHVENMKIVASEDRDLTVEERNLLSVAYKNVIGAR 55
LedodesCIP3           MPETR---EDSVYLAKLAEQAERYEEHVENMKRVASSDQELTVEERNLLSVAYKNVIGAR 57
ScerevisiaeBmh1p      --MST-SREDSVYLAKLAEQAERYEEHVENMKTVASSGQELSVEERNLLSVAYKNVIGAR 57
ScerevisiaeBmh2p      --MSQ-TREDSVYLAKLAEQAERYEEHVENMKAVASSGQELSVEERNLLSVAYKNVIGAR 57
CalbicansBMH          --MPA-SREDSVYLAKLAEQAERYEEHVENMKAVASSGQELSVEERNLLSVAYKNVIGAR 57
SpombeRAD24           --MSTTSREDAVYLAKLAEQAERYEGHVENMKSVASTDQELTVEERNLLSVAYKNVIGAR 58
SpombeRAD25           --MSN-SRENSVYLAKLAEQAERYEEHVENMKKVACSNDKLSVEERNLLSVAYKNIIGAR 57
TreeseiFTTII          --MAT-ERESKTFLARLCEQAERYDEHVTYMKEVAQLGGELSVDERNLLSVAYKNVVGTR 57
                              *. .:**:*.******: **  ** **  . .*:*:************::*:*
                         -------------    -------------       -----------------
                                α1               α2                     α3

Tharzianum14-3-3      RASWRIVTSIEQKEESKG-NSSQVTLIKEYRQKIENELAKICDDILEVLDQHLIPSAKSG 114
TreeseiFTTI           RASWRIVTSIEQKEESKG-NSSQVALIKEYRQKIEAELAKICDDILEVLDQHLIPSAKSG 114
LedodesCIP3           RASWRIVSSIEQKEESKG-NEAQVSMIKGYREKIETELAEICEDILDVLDKHLIPSAASG 116
ScerevisiaeBmh1p      RASWRIVSSIEQKEESKEKSEHQVELICSYRSKIETELTKISDDILSVLDSHLIPSATTG 117
ScerevisiaeBmh2p      RASWRIVSSIEQKEESKEKSEHQVELIRSYRSKIETELTKISDDILSVLDSHLIPSATTG 117
CalbicansBMH          RASWRIVSSIEQKEEAKG-NESQVALIRDYRAKIEAELSKICEDILSVLSDHLITSAQTG 116
SpombeRAD24           RASWRIVSSIEQKEESKG-NTAQVELIKEYRQKIEQELDTICQDILTVLEKHLIPNAASA 117
SpombeRAD25           RASWRIISSIEQKEESRG-NTRQAALIKEYRKKIEDELSDICHDVLSVLEKHLIPAATTG 116
TreeseiFTTII          RASWRIISSIEQKEESKG-SDKHVATIKEYRSKIELELEKVCEDVLNVLDTSLIPNAATG 116
                      ******::*******::  .  :.  *  ** *** **  :..*:* **.  **. * :.
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                                                                   α4

Tharzianum14-3-3      ESKVFYHKIKGDYHRYLAEFAIGDRRKDSADKSLEAYKAATEVAQTELPPTHPIRLGLAL 174
TreeseiFTTI           ESKVFYHKMKGDYHRYLAEFAIGDRRKDSADKSLEAYKAATEVAQTELPPTHPIRLGLAL 174
LedodesCIP3           ESKVFYHKMMGDYHRYLAEFATGDKRKESADKSLEAYKAASDVAVTELPPTHPIRLGLAL 176
ScerevisiaeBmh1p      ESKVFYYKMKGDYHRYLAEFSSGDAREKATNASLEAYKTASEIATTELPPTHPIRLGLAL 177
ScerevisiaeBmh2p      ECKVFYYKMKGDYHRYLAEFSSGDAREKATNSSLEAYKTASEIATTELPPTHPIRLFLAL 177
CalbicansBMH          ESKVFYYKMKGDYHRYLAEFAIAVFRKEAADLSLEAYKAASDVAVTELPPTHPIRLGLAL 176
SpombeRAD24           ESKVFYYKMKGDYYRYLAEFAVGEKRQHSADQSLEGYKAASEIATAELAPTHPIRLGLAL 177
SpombeRAD25           ESKVFYYKMKGDYYRYLAEFTVGEVCKEAADSSLEAYKAASDIAVAELPPTDPMRLGLAL 176
TreeseiFTTII          ESKVFYHKMKGDYHRYLAEFASGEKRKVAATAAHEAYKNATDVAQTELTPTHPIRLGLAL 176
                      *.****:*: ***:******: .    : ::  : *.** *:::* :**.**.*:** ***
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                                α5                       α6                 α7

