The Effects of Drugs Inhibiting Protein Secretion in the Filamentous Fungus *Trichoderma reesei*

EVIDENCE FOR DOWN-REGULATION OF GENES THAT ENCODE SECRETED PROTEINS IN THE STRESSED CELLS

To study the mechanisms of protein secretion as well as the cellular responses to impaired protein folding and transport in filamentous fungi, we have analyzed *Trichoderma reesei* cultures treated with chemical agents that interfere with these processes, dithiothreitol, brefeldin A, and the Ca^{2+}-ionophore A23187. The effects of the drugs on the kinetics of protein synthesis and transport were characterized using metabolic labeling of synthesized proteins. Cellulobiohydrolase I (CBHI, Cel7A), the major secreted cellulase, was analyzed as a model protein. Northern analysis showed that under conditions where protein transport was inhibited (treatments with dithiothreitol or brefeldin A) the unfolded protein response pathway was activated. The active form of the *hac1* mRNA that mediates unfolded protein response signaling was induced, followed by induction of the foldase and chaperone genes *pdil* and *bip1*. Concomitant with the activation of the unfolded protein response pathway, the transcript levels of genes encoding secreted proteins, like cellulases and xylanases, were drastically decreased, suggesting a novel type of feedback mechanism activated in response to impairment in protein folding or transport (repression under secretion stress (RESS)). By studying expression of the reporter gene *lacZ* under *cbh1* promoters of different length, it was shown that the feedback response was mediated through the cellulase promoter.

Filamentous fungi include species characterized by efficient synthesis and secretion of hydrolytic enzymes and thus play an important role in decomposition of biological material in their natural habitats. *Trichoderma reesei* produces a variety of cellulolytic enzymes in large quantities and in a strictly controlled manner (reviewed in Refs. 1 and 2). The enzyme production by the fungus is readily adapted to meet the requirements set by the environmental conditions and the carbon source available. In the presence of the readily utilized carbon source, glucose, the expression of cellulase and hemicellulase genes is tightly repressed. However, under conditions in which enzyme activity is required for hydrolysis, e.g. on plant derived polymers, such as cellulose, or in the presence of some oligosaccharides, like sophorose, cellobiose, and lactose, the cellulase genes are efficiently induced (3, 4). *T. reesei* is widely used as an industrial host organism for production of hydrolytic enzymes as well as for production of heterologous proteins, typically under the control of the strong cellulase promoters (2, 5).

Production of a heterologous protein or the extra-cellular fungal proteins in very large quantities generates a need to enhance the efficiency of protein folding and transport as well as the quality control of the synthesized proteins. Various stress responses are triggered in cells to alleviate the harmful effects of the limitation in the capacity of the secretory pathway. Mechanisms that sense the status of protein folding and transport in the endoplasmic reticulum (ER) have been characterized in detail in the yeast *Saccharomyces cerevisiae* and in mammalian cells (reviewed in Refs. 6–9). Accumulation of unfolded proteins in the ER activates the unfolded protein response (UPR) pathway, which leads to induction of a number of genes involved in protein folding, glycosylation, and transport, and in the degradation of misfolded proteins (7, 10). The UPR is typically activated in conditions where cells have been treated with chemical agents that prevent protein folding or transport, such as dithiothreitol (DTT), tunicamycin, or Ca^{2+}-ionophores, or in cultures overproducing mutant proteins that cannot fold properly. In *S. cerevisiae* the response is mediated by the protein kinase/endoribonuclease Ire1p (11–13) that converts *HAC1* mRNA to a translationally active form (14, 15). Hac1p acts as a positive transcription factor of the genes under the UPR control (16–18). A similar but more clearly complex UPR signaling system has been characterized in mammalian cells (reviewed in Refs. 8 and 9). ER stress signaling in mammalian cells leads also to inhibition of protein translation via phosphorylation of eIF2α (eukaryotic initiation factor 2 α) (6, 19), activation of the ER overload response (20), and induction of sterol biosynthesis (21). A stress-induced kinase with some sequence homology to Ire1, PERK/PEK, is responsible for the phosphorylation of eIF2α (6, 19). ATF6, a basic leucine zipper transcription factor, forms another important part of the mammalian UPR apparatus. The transactivation domain of this protein is cleaved upon ER stress and transported to the nucleus where it binds promoters of UPR target genes (6, 22).
Mammalian cells also have homologues of Ire1, but the substrates and the signaling pathways of these stress sensors were unknown for a long time. Recently, however, the Ire1 proteins were found to induce a HAC1-like splicing of a mRNA for XBP-1, a novel factor regulating the transcription of UPR target genes both in mammalian cells and in Caenorhabditis elegans (23–25). Furthermore, the XBP-1 mRNA is induced by ATF6 during mammalian UPR, thus providing a link between the ATF6 and Ire1 systems (25).

