Role of Endoproteolytic Dibasic Proprotein Processing in Maturation of Secretory Proteins in *Trichoderma reesei*

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Cell extracts of *Trichoderma reesei* exhibited dibasic endopeptidase activity toward the carboxylic side of KR, RR, and PR sequences. This activity was stimulated by the presence of Ca^{2+} ions and localized in vesicles of low bouyant density; it therefore exhibited some similarity to yeast Kex2. Analytical chromatofocusing revealed a single peak of activity. The dibasic endopeptidase activity was strongly and irreversibly inhibited in vitro as well as in vivo by 1 mM *p*-amidinophenylmethylsulfonyl fluoride (pAPMSF) but not by PMSF at concentrations up to 5 mM. We therefore used pAPMSF to study the role of the dibasic endopeptidase in the secretion of protein by *T. reesei*. Secretion of xylanase I (proprotein processing sequence -R-R- ↓ -R- ↓ -A-) and xylanase II (-K-R- ↓ -Q-) was strongly inhibited by 1 mM pAPMSF, and a larger, unprocessed enzyme form was detected intracellularly under these conditions. Secretion of cellobiohydrolase II (CBH II; -E-R- ↓ -Q-) was only slightly inhibited by pAPMSF, and no accumulation of unprocessed precursors was detected. In contrast, secretion of CBH I (-R-A- ↓ -Q-) was stimulated by pAPMSF addition, and a simultaneous decrease in the concentration of intracellular CBH I was detected. Similar experiments were also carried out with a single heterologous protein, ShBLE, the phleomycin-binding protein from *Streptoalloteichus hindustanus*, fused to a series of model proprotein-processing sequences downstream of the expression signals of the *Aspergillus nidulans gpdA* promoter. Consistent with the results obtained with homologous proteins, pAPMSF inhibited the secretion of ShBLE with fusions containing dibasic (RK and KR) target sequences, but it even stimulated secretion in fusions to LR, NHA, and EHA target sequences. Addition of 5 mM PMSF, a nonspecific inhibitor of serine protease, nonspecifically inhibited the secretion of heterologous proteins from fusions bearing the NHA and LR targets. These data point to the existence of different endoproteolytic proprotein processing enzymes in *T. reesei* and demonstrate that dibasic processing is obligatory for the secretion of the proproteins containing this target.

Filamentous fungi are renowned for the efficient secretion of various enzymes, such as cellulases or amylases, in large amounts and are thus also considered as potentially attractive host systems for the production of biotechnologically relevant heterologous proteins. However, so far the overall levels of heterologous (nonfungal) proteins are still considerably lower than those obtained for homologous proteins (19, 22, 46).

There may be a large number of factors that influence the final level of a secreted protein, i.e., the regulation of transcription, mRNA stability, translational initiation and elongation, translocation, protein folding, intracellular transport, and processing (16, 29). Even after secretion of the proteins into the extracellular fluid, they may be degraded by extracellular proteases (6).

Several strategies have been developed to identify and eliminate these potential bottlenecks and thus to improve protein yields (1, 29). These include the use of highly inducible promoters, the introduction of a high gene copy number, the use of protease-deficient host strains, and the fusion of heterologous genes with an endogenous gene encoding a protein secreted at high levels such as glucoamylase (6).

In many cases, the production of heterologous proteins appears to be limited at the level of secretion (28, 36). In all organisms, secretory proteins are synthesized as preprotein precursors which are N-terminally extended by a signal peptide that targets them into the secretory pathway (40, 43). However, protein maturation in eukaryotes often requires additional proteolytic processing at later stages of the secretory pathway. In *Saccharomyces cerevisiae*, the maturation of α-factor has been shown to involve three different specific peptidases: Kex1, Kex2, and a dipeptidylaminopeptidase (10, 21, 23). Calmels et al. (7) have reported that dibasic amino acid doublets, which resemble Kex2 target sites, predominate as target sequences in secretory proteins from filamentous fungi. However, apart from the demonstration of the cleavage of dibasic targets in homologous and heterologous proteins in vivo (7, 9, 32), there have been no studies on the detection and properties of a Kex2 homolog in filamentous fungi.

Here we demonstrate a dibasic endopeptidase activity in *Trichoderma reesei* and, with the aid of an irreversible inhibitor of it (*p*-amidinophenylmethylsulfonyl fluoride [pAPMSF]), we show that it constitutes an essential step in the secretion of proteins containing the respective target sequence.

MATERIALS AND METHODS

Strains and transformation. The *T. reesei* strains used in this study are listed in Table 1. They were maintained on malt agar (containing 5 mM uridine in the case of *T. reesei* TU-6) and subcultured monthly. Transformants were obtained by cotransformation with plasmid pFG1, which carries the homologous *pyr4* (previously termed *pyrG*) gene as a selectable marker (17, 18).