Tharzianum14-3-3      NFSVFYYEILNAPDQACHLAKQAFDDAIAELDTLSEESYKDSTLIMQLLRDNLTLWTSSE 234
TreeseiFTTI           NFSVFYYEILNAPDQACHLAKQAFDDAIAELDTLSEESYKDSTLIMQLLRDNLTLWTSSE 234
LedodesCIP3           NFSVFYYEILNSPDRACHLAKQAFDDAIAELDTLSEESYKDSTLIMQLLRDNLTLWTSDM 236
ScerevisiaeBmh1p      NFSVFYYEIQNSPDKACHLRKQAFDDAIAELDTLSEESYKDSTLIMQLLRDNLTLWTSDM 237
ScerevisiaeBmh2p      NFSVFYYEIQNSPDKACHLAKQAFDDAIAELDTLSEESYKDSTLIMQLLRDNLTLWTSDI 237
CalbicansBMH          NFSVFYYEILNSPDRACHLAKQAFDDAVADLETLSEDSYKDSTLIMQLLRDNLTLWT-DL 235
SpombeRAD24           NFSVFYYEILNSPDRACYLAKQAFDEAISELDSLSEESYKDSTLIMQLLRDNLTLWTSDA 237
SpombeRAD25           NFSVFYYEILDSPESACHLAKQVFDEAISELDSLSEESYKDSTLIMQLLRDNLTLWTSDA 236
TreeseiFTTII          NFSVFYYEILNSPDRACHLAKQAFDDAIAELDSLSEESYRDSTLIMQLLRDNLTLWTSSD 236
                      ********* ::*: **:* **.**:*:::*::***:**:*****************  .
                      ------          -------------          --------------
                                           α8                          α9

Tharzianum14-3-3      AETPARLMPLLRRR---PLLRL---PSRRRRAQG------- 262
TreeseiFTTI           AETSAGQVEAPPKEDTPAEAAA---PAEEPKAE-------- 264
LedodesCIP3           QDSADKPAEK------DEAADA---PADE------------ 256
ScerevisiaeBmh1p      SESGQAEDQQQQQQHQQQQ------PPAAAEGEAPK----- 267
ScerevisiaeBmh2p      SESGQEDQQQQQQQQQQQQQQQQQQAPAEQTQGEPTK----- 273
CalbicansBMH          SEAPAATEEQQQS--SQAP------AAQPTEGKADQE---- 264
SpombeRAD24           EYSAAAAGG-NTEGAQENA------PSNAPEGEREPKATHR 271
SpombeRAD25           EYNQAKEEAPAAAAASENE------HPEPKESTTDTVKA-- 269
TreeseiFTTII          SGEAEQAGEAKKDEGEAAKPAEEEPKAEEPAPEATS----- 272
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Figure 5. A ClustalW (1.81) multiple sequence alignment of fungal 14-3-3 proteins. Asterisks indicate conserved residues and dots indicate residues which share similar biochemical properties. The conserved amino acids are also highlighted in red while dissimilar amino acids in otherwise conserved positions are highlighted in blue. Dashed lines under the sequence blocks indicate the positions of putative alpha helices in three-dimensional structure.

