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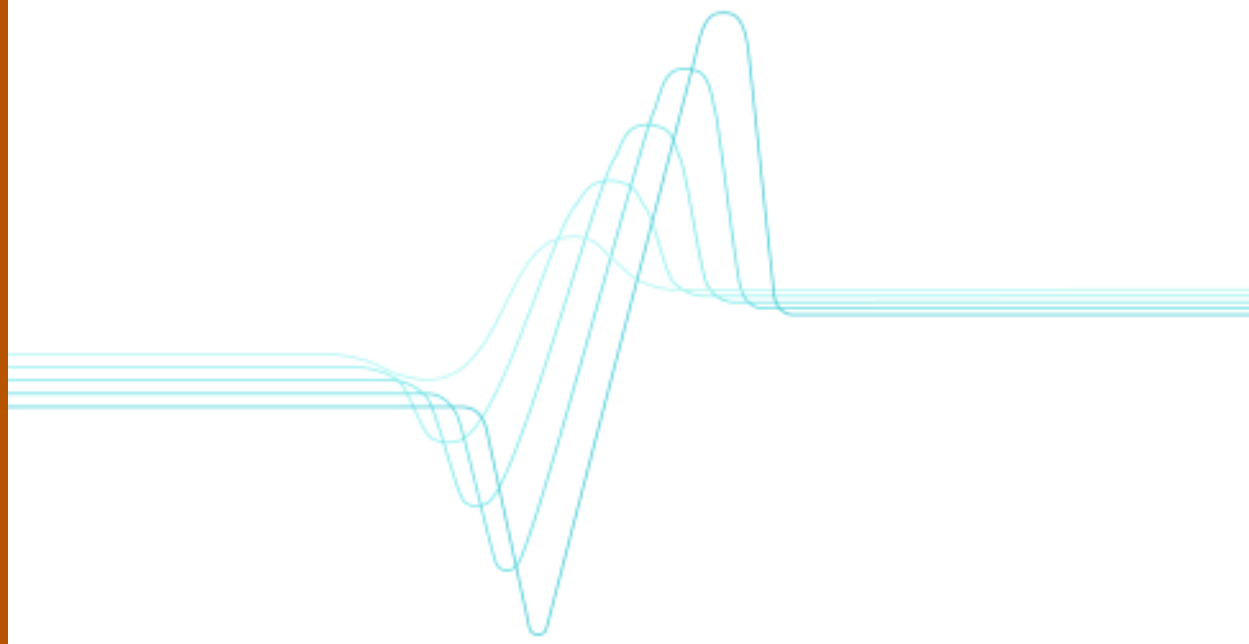
The effect of unfolded protein response on protein production



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The effect of unfolded protein response on protein production

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Abstract

This study describes the cloning and characterisation of three genes from the filamentous fungus *Trichoderma reesei*. *Snc1* encoding a putative v-SNARE was shown to be the functional homologue of *Saccharomyces cerevisiae SNC1* and *SNC2* genes that encode proteins shown to function in exo- and endocytosis. Two components of the unfolded protein response (UPR) pathway have also been identified. *Ire1* encodes a serine /threonine kinase that has been shown to be involved the sensing of the protein folding status in the ER and transferring the signal to the nucleus. *Ptc2* on the other hand, encodes a putative negative regulator of UPR. Both genes were isolated from *T. reesei* and shown to be the functional homologues of *S. cerevisiae* genes.

The effects of UPR activation on gene expression and protein production in *S. cerevisiae* and two filamentous fungi, *T. reesei* and *Aspergillus niger* var. *awamori* are also presented. The data shows that the UPR induction, either by *HacA* or *Ire1* overexpression in *A. niger* var *awamori* and *T. reesei*, respectively, induces the expression of an ER-resident foldase and an ER-resident chaperone. Moreover, the UPR induction induces the expression of genes encoding functions at different steps of the secretory pathway in both fungi. This is in correlation with results obtained in other organisms.

The UPR induction was in this study shown to induce production of secreted proteins both in *S. cerevisiae* and *A. niger* var. *awamori*. In yeast the UPR induction by constitutive over-expression of activated yeast *HAC1* and *T. reesei hac1* resulted in induction of both native and foreign protein production. On the other hand, deletion of *HAC1* resulted in decrease in protein production. In *A. niger* var. *awamori* only the production of foreign proteins was induced in *HacA* over-expressing transformants. The production of native proteins was lower in these transformants compared to the controls. The over-expression of *Ire1* in *T. reesei* had no effect on foreign protein production.

Preface

The study has been done at VTT Biotechnology. The work was supported by the Finnish Technology Agency (Tekes) and Genencor International, Inc. that are acknowledged. Executive Director, Professor Juha Ahvenainen and Research Director, Professor Hans Söderlund are thanked for creating excellent working facilities. Research Manager Tiina Nakari-Setälä is thanked for her supportive attitude.

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I feel especially lucky to have such a wonderful family. My parents, Marja and Pauli, and my darling brother, Mika, who have in part made me who I am today. Their unquestioned love and trust as well as support keeps amazing me. I feel that a big part of this achievement belongs to them. My nephews, Mikko and Tommi, as well as their mother Päivi, charming personalities, I wish to thank for all the good laughs and interesting time we have had together.

List of publications

I Valkonen, M., Pakula, T., Penttilä, M. and Saloheimo, M. 2003. Characterization of the secretory gene *snc1* from the filamentous fungus *Trichoderma reesei* encoding a v-SNARE protein involved in exocytosis: regulation at the transcriptional level. Manuscript considered for publication in Biochimica et Biophysica Acta.

II Valkonen, M., Penttilä, M. and Saloheimo, M. 2003. Characterisation of the unfolded protein response pathway from the filamentous fungus *Trichoderma reesei*. Manuscript submitted to Molecular and General Genetics.

III Valkonen, M., Penttilä, M. and Saloheimo, M. 2003. Effects of Inactivation and Constitutive Expression of the Unfolded Protein Response Pathway on Protein Production in the Yeast *Saccharomyces cerevisiae*. Applied and Environmental Microbiology. 69, pp. 2065–71.

IV Valkonen, M., Ward, M., Wang, H., Penttilä, M. and Saloheimo, M. 2003. Improvement of foreign protein production in *Aspergillus niger* var. *awamori* by constitutive induction of the unfolded protein response. To be published in Applied and Environmental Microbiology. Vol. 69, issue 12.

Contents

Appendices of this publication are not included in the PDF version. Please order the printed version to get the complete publication (http://www.vtt.fi/inf/pdf/)

List of symbols

bp	base pairs
BFA	brefeldin A
CHO	Chinese hamster ovary
DTT	dithiotreitol
ER	endoplasmic reticulum
ERAD	ER associated degradation
kDa	kilodalton
MUL	methylumbelliferyl-β-D-lactoside
NSF	N-ethylmaleimide sensitive fusion protein
ORF	open reading frame
PDI	protein disulphide isomerase
SEC	gene involved in secretion
SNAP25	synaptosome associated protein of 25 kDa
SNARE	soluble NSF attachment protein receptor
Snc	suppressor of the null allele of CAP
SRP	signal recoqnition particle
Sso	suppressor of *sec1*

VAMP	vesicle associated membrane protein
TGN	*trans*-Golgi network
UPR	unfolded protein response

1. Introduction

Recycling of organic material is an important biological process. The main components of plant biomass are cellulose, hemicellulose and lignin that are efficiently degraded by wood-rotting fungi. These fungi produce secreted hydrolytic enzymes, which degrade complex plant polymers to produce low molecular weight compounds. This is one reason why protein secretion in fungi is an attractive field of study. Because of the ability to grow on various inexpensive substrates as well as the ability to secrete large amounts of proteins, filamentous fungi have been considered as good production hosts for proteins of biotechnical interest. Considerable amount of research has been carried out in order to understand the protein synthesis and secretion processes in filamentous fungi and to understand the special problems connected to heterologous protein production. Although the information about the fungal secretion pathway has increased remarkably over the last years (reviewed by Conesa *et al*., 2001), there still is rather limited knowledge of the factors affecting secreted protein production.

Saccharomyces cerevisiae on the other hand has been widely applied in biotechnological processes such as baking. However, since yeast does not naturally secrete high levels of protein, there are quite few examples of its use in foreign protein production. *S. cerevisiae* has been used as the model organism in many biological studies including the elucidation of molecular mechanisms of protein secretion. One reason for the model organism status of *S. cerevisiae* is the availability of extensive genetic tools and methodologies.

1.1 Overview of the secretory pathway

The eukaryotic cell is composed of membrane-enclosed subcellular compartments. Proteins and other material are transported between these compartments to support vital processes like nutrient uptake and growth. The transport is needed for example for expansion of plasma membrane, endocytosis and secretion of proteins and other molecules in the exocytotic pathway.

S. cerevisiae and neuronal cell systems have been the major models for studies of the intracellular protein transport over the years. The secretory pathway of *S. cerevisiae* was originally identified genetically using temperature sensitive secretion mutants (Novick *et al*., 1981; Novick *et al*., 1995), whereas the vertebrate synaptic complexes involved in neurotransmitter release was originally characterised biochemically (Link *et al*., 1992; McMahon *et al*., 1993; Söllner *et al*., 1993).

Proteins directed to the secretory pathway, are first translocated into the lumen of the endoplasmic reticulum (ER). In the ER, the proteins fold into their final conformations and acquire the core glycosylation. After ER, the proteins are transported through the secretory pathway by a series of vesicle buddings and fusions, where vesicles bud from the donor membrane and fuse with the target membrane in a highly organised manner. From ER, the proteins are transported to the Golgi complex, where post-translational modifications of the proteins are completed. The sorting of proteins occurs in the *trans*-Golgi network (TGN), from where the proteins are targeted to endosomal/vacuolar route or secretion to the plasma membrane. To maintain the integrity of the different compartments, the proteins that are not destined for secretion undergo retrograde transport to the donor organelle.

1.1.1 Transport through the ER

Proteins are synthesised in the cytoplasm and need to be translocated across the ER membrane for routing to the secretory pathway. Targeting to the ER membrane is mediated by a signal peptide. There are two types of translocation processes in eukaryotes, cotranslational and posttranslational, the former being more common in mammalian cells, whereas both have been found to coexist in *S. cerevisiae* (reviewed in Rapoport *et al*., 1996).

In cotranslational translocation, the N-terminal signal peptide emerging from the ribosome, is recognised by a cytosolic factor, the signal recognition particle (SRP). The SRP-ribosome-complex is then targeted to the ER membrane where it is recognised by the SRP receptor. During this process, translation is arrested. After docking, the SRP dissociates from the complex and the nascent polypeptide is bound to the Sec61 complex (Rapoport *et al*., 1996). Sec61 forms

a core complex, which functions in the recognition of the signal sequence and also in the assembly of the machinery needed for the translocation channel formation (Hartmann *et al*., 1994). After the signal peptide is bound to the Sec61 complex, the chain elongation continues and the polypeptide is transferred through the translocation channel into the ER lumen. The Bip/Kar2 chaperone is needed for this translocation process. It binds to the elongating polypeptide chain thus preventing the polypeptides from misfolding or sliding back through the channel. It has been postulated that the Bip/Kar2 has an active role in the translocation by exerting a pulling force to the polypeptide across the ER membrane (Glick, 1995).

In posttranslational translocation, the translocation of nascent polypeptides across the ER membrane occurs after the polypeptide has been fully translated. In this process the SRP and its receptor are not needed and the recognition process occurs on the outer ER membrane. The signal sequence targets the polypeptide to the membrane, where it is recognised by Sec62-Sec63 protein complex together with the Sec61 complex (Ng *et al*., 1996). The Bip/Kar2 protein is needed in translocation of the polypeptide across the ER membrane. In mammalian cells the posttranslational translocation is inefficient and only quite recently the equivalents of the *S. cerevisiae* Sec62 and Sec63 proteins have been identified from mammalian cells (Meyer *et al*., 2000).

Polypeptides are modified in the ER lumen. First the signal peptidase complex removes the signal peptide (Böhni *et al*., 1988; Evans *et al*., 1986) and the oligosaccharyl transferase complex attaches an N-linked core oligosaccharide unit to asparagine residues in correct positions of the polypeptide chain (Gavel and von Heijne, 1990). The machinery needed for these processes has been localised to the ER membrane (Nilsson and von Heijne, 1993; Shelness *et al*., 1993). After the carbohydrate side chains have been added to the nascent polypeptide, they are modified by glucosidase I, glucosidase II and mannosidase in the ER. In *S. cerevisiae*, also the initiation of O-glycosylation occurs in the ER (Tanner and Lehle, 1987).

An important function of the ER is to facilitate correct folding of proteins and assembly of polypeptides into oligomers. These functions are assisted by a set of ER-resident chaperones and foldases. The ER lumen is an oxidising environment that enables the formation of disulphide bridges. Also the Ca^{+}-concentration is

high, which is important for all processes in ER: translocation, folding and glycosylation (reviewed by Meldolesi and Pozzan, 1998). Chaperones bind transiently to the polypeptides and assist in the folding and assembly of the partially folded or unassembled proteins by preventing premature misfolding and aggregation. Bip/GRP78 and GRP94 are the most abundant chaperones in the ER of mammalian cells. Protein disulphide isomerase (PDI) is one of the most abundant and important foldases in the ER. PDI and other proteins belonging to the PDI protein family, catalyse the formation of disulphide bonds on polypeptides and facilitate the correct folding of proteins (reviewed by Bardwell and Beckwith, 1993; Freedman *et al*., 1994).

Another important function of the ER is to prevent the misfolded proteins from entering the secretory pathway. In this so called ER quality control, the chaperones Bip, calnexin and calreticulin have a central role in retaining the misfolded proteins or unassembled subunits in the ER (reviewed in Hammond and Helenius, 1995). Misfolded proteins are degraded by an ER-associated degradation (ERAD) pathway (McCracken and Brodsky, 1996). The degradation takes place in the cytosol by the action of the 26S proteasome. Proteins destined to this pathway are transported out of the ER via the Sec61 translocon in an active process that consumes ATP (Pilon *et al*., 1997; Wiertz *et al*., 1996; Zhou and Schekman, 1999)

1.1.2 Transport from ER to the Golgi complex

Transport of proteins between ER and Golgi complex is mediated by recruitment of coat proteins on sites of transport vesicle formation. There are two types, COPI- and COPII-coated vesicles involved in the transport. COPII vesicles are involved in the anterograde transport from ER to Golgi. COPI vesicles function in retrograde transport as well as anterograde transport between Golgi compartments (Orci *et al*., 1997; Scales *et al*., 1997).

Formation of COPII coat starts with the activation of a small GTPase, Sar1p, at the ER membrane. Sar1p is active in GTP-bound form and the GDP-to-GTP exchange is catalysed by an ER-resident protein Sec12p (Barlowe and Schekman, 1993). Sar1p in GTP-bound form then recruits a heterodimeric complex Sec23-Sec24 to the budding vesicle. Another heterodimeric complex,

Sec13-Sec31 is bound to the Sec23-Sec24 complex (Barlowe *et al*., 1994) and this finally leads to budding of COPII-coated vesicles (Schekman and Orci, 1996). The fusion of the COPII vesicles to the target membranes requires the disassembly of the coat. This is mediated by GTP hydrolysis on Sar1, which is catalysed by Sec23 (Yoshihisa *et al*., 1993).

The problem of cargo selection has been studied intensively. The basic problem is how the ER-resident proteins are distinguished from the proteins entering the secretory pathway. It has been observed that some proteins are concentrated at specific sites before they leave the ER (Antonny and Schekman, 2001).The export of integral membrane proteins is mediated by specific sequences contained in their cytoplamic tails . Two types of ER export motifs have been isolated so far. One type contains hydrophobic and aromatic C-terminal amino acids (Iodice *et al*., 2001; Nufer *et al*., 2002). The other type contains an internal di-acidic Glu-X-Asp sequence (Nishimura and Balch, 1997). Both types can bind Sec23-Sec24 complex of the COPII coat (Belden and Barlowe, 2001b; Votsmeier and Gallwitz, 2001) thus enabling the packaging of the proteins into the transport vesicles. Some membrane proteins do not contain adequate sorting signals and their transport from ER is mediated by adapter proteins. One example of such adapter is Erv14p that is required for the localisation of a transmembrane secretory protein, Axl2p, into COPII coated vesicles. The Erv12 has been shown to bind COPII coat complex and Axl2p (Powers and Barlowe, 2002). The export of soluble cargo proteins from ER is less clear. There is evidence of both receptor mediated (Appenzeller *et al*., 1999) and bulk-flow (Wieland *et al*., 1987) export.

As mentioned above, the COPI coat functions in the retrograde transport between Golgi and ER in retrieving mislocated ER-resident proteins. Many ER proteins have been shown to contain ER-retention and -retrieval signals (Harter and Wieland, 1996). The K/HDEL is found at the C-terminus of many lumenal ER proteins. This motif binds to the KDEL receptor that functions in retrieval of the protein from the Golgi by the COPI-vesicles (Pelham, 2000). Retrieval of Type I membrane proteins on the other hand, is mediated by a KKXX signal. This signal has been shown to directly interact with the COPI coat (Jackson *et al*., 1990; Letourneur *et al*., 1994). Moreover, cysteine residues can function as ER-retention signals (Fra *et al*., 1993). The formation of COPI and COPII vesicles is mediated by similar mechanisms. The first step in the COPI coat

assembly is the activation of ARF1, a small GTP-binding protein (Donaldson *et al*., 1992). COPI coat is composed of a cytosolic protein complex called the coatomer. Unlike in the COPII coat assembly, the coatomer is assembled in the cytosol before recruitment to the vesicle (Hara-Kuge *et al*., 1994).

1.1.3 Transport from Golgi complex to plasma membrane

Secreted proteins are transported to the Golgi complex, which in higher eukaryotic cells is a series of flattened cisternae. Proteins are delivered to the *cis* face of Golgi apparatus and transferred to medial and *trans*-Golgi by non-clathrin coated vesicles. In the Golgi complex, proteins undergo a sequential series of modifications for example O-linked glycosylation, trimming of the N-linked glycans and phosphorylation. Enzymes performing these functions have been localised into specific Golgi cisternae.

In the *trans*-Golgi network (TGN), which is in close connection with the Golgi complex, proteins are sorted to their destinations. Exocytosis is the last stage of secretion leading to the release of cargo proteins from the transport vesicles to the cell surface. It also mediates addition of membrane lipids and proteins to the plasma membrane. In regulated exocytosis, transport vesicles are transported and docked to the plasma membrane and the fusion of the membranes is triggered by a stimulus. An increase in the concentration of cytosolic free calcium can function as a stimulus for the membrane fusion. In constitutive secretion, the vesicles fuse with the plasma membrane immediately after vesicle docking without an external stimulus. Studies on different cell types have shown that both constitutive and regulated pathways require cytosolic proteins and ATP for optimal function. Vesicles for both types of secretion are formed at the TGN, but have different coats on their cytoplasmic surfaces (reviewed by Lledo, 1997).

Although *S. cerevisiae* does not appear to have regulated exocytosis, secretion of proteins is regulated at the level of packaging into secretory vesicles. There are three types of secretory vesicles forming at the TGN. One vesicle type carries soluble and membrane proteins to the vacuolar system (Conibear and Stevens, 1998), while the other two are routed to the plasma membrane and have different sets of cargo proteins (Harsay and Bretscher, 1995). The secretory vesicles

derived from the TGN are transported along actin cables to the sites of membrane fusion at the plasma membrane (Finger and Novick, 1998).

1.1.3.1 SNAREs and membrane fusion

The molecular mechanisms of membrane fusion have been studied intensively. There are three major classes of proteins that are needed for the fusion of membranes, SNARE, Sec1 and Rab proteins. The main components of the machinery are conserved between the different fusion steps i.e. ER-Golgi, Golgi-plasma membrane and Golgi-vacuole transport (Aalto *et al*., 1992; Rothman, 1994; Rothman, 1996).