Escherichia coli DH5α (*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 lacU169/Φ80lacZ*-M15; Clontech Laboratories, Palo Alto, Calif.) was used for propagation of plasmids and grown under standard conditions (38).

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TABLE 1. *T. reesei* strains used in this study

Strain	Genotype or description	Source or reference
QM9414	Mutant of wild-type QM6a	31
TU-6	Δ*pyr4*	16
UT740	TU-6 (pUT740, pFG1)	This study
UT964	TU-6 (pUT964, pFG1)	This study
UT965	TU-6 (pUT965, pFG1)	This study
UT966	TU-6 (pUT966, pFG1)	This study
UT967	TU-6 (pUT967, pFG1)	This study

Medium and growth conditions. *T. reesei* was grown in conical flasks (1 liter) containing 250 ml of the medium described by Mandels and Andreotti (31), supplemented with the appropriate carbon source (1% [wt/vol]) and buffered with 50 mM sodium citrate at pH 5.0, at 30°C. Carbon sources were used as indicated for the respective experiments. To prepare cell extracts for enzyme assays, *T. reesei* QM 9414 was grown on 1% (wt/vol) xylose or 1% (wt/vol) glycerol. To induce xylanase or cellulase formation in resting mycelia, *T. reesei* QM 9414 was pregrown for 20 h with glycerol as the carbon source and then transferred to a resting-cell medium. To do this, the mycelium was washed with tap water and resuspended in minimal medium lacking a carbon source, and 3-ml aliquots were transferred to 10-ml bottles. Inducers (2 mM sophorose [30] and 4 mM xylose [27]) and inhibitors (PMSF and pAPMSF [26]; Sigma, Deisenhofen, Germany) were added to give the final concentration as indicated. Induction was started 1 h after the addition of the inhibitor, and incubation was continued for up to 20 h on a rotary shaker (250 rpm, 30°C).

Inhibitor experiments with *T. reesei* strains of the UT series were carried out as described for *T. reesei* QM 9414, except that 1% glucose was used for both pregrowth and replacement.

Plasmids and manipulation of DNA. Plasmid pFG1 (18) was obtained from the stock of the Institute of Biochemical Technology and Microbiology, Technische Universität Wien, Vienna, Austria. Construction of the recombinant plasmid pUT740, which is a pUC19 (48) derivative containing a gene fusion consisting of the *Aspergillus nidulans gpdA* promoter, a synthetic oligonucleotide for a preprosequence (see Fig. 4), the phleomycin resistance gene (*ble*) from *Streptoalloteichus hindustanus* (11), and the *A. nidulans trpC* terminator, has already been described (8). Plasmids pUT964 through pUT967 were derivatives of pUT953, which is similar to pUT740 except that it contains 1,320 bp of the constitutive *T. reesei* Tr1 promoter (35). These plasmids were constructed by insertion of custom-designed oligonucleotides into the *Xho*I-*Nco*I site between the *T. reesei* promoter and the *S. hindustanus ble* structural gene.

Standard methods were used for plasmid isolation, restriction enzyme digestion, random priming, and Southern analysis (38). A ^{32}P-labeled 366-bp *Sma*I-*Bam*HI *S. hindustanus ble* fragment derived from plasmid pGFT (8) was used to determine the copy number of pUT plasmids in *T. reesei* TU-6 transformants.

T. reesei chromosomal DNA was isolated as described previously (18).

Cell extracts for proteolytic enzyme assays and immunological analysis. Mycelia were harvested on a Buchner funnel and washed with ice-cold tap water; excess liquid was removed by blotting the material between filter paper sheets. Then 1 g of the blotted mycelium was suspended in 4 ml of 20 mM Tris-HCl buffer (pH 7.0) containing 0.1 mM PMSF, 1 mM EGTA, 1 mM 2-mercaptoethanol, and 1 μM pepstatin A or in 20 mM sodium acetate buffer (pH 5.5) containing the same additives. The suspension was further sonicated in an ice-water bath with a Sonoplus HD60 sonifier (Bandelin; four times for 90 s each with alternating 90-s cooling periods). The homogenate was centrifuged at 10,000 × *g* (15 min, 4°C), and the supernatant was kept for enzyme activity determinations.

Endopeptidase enzyme assays. Endopeptidase activity was measured as follows. First, 20 nmol of the substrate (*N-tert*-butoxycarbonyl [Boc]-GKR-4-methylcoumarin [MCA], Boc-LKR-MCA, Boc-VPR-MCA, and Boc-LRR-MCA, where GKR, LKR, VPR, and LRR indicate the peptide substrates in the amino acid one-letter code; Sigma, Deisenhofen, Germany) was incubated with 50 μl of the cell extract in 0.2 M Tris-HCl buffer (pH 7.0) or 0.2 M sodium acetate buffer (pH 5.5) and 1 mM CaCl$_2$ in a total volume of 250 μl at 37°C for 30 min. Thereafter, 3.0 ml of distilled water was added, and the amount of MCA released from the substrate was measured in a fluorescence spectrophotometer with excitation at 380 nm and emission at 460 nm. One unit of enzyme activity is defined as the amount of enzyme that can release 1 nmol of MCA from the substrate under the above assay conditions in 1 min.