4.2 Involvement of *T. reesei rho3* and *ftt1 in protein secretion*

4.2.1 Suppression of yeast late-acting secretory mutations

4.2.1.1 The exocyst components

Sec15p is a hydrophilic protein (Salminen and Novick, 1989) that associates with secretory vesicles (Guo *et al*., 1999b). It also interacts with activated Sec4p on the secretory vesicle, and mediates the effect of this Rab GTPase in vesicle tethering (Guo *et al*., 1999b) but not in vesicle transportation (Govindan *et al*., 1995; Walch-Solimena *et al*., 1997). The other interaction partner for Sec15p is the exocyst complex component Sec10p, which together with Sec5p links vesicles to the rest of the complex (Guo *et al*., 1999b). In the present study, the *T. reesei rho3* and *ftt1ΔC* genes were isolated as multicopy suppressors of temperature-sensitive *sec15-1* mutation in budding yeast. Interestingly, one of the multicopy suppressors of a *SEC4* effector loop mutation described by Brennwald *et al*. (1994), HSS43, was later shown to be *RHO3* (Adamo *et al*., 1999). Brennwald *et al*. (1994) also found that expression of HSS43 suppressed the growth of *sec15-1*.

The exocyst complex is composed of eight single-copy components, Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84 (TerBush *et al*., 1996; Guo *et al*., 1999a). It is essential in directing the secretory vesicles to the sites of exocytosis at plasma membrane subdomains (TerBush *et al*., 1996; Guo *et al*., 1999b). Sec3 protein is of central importance in spatial regulation of this membrane traffic since it has been found to localise to sites of exocytosis independently of other exocyst subunits and of the actin cytoskeleton. Based on these discoveries, it is regarded as a spatial landmark for polarised exocytosis (Finger *et al*., 1998). The results (II, III) showed that the temperature sensitive mutation of *sec3-101* was suppressed by the expression of *T. reesei rho3* or *ftt1ΔC*. In addition, overproduction of yeast Rho3p from *PGK1* promoter showed dosage toxicity in the *sec3-101* strain and overproduction from the *GAL1* promoter inhibited the growth of *sec3-101* cells (II). Both of these effects reflect genetic interaction between yeast *RHO3* and *SEC3*. Interestingly, Sec3p has been observed to interact also physically with GTP-bound Rho1p, Rho3p, Rho4p (Guo *et al*.,

2001) and Cdc42p (Zhang *et al.*, 2001). Since Rho proteins are largely connected to regulation of the actin cytoskeleton organisation (van Aelst and D'Souza-Schorey, 1997), it is not unexpected that the *sec3-101* mutation has also been found to be synthetically lethal with a profilin (actin-binding protein) mutation in yeast (Haarer *et al.*, 1996).

The assembly of the exocyst complex is thought to link the secretory vesicle, associated with Sec15p, to the specific PM exocytic region which is marked by Sec3p (Guo *et al.*, 1999b). However, Exo70p was found to be able to localise to the exocytic site independently of Sec3p (Guo *et al.*, 2001). Interestingly, Rho3p, but not the other Rho proteins, have been found to interact physically with the exocyst component Exo70p in yeast (Robinson *et al.*, 1999; Adamo *et al.*, 1999). We found that overexpression of *T. reesei rho3* suppressed, although not completely, the growth defects of *sec6-4* and *sec8-9*. We also observed dosage toxicity when yeast *RHO3* was overexpressed from *PGK1* promoter, in addition to *sec3-101* strain mentioned above, with strains *sec5-24*, *sec8-9, sec10-2* and *sec15-1*. The growth of *sec6-4* was instead only slightly retarded. The difference in the type of genetic interaction between *T. reesei rho3* and *S. cerevisiae RHO3* expressed from the *PGK1* promoter may due to differences at amino acid level; *T. reesei* RHOIII may not interact with its effector protein with the same efficiency as Rho3p in yeast and consequently, excess amount of *T. reesei* RHOIII is not toxic unlike excess amount of Rho3p. Presumably, overloading the cell with the *T. reesei* RHOIII could facilitate the targeting of secretory vesicles to the plasma membrane subdomains via direct interaction with Exo70p and Sec3p. Through these interactions RHOIII could also correct the mislocalisation defects caused by mutations in proteins closely connected to Exo70p or Sec3p, such as Sec6p, Sec8p or Sec10p. However, Cdc42p whose function has been suggested to overlap with that of Rho3p in exocytosis, does not affect exocyst localisation but presumably its conformation (Adamo *et al.*, 2001).