Soluble NSF attachment protein receptor (SNARE) proteins were originally independently isolated from *S. cerevisiae* and neuronal cells, and have been found to be well conserved between the two systems (Bennett and Scheller, 1993; Ferro-Novick and Jahn, 1994). SNAREs have since been found to be involved in most membrane fusion events in the cell (Rothman and Warren, 1994). SNAREs were originally divided into two classes according to their localisation to vesicles (v-SNARE) or target membranes (t-SNARE) (Söllner *et al*., 1993). There are two very homologous v-SNAREs in *S. cerevisiae* that function in the late secretory pathway, Snc1 and Snc2 (Gerst *et al*., 1992; Protopopov *et al*., 1993). Genetic studies have shown that these proteins are needed for membrane fusion. Deletion of both leads to the accumulation of post-Golgi vesicles and deficiencies in secretion (Protopopov *et al*., 1993). Later, the SNAREs have been classified as R-SNAREs and Q-SNAREs according to a highly conserved amino acid (arginine or glutamine, respectively) located in their ionic layer (Fasshauer *et al*., 1998). On the basis of sequence homology and domain structure, the mammalian SNAREs have further been classified into syntaxin, synaptobrevin/VAMP and SNAP-25 families (Jahn and Südhof, 1999).

A common structural feature found in SNARE proteins are the heptad repeats that are typical in α-helices that form coiled coil structures (Calakos *et al*., 1994; Chapman *et al*., 1994). The Snc proteins have been shown to interact through two α-helical regions with the t-SNAREs, Sso and Sec9 proteins (Gerst, 1997). The crystal structure of the neuronal SNARE complex has been resolved and it has been shown that the core structure is composed of a four-helix bundle

(Sutton *et al*., 1998), where synaptobrevin/VAMP and syntaxin both form one α-helix and SNAP-25 two helices (Poirier *et al*., 1998). The hydrophobic layer between the helices is interrupted by a central hydrophilic layer of one arginine and three glutamine residues (Sutton *et al*., 1998). Recent data indicates that there may be some flexibility in the SNARE complex formation. The *S. cerevisiae* exocytotic SNARE complexes can be formed and be functional even in the presence of four glutamines in the ionic layer (Katz and Brennwald, 2000). It has been postulated that proteins that regulate the SNARE complex formation bind to the grooves on the surface of the complex thus changing the affinities of the SNAREs. The core complex is extremely stable after formation and resistant to treatments like SDS denaturation, neurotoxin treatment and heating.

The specificity of SNARE complex formation was, according to the original SNARE hypothesis, achieved by binding of the SNARE proteins on the secretory vesicles (v-SNAREs) only to the specific SNAREs on the target membrane (t-SNAREs) (Söllner *et al*., 1993). This hypothesis has since been questioned as it has been found that the complex formation between different SNAREs can be rather promiscuous. For synaptic vesicle exocytosis, a v-SNARE VAMP2 binds to the t-SNAREs SNAP25 and syntaxin-1 (Hayashi *et al*., 1994; Söllner *et al*., 1993). It has been shown that VAMP2 also functions in mast cells and forms stable complexes with non-neuronal isoform of SNAP25, SNAP-23 (Foster *et al*., 1998). This indicates that VAMP2 may function in the formation of different SNARE complexes. Fasshauer *et al*. (1999) have shown that SNARE complexes can be formed between distal or closely related neuronal SNAREs with very similar biophysical properties. Yang *et al*., (1999) studied the SNARE complex formation between different members of the VAMP, SNAP-25 and syntaxin protein families localised to different cellular compartments. It was concluded that all combinations tested were stable. The *S. cerevisiae* Snc proteins can form complexes with syntaxin-like t-SNAREs Sed5 (*cis*-Golgi), Vam3 (vacuoles), Pep12 (endosomes) (Grote and Novick, 1999), Tlg1 and Tlg2 (*trans*-Golgi and endosomes) *in vitro* (Abeliovich *et al*., 1998; Holthuis *et al*., 1998), in addition to exocytic SNARE complexes formed with the t-SNAREs Sso1, Sso2 and Sec9 (Brennwald *et al*., 1994; Couve and Gerst, 1994; Rossi *et al*., 1997). It has also been observed in *S. cerevisiae* that Sed5p and Vti1p, can interact with different SNARE proteins (Fischer von Mollard and Stevens, 1999; Tsui and Banfield, 2000). Moreover, human hVti1 protein can

replace the yeast Vti1p in vesicle transport from Golgi to prevacuolar compartment and from ER to cis-Golgi (Fischer von Mollard and Stevens, 1998).

On the other hand, most SNAREs have been localised to specific membrane compartments (Advani *et al*., 1998). In a study done with the PC12 cell system, it was shown that cognate SNAREs could function more successfully than non-cognate SNAREs in a specific cellular fusion step (Scales *et al*., 2000). Recent studies on liposomes have shown pairing specificity, although some unspecificity was also observed since any R-SNARE used in the study could complex with plasma membrane Q-SNAREs (McNew *et al*., 2000). All this data would indicate that although the SNARE proteins do not generate the specificity of membrane fusion, they still may in part function in creating it. One aspect in the SNARE specificity is the fact that in *S. cerevisiae*, the t-SNAREs can be found all over the plasma membrane and yet the fusion of secretory vesicles occurs only at specific sites (Brennwald *et al*., 1994).

The specificity of the SNARE complex formation is important, since all components are transported through the secretory pathway to their correct locations as well as recycled back to cognate membranes for reuse. This requires some specificity in the formation of the SNARE complex. Tlg1 and Tlg2 are t-SNAREs that have been localised to the late-Golgi/endosomes (Holthuis *et al*., 1998). They have been shown *in vivo* to be involved in the recycling of Snc proteins from the plasma membrane to the Golgi (Gurunathan *et al*., 2002; Lewis *et al*., 2000). An interesting finding is that the endocytotic uptake of proteins form the cell surface is deficient in cells lacking the *SNC* genes. *S. cerevisiae* cells lacking functional Sncp were found to be unable to deliver a soluble dye FM4-64 to the vacuole. Also the endocytosis of an α-factor receptor, Ste2p, was blocked in these cells (Gurunathan *et al*., 2002). Thus, the Snc proteins function both in antero and retrograde protein transport between Golgi and plasma membrane.

1.1.3.2 SNARE complex assembly /disassembly

After the membrane fusion there is the problem of retrieval of the v-SNARE back to the donor compartment. Grote *et al*. (2000) have shown that after the

membrane fusion has occurred, the v-SNARE is located on the target membrane where it remains bound to the t-SNARE in a *cis*-SNARE complex. The complex needs to be disassembled and the v-SNARE targeted to an endosomal vesicle budding off from the plasma membrane. A sorting signal is required to concentrate proteins into the vesicles. The synaptic vesicle v-SNARE, VAMP2, has been shown to contain a sorting signal that targets the protein to the endosomal pathway and back to the synaptic vesicles (Grote *et al*., 1995). Mutation of a methionine-based sorting signal of the Snc proteins reduced endocytosis of both Snc proteins (Lewis *et al*., 2000).

Since the SNARE complexes are highly stable, the disassembly of these complexes requires ATP hydrolysis. The chaperone that functions in this step is the N-ethyl maleimide sensitive factor (NSF). For binding to the SNAREs, NSF needs another protein called soluble NSF attachment protein (SNAP) (Söllner *et al*., 1993). The homologues of these proteins (Sec18p and Sec17p, respectively) have been found in *S. cerevisiae*. These proteins are known to function on all SNARE complexes (Rothman, 1994) and in *S. cerevisiae* it has been shown that loss of function in *sec18-1* mutant affects secretion in multiple steps (Graham and Emr, 1991).

There are many theories regarding the function of Sec18/NSF and Sec17/SNAP. It was originally stated that disassembly of SNARE complexes triggers the fusion of membranes (Rothman and Warren, 1994). More recently, Grote *et al*. (2000) have shown in *S. cerevisiae sec18-1* mutant strain that once the *trans*-SNARE complexes have formed, the dissociation is not needed for membrane fusion. It has also been postulated that the Sec18/NSF functions in the SNARE priming step. This priming would then lead to assembly of *trans*-SNARE complexes between different membranes (Ungermann *et al*., 1998). There are differences in the binding of SNAP and NSF to the SNARE complex. It has been shown that SNAP and NSF preferentially bind to assembled SNARE complexes (McMahon and Südhof, 1995), and that they also can bind to free t-SNAREs (Hanson *et al*., 1995). This would indicate that the function of SNARE complex disassembly would be to keep the SNAREs ready for new trans-SNARE complex formation (Grote *et al*., 2000).

Proteins that affect the conformation of SNARE proteins must be able to bind them. Sec1-type proteins are hydrophilic proteins that have been shown to bind

t-SNAREs (Aalto *et al*., 1993; Pevsner *et al*., 1994a). There are contradictory results on the function of the Sec1 protein family. In mammals it has been shown that the Sec1 homologue binds to syntaxin, but not to a SNARE complex (Yang *et al*., 2000), which would indicate that Sec1 has an important role in the SNARE complex formation in preventing the formation of non-productive or premature SNARE complexes. It has also been shown that neuronal Sec1 can inhibit the binding of syntaxin to VAMP and SNAP-25 (Pevsner *et al*., 1994b). In *S. cerevisiae*, *sec1* is an essential gene and the *sec1-1* mutant has been shown to accumulate post-Golgi vesicles (Novick and Schekman, 1979). Sec1p binds to SNARE complexes and not to free Sso proteins (Carr *et al*., 1999), which would indicate a role after the SNARE complex formation perhaps in stabilising the complex. In conclusion, although Sec1 proteins seem to be essential for membrane fusion, the precise function of the protein family is not known.

It has been observed that inactivation of the SNARE complex does not seem to prevent the membrane fusion. When neurons have been treated with toxins that proteolyse the SNARE proteins at known sites and thus disassemble the SNARE complex, membrane fusion still occurs (Hunt *et al*., 1994). This indicates that SNARE complex formation is needed for vesicle docking, but not for membrane fusion. In another study it was concluded that SNARE proteins function downstream of vesicle docking since deletion of synaptic v-SNARE (synaptobrevin) or t-SNARE (syntaxin) in *Drosophila* does not prevent the vesicles from associating with the plasma membrane (Broadie *et al*., 1995). Another explanation for this could be that other SNARE proteins replace the deleted proteins in the formation of the SNARE complex. What is clear from these studies is that the SNARE proteins co-operate with various proteins and these interactions confer the specificity of membrane fusion. The protein complexes involved in the fusion of vesicles to the plasma membrane in yeast and mammalian cells are shown in Figure 1.

1.1.3.3 Membrane tethering; the function of Sec4p and the exocyst complex

Membrane tethering is a term used for the targeting of vesicles to the site of membrane fusion. Membrane tethering complexes have been found from the major transport steps, ER to Golgi, intra-Golgi, vacuole and endosome as well as exocytosis (see Waters, 1999 and references therein). Thus, it seems that

targeting of transport vesicles is regulated by specific protein-protein interactions, although the proteins involved in the different transport steps are not very well conserved. The need for Rab proteins is a common feature. Rab proteins are small GTP-binding proteins that cycle between a GTP-bound form, which is bound to membranes and GDP-bound, cytosolic form (Zerial and McBride, 2001). Rab proteins are known to function at different stages of vesicle transport: in vesicle formation, in vesicle movement along cytoskeletal elements and in the tethering process. The best known function is that large protein complexes that play a role in the tethering of vesicles are bound to the transport vesicles through the GTP-bound form of a Rab protein.

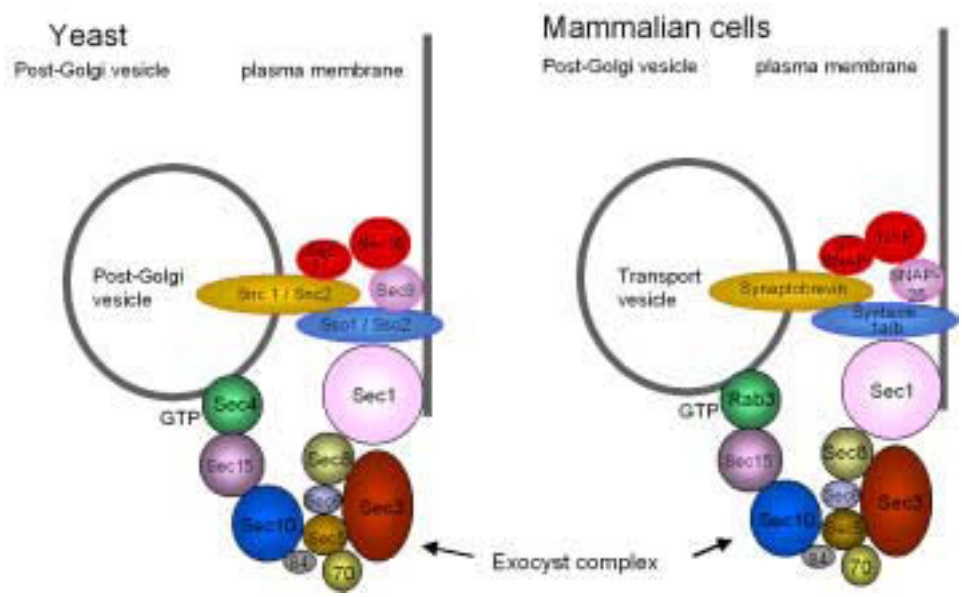

Figure 1. Schematic presentation of the docking of transport vesicles to the PM in yeast and mammalian cells. Modified from Toikkanen 1999, Jahn et al., 2003 and J. Jäntti, personal communication.

One well-studied tethering complex is the *S. cerevisiae* exocyst (TerBush *et al*., 1996) that has been found to regulate the targeting of secretory vesicles from the Golgi complex to plasma membrane in polarised secretion. Some of the components have also been isolated from mammalian cells (Hazuka *et al*., 1997; Hsu *et al*., 1996; Kee *et al*., 1997; TerBush *et al*., 1996) and the Sec6/8 complex has been found to localise to areas were exocytosis occurs (Grindstaff *et al*., 1998; Hazuka *et al*., 1999; Hsu *et al*., 1999). In *S. cerevisiae*, the exocyst is a large

protein complex containing eight proteins that have been shown to interact with each other. One of the components, Sec3p, has been found always to localise to the site of the polarised secretion, the bud tip and the bud neck (Finger and Novick, 1998). Moreover, the Sec15p has been shown to localise to the transport vesicles between the Golgi and plasma membrane. It has been postulated that Sec15p binds to a Rab protein, Sec4 that is localised to the vesicles (Guo *et al*., 1999). It has been shown that Sec4p functions upstream of the exocyst in yeast. The interaction of Sec4-GTP with Sec15p seems to cause interactions with other exocyst components in an event that eventually leads to the docking and fusion of the secretory vesicles to the plasma membrane. The mechanisms underlying this event are under investigation, but a number of other proteins like members of the Rho family of small GTPases, have been shown to interact with the exocyst. Different Rho proteins have been shown to interact with Sec3p and to affect the regulation of actin organisation, transport of vesicles to the plasma membrane and docking of the vesicles (Robinson *et al*., 1999).

1.2 The secretion pathway of filamentous fungi

Mycelial growth by apical extension of the hyphae that gives the fungal cells some motility, also requires a highly polarised transport of cell wall and membrane material to the growing tip. Protein secretion occurs mostly at the apical or subapical regions of the hyphae (Wösten *et al*., 1991). In a recent report on *Aspergillus niger*, protein secretion was studied using a glucoamylase::GFP fusion protein to localise the site of secretion. The GFP fluorescence was mainly observed at the hyphal tip (Gordon *et al*., 2000). In *T.reesei*, secretion of a foreign protein, barley cysteine endopeptidase, has been shown to occur at the apical and subapical regions of the hyphae (Nykänen *et al*., 1997). On the other hand, cellobiohydrolase I (CBHI), calf chymosin and CBHI-chymosin fusion proteins can be found from secretory vesicles all over the mycelium in *T. reesei*. This suggests that secretion can also take place in the older parts of the mycelium (Nykänen, 2002).

A structure unique to filamentous fungi, the Spizenkörper (SPK), has been found in the growing hyphal tips. This is an aggregate of vesicles that have been postulated to act as a center for vesicle supply and to direct their transport to the plasma membrane (Gierz and Bartnicki-Garcia, 2001). Another specific feature

of the fungal secretory pathway is that the Golgi complex does not exist in a form of polarised cisternal stacks, but seems to be more scattered and difficult to observe. Nevertheless, fungi are proposed to have membrane structures that are the functional equivalents of the Golgi complex (Howard, 1981; Rupes *et al*., 1995; Satiat-Jeunemaitre *et al*., 1996). Figure 2 shows a schematic presentation of the secretory pathway of *T. reesei*.

The molecular machinery for protein secretion in filamentous fungi is still largely unknown. However, a lot of research has been carried out over the recent years to resolve the basic mechanisms. Thompson *et al*. (1995) have cloned the *srpA* gene homologous to the signal recognition particle protein SRP54 of *S. cerevisiae*. This indicates that the co-translational translocation of the nascent polypeptides to the ER occurs as in other eukaryotes. Also, genes encoding proteins involved in core glycosylation of glycoproteins, *dpm1, mpg1* and *pmt1* have been cloned from *T. reesei* (Zakrzewska, *et al., 2003*; Kruszewska *et al*., 2000; Kruszewska *et al*., 1998). Another gene coding for an enzyme catalysing the first step in the assembly of dolichol-linked oligosaccharides, *gptA*, has been isolated from *A. niger* (Sörensen *et al*., 2003).

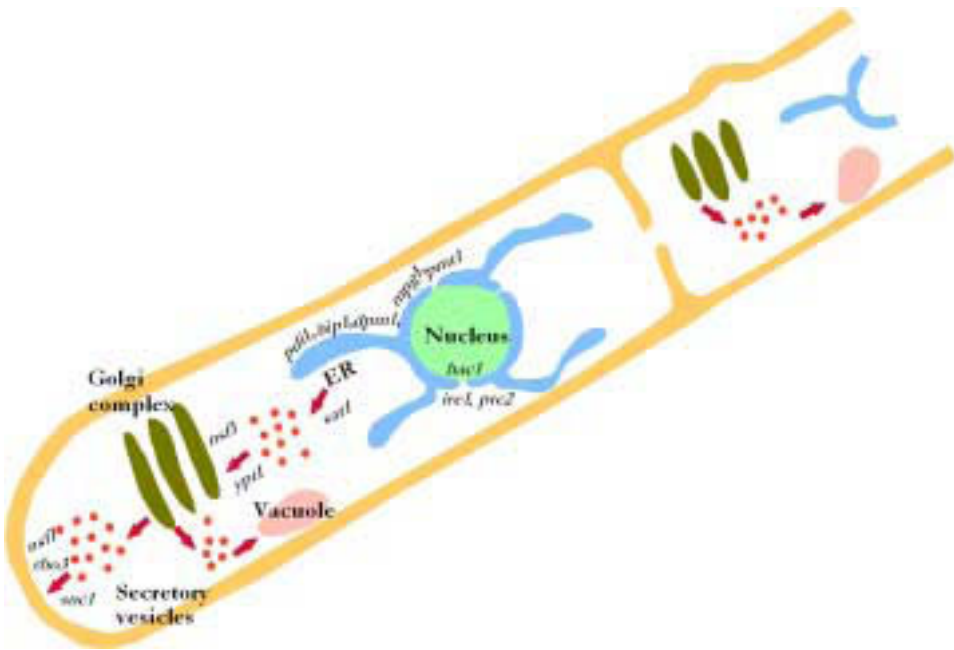

Figure 2. Proposed model of the secretory pathway of T. reesei. Genes cloned from different parts of the secretory pathway are indicated in the text.

