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Elke Kainz
University of Natural Resources and Life Sciences

Andreas Gallmetzer
University of Natural Resources and Life Sciences

Christian Hatzl
University of Natural Resources and Life Sciences

Juergen H. Nett
GlycoFi, Inc.

Huijuan Li
University of Natural Resources and Life Sciences

See next page for additional authors

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N-Glycan Modification in *Aspergillus* Species▽

Elke Kainz,[1]§ Andreas Gallmetzer,[1]§ Christian Hatzl,[1]† Juergen H. Nett,[2] Huijuan Li,[2] Thorsten Schinko,[1] Robert Pachlinger,[1]‡ Harald Berger,[1] Yazmid Reyes-Dominguez,[1] Andreas Bernreiter,[1] Tillmann Gerngross,[2,3] Stefan Wildt,[2] and Joseph Strauss[1]*

Fungal Genomics Unit, Austrian Research Centers and BOKU Vienna, Muthgasse 18, A-1190 Vienna, Austria[1]; GlycoFi, Inc., 21 Lafayette Street, Lebanon, New Hampshire 03766[2]; and Thayer School of Engineering, Dartmouth College, 8000 Cummings Hall, Hanover, New Hampshire 03755[3]

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The production by filamentous fungi of therapeutic glycoproteins intended for use in mammals is held back by the inherent difference in protein N-glycosylation and by the inability of the fungal cell to modify proteins with mammalian glycosylation structures. Here, we report protein N-glycan engineering in two *Aspergillus* species. We functionally expressed in the fungal hosts heterologous chimeric fusion proteins containing different localization peptides and catalytic domains. This strategy allowed the isolation of a strain with a functional α-1,2-mannosidase producing increased amounts of N-glycans of the $Man_5GlcNAc_2$ type. This strain was further engineered by the introduction of a functional GlcNAc transferase I construct yielding $GlcNAcMan_5GlcNac_2$ N-glycans. Additionally, we deleted *algC* genes coding for an enzyme involved in an early step of the fungal glycosylation pathway yielding $Man_3GlcNAc_2$ N-glycans. This modification of fungal glycosylation is a step toward the ability to produce humanized complex N-glycans on therapeutic proteins in filamentous fungi.

Filamentous fungi belonging to the *Aspergillus* group are well-established production hosts for extracellular enzymes of industrial importance, such as amylases, glucoamylases, pectinases, phytases, cellulases, and ligninases. (37). Glucoamylase (1,4-β-D-glucan glucohydrolase; EC 3.2.1.3) is one of the most prominent thermostable industrial enzymes (18) and is mainly produced by *Aspergillus niger* fermentation. Typical fermentation yields by *A. niger* are up to 25 g/liter of glucoamylase (3).

In addition to homologous protein production, *Aspergillus nidulans*, *A. niger*, and *Aspergillus oryzae* have offered an attractive alternative to *Escherichia coli* and yeasts, such as *Saccharomyces cerevisiae, Kluyveromyces lactis*, or *Pichia pastoris*, for the expression of recombinant, heterologous proteins, and there have been a number of attempts to program filamentous fungi for the production of such heterologous proteins (13, 14, 20, 27, 29).

Whereas posttranslational modifications of secreted heterologous proteins have been shown to influence protein stability, yield, and function, the production by filamentous fungi of therapeutic glycoproteins intended for use in mammals has been hampered by the inherent difference in protein *N*-glycosylation and by the inability of the fungal cell to modify proteins with mammalian glycosylation structures. While both fungi and mammals attach a specific oligosaccharide to asparagines in the sequence Asn-X-Ser/Thr/Cys (where X represents any amino acid except proline), the subsequent processing of the transferred glycan differs significantly between mammalian and fungal cells (47). The assembly of a lipid-linked oligosaccharide composed of three gluocose (Glc_3), nine mannose (Man_9), and two *N*-acetylglucosamine ($GlcNAc_2$) residues (referred to as $Glc_3Man_9GlcNAc_2$), followed by transfer to the nascent protein and the removal of three glucose residues and one mannose sugar to yield $Man_8GlcNAc_2$, is conserved between eukaryotes. The biosynthetic glycosylation pathways diverge between fungi and mammals once a glycoprotein leaves the endoplasmic reticulum (ER) and is shuttled through the Golgi apparatus. Yeasts and other fungi typically produce high-mannose-type N-glycans by adding up to 100 mannose sugars, including beta-linked mannoses and mannosylphosphates, whereas the formation of mammalian glycans generally involves the removal of mannose, followed by the addition of N-acetylglucosamine, galactose, fucose, and sialic acid (8, 9, 25, 31). Recently, glycoengineering in the yeast *P. pastoris* and the expression of therapeutic glycoproteins with complex "humanized" *N*-glycosylation structures have shown significant progress (4, 6, 10, 11, 15, 16, 26, 47). Far less progress has been made in glycoengineering of fungal production hosts, such as *Aspergillus* or *Trichoderma* species, although the N-glycan structures of several secreted glycoproteins have been elucidated.(1, 2, 17, 24, 28, 29, 34, 39–41, 48) and attempts were made to modify fungal glycosylation structures by the insertion of glycan structure-modifying enzymes (17, 29, 32). Introduction of rabbit *N*-acetylglucosaminyltransferase I (GnT I) into *A. nidulans* has resulted in the production of an in vitro active enzyme, but no evidence for in vivo GlcNAc transfer was found (23). When human cDNA encoding GnT I was expressed in *Trichoderma reesei*, the incorporation of *N*-acetylglucoseamine into α-1,3-linked mannose of the core oligosaccharide $Man_5GlcNac_2$ was

* Corresponding author. Mailing address: Fungal Genomics Unit, Austrian Research Centers and BOKU Vienna, Muthgasse 18, A-1190 Vienna, Austria. Phone: 43-1-36006-6720. Fax: 43-1-36006-6392. E-mail: joseph.strauss@boku.ac.at.

† Present address: Octapharma Produktionsges. m.b.H., Oberlaaerstrasse 235, A-1100 Vienna, Austria.

‡ Present address: Baxter AG, Industriestrasse 67, A-1221 Vienna, Austria.

§ E.K. and A.G. contributed equally to this work.

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TABLE 1. *A. nidulans* strains used throughout this work

Strain	Genotype[a]	Reference
YR23.5	*pyrG89 argB2 pantoA1 riboB2*	This work
YR23.5-BC10	*pyrG89 pantoA1 riboB2*	This work
YR23.5-CD28	*pyrG89 pantoA1 riboB2*	This work
YR23.5-BC10-coNA15	*pantoA1 riboB2*	This work
YR23.5-*mnnJ*-NA	*pantoA1 riboB2*	This work
YR23.5-Δ*alg*C	*pyrG89 pantoA1 riboB2*	This work

[a] *pyrG89*, pyrimidine requirement, orotidine MP decarboxylase deficient; *argB2*, arginine requirement, OTCase deficient; *pantoA1*, pantothenate requirement; *riboB2*, riboflavin requirement.

TABLE 2. *A. niger* strains used throughout this work

Strain	Genotype[a]	Reference or source
A888	*cspA1 argB13 nicA1*	FGSC[b]
T2 (*argB*$^+$ *gpdA*$_p$-K3) transformant of A888	*nicA1 cspA1 gpdA*$_p$-K3	This work
T2-BC10	*nicA1 cspA1 amdS*	This work
T2-CD28	*nicA1 cspA1 amdS*	This work
T2-BC10-coNA15	*nicA1 cspA1 amdS hph*	This work
T2-BC10-*mnnJ*-NA	*nicA1 cspA1 amdS hph*	This work
T2-ΔalgC	*nicA1 cspA1 ΔalgC*	This work

[a] *cspA1*, morphological phenotype—short conidiophores; *nicA1*, nicotinic or anthranilic acid requirement; *amdS*, amide nonutilization, acetamidase deficient; *hph*, hygromycin B resistance; *argB13*, arginine requirement, OTCase deficient; Δ*algC*, altered glycosilation, Dol-P-Man:Man$_5$GlcNAc$_2$-PP-Dol mannosyltransferase deficient; *gpdA*$_p$-K3, Kringle-3 domain of human plasminogen.
[b] FGSC, Fungal Genetics Stock Center (www.fgsc.net).

demonstrated by ^{1}H nuclear magnetic resonance analysis, but the efficiency of this incorporation was not analyzed (29). In contrast to yeast species, hyperglycosylation is not a typical feature of filamentous fungi, which most often synthesize small high-mannose-type N- and O-glycans (2). So far, no typical mammalian-like complex-type glycans have been found or generated in filamentous fungi (33). Some of the glycans found resemble mammalian high-mannose glycans [Man$_{(6-9)}$GlcNAc$_2$]. In addition, typical "fungal-type" glycans that are structurally different from the mammalian glycans have been identified on different *Aspergillus* glycoproteins [Man$_{(5-12)}$GlcNAc$_2$] (1, 29, 44, 45).