Subcellular fractionation. *T. reesei* QM 9414 was grown for 24 h on 1% (wt/vol) glycerol as the carbon source. The washed mycelial mat was thoroughly mixed with an equal volume of glass beads (diameter, 0.5 mm) and 20 mM Tris-HCl buffer (pH 7.0) containing 1 mM 2-mercaptoethanol, 1 mM EGTA, 0.1 mM PMSF, 1 μM pepstatin A, and 1 M sorbitol and was then homogenized four times for 20 s each, with alternating 2-min cooling periods, in the 75-ml chamber of a Bead-Beater apparatus (Biospec Products, Bartlesville, Okla.). The homogenate was centrifuged at 5,000 × *g* for 12 min to remove the beads, nuclei, and cell wall material. Then, 8 ml of the supernatant was layered onto a 30-ml discontinuous sucrose gradient (17.1 to 34.2% [wt/vol]). The gradient was cen-

TABLE 2. Demonstration of a dibasic endopeptidase activity in cell extracts of *T. reesei*

Substrate	Presence (+) or absence (−) of 1 mM Ca^{2+}	pH	Mean activity ± SD (U/mg)[a]
Boc-LKR-MCA	−	7.0	10.9 ± 0.6
Boc-LKR-MCA	−	5.5	15.6 ± 0.9
Boc-LKR-MCA	+	7.0	27.6 ± 1.4
Boc-LKR-MCA	+	5.5	31.9 ± 1.6
Boc-LRR-MCA	−	5.5	12.8 ± 0.8
Boc-GKR-MCA	−	5.5	10.7 ± 0.6
Boc-VPR-MCA	−	5.5	8.9 ± 0.5

[a] Units are given in nanomoles of MCA released in 1 min per milligram (wet weight) of mycelia. The enzyme assay was performed as described in Materials and Methods. The numbers represent the means and standard deviations of four independent experiments. All data were obtained with the same mycelial suspension.

trifuged for 2 h at 18,000 × *g* at 4°C and fractionated from the top into 30 fractions. One portion of each fraction was fixed for electron microscopy as described by Glaurt (15). Published methods were used to assay citrate synthase (41), acid phosphatase (24), and glucose-6-phosphate dehydrogenase (20).

Immunological analysis. Samples from the culture supernatants and from the cell extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting on nitrocellulose membranes. SDS-PAGE was carried out in 10 and 15% polyacrylamide gel slabs, as described previously (25). The protein concentration was determined as described by Bradford (3) with bovine serum albumin as a standard.

For immunostaining, a polyclonal antibody against recombinant ShBLE (CAYLA, Toulouse, France) and monoclonal antibodies against the cellobiohydrolases (33) and xylanases (XYN I [40a]; XYN II [45]) were used. Anti-mouse and anti-rabbit immunoglobulin G (Promega, Madison, Wis.)-coupled alkaline phosphatase and staining with 5-bromo-4-chloro-3-indolylphosphate as a substrate were used to detect bound monoclonal and polyclonal antibodies, respectively (33).

Analytical chromatofocusing. Portions (0.5 ml) of the cell extract were dialyzed against 25 mM imidazole-HCl buffer, pH 7.0, and applied to a Mono-P column (Pharmacia Biotechnology, Uppsala, Sweden) connected to a fast protein liquid chromatography apparatus (Pharmacia) operating at 0.5 ml/min. After elution of all nonbound protein, an automatic pH gradient was started by rinsing the column with 40 ml of 10% (vol/vol) Polybuffer 74 (Pharmacia), pH 3.5. Fractions of 1 ml were collected and assayed for dibasic endopeptidase activity with Boc-GKR-MCA as a substrate.

Purification of secreted ShBLE and N-terminal sequencing. ShBLE was purified from the culture broth as described earlier (8). N-terminal sequencing was performed on an Applied Biosystems model 477A gas-phase sequencer equipped with an automatic on-line phenylthiohydantoin derivative analyzer.