Expression of *T. reesei fttl*Δ*C* suppressed two yeast exocyst complex mutations, *sec15-1* and *sec3-101*. Presumably, the suppression mechanism of *fttl*Δ*C* is more indirect than that of *rho3*.

4.2.1.2 Proteins involved in vesicle transport

In yeast, and probably also in filamentous fungi, secretory vesicles are transported along actin filaments to the bud region/hyphal apex. Myo2p is a myosin V-like motor protein carrying out the ATP-requiring transport process of secretory vesicles. It associates with the vesicles by its carboxyl-terminal tail (Schott *et al*., 1999). It is assumed that Sec4p and its guanine nucleotide exchange factor (GEF), Sec2p (Walch-Solimena *et al*., 1997), are involved in Myo2p-vesicle contact since defects in these three genes are synthetically lethal (Govindan *et al*., 1995; Schott *et al*., 1999). In addition, *sec2-48* strain accumulates post-Golgi vesicles (Walch-Solimena *et al*., 1997), apparently detached from the motor protein (Schott *et al*., 1999). Interestingly, some 14-3-3 isoforms have been found to interact with a kinesin motor KIF1C in human, and it has been proposed that 14-3-3 proteins could facilitate the association of the motor with the membrane of transport vesicles (Dorner *et al*., 1999). It is thus tempting to speculate that excess amount of FTTIΔC could support the connection between the secretory vesicle and Myo2p in conditions where it is otherwise weak, such as in yeast cells having a *sec2-48* mutation. 14-3-3 proteins may be associated with the secretory vesicles when fulfilling this task. 14-3-3 proteins colocalise with trans-Golgi network-derived vesicles in mammalian epithelial cells (Fiedler *et al*., 1997) and with *Drosophila* synaptic vesicles (Broadie *et al*., 1997). In addition, a 12 kDa N-terminal region of 14-3-3 proteins has been found to be sufficient to bind lipids (Jones *et al*., 1995). Moreover, Sec2p, Sec4p and Sec15p are also associated with post-Golgi vesicles (Guo *et al*., 1999b), and the growth of mutants with defects in these proteins was either enhanced or suppressed by *ftt1ΔC* (III). Interestingly, another group of proteins involved in secretion in mammalian cells and able to bind lipids, the annexins (Zhuang and Stracher, 1989), displayed genetic interaction with *sec2-41*, *sec4-8* and *sec15-1* when overexpressed in yeast (Creutz *et al*., 1992).

4.2.1.3 Docking components

Secretory vesicle docking in yeast is controlled by vesicle-SNAREs (Snc1p, Snc2p) (Gerst *et al*., 1992; Protopopov *et al*., 1993) and target-SNAREs (Sso1p, Sso2p and Sec9p) (Aalto *et al*., 1993; Brennwald *et al*., 1994) present on the plasma membrane. Their interaction is controlled by specific regulatory proteins,

one of which is Sec1p (Gerst *et al.*, 1999). In mammals, Sec1 protein has been suggested to mask the t-SNAREs and inhibit unspecific binding between the vesicle and target SNAREs (Zhang *et al.*, 2000). However, Sec1p binds only with complexed SNAREs in yeast (Carr *et al.*, 1999), and indications have been found that Sec1 may have role in controlling the fusion process (Fisher *et al.*, 2001). The suppression of *sec1-1* and *sec1-11* mutations by *T. reesei rho3* is conceivable with the fact that Sec9p and Sso2p were found to act as multicopy suppressors of *rho3*Δ (Adamo *et al.*, 1999). Sec1p is known to interact with these t-SNARE components both genetically (Aalto *et al.*, 1993) and physically (Carr *et al.*, 1999; Grote *et al.*, 2000). This genetic interaction was confirmed in the present study by overexpressing the yeast *RHO3* from the *GAL1* promoter, which suppressed the *sec1-1* and *sec1-11* mutations.