Moreover, growth conditions have been found to influence the glycan modification of some proteins, possibly by modulating the expression of enzymes involved in the fungal glycosylation pathway (12).

In order to approach a solution for this important drawback in the possibility of using fungal heterologous glycoproteins for therapeutic applications (21, 28, 30), we used an approach similar to that described for *Pichia pastoris* and systematically engineered the glycosylation pathways of two *Aspergillus* species. Here, we report the introduction of heterologous fusion proteins, such as mannosidases and glycosyltransferases, as well as the deletion of a gene (*algC*) coding for an enzyme involved in an early step of the fungal glycosylation pathway.

MATERIALS AND METHODS

Strains. The *A. nidulans* and *A. niger* strains used throughout this work are listed in Table 1 and Table 2, respectively. The strain T2 carries an expression plasmid with a reporter protein and *argB*. This expression plasmid allows the purification of the reporter protein (the C-terminal six-His-tagged K3 domain of human plasminogen, as described in reference 6, and expressed from the *A. nidulans gpdA* promoter) and and will allow analysis of the glycosylation of the reporter protein in the future. *E. coli* strain JM 109 was used for routine plasmid propagation.

Cloning of pEKgnopat. From plasmid pAN52-1 (38), the ATG of the *A. nidulans gpd* promoter, as well as the NcoI site, was deleted by PCR using primers pAN52-SalF (5′-CGCAGACCGGGAACACAAGC-3′) and pAN52-BamR (5′-TAACGT TAAGTGGATCCAAGCTGATGTCTGC-3′). The resulting PCR fragment was cloned as a SalI-BamHI fragment into the SalI/BamHI-cut pAN52-1 vector. The NotI site of the vector pAN52-1 was deleted by partial digestion with NotI, filling in of the protruding ends, and subsequent ligation. Cloning sites consisting of NcoI, SwaI, AscI, and SbfI sites, as well as a PacI site, were introduced by cloning the annealed oligonucleotides Not Pac F New (5′-GATCCGCGGCCGCATTTAAAT GGCGCGCCCCTGCAGGTTAATTAAG-3′) and Not Pac R New(5′-GATCCT TAATTAACCTGCAGGGGCGCGCCATTTAAATGCGGCCGCG-3′) as BamHI fragments into the unique BamHI site of pAN52-1. Additionally, AsiSI, FseI, and PmeI restriction sites were introduced by cloning the annealed oligonucleotides pAN52 Marker F (5′-TATGGCGATCGCGGCCGGCCGTTTAAACCA-3′) and pAN52 Marker R (5′-TATGGTTTAAACGGCCGGCCGCGATCGCCA-3′) as NdeI fragments into the unique NdeI site of the vector, yielding the expression vector pEKgnopat.

Cloning of the *A. niger mnnJ* leader sequence. The putative MnnJ protein sequence was identified by a similarity search against the *A. niger* sequence database (Integrated Genomics, Chicago, IL) using *S. cerevisiae* Mnn10p (accession number YDR245W) as a query sequence. Using the *mnnJ* sequence information from the similarity search, the *mnnJ* leader sequence was amplified in a 5′ rapid amplification of cDNA ends experiment with the specific primer RT MNN10 (5′-CTTCTCCCAGCTCTCGCGCCATTCG-3′) and cloned into pGEMT-easy for sequencing. The final leader sequence was amplified from the pGEMT-easy plasmid using the forward primer An-MNN10-5 (5′GCGGCCGC CACCATGCTATTCCTCTTCCGTCGTTTCGAGCTG-3′), creating a NotI restriction site (underlined), and the reverse primer An-MNN10-3 (5′-GGCGCG CCCCCATCGTTCCACATAATTCTTCTTGTTCCA-3′), creating an AscI site (underlined). The NotI-AscI fragments were used in subsequent cloning steps.

Cloning of α-1,2-mannosidase genes into pEKgnopat. The α-1,2-mannosidase catalytic domain-leader fusions were cloned into the NotI-PacI-digested pEKgnopat as NotI-PacI fragments. In the subsequent cloning step, the *A. niger* leader sequence was cloned as NotI-AscI fragments into the NotI-AscI-digested vector.

Cloning of GlcNAc-transferase I genes into pEKgnopat. The GlcNAc-transferase I catalytic domain-leader fusions NA15 and coNA15 were obtained as a gift from GlycoFi, Inc., in vectors pPB104 and pPB104-CO. coNA15 is a codon-optimized sequence for expression in *P. pastoris*. To achieve this, the human codons have been changed to the optimal *P. pastoris* codons following the codon usage table available at http://www.kazusa.or.jp/codon/. The plasmids were constructed by fusion of sequences coding for amino acids 1 to 40 of *ScMNN9* to sequences coding for amino acids 38 to 445 of *HsGNTI* (Table 3) using the nucleotide sequences GGGCGCGCC and GGAAGAGCA, respectively, as fusion linkers in the process, adding the three amino acids GlyArgAla. In the case of pPB104, this fusion was accomplished by using the AscI site created by the additional nucleotides, and in the case of pPB104, the complete gene fusion sequence was assembled using oligonucleotides and fusion PCR. Both fusions were then released by NotI-PacI digestion and cloned into the NotI-PacI-digested pEKgnopat. Additionally, *A. niger* leader sequences were introduced into the pEKgnopat-NA15 plasmid by removing the MNN9 (no. 15) leader with a NotI-AscI digestion and cloning the *A. niger* leader as NotI-AscI fragments into the vector.

***algC* knockout constructs.** The putative *algC* protein sequence was identified by a similarity search using *S. cerevisiae* Alg3p (accession number YBL082C) as a query against the *A. nidulans* sequence database at Broad Institute (http://www.broad.mit.edu) and the *A. niger* sequence database (Integrated Genomics, Chicago, IL). Additional 3′ and 5′ sequence information on the *algC* gene of *A. niger* was obtained by probing with the *algC* fragment a minilibrary based on restriction site analysis of *A. niger* strain 888. A colony lift and subsequent hybridization experiment with the *algC* PCR product as a probe revealed two clones that yielded additional 3′ sequence information. *algC* was knocked out by a DNA fragment obtained by chimeric PCR. 3′ and 5′ fragments of the *algC* gene were amplified from chromosomal *A. niger* DNA with the primer pairs alg3 F (5′-G TCACGGTCAACGTCCTCCCTCT-3′) plus alg3_5primeR (5′-CACAACCAC CAGCGTGATCAAGTAGAG-3′) and alg3_3primeF (5′-GTTGCGATTAAG ATGACACTGTTGCTG-3′) plus alg3_3prime_R_new (5′-GGAGGTGAATT AGCTTGAGCGGAATA-3′). An *hph* fragment with *algC* overhangs was amplified from the vector pAN7 (38) using primers HmB_chimericF (5′-CTCTA CTTGATCACGCTGGTGGTTGTGTACACAGGCTCAAATCAATAAGAAG

TABLE 3. Functional-leader–catalytic domain fusion constructs

Construct	Leader (amino acid coordinates)	Organism (accession no.)	Catalytic (amino acid coordinates)	Organism (accession no.)
BC10	Pp*SEC12* (334–363)	*P. pastoris* (AF216960.1)	α-1,2-Mannosidase (81–541)	*C. elegans* (NM 059715.3)
CD28	Sc*MNN10* (1–121)	*S. cerevisiae* (YDR245W)	α-1,2-Mannosidase (187–667)	*D. melanogaster* (X82640)
NA 15 and coNA15	Sc*MNN9* (1–40)	*S. cerevisiae* (YPL050C)	GnTI (38–445)	*H. sapiens* (NM_002406)[a]
mnnJ-NA	*mnnJ* (1–250)	*A. niger* (DQ841153)	GnTI (38–445)	*H. sapiens* (NM_002406)

[a] Codon optimized with *P. pastoris* codon bias in coNA.