Several groups have also isolated genes encoding ER chaperones and foldases. *Bip* genes have been isolated from *Neurospora crassa* (Techel *et al*., 1998), *Aspergillus awamori* (Hijarrubia *et al*., 1997), black *Aspergilli* (van Gemeren *et al*., 1997) and *T. reesei* (M. Saloheimo, unpublished). Protein disulphide isomerase (PDI) family genes have been cloned from *A. niger* (Ngiam *et al*., 1997), *A. oryzae* (Lee *et al*., 1996), *T. reesei* (Saloheimo *et al*., 1999) and *Humicola insolens* (Kajino *et al*., 1994). All these seem to have similar features to their *S. cerevisiae* and mammalian homologues, a signal peptide and a carboxy-terminal ER-retention signal K/HDEL. The *T. reesei pdi1* cDNA was able to complement *S. cerevisiae PDI1* disruption (Saloheimo *et al*., 1999) thus indicating that the PDI1 proteins in filamentous fungi perform similar activities as the *S. cerevisiae* homologue. Two PDI-related genes, *tigA* and *prpA* have been cloned from *A. niger* (Jeenes *et al*., 1997; Wang and Ward, 2000). Also, a gene has been isolated from *A. fumigatus* that shows PDI activity (Nigam *et al*., 2001).

Another group of chaperones functioning in the ER are the peptidyl-prolyl isomerases (PPIase). Proteins with PPIase activity include cyclophilins and FK506 binding proteins (FKBP). A NcFKBP22 gene has been identified from *N. crassa* (Solscheid and Tropschug, 2000) and cyclophilin homologue *cypB* from *A. nidulans* (Joseph *et al*., 1999) and *A. niger* (Derkx and Madrid, 2001). Calnexin and calreticulin are also ER resident chaperones that have been shown to be involved in the quality control of the glycoproteins in mammalian cells. A calnexin homologue has been cloned from *A. niger* (Conesa *et al*., 2002; Wang *et al*., 2003) and found to exist in the genomes of *N. crassa* and *A. nidulans* (Conesa *et al*., 2001 and references therein).

Also, genes encoding protein involved in other stages of the secretory pathway have been isolated from filamentous fungi. Veldhuisen *et al*. (1997) have isolated a functional homologue of the *S. cerevisiae SAR1* gene from *A. niger* and *T. reesei*. The cDNAs from both fungi were able to complement *S. cerevisiae SAR1* and *SEC12* deficiencies indicating that genes that function in ER to Golgi transport may be conserved between filamentous fungi and *S. cerevisiae*. *S. cerevisiae* Sar1p is a small GTP-binding protein that functions in the formation and uncoating of ER-derived transport vesicles that have COPII coats (Barlowe *et al*., 1994).

Genes encoding Rab proteins functioning in different steps of the secretory pathway have been isolated form *N. crassa* and *A. niger*. From *N. crassa*, the Rab gene functioning in the ER to Golgi transport, *ypt1*, has been isolated by Heintz *et al*., (1992). Punt *et al*. (2001) have isolated genes encoding five secretion-related Rab proteins. One of these, SrgA, had low identity to *S. cerevisiae* Sec4p and another, SrgB, to Ypt1p. The *srgA* was not able to complement *S. cerevisiae* Sec4p deficiency and unlike in *S. cerevisiae*, its disruption was not lethal. It was observed though, that the *srgA* mutants grew slowly, had altered hyphal morphology and secreted less glucoamylase. The authors suggested this to be an indication of two secretory routes occurring in *A. niger*, one constitutive that needs the *srgA* function and another inducible, less dependent of *srgA*. The *nsf1* and *nsfA* genes encoding homologues of *S. cerevisiae SEC18*, have been cloned from *T. reesei* and *A. niger* var. *awamori* (Saloheimo *et al.,* 2003b). In the same study, identification of a *S. cerevisiae YPT1* homologue, y*pt1*, from *T. reesei* is presented. The YPTI protein of *T. reesei* was able to complement the yeast Ypt1p deficiency. Vasara *et al*. (2001) have cloned the *rho3* gene encoding a small GTP-binding protein, from *T. reesei* and shown that it was able to complement the yeast Rho3-Rho4p depletion and to improve actin organisation and chitin localisation when expressed in *S. cerevisiae* cells. In *T. reesei*, it was observed that the disruption of *rho3* did not change the morphology or cellular cytoskeleton organisation. Interestingly, both growth and protein secretion were decreased in *rho3* disruptant strains in conditions that induce protein production indicating a function in secretion of proteins (Vasara *et al*., 2001). Also, two genes, *ftt1* and *ftt2* encoding 14-3-3-proteins have been isolated from *T. reesei* . The *ftt1* was proposed to have a role in protein secretion (Vasara *et al.,* 2002).

So far, very few fungal SNARE proteins have been characterised. Wedlich-Söldner *et al*. (2000) have identified a putative t-SNARE *yup1* from *Ustilago maydis* that seems to have a function both in exocytotic and endocytotic pathways. Loss of Yup1 function resulted in defects in polar delivery of cell wall components and altered the morphology of the hyphae, which would indicate localisation to membranes of the late secretory pathway. It was observed that the Yup1-GFP is localised to endosomal vesicles. Thus, the authors suggest that the exocytotic and endocytotic pathways are linked through the function of Yup1 and that the membrane recycling via early endosomes supports polar growth. By using *S. cerevisiae* anti-Sso2 antibodies, Gupta and Heath (2000) have been able

to immunoprecipitate a 39 kDa protein, a putative plasma membrane t-SNARE from *Neurospora crassa*. Unlike *S. cerevisiae* homologues, this protein was localised to the plasma membrane at the hyphal apex and intracellular exocytotic apical wall vesicles. In *S. cerevisiae* the SNAREs are localised all over the plasma membrane and only part of them are activated for polarised secretion (Finger and Novick, 1998).

Available fungal genome databases (*S. cerevisiae, S. pombe, C. albicans, N. crassa, A. fumigatus* and *P. chrysoporium*) have been searched for homologues of yeast SNARE and Rab proteins. Eighteen different SNARE helices were found in all the fungi studied. The identity to the *S. cerevisiae* SNAREs was 30-70 % in the SNARE domains (Gupta and Heath, 2002). From *S. cerevisiae*, 20 SNARE genes encoding functions in different steps of membrane trafficking have been identified. These genes encode proteins displaying 22 different SNARE helices (Pelham, 2001). There seems to be more variation in the numbers of different Rab proteins between different fungal species putatively reflecting differences in the regulation of membrane traffic (Gupta and Heath, 2002). It seems that if there is more complex intracellular transport and a larger genome, also the number of Rab proteins is larger (Bock *et al*., 2001) indicating a greater need for cellular differentiation. From the fungal genomes, 9–12 different putative Rab proteins were identified, some of which had been identified before (Heintz *et al*., 1992; Punt *et al*., 2001). Six of these were conserved in all fungi and had 50–85% identity to the *S. cerevisiae* Rab proteins. The remaining Rab proteins found from the genomes of the filamentous fungi seem to have no homologues in the yeast genome. From these, Rab2 and Rab4 can be found from animal cells. Rab2 is involved in the transport between ER and cis-Golgi and back (Tisdale, 1999) and Rab4 in recycling of material from early endosomes to the plasma membrane (Mohrmann and van der Sluijs, 1999). On the other hand, there seems to be two Rab proteins in *S. cerevisiae* that are not found from filamentous fungi, Ypt10p and Ypt11p (Gupta and Heath, 2002). Almost all members of the exocyst complex were also found form the fungal genomes, although the identities between the possible orthologs were rather low, 15–40%.

It should be noted that the data by Gupta and Heath, (2002) is still preliminary, since the fungal genomes used in the study were not complete. Although the functionality of all the genes identified still needs to be verified, this data gives

indication that in filamentous fungi the secretory pathway functions much in same way as in other eukaryotes. Some features may be conserved in all organisms and others seem to be more restricted to fungi. One would expect to see more efficient protein transport and secretion machinery in filamentous fungi because of their ability to transport proteins efficiently over larger distances across the hypha.

1.3 Regulation of genes involved in secretion

The regulation of secretory pathway genes other than the ones encoding for ER resident chaperones and foldases has not been studied very extensively. There is indication in *S. cerevisiae* that the genes encoding different functions in the secretory pathway are not regulated at the transcriptional level. Vahlensieck *et al*. (1995) have studied the transcription of eight *SEC* genes, including *SAR1, SEC1, SEC14, SEC17, SEC18, SEC23 SEC62* and *YPT1* in conditions that changed the secretory activity and found no differences in their expression levels. On the other hand, it has been reported that the regulation of most of the *YPT* genes encoding Rab proteins was induced in *S. cerevisiae* by expression of a heterologous protein (Sagt, 2000). The transcriptional regulation of five *A. niger srg* genes has been studied and their expression was found to be independent of the carbon source used for growth. Most genes were found to be expressed at higher level in the early growth phase after which their expression decreased. However, the *srgA* and *srgB* genes were expressed constitutively (Punt *et al*., 2001). Contrary results have been obtained from the regulation of the *CLPT1* gene from the phytopathogenic fungus *Colletotrichum lindemuthanium*. The gene codes for a protein with homology to *S. cerevisiae* Sec4p and was shown to complement the *S. cerevisiae sec4-8* mutation (Dumas *et al*., 2001). It was observed that *CLPT1* expression was strongly increased on pectin, which induces the production of extracellular pectinases by the fungus. On glucose, a carbon source that represses the expression the extracellular glycan hydrolase genes, the expression of *CLPT1* gene was not detectable (Dumas *et al*., 2001). This indicates that the gene is regulated at transcriptional level according to the amount of secreted proteins on the secretory pathway.

The expression pattern of the *T. reesei pdi1* gene on different carbon sources resembles that of all the major secreted hydrolase genes, which are known to be

tightly regulated by the available carbon source. A major difference between *pdi1* gene expression and the expression of the hydrolase genes was that the *pdi1* gene does not seem to be under the regulation of glucose repressor CREI (Saloheimo *et al*., 1999).

1.3.1 Unfolded protein response

A signal transduction pathway called the unfolded protein response (UPR) has been shown to be activated when increased levels of misfolded proteins or incorrectly assembled subunits accumulate in the ER. Components of the UPR-pathway have been identified from different organisms including yeast, filamentous fungi, mammals and plants (Cox *et al*., 1993; Koizumi *et al*., 2001; Mori *et al*., 1993; Noh *et al*., 2002; Saloheimo *et al*., 2003a; Tirasophon *et al*., 1998; Wang *et al*., 1998a), thus the basic mechanisms seem to be evolutionally conserved. Conditions that perturb the ER function include inhibition of protein glycosylation or disulphide bond formation, over-expression of mutant proteins that accumulate into the ER (Umebayashi *et al*., 1999) or depletion of ER Ca^{2+} with Ca^{+} ionophore. Production of heterologous proteins has also been shown to induce UPR (Saloheimo *et al*., 1999). The UPR pathways of different eukaryotes are outlined in Figure 3.

1.3.1.1 UPR in S. cerevisiae

Identification of the components of the *S. cerevisiae* UPR pathway started with genetic screens, where the unfolded protein response element (UPRE) of *KAR2* fused to *lacZ* reporter gene was used (Cox *et al*., 1993; Mori *et al*., 1993). In these screens, the *IRE1/ERN1* gene was isolated and was postulated to be the proximal sensor of the pathway. Later, Cox and Walter (1996) were able to isolate the transcription factor *HAC1* in a screen for factors downstream of Ire1p in an *IRE1* deletion strain. *HAC1* had previously been isolated as a basic leucine zipper (bZIP) protein that can suppress *cdc10* mutation (Nojima *et al*., 1994).

Ire1 is the most upstream component of the UPR. It is a transmembrane serine/threonine kinase that functions in sensing the unfolded proteins in the ER and transferring the signal to Hac1p. It is not an essential gene under normal growth conditions, but under ER stress it is needed for cell viability. It has been shown that deletion of Ire1p results in the loss of UPR induced transcriptional

activation of *KAR2* and *PDI1* (Cox *et al*., 1993). Ire1 has three domains, an amino terminal domain that resides in the ER lumen, transmembrane domain and carboxy terminal domain that is cytoplasmic/nuclear and functions as the effector domain (Figure 4). Accumulation of unfolded proteins in the ER lumen is sensed by the amino terminal domain, which induces the Ire1p oligomerisation that initiates the signalling downstream from ER. This model was first proposed by Mori *et al*. (1993) and later verified by Shamu and Walter (1996). The C-terminal domain has homology to both Ser/Thr protein kinases (Cox *et al*., 1993; Mori *et al*., 1993) and RNaseL (Sidrauski and Walter, 1997). It has been shown that *trans*-phosphorylation of the Ire1 kinase domain is needed for the activation of the UPR and a mutation in the kinase domain causes inactivation of the response (Shamu and Walter, 1996). The lumenal domain has been shown to dimerise and activate the kinase domain (Liu *et al*., 2000). Oligomerisation induces the *trans*-autophosphorylation of the kinase domain with subsequent activation of the kinase and RNase activities (Sidrauski and Walter, 1997; Welihinda and Kaufman, 1996). In unstressed conditions Ire1 in found in monomeric and thus inactive state.

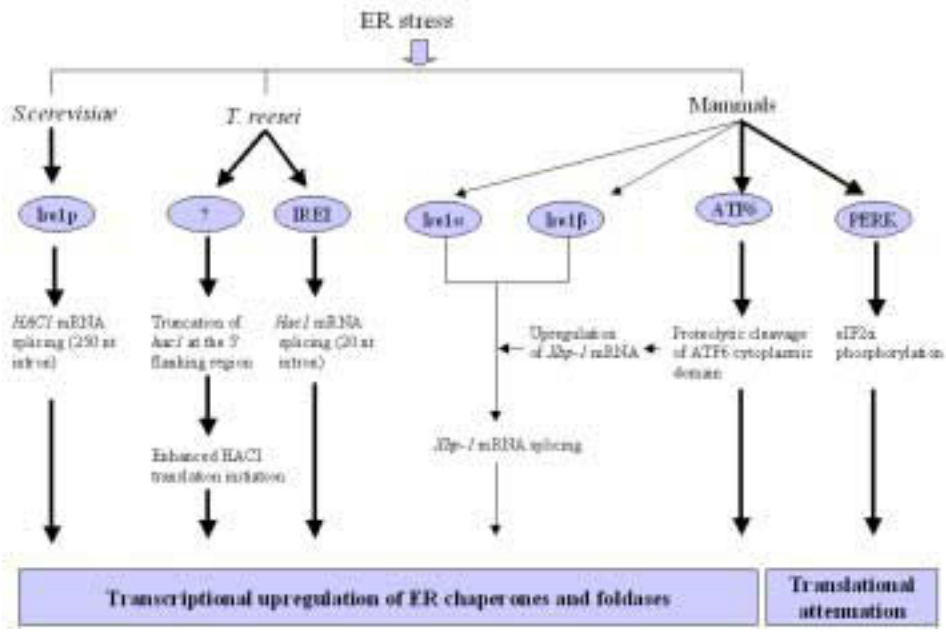

Figure 3. Comparison of the UPR-pathways of S. cerevisiae, T. reesei and animal cells. Modified from Ma and Hendershot, (2001) and M . Saloheimo, personal communication. See text for abbreviations.

Kohno *et al*. (1993) first showed that the UPR is initiated by a decrease of the level of the free ER chaperone Kar2p. Since then it has been shown that in the absence of ER stress, Kar2p binds to the lumenal domain of Ire1p. It has been shown that Kar2/Bip binding keeps Ire1 in an inactive, monomeric form (Bertolotti *et al*., 2000). As unfolded proteins accumulate during ER stress, Kar2/Bip is bound to the hydrophobic surfaces on unfolded proteins. This reduces the binding to Ire1, which is then free to oligomerise and undergo autophosphorylation thus causing UPR induction (Okamura *et al*., 2000).

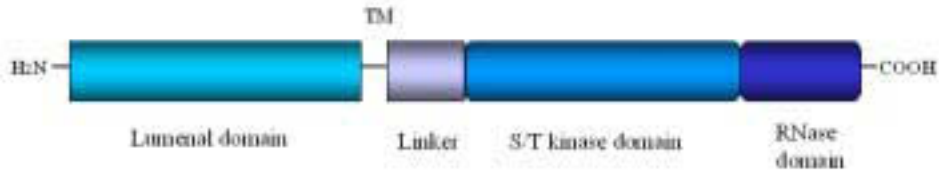

Figure 4. Domain structure of the Ire1 protein. TM, transmembrane domain; S/T kinase, Serine-Threonine kinase. Modified from Kaufman et al. (2002).

HAC1 mRNA is so far the only known substrate for Ire1 endoribonuclease activity in *S. cerevisiae*. Hac1p belongs to the basic leusine zipper (bZIP) protein family of transcription factors and has been shown to activate the transcription of UPR target genes. *HAC1* mRNA is spliced in a non-conventional way by Ire1p (Kawahara *et al*., 1998; Sidrauski *et al*., 1996). It has been shown that a 252 bp intron near the 3´end of the coding region is cleaved off from the *HAC1* mRNA during UPR induction (Cox and Walter, 1996; Kawahara *et al*., 1997). The splicing did not occur in an *IRE1* deletion strain. It was also shown that UPR was constitutively induced in an *IRE1* deleted strain, when an intronless *HAC1* cDNA was expressed (Cox and Walter, 1996). The non-spliced form of *HAC1* encodes a 230 amino acid protein (Cox and Walter, 1996). Splicing causes a replacement of the C-terminal portion of the Hac1p resulting in a mature Hac1p of 238 amino acids. It was originally proposed that the intron splicing would alter the stability of the protein and thus only the induced form of Hac1p can be detected in the cells (Cox and Walter, 1996). This proved not to be the case since both uninduced and induced forms of Hac1p are equally unstable. Later, it was shown that the unconventional intron blocks the translation of the non-spliced *HAC1* mRNA (Chapman and Walter, 1997; Kawahara *et al*., 1997). The attenuation of translation of unspliced *HAC1* mRNA has been shown to

require a base-paring interaction between the intron and the 5' untranslated region (Ruegsegger *et al*., 2001). Splicing of the *HAC1* intron has been shown to replace the last C-terminal amino acids with a 18-aa fragment containing a potent activation domain. The UPR induction is weaker by the expression of the uninduced form of Hac1p, because it has no activation domain (Mori *et al*., 2000).

The unconventional *HAC1* intron is removed by two specific cleavages that have been shown *in vitro* to be executed by Ire1p and can occur even if the spliceosomal function is blocked (Sidrauski *et al*., 1996; Sidrauski and Walter, 1997). Small stem-loop structures are predicted at the 5´and 3´splice sites of the uninduced *HAC1* mRNA. It was proposed that Ire1p recognises these structures and is thus able to cleave the mRNA at specific sites. It was proposed in the same study that Ire1 leaves 2´,3´-cyclic phosphates and that the exons remain associated by base pairing thus enabling the religation of the exons (Gonzalez *et al*., 1999). The ligase that functions in the ligation has been shown to be Rlg1p, a tRNA ligase (Sidrauski *et al*., 1996).

Using promoter analysis, the induction of the *S. cerevisiae KAR2* gene by UPR was observed to require a 22 bp element called UPRE (Kohno *et al*., 1993). The *S. cerevisiae* UPRE contains an imperfect palindrome of CNCAGcGTGNG that has been shown to bind Hac1p. The palindrome has been found from the promoters of *KAR2, PDI1, EUG1, FKB2* and *LHS1* and it has been shown to be necessary and sufficient for UPR induction (Kohno *et al*., 1993; Mori *et al*., 1993; Mori *et al*., 1998)

Another regulator of the *S. cerevisiae* UPR pathway has been shown to be a protein serine/threonine phosphatase, Ptc2p, which in *S. cerevisiae* interacts with Ire1p and causes its dephosphorylation. This leads to the inactivation of Ire1p and thus to attenuation of UPR response, indicating that Ptc2p is a negative regulator of UPR (Welihinda *et al*., 1998). It was shown in a reporter gene assay that deletion of Ptc2 increases the UPR induction by three to four fold and that over-expression reduces the level of spliced *HAC1* mRNA (Welihinda *et al*., 1998). In the same study it was shown that Ptc2 interacts directly with the phosphorylated from of Ire1p.