RESULTS

Evidence for a dibasic endopeptidase activity in *T. reesei*. To demonstrate the presence of an endoproteinase with specificity for basic amino acid doublets in *T. reesei*, cell extracts were tested for activity against four fluorogenic substrates containing appropriate target sites (KR, RR, and PR [13, 39, 42]) (Table 2). The relative activities against the various substrates coincide well with the activities of yeast Kex2 (5, 33). In support of this similarity, the dibasic endopeptidase activity was optimal in the presence of 1 mM CaCl$_2$ (Table 2) and was inhibited by EDTA at pH 7.0 (data not shown). In contrast, however, it had a higher activity at pH 5.5 than at pH 7.0 and, unlike in yeast cells (5), this was not due to limited stability. Subcellular fractionation localized the highest activity at a bouyant density of 1.1 g/cm^3, which was juxtaposed by the activity of acid phosphatase, an enzyme secreted by *T. reesei* (Fig. 1). The relative appearance of marker enzymes for cytosol (glucose-6-phosphate dehydrogenase) and mitochondria (citrate synthase) on the gradient suggested that the fraction with the highest dibasic target cleaving activity consisted of smaller vesicles and/or membrane fragments, and this was supported by electron microscopy (data not shown). The presence
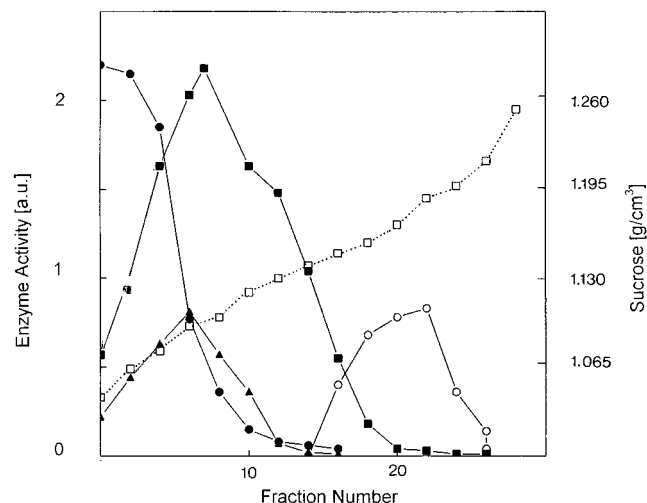
FIG. 1. Subcellular localization of the Kex2-like activity in *T. reesei*. *T. reesei* mycelia were homogenized as described in Materials and Methods, cell debris and nuclei were removed by centrifugation, and the supernatant was loaded onto a 30-ml discontinuous sucrose gradient (17.1 to 34.2% [wt/vol]). The gradient was centrifuged for 2 h at 18,000 × *g* and fractionated from the top into 30 fractions. Symbols: □, the actual bouyant density of individual fractions; ■, the dibasic endopeptidase-like activity of individual fractions; ●, glucose-6-phosphate dehydrogenase activity; ○, citrate synthase activity; ▲, acid phosphatase. All enzyme activities are given in arbitrary units (a.u.) only, so they could be plotted on the same graph.

of acid phosphatase further indicates involvement of these vesicles in protein secretion. Hence, we conclude that this activity is localized on membranes and/or smaller subcellular particles in *T. reesei*.

In order to find out whether this activity was due to one or more enzyme proteins, cell extracts were analyzed by chromatofocusing (Fig. 2). The dibasic endopeptidase activity eluted as a sharp, symmetrical peak at pH 5.8 to 6.0. This result supports the presence of a single dibasic endopeptidase in *T. reesei*.

Irreversible inhibition of enzyme activity with pAPMSF in vitro and in vivo. To confirm that the processing enzyme is a
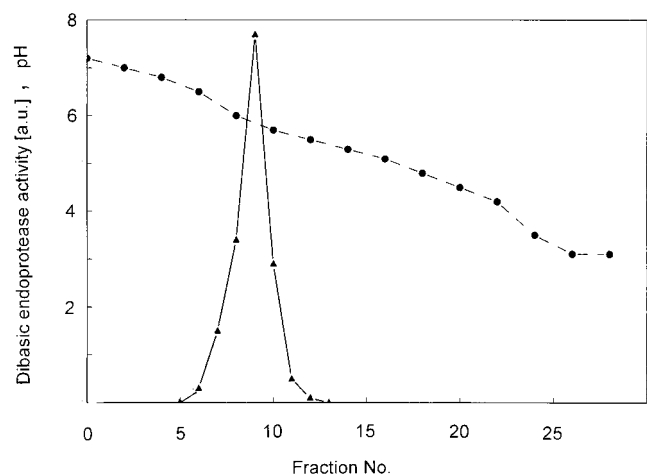

FIG. 2. Analytical chromatofocusing of the dibasic endopeptidase activity of *T. reesei*. Chromatofocusing was performed as described in Materials and Methods. Symbols: ●, pH of every second fraction; ▲, dibasic endopeptidase activity (with Boc-LKR-MCA as a substrate).