AACG-3′) and HmB_chimericR (5′-CAGCAACAGTGTCATCTTAATCGCAA CTAGATGTGGAGTGGGCGCTTACAC-3′) and a long-run PCR kit (MBI Fermentas). The three fragments were purified via gel excision and assembled in a subsequent PCR, in which the overhangs of the *hph* fragment functioned as a primer. Amplification of the chimeric PCR product was performed with the primers alg3 F (5′-GTCACGGTCAACGTCCTCCCTCT-3′) and alg3_3prime_R_new (5′-GGAGGTGAATTAGCTTGAGCGGAATA-3′). Fifteen microliters of the PCR product was used directly for transformation.

To knock out the *algC* gene in *A. nidulans*, a recombinant DNA fragment was constructed via chimeric PCR. 5′ and 3′ fragments of the *algC* gene were amplified from chromosomal *A. nidulans* DNA with the primer pairs ANalgC5′_fwd (5′-GC TGTTGGAGGCTCTGGATAGAAA-3′) plus ANalgC5′_rev (5′-TGCCATTGTA GTTGATGAAGAAGAAGA-3′) and ANalgC3′_fwd (5′-GGACAACGTACATG CAACAGGTCA-3′) plus ANalgC3′_rev (5′-CCCGCGCAATTCCTTCTTTAG-3′). An *argB* fragment with *algC* overhangs was amplified from the vector pFB39 (43) using the primers chimeric-argB_fwd (5′-CTCTTCTTCTTCATCAACTAC AATGGCATATTTCGCGGTTTTTTGGGGTAGT-3′) and chimeric-argB_rev (5′-TGACCTGTTGCATGTACGTTGTCCTAGCCATTGCGAAACCTCA GAAG-3′) and a long-run PCR kit (Roche). The three fragments were purified and assembled in a subsequent PCR, in which the overhangs of the *argB* fragment functioned as primers, and amplification of the chimeric nested PCR was performed with the primers ANalgCnested_fwd (5′-GTGTATGGAAACGAG ACGATCATCTTC-3′) and ANalgCnested_rev (5′-GCAGCAGATATTCCAT TCAACCAAAG-3′) in one PCR; 15 μl of the PCR product was used directly for transformation.

***Aspergillus* transformation.** Transformation protocols basically followed the procedures published by Tilburn and colleagues (42). Transformants were selected according to the selection marker used (Tables 1 and 2). The transformants were then checked by PCR and/or dot blot hybridization, as well as by Southern blot analysis. Replacement of *A. nidulans algC* by the marker gene was verified by PCR, using primers ANalgC5′_fwd (5′-GCTGTTGGAGGCTCTGG ATAGAAA-3′) and ANalgC3′_rev (5′-GGACAACGTACATGCAACAGGTC A-3′), which anneal to *algC* 5′ and *algC* 3′ sequences not present on the knockout construct, giving rise to a larger PCR product (4,881 bp) than an intact *algC* gene (2,923 bp).

Replacement of *A. niger algC* by the marker gene used a similar strategy, employing the *algC* 5′primers alg3_KO_PCR_F (5′-CGCCGCATCTACATCC CCG-3′) and alg3_KO_gpdA_R (5′-TTGGGACGATGCAAGATATAAACGA A-3′), which anneal to the *gpdA* promoter of the selection marker on the knockout construct. Additionally, the absence of *algC* coding sequence was verified by PCR with specific primers and by Southern analysis using an *algC* open reading frame fragment of either *A. nidulans* or *A. niger*.

Culture conditions. Strains were grown for 20 h at 37°C on 250 ml LB medium containing supplements according to the auxotrophy markers of the strain and ammonium tartrate (10 mM) as an additional nitrogen source. The mycelium was harvested by filtration, washed with ~500 ml of tap water, and ground to fine powder under liquid nitrogen, and the powder was subsequently lyophilized. The lyophilized biomass was used in glycan digestion and subsequent mass spectrometry (MS) analysis to determine the whole-cell glycosylation.

Glucoamylase production. Strains were grown on *Aspergillus* complete medium containing supplements according to the auxotrophy markers of the strain and ammonium tartrate (10 mM) as an additional nitrogen source for 50 h at 200 rpm and 37°C. The mycelium was harvested by filtration and washed with sterile water. Subsequently, the mycelium was transferred to fresh expression medium (70 g/liter trisodium citrate dihydrate, 20 ml/liter *Aspergillus* salt solution, and 10 g/liter maltodextrin, plus the respective supplements and ammonium tartrate [10 mM] as a nitrogen source) and cultivated for about 52 h at 200 rpm and 30°C. The culture supernatant (containing glucoamylase) was harvested and stored at −80°C. Glucoamylase was purified from the culture supernatant by size exclusion chromatography (PD-10 columns; Amersham Biosciences), using Tris, pH 8.0, according to the manufacturer's manual. The eluate was concentrated via ultrafiltration (Vivaspin 20-ml concentrator; 5,000 MWCO PES; VivaScience) and further purified via strong basic anion-exchange chromatography (Vivapure Q Mini H; VivaScience), using 25 mM Tris, pH 8.0, for equilibration and 1 M NaCl-25 mM Tris, pH 8.0 for elution. The purified protein was lyophilized.

Total cellular protein extraction and release of N-linked glycan. Total cellular protein was extracted by dissolving 50 mg of lyophilized and ground cell pellet in 0.5 ml RCM buffer (8 M urea, 360 mM Tris, and 3.2 mM EDTA, pH 8.6) at room temperature. Cell debris was removed by centrifugation for 15 min at 2,500 rpm in a Beckmann Coulter Allegra 6 centrifuge. The protein content of the supernatant was determined by protein assay (Bio-Rad, Hercules, CA). The glycans were released and separated from the glycoproteins by a modification of previously reported methods (4, 35). Briefly, 50 μg of solubilized proteins of various samples were adsorbed onto a polyvinylidene difluoride membrane in a 96-well MultiScreen IP plate (Millipore, Bedford, MA). After the proteins were reduced and carboxymethylated and the membranes were blocked, the wells were washed three times with water. The proteins were deglycosylated by the addition of 22 μl of 10 mM NH_4HCO_3, pH 8.3, containing 1 mU PNGase F (New England Biolabs). After 16 h at 37°C, the solution containing the glycans was removed to a clean 96-well plate by centrifugation and evaporated to dryness.

MS analysis. The molecular weights of the glycans were determined using a Voyager DE PRO linear matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometer (Applied Biosciences) as described previously (6). All spectra were generated with the instrument in the positive ion mode. In this MALDI-TOF ion mode, it is possible that phosphorylated glycans could not be detected, and the influence of the expression of α-1,2-mannosidase on phosphorylated fungal glycans has not been studied here. All of the masses obtained in MS analysis are summarized in Table 4.

TABLE 4. Molecular weights of glycans detected by MALDI-TOF[a]

Proposed structure	Composition	Theoretical mol wt[b]	Mol wt detected by MALDI-TOF[c]
Hex_3	$GlcNAc_2$ Hex_3	933 (949)	934 (950)–936 (952)
Hex_4	$GlcNAc_2$ Hex_4	1,095 (1,111)	1,096 (1,112)–1,098 (1,114)
Hex_5	$GlcNAc_2$ Hex_5	1,257 (1,273)	1,258 (1,274)–1,261 (1,277)
Hex_6	$GlcNAc_2$ Hex_6	1,419 (1,435)	1,419 (1,435)–1,425 (1,441)
Hex	$GlcNAc_2$ Hex_7	1,581 (1,597)	1,581 (1,597)–1,586 (1,602)
Hex_8	$GlcNAc_2$ Hex_8	1,743 (1,759)	1,743 (1,759)–1,749 (1,765)
Hex_9	$GlcNAc_2$ Hex_9	1,905 (1,921)	1,905 (1,921)–1,912 (1,928)
$GlcNAcM_5$	$GlcNAc_2Man_5$ GlcNAc	1,460 (1,476)	1,461 (1,477)–1,465 (1,481)

[a] Molecular weight masses hexose, 180 (162, loss of H_2O); GlcNAc, 221 (203, loss of H_2O).
[b] Molecular weight with Na adduct (K adduct in parentheses).
[c] Molecular weight range from multiple spectra.

In vitro α-1,2-mannosidase digestion. *T. reesei* α-1,2-mannosidase (a gift from R. Contreras, Unit of Fundamental and Applied Molecular Biology, Department of Molecular Biology, Ghent University, Ghent, Belgium) digestions were performed as described previously (4).

