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A Novel β-(1–3)-Glucanosyltransferase from the Cell Wall of *Aspergillus fumigatus**

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Cell wall transferases utilizing β-(1–3)-glucan chains as substrates may play important roles in cell wall assembly and rearrangement, as β-(1–3)-glucan is a major structural component of the cell wall of many fungi. A novel β-(1–3)-glucanosyltransferase was purified to apparent homogeneity from an autolysate of the cell wall of *Aspergillus fumigatus*. The enzyme had a molecular mass of 49 kDa and contained approximately 5 kDa of *N*-linked carbohydrate. The enzyme catalyzed an initial endo-type splitting of a β-(1–3)-glucan molecule, followed by linkage of the newly generated reducing end to the nonreducing end of another β-(1–3)-glucan molecule. Laminarioligosaccharides of size G_{10} and greater were donor substrates for the transferase. Laminarioligosaccharides of size G_5 and greater formed acceptors. The enzyme was able to reuse initial transferase products as donors and acceptors in extended incubations, resulting in the formation of increasingly larger transferase products until they became insoluble. The major initial products from an incubation of the transferase with borohydride-reduced G_{11} (rG_{11}) were rG_6 and rG_{16}. ^1H NMR analysis of the rG_{16} transferase product showed it was a laminarioligosaccharide, indicating that the enzyme forms a β-(1–3)-linkage during transfer. The enzyme may have a key function *in vivo* by allowing the integration of newly synthesized glucan into the wall and promoting cell wall expansion during cell growth.

The fungal cell wall is a highly dynamic structure and despite decades of research elucidating its composition, very little is known about the processes involved in its assembly and subsequent rearrangements during cell growth. The wall is composed of a complex of macromolecules that protects the cell and resists the high turgor pressure of the enclosed protoplast, thereby giving the cell its shape. Glucans are the most abundant polysaccharides present in fungal walls (1), and the wall's inherent mechanical strength is provided by the major structural component, β-(1–3)-glucan, together with varying proportions of chitin depending on the species and morphology. In most fungi, β-(1–3)-glucan is branched to varying degrees with β-(1–6)-linkages at the branchpoints (1–5). Studies carried out in yeast have shown that β-(1–3)-glucan is synthesized vectorially by a plasma membrane-bound enzyme complex, which utilizes UDP-glucose as substrate and extrudes its linear product through the membrane into the wall (6–8). The assembly and rearrangement of β-(1–3)-glucan once external to the plasma membrane must involve the coordinated action of many different cell wall enzymes, and these may play a direct role in events such as the integration of the newly synthesized β-(1–3)-glucan into the existing wall glucan, branching of β-(1–3)-glucan via β-(1–3)/(1–6)-branchpoints, as well as the cross-linking of β-(1–3)-glucan to other wall components such as chitin or proteins. Chemical analyses over the last decade or more have provided evidence that all of the above events must occur, but no enzymes have yet been described that can catalyze any of the necessary reactions. It is likely that many of these reactions will involve cell wall transferases.

Recent studies with *Saccharomyces cerevisiae* have shown that the alkali-soluble β-(1–3)-glucan and the chitin chains produced by chitin synthase 3 (9) serve as precursors for the alkali-insoluble glucan/chitin complex (10, 11). Cabib and coworkers (11) have shown that some of the cell wall chitin is linked through a β-(1–4)-linkage from the terminal residue to the nonreducing end of β-(1–3)-glucan chains. Analysis of an alkali-insoluble glucan fraction in *Candida albicans* has provided evidence for a glycosidic linkage between C-1 of the terminal residue of β-(1–6)-glucan and C-6 of the nonreducing end of chitin (12). In addition, studies in *Schizophyllum commune* have provided evidence for a covalent linkage between β-glucan and chitin involving amino acids, of which lysine was the most predominant (13). Also in *S. cerevisiae*, Klis and co-workers have found glucomannoproteins consisting of β-(1–6)-glucan covalently linked to mannoproteins via a novel type of side chain (14) involving a phosphodiester bridge (15). These proteins were isolated from the wall by β-(1–3)-glucanase digestion, suggesting that these glucan-mannoprotein complexes are covalently linked to β-(1–3)-glucan in the wall (5, 15).

Recently, a secreted β-(1–3)-glucanosyltransferase (referred to as BGT1 in this paper) was isolated from *C. albicans*, which catalyzes the transfer of a segment of β-(1–3)-glucan to the nonreducing end of another β-(1–3)-glucan chain, forming a new, intrachain β-(1–6)-linkage in the process (16, 17). The enzyme's function in the wall is unknown since disruption of the *BGL2* gene encoding the homolog in *S. cerevisiae* did not show a distinct phenotype (18, 19). β-(1–3)-Glucan has been shown to undergo some turnover in the wall of exponentially growing cells of *S. cerevisiae*, and newly synthesized glucan more so than pre-existing glucan (20).

We have recently undertaken a study of enzymes involved in the organization of the cell wall of the filamentous, opportunistic human pathogen, *Aspergillus fumigatus*. Its hyphal wall contains predominantly β-(1–3)-glucan, chitin, and α-(1–3)-glucan, with lesser amounts of galactomannan and protein (21,

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22). During the search for enzymes that utilize β-(1–3)-glucan chains as substrates, we found two β-(1–3)-glucanosyltransferase activities (BGT1 and BGT2). One catalyzed a transferase reaction[1] virtually identical to that previously described for *Candida* BGT1 (16). The other enzyme represents a novel β-(1–3)-glucanosyltransferase (BGT2), which is described in this paper.