TABLE 3. Effect of pAPMSF and PMSF on the *T. reesei* dibasic endopeptidase activity in vitro and in vivo

Substrate	Presence (+) or absence (−) of:		Condition	Mean activity ± SD (U/mg)[a]
	pAPMSF	PMSF		
Boc-LKR-MCA	−	−	In vitro	100 ± 6.2
	+	−	In vitro	12.9 ± 5.1
	−	+	In vitro	100 ± 6.3
	−	−	In vivo	100 ± 5.9
	+	−	In vivo	19.4 ± 2.9
	−	+	In vivo	100 ± 5.8
Boc-LRR-MCA	−	−	In vitro	81.7 ± 5.7
	+	−	In vitro	8.6 ± 3.4
	−	+	In vitro	82.6 ± 6.8
Boc-GKR-MCA	−	−	In vitro	72.0 ± 6.0
	+	−	In vitro	6.9 ± 1.9
	−	+	In vitro	71.4 ± 4.0

[a] Values are given as the percentage of activity with Boc-LKR-MCA in the absence of any inhibitor. Cell extracts (in vitro) or mycelia were incubated with the inhibitors (1 mM pAPMSF or 5 mM PMSF, final concentration, respectively) for 1 h. The mycelia were then used to prepare the cell extracts (in vivo). The numbers indicate the means and standard deviations of four independent experiments. All data were obtained with the same mycelial suspension.

serine protease, we used pAPMSF, which specifically and irreversibly inactivates proteases with substrate specificity for positively charged amino acid side chains (26). A time course of inhibition showed that half-maximal inactivation appeared to have occurred after 30 to 40 min and that complete inactivation occurred within 2 h of incubation (data not shown); all data are thus reported for incubation times with the inhibitor of 1 h. Similar results were obtained when the activity was assayed with Boc-LKR-MCA, Boc-LRR-MCA, or Boc-GKR-MCA (Table 3). In contrast, 1 mM PMSF had no effect on *T. reesei* dibasic endopeptidase activity (Table 3).

pAPMSF also inactivated the dibasic endopeptidase activity when added to mycelia in vivo: when *T. reesei* cultures were pulsed with 5 mM pAPMSF (final concentration) and cell extracts were prepared 1 h later, only 19.4% of the original activity was detected (Table 3). The higher concentrations of pAPMSF needed are most probably due to transport and compartmentation problems. Control experiments without pAPMSF resulted in 100% ± 13% of the original activity. The addition of PMSF concentrations of up to 10 mM in vivo had no effect on the activity of the dibasic endopeptidase.

Effect of pAPMSF and PMSF on maturation and secretion of cellulases and xylanases in vivo. The results described above showed that the dibasic endopeptidase can be inactivated in vivo by addition of pAPMSF, thus providing us with a tool to study whether this proprotein-processing activity is required for secretion of *T. reesei* proteins in vivo. To accomplish this, we chose the cellulases cellobiohydrolase I (CBH I) and CBH II and the xylanases XYN I and XYN II as model secretory proteins. A comparison of their nucleotide sequence-deduced putative amino acid sequences with that derived from N-terminal sequencing of the purified protein revealed different potential target sites for posttranslational proteolytic modification, e.g., -R-A- ↓ -Q-, CBH I; -E-R- ↓ -Q-, CBH II; -R-R- ↓ -R- ↓ -A-, XYN I; and -K-R- ↓ -Q-, XYN II (Fig. 3A). Therefore, processing of XYN I and XYN II, but not of CBH II or CBH I, should occur by the dibasic endopeptidase and thus be inhibited by pAPMSF.

To investigate this, we pregrew *T. reesei* on glycerol and

A

XYN I   MVAFSSLICALTS<u>IAS</u>TLAMPTGLEPESSVNVTARGMYFVLGAHNDRR ⇓ R ⇓ A-

XYN II   MVSFTSL<u>LAA</u>PAAEVESVAVEKR ⇓ Q-

CBH II   MIVGILTT<u>LATLATLAA</u>SVPLEER ⇓ Q-
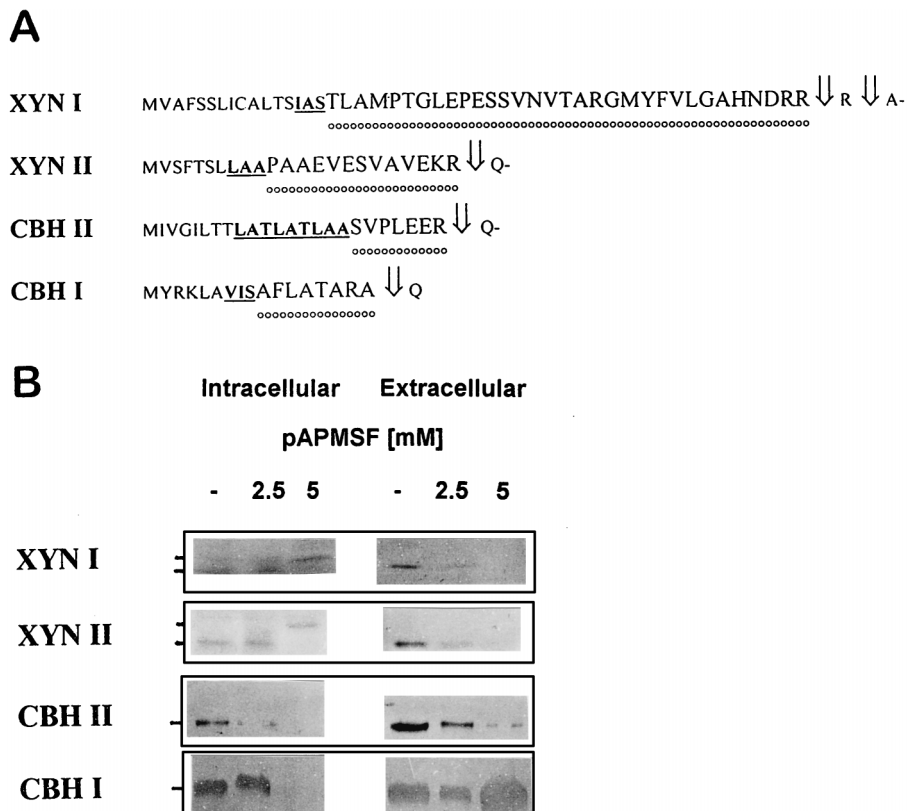
CBH I   MYRKLA<u>VIS</u>AFLATARA ⇓ Q