The UPR signaling has also been studied in filamentous fungi. Elevated expression levels of foldase and chaperone genes have been observed in fungal cultures under conditions in which malfolded proteins are expected to accumulate in the ER, in cultures expressing heterologous proteins (26, 27), or in cultures treated with DTT or tunicamycin (26, 28, 29). The UPR transcription factor gene hac1/hacA has been cloned from T. reesei and Aspergillus nidulans (30). The activation of these genes includes two steps, splicing of an unconventional intron as in the case of yeast HAC1 and animal XBP-1, and truncation of the mRNA at the 5′ flanking region. This truncation removes from the mRNA an upstream open reading frame that is involved in translational regulation of the gene.

Treatment of cell cultures with chemical agents interfering either with protein folding or transport has been widely used as a tool to study protein secretion as well as the cellular stress responses under these conditions, especially in mammalian cells and in S. cerevisiae. To be able to use this approach in studies on protein transport in Trichoderma, we have here analyzed the effects of selected drugs in T. reesei cultures. DTT is a reducing agent known to inhibit formation of the intra-chain disulfide bonds, thus preventing correct folding of proteins in the ER and their further transport from the ER (31–33). Brefeldin A (BFA) has been widely used to elucidate the mechanisms of protein transport in mammalian cells. In many cell types the structure of the Golgi compartment is disrupted during BFA treatment, and the transport between the ER and Golgi is blocked (34). The specific targets of BFA have been shown to be the guanine nucleotide exchange factors of ARF during BFA treatment, and the transport between the ER and other organelles is thought to be mediated by the BARS protein suggested to be important for the integrity of Golgi structure (38). In addition, the ionophore A23187 was included in the work presented here. In mammalian cells treated with this ionophore, the perturbation of the Ca2+ homeostasis of the cellular compartments has been reported to hamper protein folding and exit from the ER, to induce UPR, to increase protein degradation within ER, and to inhibit translation initiation via phosphorylation of the initiation factor eIF2α (39–43). The results of the present study provide fundamental information on the effects of DTT, BFA, and A23187 on protein secretion in T. reesei. Most importantly, we provide evidence for a novel type of feedback mechanism that functions during secretion stress and down-regulates the transcript levels of genes coding for endogenous secreted proteins (repression under secretion stress (RESS)).

**EXPERIMENTAL PROCEDURES**

*Strains and Cultivation Conditions—* T. reesei strain Rut-C30 (44) was cultivated on minimal medium (NH₄)₂SO₄ 7.6 g l⁻¹, KH₂PO₄ 15.0 g l⁻¹, MgSO₄·7H₂O 0.5 g l⁻¹, CaCl₂·H₂O 0.2 g l⁻¹, (final concentration of 10 μM), BFA (50 μg ml⁻¹), or Ca²⁺-ionophore A23187 (5 μM). A corresponding volume of the solvent of the drug stock solutions was added into the untreated control cultures (0.2% and 0.5% Me₂SO in the control cultures for A23187 and BFA treatment, respectively, and double distilled water in the control cultures for DTT treatment. In some experiments 250 mg ml⁻¹ of cycloheximide (CHX) was added in the cultures. The strain QM9414 (45) and its derivatives transformed with expression cassettes pM134 and pMLO16 containing *Escherichia coli lacZ* under *cbh1* promoter (46) were cultivated in the minimal medium containing sorbitol (20 g l⁻¹) as a carbon source and supplemented with 0.85% protease peptone. 8 × 10⁵ spores were inoculated per 200 ml of growth medium, and the cultures were grown in conical flasks at 28 °C with shaking at 200 rpm. α-Sorbose (1 mM) was added after 23 h and after 32 h of cultivation to induce cellulse gene expression on sorbitol medium (46). Treatment of the cultures with 10 μM DTT was started after 40 h of cultivation. The same amount of water was added to control cultures. Dry weight of the cultures was measured before the sorbitol induction and at the end of the treatment either with DTT, BFA, or the ionophore by filtering and drying mycelium samples at 105 °C to constant weight (24 h). The dry weight in the cultures was 1.1–1.4 g l⁻¹ at the beginning of the treatment with A23187, DTT, or BFA.