EXPERIMENTAL PROCEDURES

Preparation of Cell Walls and Autolysis—*A. fumigatus* CBS 144–89 was grown in a 15-liter fermenter in 2% glucose, 1% mycopeptone (Biokar Diagnostics) plus 0.1% silicone antifoam 426R (Rhodorsil) at 25 °C, 500 rpm, 8 liters of air·min^{-1} for 42 h. A culture grown for 3 days in a 2-liter fermenter under the same conditions was used as inoculum (8% (v/v)). The mycelia were collected by filtration under vacuum and broken by passing through a Dyno-mill (W. A. Bachofen AG, Basel, Switzerland) in the presence of glass beads (0.5–0.75 mm diameter). The progress of cell disruption was followed by microscopic examination. The broken mycelial suspension was centrifuged (8,000 × *g*, 15 min) and the cell wall pellet washed three times with water and once in 50 mM sodium acetate, pH 5.6, containing 5 mM sodium azide before being resuspended in the same buffer (250 g wet weight/liter of buffer) and incubated (200 rpm agitation) at 37 °C. After 72 h, the suspension was centrifuged (10,000 × *g*, 15 min) and the supernatant was placed in dialysis tubing, concentrated 5–10 fold with polyethylene glycol 20,000, dialyzed against 5 mM sodium acetate, pH 5.6, recentrifuged (10,000 × *g*, 15 min), and filtered (0.45-μm filter). This preparation is referred to as the autolysate.

Enzyme Purification—Fractions collected during every chromatography step were assayed for enzyme activity using the nonradioactive transferase assay (see below). The concentrated, dialyzed autolysate was applied to a column (4 × 18 cm) of DEAE-Sepharose Fast-Flow (Pharmacia Biotech Inc.) equilibrated in 5 mM sodium acetate, pH 5.6, and eluted with a linear gradient of 0–1 M NaCl (1000 ml) at a flow rate of 240 ml h^{-1}. Fractions containing the transferase activity were pooled, dialyzed against 10 mM β-mercaptoethanol, 5 mM EDTA, 10 mM sodium acetate buffer, pH 4.0, and applied to a Mono S column (HR 5/5, Pharmacia) and eluted with a linear NaCl gradient (0–300 mM in 40 min) at a flow rate of 0.8 ml min^{-1}. The fractions containing the transferase activity were pooled, dialyzed against 10 mM Tris/HCl, pH 7.0, and deposited on a DEAE-5PW column (8 × 75 mm, TosoHaas) and eluted with a linear NaCl gradient (0–300 mM in 60 min) with a flow rate of 0.75 ml min^{-1}. Fractions containing the transferase activity were pooled, dialyzed against 10 mM β-mercaptoethanol, 5 mM EDTA, 10 mM sodium acetate, pH 4.0, and deposited on a CM-5PW column (8 × 75 mm, TosoHaas) and eluted with a linear NaCl gradient (0–300 mM in 60 min) at a flow rate of 0.8 ml min^{-1}. Transferase-containing fractions were pooled and concentrated by SpeedVac and fractionated on a Superdex HR75 column (Pharmacia) equilibrated in 10 mM Tris/HCl, pH 7.0, containing 150 mM NaCl, at a flow rate of 0.75 ml min^{-1}. Fractions containing the purified transferase were pooled, dialyzed against 5 mM sodium citrate, pH 5.0, concentrated by SpeedVac, and stored at −20 °C until used.

Transferase Assays—Enzyme fractions were assayed for the presence of transferase activity by incubating in 50 mM sodium citrate, pH 5.0, at 37 °C (10 μl assay volume) with a borohydride-reduced laminarioligosaccharide (8 mM final) of at least G_{10} in size. Samples (3 μl) were taken at different times, added to ice-cold 50 mM NaOH (47 μl) to terminate the reaction, and frozen until analyzed by high performance anion exchange chromatography (HPAEC).[2] Because of variations in peak intensities from day to day with pulsed electrochemical detection detection, transferase activity was quantitated using ^{3}H-labeled reduced laminarioligosaccharides as substrates and measuring the appearance of label in the products after separation by HPAEC, using an on-line Radiomatic flow scintillation analyzer (model 150TR; Packard). Unless stated otherwise, assays for enzyme characterization studies were performed as above with 0.25 μg of purified transferase.

Colorimetric Assays—β-Glucanase activity in protein fractions was measured by a reducing sugar assay using the *p*-hydroxybenzoic acid hydrazide reagent with borohydride-reduced laminarin instead of carboxymethyl pachyman as substrate (23). Exo-β-glucanase/β-glucosidase activities were measured by incubation of enzyme fractions with *p*-nitrophenyl-β-D-glucopyranoside (16). Protein was estimated using the Bio-Rad protein assay according to the manufacturer's instructions, with bovine serum albumin as standard.

High Performance Anionic Exchange Chromatography—Carbohydrate samples from transferase assays were analyzed on a CarboPac PA1 (4 × 250 mm) Dionex analytical column (with a PA1 guard column) on a Dionex HPAEC system with pulsed electrochemical detection (PED-2 cell), fitted with a combination pH-Ag/AgCl reference electrode and using a potential of 0.4 V for the first 0.5 s of detection. Oligosaccharides were eluted with a 10–175 mM sodium acetate gradient in 50 mM NaOH (35 min) at a flow rate of 1 ml min^{-1}.