4.2.2 Possible mechanisms of multicopy suppression

Suppression cloning in yeast is a way to reveal genetic interactions between molecules involved in the same cellular process or signal transduction pathway, even from species other than *S. cerevisiae*. By suppression cloning in the *sec15-1* strain, genes encoding the rat synaptotagmin II (Damer and Creutz, 1996) and a RING finger motif protein Rma1p from *Arabidopsis thaliana* (Matsuda and Nakano, 1998) were cloned. Synaptotagmin II is involved in vesicle targeting similarly to Sec15p.

There are several possible mechanisms for multicopy suppression of conditional mutations: 1) a suppressing gene may encode the wild type counterpart of the mutated protein and thus complement the mutation or 2) elevated levels of a protein can compensate its lowered affinity to the mutated protein. However, multicopy suppression does not necessarily mean physical interaction, and thus other possible mechanisms could be: 3) a suppressor may, in excess amount, activate the downstream pathway which is perturbed by the mutated protein, either being a downstream component itself or a regulatory protein, or 4) overproduction of a protein may bypass the mutation through enhancing the function of a parallel pathway (Nelson and Lemmon, 1993).

The suppressors isolated and characterised in this work (I–III) do not encode the corresponding proteins whose mutations they suppressed. Instead, *T. reesei*

RHOIII may interact with mutated Sec3p but unlikely with the other mutated proteins used in the present study. Rather, RHOIII may act as a regulatory protein of a downstream component when suppressing *sec15-1*, e.g. through interaction with Exo70p, and when suppressing *sec6-4* or *sec8-9*, via interaction with Sec3p. The mechanism of *T. reesei rho3* in suppression of *sec1* mutations is more difficult to explain.

T. reesei ftt1ΔC also suppressed many late-acting secretory mutations and, unexpectedly, one mutation (*sec7-1*) which is suggested to affect the ER-Golgi stage of secretion (Lupashin *et al*., 1996). The full-length *ftt1* or *ftt2* did not exhibit suppressor activity. The *Trichoderma fttΔC* lacks more than one third of the coding region and thus the truncated FTTI protein lacks the entire hydrophobic wall of the putative amphipathic groove within the protein structure (Liu *et al*., 1995). Most of the mutational and crystallisation studies carried out have shown that a cluster of basic amino acids in the groove is important for the interaction of 14-3-3 proteins with diverse ligands (Fu *et al*., 2000, and references therein). If the fungal 14-3-3 proteins play a role in exocytosis, one way to explain the pattern of the suppressor activities could be that the wild type protein is unable to interact with (a) mutated Sec protein(s) that may be structurally distorted, while with the truncated FTTI form the binding is re-established due to its altered binding properties caused by the deletion. On the other hand, the wild type FTTI may, even though it does not suppress a mutation, interact with the wild type protein and thus enhance secretion (III). Therefore, FTTIΔC may interact directly with the mutated proteins in yeast, especially Sec2p, Sec4p and Sec15p. However, it is well possible that the suppression of the *sec* mutations occurs by another mechanism than direct binding between the Sec and FTTIΔC proteins.

4.2.3 Lack of RHOIII retards highly induced secretion in *T. reesei*

Many filamentous fungi produce extracellular enzymes which degrade complex plant polymers, such as cellulose, into monomers. *T. reesei* is one of the most efficient cellulolytic organisms and protein secretors. The major secreted protein is cellobiohydrolase I (CBHI) that may comprise up to 60% of the secreted proteins.

Disruption of *rho3* gene was found to retard the growth and secretion of *T. reesei* on a cellulose-containing medium but not on a cellobiose- or glucose-containing medium (II). It is probable that the *rho3* gene disruption in the *T. reesei* genome affects specifically secretion and not growth, *per se*, because the defect in growth and secretion was observed only in *T. reesei* cultures grown under inductive conditions. In the presence of cellulose the fungus produces high levels of hydrolytic enzymes which degrade the crystalline cellulose into glucose (Bisaria and Misra, 1989). During reduced secretion the growth is also disturbed due to a reduced amount of available carbon. Consistent with this, no differences in growth between the parental strain and *rho3* disruptants could be detected in cultures carried out in a glucose-containing medium. In the presence of a moderate cellulase inducer, cellobiose (Ilmén *et al.*, 1997; Margolles-Clark *et al.*, 1997), no effects on growth or secretion could be detected either. In cellobiose-containing medium, massive protein secretion is probably not necessary for growth, since β-glucosidase is the sole enzyme required for the cleavage of cellobiose into glucose. Furthermore, cellobiose can be taken up as such into the cell (Kubicek *et al.*, 1993) and cleaved by an intracellular β-glucosidase (Inglin *et al.*, 1980). In conclusion, the growth of *T. reesei* in glucose or cellobiose-containing media does not demand high levels of extracellular protein secretion while in the presence of cellulose, cellulase secretion is essential for growth.