*Metabolic Labeling and Analysis of the Labeled Proteins—* Metabolic labeling of the proteins was carried out essentially as described (47). The labeling experiment was started 10 min after addition of DTT or A23187 or 15 min after addition of BFA. 1 μCi of [³⁵S]methionine (Amersham Biosciences) was added to a 50-ml aliquot of the cultivation, and samples of 2 ml were collected with short time intervals from the labeled culture. Untreated cultures were labeled in parallel in a similar manner. The mycelia in the samples was separated from the culture medium by filtering through Millipore HVLP02500 filters, washed with 10 ml of double distilled water, and the samples were frozen immediately in liquid nitrogen. Cell extracts were prepared as described previously (47). Total proteins in the cell extracts and in the culture supernatant were precipitated in 20% TCA, and 0.1% (w/v) bovine serum albumin (47). The precipitate was heated at 95 °C for 10 min in 5% (w/v) TCA to release labeled tRNA (48) and washed with 5% (w/v) TCA. Radioactivity in the protein samples was measured by scintillation counting.

Preparation of protein samples and two-dimensional gel electrophoresis were carried out essentially as described (47). Equal amounts of total protein from the different time points were loaded in the gels. The protein amount loaded was confirmed to be in a range giving a linear response between the CBHI signal and the sample volume. Typically, 20 μg of intracellular protein or 3 μg of extra-cellular protein was loaded in the gels. The gels were analyzed using a PhosphorImager (Amersham Biosciences). Identical exposure times were used for quantification of the signals in the gels. For reproduction of the figures the signal scale of the images was adjusted for optimal inspection of the pI pattern of the proteins in the different conditions.

*Parameters Describing Protein Synthesis and Secretion—* The rate of protein synthesis and secretion, the average synthesis time of specific proteins as well as minimum secretion time of the molecules were determined essentially as described previously (47). The amount of labeled protein in cell extract and culture supernatant was quantified (either using scintillation counting of TCA insoluble material for total protein or quantification of specific labeled proteins in two-dimensional gels using a PhosphorImager) and plotted against the time scale. The rate of protein synthesis was determined as the slope of the linear part of the curve representing the intracellular labeled protein at the early time points where protein secretion is not yet detectable. The rate of protein secretion was determined as the slope of the linear part of the curve representing extra-cellular labeled protein. The average synthesis time of a specific protein was determined by extrapolating the linear part of the curve representing the amount of the labeled protein in the cell extract to the abscissa. The intercept of the curve and the abscissa corresponds to the half of the synthesis time of the protein (see also Refs. 48–50). The minimum secretion time of the protein was determined as the distance of the intercepts of the intracellular and extra-cellular protein curves extrapolated to the abscissa (for details see Refs. 48, 49).

*Northern Analysis of the Cultures Treated with DTT, BFA, or Ca²⁺-ionophore A23187—* Mycelial samples of 50 ml were collected from cultures treated with DTT, BFA, or A23187 or from the untreated control cultures after 0, 15, 30, 60, 90, 120, 240, and 360 min of the treatment. The first sample (the time point 0 min) was withdrawn immediately prior to addition of DTT, BFA, or A23187. The mycelia were washed with an equal volume of 0.7% NaCl, frozen immediately in liquid nitrogen, and stored at −80 °C. Total RNA was isolated using the Trizol™ Reagent (Invitrogen) essentially according to manufacturer’s instructions. Northern blotting and hybridization on nitrocellulose fil-
Total protein synthesis and secretion in cultures treated with DTT, BFA, or A23187. The cultures were labeled with [35S]methionine during treatment with DTT, BFA, or A23187. The amount of radioactivity incorporated into TCA insoluble material in cell extract and in culture medium was measured at different time points of labeling (x-axis, min after addition of the labeled precursor). The treatments with DTT or BFA were started 10 min prior to the addition of the labeled methionine and the treatment with BFA 15 min before the labeling. The radioactivity in samples prepared from cultures treated with the drugs are shown by open squares (○), and the corresponding values for samples prepared from the untreated control culture are in each panel shown by black squares (■). (DW, dry weight of biomass.)

**Results**

The Effect of DTT, BFA, and the Ca²⁺-ionophore A23187 on Total Protein Synthesis and Secretion in T. reesei—Protein synthesis and secretion were analyzed in T. reesei cultures treated with DTT, BFA, or A23187 by metabolic labeling of the proteins, and parameters describing the efficiency of the processes under these conditions were deduced from the data. Logarithmic phase shake flask cultures were subjected to treatment with 5 μM A23187 or 10 μM DTT 10 min prior to the addition of [35S]methionine into the cultures or with 50 μg/ml BFA 15 min before the labeling.

