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Shotgun Proteomics of *Aspergillus niger* Microsomes upon D-Xylose Induction▽†

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Protein secretion plays an eminent role in cell maintenance and adaptation to the extracellular environment of microorganisms. Although protein secretion is an extremely efficient process in filamentous fungi, the mechanisms underlying protein secretion have remained largely uncharacterized in these organisms. In this study, we analyzed the effects of the D-xylose induction of cellulase and hemicellulase enzyme secretion on the protein composition of secretory organelles in *Aspergillus niger*. We aimed to systematically identify the components involved in the secretion of these enzymes via mass spectrometry of enriched subcellular microsomal fractions. Under each condition, fractions enriched for secretory organelles were processed for tandem mass spectrometry, resulting in the identification of peptides that originate from 1,081 proteins, 254 of which—many of them hypothetical proteins—were predicted to play direct roles in the secretory pathway. D-Xylose induction led to an increase in specific small GTPases known to be associated with polarized growth, exocytosis, and endocytosis. Moreover, the endoplasmic-reticulum-associated degradation (ERAD) components Cdc48 and all 14 of the 20S proteasomal subunits were recruited to the secretory organelles. In conclusion, induction of extracellular enzymes results in specific changes in the secretory subproteome of *A. niger*, and the most prominent change found in this study was the recruitment of the 20S proteasomal subunits to the secretory organelles.

Aspergillus niger is a soil-dwelling filamentous fungus with a high capacity for decomposing plant materials. Many of the secreted depolymerizing enzymes have important biotechnological applications, e.g., to modify plant-derived food products. Homologous protein secretion in filamentous fungi may yield up to 20 g per liter of extracellular medium (14). For this reason, *A. niger* has been used intensively as a cell factory for enzyme production (3, 14, 32). In contrast to this, yields are much lower for heterologous protein secretion, with exceptions (11, 18, 35, 59).

The secretory potential of *A. niger* is not well understood, and only a limited number of functional studies have been performed to investigate the major components of the fungal secretion pathway. These studies have addressed overall transcriptional and translational responses in *A. niger* by studying the impact of secretion stress-inducing chemicals, temperature shifts, protein overproduction, or growth on carbon sources that induce changes in secretory states (20, 23, 26).

In recent years, high-throughput shotgun proteomics has been used to study cell organelle makeup and function. Through the combined use of liquid chromatography and tandem mass spectrometry (LC-MS/MS), various studies have identified many organelle-specific proteins, including proteins related to protein secretion (18, 24, 29, 51, 51). In the case of aspergilli, such studies have focused mainly on the secretome and not on the actual components of the secretory pathway (34, 36, 55).

In a previous study, we established defined culture conditions for the induced expression of the cellulase and hemicellulase enzyme system of *A. niger* (56). The expression of the corresponding genes is controlled by the dedicated transcriptional activator XlnR and its inducer, D-xylose (58). Using identical conditions with a different strain of *A. niger*, we applied shotgun proteomics for the analysis of enriched microsomal fractions containing endoplasmic reticulum (ER) membranes with associated ribosomes and Golgi membranes. The microsomal fractions were isolated from D-xylose-induced and non-D-sorbitol-induced mycelium. Following this approach, we were able to identify major protein components of the secretory organelles and to estimate their relative abundances in enriched microsomal samples.

MATERIALS AND METHODS

Strain and culture conditions. The fungal strain used in this study was *A. niger* wild type N400 (CBS 120.49). For preculture, 1.0×10^6 spores per milliliter were inoculated into 2.5-liter fermentors (Applikon) containing 2.2 liters of minimal medium (44) with 0.05% yeast extract and 100 mM D-sorbitol at 30°C. Spore germination in bioreactors was as described previously (56), with headspace aeration and a stirring speed of 300 rpm; when dissolved-oxygen levels were below 60%, the stirring speed was increased to 750 rpm and aeration was switched to the sparger inlet. This moment was defined as the actual culture starting point ($t = 0$). At 14 h from the starting point, D-xylose or D-sorbitol (10 mM) was added to each culture, and at a t of 16 h, mycelia were harvested.

Subcellular fractionation and marker enzyme assays. Subcellular fractionation was based on the work of Record and coworkers (46) with important modifications. Mycelia were harvested by Büchner filtration on nylon gauze and washed with sterile cold 0.25 M sucrose. From that moment until storage of the samples, all procedures were conducted at 4°C. Mycelia were press dried, and under each of the two conditions (addition of D-xylose or D-sorbitol), 6 g of mycelial sample was added to 80 ml of homogenization buffer (0.25 M sucrose,