The effect of *rho3* disruption was detected in the total extracellular protein levels, in activities of the two major cellulase components, CBHI and EGI, and in extracellular protease levels (II, Figure 7). Since protease activity is regulated in a different way than the cellulases (Haab *et al.*, 1990) the *rho3* disruption appears to affect all secreted proteins and not only those regulated by the carbon source. The secretion is not, however, entirely blocked by *rho3* disruption but rather retarded. Therefore RHOIII appeared not to be essential for secretion in *T. reesei*.

In the dimorphic fungus *Ashbya gossypii* the disruption of *rho3* gene resulted in morphological abnormalities and defects in actin organisation and in growth (Wendland and Philippsen, 2001), in contrast to our results in *T. reesei* (II). Similarly, disruption of *RHO3* in *S. cerevisiae* causes a growth delay (Matsui and Toh-E, 1992a) and delocalisation of actin patches together with disappearance of actin cables (Kagami *et al.*, 1997). We found that the yeast

cells lacking Rho3p activity rather exhibit a severe chitin delocalisation defect which indicates vesicle delivery problems and only slight disturbance of the actin cytoskeleton. These defects were both repaired by *T. reesei rho3* expression (I). However, *RHO3* effector loop mutations have been shown to affect both secretion and actin organisation in yeast (Adamo *et al*., 1999). Therefore, *T. reesei rho3* might have redundant functions with another rho-type gene in a similar way as the yeast *RHO3* that is partially redundant with *RHO4* (Matsui and Toh-E, 1992a). The potential gene redundant with *rho3* in *T. reesei* could be predominantly responsible for the interactions with actin as *rho3* disruption did not affect actin organisation in *T. reesei*.

4.3 The maintenance of polarity in fungal cells

In both unicellular and multicellular organisms, cell polarity is of central importance for various cellular processes such as morphogenesis, differentiation, reproduction and motility. Polarised cell growth in fungi is a complex series of hierarchical events: initial response to endo/exogenous signals, establishment of the axis of polarity, asymmetric organisation of cytoskeleton and organelles, vectorial membrane transport, and local membrane growth and cell wall remodelling. Virtually all of these aspects derive from the polarity of the actin cytoskeleton (Pruyne and Bretscher, 2000). The major difference in the asymmetric growth between *S. cerevisiae* and filamentous fungi is that the axis of polarity in yeast is reoriented after each cell cycle but the axis of polarity in filamentous fungi remains fixed when once established in the beginning of germ tube growth. A new axis of polarity is created in filamentous fungi only when a branch point of the mycelium is determined (Torralba and Heath, 2001).

Filamentous actin is involved in both polarity establishment and maintenance in filamentous fungi (see Torralba and Heath, 2001, and references therein). It participates in the transport of exocytic vesicles that deliver plasma membrane and cell wall material to the hyphal tips (Heath, 1990), controls the localisation of organelles, such as Spitzenkörper, in the cell, and regulates plasma membrane protein positioning, such as that of t-SNAREs, in the tips (see Torralba and Heath, 2001, and references therein). In contrast, the polarised localisation of Sec3p, spatial landmark of polarised exocytosis in yeast, is not dependent on filamentous actin (Finger *et al*., 1998). Nevertheless, it is dependent on small

GTPase Rho1p (Guo *et al.*, 2001) which also regulates actin organisation (Kohno *et al.*, 1996; Helliwell *et al.*, 1998). Similarly, Adamo *et al.* (1999) found that the effects of Rho3p on the actin cytoskeleton and exocytosis are separable. These Rho-regulated pathways could, however, be parallel in polarisation of exocytosis, like it has been found in mast cells (Norman *et al.*, 1996). Thus, the numerous genetic/physical interactions between the secretory pathway and (regulators of) the actin cytoskeleton, detected in this work and other studies, are not unexpected.