Total protein synthesis was measured as incorporation of the labeled precursor into the TCA insoluble pool of total cellular protein. The amount of radioactivity in the TCA insoluble material in the cell extract was plotted against time (Fig. 1), and the rate of protein synthesis was determined as the slope of the linear part of the curve at the early stages of labeling before the onset of the appearance of labeled proteins into the medium. Treatment of the cultures with either DTT or BFA did not have a significant effect on the rate of protein synthesis, whereas in cultures treated with A23187 the protein synthesis rate was clearly reduced as compared with the rate in the control cultivation. In the presence of A23187 the synthesis rate was reduced to 51% of the rate in the control cells, and in the presence of DTT or BFA the rate was 105 or 100% of the rate in control cultivation, respectively (Table I). The uptake of the labeled precursor into the cells was not significantly affected at the concentrations of the drugs used in the experiment, and thus does not interfere with the comparison of the protein synthesis rates. However, in cultures treated with higher concentrations of A23187 (10 μM), a reduction in the uptake of [35S]methionine was observed (data not shown).

On the other hand, production of the labeled proteins into the culture medium was efficiently inhibited in the presence of DTT or BFA. The secretion rate of total labeled proteins into the culture medium was efficiently inhibited in the presence of DTT or BFA. The secretion rate of total labeled proteins in the culture medium per time unit, was only 5% of that in the control cells (Fig. 1 and Table I). In cultures treated with the ionophore A23187, the rate of secretion of labeled proteins into the culture medium was reduced to 23% of that in the non-treated cultures.
ity in the pI of the protein, and that glycan structures play an important role in the formation of the pI forms (47). The synthesis of the first nascent forms of CBHI is followed by formation of additional more acidic pI forms, and finally the protein is secreted into the medium space as 7–8 different pI forms, detectable in two-dimensional gels (47). To compare maturations of the pI pattern of CBHI in cultures treated with DTT, BFA, or A23187, the labeled protein samples of cell extract and culture medium were subjected to two-dimensional gel electrophoresis (Fig. 2). In cultures treated with DTT, the intracellular pI forms of CBHI were limited to the two very early forms of the protein, and there was no detectable production of the labeled protein into the culture medium. In a similar manner, the pI pattern of intracellular CBHI in the BFA-treated cultures displayed only the early pI forms. As treatment with BFA hindered CBHI transport almost completely, only a minute amount of labeled CBHI was detectable in the culture medium at the late stages of the labeling experiment. However, this extra-cellular CBHI fraction had gained the full pI pattern, indicating that a minor portion of the protein is modified and transported in a normal manner, although the amount of the fully processed pI forms in the cell extracts is too low to be detected. The effect of the treatment with A23187 on CBHI transport was less pronounced. The full array of pI forms was detected in the cells, although the maturation of the pI pattern was delayed by 15–20 min as compared with the control cells, and CBHI with full pattern of the pI forms was secreted into the culture medium, albeit with a delay.

To study the kinetics of CBHI synthesis and secretion in more detail, the labeled CBHI analyzed in the two-dimensional gels was quantified at different time points (Fig. 3), and parameters describing protein synthesis and secretion were deduced from the data (Table I). The average synthesis time of full-length CBHI was not significantly affected in the DTT- or BFA-treated cultures, being in accordance with the result that total protein synthesis is not affected by these treatments. The minimum secretion time of CBHI measured in the BFA-treated cultures was markedly longer (69 min) than in control cultures (11 min). In the DTT-treated cultures the parameter could not be determined due to the very low amount of extra-cellular protein produced in these conditions. Treatment with A23187 affected both the synthesis and the transport of CBHI. The average synthesis time of the protein was increased by 3–4 min in cultures treated with A23187 as compared with the control cultures, and the minimum secretion time was 10 min longer than for the control cultures (Fig. 3 and Table I).

Surprisingly, although the treatment with DTT or BFA did not reduce the rate of total protein synthesis or prolong the time required for the synthesis of CBHI molecules, the rate of CBHI synthesis (the amount of labeled CBHI synthesized per time unit) was reduced in cultures treated with these drugs. In DTT-treated cultures the CBHI synthesis rate was 14% and in BFA-treated cultures 52% of that measured for control cultures (Table I). Most of the CBHI synthesized remained intracellular. In BFA-treated culture the rate of CBHI secretion into the culture medium (the amount of labeled CBHI produced into the culture medium per time unit) was 4% of that measured for control cultures; in DTT-treated cultures the production into the culture medium was too low to be measured. Also in cultures treated with the ionophore A23187 the rate of CBHI synthesis was affected to a greater extent than the total protein synthesis rate (Table I). The CBHI secretion rate was in A23187-treated cells reduced to the same extent as the synthesis rate. This indicates that the reduction in the amount of extra-cellular CBHI produced was mainly due to the reduced synthesis in A23187-treated cells.