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1 mM EDTA, 20 mM HEPES, pH 7.6) containing 1% (vol/vol) protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Cells were disrupted by French press cell homogenization at 3,000 lb/in^2 (20.6 MPa). The resulting homogeneous suspension was filtered through two layers of nylon gauze, and the homogenate suspensions (60 ml per condition) were centrifuged at 6,500 × *g* for 15 min. The supernatants were centrifuged at 29,000 × *g* for 18 min. The mitochondrial pellets were resuspended in 2 ml of homogenization buffer with protease inhibitors, and the supernatants were centrifuged at 100,000 × *g* for 1 h 12 min. The microsome-enriched pellets were resuspended in 2 ml of 0.4 M sucrose, 1 mM EDTA, 20 mM HEPES, pH 7.6, in a Dounce homogenizer (10 gentle strokes with a loose piston), and 200 μl of these suspensions (1 mg protein) was loaded onto each linear 9-ml buffered sucrose gradient (1 mM EDTA, 20 mM HEPES, pH 7.6; densities, 1.06 to 1.17 g · ml^{-1}) and centrifuged at 130,000 × *g* for 13 h 42 min at 4°C. Fractions of 360 μl were collected, and samples were stored at −80°C until further processing was done. Protein concentrations were estimated using the Bradford method (7), with bovine serum albumin as a standard. The NADPH-cytochrome *c* reductase assay, as a marker enzyme for microsomes, was done using a commercially available kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions.

Sample preparation for LC-MS/MS. Enriched microsomal fractions from ultracentrifugation were pooled in two groups according to density (one of higher density and one of lower density) and loaded onto Microcon YM-10 columns (cutoff, 10 kDa; Millipore, Eschborn, Germany). The concentrated enriched samples were run on 12% SDS-polyacrylamide gels, and the gels were stained according to the manufacturer's instructions using Colloidal Blue staining (Invitrogen, Carlsbad, CA). Each gel lane was cut into five slices, and the slices were cut into smaller pieces of about 1 mm^3 and washed with ultrapure water and 50% acetonitrile in 50 mM NH_4HCO_3, pH 8.0. The gel samples were reduced with 50 mM dithiothreitol (DTT) in 50 mM NH_4HCO_3, pH 8.0, for 2 h at 60°C. Subsequently, the DTT solutions were removed and samples were alkylated with 100 mM iodoacetamide in NH_4HCO_3, pH 8.0, for 1 h at room temperature in the dark with occasional mixing. The iodoacetamide solutions were removed, and samples were washed with NH_4HCO_3, pH 8.0. Gel pieces were frozen at −20°C and thawed at least three times to increase the accessible area for trypsin digestion. All gel pieces were rehydrated in 25 ng · μl^{-1} trypsin (sequencing-grade modified trypsin; Promega, Madison, WI) and digested overnight at room temperature. In order to maximize peptide extraction, all solutions from trypsin digestion were transferred to new tubes, and the gel pieces were subjected to two rounds of 1 min of sonication, the first round with 10% trifluoroacetic acid (TFA) and the second round with 5% acetonitrile, 1% TFA. After each of these two rounds, the solutions were removed and added to the original trypsin digests. The final pH was adjusted to 2.5 by addition of 10% TFA.

Liquid chromatography-tandem mass spectrometric analysis. LC-MS/MS was performed at Biqualys, as described previously (57). Briefly, samples were loaded on a preconcentration column, and peptides were eluted to an analytical column with an acetonitrile gradient and a fixed concentration of formic acid. The resulting eluent was subjected to an electrospray potential via a coupled platinum electrode. MS spectra were measured on an LTQ-Orbitrap (Thermo Electron, San Jose, CA), and MS scans of the four most abundant peaks were recorded in data-dependent mode.

Mass spectrometry database searching. The resulting spectra from the MS analysis of the *A. niger* samples were submitted to a local implementation of the open mass spectrometry search algorithm (OMSSA) search engine (15). MS/MS spectra were independently searched against peptide databases derived from the predicted proteomes of *A. niger* strain CBS513.88 and strain ATCC 1015, and a decoy reverse database was constructed from the reverse CBS513.88 proteome.

All OMSSA searches used the following parameters: a precursor ion tolerance of 0.2 Da, a fragment ion tolerance of 0.3 Da, a missed-cleavage allowance of ≤2, fixed carbamide methylation, variable oxidation of methionine, and deamination of glutamine and asparagine.

The E value threshold was determined iteratively from the false-discovery rate (FDR) and was set to 0.01. With this setting, an FDR of <5% is expected.

FDR calculation was done as follows. Peptide spectrum matches with each individual peptide database were ranked by their E values for each identified spectrum with a threshold E value of <0.01, and the top hit identified peptide sequence was selected. For FDR calculation, top hit spectral matches to peptides in the reversed database rCBS were taken, and the number of false positives was divided by the number of total positives.

Functional annotation groups and sequence analysis. Proteins predicted to play a role in secretion were grouped in functional annotation groups guided by previously published functional classification schemes (16), the Functional Catalogue (FunCat) annotation scheme, and the predicted molecular function as provided with the *A. niger* CBS531.88 genome annotation (42). Proteins considered to be involved in secretion were used for a BLAST search (NCBI) against *Saccharomyces cerevisiae* and *Homo sapiens* protein databases. Only the yeast and human reference sequence proteins that presented the lowest E values and highest percentages of positives were included in this study. Protein similarity analyses were done using the ClustalW and Jalview version 2 tools (54, 60). Transmembrane domains (TMD) were assessed using the TMHMM tool (50).