It also appears reasonable to assume that such a large complex as the exocyst would interact with numerous proteins involved in polarisation. The interactions between Sec3p or Exo70p with Rho-type GTPases in yeast were described by several groups (Adamo *et al.*, 1999; Robinson *et al.*, 1999; Guo *et al.*, 2001; Zhang *et al.*, 2001). Moreover, expression of activated mutant form of the Rab8 GTPase caused formation of protrusions in BHK cells as a result of reorganisation of actin filaments and microtubules (Peränen *et al.*, 1996). Similarly, overexpression of the relatively hydrophobic carboxy-terminus of Sec10p in yeast was not found to affect exocytosis but resulted in elongated cell phenotype indicating disturbance of reorientation of secretion during the cell cycle (Roth *et al.*, 1998). Overexpression of human Sec10 in MDCK cells, in turn, increased both delivery and synthesis of basolateral secretory proteins. Consequently, Lipschutz *et al.* (2000) suggested that part of the exocyst may translocate to the nucleus and influence protein synthesis. Quite recently, *exo84-2* mutation was shown to decrease both splicing and total level of actin mRNAs in yeast (Awasthi *et al.*, 2001). These effects may imply that exocyst complex components are, besides responsive for polarity cues mediated by signalling proteins, also able to regulate the directionality of growth through actin organisation or transcriptional control. Therefore, the knowledge of proteins interacting with exocyst components may reveal attractive signalling pathways.

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Title

Functional analysis of the RHOIII and 14-3-3 proteins of *Trichoderma reesei*

Abstract

The filamentous fungus *Trichoderma reesei* is a biotechnologically important organism having an excellent capability to secrete hydrolytic enzymes e.g. cellulases. Allthough the protein production technology is well established, the protein secretion apparatus of *T. reesei* is still insufficiently known.

To isolate and characterise genes involved in secretion in *T. reesei*, a complementation screening in a *sec15-1 Saccharomyces cerevisiae* strain with a *T. reesei* expression cDNA library was performed. Sec15p is a component of the exocyst complex that is essential for the orientation of the secretion pathway and thus for polarised growth and secretion. Two signal transduction genes encoding the RHOIII and a truncated 14-3-3 protein (FTT1ΔC) were obtained as suppressors of the yeast *sec15-1* mutation. Two full-length FTT genes (FTTI and FTTII) were also cloned from *T. reesei* but they did not suppress this mutation.

Several other genetic interactions between late-acting secretory genes of *S. cerevisiae* and *T. reesei rho3* or *ftt1ΔC* were also detected and this further implies the involvement of these genes in regulation of protein secretion. The expression of *rho3* gene was shown to rescue the growth of mutations in genes encoding other exocyst components and Sec1p that is a regulator of secretory vesicle docking to the plasma membrane. *Ftt1ΔC* appeared to interact with genes involved in vesicle targeting along actin cables in yeast.

These *T. reesei* genes were also able to suppress the secretion defects of *sec15-1* or *sec2-41* strains of *S. cerevisiae*. The full-length *ftt1* enhanced the secretion of invertase in a wild type yeast although it could not suppress the growth of the yeast secretion mutations. The *ftt2* did not have any clear effect on secretion in yeast. Moreover, expression of *ftt1* slightly retarded the growth of wild type yeast cells while expression of *ftt2* enhanced it.

The *rho3* gene was disrupted in the *T. reesei* genome. The disruptants were able to grow as well as the parental strain in media with either glucose or cellobiose as the carbon source. However, in a medium with cellulose as the carbon source both growth and secretion were reduced in the disruptants. The morphology or actin organisation was not affected by the disruption. Therefore, the *rho3* gene would appear to be more involved in the regulation of secretion than morphogenesis in *T. reesei*.

Keywords

Trichoderma reesei, *Hypocrea jecorina*, GTP-binding proteins, *rho3*, *ftt1*, *ftt2*, protein secretion, exocytosis, exocyst complexes, gene expression

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