Functional genomics to study protein secretion stress in Aspergillus niger

Silva Pinheiro Carvalho, N.D.

Chapter 6

General Discussion

6.1. General discussion

Aspergillus niger is a widely used host for the industrial production of enzymes as a result of its natural and high secretion capacity of proteins to the extracellular environment. As a result of many years of investigation and strain improvement, the production of many *A. niger* native enzymes have successfully improved resulting in high production levels, whereas researchers still face several problems in reaching satisfactory production levels of heterologous proteins. Many studies have been focusing on the understanding of the metabolism and physiology alongside with genetic engineering of many steps of the secretory pathway (reviewed in Fleissner and Dersch, 2010). A main bottleneck for heterologous protein production is attributed to difficulties of the host in the folding and maturation of foreign proteins in the ER. An increase bulk and flux of proteins in the ER often results in protein misfolding and/or misassemble, triggering an increase in the ER folding machinery and quality control mechanisms, a condition known as ER stress or protein secretion stress (Guillemette *et al*., 2007). This thesis investigates the effects of the modulation of different components of the *A. niger* secretory pathway, with special emphasis on the Unfolded Protein Response (UPR) and Endoplasmic Reticulum Associated Degradation (ERAD) pathways and its relation to protein secretion efficiency. The main conclusions and arisen questions from our research will be addressed and discussed in more detail in the following sections.

6.1.1. The double-edged sword of HacA activation

The UPR is a signal transduction network activated by ER stress or perturbations in the ER homeostasis. Removal of a 20 nt intron from the *hacA* mRNA by IreA activates the UPR pathway (Mulder *et al*., 2004), which affects a large number of genes (Guillemette *et al*., 2007). In Chapter 5 we have explored the physiological and transcriptomic consequences of an *A. niger* strain bearing a constitutive active *hacA* form, therefore expecting a full activation of genes under HacA control and other components of the UPR pathway. In Fig. 1 is depicted a schematic representation of pleiotropic effects of HacA in *A. niger* and its presumed involvement in the down- regulation of genes encoding secretory enzymes.

Like in the *S. cerevisiae* UPR, HacA binding results in a direct activation and transcriptional up-regulation of folding enzymes (Travers *et al*., 2000; Gasser *et al*., 2007). Mulder and co-workers (2006) have define a set of chaperones and foldases that contain putative UPR elements or UPR binding sites in their promoter region, which include *bipA*, *cypB*, *pdiA*, *prpA*, *tigA* and the transcription factor *hacA* itself. Studies in *S.cerevisiae*, *A. niger* and in mammalian cells where Hac ortologs (Hac1/HacA/XBP1) and the UPR have been studied have shown that this pathway is not solely a linear response engaging transcription factor activation followed by folding machinery up-regulation, but involves the up-regulation of many other genes involved in different processes throughout the secretory pathway, such as glycosylation, intracellular vesicular transport between organelles, membrane proliferation, ERAD, among others (Travers *et al*., 2000; Shaffer *et al*., 2004; Kimata *et al*., 2006; Arvas *et al*., 2006; Gasser *et al*., 2007; Guillemette *et al*., 2007; Bommiasamy *et al*., 2009). Analysis of our data set

(Chapter 5) revealed that only about 10% of the HacA^{CA} up-regulated genes seem to contain an UPRE sequence in the proximity of the start codon (≤-400bp), making it unlikely that the large set of HacA^{CA} induced genes will be under direct HacA regulation. From this point of view, it seems more plausible that more regulators will have a role in activating different secretory machinery through a cascade signal activated by HacA or unidentified HacA target(s). Following research should focus on the identification of regulatory elements that coordinate the expression of the genes involved in protein synthesis and secretion.