Relative protein abundance and statistics. A normalized spectral abundance factor was used as a parameter to estimate relative protein abundance using the following equation (63):

$$(\mathrm{NSAF})_k = \frac{(\mathrm{SpC}/L)_k}{\sum_{i=1}^{N}(\mathrm{SpC}/L)_i}$$

where $(\mathrm{NSAF})_k$ is the normalized spectral abundance factor for a protein *k*, SpC is the number of spectral counts (the total number of MS/MS spectra) for a given protein plus a pseudocount factor of 0.5, and *L* is the protein's length. The pseudocount was necessary to analyze the differential distribution of secretion-related proteins between the two conditions (addition of xylose or sorbitol). For the proteins predicted to be involved in secretion, significant differences in NSAF values were evaluated by applying a likelihood ratio test (G test) for independence using a null hypothesis of equal protein distribution between the two conditions.

QPCR. RNA isolation, cDNA synthesis, quantitative real-time PCR (QPCR), and data analysis were performed as described previously (37). In brief, total RNA was extracted, quantified, and mixed with an exogenous RNA reference transcript. After enzymatic DNA degradation, oligo(dT)-mediated cDNA synthesis was performed. QPCR was performed using QPCR SYBR green mix (ABgene, Epsom, United Kingdom) and specific oligonucleotide primers (see Table S1 in the supplemental material). Two independent PCR runs were performed per cDNA sample from each biological sample (xylose or control sorbitol addition). The Pfaffl method (43) was used to calculate relative gene expression levels double normalized to the added exogenous RNA transcript and to the standard condition, i.e., the average normalized expression of the two biological samples from the sorbitol control condition.

RESULTS

Global protein analysis of the secretory machinery of *A. niger*. Shotgun proteomics was applied to analyze the secretory machinery of *A. niger* under induction of secretion, i.e., induction of cellulases and hemicellulases by D-xylose. For each of the two conditions, addition of D-xylose (induced) or D-sorbitol (noninduced), biological duplicates were taken and proteins from different pooled gradient fractions were analyzed (Fig. 1) to increase the resolution, similarly to previous studies (17, 29). Overall, between 32,000 and 50,000 MS/MS spectra were obtained per condition, and 25 to 30% of these spectra could be assigned to proteins. In total, 1,081 proteins were identified: 638 proteins were present under both conditions, 282 proteins were found only in the D-xylose-induced samples, and 161 proteins were found exclusively in the samples from sorbitol addition. A protein database is available for *A. niger* CBS120.49, the strain used in this work (52). However, despite the improved annotation, many open reading frames (~5,672) lack a translation start, resulting from errors in the genome sequence. Although not fully identical to the strain used in our laboratory, the *A. niger* CBS513.88 genome-derived database was used, since from this genome, an additional 3,000 proteins are predicted compared to the other high-coverage genome-sequenced strains, such as *A. niger* ATCC 1015 (5). For a limited number of spectra, no proteins could be matched to the CBS513.88 proteome, and in these cases, the *A. niger* ATCC 1015 database was used. The proteins identified in this way were included in this study (see Table S2 in the supplemental

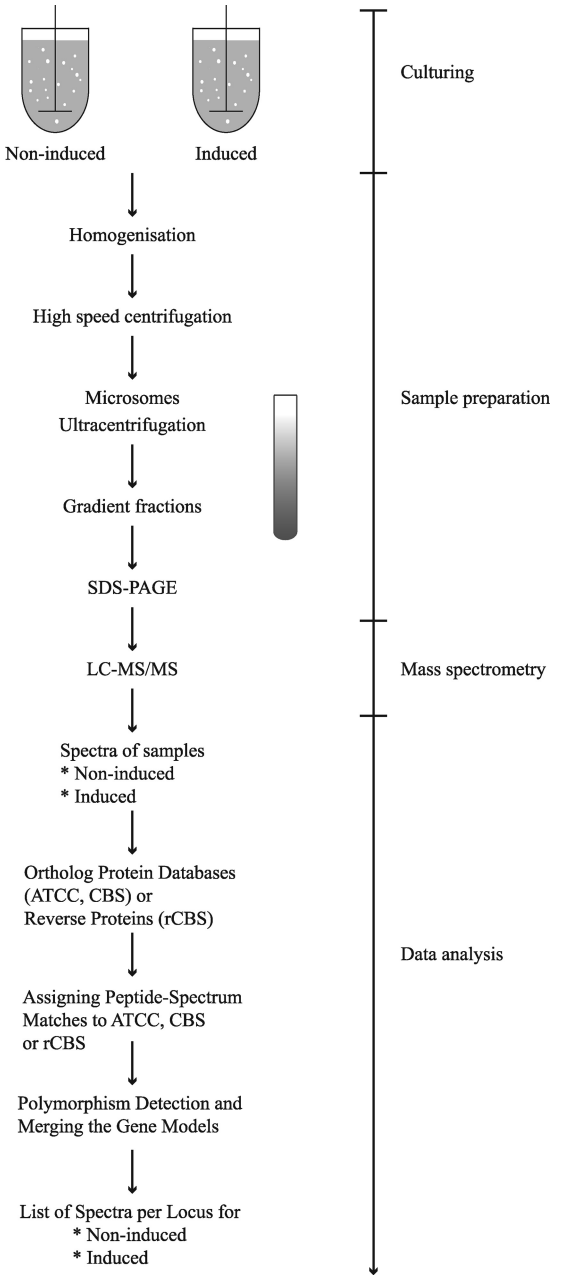

FIG. 1. Workflow of the subcellular organelle sample preparation and data analysis.