**Table I**

|                  | Total protein synthesis rate | Total protein secretion rate | CBHI synthesis rate | CBHI secretion rate |
|------------------|-----------------------------|------------------------------|---------------------|---------------------|
| Untreated cells  | 100                         | 100                          | 100                 | 100                 |
| DTT              | 105                         | 5                            | 14                  | n.d.                |
| BFA              | 100                         | 5                            | 52                  | 4                   |
| A23187           | 51                          | 23                           | 26                  | 27                  |

Average time of CBHI synthesis Minimum time of CBHI secretion

|                  | min | min |
|------------------|-----|-----|
| Untreated cells  | 5.7 | 11  |
| DTT              | 4.3 | n.d.|
| BFA              | 4.6 | 69  |
| A23187           | 9.0 | 21  |

**Fig. 2.** Two-dimensional gel analysis of CBHI from cultures treated with DTT, BFA, or A23187 and labeled with [35S]methionine for different time periods. A, cell extracts from the labeled cultures were subjected to two-dimensional gel analysis, and the gels were scanned using a PhosphorImager (Amersham Biosciences). The region of the gel showing CBHI at different time points of the labeling experiment is shown. The pH range covered in each of the panels is approx. 3.5–4.5 (from left to right in the figure). The labeling time point is indicated as minutes from addition of the labeled precursor. Samples of labeled untreated cultures are included as a control. B, CBHI from medium of the labeled cultures was analyzed in two-dimensional gels. Extra-cellular labeled CBHI from cultures treated with BFA or A23187 and labeled for 180 min is shown. In DTT-treated cultures the amount of labeled CBHI was too low to be detected.
The half-life of the cbh1 mRNA under cellulase-inducing conditions was determined as 14.7 min ± 1.1 min by analyzing the cbh1 transcript level after subjecting the cultures to a treatment with a transcription inhibitor 1,10-phenanthroline (Fig. 7). The half-life measured in a pulse-chase labeling experiment with [3H]uridine was 12.5 min. In the DTT-treated cultures the reduction kinetics of the cbh1 transcript is in accordance with the measured half-life of the transcript, whereas the reduction in the BFA-treated cultures took place with somewhat slower kinetics.

Trichoderma cultures were also treated with a protein synthesis inhibitor, CHX, in combination with DTT, to see whether a reduction in protein synthesis and thus in the ER protein load would affect UPR induction and the down-regulation of the genes encoding secreted proteins. Northern analysis showed there was no detectable induction of the short form of the hac1 transcript or induction of the UPR target gene pdi1 in the cultures treated with both CHX and DTT (Fig. 8), whereas in the cultures treated with DTT alone there was a clear induction of both hac1 and pdi1. The cbh1 transcript was expressed at all time points in the cultures treated with both CHX and DTT, but the level of the transcript was slightly reduced at the late time points of the treatment. However, the reduction in the cbh1 transcript level was not as strong as in cultures treated with DTT alone (Fig. 8). A significant decrease in the transcript levels of cbh1 and pdi1 was also observed at the late time points (240–360 min) of the treatment with CHX alone, indicating that prolonged treatment of the cultures with CHX as such had a down-regulating effect on the transcript levels. The results from the earlier time points (0–120 min) suggest that the reduction in protein synthesis and ER protein load in the CHX-treated cultures, even in the presence of DTT, abolished UPR induction and resulted in less pronounced down-regulation of transcripts encoding secreted proteins.

Analysis of Expression of the lacZ Reporter Gene under the cbh1 Promoter under Conditions of Secretion Stress—To study whether the observed feedback regulation of the mRNAs encoding the endogenous secreted cargo proteins was mediated by
the promoter sequence of the gene involved, a reporter gene system was used. A schematic view of the reporter gene expression cassettes is shown in Fig. 9A. The E. coli lacZ gene was expressed in T. reesei under either a full-length 2.2 kb cbh1 promoter (pMLO16) or a shortened promoter of 161 bp (pMI34). The short promoter contains the putative TATA-box and the transcription start sites and is still functional and inducible with sophorose in vivo (46). The lacZ mRNA expression levels during DTT treatment of the strains were studied by Northern analysis (Fig. 9B). The quantification of the lacZ signal normalized with the signal of gpd1 (encoding glyceraldehyde-3-phosphate dehydrogenase), which remained highly similar in all specimens. The black bars indicate the signal during the treatment and the white bars the signal in corresponding samples of the control cultivation.