Preparation of Reduced Substrates—Laminarioligosaccharides were obtained by a partial acid hydrolysis (6.5 M trifluoroacetic acid, 15 min, 100 °C, followed by 1 M trifluoroacetic acid, 45 min, 100 °C) of curdlan (Serva). Trifluoroacetic acid was removed by rotary evaporation in the presence of methanol. Oligosaccharides were reduced overnight with $NaBH_4$ (1:0.5 (w/w) in 0.1 M NaOH at room temperature). Reducing end ^{3}H-labeled laminarioligosaccharides were similarly prepared by reduction with NaB^3H_4 (Amersham, 20–40 Ci/mmol, 10 mCi/mg of oligosaccharide) overnight, followed by further reduction with $NaBH_4$ as before. Excess $NaBH_4$ was destroyed by the addition of acetic acid to pH 5–6, and borate was removed by rotary evaporation in the presence of methanol. The reduced oligosaccharides were desalted by gel fitration on a Sephadex G15 column (1.2 × 80 cm, 8 ml h^{-1}, equilibrated in water) and collected after detection by the orcinol-sulfuric acid method (24). The laminarioligosaccharides were separated by HPAEC on a CarboPac PA1 (9 × 250 mm) preparative column (Dionex) with a 15–350 mM sodium acetate gradient in 50 mM NaOH (45 min) at a flow rate of 4 ml min^{-1}. Collected oligosaccharide fractions were neutralized by acetic acid, desalted by gel fitration on a Sephadex G15 column as before, and freeze-dried. Laminarin (Sigma) was similarly reduced, but desalted by dialysis against 0.5% acetic acid, followed by dialysis against water, and then freeze-dried. Gentiooligosaccharides were prepared as above (without reduction) from pustulan (Calbiochem), which had been finely ground by mortar and pestle. Maltoheptaose and cellopentaose were from Boehringer Mannheim and Sigma, respectively. Chitohexaose was a generous gift from Dr. A. Domard (Université Claude Bernard, Villeurbanne, France). Borohydride-reduced G_{10} containing an intrachain β-(1–6)-linkage at the 6th linkage from the reducing end (rG_{10}*) was obtained by incubating reduced laminarihexaose (rG_6) with an enzyme homolog to *Candida* BGT1 (16) purified from *A. fumigatus*.[1] The rG_{10}* transferase product was separated and purified as for the laminarioligosaccharides above.

SDS-Polyacrylamide Gel Electrophoresis—Protein samples were analyzed by SDS-PAGE (25) using 10% separating gels and 4% stacking gels. Proteins bands were visualized by staining with Coomassie Blue. De-*N*-glycosylation of glycoprotein was done using recombinant *N*-glycosidase F (Oxford GlycoSystems) according to the manufacturer's instructions.

^{1}H NMR Spectroscopy—Two samples were analyzed: a reduced G_{10} laminarioligosaccharide used as a standard and a reduced G_{16} oligosaccharide, which was obtained after incubation of rG_{10} with the transferase and purified by HPAEC. Dry samples were deuterium-exchanged by freeze-drying and dissolving in D_2O (99.95%, Solvants Documentation Synthese, Peypin, France). Spectra were recorded at 300 and 318 K on a Varian Unity 500 spectrometer operating at a proton frequency of 500 MHz. The OH resonance of residual water was suppressed by selective irradiation during the relaxation delay. Sodium 3-trimethylsilyl propionic acid was used as an external reference.

RESULTS

Purification of the 49-kDa Protein—In order to study cell wall-associated β-glucanosyltransferase activities in *A. fumigatus*, a HPAEC assay was developed using borohydride-reduced laminarioligosaccharides as substrates. A novel β-(1–3)-glucanosyltransferase (BGT2) activity was detected (see below) in semipurified fractions from a cell wall autolysate of *A. fumigatus*, which remained associated with a protein of 49 kDa throughout its purification. The protein was purified to apparent homogeneity by four conventional ion-exchange chromatography steps and a gel fitration step (see "Experimental Procedures"). The transferase activity was clearly detectable only after the second chromatography step (Mono S), after which most of the contaminating β-glucanase activity had been re-

[1] R. P. Hartland, T. Fontaine, J.-P. Debeaupuis, C. Simenel, M. Delepierre, and J.-P. Latgé, manuscript in preparation.

[2] The abbreviations used are: HPAEC, high performance anion exchange chromatography; PAGE, polyacrylamide gel electrophoresis; Gn, oligosaccharide containing *n* β-(1–3) glucose residues; rGn, reduced oligosaccharide containing *n* β-(1–3) glucose residues.

moved. SDS-PAGE analysis of the purified fraction showed one major band at 49 kDa (Fig. 1, *lane b*). To determine whether it contained *N*-linked carbohydrate, the protein was digested with *N*-glycosidase F. The digested protein ran on SDS-PAGE as a 44-kDa protein (Fig. 1, *lane c*), indicating that it contained about 5 kDa of *N*-linked carbohydrate.