material). Upon initial curation, the total number of peptides per condition could be primarily divided in the following manner: related to membrane traffic and protein secretion, 26%; mitochondrial, 15%; ribosomal and translation, 13%; metabolism, 13%; lipid biosynthesis and cytochrome P450 enzymes, 10%; cargo proteins, 5%; nuclear, 3%; and other functions or unknown, 15%. Proteins from nonsecretory organelles, i.e., the nucleus and the mitochondrion, are listed in Table S3 in the supplemental material.

Many secretion components in *A. niger* are highly similar to mammalian proteins. Many of the membrane traffic and secretion components identified were found to be more similar to mammalian than to yeast proteins (see Tables S4 to S7, boldface, in the supplemental material). These proteins (Fig. 2) were predicted to be involved in (i) N-glycan biosynthesis and transfer to asparagine in normally glycosylated target proteins, (ii) ER-to-Golgi network anterograde and retrograde transport, (iii) processes mediated by Rab GTPases and interacting factors, (iv) microtubule-related processes, and (v) the early checkpoints of the ER-associated degradation (ERAD) pathway. Furthermore, proteins, such as UDP-glucose-glycoprotein glucosyltransferase, were found to be much more similar to human than to yeast counterparts, particularly at the catalytic core (see Fig. S1 in the supplemental material). In addition, we were able to detect five secretory pathway candidate proteins from *A. niger* that lacked a corresponding yeast homologue: a protein for utilization of Dol-P-mannose (An04g03130), a UDP-galactose transporter (An08g10400), a tetratricopeptide repeat domain 35 protein (54765; JGI), annexin AnxC3.1 (An02g05210), and a dynamin-related Ras GTPase (An06g02180).

Secretory pathway proteins of *A. niger*. From all proteins identified in this study, 254 proteins were predicted to play a direct role in membrane traffic and protein secretion (see Tables S4 to S7 in the supplemental material). The secretion-related proteins with the highest abundances identified in this study were molecular chaperones (e.g., PdiA and BipA), the structural molecules actin and tubulin, oligosaccharyltransferase subunits, and small GTPases (Table 1). Of the 34 high-abundance proteins identified, 11 have been previously characterized (Table 1, boldface). What is more, 21 secretory pathway proteins were differentially expressed in microsomal samples after the addition of D-xylose or D-sorbitol (Table 2; see Table S2 in the supplemental material). The secretion-related proteins overexpressed after D-xylose induction comprised the subunits of the 20S core particle of the proteasome, the ER retrotranslocation ATPase Cdc48, Gpi12 for glycosylphosphatidylinositol (GPI) anchor biosynthesis, the GTPases RhoA and SrgA, and a homologue of the human Rab5C protein. For the D-sorbitol condition, the cyclophilin CypA and proteins involved in targeting to the ER were found to be more abundant in the microsomal fractions.

D-Xylose induction leads to 20S proteasome recruitment to the microsomal fraction. In a previous study, we analyzed transcript levels resulting from D-xylose induction by microarray analysis (56). In this data set, no significant change in the transcript levels of the gene encoding the 20S core particle of the proteasome was found. Since the strain used in our previous study was derived from *A. niger* CBS 120.49, we determined whether the significant increase in abundance of 20S proteasome complex proteins under the secretion-induced conditions was a result of increased transcription levels. For this, we compared the transcription levels of three distinct types of genes by quantitative PCR: the D-xylose reductase gene, which is strongly induced by D-xylose (21); the vacuolar serine proteinase C gene, which is stably transcribed under a wide range of conditions (25); and the homologues of the yeast genes *PRE7* and *CDC48*. The corresponding proteins Pre7 and Cdc48 showed the largest difference in relative protein abundance in microsomes under the xylose-induced condition compared to the sorbitol control condition (Table 2). However, transcript levels for the *A. niger* homologues of yeast *PRE7* and