The steady-state mRNA levels of hac1, bip1, pdi1, and gpd1 in cultures treated with DTT, BFA, or A23187 and in untreated control cultures. A, total RNA from samples collected at different time points (indicated above the lanes) during the drug treatments was subjected to Northern analysis. Ctrl, control. B, the steady-state mRNA levels of the uninduced long form of hac1 and the induced short form (indicated as hac1U and hac1I, respectively), and bip1 and pdi1 were quantified from the Northern blots using a PhosphorImager. For hac1U and hac1I, the proportion of the measured signal from the total hac1 signal is shown. All the signals were normalized using those of gpd1 (encoding glyceraldehyde-3-phosphate dehydrogenase), which remained highly similar in all specimens. The black bars indicate the signal during the treatment and the white bars the signal in corresponding samples of the control cultivation.
shortened cbh1 promoter was used for lacZ expression, only a mild transient decrease of the mRNA level was detected at 30 min of DTT treatment, and at the other time points the transcript was detectable at normal or increased quantity. The egl1 mRNA level was analyzed in both of these strains in parallel, to control that the down-regulation mechanism was functional in the specific strains under the conditions used. The results indicate that sequence elements located between nt -2200 and -161 in the cbh1 promoter are required for the down-regulation.

**DISCUSSION**

In the present study we have used selected chemical agents that interfere either with protein folding or transport, to study protein secretion and the cellular stress responses in the drug-treated T. reesei cells. Treatment of cultures with DTT or BFA strongly inhibited secretion of total protein (Fig. 1) and the major cellulase CBHI (Fig. 3), in the absence of significant effects on total protein synthesis. The two-dimensional gel analysis of intracellular CBHI showed that in these conditions also the maturation of the full pl pattern of the protein was prevented, indicating lack of post-translational modifications typically taking place during the transport. The increasing heterogeneity in the pl pattern of the protein occurring after synthesis has been explained by modification of glycan structures and/or addition of new glycan moieties on the protein during transport (47). In the presence of DTT or BFA, only the pl forms corresponding to the very early nascent forms of the protein were detected in the cell extracts, indicating a secretion block at the early stages of transport (see also Ref. 47). The result is consistent with data obtained in other organisms. In both mammalian and yeast cells, the treatment of cultures with DTT has been shown to inhibit protein folding and leads to accumulation of the protein in the ER compartment (31–33). BFA treatment has been reported to cause disruption of the Golgi compartment and fusion of the Golgi components with the ER (34). In *Aspergillus niger* a glucoamylase-green fluorescent protein fusion protein was reported to accumulate in reticular ER-like structures at early times after BFA addition, but was later found in vacuoles in which it was most probably degraded (54).

One of the well characterized cellular responses to ER stress and inhibition of protein folding and transport in mammalian cells is inhibition of translation initiation, which is mediated by the PERK kinase and phosphorylation of initiation factor eIF2α (e.g. in A23187- or DTT-treated cultures) (6, 19, 40). However, in the present study protein synthesis was unaffected
in the DTT- or BFA-treated *Trichoderma* cultures, although protein folding and transport were clearly inhibited. This indicates that translational inhibition may not play an important role in the defense mechanisms activated under ER stress in *Trichoderma*. The kinase PERK has not been characterized in filamentous fungi, and the *S. cerevisiae* genome seems to lack the corresponding gene (8). Our BLAST search in the *Neurospora crassa* genome did not reveal clear PERK homologues either.

The effect of the ionophore A23187 on protein transport under the conditions used was less pronounced in *Trichoderma* cultures as compared with the treatments with DTT or BFA. Protein transport was only mildly retarded as shown by the delay in maturation of the pi pattern of newly synthesized CBHI molecules during the metabolic labeling and by the prolonged minimum time of CBHI secretion as compared with the untreated control cultures. The primary effect of the ionophore was to reduce total protein synthesis activity in the cells. Perturbation of cellular Ca$^{2+}$ homeostasis by A23187 has been shown to inhibit protein synthesis also in mammalian cells (43, 55). In this respect the ionophore has thus similar impact on *Trichoderma* and mammalian cells, but the reduction of translation activity observed in this study is likely to be mediated by other means than the PERK pathway functioning during secretion stress.