Enzyme Activity of the 49-kDa Protein—Analysis by HPAEC of the products resulting from the incubation of the 49-kDa protein with a borohydride-reduced laminarioligosaccharide (rG_n) of size G_{10} or greater allowed the characterization of a novel glucanosyltransferase activity. The major initial products from the incubation with rG_{11} were rG_6 and rG_{16}; rG_{12} gave rG_6+rG_7 and rG_{17}+rG_{18}; rG_{13} gave rG_6 to rG_8 and rG_{18} to rG_{20}; and rG_{14} resulted in the formation of rG_6 to rG_9 and rG_{19} to rG_{22} (Fig. 2). Importantly, there were no nonreduced laminarioligosaccharide products detected, confirming the absence of any endo-β-(1–3)-glucanase activity. The presence of such an activity would have resulted in the formation of a mixture of reduced and nonreduced hydrolysis products, the latter having quite different retention times. In addition, there was no glucose detected and, together with the absence of both *p*-nitrophenyl-β-glucopyranoside hydrolysis and net reducing-sugar formation from borohydride-reduced laminarin in the corresponding colorimetric assays, confirmed the absence of exo-β-(1–3)-glucanase and β-glucosidase activity. The pattern of products obtained (Fig. 2) is consistent with an endo-type glucanosyltransferase activity in which the glucan chain is cleaved in an endolytic fashion, releasing the reducing end portion, and the remainder is transferred to another glucan chain, forming a larger transferase product. Thus, in the simplest reaction with rG_{11}, the enzyme cleaves the substrate releasing rG_6 from the reducing end of the substrate molecule, and the remaining G_5 is then transferred to another rG_{11} molecule acting as an acceptor, to form the rG_{16} transferase product, where *E* represents the enzyme.

$$E + rG_{11} \rightarrow E \cdot G_5 + rG_6$$

$$E \cdot G_5 + rG_{11} \rightarrow E + rG_{16}$$

REACTIONS 1 AND 2

The transferase cleaves rG_{12} in two different ways, resulting in two different transferase products.

$$E + rG_{12} \rightarrow E \cdot G_6 + rG_6$$

$$\rightarrow E \cdot G_5 + rG_7$$

$$E \cdot G_6 + rG_{12} \rightarrow E + rG_{18}$$

$$E \cdot G_5 + rG_{12} \rightarrow E + rG_{17}$$

REACTIONS 3–5

Similarly, with rG_{13} and rG_{14}, the transferase cleaves in three and four different ways, respectively, each time transferring the nonreducing end portion to another rG_{13} or rG_{14} acceptor molecule.

Further analyses of incubations of the 49-kDa transferase with smaller, reduced laminarioligosaccharides showed that the reaction with rG_{10} gave rG_5+rG_6 and rG_{14}+rG_{15} as the major initial products, whereas the reaction with rG_9 was extremely slow, forming small peaks at rG_5 to rG_8 and rG_{10} to rG_{13} (data not shown). There were no products detected upon incubation with reduced laminarioligosaccharides of size G_8 and smaller.

To determine the enzyme's relative rate of reaction with varying sized laminarioligosaccharides, the 49-kDa enzyme (0.25 μg) was incubated separately with 8 mM ^{3}H-labeled rG_{10} to rG_{15} and the rate of formation of labeled products was measured. The rate with rG_{10} (328 nmol·min^{-1}·mg protein^{-1}) was approximately 50% of that of the larger substrates and there appeared to be no significant difference between the reaction rates for rG_{11} to rG_{15} (648 ± 46 nmol·min^{-1}·mg protein^{-1}).

Analyses of extended incubations of purified enzyme with a reduced laminarioligosaccharide of at least G_{10} in size showed

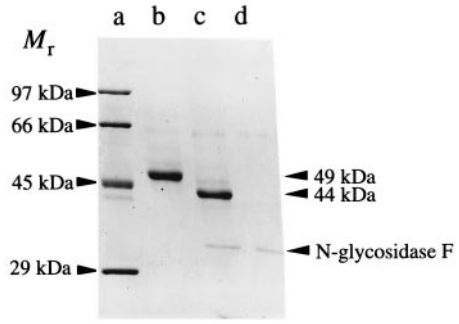

FIG. 1. **SDS-PAGE analysis of the purified 49-kDa protein.** The 49-kDa protein was purified as described under "Experimental Procedures." *Lane a*, molecular size standards; *lane b*, purified 49-kDa protein (1.5 μg); *lane c*, purified 49-kDa protein (1.5 μg) after treatment with *N*-glycosidase F; *lane d*, *N*-glycosidase F only. The molecular masses of the protein bands and *N*-glycosidase F are indicated.

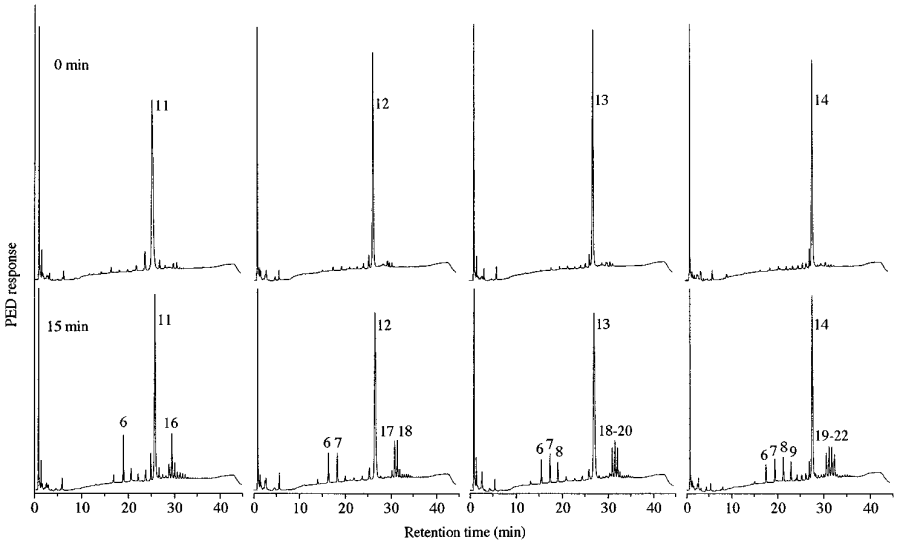

FIG. 2. **HPAEC analysis of products from the incubation of the 49-kDa enzyme with reduced laminarioligosaccharides.** Purified 49-kDa protein was incubated with 8 mM reduced laminarioligosaccharide of size G_{11}, G_{12}, G_{13}, or G_{14} and the HPAEC profiles from samples taken at zero time and 15 min are shown, with the size of the major products indicated.