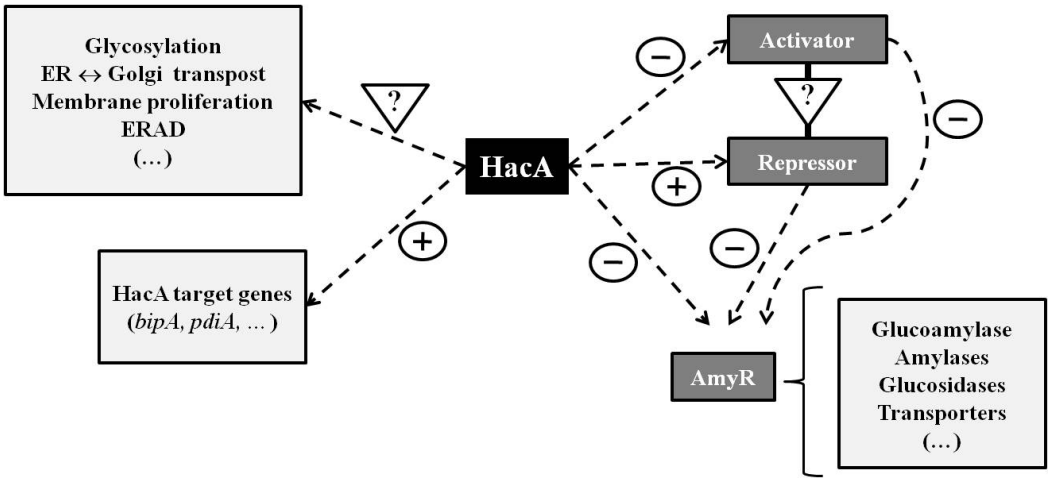

Figure 1. Schematic overview of the consequences of a constitutive activation of the UPR transcriptional regulator HacA and putative models for its role on RESS.

The repression of extracellular enzymes transcription has also been related to the activation of the UPR pathway and describes the phenomena known as response to secretion stress (RESS). The down-regulation of secreted enzymes such as glucoamylase (Al-Sheikh *et al*., 2004), xylanases and cellulases (Pakula *et al*., 2003) reflects an effort from the fungi to prevent the entry and overload of newly synthesized proteins into the ER. In our study (Chapter 5) we have shown that down-regulation is not confined to single carbohydrate degrading enzymes, like glucoamylase, but extends to the transcription factor and its regulon, in our case the AmyR regulon. These results obtained by transcriptomic analysis could be experimentally shown by the inability of HacA^{CA} to grow on starch (Chapter 5, Fig. 7). The mechanism(s) that regulates the repression of AmyR and its target genes under constitutive HacA activation is not known but some hypothesis can be considered. Al-Sheikh and co-workers (2004) have shown that repression involves a 1.0-2.0 kb promoter region of the *glaA* gene. As no UPRE were described in this region, a direct binding and repression by HacA to these secreted enzymes, transporters or *amyR* itself seems unlikely. We speculate that AmyR repression might come from the activation of an unidentified repressor, directly or indirectly involving HacA. Alternatively, the down

regulation of an unidentified AmyR activator also seems plausible (Fig. 1). These putative repressor or activator proteins may be present in the large set of genes of unknown function found to be differentially expressed in the $HacA^{CA}$ strain (Chapter 5, Supplementary Table S1). The RESS mechanism resembles the mammalian UPR branch regulated by PERK. Upon UPR activation, this transcription factor mediates a mechanism which prevents the influx of new proteins into the ER by mRNA translation attenuation (Fels and Koumenis, 2006).

Although we did not obtain evidence for ER membrane proliferation in the $HacA^{CA}$ strain (unpublished data), up-regulation of lipid-biosynthesis related genes in the $HacA^{CA}$ strain suggests an expansion of ER to harbour and increase the protein folding capacity in this organelle. The involvement of XBP1 (the HacA orthologue in mammalian cells) in events beyond ER stress response has been well described in professional secretory mammalian systems, suggesting a broader role for XBP1 (Shaffer *et al*., 2004; Bommiasamy *et al*., 2009). The idea of a "physiological UPR" activated in secretory cells different from a "stress UPR" activated by a disturbed ER homeostasis, as described for secretory mammalian systems (Gass *et al*., 2002), might represent a new UPR paradigm in filamentous fungi and has not been associated with the *S. cerevisiae* UPR. Recent evidence for a "physiological UPR" in *A. niger* comes from the study of Jørgensen and co-workers (2009). The high secretion capacity of extracellular enzymes by *A. niger* comes from its saprophyte lifestyle. The transcriptomic response when growing on maltose (Jørgensen *et al*., 2009) much resembles the "physiological" UPR in mammalian systems (Gass *et al*., 2002; Shaffer *et al*., 2004) and comprises a large overlap of the gene set observed under constitutive *hacA* activation (Chapter 5). Hence, the transcriptional regulation of secretory pathway genes reflects a general mechanism for modulation of secretion capacity in response to the conditional need for extracellular enzymes. Hence, although *A. niger* shares many common features with the *S. cerevisiae* UPR, new evidences point to a closer relation to the UPR system as it has been described secretory mammalian cells. The impaired growth of Δ*hacA* (Chapter 2) and reduced growth of $HacA^{CA}$ (Chapter 5) suggests that the activity of HacA should be fine tuned and controlled to sustain optimal growth.