RNA isolation. Strains were grown overnight on minimal medium containing the appropriate supplements at 37°C and 180 rpm. The mycelium was harvested and frozen in liquid nitrogen. RNA was isolated using Trizol according to the manufacturer's instructions (Gibco BRL).

qRT-PCR. Quantitative reverse transcription-PCR (qRT-PCR) for gene expression was performed with Platinum Sybr green qPCR SuperMix-UDG (Invitrogen) and a SuperScript III Platinum Two-Step qRT-PCR kit with Sybr green (Invitrogen) and was carried out on an iCycler thermal cycler with a MyiQ single-color real-time PCR detection system (Bio-Rad). Reactions were run in duplicate, and a mean value of the two samples was calculated. The results were calculated as the relative amount of product adjusted for the level of internal control amplification (actin mRNA) obtained for the sample. The primers used for quantification of BC10 in *A. nidulans* and *A. niger* were BC10fwd (5′-ACT GCCGGATACTCTGGAATC-3′) and trpCrev (5′-GATTTCAGTAACGTTA AGTGGATCC-3′), those for CD28 in *A. nidulans* and *A. niger* were CD28fwd (5′-TCGCCGAAACGCTTAAGTAC-3′) and trpCrev, those for coNA15 in *A. nidulans* and *A. niger* were coNA15 F (5′-CAAAACACAATGTCACTTTCTC TTGTATCCTAC-3′) and trpCrev, and those for *mnnJ*-NA in *A. nidulans* and *A. niger* were GNT F (5′-CTTGTATCGTACCGCCTAAGAAAGAACC-3′) and trpCrev. For amplification of the *A. niger* actin gene (accession no. AAU11333), the primers actNigR (5′-TATCTGAGGGTGAGGATACCACG-3′) and actNigerF (5′-GACAATGGTTCGGGTATGTGC-3′) were used. For amplification of the *A. nidulans* actin gene (accession no. AN6542.3), the primers actin F (5′-GATCGGTATGGGTCAGAAGGA-3′) and actin R (5′-CGATGTTGCCG TACAGATCC-3′) were used.

Nucleotide sequence accession numbers. The *mnnJ* leader sequence has been deposited with NCBI under accession number DQ841153. The *A. niger algC* sequence has been deposited under accession no. DQ841152. The accession number for *A. nidulans algC* is AN0104.3.

RESULTS AND DISCUSSION

Expression of functional α-1,2-mannosidase fusion proteins results in trimming of terminal mannose residues to putative $Man_5GlcNAc_2$ structures. α-1,2-Mannosidase hydrolyzes terminal 1,2-linked α-D-mannose residues in the oligomannose oligosaccharide $Man_9GlcNAc_2$ in a calcium-dependent manner to produce $Man_8GlcNAc_2$ in a first step and $Man_5GlcNAc_2$ *N*-glycans on glycosylated proteins in a second step (19). Generation of $Man_5GlcNAc_2$ is one of the critical steps in obtaining complex glycoproteins because this substrate is the precursor for all subsequent downstream processing steps (11). In an attempt to express functional α-1,2-mannosidase in *Aspergillus*, cDNAs coding for leader sequences from enzymes known to target the protein to the ER or Golgi apparatus from *S. cerevisiae, P. pastoris*, or *A. niger* were combined in the pEKgnopat *Aspergillus* expression vector (see Materials and Methods) with different catalytic domains derived from *Caenorhabditis elegans, Drosophila melanogaster, Mus musculus*, and *T. reesei*. Leader sequences from putative *A. niger* orthologues of *S. cerevisiae* Gls1p, Mns1p, Mnn10p, Mnn11p, and Anp1p were identified and cloned by similarity searches against an *A. niger* sequence database (data not shown). Among all putative *A. niger* leaders, only the Mnn10p orthologue was found to be functional in combination with heterologous catalytic domains (see below). The combinatorial fusion constructs were individually transformed into *A. niger* strain T2 and *A. nidulans* strain YR23.5 (in *A. nidulans*, only constructs BC10 and CD28 were transformed), and the integration events were verified by PCR and Southern blotting (results not shown). The copy numbers of constructs integrated into the genome varied from one copy to several copies in our Southern analysis, potentially influencing the expression levels of the integrated genes. We determined the expression levels in at least two single-copy strains of each transformation event in *Aspergillus* complete medium (36) by qRT-PCR. Expression levels were monitored by a combination of α-1,2-mannosidase-specific and *trpC* terminator-specific primers, and the transcript levels obtained were normalized to the *A. nidulans* or *A. niger* actin gene *acnA* (see Materials and Methods). Additionally, the actin-normalized expression levels of the transgenes were compared with the actin-normalized mRNA levels of the endogenous *gpdA* genes of *A. niger* or *A. nidulans*. In single-copy transformants, similar mRNA levels (with differences of less than 20%) were recorded (data not shown). Such strains were selected for further analysis, for which the cultures were processed as described in Materials and Methods, and whole-cell glycans were released by PNGase digestion and analyzed by MALDI-TOF analysis.

Structure assignments were based on the masses (summarized in Table 4) and sensitivity to α-1,2-mannosidase. However, we cannot rule out the possibility of other hexoses. For example, in *A. niger* N-linked glycans, the presence of galactose in a galactofuranose conformation has been established (44, 46). In this work, we did not analyze for their appearance by digestion with alternative mannosidases, such as an α-1,2/3/6-mannosidase.

Therefore, the masses of N-linked glycans released by PNGase F digestion of proteins obtained by MALDI-TOF are referred to as hexoses. For example, in the figures, we refer to a $GlcNAc_2Hex_6$ structure as Hex_6.

The BC10 construct (a *P. pastoris SEC12* leader fused to a *C. elegans* α-1,2-mannosidase lacking a transmembrane domain [Table 3]) was the only one found to be functional in *A. niger* and resulted in a considerable shift of glycan composition from mainly $Man_8GlcNAc_2$ (Fig. 1A, right) to strongly elevated amounts of $Man_5GlcNAc_2$ (Fig. 1B, right). All other constructs were not functional in modifying the N-glycan masses; one example is shown in Fig. 1C, right, for the CD28 fusion construct (an *S. cerevisiae MNN10* leader fused to a *D. melanogaster* α-1,2-mannosidase lacking a transmembrane domain). Of these two fusion proteins, only the expression of BC10 resulted in a different glycosylation pattern at the level of whole-cell glycans in *A. nidulans*. Figure 1, left, compares the glycan masses obtained by MALDI-TOF analysis for the *A. nidulans* recipient strain YR23.5 (Fig. 1A) with the spectra obtained from the strain expressing the BC10 construct (Fig. 1B) and an example of a typical spectrum obtained from a strain carrying a nonfunctional fusion construct (CD28) (Fig. 1C). The mass spectrum of BC10 clearly shows elevated masses consistent with $Man_5GlcNAc_2$ structures. Ions appearing with low abundance between Hex6 and Hex7 that have masses similar to $GlcNAcMan_5GlcNAc_2$ in Fig. 1A and B are probably a matrix artifact and not N-linked glycans. The glycan patterns of *A. nidulans* (Fig. 1D, left) and *A. niger* (Fig. 1D, right), which were obtained after in vitro digestions using purified α-1,2-mannosidase on whole-cell extracts, show an almost complete reduction of any N-glycan other than $Man_5GlcNAc_2$, and this result shows that the in vivo functionality of the α-1,2-mannosidase fusion constructs is only partial.

In addition to whole-cell glycan analysis, the N-glycans of a prominent secreted *A. niger* protein were analyzed. Glucoamylase, an enzyme secreted under starch or maltodextrin induc-