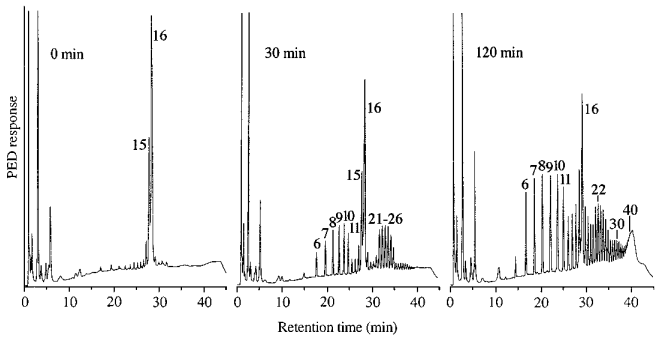

FIG. 3. **HPAEC analysis of products from the incubation of the 49-kDa enzyme with rG_{16}.** The 49-kDa protein was incubated with 8 mM rG_{16} and the HPAEC profiles from samples taken at zero time, 30 min, and 120 min are shown, with the size of the major products indicated.

that the initial transferase products can be reused subsequently either as donors or as acceptors, resulting in the formation of products of increasing size, until they drop out of solution because of their insolubility in aqueous (buffer). A 30-min incubation with rG_{16} (containing some contaminating rG_{15}) resulted in the formation of reduced, major initial products of sizes G_6 to G_{11} and G_{21} to G_{26} (Fig. 3), but after 120 min, larger transferase products had appeared of up to at least G_{40} in size (Fig. 3). The products of G_{29} in size and larger had precipitated in the bottom of the incubation tube, as these were missing when the reaction mixture was briefly centrifuged and the supernatant was analyzed (data not shown). Incubation of purified enzyme with reduced laminarin resulted in the production of smaller and larger products (data not shown), indicating that soluble oligosaccharides of G_{30} in size and larger could act both as donors and acceptors in the reaction.

To determine the smallest laminarioligosaccharide that could act as an acceptor, the purified transferase was incubated with 4 mM rG_{11} as the donor plus 16 mM rG_8 or smaller as an acceptor. Analyses of the incubations containing rG_{11} plus rG_4 or smaller showed the formation of rG_6 and rG_{16} as the only major initial products (data not shown), indicating that only rG_{11} was being used as an acceptor. However, incubations containing rG_{11} plus rG_5 to rG_8 showed additional transferase products consistent with the latter oligosaccharides being used as acceptors (data not shown). For example, the reaction of rG_{11} plus rG_7 resulted in the initial formation of rG_6, rG_{12} and rG_{16} consistent with the following reactions.

$$E + rG_{11} \rightarrow E \cdot G_5 + rG_6$$

$$E \cdot G_5 + rG_{11} \rightarrow E + rG_{16}$$

$$E \cdot G_5 + rG_7 \rightarrow E + rG_{12}$$

REACTIONS 6–8

The relative rate of the reaction with these acceptors was determined by using 2 mM rG_{11} as the donor plus 32 mM ^{3}H-labeled reduced acceptor and measuring the formation of labeled transferase product (Table I). Under these conditions, the reaction with rG_{11} being used as an acceptor is negligible. The reaction rate increased with increasing chain length, indicating that the 49-kDa enzyme prefers larger laminarioligosaccharide acceptors.

The transferase showed no activity toward gentiooligosaccharides (G_{3-8} in size), chitohexaose, cellopentaose, or maltoheptaose in either the presence or absence of rG_{11} (data not shown), suggesting the enzyme uses exclusively β-(1–3)-linked substrates. The requirement for a linear β-(1–3)-glucan as a donor was shown by using a "kinked" reduced G_{10} (rG_{10}*) similar to laminaridecaose, except that the 6th linkage from the reducing end is a β-(1–6)-linkage. Incubation of the 49-kDa enzyme with 8 mM rG_{10}* gave no products, indicating that it was not a donor. However, a similar incubation in the presence of 2 mM rG_{11} resulted in the formation of rG_6 and a peak eluting at the position of rG_{15} as the major initial products, showing that rG_{10}* could act as an acceptor (data not shown).

TABLE I
Rate of reaction of the 49-kDa enzyme with different sized acceptors with rG_{11} as the donor

The 49-kDa protein was incubated with 2 mM rG_{11} plus 32 mM ^{3}H-reduced acceptor of size G_{5-8} using the standard assay conditions described in the Methods. The transferase reaction with the initial rate of formation of the transfer product is shown.