Induction of foldase/chaperone genes has been reported to occur in DTT-treated cultures of the filamentous fungi *Trichoderma* and *Aspergillus*, indicating activation of the UPR pathway under these conditions (26, 56, 57). Our results confirm these findings and show that the impaired protein transport results in UPR activation also in BFA-treated *T. reesei* cultures. This was demonstrated by the expression of the active form of the *hac1* mRNA, which was followed by induction of the foldase gene *pdi1* and the chaperone gene *bip1*. The data shows that DTT affected the *hac1* expression levels more rapidly than BFA. Treatment of the cultures with the ionophore A23187 lead only to a weak, transient activation of *hac1*, which did not result in activation of the genes *pdi1* or *bip1*, possibly due to a balancing effect of the observed reduction in total protein synthesis and thus also in ER load.

It has been recently shown that activation of the ER-associated protein degradation required to remove misfolded polypeptides from the ER is also controlled by the UPR pathway (10). In the *Trichoderma* cultures treated with BFA, the intracellular accumulation of the labeled CBHI continued for the first 40 min (55 min from the start of the drug treatment), after which the amount of the protein in the cell extracts declined. The clearance of CBHI from the cell extracts could not be explained by the low amount of the protein produced into the medium, but was most likely due to degradation of the protein. The treatment with BFA might allow the transport of the protein into the vacuoles as in *A. niger* (54) or the degradation might be mediated by the ER-associated degradation system known to be induced in the yeast *S. cerevisiae* upon UPR activation (10). The same trend of decreasing amounts of the labeled intracellular CBHI continued during the later stages of the labeling experiment as well seen in the DTT-treated *Trichoderma* cultures.

An intriguing find was that under conditions in which protein transport was severely impaired but the total protein synthesis was unaffected (during DTT or BFA treatments), clearly reduced amounts of secreted cargo protein, CBHI, were synthesized. The average time of synthesis of full-length CBHI
molecules was equal to the values measured for control cells, but the synthesis rate of CBHI (the amount of CBHI synthesized per time unit) was markedly reduced. In addition, also in the A23187-treated cultures where protein transport was only mildly retarded, the rate of CBHI synthesis was affected to a greater extent than the total protein synthesis rate.

Northern analysis revealed that the transcript level of the cbh1 gene decreased rapidly during the drug treatments, which most probably led to reduction in CBHI synthesis rate. In the DTT-treated cultures the response was detectable soon after the onset of the drug treatment and the kinetics of the decrease was in accordance with the half-life of the transcript (14.7 ± 1.1 min), as measured using a transcription inhibitor. In the BFA-treated cultures the reduction in the mRNA level of cbh1 occurred with a slight delay and followed slower kinetics compared with the response in the DTT-treated cultures. As a result, the higher amount of mRNA is likely to enable production of CBHI in a higher quantity as compared with the cultures treated with DTT.

The down-regulation of the messenger was not only characteristic of the cbh1 mRNA, but also other transcripts coding for extra-cellular proteins, such as eg1, cbh2, eg2, xyn1, and hfb2, were down-regulated. An interesting finding was that the transcript level of bg22 encoding an intracellular β-glucosidase was not down-regulated in response to impairment in protein transport, even though the gene is regulated on different carbon sources in a similar manner as the genes coding for extra-cellular cellulases (53). This suggests that the down-regulation mechanism involves specifically genes encoding secreted extra-cellular proteins. In addition to the genes up-regulated under the control of UPR (pdi1 and bip1), several examples of genes whose expression levels remained unchanged in the DTT-treated cultures were found. These include mRNAs coding for glyceraldehyde-3-phosphate (gpd1), 14–3–3 protein (ftl1), aminobutyrate transaminase (abt1), the glucose repressor (cre1), as well as the components of vesicle transport machinery, sar1 and ypt1.

Repression of genes encoding secreted proteins in response to secretion stress (RESS) is a novel finding in fungal cultures. However, the phenomenon is not only limited to Trichoderma. Subsequent work in progress has shown that a similar down-regulation mechanism is functional also in the filamentous fungus A. niger. In higher eukaryotes, transcriptional profiling has revealed that DTT or tunicamycin treatment of Arabidopsis plants resulted in down-regulation of a large group of genes encoding secreted proteins, indicating a similar type of response to secretion stress (58). Expression of genes encoding secreted proteins in yeast does not seem to display such a co-ordinate regulation under secretion stress. DNA microarray data of S. cerevisiae cultures treated with DTT or tunicamycin and strains expressing a heterologous protein (major histocompatibility complex class I heavy chain) showed examples of genes encoding secreted proteins that are down-regulated as well as those that are not affected under the conditions studied (10, 59). However, decreased transcription of genes encoding ribosomal components and tRNAs has been reported in S. cerevisiae strains with secretory mutations (60).