B

FIG. 3. Effect of pAPMSF on formation and secretion of some cellulases and xylanases by *T. reesei*. (A) Preprosequences of the enzymes investigated. The arrows indicate the N-terminal amino acid of the secreted protein according to published evidence (2, 39, 42, 44). (B) Demonstration of the intra- and extracellular levels of these enzymes by SDS-PAGE and immunostaining with specific monoclonal antibodies. Experiments were carried out as described in Materials and Methods. The marks in the left margin indicate the positions of the unprocessed (higher mark) and processed (lower mark) enzyme form. Blots for extracellular and intracellular samples from the same protein are placed so that the relative positions of these two enzyme forms are comparable. For intracellular samples, equal amounts (40 μg) of protein were loaded onto the gels; equal volumes (20 μl) of the culture broth were loaded for the extracellular samples. Only a single mark is shown where only the processed form can be observed (CBH I and CBH II). Results shown are representative of at least three separate experiments.

placed it onto a resting-cell medium containing the respective inducers of cellulases and xylanases (sophorose and xylose). pAPMSF was added to one batch of these cultures after 2 h of incubation with the inducer. In addition, control experiments were done with the general serine protease inhibitor PMSF and with the solvents for the inhibitors only (acetone for pAPMSF, dimethyl sulfoxide for PMSF).

Figure 3B documents that 2.5 mM pAPMSF strongly inhibited the secretion of both XYN I and XYN II, and neither enzyme could be detected after the addition of 5 mM pAPMSF. At the latter concentration, the intracellular accumulation of larger enzyme precursors (i.e., a 22.3-kDa form of XYN II and a 21-kDa form of XYN I) was detected. These M_r would be consistent with the sizes of the XYN I and XYN II proproteins from which only the signal peptide has been removed and which are thus 19 (2.0 kDa) and 11 (1.3 kDa) amino acids larger than the secreted, mature proteins. Controls with acetone or dimethyl sulfoxide did not show any effect on secretion levels. PMSF at 10 mM inhibited the secretion of XYN I and XYN II but without the concomitant accumulation of intracellular enzymes (data not shown), suggesting a nonspecific effect on cell viability.

The addition of pAPMSF influenced the secretion of enzymes containing different target sites in completely different ways: the secretion of CBH II, which contains an acidic-basic instead of a dibasic target site (-E-R- ↓ -Q-), was much less inhibited by 2.5 mM pAPMSF than were the two xylanases

(Fig. 3B) but was completely inhibited by 5 mM pAPMSF. However, in contrast to the situation with XYN I and XYN II, no accumulation of intracellular precursors was observed. CBH II secretion was also inhibited by PMSF. The secretion of CBH I, which contains a processing target consisting of a basic-hydrophobic amino acid sequence (-R-A- ↓ -Q-), not only was not inhibited by the addition of 5 mM pAPMSF but was even enhanced by it. Concomitantly, a decrease in the concentration of the intracellular CBH I protein was monitored. Secretion of CBH I was also completely inhibited by 5 mM PMSF (data not shown).