Transferase reaction	Rate of transfer
	nmol · min^{-1} · mg protein^{-1}
rG_{11} + [^{3}H]$rG_5 \rightarrow rG_6$ + [^{3}H]rG_{10}	203
rG_{11} + [^{3}H]$rG_6 \rightarrow rG_6$ + [^{3}H]rG_{11}	387
rG_{11} + [^{3}H]$rG_7 \rightarrow rG_6$ + [^{3}H]rG_{12}	484
rG_{11} + [^{3}H]$rG_8 \rightarrow rG_6$ + [^{3}H]rG_{13}	586

^{1}H NMR Analysis of the Reduced G_{16} Transferase Product—To determine whether the 49-kDa transferase produced a new linkage type during transfer, the rG_{16} transferase product was purified from an incubation of the transferase with rG_{11}. Approximately 300 μg of the product was analyzed by ^{1}H NMR. The one-dimensional spectrum of the rG_{16} transferase product presented three chemical shifts in the anomeric region: δ = 4.68 ppm corresponding to the glucose residue linked to the glucitol group; δ = 4.75 ppm corresponding to the glucose residue of the nonreducing end; δ = 4.80 ppm corresponding to the intrachain glucose residues linked in β-(1–3). The relative intensity of the anomeric signals indicated 1, 1, and 13 protons, respectively. Since glucitol does not give any signal in the anomeric region, it confirmed the length of the oligosaccharide (16 residues). Coupling constants measured on these signals were in agreement with β-linked glucose residues ($^3J_{1,2}$ = 7.9 Hz). The presence of a single glucose unit at the nonreducing end indicated that the 49-kDa protein transferred onto the nonreducing end of the β-(1–3) glucan acceptor. The one-dimensional spectrum of the rG_{16} product was identical, except for the relative intensity of the 4.80 ppm signal to that of a rG_{10} laminarioligosacharide standard. In addition, no chemical shift characteristics of glucose residue linked in (1–2), (1–4), or (1–6) were seen, confirming that the rG_{16} was a reduced laminarihexadecaose. The coelution of the rG_{16} product with a rG_{16} reference on HPAEC and the insolubility of the larger product were consistent with the production of a β-(1–3) linkage during transfer.

Effect of Substrate Concentration on the Reaction Products—To determine whether lowering the acceptor concentration would promote hydrolysis reactions, the 49-kDa transferase was incubated with 3 μM [^{3}H]rG_{11} plus decreasing amounts of unlabeled rG_{11}. There was an observed shift from (i) transfer to (ii) hydrolysis (Fig. 4, *inset*).

$$E + [^3H]rG_{11} \rightarrow E \cdot G_5 + [^3H]rG_6$$

$$(i)\ E \cdot G_5 + [^3H]rG_{11} \rightarrow E + [^3H]rG_{16}$$

$$(ii)\ E \cdot G_5 + H_2O \rightarrow E + G_5$$

REACTION 9

The percent transfer was determined by measuring the formation of labeled rG_{16} (transfer only) compared to that of labeled rG_6 (transfer plus hydrolysis) in the reaction. At 3 mM rG_{11}, only transfer was detected. As the substrate concentration decreased to 18 μM, the percent transfer leveled out to about

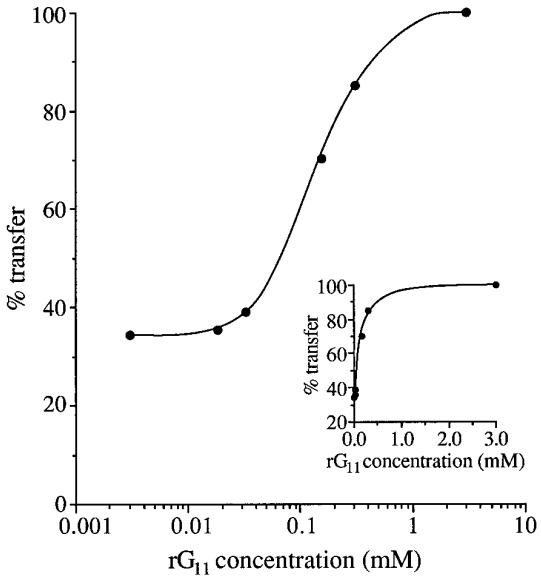

FIG. 4. **Effect of varying substrate concentration.** The 49-kDa transferase was incubated with 3 μM [^{3}H]rG$_{11}$ (1 × 10^6 cpm) plus varying amounts of unlabeled rG$_{11}$. The percent transfer was determined by comparing the proportion of label formed as rG$_6$ and rG$_{16}$. The *inset* shows the same data with the substrate concentration presented with a linear scale.

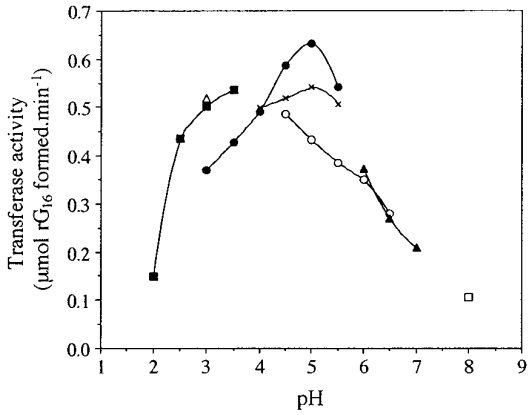

FIG. 5. **Effect of pH on the rate of transfer.** The 49-kDa transferase was incubated with 8 mM [^{3}H]rG$_{11}$ (1 × 10^6 cpm). The reaction rates were determined by measuring the amount of label formed as rG$_{16}$ product. The buffers used were: ●, sodium citrate/citric acid; ○, imidazole/citric acid; ×, sodium acetate/acetic acid; ■, Tris/glycine; ▲, phosphate/NaOH; □, Tris/acetic acid; △, glycine/HCl.