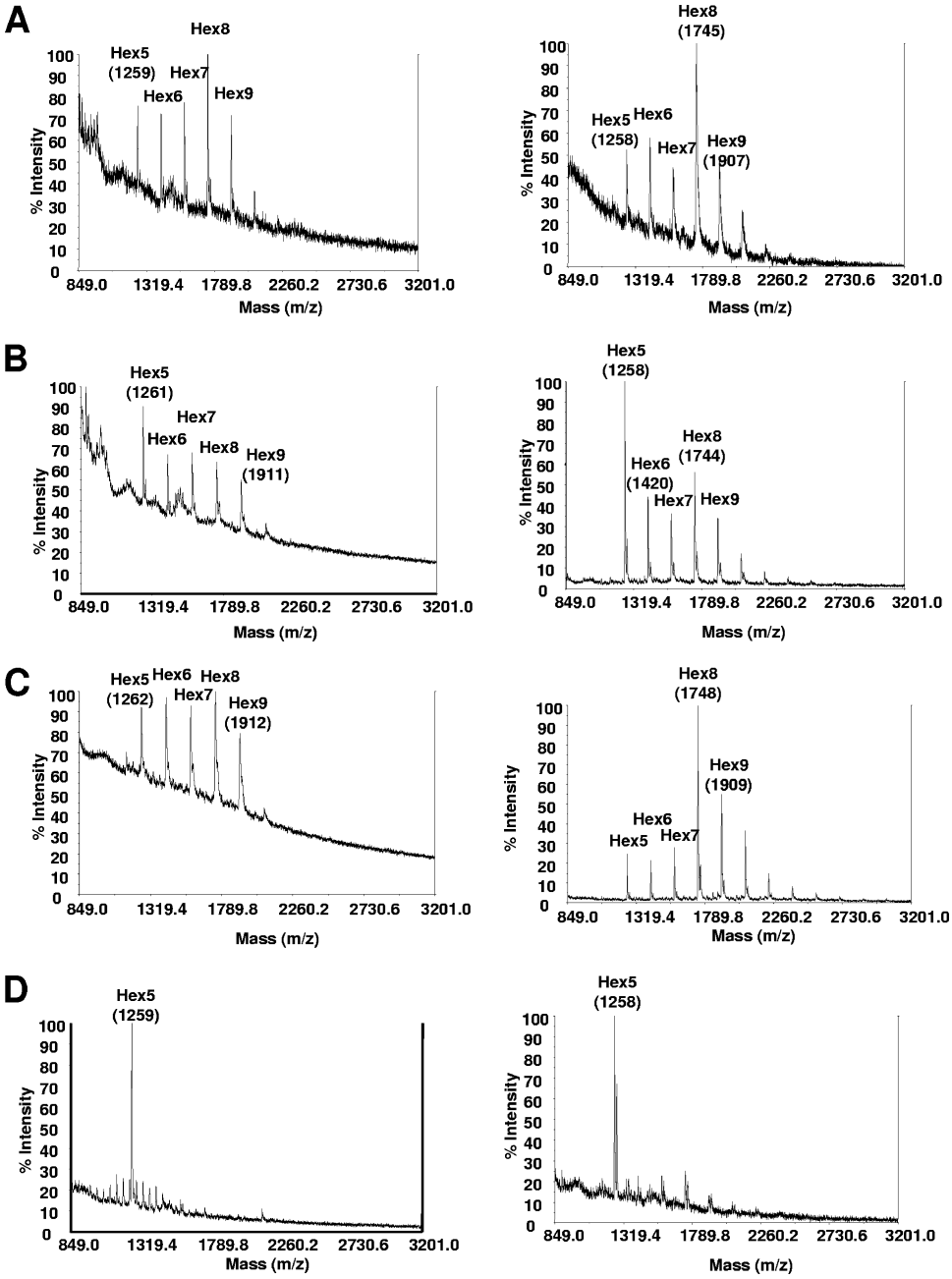

FIG. 1. Whole-cell N-glycan analysis of *Aspergillus* strains expressing engineered α-1,2-mannosidases. The images on the left compare whole-cell N-glycans from *A. nidulans* recipient strain YR23.5 (A) with strain YR23.5-BC10 (B) and strain YR23.5-CD28 (C). The images on the right show whole-cell N-glycan spectra obtained from *A. niger* recipient strain T2 (A), strain T2-BC10 (B), and strain T2-CD28 (C). (D) N-glycan spectra obtained after in vitro PNGase F digestion of *A. nidulans* (left) or *A. niger* (right) whole-cell extracts with α-1,2-mannosidase. Preparation and analysis of N-glycans was performed as described in Materials and Methods. Structure assignments in this figure and all subsequent figures are based on MALDI-TOF masses (*m/z*) of individual PNGase F-released ions sensitive to α-1,2-mannosidase, and the relative intensities of the masses are shown (% intensity).

tion, was purified (see Materials and Methods) and analyzed for glycan composition. The glycan pattern of glucoamylase was comparable to that of the whole-cell glycans, with a shift from $Man_8GlcNAc_2$ to high levels of $Man_5GlcNAc_2$ (Fig. 2).

Interestingly, only one construct (BC10) was shown to be functional in both *Aspergillus* species at the level of whole-cell glycans (*A. nidulans* and *A. niger*) and on secreted glucoamylase (*A. niger*). As in *P. pastoris*, the catalytic domain of *C. elegans* worked best in both *Aspergillus* species. In *P. pastoris*, when combined with the *S. cerevisiae* leaders MNS1 and MNN10, the catalytic domain of *C. elegans* produced predominantly $Man_5GlcNAc_2$ on the *Pichia* reporter protein K3 (6). A slightly lower rate, as estimated from MALDI-TOF analysis, was observed in *A. niger*. Other constructs shown to be func-

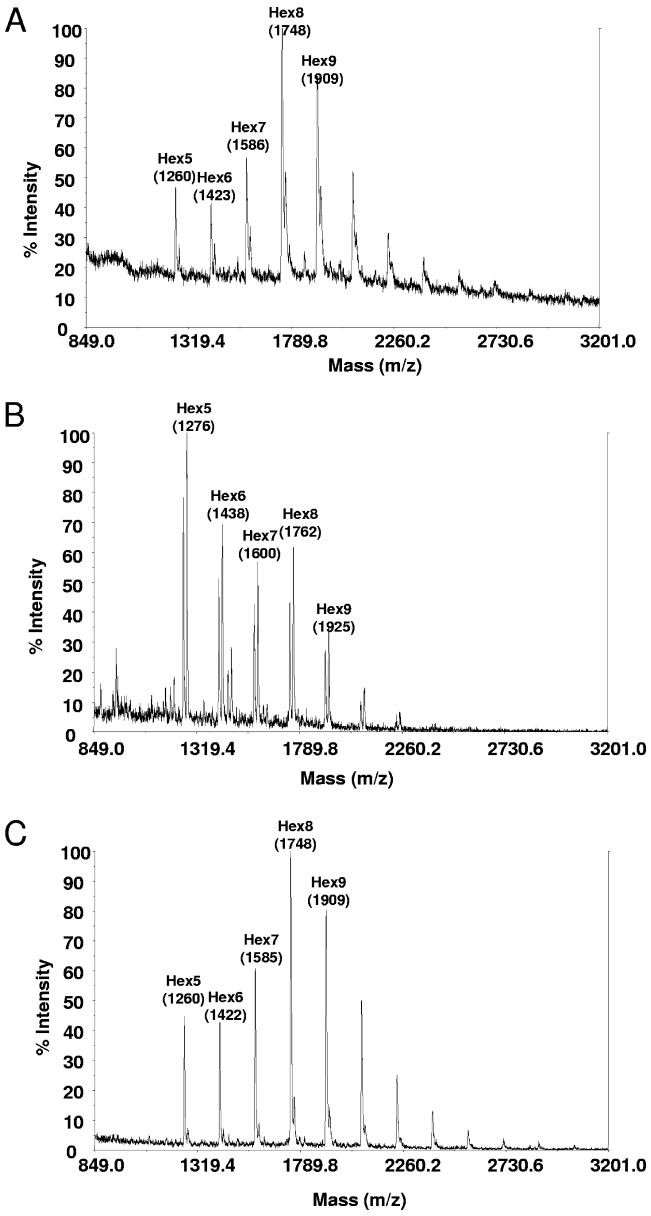

FIG. 2. MALDI-TOF spectra of N-glycans derived from purified *A. niger* glucoamylase of the recipient strain T2 (A), strain T2-BC10 (B), or strain T2-CD28 (C).

tional in *Pichia* were not successful in *Aspergillus*. Even though previous studies established a correlation between certain domains and targeting events, there is currently no tool available that allows one to predict the behavior of a given targeting peptide across different hosts. Accordingly, we were not able to predict from comparison of the primary protein sequences of functional versus nonfunctional mannosidases which of the constructs tested would work in *Aspergillus*.

Human β-1,2-*N*-acetylglucosaminyltransferase I (GNT I) is functional in vivo. The transfer of *N*-acetyl-D-glucosamine to $Man_5GlcNAc_2$ substrates generated by the previous $Man_8GlcNAc_2$-to-$Man_5GlcNAc_2$ trimming step was investigated. The GNT I enzyme localizes to the luminal face of the Golgi apparatus, allowing the sequential processing of glycoproteins as they are shuttled through the secretory pathway. In one construct, the catalytic domain of human GNT I (abbreviated as NA) was fused to the MNN9 leader (abbreviated as 15) from *S. cerevisiae* to yield construct NA15. In a second construct, a fungal codon-optimized form (coNA; see Materials and Methods for details) was combined with the same MNN9 leader to yield construct coNA15. Yet another construct used a leader sequence from a putative *A. niger* gene with similarity to the *S. cerevisiae MNN10* gene, designated *mnnJ* according to the *Aspergillus* nomenclature (for the *mnnJ* accession number and all construct details, see Materials and Methods and Table 3). Fusion of the *mnnJ* leader sequence with NA yielded *mnnJ*-NA, which was, like all the other fusion constructs, subsequently integrated into the same expression vector backbone. These expression vectors were stably introduced by transformation into *A. nidulans* and *A. niger* strains already carrying the BC10 α-1,2-mannosidase construct, which showed the highest in vivo activity. Integration events were verified by PCR and Southern analysis, and expression of the constructs was tested by qRT-PCR (see Materials and Methods).