1.3.1.2 UPR in animal cells

The mammalian UPR-pathway has been shown to be more complex than the yeast system. The transcription of genes encoding ER-resident foldases, chaperones and components of the ERAD pathway is increased in order to deal with the increased load of unfolded proteins in the ER. On the other hand, also translational repression through phosphorylation of translation initiation factor 2α (eIF-2α) is induced (Prostko *et al*., 1993). A PKR-like ER-kinase (PERK) has been shown to phosphorylate eIF-2α under ER-stress thus inhibiting protein synthesis. PERK is a serine/threonine kinase that has been localised to ER membranes and the luminal domain has sequence homology to IRE1 (Harding *et al*., 1999). Another link between UPR and translational repression is created by the observation that over-expression of human IRE1β induced the cleavage of 28S ribosomal RNA in response to ER-stress (Iwawaki *et al*., 2001).

It has been observed that the induction of UPR leads to the onset of programmed cell death through the induction of a transcription factor CHOP/CADD153. Known inducers of UPR, tunicamycin, thapsigargin, calcium ionophore A23187 and DTT, induce CHOP (Halleck *et al*., 1997; Price and Calderwood, 1992). It has also been shown that Bip over-expression attenuates CHOP induction in response to ER-stress (Wang *et al*., 1996) and that deletion of CHOP in mammalian cells leads to decreased apoptosis in response to agents that induce UPR (Zinszner *et al*., 1998). A more direct link between UPR and apoptosis has come from the studies of Wang *et al*., (1998), who showed that over-expression of the murine homologue of yeast *IRE1* in mammalian cells activates both Bip and CHOP-encoding genes and that a dominant-negative form of Ire1 blocks the UPR-induction of both genes.

Mice and humans have two Ire1 proteins, Ire1α and Ire1β (Tirasophon *et al*., 1998; Wang *et al*., 1998a). Either isoform, when over-expressed, can activate the *Bip* promoter (Tirasophon *et al*., 1998; Wang *et al*., 1998a). Two Ire1 isoforms have also been isolated from *Arabidopsis thaliana* (Koizumi *et al*., 2001) (Noh *et al*., 2002). Some differences in the expression patterns of the mammalian Ire1 proteins have been observed, the α-isoform is expressed in all cell types and the expression is essential for normal development (Tirasophon *et al*., 1998), whereas the expression of the β-isoform is restricted to the epithelia of the gut (Urano *et al*., 2000b). The *A. thaliana* genes seem to be expressed in all tissues

with only small differences in the expression patterns (Noh *et al*., 2002). Both mammalian and plant Ire proteins are structurally similar to *S. cerevisiae* Ire1p having the conserved domain structure and being localised to the ER membrane (Koizumi *et al*., 2001; Tirasophon *et al*., 1998; Wang *et al*., 1998b). The Ire1 proteins have also been shown to have kinase activity and to be *trans*-autophosphorylated (Koizumi *et al*., 2001; Niwa *et al*., 1999; Tirasophon *et al*., 1998). Over-expression of human Ire1α increased the expression of a reporter gene that was under the rat *Bip* promoter and a mutation in the kinase domain of Ire1α resulted in the loss of UPR induction of the *Bip* gene during tunicamycin treatment (Tirasophon *et al*., 1998). The over-expression of the β-isoform has been shown to induce the expression of genes encoding both Bip and CHOP, whereas the expression of dominant-negative form inhibits the response of both genes to ER stress (Wang *et al*., 1998b).

ER stress has been shown to induce dimerisation of the amino-terminal ER lumenal domains (NLDs) of mammalian Ire1 and PERK proteins, a process necessary for their activation (Liu *et al*., 2000; Liu *et al*., 2002). Interestingly, the NLD of *S. cerevisiae* Ire1 can be replaced by mammalian Ire1α, Ireβ or PERK NLDs and without affecting its functionality in UPR-signalling (Liu *et al*., 2000). The biological function of NLDs was further verified in a study where the human Ireα NLD was produced and characterised. It was found that the NLDs are able to form stable dimers and to interact with Bip (Liu *et al*., 2002). How is this binding regulated? It has been shown that Bip over-expression causes a block in the activation of UPR in both mammalian and *S. cerevisiae* cells (Dorner *et al*., 1992; Kohno *et al*., 1993). Liu *et al*. (2000) proposed that the blockage is caused by a negative regulator that binds Ire1 and keeps it as a monomer under normal conditions. In ER stress, the negative regulator is bound to unfolded proteins and Ire1 is released for dimerisation. Further evidence for this model was presented by Bertolotti *et al*. (2000), who showed that the lumenal domains of both Ire1 and PERK form a stable complex with Bip in nonstressed cells. Accumulation of unfolded proteins causes the dissociation of Bip from the Ire1 and PERK and correlates with the formation of high-molecular-mass complexes. In cells that over-express Bip, the amount of Bip bound to Ire1 or PERK is greater than in parental cells (Bertolotti *et al*., 2000).

Further evidence for the similarities between mammalian and *S. cerevisiae* UPR pathways has been gained from studies showing that the mammalian IRE1

proteins have endonuclease activity. The kinase/nuclease domains of human Ire1α and Ire1β have been shown to cleave the *S. cerevisiae HAC1* mRNA both at the 5´and 3` splice sites *in vitro*. It was also shown by RT/PCR that when HeLa cells were transfected with the *S. cerevisiae HAC1* gene, a band of the expected size for spliced *HAC1* appeared in UPR induced cells (Niwa *et al*., 1999). On the other hand, it was shown that the unspliced form of the *S. cerevisiae* Hac1p can be expressed in mammalian cells as stably as the spliced form, and that the unspliced and spliced forms are equally efficient in the induction of UPR (Foti *et al*., 1999). Further, it was shown in the same study that the *S. cerevisiae HAC1* mRNA is not processed under ER stress in COS cells. These are quite contradictory results, but nevertheless it has been shown that the *S. cerevisiae* Hac1p can bind to a CCAGC motif, which is included in the binding site shown to be important for mammalian UPR (Foti *et al*., 1999).

In mammals, the *bip/grp78* gene is the best-characterised UPR target gene. The Rat *grp78* promoter has been shown to contain three ER-stress elements (ERSE) (Yoshida *et al*., 1998). ERSEs have also been found in the promoters of other ER stress inducible genes and are sufficient for UPR induction (Roy and Lee, 1999; Yoshida *et al*., 1998). The consensus sequence is CCATT(N_9)CCACG and the ERSE has been shown to contain binding sites for the ubiquitous transcription factors YY1 and NF-Y (Foti *et al*., 1999; Roy and Lee, 1999). Specific activation was suggested to result from the binding of a bZIP transcription factor ATF6 (Haze *et al*., 1999). ATF6 has been shown to be regulated at the post-translational level. Under normal conditions ATF6 is constitutively expressed as an ER membrane protein, which is inactive as a transcription factor. Under ER-stress the cytosolic domain, containing a DNA binding and a transcriptional activation domain, is cleaved off and transported to the nucleus where it binds to the promoters of the target genes in the presence of NF-Y (Yoshida *et al*., 2000). The cleavage of ATF6 is dependent on Site-1 protease (S1P) and Site2 protease (S2P) (Ye *et al*., 2000). S1P and S2P also process sterol regulatory element binding proteins (SREBPs) (Duncan *et al*., 1997; Duncan *et al*., 1998).

For a long time, the function of Ire1 in mammalian cells was not clear. It apparently functioned upstream of ATF6 since the over-expression of Ire1 resulted in the induction of UPR target genes (Tirasophon *et al*., 1998; Wang *et al*., 1998b) and also induced an artificial promoter containing several ATF6-

binding sites. To further support the theory, ATF6 signalling was blocked by an IRE1α dominant-negative mutant, where the C-terminal kinase and RNase domains were deleted (Wang *et al*., 2000). On the other hand, it has been shown that cells deleted for Ire1α have no defect in Bip induction (Urano *et al*., 2000b). Moreover, deletion of either or both IRE1 isoforms did not inhibit the UPR in mammalian cells (Lee *et al*., 2002; Urano *et al*., 2000a; Urano *et al*., 2000b).

When looking for targets of the Ire1p in mammalian cells, it was shown that IRE1 proteins are intimately associated with RNA fragments in an assay in which either UPR induced or uninduced cells were UV crosslinked. The amount of RNA bound to IRE1 was higher in DTT-treated cells and also the RNA fragments were shorter than in nonstressed cells (Bertolotti and Ron, 2001). Identification of the RNA fragments was not possible in this assay, but it indicated that the nuclease activity of IRE1 proteins is relevant for their function. Further verification for IRE1 function was obtained from recent studies with *Caenorhabditis elegans* that showed that a mutation in either *Ire1* or *Xbp1*genes resulted in loss of UPR induction (Calfon *et al*., 2002). Mammalian XBP-1 is a transcription factor essential for immunoglobulin secretion (Reimold *et al*., 2001) and high levels of *Xbp-1* mRNA are found in specialised secretory cells (Clauss *et al*., 1993). In *C. elegans*, ER-stress caused the appearance of 54 kDa form of XBP-1 protein and in cells where IRE1 was deleted, the induction of this form was not seen. An intron of 23 bp was spliced from the *Xbp-1* mRNA upon UPR induction. The intron was shown to form similar structures as the yeast *HAC1* mRNA around the cleavage sites. IRE1β was shown to cleave the intron of the unspliced *Xbp-1* mRNA *in vitro*. Re-ligation caused a shift in the reading frame and produced 54 kDa protein instead of 33 kDa, which would be generated from an unspliced mRNA (Calfon *et al*., 2002). In support of these findings, Lee *et al*., (2002) have shown that the mammalian IRE1α was needed for *Xbp1* mRNA splicing in the Chinese hamster ovary (CHO) cells. This splicing converts XBP1 to a potent UPR transcription factor. They were also able to show that the RNA cleavage specificity is conserved between mammals and *S. cerevisiae*. To bring all these findings together, they proposed a model for UPR induction where in essence there are two pathways that sense the state of ER, one through IRE1 and other through ATF6. These pathways meet in the regulation of XBP1. In this model both ATF6 and XBP1 are needed for efficient induction of UPR target genes (Lee *et al*., 2002).

1.3.1.3 UPR in filamentous fungi

Although many of the components of the UPR have been cloned from higher eukaryotes, no Hac1p homologue has been found in mammals. The work carried out in filamentous fungi has revealed functional homologues of *S. cerevisiae HAC1* from *T. reesei* and *Aspergillus nidulans*, *hac1* and *hacA* respectively. The induction of these genes differs in some respects from the mechanisms described in *S. cerevisiae* and involves two steps. These are splicing of an intron of 20 bases in length and truncation of the mRNA at the 5' flanking region (Saloheimo *et al*., 2003a). The truncation removes an upstream open reading frame from the mRNA, apparently increasing translation initiation at the correct start codon. This is a novel finding indicating that the induction of UPR in filamentous fungi has similar features as in *S. cerevisiae*, but that the mechanism appears to be more complex. The comparison of *Hac1/A* splicing in yeast and filamentous fungi is presented in Figure 5.

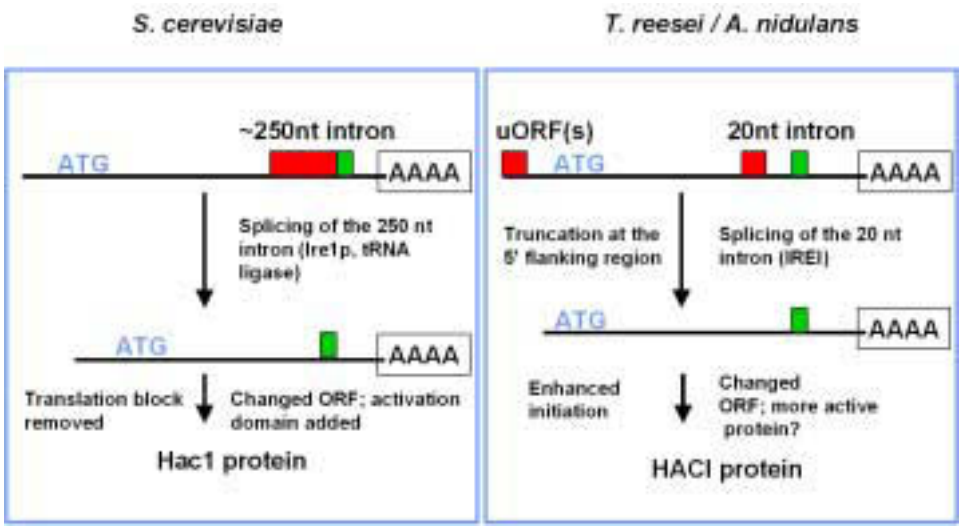

Figure 5. Mechanisms of Hac1 splicing in yeast (left) and filamentous fungi (right).

It has been observed that the expression of foreign proteins activates the UPR in filamentous fungi. Production of an antibody Fab fragment in *T. reesei* resulted in a notable increase in the *pdi1* expression, which is possibly caused by the accumulation of unfolded proteins in the ER and activation of the UPR

(Saloheimo *et al*., 1999). Also, expression of tissue plasminogen activator t-PA in *A. niger* has been found to induce UPR and increase the expression of *bipA*, *pdiA* and *cypB* by 1.5-2-fold (Wiebe *et al*., 2001). It can be concluded from these and other experiments that overproduction of heterologous proteins causes stress in the ER that is seen in the increased transcription of ER-resident foldases and chaperones. *S. cerevisiae* cells have also been shown to display UPR when heterologous proteins are expressed. For example, cutinase mutant proteins from *F. solani pisi* have been shown to be retained in the ER in association with Bip (Sagt *et al*., 1998).

1.3.1.4 Targets of the UPR regulation

The UPR was first observed in mammalian cells, where the expression genes encoding ER resident chaperones and foldases Bip, GRP94, PDI and ERp72, were found to be induced under different stress conditions (Dorner *et al*., 1990; Kozutsumi *et al*., 1988). Since then several other genes encoding ER resident foldases and chaperones have been shown to be induced by UPR both in mammals and *S. cerevisiae* (reviewed by Kaufman, 1999). *KAR2* induction has been shown to occur in *S. cerevisiae* as a result of a misfolded ER membrane protein, cytochrome P450 (Zimmer *et al*., 1999) as well as by overproduction of cytochrome P450 and invertase (Menzel *et al*., 1997). *S. cerevisiae* cells lacking a functional p24 complex, which functions in the sorting during transport between ER and Golgi, have been shown to accumulate secretory proteins in the ER and increase the secretion of ER resident proteins. This leads to the activation of UPR and increased *KAR2* expression (Belden and Barlowe, 2001a).

The cloning and characterisation of ER resident chaperones and foldases has revealed that the expression of these genes can be induced by treatments with agents that cause ER-stress. The expression of the *pdiA* gene from *A. niger* as well as the *pdi1* gene from *T. reesei* have been shown to be induced in tunicamycin and DTT-treated cells (Ngiam *et al*., 1997; Ngiam *et al*., 2000; Saloheimo *et al*., 1999). Also, a PDI1-family member, the *tigA* gene and a chaperone, the *bipA* gene from *A. niger* have been shown to be induced after tunicamycin treatment (Jeenes *et al*., 1997).

Travers *et al*. (2000) have shown by transcriptional profiling that under ER stress, the unfolded protein response (UPR) pathway regulates transcription of 381 genes in *S. cerevisiae* . Of those functionally characterised, 103 are involved in secretion process or biogenesis of secretory organelles. The genes that were found to be induced under ER-stress included quite expectedly genes for ER-resident chaperones and foldases. More surprising was that genes encoding proteins functioning throughout the whole secretory pathway were also induced. These included functions associated with protein translocation, lipid metabolism, glycosylation, ER associated protein degradation (ERAD), ER to Golgi transport and protein targeting to plasma membrane and vacuole (Travers *et al*., 2000). Another group has shown by using the DNA microarray technology that under ER-stress the expression of genes involved in cell wall biogenesis, protein secretion and processing in the ER are induced (Gasch *et al*., 2000).

ERAD is an important function in regulating the status of the ER. The misfolded proteins are recognised and transported out of the ER lumen to the cytosol to be degraded. Many of the ERAD components have been found to be regulated by UPR and it has been also shown that the UPR is needed for efficient ERAD since misfolded proteins are stabilised in Ire1 deleted strains (Casagrande *et al*., 2000; Friedlander *et al*., 2000; Ng *et al*., 2000; Travers *et al*., 2000). It has been shown by Friedlander *et al*. (2000) that mutations affecting both ERAD and UPR cause synthetic lethal phenotypes in *S. cerevisiae* whereas deletion of either pathway alone does not destroy viability. This indicates that cells need some pathway to deal with the unfolded proteins and that the two pathways, UPR and ERAD, are closely linked.

UPR has been shown to function under nitrogen starvation in *S. cerevisiae*. Nitrogen starvation-induced pseudohyphal growth and sporulation have been shown to be repressed when UPR is induced (Schröder *et al*., 2000). UPR is induced under nitrogen-rich conditions and the authors postulated this to occur because of a high protein synthesis rate and accumulation of large amounts of unfolded proteins in the ER. In mammalian cells it has been observed that the UPR induces the expression of an asparagine synthase gene indicating that some aspect of nitrogen metabolism is regulated by the UPR (Barbosa-Tessmann *et al*., 1999). Moreover, the dolichol pathway that is needed for protein glycosylation (Doerrler and Lehrman, 1999) and the sarco/ER calcium-ATPase

2b, which functions in translocating Ca^{2+} from the cytosol into the ER lumen (Caspersen *et al*., 2000) are regulated by the UPR in mammalian cells.

The *S. cerevisiae IRE1* gene was originally isolated as a gene required for inositol prototrophy (Nikawa and Yamashita, 1992). Also cells deleted of *HAC1* exhibit inositol auxotrophy (Nikawa *et al*., 1996). The expression of active Hac1p is needed for the synthesis of inositol through activation of the transcription of the *INO1* gene that is essential in the inositol biosynthesis (Cox *et al*., 1997). This led the researchers to think that the active UPR is needed for inositol synthesis and membrane biogenesis. The relationship between the two pathways is not straightforward since Mori *et al*. (2000) have shown that the unspliced form of Hac1p is also able to suppress the inositol requirement in *HAC1* deleted cells. Menzel *et al*. (1997) have shown that ER membrane proliferation caused by cytochrome P450 over-expression was not blocked in *IRE1* deleted cells. Similar results were obtained in a study by Umebayashi *et al*. (1999), who showed that ER membrane proliferation continued in Δ*ire1* cells over-expressing a mutated foreign secretory proteinase that accumulated into the ER lumen. On the other hand, it has been shown that the expression of a hydrophobic cutinase mutant induces the UPR and causes aberrant ER morphology (Sagt *et al*., 2002). Thus, it may be that the inositol response is controlled by both Ire1-dependent and Ire1-independent pathway, as proposed by (Stroobants *et al*., 1999).

1.4 Production of heterologous proteins in fungi

1.4.1 Fungi as production hosts

Filamentous fungi have been used for expression of both native and foreign proteins. The fact that fungi secrete enzymes into the culture medium has a clear advantage in producing proteins that might be toxic if they accumulate inside the cells. Secretion is also beneficial for the purification of the desired products since there is no need to break the cells and to get rid of all the intracellular proteins, a process that is often tedious and expensive. Moreover, fermentation techniques are well developed and fungi can be grown in large quantities in inexpensive media.

The secreted levels of native proteins are high in optimal production conditions, *T. reesei* can produce hydrolases at grams per litre quantities (Durand *et al*., 1988) and *A. niger* has been shown to produce more than 20 grams of glucoamylase per litre (Berka *et al*., 1991b). Studies on production of calf chymosin in both *A. awamori* and *T. reesei* (Dunn-Coleman *et al*., 1991; Harkki *et al*., 1989) as well as antibody Fab fragments in *T. reesei* (Nyyssönen *et al*., 1993) have shown that filamentous fungi are potential hosts also for foreign protein production.

S. cerevisiae has often been used as a production host for foreign proteins. It has GRAS-status (Generally Regarded As Safe) and can be cultivated easily and inexpensively. *S. cerevisiae* has certain advantages over bacterial systems. Since it is a eukaryote, most of the post-translational modifications that normally take place in animal cells, also occur in *S. cerevisiae* cells. Moreover, the purification of secreted proteins from the culture supernatants is relatively easy due to low amounts of native proteins secreted by *S. cerevisiae*, However, proteins produced in *S. cerevisiae* are often overglycosylated (Romanos *et al*., 1992) and the yields of heterologous proteins tend to be too low for industrial applications. Therefore, filamentous fungi with moderate glycosylation and excellent secretion capacity, would be a preferred choice as hosts for foreign protein production.