Effect of pAPMSF and PMSF on the secretion of a heterologous protein fused to various propeptide sequences. The findings presented above are in accordance with an essential role of the pAPMSF-inhibitable dibasic endopeptidase in the secretory pathway of *T. reesei* proteins containing dibasic processing targets. However, a direct comparison of the results obtained with these four different proteins is hampered by the possibility that they have different rates and kinetics of secretion. In order to overcome this difficulty, we have reassessed the effect of pAPMSF on secretion of a single heterologous protein fused to different propeptide sequences. We have chosen the phleomycin-binding protein from *S. hindustanus* (ShBLE [11]) for this purpose and fused it to various preprosequences as shown in Fig. 4. The respective constructs were introduced into *T. reesei* TU-6 by cotransformation with the plasmid pFG1 containing the *pyr4* gene as a selection marker
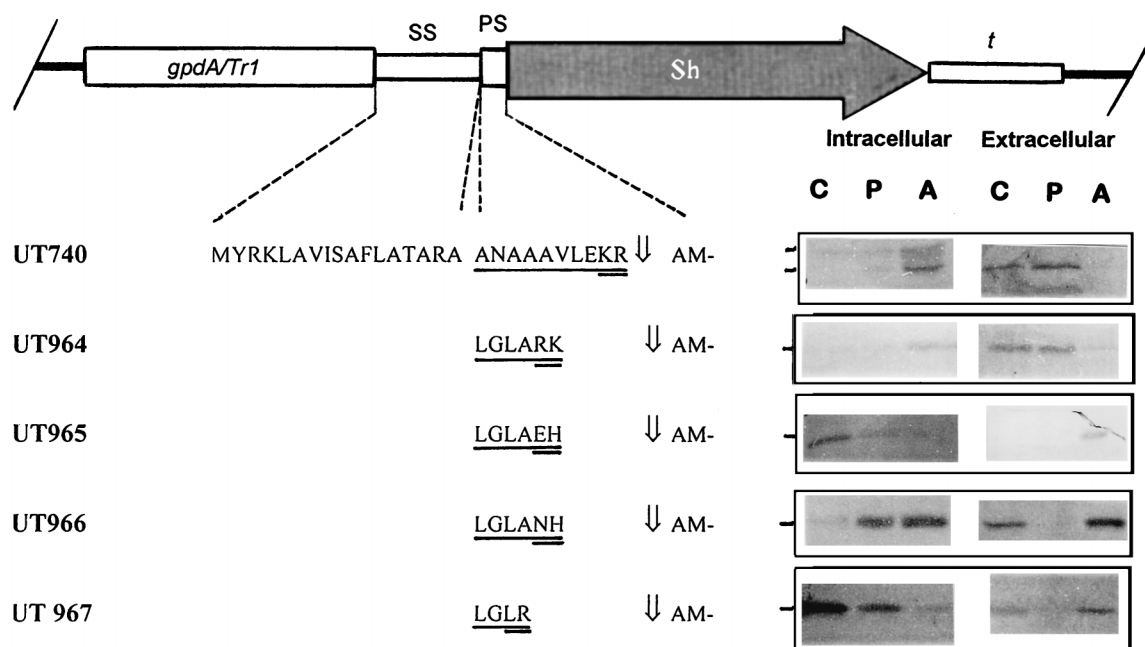
FIG. 4. Effect of pAPMSF on formation and secretion by *T. reesei* of the phleomycin-binding protein fused to different proprotein sequences. The N-terminal amino acid of the secreted protein was determined by Edman degradation and is marked by an arrow. The UT numbers of the respective recombinant strains are given in the left margin. The construct is shown on top (*gpdA/Tr1*, promoter; SS, signal sequence; PS, prosequence; Sh, the phleomycin-binding protein-encoding sequence; *t*, TrpC terminator). Sequences differing between the different constructs are underlined, and the putative cleavage targets are double underlined. Detection of intracellular and extracellular levels of ShBLE (13.7 kDa) by SDS-PAGE and immunostaining with a polyclonal antibody are shown to the right of the amino acid sequences. Columns: C, control (no inhibitor added); P, PMSF; A, pAPMSF. The markers on the left of the blots indicate the positions of the mature proteins. For intracellular samples, equal amounts (40 μg) of protein were loaded onto the gels; equal volumes (20 μl) of the culture broth were loaded for extracellular samples. The blots shown are typical for the results obtained in three independent experiments.

(see Materials and Methods). Mitotically stable transformants were selected, and the copy number of plasmids integrated into the genome was determined to allow a comparison of the obtained results. Whenever possible, transformants with two integrated copies were chosen for further experiments.

The effect of pAPMSF and PMSF on the secretion of ShBLE was investigated by pregrowing the strains for 20 h on 1% (wt/vol) glucose, replacement into the same fresh medium, and subsequent addition of the inhibitors. Purification of ShBLE secreted into the medium and analysis of its N-terminal amino acid sequence proved that the artificial proprotein target sequence was correctly cleaved in all cases (data not shown). The effects of pAPMSF and PMSF on the secretion of ShBLE from the various constructs are shown in Fig. 4: when fused to the dibasic target site -K-R- ↓ -A-, secretion was completely inhibited by the addition of 5 mM pAPMSF, and cell extracts prepared from these mycelia revealed the accumulation of an intracellular precursor. No differences between the unprocessed and processed enzymes could be detected in these experiments because of the small size differences between the prepropeptides and propeptides. Similar results were obtained with fusions to the dibasic site -R-K- ↓ -A-. PMSF at 5 mM had no effect on the secretion of ShBLE from these two fusions.