Whole-cell N-glycan analysis of *A. niger* BC10-transformed strains revealed that expression of the fusion construct coNA15 (Table 3) resulted in efficient transformation of $Man_5GlcNAc_2$ to $GlcNAcMan_5GlcNAc_2$ N-glycans, as seen from mass analysis (Fig. 3, right). It is intriguing that in the *A. niger* T2-BC10 recipient strain a considerable portion of higher-mannose N-glycans, such as $Man_6GlcNAc_2$, $Man_7GlcNAc_2$, and $Man_8GlcNAc_2$, still existed in parallel with a large amount of $Man_5GlcNAc_2$ (Fig. 3A, right). In the strain additionally expressing the GNT I enzyme (Fig. 3B, right), these higher-mannose glycans were significantly reduced.

The N-glycans released from the purified *A. niger* glucoamylase were subsequently analyzed in strains T2-BC10-coNA15 und T2-BC10-*mnnJ*-NA. The glycan pattern of glucoamylase was comparable to that of the whole-cell glycans, with a shift from $Man_5GlcNAc_2$ to $GlcNAcMan_5GlcNAc_2$ (Fig. 4). Whereas at the level of whole-cell glycans, the construct *mnnJ*-NA was not functional at all (Fig. 3C, right), some GlcNAc transfer was detected on glucoamylase (Fig. 4C).

On glucoamylase of *A. niger* strain T2-BC10-coNA15, predominantly masses corresponding to $GlcNAcMan_5GlcNAc_2$ were found, but $(Man_5\text{-}Man_9)GlcNAc_2$ and a mass consistent with $GlcNAcMan_6GlcNAc_2$ were also detected. It remains to be determined whether introduction of a GlcNAc transporter would shift the glycosylation of secreted protein in *A. niger* toward $GlcNAcMan_5GlcNAc_2$, as was reported for *P. pastoris* (6).

Whole-cell glycan analysis of the transformed strains, along with the *A. nidulans* BC10 recipient strain (Fig. 3A, left), was carried out by mass spectrometry following glycan digestions. Of all transformants tested, the strain carrying construct coNA15 showed a new peak corresponding to the mass of $GlcNAcMan_5GlcNAc_2$ structures (Fig. 3B, left). Interestingly, the construct *mnnJ*-NA showed some activity in *A. nidulans* (Fig. 3C, left) but no activity in *A. niger*.

MnnJ is the putative orthologue of *S. cerevisiae* Mnn10p, which functions in a complex with MNN9p in a subunit of the yeast Golgi apparatus mannosyltransferase complex, also containing Anp1p, Mnn11p, and Hoc1p, that mediates elongation of the polysaccharide mannan backbone (22). Interestingly, the

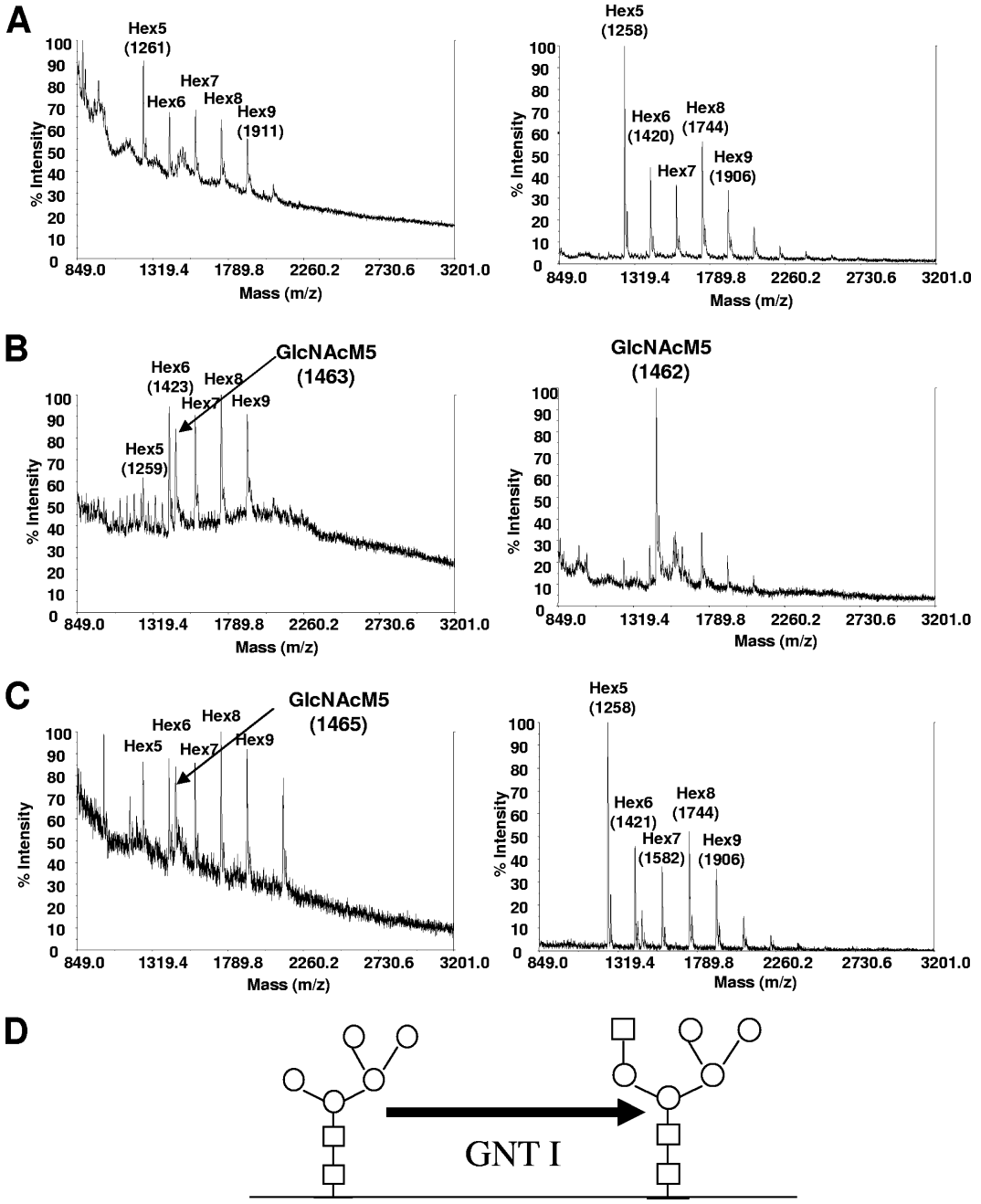

FIG. 3. Whole-cell N-glycan analysis of *Aspergillus* strains expressing engineered GNT I. (A, B, and C) The images on the left compare whole-cell N-glycans from *A. nidulans* strain YR23.5-BC10, which served as a recipient for the GNT I constructs (A) (shown in Fig. 1B, left, and repeated here for clarity), with strain YR23.5-BC10-coNA15 (B) and strain YR23.5-BC10-mnnJ-NA (C). The images on the right show whole-cell glycan spectra for *A. niger* strain T2-BC10, which served as a recipient strain for the GNT I constructs (A), compared to N-glycans obtained from strain T2-BC10-coNA15 (B) and strain T2-BC10-mnnJ-NA (C). (D) Schematic diagram representing the function of GNT I, which generates $GlcNAcMan_5GlcNAc_2$ structures by the addition of one GlcNAc moiety on $Man_5GlcNAc_2$. GlcNAc residues are represented by squares and mannose (hexose) residues by circles.

MnnJ leader sequence was the only fungal leader with significant activity out of five putative proteins derived from the Golgi apparatus mannosyltransferase complex.