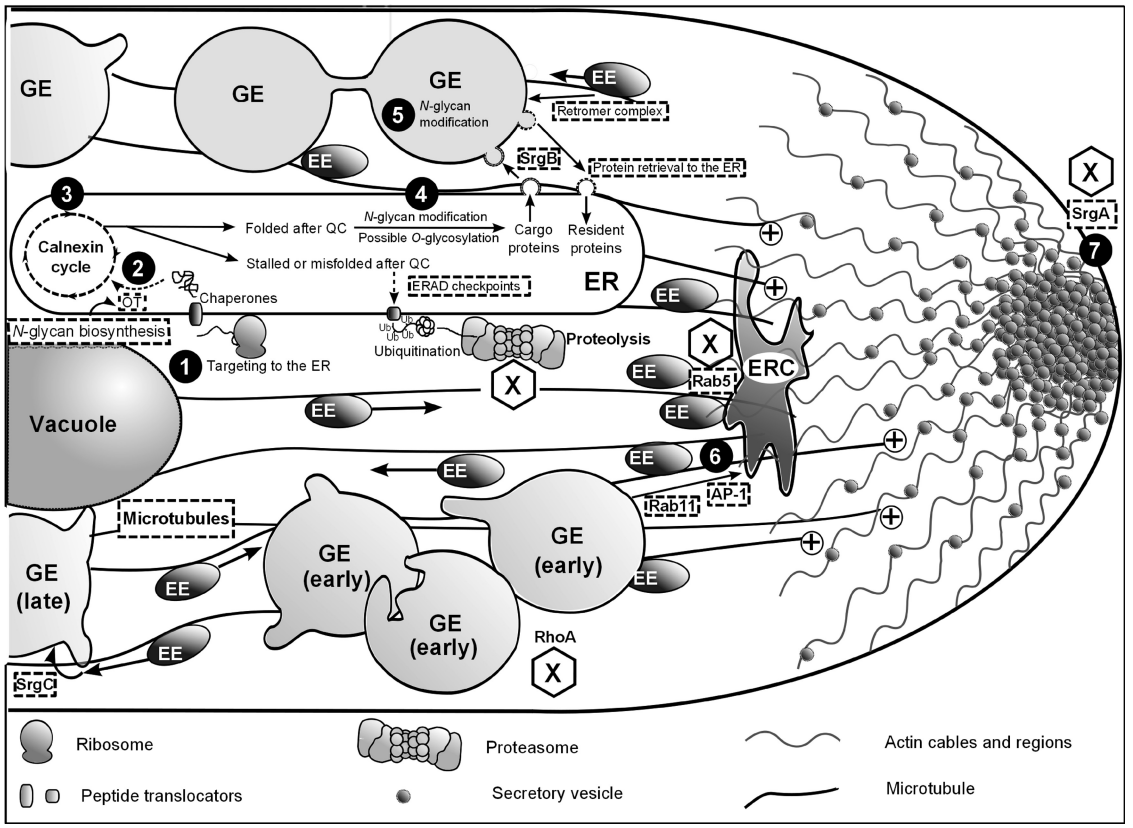

FIG. 2. Secretory processes controlled by mammalian-like proteins in *A. niger*. The dashed arrows and text boxes indicate processes that are carried out by a majority of proteins more similar to human than to yeast proteins. 1, peptide biosynthesis and translocation to the ER; 2, addition of N-glycan; 3, glycoprotein folding; 4, N and O glycosylation; 5, N glycosylation after transport to Golgi equivalents (GEs); 6, protein sorting and formation of secretory vesicles; 7, vesicle fusion with the plasma membrane; EE, early endosomes; ERC, endosomal recycling compartment; OT, oligosaccharyltransferase complex; QC, quality control; X, proteins abundant after D-xylose induction.

CDC48 were identical under xylose-induced and sorbitol control conditions (Fig. 3). In contrast to this and as expected, the transcript levels of the xylose reductase gene were strongly increased in the presence of D-xylose, while there was no significant change in the transcript levels of *pepC*.

DISCUSSION

Protein secretion is a fundamental feature of filamentous fungi that is strongly related to the fungal saprophytic lifestyle. Hyphal growth requires coordination of protein secretion with cell wall synthesis, polarized growth, recycling by endocytosis, and transport of extracellular hydrolytic enzymes. Ironically, protein secretion in the kingdom *Fungi* has been best described for typically modest secretors, such as the yeast *S. cerevisiae*. Thus, compared to yeast, more sophisticated secretory mechanisms exist in the protein secretor *A. niger*. Here, we aimed to identify candidate microsomal proteins involved in intracellular membrane traffic and protein secretion upon cellulase and hemicellulase induction by D-xylose.

In order to decrease false-positive identifications of peptides, experiments were done in duplicate, and a conservative criterion was applied for peptide identification (at least 2 unique peptides in one of the biological samples and another unique peptide in the replicate), resulting in the identification of 30% of ~40,000 MS peptide spectra per condition. Organelle isolation is prone to contamination by cell components other than the target organelle, as has become clear after the use of sensitive methods such as mass spectrometry (22, 31). Apart from technical challenges, other factors may obscure shotgun proteomic analyses, such as the multiple localization of a given protein or the direct physical interaction of target organelles with other organelles or intracellular structures (33, 47, 48).