6.1.2. Can heterologous protein production benefit from constitutive *hacA* activation?

Aspergillus niger is frequently used as a microbial cell factory for the production of heterologous proteins, not only for being able to take care of post-translational modifications essential for protein activity, but mostly because of its natural high secretion capacity. However, producing a foreign proteins alongside with the complexity of folding, trafficking and secretion processes often results in undesirable lower yields and/or accumulation of unfolded proteins in the ER, which can be toxic for the cells. Increasing knowledge on the UPR pathway and its association with the increase of folding related enzymes, ER proliferation and overall secretory pathway expansion (Travers *et al*., 2000; Arvas *et al*., 2006; Guillemette *et al*., 2007; this thesis), led to the study and modulation of individual UPR components to increase heterologous protein production. Some studies in *S. cerevisiae* have shown, for example, that overproducing BiP

increases the secretion yields of several heterologous proteins (Shusta *et al*., 1998; Harmsen *et al*., 1996; Kim *et al*., 2003). Similar results were found in other fungi, not only when overexpressing BiP (Lombraña *et al*., 2004), but also other folding enzymes like Pdi (Robinson et *al*., 1994; Moralejo *et al*., 2001; Smith *et al*., 2004) and calnexin (Conesa *et al*., 2002; Klabunde *et al*., 2007). On the other hand, some studies revealed that is not always the case that increasing the folding machinery has benefits on protein production (Harmsen *et al*., 1996; Robinson *et al*., 1996; van Gemeren *et al*., 1998; Ngiam *et al*., 2000; Smith *et al*., 2004) and can even be detrimental (van der Heide *et al*., 2002). These studies show that the yields of heterologous protein obtained is variable and much depends on the properties of the protein expressed. Other approaches have focused on enhancing the secretory pathway by overexpressing the UPR transcriptional regulator HAC1/HacA. In *S. cerevisiae*, overexpressing *T. reesei* HAC1 improved the secretion yields of *Bacillus* α-amylase, but had no effects on *T. reesei* endoglucanase I secretion; whereas disruption of *HAC1* in *S. cerevisiae* decreased the secretion of these two heterologous proteins (Valkonen *et al*., 2003a). In *P. pastoris*, expressing *S. cerevisiae HAC1* increased the secretion rate of a Fab antibody fragment (Gasser *et al*., 2006). More recently, Guerfal and co-workers (2010) have shown that overexpressing Hac1p in *P. pastoris* could slightly improve the surface expression levels of mouse interferon-γ, human thrombomodulin and human erythropoietin but not the levels of human interferon-β. Overproduction of *hacA* in *A. awamori* increased *T. versicolor* laccase and *preprochymosin* production (Valkonen *et al*., 2003b).

We previously showed that the expression of a Glucoamylase-Glucuronidase (GlaGus) fusion construct in *A. niger* did not lead to a successful secretion of this protein into the growth medium (Chapter 4). To examine whether constitutive activation of HacA had a positive effect on the secretion of the GlaGus protein the GlaGus fusion construct was transformed to the $HacA^{CA}$ and $HacA^{WT}$ strains (Chapter 5). For both strains a transformant was selected that contained a single copy of the GlaGus construct (pBB19-3pyrG*, Chapter 4). Expression is driven by the *gpdA* promoter. Western blot analysis (Fig. 2) was performed on medium samples and total protein extract samples. No GlaGus protein could be detected in the culture medium in both strains using the Gus antibody (data not shown). Fig. 2 illustrates that when using an antibody against Gus, relatively low amounts of intracellular GlaGus could be detected in both $HacA^{CA}$ and $HacA^{WT}$ strains. No activity could be measured in protein extracts or culture media of either strains expressing GlaGus (data not shown). This suggests that production of this heterologous fusion protein is difficult (low amounts) in the wild-type strain and doesn't seem to be much improved by a constitutive activation of HacA. Furthermore, the Gus antibody recognized smaller proteins in the protein extract of the $HacA^{CA}$ strain which might suggest that the proteolytic fragments are more stable in the $HacA^{CA}$ strain. A possible explanation might be the increased abundance ER-chaperones in the $HacA^{CA}$ strain that prevent rapid degradation. We cannot rule out the possibility that glycosylation of Gus might interfere with its enzymatic activity.