The fusion with the monobasic target site in UT967 (-L-R- ↓ -A-) was enhanced by pAPMSF, and the intracellular precursor pool was correspondingly decreased. A similar finding was observed with the constructs UT965 and UT966, which contained -E-H- ↓ -A- and -N-H- ↓ -A-, respectively, as target sequences and in which addition of pAPMSF stimulated the secretion of ShBLE (Fig. 4). Secretion of ShBLE in the absence of pAPMSF was poor or moderate, respectively. The

addition of 5 mM PMSF inhibited secretion in all three cases and also decreased the intracellular precursor pool in UT967.

DISCUSSION

The presence of a dibasic, proprotein-processing endopeptidase in filamentous fungi has been assumed previously on the basis of comparison of proprotein sequences of secretory proteins (7) and because of detection of free interleukin-6 of the correct size in extracellular culture supernatants of recombinant strains producing a fusion of interleukin-6 to CBH I, separated by a Kex2 cleavage site (8, 31). The data presented here provide experimental support for this assumption. The pAPMSF-inhibitable substrate specificity, the Ca^{2+} dependency of the reaction, and its localization in a vesicular fraction are all very similar to the properties of yeast Kex2 protease (5, 14, 34, 37). However, the pH optimum was strikingly different. Although attempts to clone a *kex2* homolog from *T. reesei* have been carried out in several laboratories, they were unsuccessful (15a, 34a, 37a). It is thus uncertain whether the enzyme catalyzing the endopeptidase studied in this work actually justifies the name Kex2, and we are at this point referring to it consequently as a dibasic endopeptidase only.

The finding that pAPMSF inhibited the *T. reesei* dibasic endopeptidase irreversibly when added in vivo provided us with an alternative strategy to manipulate the activity of this enzyme and thus to study its effect on the secretion of proteins by *T. reesei*. We consider this approach even more straightforward, since the disruption of *KEX2* in *S. cerevisiae* or *Yarrowia lipolytica* results in severe growth defects (13, 21), thus making the investigation of effects on protein secretion rather difficult.

Furthermore, pAPMSF may offer an attractive means for isolating mutants overexpressing the dibasic endopeptidase.

Results from both homologous and heterologous systems show that pAPMSF efficiently blocks the secretion of proteins containing dibasic target sites. The simultaneous accumulation of intracellular protein forms with higher M_r values suggests that this is not due to a nonspecific general effect but rather that the inhibition of proprotein processing by the dibasic endopeptidase blocks further—or at least decreases the rate of—protein secretion. This is in contrast to results with *Y. lipolytica*, where alkaline protease was not processed, yet it was secreted (13). At first sight, these data also contrast with findings in *Aspergillus niger* and *A. awamori* that artificial gene constructs of restrictocin and cutinase lacking a prosequence ending with a dibasic amino acid motif were efficiently secreted (4, 47). These data suggest that processing by the dibasic endopeptidase in itself is not a prerequisite for secretion in filamentous fungi, yet if the respective target sequence is present in the protein its cleavage appears to be essential.

Secretion of CBH II, which contains a monobasic target site, was inhibited only by much higher concentrations of pAPMSF, but this effect was also seen with PMSF and thus was not specific. Thus, we conclude that this site is processed not by the dibasic endopeptidase but probably by other serine proteases. In yeast, Yap3p, which cleaves after a single R, which would perfectly match the sequence present in CBH II, is responsible for the processing of monobasic proprotein target sites (12). No intracellular accumulation of unprocessed CBH II was detected in the presence of pAPMSF. However, the prosequence of CBH II is very small (42), and the difference between the proprotein and the mature protein may not be detectable by SDS-PAGE. Because of the results with XYN I and II (see above) and with CBH I (see below), we consider it rather unlikely that pAPMSF exerts a general inhibitory effect on CBH II formation.

The processing of CBH I, whose apparent proprotein cleavage site does neither correspond with a dibasic endopeptidase or a Yap3 target site, was not at all affected by the addition of pAPMSF, thus providing support for the hypothesis that pAPMSF at the concentration used has no general effects on secretion. Intriguingly, the secretion of CBH I was enhanced by pAPMSF addition, the secretion of ShBLE UT965 was only detected in the presence of 5 mM pAPMSF, and inverse observations were made for the intracellular precursor pool. One explanation for these findings would be that dibasic endopeptidase-dependent and dibasic endopeptidase-independent secretory proteins compete for further transport subsequent to the dibasic endopeptidase processing step. If this assumption is correct, it would indicate that the prosequence can influence the rate of protein secretion, which would be an interesting focus for further studies, offering as it does the possibility to alter the efficacy of protein secretion in *T. reesei* and perhaps other fungi by an alteration of its processing peptidases.

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