1.4.2 Enhancement of foreign protein production in filamentous fungi

A lot of research has been carried out to overcome problems encountered in the foreign protein production in filamentous fungi. It has been observed that bottlenecks can occur at any level of protein production: transcription, translation, secretion or extracellular degradation. The first attempts to produce foreign proteins in filamentous fungi were made with proteins of fungal origin (Fincham, 1989). Since then, many industrially important fungal proteins have been over-expressed in various production hosts. One example is the production of *Rizomucor miehei* aspartic protease in *A. oryzae* (Christensen *et al*., 1988), where production levels of 3 g/litre were obtained.

In contrast to the production of fungal proteins, the yields of non-fungal proteins in filamentous fungi can be very low (e.g., in the low milligrams per litre range) which is unattractive for industrial production (Gouka *et al*., 1997a; van den Hondel *et al*., 1991). Many strategies have been used to increase the yields of secreted foreign proteins in fungi. A good example is the enhancement of bovine chymosin production in *Aspergillus niger*. The applied approach has showed that modern gene technology and classical mutagenesis work hand in hand to improve the yields of secreted heterologous proteins. The process is presented in Table 1. The strains obtained in this way can be further used as hosts for production of other proteins (Ward *et al*., 1993).

Table 1. Enhancement of bovine chymosin production in A. niger. (GlaA, Glucoamylase A)

Method	**Yield**	**Reference**
glaA promoter + prochymosin	14 mg/l	(Ward, 1989)
glaA promoter + *glaA*-prochymosin fusion	47 mg/l	(Ward *et al*., 1990)
optimisation of cultivation conditions	150 mg/l	(Ward *et al*., 1990)
protease negative, *glaA* negative host	300 mg/l	(Berka *et al*., 1990)
mutagenesis of the strain, six rounds	750 mg/l	(Dunn-Coleman *et al*., 1991)
mutagenesis, 2-D-glc resistance	~ 1 g/l	(Dunn-Coleman *et al*., 1991)

Production systems usually use strong, either inducible or constitutive, promoters and/or multiple copies of the gene to be expressed inserted in the genome in order to facilitate high expression of the desired gene. Well-known inducible promoters used in many applications are those of the cellobiohydrolase I (*cbh1*) from *T. reesei* (Harkki *et al*., 1991), glucoamylase A (*glaA*) from *A. niger* (Smith *et al*., 1990), TAKA-amylase (*amyA*) from *A. oryzae* (Tsuchiya *et al*., 1992) and xylanase (*exlA*) from *A. awamori* (Gouka *et al*., 1996a). Among constitutively expressed genes, the *A. nidulans gpd*-promoter has been shown to

function in many filamentous fungi. A high copy number of the heterologous expression cassette has in some cases been shown to increase the production of foreign proteins. However, there seems to be no clear correlation between the copy number and the amount of proteins produced (Fowler *et al*., 1990; Punt *et al*., 1991). The differences seen in the expression of exogenous genes may partly be caused by the effect of the integration site of the expression cassette in the genome (Graessle *et al*., 1997).

The use of signal sequences from well-secreted proteins can improve the production of some heterologous proteins (van den Hondel *et al*., 1991). On the other hand, the origin of the signal sequence does not seem to make a difference to the production of other proteins. For example, in *A. nidulans*, the use of the signal sequence of either *glaA* or chymosin resulted in similar amounts of secreted chymosin (Cullen *et al*., 1987). The production of the *Fusarium solani pisi* cutinase in *A. awamori* did not require the use of cutinase presequence since the use of the endogenous *exlA* leader peptide led to similar levels of production (van Gemeren *et al*., 1996). The effect of naturally occurring pro-sequences on secretion levels has been studied. A positive effect of a prosequence on secretion has been observed in the case of restrictocin (Brandhorst and Kenealy, 1995) and a negative effect in the production of cutinase (van Gemeren *et al*., 1996) and chymosin (van Hartingsveldt *et al*., 1990).

The gene fusion strategy has been very useful in different expression systems. The best-characterised fusions are those with the *T. reesei* CBHI and with *A. niger* or *A. awamori* glucoamylase (*glaA*). This strategy has been used efficiently in the production of bovine chymosin (Dunn-Coleman *et al*., 1991), human interleukin 6 (Broekhuijsen *et al*., 1993), porcine pancreatic phospholipase A_2 (Roberts *et al*., 1992), hen egg-white lysozyme (Jeenes *et al*., 1993), human lactoferrin (Ward *et al*., 1995) and the catalytic subunit of bovine enterokinase (Svetina *et al*., 2000). The production of antibody fragments in *T. reesei* as a CBHI fusion, resulted in more than 150-fold increase in the yield (Nyyssönen and Keränen, 1995). On the other hand, Conesa *et al*. (2000) have shown that the expression of two fungal peroxidases, *Phanerochaete chrysosporium* lignin peroxidase H8 (*lipA*) and manganase peroxidase (*mnp1*), was not affected by fusion to the *glaA* gene. In all these cases the fusion has been made to the N-terminus of the foreign protein. It has been proposed that the N-terminal fusion partner functions as a carrier that enhances protein

translocation and folding as well as protects the foreign protein from degradation. Indeed, Gouka *et al*. (1997b) showed that *glaA* fusion to the 5´end of the human interleukin 6 gene resulted in an increase of the hIL-6 production, whereas *glaA* fusion to the 3´end greatly reduced the production of the fusion protein. As the steady state mRNA levels were similar with both constructs, the authors suggested that the production of the *hil6-glaA* fusion is blocked at translational or post-translational level.

It has been observed that AT-rich stretches can cause premature termination of transcription (Gouka *et al*., 1997b; Schuren and Wessels, 1998), a problem that can be overcome by changing codon usage (Romanos *et al*., 1991; Te'o *et al*., 2000). Problems at the transcriptional level have been observed in some cases where non-fungal proteins have been expressed in filamentous fungi. Analysis of human interleukin 6 and guar α-galactosidase mRNA levels indicated that there were only small amounts of mRNA present in cells expressing the two proteins. This was probably due to lower stability of the transcripts when compared to that of the endogenous mRNAs (Gouka *et al*., 1996b). However, although there may be problems at the transcriptional level in heterologous expression, transcript levels have usually been shown to be sufficient for efficient production. It has been observed in studies of hen egg white lysozyme in *A. niger* that there was a direct relationship between HEWL copy number, transcript level and the amount of secreted protein (Jeenes *et al*., 1994).

Even though the foreign proteins may be protected from the intracellular proteases when expressed as fusion proteins, they can be very sensitive to the extracellular proteases present in the culture medium. Proteases have been thought to be one of the major limiting steps in the industrial production of enzymes (Gouka *et al*., 1997a; van den Hombergh *et al*., 1997b). As mentioned before, the production of chymosin as well as porcine phospholipase 2 was increased in *A. awamori* by deletion of the *pepA* gene encoding the aspartic proteinase aspergillopepsin A (Berka *et al*., 1990; Roberts *et al*., 1992). The applicability of this strategy varies due to different sensitivities of foreign proteins to proteases produced by the host. For example, it has been shown that hIL6 is very sensitive to protease activity (Broekhuijsen *et al*., 1993), whereas hen egg white lysozyme and porcine pancreatic phospholipase A_2 have different sensitivities to aspergillopepsin A in *A. niger* (Archer *et al*., 1992). The findings that extracellular proteases can decrease the amount of foreign protein have lead

to the construction of protease-deficient strains. A strain of *A. niger* that lacks *pepA*, *pepB* and *pepE* has been constructed (van den Hombergh *et al*., 1997a) and at least *in vitro* the protease activities were lower than in the control strain. Changing the production conditions can also decrease the protease activity to some extent. Inhibition of protease activity and increased heterologous protein production can be accomplished by inducing pelleted growth in *A. niger* (Xu *et al*., 2000).

There are some indications that glycosylation has a role in the production of heterologous proteins. N-glycosylation has been shown to increase the amount of secreted chymosin in *A. awamori*, although the specific activity of the chymosin was reduced (Ward, 1989). In *T. reesei*, it has been shown that the over-expression of mannosylphosphodolichol synthase encoding gene from *S. cerevisiae*, which is required for O-glycan precursor synthesis, increased the production of CBHI (Kruszewska *et al*., 1999). As for foreign proteins, it has been shown that *Candida antarctica* lipase produced in *A. oryzae* (Heogh *et al*., 1995) and *Hormoconis resinae* glucoamylase P produced in *T. reesei* (Joutsjoki *et al*., 1993) are overglycosylated. These findings could indicate that the problems encountered in glycoprotein production could be reduce by manipulating glycosylation.

It has been observed that misfolded foreign proteins accumulate in the ER (Marquardt and Helenius, 1992). Thus, it would seem that the efficiency of protein folding and assembly of subunits in the ER is often the step that determines the efficiency of foreign protein production. In order to enhance the foreign protein yields, modification of the levels of ER chaperones and foldases has been tested in several production systems (Dorner *et al*., 1988; Dorner *et al*., 1989; Dorner *et al*., 1992; Hsu and Betenbaugh, 1997). In a study where the production of several homologous and heterologous proteins fused to *glaA* was investigated, it was shown that the over-expression of *bipA* resulted in increased levels of unprocessed fusion proteins in the cell lysates in *A. niger* (Punt *et al*., 1998). However, no improvement of the secretion of the fusion proteins was observed. Similar results were obtained in *A. awamori*, where Bip over-expression had no effect on the production of cutinase variants (van Gemeren *et al*., 1998). In another study, *bipA* over-expression had no effect on the production of a fungal peroxidase, MnP, but over-expression of another ER

chaperone, calnexin, resulted in four to five-fold increase in the secretion of MnP (Conesa *et al*., 2002).

As for the function of foldases in the heterologous protein production in filamentous fungi, Moralejo *et al*. (2001) have shown that the level of PDIA was critical for production of thaumatin in *A. awamori*. A peak level of thaumatin (five-fold increase) was obtained in a strain with two to four fold more PDIA than in the parental strain. Production of the native proteins acid phosphatase and α-amylase was not affected by the varying PDIA levels. Increased levels of *pdiA*, *tigA* and *bipA* were detected in *A. awamori* strains over-expressing hen egg white lysozyme (HEWL). Over-expression of PDIA did not increase the secretion of HEWL, although downregulation of *pdiA* by applying an antisense strategy lowered the level of secreted glucoamylase (Ngiam *et al*., 2000). Characterisation of the PDI-like gene *prpA* of *A. niger* var. *awamori* has also shown that although the *prpA* gene was induced in cells secreting bovine prochymosin, the overproduction of *prpA* had no effect on chymosin production (Wang and Ward, 2000).

1.4.3 Enhancement of protein production in *S. cerevisiae*

Some of the methods that have been used in filamentous fungi to increase the production of foreign proteins have been applied to *S. cerevisiae* as well. These include the use of a suitable promoter and signal sequence (Ruohonen *et al*., 1995), modification of the expression vector copy number (Ruohonen *et al*., 1991), mutagenesis and screening for supersecretory mutants (Sleep *et al*., 1991). Expression level tuning in *S. cerevisiae* has been shown to be a potent solution in the expression of foreign proteins. It has been suggested that an optimum expression level exists for the secretion of any foreign protein, which is due to saturation of the folding capacity of the ER (Wittrup *et al*., 1994). Parekh and Wittrup (1997) have shown that there is an optimum synthesis level for secretion of bovine pancreatic trypsin inhibitor in *S. cerevisiae*. The optimal secretion levels were determined using a vector with inducible *GAL-10* promoter. It was concluded in this study that the maximum productivity is determined by the protein folding capacity of the ER.

Proteolytic degradation has been a problem in the production of foreign proteins also in *S. cerevisiae* and attempts have been made to avoid this. Several proteinase-deficient strains have been constructed that have increased protein yields (Suzuki *et al*., 1989; Wingfield and Dickinson, 1993). Another strategy used has been the disruption of the *YAP3* gene encoding an aspartyl protease (Kerry-Williams *et al*., 1998). This was shown to reduce the degradation of secreted recombinant human albumin and thus increase the secreted yields of the protein. Also, modification of foreign protein glycosylation has been shown to increase secretion. Secretion of cutinase and llama V_{HH} antibody fragments by both *S. cerevisiae* and *Pichia pastoris* was improved by addition of N-glycosylation sites (Sagt *et al*., 2000). The success of this strategy was dependent on the site of glycosylation used; C-terminal glycosylation increased the secretion of cutinase only 1.8-fold, whereas N-terminal glycosylation enhanced production five-fold. The authors' interpretation was that glycosylation shields hydrophobic patches in the nascent polypeptides and prevents the aggregation of the cutinase in the ER.

Many of the secretion-related genes from *S. cerevisiae* have been cloned as a result of a secretion block caused by a mutation. Only a few of these genes have been shown to increase the protein secretion when over-expressed. One example is provided by the *S. cerevisiae SSO* genes encoding t-SNAREs of the exocytosis step. Over-expression of these genes was shown to increase the secretion of a foreign protein, *Bacillus* α-amylase, and a native protein invertase (Ruohonen *et al*., 1997). Moreover, overexpression of Sso2p resulted in S. cerevisiae strain producing Trametes versicolor laccase, resulted in two-fold increase in laccase activity (Larsson *et al*., 2001). An interesting finding is also that the expression of a mammalian integral ER ribosome receptor (p180) led to proliferation of rough ER membranes and increased secretory capacity in *S. cerevisiae* (Becker *et al*., 1999).

Over-expression of ER resident chaperones and foldases with the aim of improving foreign protein secretion has been reported in several studies. The results are quite contradictory indicating that the effect may be dependent on the protein being produced. The secretion of five single-chain antibody fragments was increased in *S. cerevisiae* upon over-expression of *KAR2* or *PDI1* by two to eight-fold. It was observed that a synergistic increase occurs when Kar2 and PDI are co-expressed (Shusta *et al*., 1998). Harmsen *et al*. (1996) have shown that

the over-expression of *KAR2* causes a 20-fold increase in the secretion of prochymosin. Interestingly, disruption of *PMR1* together with the over-expression of *KAR2* gene resulted in the secretion of all the prochymosin expressed. On the other hand, no effect on secretion of thaumatin was observed in the same study. *PMR1* is a Golgi localised Ca2+ ATPase that has been shown to affect the transit of proteins through the secretory pathway (Rudolph *et al*., 1989). Disruption of the gene has been shown to result in a clear increase in the secretion of bovine growth hormone and nonglycosylated variants of human urinary plasminogen activator (Rudolph *et al*., 1989). Robinson *et al*. (1996) have shown that the five fold over-expression of Kar2p had no effect on the secretion of human granylocyte colony-stimulating factor (GCSF), *Schizosaccharomyces pombe* acid phosphatase or bovine pancreatic trypsin inhibitor in *S. cerevisiae*. For all three proteins, though, the reduction of *KAR2* expression reduced their secretion. Interestingly, it has been shown that constitutive expression of GCSF, *S. pombe* acid phosphatase and human erythropoietin reduced the levels of Kar2 and PDI1 in the ER (Robinson and Wittrup, 1995).

Over-expression of *PDI1* has been shown to result in ten fold increase in human platelet derived growth factor B homodimer and a four-fold increase in *S. pombe* acid phosphatase production in *S. cerevisiae* (Robinson *et al*., 1994). Moreover, antistasin as well as human lysozyme (h-LZM) production have been shown to be increased when PDI1 is over-expressed (Hayano *et al*., 1995; Schultz *et al*., 1994). The h-LZM expression was also increased in a strain expressing PDI1 mutant, where both active sites had been disrupted, which indicates that the effect of PDI1 to the production of this protein was due to chaperone action rather than foldase action (Hayano *et al*., 1995).

In summary, the data obtained from *S. cerevisiae* and filamentous fungi indicate that there seems to be no general way to improve the production of all proteins in these systems. Rather it seems that the improvement is more dependent on the properties of the proteins being produced. Different production systems have of course different capability to transport proteins, but to enhance the production of a given protein, the step causing the problem in the production should be assessed for each individual protein in order to get maximal yields.

1.5 Aims of the study

The first aim of the study was to clone and characterise genes from the secretory pathway of the filamentous fungus *Trichoderma reesei*. An important aspect of the work was to study their functions in correlation with what is known in other eukaryotes. The genes isolated and studied in this work were the *snc1* encoding a putative v-SNARE, *ire1* encoding a serine-threonine kinase and *ptc2* encoding a serine-threonine phosphatase.

The other major aim was to study the relationship between unfolded protein response and protein production in both *S. cerevisiae* and the filamentous fungi *T. reesei* and *A. niger* var. *awamori*. The approach taken was to demonstrate the effects of manipulation of the UPR on protein production in *S. cerevisiae* and to analyse the effect of the enhancement of the UPR on protein production in filamentous fungi. A goal important from a biotechnical point of view was to improve foreign protein production in filamentous fungi in a system, where the UPR pathway was constitutively activated.

2. Materials and methods

Method	**Used in**
Brefeldin A treatment of *T. reesei*	I
Complementation of yeast disruptions	I, II
Determination of enzyme activity against MUL	IV
Fungal and yeast DNA extraction and Southern hybridisation	I, II, III, IV
Dry weight measurements	II, IV
DTT treatment of *T. reesei*	I
IREI autophosphorylation assay	II
Fungal transformation and cultivation	II, IV
Measurement of α-amylase activity	III, IV
Measurement of β-galactosidase activity	II
Measurement of β-glucosidase activity	IV
Measurement of endoglucanase activity	III
Measurement of invertase activity	I, III
Measurement of laccase activity	II, IV
Measurement of chymosin activity	IV

Polymerase chain reaction	II, III, IV
Fungal and yeast RNA extraction and Northern hybridisation	I, II, III, IV
Suppression of yeast temperature sensitive mutations	I
Total protein measurement	IV
Tunicamycin treatment of *S. cerevisiae*	II
Yeast transformation and cultivation	I, III

3. Results and discussion

3.1 Cloning and characterisation of the SNARE protein SNCI from *T. reesei* (I)

The secretory pathway has been shown to be conserved in many aspects between *S. cerevisiae* and higher eukaryotes (Bennett and Scheller, 1993). Since the secretory pathway of filamentous fungi is quite poorly characterised, we started characterising its central components from *T. reesei.* The *S. cerevisiae* Sso proteins, when over-expressed, enhance the production of a foreign protein, *Bacillus* α-amylase (Ruohonen *et al*., 1997). Since we were also interested in studying the enhancement of the production of foreign proteins in *T. reesei*, Sso proteins were identified as a potent targets for these studies. Cloning of the genes encoding homologues for *S. cerevisiae SSO1* and *SSO2* proteins was attempted by complementation of *S. cerevisiae SSO2* temperature sensitive (ts) mutation. In S. *cerevisiae*, the Sso1 and Sso2 proteins have been characterised as the plasma membrane t-SNAREs that are needed for exocytotic vesicle fusion (Aalto *et al*., 1993). The two proteins are very similar to each other with 74% identity in amino acid sequence. Recently Jäntti *et al*. (2002) have shown that there is functional difference between the Sso1 and Sso2 proteins so that Sso1 is specifically required for sporulation.

In the complementation screening with the *S. cerevisiae SSO2* temperature sensitive strain transformed with *T. reesei* cDNA library. Several clones were found that were able to grow at the restrictive temperature. The cDNA isolated from two of these transformants was homologous to *S. cerevisiae SNC1* and *SNC2*. The Snc proteins are v-SNAREs localised in the secretory vesicles that fuse with the plasma membrane (Gerst *et al*., 1992; Protopopov *et al*., 1993) and have also been shown to be involved in endocytosis (Gurunathan *et al*., 2002). *S. cerevisiae* has two Snc1 proteins that are functionally redundant (Protopopov *et al*., 1993) and very similar, with 77% identity. Therefore, the genes encoding Snc proteins have been thought to be among those that have been duplicated in *S. cerevisiae*.