Despite these limitations, we were able to identify key components of the secretory pathway of *A. niger* and to estimate their relative abundances. With regard to the relative abundance in microsomal samples, only 11 of the 34 most abundant protein components found have been previously characterized or described for *A. niger* (Table 1, boldface). Besides ranking high-abundance proteins, NSAF calculation also allows the estimation of the stoichiometry of protein subunits in protein complexes. Many protein complexes in *A. niger* were found present with the same stoichiometry as the ones observed in yeast protein complexes *in vivo* (Table 3). These protein complexes are predicted to be involved in a diversity of biological processes, namely (i) protein targeting to the ER by the Sec63-Sec66-Sec62 complex; (ii) addition of N-glycan to the protein asparagine by the oligosaccharyltransferase complex; (iii) ER-to-Golgi network transport by the complexes formed by Sec23-

TABLE 1. Most abundant secretory pathway proteins

A. niger locus tag[a]	Yeast homologue	Specific process and function	NSAF (10^4)[b]	
			S	X
An02g14800	PDI1	Protein disulfide isomerase PdiA: formation and unscrambling of S-S bonds (essential)	75.6	59
An07g09990	SSA4	Hsp70 protein: involved in SRP-dependent protein targeting to the ER	72.5	40
An11g04180	KAR2	ER ATPase BipA: protein import, protein folding; regulates UPR[d] via Ire1; role in karyogamy	71.8	52.7
An07g08300	CPR1	Peptidyl-proline *cis-trans* isomerase CypA: cyclophilin	65.1	32.7
An17g01420	YOP1	Membrane protein: Yip1 interaction; ER tubular morphology	56.2	40
An15g00560	ACT1	Actin structural protein ActA: cell polarity, endocytosis	53.8	42.1
An07g04570	HYP2	HexA protein: assembly of Woronin body (septal pore sealing)	50	28
An09g06590	HSC82	Hsp90 chaperone SspB: nearly identical to Hsp82	46.9	38.7
An01g08420	CNE1	Integral membrane ER chaperone ClxA: folding/QC[c] of glycoproteins (calnexin cycle)	42.4	46.4
An07g04190	WBP1	OT[e] complex β subunit (essential)	42.3	38
An02g14560	OST1	OT complex α subunit OstA: ribophorin I (essential)	41.1	35.7
An01g04040	SAR1	ARF[f] GTPase SarA: ER-to-Golgi vesicle formation	39.2	26.2
An04g02020	CPR1	ER peptidyl-prolyl *cis-trans* isomerase CypB: cyclophilin	37.4	41.3
An01g12810	YET3	Mutant that decreases level of secreted invertase	34.6	20.7
An08g03190	TUB2	Structural protein TubB: β-tubulin forms dimers with α-tubulin, and these form microtubules	34.1	29.3
An18g05980	RHO1	Ras GTPase RhoA: Rho subfamily; involved in cell polarity	33	61.2
An18g02020	PDI1	Protein disulfide isomerase TigA: S-S bonds	33	25.9
An04g02070	CHC1	Structural molecule: clathrin heavy chain	31.4	15
An07g07760	BMH1	14-3-3 protein similar to ArtA: DNA/protein binding, exocytosis, Ras/MAPK signaling	30.5	29.5
An18g06270	BMH2	1 14-3-3 protein ArtA: DNA/protein binding, exocytosis, Ras/MAPK signaling	30.1	28.8
An09g05880	ROT2	ER glucosidase II α subunit: sequential removal of 2 Glc residues (calnexin cycle)	27.6	28.9
An01g04600	MPD1	Protein disulfide isomerase PrpA: N terminus similar to Mpd1, C terminus unknown	26.6	26.2
An04g07440	SHR3	ER packaging chaperone: incorporation of amino acid permeases into COPII-coated vesicles	26.4	15.5
An16g04330	DPM1	ER dolichyl-phosphate β-D-mannosyltransferase, also required for O mannosylation	26	23.9
An05g00140	SRP102	Signal recognition particle (SRP) receptor β subunit: anchors Srp101 to the ER membrane	25.6	11.6
An01g03820	SBH2	GEF[g] for ARFs: protein translocation to the ER	25.6	7.8
An09g06790	YPT1	Rab GTPase SrgB: ER-to-Golgi traffic	24	21.1
An12g00820	UGP1	UGPase: Glc-1-P + UTP ↔ UDP-Glc	23.2	26.5
An01g02500	TRX1	Thioredoxin: ER-Golgi transport and vacuole inheritance	22.9	9.2
An03g04340	SEC61	ER SRP-dependent and -independent protein targeting, P-P bond hydrolysis-driven transporter	22.1	12.3
An01g06060	YPT31	Rab GTPase: intra-Golgi network traffic, budding of post-Golgi vesicles	21.6	24.4
An15g05740	YPT6	Rab GTPase SrgC: fusion endosomal vesicles with the late Golgi network	20.5	15.1
An08g04480	SEY1	Dynamin GTPase: ER morphology	20.2	19.9
An01g13220	LHS1	ER nucleotide exchange factor for Kar2/BipA (LhsA): UPR-regulated translocation/folding	20.2	22.1

[a] Boldface indicates characterized genes.
[b] S, sorbitol addition; X, xylose addition.
[c] QC, quality control.
[d] UPR, unfolded protein response.
[e] OT, oligosaccharyltransferase.
[f] ARF, ADP ribosylation factor.
[g] GEF, guanine nucleotide exchange factor.