35% and did not decrease significantly with a lower substrate concentration (3 μM) (Fig. 4). Decreasing the buffer concentration to 10 mM did not alter the percent transfer at any of the substrate concentrations (data not shown). It appears that, under the given conditions, the 49-kDa transferase was unable to catalyze more than about 65% hydrolysis by simply reducing the substrate concentration to very low levels.

pH Optimum and Stability—The 49-kDa enzyme was assayed at different pH values, checked for stability upon storage, and the de-*N*-glycosylated enzyme assayed for activity. The enzyme was active over a broad range of acidic pH, showing activity of more than 50% of maximum between pH 2.5 and 6.0. The enzyme displayed a pH optimum of about 5.0 in citrate buffer (Fig. 5). The enzyme was very stable and could be kept at 4 °C in 10 mM citrate buffer, pH 5.0, for many weeks, or dried by SpeedVac and later resuspended in buffer, or stored at −20 °C without significant loss of activity. The de-*N*-glycosylated 44-kDa enzyme prepared under nondenaturing conditions was as active as the native, glycosylated enzyme when incubated with 8 mM rG$_{11}$ (data not shown).

Kinetic Analysis—The 49-kDa enzyme catalyzes its transferase reaction via a bi-reactant (two-step) mechanism with initial hydrolysis of the substrate to release the reducing end portion, and subsequent transfer of the remainder to the nonreducing end of a substrate molecule playing the role of acceptor molecule. Using rG$_{11}$ as the substrate, it was impossible to calculate an apparent K_m accounting for both reaction steps. To determine a K_m for the donor site, we used an acceptor that was not a donor. [^{3}H]rG$_7$ (1 × 10^6 cpm) was used as the acceptor at high concentration (64 mM), with varying concentrations of rG$_{11}$ kept below 8 mM. Under these conditions the reaction proceeded with rG$_{11}$ as the donor and rG$_7$ being used in favor of rG$_{11}$ as the acceptor, as determined by the absence of formation of the rG$_{16}$ transferase product (data not shown). The initial rate of the reaction was determined by measuring the appearance of labeled rG$_{12}$. An apparent K_m of 5.3 mM was obtained from the double-reciprocal plot (r^2 value = 0.997) (data not shown).

DISCUSSION

The 49-kDa transferase was released from cell walls of *A. fumigatus* by autolysis, a relatively common method used for the release of wall-associated proteins in yeast and other fungi and involves the action of endogenous hydrolases and proteases (3, 26). The 49-kDa transferase was also found in the culture medium, but its abundance varied considerably, and generally more was found in the medium of older cultures (older than 40 h) (data not shown). This suggests that the enzyme may be slowly released from the cell wall by autolysis, which might occur during normal growth in a fermenter. The protein's association with the cell wall was further demonstrated by showing that after 15 min of incubation at 4 °C in 5 mM sodium acetate, pH 5.6, the enzyme bound specifically to the alkali-insoluble wall fraction from *A. fumigatus* (a β-glucan-chitin complex; Ref. 21), as shown by the absence of the protein in the supernatant following incubation. This association could be prevented by the addition of 10 mM water-soluble glucan, laminarin, suggesting that the enzyme bound to β-(1–3)-glucan in the wall (data not shown). Interestingly, the enzyme did not bind to the water-insoluble β-(1–3)-glucan, curdlan, and this may reflect the importance of conformational differences of different β-(1–3)-glucans (27, 28).

With the inclusion of the present study, there are now two types of cell wall-associated β-(1–3)-glucanosyltransferases known in fungi, each with a distinct action pattern. Both enzymes cleave a β-(1–3)-glucan chain and transfer the nonreducing end portion to the nonreducing end of another β-(1–3)-glucan chain. The first type (BGT1) specifically hydrolyzes a laminaribiose unit from the reducing end of a β-(1–3)-glucan chain and then transfers the remainder to the nonreducing end of another β-(1–3)-glucan chain, forming a new intrachain β-(1–6)-linkage during transfer (16, 17, 19). This transferase has been found in *C. albicans* (16) and in *S. cerevisiae* and both are encoded by the *BGL2* gene (19). We have now found a BGT1 homolog in *A. fumigatus*.[1] The second β-(1–3)-glucanosyltransferase is described in this paper, and we propose to designate this novel enzyme a β-(1–3)-glucan: β-(1–3)-glucan 3-β-glucanosyltransferase (EC 2.4.1.-), abbreviated as β-(1–3)-glucanosyltransferase 2 (BGT2). The *A. fumigatus* BGT2 catalyzes an endo-β-(1–3)-glucanosyltransferase reaction summarized in Table II and consistent with the following general scheme, where $a + b = x$ and E = enzyme.

$$E + \mathrm{rG}_{11+x} \rightarrow E \cdot \mathrm{G}_{5+a} + \mathrm{rG}_{6+b}$$

$$\mathrm{E{\cdot}G}_{5+a} + \mathrm{rG}_{y} \rightarrow E + \mathrm{rG}_{5+a+y}$$

REACTIONS 10 AND 11

TABLE II
Summary of transferase action and products formed from reduced laminarioligosaccharides

Results are summarized from reactions performed with reduced laminarioligosaccharides using the conditions described in the legend to Fig. 2.