3.1.1 Sequence analysis of *T. reesei* SNCI

T. reesei snc1 encodes a protein of 111 amino acids and sequencing of the genomic gene isolated from a λDNA library clone revealed two introns. In Blast-searches, the SNCI protein was found to be most similar with a synaptobrevin homologue of *Aspergillus paraciticus* (accession number O13312) in the translated EMBL database . The identity between the two proteins was 61%. The identity of *T. reesei* SNCI to *S. cerevisiae* Snc1 and Snc2 proteins was at a similar level, 53% and 61%, respectively. The alignment of the *T. reesei* SNCI with *A. paraciticus* synaptobrevin homologue, *S. pombe* Snc2 and *S. cerevisiae* Snc1 and Snc2 proteins is shown in Figure 6.

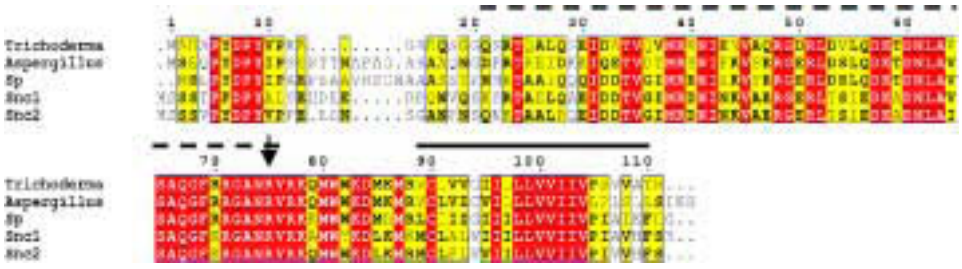

Figure 6. The alignment of the different Snc fungal proteins. Synaptobrevin signature (- -), putative transmembrane region (──), conserved arginine (➔). Sp; S. pomb; Snc1, Snc2, S. cerevisiae proteins. Amino acids shown in red are strictly conserved and the positions shown in yellow have similar amino acids.

The most conserved area between the proteins is the synaptobrevin signature sequence. According to protein structure prediction on the PSIPRED server (McGuffin *et al*., 2000), SNCI is predicted to form two α-helices. The putative cytoplasmic helix may be involved in the formation of a SNARE complex as has been shown in *S. cerevisiae* (Gerst, 1997). The arginine at position 48 of the *T. reesei* SNCI is conserved between different R-SNAREs.

In order to find out if there would be another *snc* gene in *T. reesei,* heterologous Southern analysis was carried out in non-stringent conditions. The result was that there may be a homologue of *snc1* in *T. reesei* genome although the sequence conservation is likely to be rather low (I, Figure 2). This implied the

existence of either a duplicate gene of *snc1* or perhaps a similar gene with a different function e.g. in membrane fusion in another cellular location. In general, very few SNARE proteins have been cloned form filamentous fungi. The cloning of a putative t-SNARE from *U. maydis* (Wedlich-Söldner *et al*., 2000) and a synaptobrevin homologue (v-SNARE) from *A. paraciticus* as well as the finding of genes encoding SNARE helices from the genomes of different fungi (Gupta and Heath, 2002) indicate that membrane fusion probably occurs through similar structures in filamentous fungi as in other eukaryotes.

3.1.2 Functional analysis of *T. reesei* SNCI

Sequence conservation between *T. reesei* SNCI and *S. cerevisiae* Snc proteins indicated that they were functionally equivalent. But in order to verify this, complementation of a *S. cerevisiae* Snc1p-Snc2p depletion strain was studied. Deletion of the genes encoding Snc1 and Snc2 proteins in *S. cerevisiae* causes temperature sensitivity in a synthetic medium and inability to grow in a rich medium (Protopopov *et al*., 1993). The complementation was done using a yeast strain (JG8 T15:85), where both genomic *SNC* genes are deleted and *SNC1* is under the inducible *GAL10* promoter in a plasmid. The yeast transformants with the *T. reesei snc1* cDNA were able to grow at 30°C on rich YPD-plates as well as on plates containing synthetic medium with glucose as the carbon source. The strain transformed with the expression vector alone was not able to grow under these conditions (I, Figure 3A).

Secretion of invertase in *S. cerevisiae* is also impaired in a strain deleted for both *SNC* genes. The ability of *T. reesei* SNCI to suppress this deficiency was tested. The yeast Snc1/2p deletion strain transformed with the *T. reesei snc1* expression construct or the expression vector alone was cultivated in a medium with glucose as the carbon source at permissive and restrictive temperatures. The cultures were diluted repeatedly in order to clear the cells of the endogenous Snc1 protein. After this, the cells were shifted to low glucose concentrations to derepress the invertase gene. Invertase activities measured from the transformant and control cultures showed that the *T. reesei* SNCI was able to suppress the *S. cerevisiae* Snc protein depletion. The difference in the invertase production between the transformants and the control was about two-fold at the restrictive temperature (I, Figure 3B).

The results show that the *T. reesei* SNCI is able to complement both growth defect and secretion deficiency of the *S. cerevisiae* Sncp depletion. This confirms that the *T. reesei* SNCI is a functional homologue of the *S. cerevisiae* Snc proteins. Although Gupta and Heath (2002) were able to find Snc homologues from the genomes of various filamentous fungi and Snc homologues can also be found from the *A. nidulans* EST database, the current study provides the first indication that a SNARE mediated membrane fusion occurs in filamentous fungi through mechanisms similar to other eukaryotes.

The *T. reesei snc1* gene was originally cloned as a suppressor of the temperature-sensitive *SSO2* mutation. Because of this, the mechanism of membrane fusion was further studied by attempting to suppress *S. cerevisiae SSO* and *SEC9* depletion with the *T. reesei* s*n*c1 cDNA. The *T. reesei snc1* cDNA was transformed into three strains, a strain with an *sso2* ts-mutation and *SSO1* gene deleted, a strain where both *SSO* genes were deleted and *SSO1* is under control of the *GAL10* promoter and a strain with a *sec9* ts-mutation. Sso and Sec9 proteins are plasma membrane t-SNAREs that have been shown to form a SNARE complex with Snc proteins (Couve and Gerst, 1994; Rossi *et al*., 1997). It has been shown in *S. cerevisiae* that Snc1 overexpression can partially suppress the temperature sensitivity of both *sec9* (Couve and Gerst, 1994) and *sso2* (Gerst, 1997). Therefore the cloning of the *T. reesei snc1* in the Sso2 ts screening was not unexpected. Despite several attempts the suppression could not be repeated in the *SSO2* mutant strain and neither could we show suppression in Sso1p-Sso2p deletant or Sec9p temperature sensitive strains. This may indicate that the *T. reesei* SNCI protein is inefficient in the formation of the SNARE complex with the *S. cerevisiae* Sso and Sec9 proteins and was thus only capable of transient suppression of the Sso2 ts mutant when it was cloned. It could also be that the regulation of the complex formation is impaired due to inefficient binding of yeast regulatory proteins to *T. reesei* SNCI.

3.1.3 Regulation of the *T. reesei snc1* gene expression

Only a little work has been done to analyse the expression of secretion related genes in *S. cerevisiae* and filamentous fungi on different carbon sources. In *S. cerevisiae* the expression of several genes related to different steps of the secretory pathway was studied in different culture conditions and was shown not

to be regulated at the transcriptional level (Vahlensieck *et al*., 1995). Similar results have been obtained from studies with *A. niger srg* genes that encode small secretion-related GTPases. The expression of two genes, *srgA*, a homologue of *S. cerevisiae SEC4* and *srgB*, a homologue of *S. cerevisiae YPT1* was found not to be regulated according to the available carbon source (Punt *et al*., 2001). On the other hand, it has been observed in *T. reesei* that the expression of the *pdi1* gene is regulated by the available carbon source (Saloheimo *et al*., 1999), being highest under conditions where a large amount of protein is secreted. Further evidence on carbon source regulation has come from work done with the phytopathogenic fungus *Colletotrichum lindemuthianum*, where it was shown that the homologue of *S. cerevisiae SEC4*, *CLPT1,* is differentially expressed on different carbon sources that also affected the amounts of protein secreted (Dumas *et al*., 2001).

We have cloned and characterised several genes encoding proteins in the secretory pathway of *T. reesei*. In addition to *snc1* discussed earlier, we have cloned the *ypt1* gene encoding a small GTPase and *nsf1*, a homologue of *S. cerevisiae SEC18* known to function in different steps of vesicle fusion (Rothman and Warren, 1994). Expression of these three genes was studied on different carbon sources and it was observed that none of them was regulated at the transcriptional level by the available carbon source (Saloheimo *et al*., 2003b). It would be logical to assume that the expression of secretion-related genes would react to the changing need of protein transport in the secretory pathway. In *T. reesei*, this seems not to be the case for most of the secretion-related genes. This could indicate that the secretory genes are transcribed under normal growth conditions at high enough levels to deal with differences in secretory load.

We have also studied the effect of brefeldin A (BFA), a protein trafficking inhibitor (Klausner *et al*., 1992), and dithiothreitol (DTT), a folding inhibitor that prevents protein transport from the ER (Jämsä *et al*., 1994), on the expression of the *snc1* gene in *T. reesei*. Both DTT and BFA have been shown to strongly inhibit protein secretion without affecting total protein synthesis in *T. reesei* (Pakula *et al*., 2003). There are also indications that both DTT and BFA block secretion at the early stages of the secretory pathway in *T. reesei* (Pakula *et al*., 2003; Pakula *et al*., 2000). It was shown in the current study that both BFA and DTT induced the expression of the *snc1* gene and that the induction by

BFA treatment was more pronounced (I, Figures 4 and 5). In contrast, the *ypt1* and *nsf1* genes were induced by BFA treatment to a much higher extent than the *snc1* gene (Saloheimo *et al*., 2003b). One explanation as to why *ypt1* and *nfs1* are more induced by the BFA treatment than *snc1* could be that in BFA treated cells the transport of proteins may be directed to vacuoles as has been shown to occur in *A. niger* (Khalaj *et al*., 2001). This could cause the induction of genes like *nsf1* and *ypt1* that function in the early secretory pathway, under conditions where cells try to clear the excess of unfolded protein, while the late secretory pathway is affected only mildly.

The induction of *snc1* expression in the DTT treated samples was highest 60–90 minutes after DTT addition, at which time the *snc1* expression level was about two fold higher than in the control (I, Figure 4). A search of the webminer database (Heiman and Walter, 2000) at http://webminer.ucsf.edu showed that *S. cerevisiae SNC1* is induced 2–3-fold when UPR pathway is induced by DTT. This induction may be dependent on the UPR pathway components Hac1p and Ire1p since the expression of the gene was lower in strains where *HAC1* or *IRE1* were deleted. The *pdi1* gene encoding an ER-resident foldase has been shown to react faster and to a larger extent during DTT treatment (Saloheimo *et al*., 1999), being induced already after 30 minutes of DTT treatment. The *snc1* level is clearly increased only after 60 minutes of treatment. Thus, it may be that the induction of *snc1* is a secondary effect of the UPR induction. This proposal is further supported by the fact that no putative UPR-element was found in the 5' non-coding region of the *snc1* gene.

3.2 Cloning and characterisation of two factors from the UPR-pathway from the filamentous fungus *T. reesei* (II)

The unfolded protein response pathway has been best characterised in *S. cerevisiae* where the basic factors involved have been identified and the mechanisms studied in detail. The *IRE1* function was established in *S. cerevisiae* in screens for factors required for *KAR2* induction under ER stress (Cox *et al*., 1993; Mori *et al*., 1993). Deletion of IRE1 resulted in a loss of activation of the UPR in cells treated with tunicamycin (Casagrande *et al*., 2000; Friedlander *et al*., 2000; Travers *et al*., 2000). *IRE1* homologues have been cloned from mammals and plants and the mechanism of their activation has been shown to be

conserved (Koizumi *et al*., 2001; Noh *et al*., 2002; Tirasophon *et al*., 2000; Wang *et al*., 1998b). The Ptc2p protein phosphatase, on the other hand, has been shown to be a negative regulator of the UPR pathway only in *S. cerevisiae* (Welihinda *et al*., 1998).

The *ire1* and *ptc2* genes from the filamentous fungus *T. reesei* were cloned by heterologous hybridisation using *A. nidulans* gene fragments as probes. The *A. nidulans* genes were identified in searches with the *S. cerevisiae* Ire1p and Ptc2p sequences against a public *A. nidulans* EST database. The EST clone u4h01a1 was most similar to *S. cerevisiae* Ire1p and i2c04a1 to *S. cerevisiae* Ptc2p, although the similarity of i2c04a1 to *S. cerevisiae* Ptc3p was also clear.

3.2.1 Sequence analysis

The *T. reesei ire1* gene is about 4.5 kb in length and the open reading frame encodes a protein of 1243 amino acids. The *T. reesei* IREI protein starts with a predicted signal sequence of 25 amino acids. Interestingly, there are two short upstream open reading frames (uORF') (14 amino acids and 1 amino acid) before the start of the coding region. A putative transmembrane segment is located at positions 584–606 of the open reading frame (Figure 7). The N-terminal domain (before the transmembrane segment), presumably facing the lumen of the ER, has 25% identity and 43% similarity over an area of 367 amino acids with *S. cerevisiae* Ire1p. There is a protein kinase domain at the C-terminus of the predicted IREI protein (amino acids 809–1105) that contains a conserved subdomain of Serine/Threonine kinases. The C-terminal part with the kinase domain and the RNase domain at the C-terminus is 41% identical and 56% similar over 482 amino acids to *S. cerevisiae* Ire1p. The C-terminal 232 amino acid segment is 22% identical and 38% similar over 227 amino acids to human Ribonuclease L (RNase L). There is a putative nuclear localisation signal at positions 732–751 in the *T. reesei* IREI protein possibly indicating nuclear

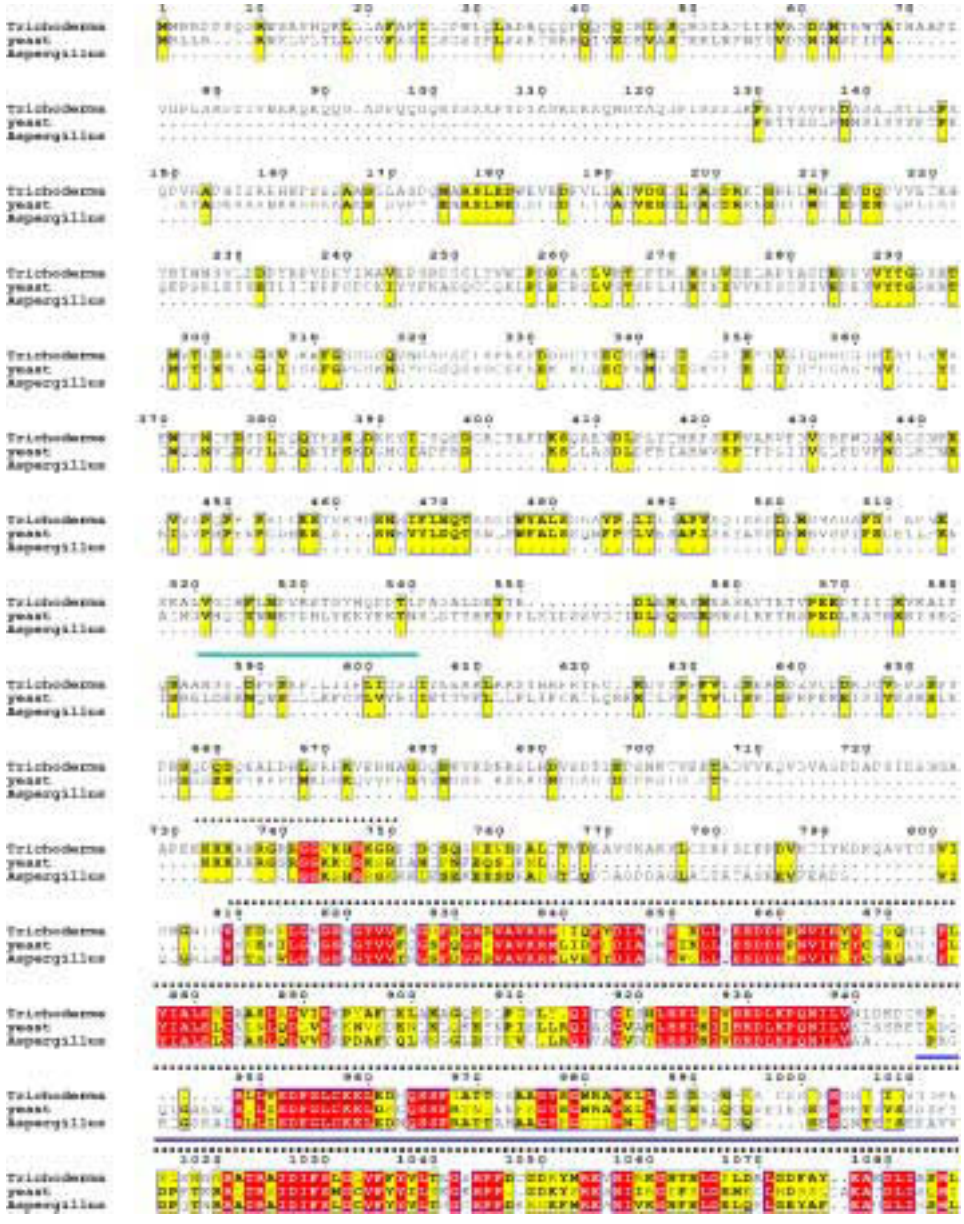
Trichoderma
yeast
Aspergillus

Figure 7. The alignment of the Ire1 proteins. The kinase domain is marked with dashed line, the putative RNase domain with blue line, the putative transmembrane domain with green line and the putative nuclear localisation signal with dotted line.

localisation of the C-terminus of the protein. In conclusion, the IREI protein is a type I transmembrane protein with clear conservation of the C-terminus compared to the other Ire1 proteins. The N-terminus of the *T. reesei* IREI protein has quite low homology to the other Ire1 proteins, but in general the N-terminus is less conserved between the different Ire1 proteins. In fact it has been shown that it can be replaced by the N-terminus of an other kinase PERK or even with a functional leucine zipper dimerisation motif without losing the kinase activity (Liu *et al*., 2000). Figure 7 shows the alignment of *T. reesei* IREI protein with *S. cerevisiae* Ire1p and the cloned *A. nidulans* IreA fragment.

T. reesei ptc2 encodes a protein of 438 amino acids. The predicted protein contains a protein phosphatase 2C signature (amino acids 62–70) included within a domain (amino acids 1–297) that is found in protein phosphatases of type 2C. There are two PP2C-type serine-threonine phosphatases in *S. cerevisiae* that are very similar to each other. The gene that we have cloned from *T. reesei* encodes a protein that is more homologous to the *S. cerevisiae* Ptc2p than to Ptc3p. The putative *T. reesei* PTCII protein has 48% identity to S. *cerevisiae* Ptc2p and 58% identity to a putative PTC2 protein from *S. pombe*. Comparison of *T. reesei* PTCII to *S. cerevisiae* Ptc2p and Ptc3p is shown in Figure 8.