One GNT I construct was shown to be particularly active in *A. niger* and to shift the equilibrium of the enzymatic reaction toward $GlcNAcMan_5GlcNAc_2$. Interestingly, in the strain expressing only α-1,2-mannosidase from the BC10 construct, a considerable number of $Man_7GlcNAc_2$ and $Man_8GlcNAc_2$ glycan structures still existed in parallel with the large amount of $Man_5GlcNAc_2$ (Fig. 3A, right), but in the strain additionally expressing the GNT I enzyme (Fig. 3B, right), these higher-mannose structures were almost completely lost. These results suggest that removal of $Man_5GlcNAc_2$, produced by α-1,2-mannosidase, by GNT I, which uses $Man_5GlcNAc_2$ as a

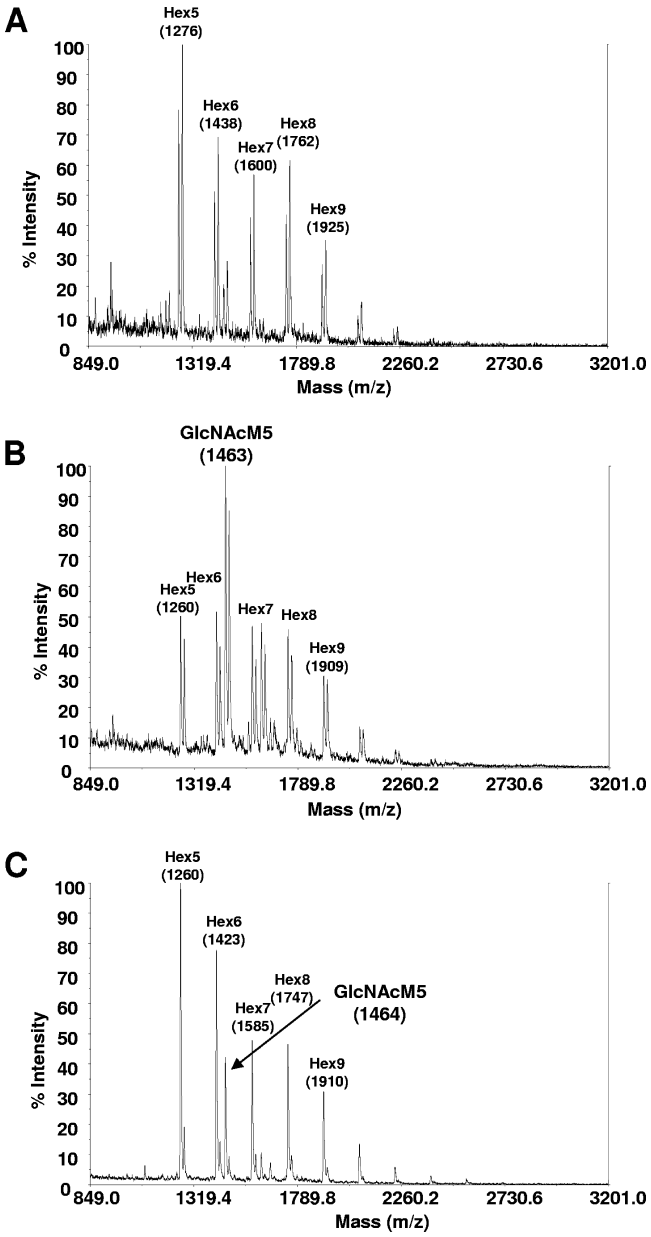

FIG. 4. N-glycan analysis of purified *A. niger* glucoamylase from strain T2-BC10, which served as a recipient for the GNT I constructs (A) (shown in Fig. 2B and repeated here for clarity), compared to strains T2-BC10-coNA15 (B) and T2-BC10-mnnJ-NA (C).

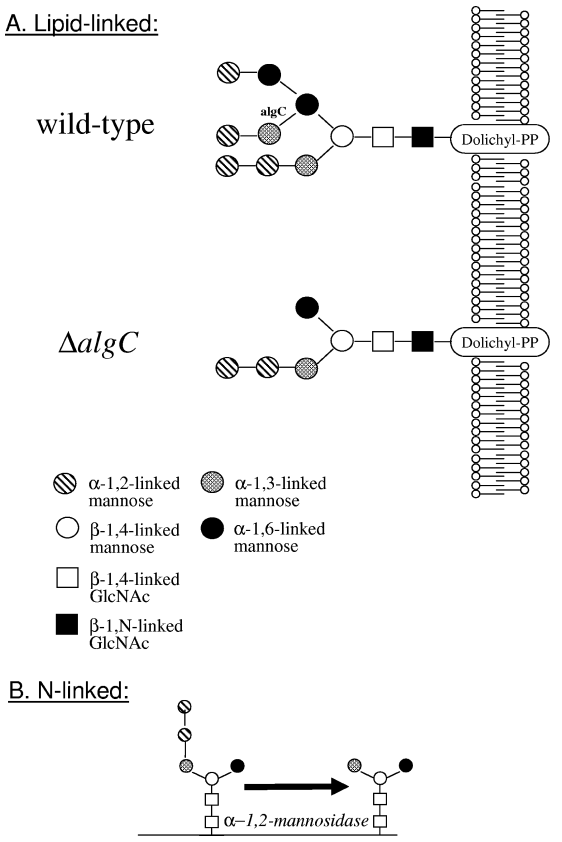

FIG. 5. The function of Alg3p. (A) The yeast *ALG3* gene encodes the Dol-P-Man:$Man_5GlcNAc_2$-PP-Dol mannosyltransferase, which converts $Man_5GlcNAc_2$-Dol-PP to $Man_6GlcNAc_2$-Dol-PP. (B) Knockout of *ALG3* leads to $Man_5GlcNAc_2$ structures, which can be trimmed to $Man_3GlcNAc_2$ by α-1,2-mannosidase.

substrate, shifts the substrate-product equilibrium for α-1,2-mannosidase, which in turn results in a more active α-1,2-mannosidase enzyme along the glycosylation line. Additionally or alternatively, removal of $Man_5GlcNAc_2$ could also remove the substrate for alternative resident glycosyltransferases. Moreover, in a GNT I strain expressing a nonfunctional α-1,2-mannosidase fusion construct (e.g., T2-CD28), no $GlcNAcMan_5GlcNAc_2$ was detected, and therefore, it seems that the endogenous *A. niger* α-1,2-mannosidase is not sufficient to provide enough substrate for a functional GNT I enzyme. As already seen with the α-1,2-mannosidase fusion construct BC10, the GNT I fusion construct already shown to be functional in *Pichia pastoris* also worked best in *Aspergillus*. In contrast to the α-1,2-mannosidase, which showed only partial activity in *Aspergillus*, the GNT I construct led almost exclusively to $GlcNAcMan_5GlcNAc_2$, as reported for *P. pastoris* (6). Our results on whole-cell glycan structures also suggest that the α-1,2-mannosidase fusion construct BC10 is correctly localized in *Aspergillus* and that α-1,2-mannosidase trimming occurs in the ER and early Golgi apparatus and not through secreted α-1,2-mannosidase in the medium. This was of concern, since leakage of α-1,2-mannosidase into the medium was reported by other groups (5, 6).

Generation of $Man_3GlcNAc_2$ structures by knockout of the *algC* gene. The formation of N-glycosidic linkages of glycoproteins involves the ordered assembly of the common $Glc_3Man_9GlcNAc_2$ core oligosaccharide on the lipid carrier dolichyl pyrophosphate. Whereas early mannosylation steps occur on the cytoplasmic side of the ER with GDP-Man as the donor, the final reactions from $Man_5GlcNAc_2$-PP-Dol to $Man_9GlcNAc_2$-PP-Dol on the luminal side use Dol-P-Man. The *ALG3* gene encodes the Dol-P-Man:$Man_5GlcNAc_2$-PP-Dol mannosyltransferase, which converts $Man_5GlcNAc_2$-Dol-PP to $Man_6GlcNAc_2$-Dol-PP. Knockout of *ALG3* in *S. cerevisiae* and *P. pastoris* leads to specific $Man_5GlcNAc_2$ structures, which can be trimmed to $Man_3GlcNAc_2$ by α-1,2-mannosidase (Fig. 5). We have previously shown that the distinct Δ*alg3* $Man_5GlcNAc_2$ can be trimmed by α-1,2-mannosidases to obtain paucimannose

$Man_3GlcnNAc_2$. Paucimannose then can serve as a substrate for GnT I. (7).