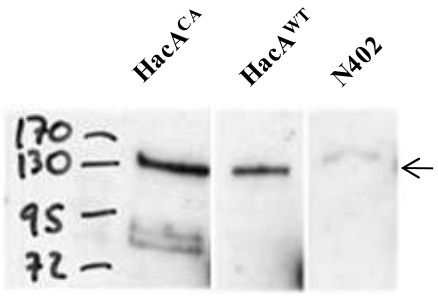

Figure 2. Effects of a constitutive activation of HacA on the amounts of GlaGus fusion protein in total intracellular protein extracts. Western analysis was performed on samples from HacA^{CA}, HacA^{WT} and N402 (negative control) grown in CM for 24h at 30°C. Ten micrograms of total protein were separated by gel electrophoresis and immunodetected with an anti-Gus antibody. Detection was carried out through a chemiluminescence reaction for 30 minutes. Arrow indicates the expected GlaGus band size.

Can heterologous protein production benefit from artificial activation of HacA? Results from literature are controversial but most seem to point out a beneficial effect of HacA activation on production levels. The results presented in Fig. 2 are preliminary but suggest that β-glucuronidase could be one of the heterologous where the production and secretion cannot be improved by manipulating *hacA* activation. On the other hand, even though the HacA^{CA} shows a limited growth phenotype and most of the central metabolism is repressed (Chapter 5); it is still capable to support similar levels of heterologous protein (GlaGus) as observed in the HacA^{WT} strain (Fig. 2), and therefore this topic deserves further attention. It is clear from previous studies and this preliminary study that constitutive activation of *hacA* does not per definition results in higher production levels. It is likely that each heterologous protein to be produced is unique and will challenge the host in a different way.

6.1.3. ERAD: the only way to destruction?

The UPR and the ERAD are two pathways which have been shown to be linked and cooperatively work when the ER is overloaded with misfolded proteins (Travers *et al.*, 2000; Casagrande *et al.*, 2000; Ng *et al.*, 2000; Friedlander *et al.*, 2000). The ERAD pathway is part of a complex ER quality control (ERQC) system that monitors protein folding and assembly as well as detects and targets terminally misfolded proteins for destruction (reviewed in Sayeed and Ng, 2005). Studies in *S. cerevisiae* and mammalian systems have identified several ERAD components and collectively infer that ERAD substrates are recognized, targeted, retrotranslocated, polyubiquitylated and finally degraded by the 26S proteasome (reviewed in Vembar and Brodsky, 2008). In *A. niger* the ERAD pathway is still poorly understood. In Chapter 4 we have given the first steps on the identification and characterization of ERAD components that act at different levels within this pathway. Surprisingly, none of the ERAD genes studied – *derA*, *mnsA*, *mifA*, *doaA* and *hrdC* – was found to be essential in *A. niger*. Moreover, phenotypic differences from the deletions and the wild-type strains were limited to the Δ*doaA* strain. Deletion of *doaA* in *A. niger* resulted in an irregular morphology and reduced

sporulation phenotype. In *S. cerevisiae*, Doa1p plays a role in the ubiquitin-dependent protein degradation by a direct interaction with Cdc48p (Ogiso *et al*., 2004; Mullally *et al*., 2006). It has also been shown in *S. cerevisiae* that Doa1 has a role in cell morphology (Kunze *et al*., 2007) and in the response to DNA damage (Hanway *et al*., 2002); therefore, the Δ*doaA* phenotype observed may not entirely relate to ERAD. These (phenotypic) results did not vary when the deletion strains were challenged to express or over-express a heterologous protein, although degradation seemed to be compromised in some strains (Chapter 4). The lack of a clear phenotype when deleting individual ERAD genes seems coherent with the idea that under normal growth and no ER stress conditions this pathway is not activated (only basal levels). However, the production of heterologous proteins is usually associated with ER stress; and the often low protein production yields obtained are attributed, to some extent, to degradation mechanisms by the host (Gouka *et al*., 1997).