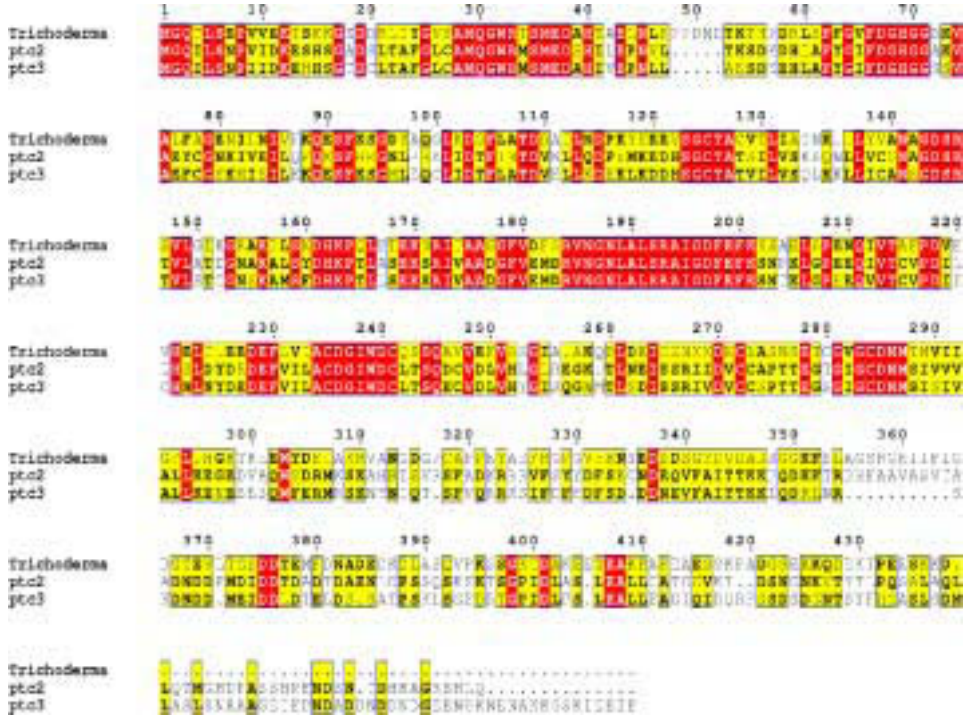

Figure 8. Alignment of S. cerevisiae ptc2, ptc3 and T. reesei PTCII (Tr) proteins.

3.2.2 Functional studies of *T. reesei* IREI and PTCII

To study the properties of the *T. reesei* IREI protein *in vitro*, a fragment from the *ire1* gene encoding the kinase and RNase domains (amino acids 607–1243) was fused with the glutathione S-transferase (GST) gene. The purification resulted in a protein of the expected size that reacted with the anti-GST-antibody (II, Figure 1B).

Autophosphorylation activity of the purified IREI-GST fusion was tested in an *in vitro* kinase assay (Welihinda and Kaufman, 1996). The assay shows that although some label was associated with the control GST, IREI-GST was efficiently autophosphorylated (II, Figure 1B). This indicates that *T. reesei* IREI protein has intrinsic protein kinase activity as do other Ire1 proteins (Mori *et al*.,

1993; Tirasophon *et al*., 2000; Tirasophon *et al*., 1998; Welihinda and Kaufman, 1996). Unfortunately the yield of the fusion protein was too low to perform *T. reesei hac1* mRNA splicing assays or dephosphorylation assay with *T. reesei* PTCII.

To test the functionality of the *T. reesei* IREI protein, the full-length *ire1* cDNA without two short upstream open reading frames was cloned into a yeast expression vector. The expression vector and the control vector were transformed into a yeast strain where the *IRE1* gene has been deleted. The transformants were screened for their requirement for inositol for growth (Nikawa and Yamashita, 1992). The strain expressing *T. reesei ire1* cDNA was able to grow in the absence of inositol, whereas the strain transformed with the vector alone was not (Figure 9). This showed that the *T. reesei ire1* was able to complement yeast *IRE1* deficiency, thus indicating that it is a functional homologue of the yeast protein.

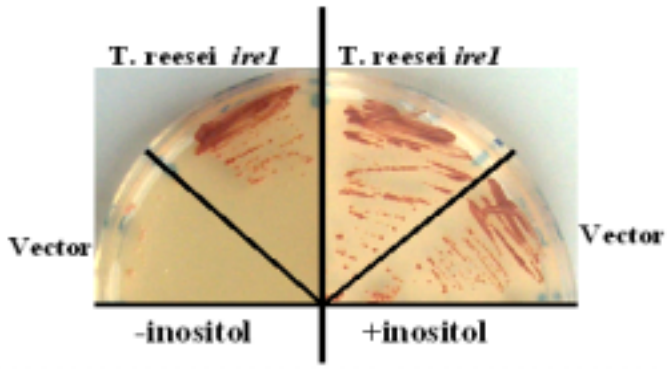

Figure 9. Complementation of the yeast IRE1 deficiency by T. reesei ire1.

To test the functionality of the *T. reesei ptc2* gene, complementation of *S. cerevisiae PTC2* disruption was tested. The *T. reesei ptc2* cDNA in a yeast expression vector and the expression vector alone were transformed into a yeast Δptc2p-strain (AWY506) and its parental strain (AWY500) both containing a reporter construct where *lacZ* gene is under a minimal promoter with a UPR element (strains obtained from Dr. R. Kaufman (Welihinda *et al*., 1998)). The glycosylation inhibitor tunicamycin (Tm) was used in the experiment as an inducer of the UPR pathway. Induction of UPR by tunicamycin resulted in a clear increase in the β-galactosidase activity in the strain with the wild-type

yeast *PTC2* gene. In the yeast *ptc2* disruptant strain, UPR induction by tunicamycin was almost twice as high as in the parental strain. When the *T. reesei ptc2* cDNA was expressed in the *ptc2* disruptant strain, the extent of UPR induction was about the same as in the yeast strain having an intact native *PTC2* gene. Yeast Ptc2p is a negative regulator of the UPR (Welihinda *et al*., 1998) and *T. reesei ptc2* could restore its negative effect on the extent of UPR induction in the *ΔPTC2* cells. Thus the results show that the *T. reesei* PTCII can complement the *S. cerevisiae* ptc2p deficiency (Figure 10).

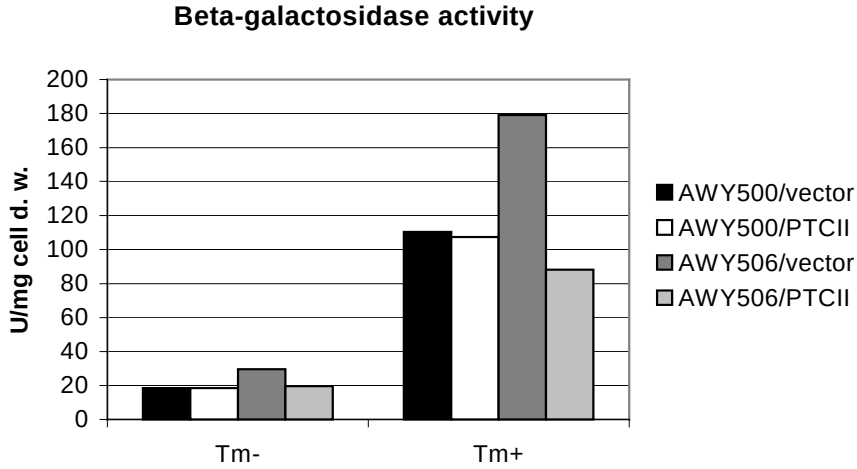

Figure 10. Complementation of the yeast PTC2 deficiency by T. reesei ptc2.Tm, tunicamycin.

Despite the fact that the *T. reesei* PTCII was able to complement the *S. cerevisiae* Ptc2p deficiency, the function of PTCII in the UPR of filamentous fungi still needs further evidence. The best way to gain such evidence would be to disrupt the gene in *T. reesei* and examine the phenotype of the disruption.

3.2.3 Regulation of *ire1* and *ptc2* in *T. reesei*

The expression of *ire1* and *ptc2* was studied in UPR-activated cells in order to reveal their possible autoregulation by the UPR pathway. *T. reesei* mycelia were treated with dithiothreitol (DTT) and Northern hybridisation from DTT-treated

and control mycelia was done with *ire1* and *ptc2* probes. There is low basal expression of the *ire1* gene both in the control and DTT-treated samples. In the DTT-treated samples, there is an initial decrease in the *ire1* signal levels, but after 30 minutes of treatment the expression levels begin to increase. The expression of the *ptc2* gene decreases until 90 minutes of treatment after which there is a slight increase in the expression (II, Figure 5). In the case of *ire1*, the initial decrease could be an indication of down-regulation similar to what has been observed in mammals. Recently it has been shown that the RNase activity of IRE1α down-regulates the *Ire1α* mRNA expression by cleavage at three specific sites at the 5′end of *Ire1α* mRNA (Tirasophon *et al*., 2000). The mechanism of the possible *ire1* regulation would appear not to be similar in *T. reesei* though, since no additional band is seen in the DTT-treated samples when compared to the control. From the Northerns of the *ire1* over-expression strains, it is difficult to conclude whether the endogenous *ire1* is expressed at a reduced level in the transformants than in the parental strain, because the two bands run so close to each other and the endogenous *ire1* is expressed at a very low level (II, Figure 2A).

The short upstream open reading frames (uORFs) found from the *ire1* cDNA may be involved in the regulation of the IREI translation. Short uORFs have been shown to affect the translation of some proteins when found in the transcript. The classical example is translational regulation of the *S. cerevisiae GCN4* encoding a transcriptional activator of genes involved in amino acid biosynthesis. It has been shown that there are four short uORFs in the leader of *GCN4* mRNA. Deletion of all these results in high level expression of the gene (Hinnebusch, 1997 and references therein). It is noteworthy that the *T. reesei hac1* encoding the UPR transcription factor has two uORFs that have a negative effect on formation of the HACI protein (Saloheimo *et al*., 2003a). The function of the short upstream uORFs in *ire1* mRNA remains to be elucidated.

3.3 Effects of UPR-pathway manipulation (II, III, IV)

Many methods have been used to increase the amounts of secreted foreign proteins both in *S. cerevisiae* and in filamentous fungi. Limiting amounts of ER-chaperones and foldases is one factor that may cause problems in foreign protein production. For this reason, the effects of over-expression of individual

chaperones or foldases on heterologous protein production have been studied intensively (Harmsen *et al*., 1996; Hayano *et al*., 1995; Robinson *et al*., 1994; Schultz *et al*., 1994; Shusta *et al*., 1998; Conesa *et al*., 2002; Moralejo *et al*., 2001; Ngiam *et al*., 2000; Punt *et al*., 1998; van Gemeren *et al*., 1998; van Gemeren *et al*., 1997; Wang and Ward, 2000).

In this study the effects of UPR on native and foreign protein production as well as gene expression were examined by constitutive activation of the UPR pathway. This method was used both in *S. cerevisiae* and in two filamentous fungi, *A. niger* var. *awamori* and *T. reesei*. The effects of *hac1* deletion were studied in *S. cerevisiae*.

3.3.1 Constitutive UPR induction by *hac1* expression (III, IV)

3.3.1.1 *S. cerevisiae*

The UPR pathway was induced in *S. cerevisiae* in two ways. Firstly, the spliced version of yeast *HAC1* was over-expressed. This *HAC1* form was lacking the 250 base intron that in normal conditions prevents the translation of the mRNA. Secondly, the *T. reesei hac1* without its 5' flanking region and the small intron was expressed in *S. cerevisiae*. Both constructs were transformed into strains producing *Bacillus amyloliquefaciens* α-amylase from an integrated construct. In both cases, the expression of the active Hac1 protein retarded the growth of the strains as has been observed also by others (Cox and Walter, 1996; Kawahara *et al*., 1997). The growth rate of the strain over-expressing *S. cerevisiae HAC1* was slightly retarded, whereas the growth of the strain expressing *T. reesei hac1* was clearly slower compared to the control (III, Figures 2A and 4A). An explanation for this may be that the *T. reesei hac1* construct induced the UPR to a higher extent than the yeast *HAC1* construct (see below).

To verify that the UPR was induced, Northern hybridisation was carried out with the yeast strains expressing *S. cerevisiae HAC1* or *T. reesei hac1*. It has been shown that the *KAR2* gene is a target for the UPR regulation, and therefore the Northern filters were probed with a *KAR2* probe and a *TDH1* probe for normalisation. The *S. cerevisiae HAC1* induced the *KAR2* expression less than

two-fold as compared to the parental strain, whereas the *T. reesei hac1* caused at highest over two fold induction (III, Figure 3D and 5D). The *KAR2* signal intensities in both cases increased after the third cultivation day in the parental strain, which may be due to the induction of the endogenous UPR pathway by production of the heterologous α-amylase or by general stress induction in the late growth stages. Expression of foreign proteins as well as over-expression of native proteins has been shown to induce the UPR pathway (Dorner *et al*., 1989; Menzel *et al*., 1997; Punt *et al*., 1998; Saloheimo *et al*., 1999). The higher UPR induction observed with the *T. reesei hac1* than with the yeast *HAC1* might be because this gene was expressed from a multicopy plasmid, whereas the *S. cerevisiae HAC1* was expressed from a single copy plasmid. Furthermore, the yeast *HAC1* construct did not contain the suggested 18 aa activation domain region located downstream of the 250 bp intron (Mori *et al*., 2000).

Secretion of the *Bacillus* α-amylase was studied in the *S. cerevisiae* transformants over-expressing *HAC1*. It was found that the average production level by the *HAC1* transformants was two-fold higher than by the strain transformed with the expression vector only (III, Figure 2B). Also, the effect of *HAC1* over-expression on *T. reesei* EGI production was tested, but no increase was obtained. Thus, it seems that the two foreign proteins behave differently under UPR induction. The periplasmic invertase levels were also measured and about two-fold increase was observed in the transformants with the *HAC1* construct (III, Figure 2C).

The heterologous α-amylase activity in the yeast transformants expressing *T. reesei hac1* was higher than by the strain transformed with the expression vector only from day three until the end of the culture. The difference between the transformants and the control was highest on the sixth day. At that time point the *hac1* transformants had produced 2.4-fold more active enzyme (III, Figure 4B). Again, invertase activities were measured from the strains. The transformants expressing *T. reesei hac1* produced slightly more invertase than the control strain, but the difference between the transformants and the controls was not very obvious (III, Figure 4C). Thus it seems that invertase production may be more sensitive to the extent of UPR induction and a balance needs to be found for optimal induction.

The question why more heterologous α-amylase was produced at the end of the culture, even though the UPR induction is more prominent in the early stages, is interesting. Since induction of components of the secretory machinery has been observed in *S. cerevisiae* in UPR-induced cells (Travers *et al*., 2000), it may be that the Hac1p mediated induction comes from the induction of the whole secretory pathway, rather than through induction of ER functions only. This effect could also be a secondary effect of UPR induction since it occurs later than the induction of the *KAR2* gene. On the other hand, the levels of the UPR-induced proteins may have remained at an increased level for a longer time than the corresponding mRNAs.

3.3.1.2 *A. niger* var. *awamori*

The effect of UPR pathway induction on protein production by the filamentous fungus *A. niger* var. *awamori* was investigated. The UPR-induced form of the *hacA* cDNA was expressed in strains producing *Trametes versicolor* laccase or bovine preprochymosin. The induced form was created by deletion of the unconventional 20-base intron and truncation of about 150 bp from the 5' flanking region. These two modifications of the *hacA* mRNA occur during UPR induction, and the modified *hacA* mRNA is likely to produce an active HACA protein. It has been shown in *T. reesei* and *A. nidulans* that the activation of the *hac1* and *hacA* genes, respectively, requires both truncation of the mRNA at the 5'-flanking region and unconventional splicing of a small intron (Saloheimo *et al*., 2003a). The transformants were first analysed by Southern hybridisation and a band of expected mobility derived from the expression construct was observed in the transformants. Transformants expressing constitutively active *hacA* were subsequently analysed by Northern analysis using non-transformed laccase and chymosin producing strains as controls. A *hacA* signal of expected size derived from the expression cassette was seen in all the transformants in addition to the signal derived from the endogenous *hacA* (IV, Figure 3). In all Northerns, there was a faint smaller band also seen in the control strains, possibly showing that the UPR was partially induced by the production of the foreign protein in these strains.

To examine the possible UPR induction in the *hacA* transformants, the Northern filters from the laccase producing strains were also probed with *A. niger* var.

awamori bipA and *pgkA* probes. In the early time points, the *bipA* gene was induced in the transformants 2–4-fold as compared to the control strain (IV, Figure 3). In the samples from the seventh day the difference had decreased and only 1.3–2-fold enhancement in *bipA* expression was observed in the transformants as compared to the controls (data not shown). This could be caused by the induction of the endogenous *hacA* gene or general stress responses in the late culture stage. A similar observation was made in *S. cerevisiae* (section 3.3.1.1). There is variation in the *bipA* levels in the different transformants that is in accordance with the *hacA* expression levels. The filter of the second day samples was also probed with *T. versicolor lcc1* probe (IV, Figure 3). Surprisingly, the laccase expression level was lower in the control strain than in any of the transformants in the time points studied. This could be partly due to titration of transcription factors affecting the *glaA* gene in the transformants, since both laccase and *hacA* are expressed from the *glaA* promoter (Verdoes *et al*., 1994). Probing with the *glaA* gene fragment showed that the gene was expressed at higher level in the control strain as compared to the transformants (IV, Figure 4D). On the other hand, the two genes, *amyA* and *amyB* encoding for α-amylases, were not affected by *hacA* over-expression (IV, Figure 4D). Therefore, general down-regulation of genes encoding secreted proteins in the *hacA* transformants was not apparent, even though this has been shown to occur in cells exposed to secretion stress in both *T. reesei* (Pakula *et al*., 2003) and *A. niger* (Watson *et al*., submitted). This would further indicate that the cause for lower *glaA* mRNA is the titration of the transcription factors.

The Northern filters made from the UPR-induced *A. niger* var. *awamori* strains were probed with novel genes encoding proteins with different functions in the secretory pathway. The orthologs of these genes in yeast have been shown to be induced by UPR (Travers *et al*., 2000). The *ino1* gene encoding inositol-3-phosphate synthase involved in lipid biosynthesis and *snc1* were not significantly affected in the *hacA* over-expressing transformants (IV, Figure 4B). The *ktr1* gene encoding mannosyltransferase involved in protein glycosylation in Golgi complex and *sec61* encoding a major component of the translocon complex, were clearly induced in all the transformants as compared to the parental strain. The induction of these genes was 1.5–3-fold in the transformants (IV, Figure 4C). The *nsfA* gene that is the homologue of yeast *SEC18* encoding a general vesicle fusion factor functioning at different steps of vesicle transport,

was also slightly induced in the transformants as compared to the parental strain (IV, Figure 4C).

The growth rates of the *hacA*-transformants and their parental strain derived from the *T. versicolor* laccase-producing strain were studied in shake flask cultures. The pH and the dry weight measurements both showed that the parental strain grew faster than the transformants for the first four days of the cultivation. After a lag period the transformants grew about as fast as the parental strain, finally reaching a slightly higher biomass than the control (IV, Figures 5B and 5C). The reduced growth of the hacA transformants is in agreement with results from *S. cerevisiae*, where constitutive UPR induction retards growth (III, Cox and Walter, 1996; Kawahara *et al*., 1997).

The laccase activities produced by the *hacA* transformants and the control strain were analysed. All the transformants produced more laccase than the control strain, but the levels varied between the transformants (Figure 11A). The production levels were highest in the transformants at day five, where the difference between the transformants and the controls was 3−7.6 - fold. By the end of the cultivation the differences between the transformants and the controls levelled out so that the laccase production of the transformants decreased while the parental strain still produced laccase into the medium. Thus, the transformants produced laccase much faster than the parental strain. An even more striking observation is that lower *lcc1* mRNA levels and somewhat lower amounts of biomass in the transformants were sufficient to produce much more of active laccase. This indicates that the posttranscriptional events of laccase production are clearly enhanced in the transformants. Chymosin production of *hacA*-transformants and the preprochymosin-producing control strain was measured on day five of the cultivation. The results showed that the production of chymosin was not increased to the same degree as observed for the laccase production. Still, most of the *hacA*-transformants produced 1.8−2.8-fold more active chymosin than the control strain (Figure 11B).

The levels of total secreted proteins and two native enzyme activities were measured from the *hacA*-transformants and the control of the *T. versicolor* laccase producing strain. The control strain accumulated 1.5−4-fold more total protein in the culture medium than any of the transformants (IV, Figure 6A). The activities of the two native enzymes, α-amylase and β-glucosidase, were higher

in the control strain. The *hacA*-transformants produced 3–7-fold less α-amylase in all time points tested (IV, Figure 6B). As for the β-glucosidase production, there was not much difference between the *hacA*-transformants and the control strain until day seven. In the day ten samples, the activity in the parental strain had increased almost four-fold, whereas the activity in the transformants had remained at about the same level (IV, Figure 6C).