Sec24, Emp24-Erv25, and Erv41-Erv46; (iii) Golgi network protein sorting by the Apl2-Apm1-Apl4 complex; and (iv) endosome-to-Golgi network transport by the Pep8-Vps29-Vps35 complex. On the other hand, one protein complex was not completely identified in this study. This complex, termed the chaperonin-containing T complex (CCT), is required in yeast for the cytosolic assembly of actin and tubulins *in vivo*. This discrepancy could be the result of selective peptide picking during MS, incorrect gene models to identify peptides, different stoichiometry in *A. niger*, or undersampling, as the NSAF values of the identified CCT subunits were low compared to the NSAF values of the aforementioned protein complexes found with the expected stoichiometry. Moreover, we were unable to detect a few other protein complexes predicted to play a role in membrane traffic or protein secretion in *A. niger*, such as the HOPS complex for vacuole fusion and vacuole protein sorting, the GARP complex for traffic from the early endosome to the late Golgi, the ESCRT endosomal sorting complexes, and the Exocyst complex for targeting of vesicles to active sites of exocytosis. It is possible either that these com-

TABLE 2. Relative abundances of secretion-related proteins in microsomes after addition of D-xylose or D-sorbitol

Overrepresentation in microsomes	Locus tag	Description	G score[a]
D-Xylose addition	An18g06700	Pre7 20S CP β subunit of the proteasome	26.20
	An04g09170	Cdc48 retrotranslocation ATPase	20.47
	An15g00510	Pre5 20S CP α subunit of the proteasome	16.83
	An02g10790	Pre6 20S CP α subunit of the proteasome	11.95
	An11g06720	Pre9 20S CP α subunit of the proteasome	9.91
	An05g02300	Gpi12 ER protein: GPI anchor assembly	9.47
	An02g07040	Scl1 20S CP α subunit of the proteasome	9.23
	An18g05980	RhoA Ras GTPase: cell polarity	8.56
	An18g06800	Pre10 20S CP α subunit of the proteasome	8.52
	An07g02010	Pre8 20S CP α subunit of the proteasome	8.36
	An02g03400	Pup2 20S CP α subunit of the proteasome	7.48
	An04g02470	Rab5-like GTPase: endocytosis, protein sorting	6.39
	An14g00010	SrgA Rab GTPase: exocytosis	5.70
D-Sorbitol addition	An07g08300	PPIase CypA: cyclophilin	10.95
	An01g03820	Shb2/Sbh1: protein translocation to the ER	9.99
	An07g09990	Ssa4/3/1/2: SRP-dependent targeting to the ER	9.49
	An07g04570	HexA protein: assembly of Woronin body	6.31
	An01g02500	Thioredoxin: ER-Golgi network transport	6.05
	An04g02070	Clathrin heavy chain: structural	5.91
	An05g00140	SRP receptor β subunit: binds Srp101 to the ER	5.45
	202202 (JGI)	Sac6 actin-bundling protein	4.13

[a] Normalized spectral abundance factors were compared by G test ($P < 0.05$).

plexes rarely occur in secretory organelles in *A. niger* or that they are more present in vesicle subpopulations that were incompletely covered during organelle enrichment procedures.

In *A. niger*, specific secretory processes are carried out by proteins more similar to mammalian proteins than to yeast proteins, a phenomenon that has been reported for filamentous fungi in general (8, 30). For example, most *A. niger* Rab GTPases identified here show higher similarity to mammalian than to yeast proteins. Since these proteins are involved in vesicle transport and fusion, it is very likely that this similarity to mammalian proteins would explain some fundamental differences in protein secretion of yeast and *A. niger*. Moreover, the controlled interplay between exo- and endocytosis is thought to be necessary for adequate polarized growth in filamentous fungi, and this interplay must in part be determined by the primary sequence of secretory pathway proteins.

In addition to the differences in terms of protein sequence in comparison to yeast, this study also reveals that important changes in the composition of the secretory pathway may be explained by different relative protein abundances in secretory organelles. During induction of cellulase and hemicellulase secretion by D-xylose, hydrolytic enzymes are efficiently synthesized and exported from the cell. These enzymes include proteins previously reported to be significantly upregulated by D-xylose induction, namely, β-xylosidase, a protein similar to bacterial xylosidase S (AxlA; An09g003300), and β-glucosidase (56). From the current study, it becomes clear that in addition to bringing changes in the amount of cargo hydrolytic enzymes, the presence of D-xylose also promotes changes in the relative protein abundance of specific components within the secretory organelles. Unexpectedly, proteins involved in targeting to the ER or quality control of protein glycosylation and folding were not overrepresented after D-xylose induction. On the contrary, these proteins were in some cases even underrepresented in comparison to the noninduced state of D-sorbitol addition. In this study, it was found that D-xylose induction is associated with an increase of three GTPases that are predicted to play roles in endocytosis, exocytosis, and polarized growth and with an increase of specific components of protein degradation pathways. With regard to the three overexpressed GTPases, the RhoA and SrgA GTPases have been described as necessary for polar growth and proper hyphal branching (4, 19, 38, 45), whereas Rab5 is known to play a role in early endocytosis in eukaryotes (7, 8). This important role in endocytosis has also been investigated recently in *Aspergillus* (1). From this study, it has become clear that Rab5-rich endosomes cycle rapidly