According to *S. cerevisiae* model, Hrd3p is responsible for the activity and stability of Hrd1p (E3 ubiquitin ligase) (Plemper *et al*., 1999b; Gardner *et al*., 2000). Der1p is part of the Hrd1 complex and it has been attributed a role in the retrotranslocation of targeted proteins to be delivered to the proteasome (Lilley and Ploegh 2004; Goder *et al*., 2008). The Sec61p is the retrotranslocation channel associated with ERAD substrates during degradation (Gillece *et al*., 2000). In the absence of DerA, Sec61p might act as an alternative retrotranslocon for Der/Hrd substrates. Additionally, in *S. cerevisiae*, some substrates of this complex have been shown to be targeted to destruction by a HRD/DER independent pathway (Haynes *et al*., 2002). If such pathway exists in *A. niger* remains to be investigated but could account for an alternative degradation pathway if HrdC fails to activate the HdrA complex.

In mammalian cells, *Mif1* is an UPR target and contains an ER stress-responsive element (van Laar *et al*., 2000). Its function seems to be to mediate the translocation of the 26S proteasome from the cytoplasm to the ER membrane upon ER stress (van Laar *et al*., 2001). Moreover, studies have shown MIF1 forms a complex with HDR1 (Schulze *et al*., 2005) and that the knockdown of this gene leads to the stabilization of ERAD substrates (Hori *et al*., 2004). *Mns1* codes for a α1,2-mannosidase which is responsible for the cleavage of the α1,2-mannose from misfolded glycoproteins, an event that will lead the protein to be eliminated by the ERAD machinery (Cabral *et al*., 2001; Termine *et al*., 2009). *Mns1* disruption or inhibition has shown to stabilize glycoproteins (Jakob *et al*., 1998; Liu *et al*., 1999). If both *mifA* and *mnsA* deletions also stabilize ERAD substrates in *A. niger*, it is possible that these substrates are no longer recognized as misfolded and continue their journey to the following organelle. On the other hand, under Δ*mifA* and Δ*mnsA* conditions, ERAD substrates might accumulate in ER sub-compartments, as the ERACs (ER-associated compartments) proposed for *S. cerevisiae* (Huyer *et al*., 2004). These ERACs are suggested to be retaining sites where some misfolded proteins are targeted to in order not to interfere with normal cellular functions (Huyer *et al*., 2004). Similar structures (Quality Control compartments) have been described in some mammalian cells but are often associated with the development of diseases (Kopito, 2000; Markossian and Kurganov, 2004; Rodriguez-Gonzalez *et al*., 2008). In *S. cerevisiae* , the ERACs have no negative effects on protein traffic

through the ER nor lead to UPR induction; and the retained substrates will eventually be degraded via the proteasome (Huyer *et al*., 2004) or autophagy (Fu and Sztul, 2009).

Alongside with the ERAD-proteasome, vacuoles represent, among other functions, the second degradation system (Klionsky *et al*., 1990). Vacuoles contain many proteases (e.g. vacuolar proteases proteinase A (Pep4p), carboxypeptidase Y (CPY), proteinase B (Prb1p), carboxypeptidase S (CPS)) that are relatively nonspecific and directed toward degradation of a large variety of substrates (reviewed in van den Hazel., 1996). Substrates are directed to the vacuole for degradation by different routes, e.g., plasma membrane proteins are targeted to the vacuole in both a constitutive manner, as a mean of continually refreshing the amino acids population; or a signal dependent manner, in order to down-regulate signalling or transport functions (reviewed in Li and Kane, 2009). Vacuoles are also involved in constitutively removing cytosolic and organellar proteins, and under starvation conditions basal levels of autophagy possibly account for the degradation of these proteins (Mizushima and Klionsky, 2007). To understand the possible role of vacuole in the degradation of heterologous proteins, it will of interest to identify mutants in which the biosynthesis or transport step to the vacuole is hampered. As the vacuole is an essential organelle such approaches require the generation of conditional mutants in which the vacuolar function can be blocked temporarily.

Impairing the ERAD pathway results in ER-retention of degradation-substrates (Chapter 4) and this could become toxic for the cells. The fact that *A. niger* is able to cope with this may suggest the existence of alternative mechanisms or by-pass pathways to relief the cells from the accumulation of misfolded proteins. The strategy of modulating the ERAD pathway as an approach of strain improvement for heterologous protein production requires a deeper knowledge on “if” and “how” these alternative mechanisms in filamentous fungi co-operate to deliver substrates for destruction.