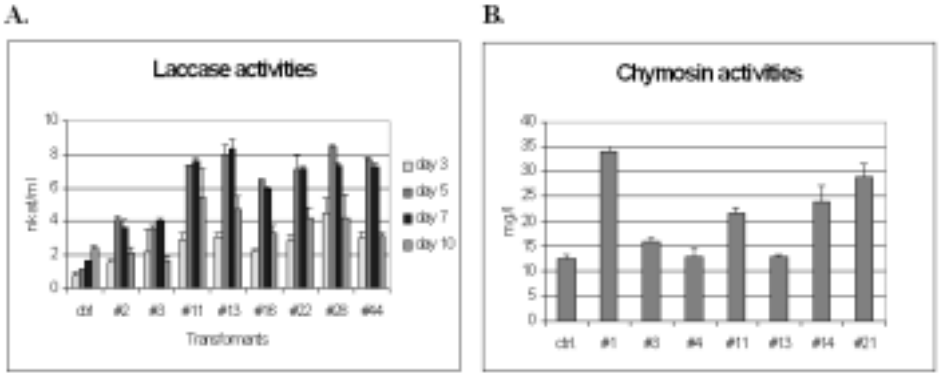

Figure 11. Laccase A) and chymosin B) activities measured from the culture supernatants of the A. niger var. awamori hacA over-expressing transformants and the parental strain. Chymosin activities are shown from the fifth culture day. Results shown are averages from two parallel cultures (+/-SD).

The differences seen in the total and native protein production can be partly explained by the slower growth of the *hacA*-transformants. In the case of the α-amylase activity, the test also measures glucoamylase activity. As stated before this is probably due to lower level of *glaA* transcript in the transformants. The strains were grown on maltose that has been shown to repress β-glucosidase gene expression. Since the control strain grew slightly faster that the transformants, it could be that the maltose was consumed faster by the control strain and the β-glucosidase gene is released from repression sooner than in the transformant cultures.

3.3.2 Constitutive UPR induction in *T. reesei* by *ire1* expression (II)

The UPR-pathway was induced in *T. reesei* by constitutive expression of the *ire1* cDNA without the two short uORFs, in a strain producing *Phlebia radiata* laccase. The transformants were first analysed by Southern blotting and four clones containing the *ire1* over-expression cassette were analysed further.

Probing of Northerns with *ire1* showed a single band of about 4.5 kb in the control strain and an additional mRNA of about 4 kb in the *ire1*-transformants, derived from the expression construct. In two *ire1*-transformants (2, 49) there was a very clear increase in the *ire1* mRNA levels when compared to the control strain. Also the *pdi1* and *bip1* expression levels were increased in these transformants, indicating strong UPR induction. The other two transformants (100, 137) had about two-fold *ire1* over-expression and a very modest *pdi1* or *bip1* induction as compared to the parental strain. The *hac1* probing showed a mRNA of about 2.5 kb in all the samples and a smaller band that represents the UPR-induced form of the *hac1* mRNA. This band occurred in all the transformants, but not in the parental strain. In transformants 2 and 49 the signal intensity of the smaller *hac1* band was much higher than in the other two transformants, which is in correlation with the extent of *pdi1* and *bip1* induction (II, Figure 2A).

The analysis of the *ire1*-transformants supports the idea that the *T. reesei* IREI is a functional homologue of the known Ire1 proteins. The over-expression of IRE1 in mammals has been shown to induce the expression of the *bip* promoter (Tirasophon *et al*., 1998; Wang *et al*., 1998b). Also, it has been shown that the Ire1 protein binds the chaperone Bip in the ER lumen and UPR induction is triggered by dissociation of Bip from Ire1 as excessive unfolded protein accumulates in the ER (Bertolotti *et al*., 2000; Okamura *et al*., 2000). This is consistent with our finding that a certain threshold level of *ire1* over-expression has to be exceeded before UPR induction can occur. Above this threshold the amount of IREI protein probably exceeds the amount of free BIPI protein in the ER.

The Northern filters made from the *ire1* over-expression strains were also probed with *T. reesei snc1*, *pmi40*, *lhs1*, *sec61* and *ino1* genes that represent functions from different parts of the secretory pathway. All these genes have been shown to be induced by the UPR in yeast (Travers *et al*., 2000). Yeast

pmi40p catalyses the interconversion of fructose-6-P and mannose-6-P in the early steps of protein mannosylation. Yeast lhs1p is an ER-resident chaperone that is required for efficient translocation of protein precursors across the ER membrane. Ino1p is involved in the rate-limiting step of inositol biosynthesis. The *T. reesei sec61* and *lhs1* genes were clearly induced in the two *ire1*-transformants with a strongly induced UPR. The other genes were less clearly induced in the *ire1*-transformants, but all genes showed at least a slight increase in expression when compared to the parental strain (II, Figure 2B and C).

The effect of *ire1* over-expression on protein production was analysed from cultures that were grown for nine days. The total protein measurements showed that the control strain secreted more protein to the supernatant than any of the *ire1*-transformants at all time points measured. The difference was at highest two-fold (II, Figure 3C). As the transformants grew slightly more slowly than the control strain (II, Figure 3B), the lower production of native protein might be partly explained by differences in growth. Alternatively, it could be caused by down-regulation of genes encoding secreted proteins that has been demonstrated in *T. reesei* during secretion stress (Pakula *et al*., 2003). However, a direct link between IREI and this down-regulation has not been shown.

The effect of *ire1* over-expression on foreign protein production was also studied. The strains were grown in buffered medium to maintain the laccase activity in the supernatant. Laccase activity measurements showed that there were no major differences in laccase production levels between the transformants and the control strain (data not shown).

3.3.3 The range of UPR regulation in filamentous fungi

The UPR pathway regulates many functions throughout the secretory pathway in yeast (Gasch *et al*., 2000; Ng *et al*., 2000; Travers *et al*., 2000). It has also been shown that there is a genetic link between the UPR and vesicle formation from the ER since *IRE1* over-expression suppresses yeast mutants defective in vesicle budding from ER (Higashio and Kohno, 2002; Sato *et al*., 2002). In accordance with this result, *HAC1* was identified in a screen where factors affecting the vesicle formation in the ER were searched (Higashio and Kohno, 2002). Recently it has also been shown in *Arabidopsis*, that a large number of genes

encoding proteins functional at different parts of the secretory system including chaperones, vesicle transport proteins, and ER-associated degradation proteins are upregulated in UPR-induced cells (Martinez and Chrispeels, 2003). A microarray study with mammalian cells, however, showed that the only UPR-inducible genes were either ER-resident chaperones and foldases or PERK-controlled genes (Okada *et al*., 2002). On the other hand, the lectin ERGIC-53, a marker for the ER-Golgi intermediate compartment that functions as a cargo receptor, has been shown to be induced by effectors of UPR. This indicates that also in mammalian cells at least some functions downstream from the ER in the secretory pathway are affected by the UPR (Nyfeler *et al*., 2003).

The results from our Northern analysis support the idea that at least a limited number of functions throughout the whole secretory pathway can be induced by constitutive UPR induction in filamentous fungi. This was seen by induction of genes that encode functions at different steps of the secretory pathway. Our results also indicate that there are differences in the regulation of the secretion-related genes between yeast and filamentous fungi. The fact that *ino1* was not clearly induced in the two filamentous fungi *T. reesei* and *A. niger* var. *awamori* was surprising, since in yeast this gene was among those that were most highly up-regulated during UPR induction (Travers *et al*., 2000). There is some contradiction in studies on the effects of UPR induction on *INO1* expression in yeast. It has been shown that a functional UPR is required for sustained expression of *INO1* in the absence of inositol (Cox *et al*., 1997). On the other hand, it may be that the UPR pathway is not directly involved in transcriptional regulation of *INO1*, because *INO1* expression was only slightly affected in wild-type cells treated with tunicamycin and grown in inositol-containing medium, whereas UPR was clearly induced in these cells (Chang *et al*., 2002). Mori *et al*., (2000) have shown that the unspliced Hac1p is able to suppress inositol auxotrophy of *Δhac1*, also indicating that the UPR induction is not directly needed for *INO1* expression.

The data obtained in this work of UPR induction of genes involved in protein secretion in filamentous fungi is consistent with the data published of *S. cerevisiae*. For example, the yeast *SEC18* gene is induced 2.7-fold by DTT-treatment and the *A. niger* var. *awamori* ortholog, *nsfA*, at the highest about 2-fold by *hacA* over-expression. In *T. reesei*, the *nfs1* gene is induced about 2-fold by DTT-treatment (Saloheimo *et al*., 2003b). The differences in the extent of

UPR induction in the yeast data and our study could be due to differences in the methods applied. In the yeast experiments, the UPR induction was obtained with DTT- or tunicamycin-treatments and in our studies with constitutive expression of the two UPR key factors. Also, since yeast and filamentous fungi are very different in their ability to secrete proteins, there may be differences in the regulation of the secretion-related genes.

3.3.4 Improvement of protein production

Our strategy to improve heterologous protein production involved upregulation of the ER folding machinery and a number of genes encoding functions over the entire secretory pathway by constitutive induction of the UPR. In this work (III), the secretion of both *Bacillus* α-amylase and *S. cerevisiae* invertase was increased in *S. cerevisiae* strains where the UPR was induced. Thus, the method used was successful not only in the enhancement of production of a foreign protein, but also native protein production was increased. In *A. niger* var. *awamori*, the induction of UPR pathway by *hacA* over-expression resulted in the enhancement of *T. versicolor* laccase and bovine chymosin production. Lignolytic enzymes have generally been difficult to produce in active form in heterologous systems, and likely reason for this is their poor folding and incorporation of prostethic groups, or poor secretion. Thus, it may be that the improvement of production seen in our study (IV) is due to enhancement of these processes. Unfortunately we did not have an antibody against the *T. versicolor* laccase so it could not be concluded if there was more of the protein produced or if the produced protein was more active in the *hacA* transformants. It has been shown that the secretion of chymosin is inefficient because the protein remains associated with the cell (Berka *et al*., 1991a). From our Western analysis, it seems that there is more mature chymosin produced in the *hacA* transformants than in the control strain indicating that the folding and/or secretion of the protein is enhanced. Why is the chymosin production less induced than *T. versicolor* laccase? One possible explanation could be that there are differences in the folding complexity of chymosin and laccase proteins. Chymosin is a smaller protein (381 aa), compared to the *T. versicolor* laccase (519 aa), but contains one disulphide bond more than laccase. Chymosin consists of one protein domain whereas laccase has three β-barrel domains folding together. In addition, laccases contain bound copper ions as co-factor and these

are thought to be added to the protein within the secretory apparatus (reviewed by O'Halloran and Culotta, 2000). Still, although the crystal structure of both proteins has been solved (Gilliland *et al*., 1991; Piontek *et al*., 2002), it is difficult to conclude what features might affect their secretion.

In *T. reesei*, on the other hand, the production of calf preprochymosin and CBHI-chymosin fusion protein were not improved by constitutive expression of active form of *hac1*. The levels of *pdi1* and *bip1* were elevated in the *hac1*-transformant strains as compared to the control strain, which indicates that the UPR was induced (M. Saloheimo, in preparation). As shown in this work (II) the *ire1* over-expression had no effect on production of *P. radiata* laccase in *T. reesei*. The laccases from *T. versicolor* and *P. radiata* are both from basidiomycetes and rather similar in sequence (64% identical). It has been indicated that the *T. versicolor* laccase structure is quite similar to other known enzymes of the same family (Piontek *et al*., 2002). Thus, it seems likely that also the *P. radiata* laccase is similar in structure as well. This does not rule out the possibility that secretion of these two laccases is limited at different stages of production or at different steps of the secretory pathway. Another explanation could be that the observed differences seen in the effect of UPR induction on protein production are caused by differences in the secretory pathways of the two filamentous fungi.

As it has already been stated, the method used in this work for increasing foreign protein production is not suited for all foreign proteins. An example of this was the difference seen in *S. cerevisiae* in the secretion of *Bacillus* α-amylase and *T. reesei* EGI. The production of EGI could not be improved by the activation of UPR, whereas there was a clear increase in α-amylase production. This can be due to differences in the folding or secretion processes of the two proteins in yeast. In conclusion, improvement of foreign proteins with any given approach appears to be successful only with certain protein-host combinations.

There was a clear difference between *S. cerevisiae* and filamentous fungi in the effect of UPR induction on the production of native proteins. It is interesting that the invertase production was more enhanced in the *HAC1* over-expressing strain than in the *T. reesei hac1* expressing yeast strain (III, Figures 2C and 4C). This was probably due to differences in the extent of UPR induction between the two strains. On the other hand, the levels of native proteins were decreased in UPR-

induced strains of *A. niger* var. *awamori* (IV) and *T. reesei* (II). This could partly be due to slower growth of the transformants caused by the constitutive induction of the UPR pathway. There are other possible explanations. One could be that the induction of UPR somehow favours the secretion of foreign proteins over native ones. Also it is possible that the genes encoding secreted proteins are down-regulated in UPR-induced strains. It has been shown in *T. reesei* that the expression of genes encoding secreted proteins was decreased in conditions that induce UPR (treatments with DTT and brefeldin A) (Pakula *et al*., 2003), and similar findings have been made in *A. niger* (Watson et al., submitted). There is preliminary data indicating that the mRNA levels of *xyn1* encoding xylanase 1 and *egl1* encoding endoglucanase 1 were decreased in the early culture stages of the *T. reesei ire1* over-expressing strains (days 2 and 3). In later time points the downregulation of these genes is not observed any more (data not shown). We did not get evidence for the down-regulation in *A. niger* var. *awamori hacA* overproducing strains, since the two genes encoding α-amylases are not affected by *hacA* over-expression. However, it has not been directly shown that these genes would be down-regulated during secretion stress. Although the data is still preliminary, there seems to be differences in the regulation of genes encoding secreted proteins between these two fungi under UPR induced-conditions.

3.3.5 Effects of *HAC1* deletion in *S. cerevisiae* (III)

The *HAC1* gene was disrupted from the *S. cerevisiae* genome by replacing it with a marker gene. The disputants were selected on the basis of their requirement of inositol for growth (Nikawa *et al*., 1996). The inositol-dependent clones were analysed by Southern hybridisation to verify the disruption. *Bacillus* α-amylase and *T. reesei* EGI were then expressed in *HAC1* disruption strain and the effect of the disruption on protein production was tested. There was a clear reduction in the production of both proteins in the disruptant strains: the α-amylase was produced at 70–75% lower level than in control strains transformed with the empty vector (III, Figure 1B). The EGI production was about 50–60% of the level of the control strain (III, Figure 1D). Growth of the strains was not impaired when compared to the control strains (III, Figures 1A and 1C).

The results of these experiments show that an intact UPR-pathway is beneficial for the production of Bacillus α-amylase and *T.* reesei EGI in yeast. They further

suggest that the expression of foreign proteins induce the yeast UPR at least to some extent. Since no UPR induction occurs in a *HAC1* disruptant strain (Cox and Walter, 1996; Nikawa *et al*., 1996), the reduction seen in the production of the model proteins is likely to be caused by limitation of some function that is under the control of the UPR pathway. It is likely that ER-associated degradation of the unfolded protein forms occurs at a considerable rate in the *HAC1* disruptant strain, although it has been shown that the ERAD pathway requires the active UPR to be fully functional (Ng *et al*., 2000; Travers *et al*., 2000). Thus, it could be that a major part of the foreign protein is lost due to ERAD, if e.g. folding or transport of the proteins becomes compromised in the *HAC1* disruptant. It may also be that there is a general slow down of the secretory functions due to the loss of UPR in the *HAC1* deleted strain. However, this would have to be targeted selectively towards the foreign proteins since the growth of *S. cerevisiae* was not affected and thus the secretion of cell wall material and membrane proteins is likely to occur at similar rate as in the control. It has been reported that *S. cerevisiae* has different secretory vesicle populations (David *et al*., 1998; Harsay and Bretscher, 1995; Roberg *et al*., 1997). It is, at least in theory, possible that the native proteins needed for cell growth and the foreign proteins are transported in the different vesicle populations and that the different populations are differentially regulated.

4. Conclusions and future perspectives

The present data on genes encoding components of the secretory pathway from *T. reesei* and other filamentous fungi indicate that the basic machinery for vesicle trafficking in these organisms is similar to other eukaryotes. The functional tests made in this study further indicate that the vesicle trafficking system operates through similar mechanisms. The responses of secretory genes to stress conditions caused by impaired protein folding or secretion show both similarities and differences to the corresponding mechanisms in the major experimental systems, *S. cerevisiae* and animal cells.

The present study gives further insight into the UPR-pathway of filamentous fungi. The complementation of yeast IRE1 and PTC2 disruptions by *T. reesei ire1* and *ptc2* shows that they are functional homologues of these *S. cerevisiae* genes. Induction of a number of potential UPR target genes by over-expression of *ire1* in *T. reesei* confirmed that it is a component of this regulatory pathway. The constitutive expression of *A. niger* var. *awamori hacA* also showed that this gene encodes the UPR transcription factor. Analysis of the modified *T. reesei* and *A. niger* var. *awamori* strains with constitutive UPR induction suggested that UPR regulates a large number of genes involved in different functions of the secretory pathway.

Due to the failure to produce sufficient amounts of T. reesei IREI protein in this study, we were not able to show splicing of the *T. reesei hac1* mRNA by IREI and dephosphorylation of IREI by PTCII. For future studies, it would be highly interesting to investigate in more detail these mechanisms, especially since the involvement of Ptc2p in the regulation of the UPR-pathway has so far been shown only in *S. cerevisiae*. It would also be interesting to study the protein levels of ER chaperones and foldases in the cells to see how they react to UPR induction, for example, because it has been shown that BiP expression is controlled at the translational level in animal cells.

The most important novel finding in this study was that we were able to increase protein production in *S. cerevisiae* and *A. niger* var. *awamori* by constitutive induction the UPR-pathway. The study showed that there are differences in the applicability of UPR activation as a means to improve foreign protein

production. A closer study of the secretion of different proteins in the UPR-induced cells might show if there is a differential need for folding or chaperone functions that could affect protein production. This could facilitate designing successful host - protein combinations to achieve optimal yields of a protein of interest.

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Functional studies of the secretory pathway of filamentous fungi

The effect of unfolded protein response on protein production

Abstract

This study describes the cloning and characterisation of three genes from the filamentous fungus *Trichoderma reesei*. *Snc1* encoding a putative v-SNARE was shown to be the functional homologue of *Saccharomyces cerevisiae SNC1* and *SNC2* genes that encode proteins shown to function in exo- and endocytosis. Two components of the unfolded protein response (UPR) pathway have also been identified. *Ire1* encodes a serine /threonine kinase that has been shown to be involved the sensing of the protein folding status in the ER and transferring the signal to the nucleus. *Ptc2* on the other hand, encodes a putative negative regulator of UPR. Both genes were isolated from *T. reesei* and shown to be the functional homologues of *S. cerevisiae* genes.

The effects of UPR activation on gene expression and protein production in *S. cerevisiae* and two filamentous fungi, *T. reesei* and *Aspergillus niger* var. *awamori* is also presented. The data shows that the UPR induction, either by *HacA* or *Ire1* overexpression in *A. niger* var *awamori* and *T. reesei*, respectively, induces the expression of ER-resident foldase (protein disulphide isomerase, *PDI*) and chaperone, *Bip*. Moreover, the UPR induction induces the expression of genes encoding functions at different steps of the secretory pathway in both fungi. This is in correlation with results obtained in different organisms.

The UPR induction was in this study shown to induce production of secreted proteins both in *S. cerevisiae* and *A. niger* var. *awamori*. In yeast the UPR induction by constitutive over-expression of activated yeast *HAC1* and *T. reesei hac1* resulted in induction of both native and foreign protein production. On the other hand, deletion of *HAC1* resulted in decrease in protein production. In *A. niger* var. *awamori* only the production of foreign proteins was induced in *HacA* over-expressing transformants. The production of native proteins was lower in these transformants compared to the controls. The over-expression of *Ire1* in *T. reesei* had no effect on foreign protein production.

Keywords
filamentous fungi, *Trichoderma reesei*, *Saccharomyces cerevisiae*, cloning, characterization, genes, unfolded protein response, gene expression, protein synthesis, protein secretion

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