We cloned the *ALG3* orthologues of both, *A. nidulans* and *A. niger* (see Materials and Methods) and designated the corresponding genes *algC*. Alignments of the *A. nidulans* (AN0104.2), *A. niger* (DQ841152), and *A. terreus* (EAU37037) AlgC proteins with *S. cerevisiae* Alg3p (YBL082C) showed high similarity among the three proteins only in the N-terminal part. The *A. nidulans* protein is around 200 amino acids smaller than the yeast Alg3p and showed a similarity score of only 8e-33 in a BLAST search against the *A. nidulans* genome database (http://www.broad.mit.edu) using *S.c.*Alg3p as the in silico probe. High overall similarity can be observed only among the putative AlgC proteins of filamentous fungi (e.g., *A. oryzae*, *Aspergillus fumigatus*, and *Neurospora crassa*), but yeasts and basidiomycetes show only very limited overall similarity in their putative Dol-P-Man:$Man_5GlcNac_2$-PP-Dol mannosyltransferases (not shown).

Using roughly 1 kb of the nucleotide sequence upstream and downstream of the *algC* open reading frame, we generated by hybrid PCR two sets of deletion constructs. One construct carried between the upstream and downstream sequences the auxotrophic marker gene *argB* (for *A. nidulans*), while the other harbored the dominant hygromycin resistance marker gene *hygB* (for *A. niger*). We then generated *algC* knockout strains (Δ*algC*) for both organisms. Morphologically, *A. nidulans* strains YR23.5 and YR23.5-Δ*algC* did not show any significant differences. The knockout strain did not show any growth defects on minimal or complete medium and sporulated as well as the recipient and other *algC*$^+$ strains (not shown). Analysis of the whole-cell glycans of the *A. nidulans* strain YR23.5-Δ*algC* showed a shift of the whole-cell glycan pattern to lower-mannose-type glycosylation. Additional masses, such as $Man_3GlcNAc_2$ and $Man_4GlcNAc_2$, previously not seen in the parental strain were observed (Fig. 6A). Structures larger than $GlcNAcMan_3GlcNAc_2$ could be the result of endogenous glycosyltransferase activity capping the free α-1,6-mannose arm. However, knockout of *algC* efficiently inhibited core oligosaccharide assembly in *A. nidulans*, confirming that indeed we had for the first time cloned and deleted an *ALG3* homologue of a filamentous fungus. We then tested whole-cell glycans in the *A. niger* T2-Δ*algC* knockout strain. As in *A. nidulans*, the lack of AlgC activity led to a shift of the whole-cell glycan pattern to lower-mannose-type glycosylation, like $Man_3GlcNAc_2$ and $Man_4GlcNAc_2$ (not shown). In vitro digestion of N-glycans released from the *A. niger* Δ*algC* strain with purified α-1,2-mannosidase led to an almost complete trimming of the $Man_4GlcNAc_2$, $Man_5GlcNAc_2$, and $Man_6GlcNAc_2$ structures to $Man_3GlcNAc_2$ (Fig. 6C). When N-glycans released from glucoamylase purified from this strain were analyzed (not shown), the mass spectra showed a peak composition similar to that seen for *A. niger* whole-cell glycans, i.e., a loss of higher masses and the appearance of masses corresponding to $Man_3GlcNAc_2$ to $Man_6GlcNAc_2$ (Fig. 6B).

Knockout of *algC* (AN0104.4) in *A. nidulans* led to some $Man_3GlcNAc_2$, in addition to $Man_4GlcNAc_2$ and $Man_5GlcNAc_2$, which was the dominant glycan, as well as $Man_6GlcNAc_2$. In *A. niger*, the knockout of *algC* also led to the generation of masses consistent with $Man_3GlcNAc_2$ and $Man_4GlcNAc_2$, structures not present in the parent strain (compare Fig. 1). Similarly to *A. nidulans*, $Man_5GlcNAc_2$ and $Man_6GlcNAc_2$ were additional glycans, in this case dominated by $Man_6GlcNAc_2$. In vitro digestion of T2-Δ*algC* whole-cell glycans led almost exclusively to $Man_3GlcNAc_2$ paucimannose glycans. Previously observed larger glycan masses recalcitrant to in vitro α-1,2-mannosidase treatment in a *P. pastoris* Δ*alg3* Δ*och1* strain (7) were not detected in *A. niger*. Such larger structures can be formed by endogenous glycosyltransferases, which may add hexoses to various glycan structures (such as the unusual Δ*algC* glycan created here) in vivo. Digestion of N-linked glycans derived from the Δ*algC* strain with purified α-1,2-mannosidase led to an almost complete trimming of the masses consistent with $Man_4GlcNAc_2$, $Man_5GlcNAc_2$, and

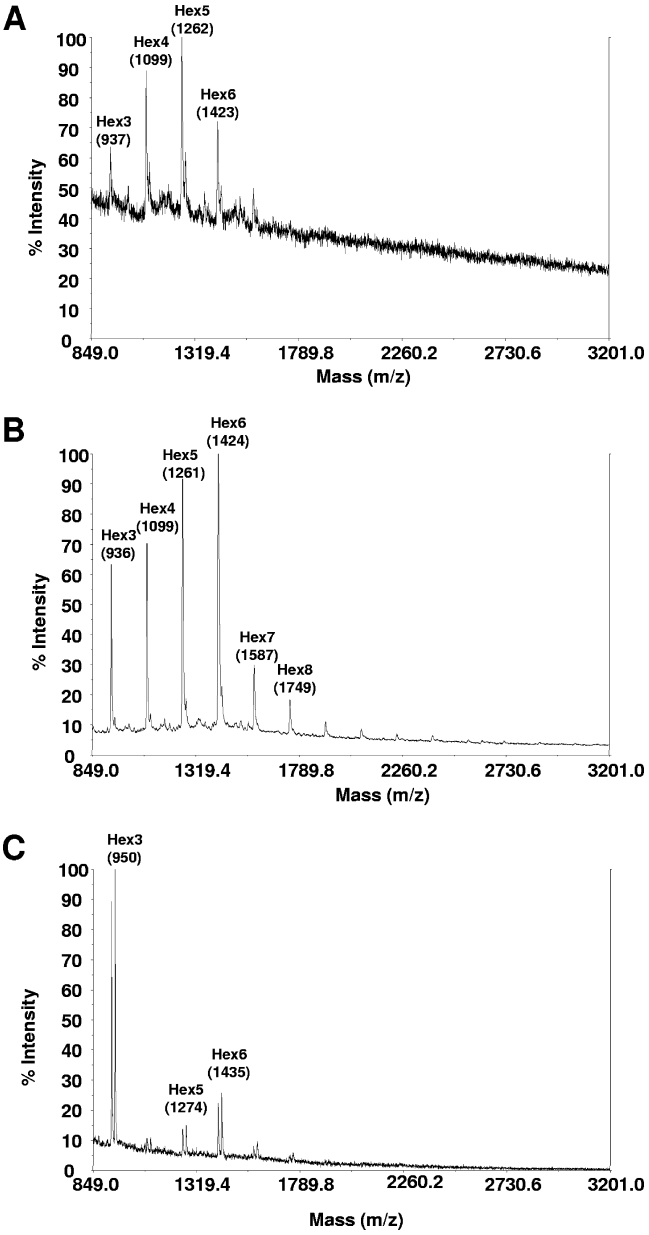

FIG. 6. Whole-cell N-glycan analysis of *Aspergillus* strains lacking AlgC activity. (A and B) Whole-cell N-glycan structures of the *A. nidulans* strain YR23.5-Δ*algC* (A) and *A. niger* strain T2-Δ*algC* (B). (C) N-glycan spectrum obtained after in vitro digestion of *A. niger* T2-Δ*algC* whole-cell extracts with purified α-1,2-mannosidase.

$Man_6GlcNAc_2$ structures to $Man_3GlcNAc_2$. Similar larger-size oligosaccharides were observed after the cloning and deletion of the ALG3 gene in the methylotrophic yeast *P. pastoris*. This finding implies the existence of endogenous glycosyltransferases capable of transferring sugars to the canonical *alg3* Man_5 N-glycan (7). Subsequent experiments, such as the analysis of lipid-linked oligosaccharides, should allow the elucidation of these novel structures and will in turn provide a deeper understanding of the substrate specificity of glycosyltransferases residing in the secretory pathway in *Aspergillus*.

In sum, the findings presented here suggest, as also previously demonstrated for the methylotrophic yeast *P. pastoris* (4), that the deletion of *algC* can indeed provide a promising route in *Aspergillus* for the addition of one additional GlcNAc residue to produce $GlcNAcMan_3GlcNAc_2$, and hence, to drive further the production of humanized complex glycoproteins in filamentous fungi.

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