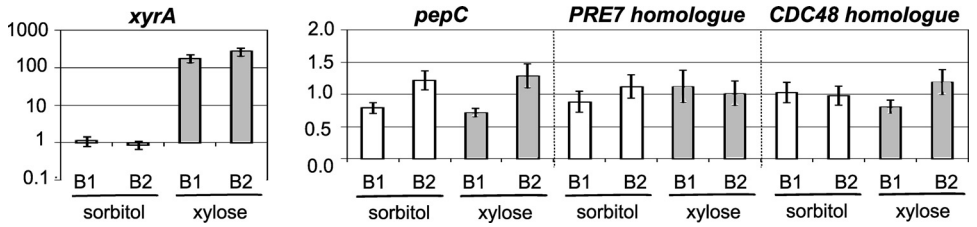

FIG. 3. Relative gene expression of the *A. niger* homologues of the yeast genes *PRE7* and *CDC48*. B1, biological replicate 1; B2, biological replicate 2. Relative levels of *xyrA* transcripts are given in logarithmic scale. The bars represent the mean relative gene expression ± standard error.

TABLE 3. Secretory pathway and membrane-trafficking protein complexes identified in this study and protein subunit relative abundances

Complex[a]	Reference	*S. cerevisiae* protein	*A. niger* locus tag	NSAF (10,000)[b]	
				Sorbitol	Xylose
Sec62/Sec63	13	Sec63	An01g13070	16.9	14.8
		Sec66	An16g08830	19.8	16.4
		Sec62	An02g01510	14.3	9.1
OT subcomplexes	28	Stt3	An16g08570	11.4	15.7
		Ost3	An02g14930	8.2	15.3
		Ost4[c]	An08g07485 (N terminus)	ND	ND
		Ost1	An02g14560	41.1	35.7
		Wbp1	An07g04190	42.3	38.0
Sec23-Sec24 heterodimer	12	Sec23	An01g04730	9.0	4.1
		Sec24	An08g10650	6.4	6.6
p24	6	Emp24	An08g03590	12.2	17.3
		Erv25	An01g08870	16.7	22.7
Erv41/Erv46	39	Erv41	An03g04940	7.5	11.1
		Erv46	An01g04320	12.5	14.9
AP-1 adaptor	62	Apl2	An16g02490	2.6	0.8
		Apm1	An07g03200	2.8	1.5
		Apl4	An01g02600	2.6	1.4
Retromer complex inner shell	49	Pep8	An01g07320	9.2	4.4
		Vps29	An08g01030	10.0	5.1
		Vps35	An16g04270	11.3	9.5
Chaperonin-containing T complex	27	Tcp1	An15g06370	ND	ND
		Cct2	An01g06480	1.2	1.2
		Cct3	An02g08920	ND	ND
		Cct4	An02g12750	2.2	1.2
		Cct5	An11g02360	ND	ND
		Cct6	An16g03100	ND	ND
		Cct7	An18g05770	0.2	1.0
		Cct8	An04g07340	ND	ND

[a] OT, oligosaccharyltransferase.
[b] ND, not detected.
[c] *S. cerevisiae* Ost4 is 36 amino acids long, too small for the 10-kDa cutoff used in this study.

along the hyphal cell in a microtubule-dependent manner. It is plausible that an increased state of protein secretion, such as induction by D-xylose in *A. niger*, increases the number of Rab5-rich early endosomes, thereby facilitating exchange of material with the Golgi apparatus equivalents and the hyphal tip. One observation that confirms this hypothesis is that Golgi apparatus equivalents in *Aspergillus* are relatively slow in motion and are usually found in a gradient across the hyphal cell, more concentrated near the hyphal tip but excluded from the apical body (41, 53).

Previous microarray analyses investigated the effects of D-xylose on the transcriptome of *Aspergillus* species and linked it to metabolic pathways or cellular processes (2, 26, 40, 56). In our study, and in agreement with the above-mentioned transcriptome studies, at the proteome level, D-xylose induction did not affect the microsomal abundance of most proteins belonging to the ERAD pathway. However, the presence of D-xylose did increase the protein microsomal abundance of Cdc48 homologue and the proteasomal 20S CP. Since there was no increase in the transcription levels of the proteasomal 20S CP, both previously by microarray analysis of a daughter fungal strain (56) and in this study by quantitative PCR, this strongly suggests that these proteins are recruited and that they can attach and detach from microsomes according to the cell's needs.

This study shows different strategies that *A. niger* undertakes when forced into a secretory state. Some functional routes of the cell's metabolism show differences between induced and noninduced states via different gene expression profiles, like the cargo proteins, followed by changes in the protein abundances. In other cases, no change in the transcriptional status of the organism was found, and changes in the protein abundance in the secretory organelles can be attributed to specific recruitment of functional complexes (e.g., the proteasome). Together, this shows that secretion is a dynamic and interacting process, several aspects of which can be studied using proteomic analyses.

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