Decreased mRNA stability is a common control mechanism of protein expression in various organisms. A well studied example of this is regulation of the S. cerevisiae genes subject to glucose repression (61). To study whether the down-regulation of cbh1 is a transcriptional response mediated by the promoter sequence or whether it is solely due to instability of the transcript, we analyzed the expression of a lacZ reporter under the cbh1 promoter. The results show that lacZ was down-regulated during treatment with DTT when expressed under the full-length cbh1 promoter, but not under a shortened cbh1 promoter containing the putative TATA-box and the transcription start sites. Thus, the regulatory elements of cbh1 are able to render a heterologous gene susceptible to the down-regulation in DTT-treated cultures. Further analysis is now required to

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**Fig. 9. Dependence of the cbh1 mRNA down-regulation on the promoter region of the gene.** A, schematic presentation of the lacZ expression cassettes used for the expression studies. lacZ is expressed under the full-length cbh1 promoter in the strain transformed with the cassette pMLO16, and under a truncated promoter in the strain transformed with pMI34. B, Northern analysis of lacZ, eg1, and gpd1 in cultures of the strains treated with 10 mM DTT and in untreated control cultures (ctrl). The time point of the drug treatment is indicated above the lanes. C, the steady-state mRNA signals of lacZ and eg1 during the DTT treatment was quantified from the Northern blots using a PhosphorImager, and the signals were normalized using those of gpd1. The black bars indicate the signal during the DTT treatment and the white bars in the untreated control cultivations. The error bars indicate the standard error of the mean. (tsp, transcription start point.)

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identify the specific sequences in the promoter responsible for the novel regulatory response. Binding sites of several transcriptional regulators have already been analyzed in the cbh1 promoter (46, 62). The down-regulation during secretion stress seems, however, to be distinct from the creI-mediated glucose repression system (3, 46). Strains either with a wild type or defective creI glucose repression system display down-regulation of the cellulase transcription in response to secretion stress to the same extent (data not shown). The down-regulation is typically observed under the same conditions that activate UPR, in cultures treated with chemical agents interfering with protein folding or transport, e.g., DTT or BFA. In addition, treatment of the cultures with a protein synthesis inhibitor, cycloheximide, in combination with DTT both abolished UPR activation and caused significantly less severe down-regulation of the transcript level of the cellulase gene, cbh1, as compared with the treatment with DTT alone (Fig. 8). The results indicate that inhibition of protein synthesis in CHX-treated cultures led to reduced ER protein load, thus preventing activation of UPR and RESS even in the presence of DTT. However, it is at present not known whether RESS directly involves components of the UPR regulatory pathway. The down-regulation of the cellulase mRNAs was significant in cultures treated with A23187, even though the treatment with the drug resulted only in a slight effect on UPR. Similarly, tunicamycin treatment results in cellulase down-regulation, although tunicamycin is a poor inducer of UPR in the fungal strain.5 Furthermore, the cbh1 promoter does not contain consensus UPR elements, and the effect is thus unlikely to be mediated by the UPR transcription factor HAC1. However, it is possible that this feedback mechanism could share regulatory components with the UPR that are more upstream in the signaling pathway. Further studies are required to elucidate whether the UPR mediator Ire1 plays a role in the phenomenon, or whether there are other factors mediating the response, either components participating in the UPR activation or ones acting independently.

T. reesei is an organism capable of producing extra-cellular proteins in extremely high quantities. The protein production by the fungus is strictly controlled by the carbon source available, switching from production from tens of grams of protein to the medium to almost complete shut-off of protein production. Therefore, T. reesei must have developed efficient regulatory mechanisms enabling the cells to rapidly adapt to different requirements of protein synthesis and transport. One can envision that secretory stress ensues in a phase of high-activity protein secretion, the T. reesei cells need exceptionally powerful tools to be able to avoid severe damage. The unfolded protein response is activated to inhibit and dissolve the accumulation of misfolded protein in the ER lumen. The present feedback mechanism down-regulating the transcription of genes for secreted cargo proteins may in this organism form another important part of the mechanism alleviating ER stress.

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