Substrates showing sites of initial bond cleavage	Major initial products formed
rG_{19}	$rG_{6\text{-}14} + rG_{24\text{-}32}$
rG_{14}	$rG_{6\text{-}9} + rG_{19\text{-}22}$
rG_{13}	$rG_{6\text{-}8} + rG_{18\text{-}20}$
rG_{12}	$rG_{6\text{-}7} + rG_{17\text{-}18}$
rG_{11}	$rG_6 + rG_{16}$
rG_{10}	$rG_{5\text{-}6} + rG_{14\text{-}15}$

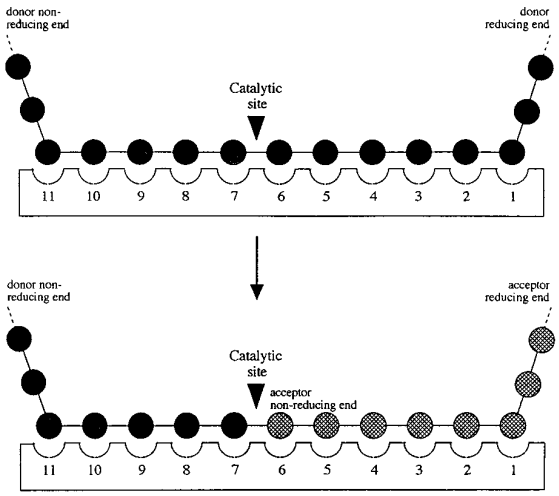

FIG. 6. **Active site model for the 49-kDa transferase.** The active site is proposed to consist of 11 glucose-binding sites with the catalytic site situated between binding sites 6 and 7 from the reducing end. A large donor is shown ($>G_{11}$); however, the transferase is able to use donors as small as G_{10} in size where glucose-binding site 1 or 11 is unoccupied. In addition, a large acceptor is shown ($>G_6$); however, the enzyme is capable of transferring to acceptors as small as G_5 in size where glucose-binding site 1 is unoccupied.

rG_{11+x} represents a reduced donor laminarioligosaccharide ($x \geq -1$), and rG_y represents a reduced acceptor glucan of length y, where $y \geq 5$. Unlike BGT1, BGT2 produces a β-(1–3)-linkage during transfer. Consequently, transferase products may be reutilized as donors and acceptors. The observation that larger transferase products drop out of solution is consistent with the low solubility properties of large laminarioligosaccharides in aqueous conditions (29). An important difference between the two transferases is that BGT1 requires a β-(1–3)-chain with a free reducing end before it can act, whereas BGT2 does not have this limitation. Such donors with a free reducing end would be created by the action of endo-β-glucanases.

Based on the specificity of action on different sized laminarioligosaccharides, BGT2 is proposed to have an active site composed of 11 glucose-binding sites, with its catalytic site situated between binding sites 6 and 7 from the reducing end (Fig. 6). Interestingly, the rG_{10}* transferase product, containing a β-(1–6)-intrachain linkage between the 6th and 7th glucosyl residue from the reducing end, is not a donor for BGT2 but will act as an acceptor, indicating that an intrachain β-(1–6)-linkage is acceptable between glucose-binding sites 2 and 3 in a β-(1–3)-glucan acceptor, but is unacceptable in a donor between binding sites 6 and 7 or 7 and 8 from the reducing end.

The determination of kinetic parameters for BGT2 presented a problem because β-(1–3)-glucan donors could also act as acceptors. To determine a K_m for the acceptor site, it was necessary to use a donor that was not an acceptor; however, such a molecule was unavailable. Future attempts may follow an alternative method, which has been used in kinetic studies of the transglycosylation reaction of cyclodextrin glucanotransferase in which a maltooligosaccharide donor was used with a blocked non-reducing end C4-hydroxyl group, so that cyclodextrin glucanotransferase could not use it as an acceptor (30).

Analysis by HPAEC pulsed electrochemical detection of a semipurified fraction derived from the culture filtrate of *C. albicans* and incubated with rG_{11} revealed a transferase activity similar, but not identical to, that of *Asp*BGT2, producing $rG_6 + rG_7$ and $rG_{15} + rG_{16}$ as the major initial products (data not shown). Incubation with rG_{10} resulted in the initial formation of rG_6 and rG_{14}, suggesting that this enzyme has an active site composed of 10 glucose-binding sites with the catalytic site also situated between binding sites 6 and 7 from the reducing end. The finding of a similar enzyme activity in a yeast suggests that this cell wall activity may be conserved among fungi.

Most, if not all hydrolases acting on pyranosides with overall retention of configuration are also able to transfer a glycosyl moiety from a substrate (donor) to the hydroxyl group of acceptors other than water, resulting in a transferase reaction rather than hydrolysis (31, 32). The secreted exo-β-glucanase from *C. albicans* (33) as well as hen egg white lysozyme (34) have been reported to catalyze transferase reactions under conditions of high substrate concentration or in the presence of ammonium sulfate. Conversely, certain transferases are capable of catalyzing hydrolysis reactions under low substrate conditions. The 49-kDa transferase was able to catalyze hydrolysis reactions at low substrate concentrations (Fig. 4). Similarly, *Can*BGT1 has been observed to function like a β-glucanase under these conditions (19). However, the latter transferase catalyzed reactions with 100% hydrolysis below 10 μM G_5 and shifted toward glycosyl transfer above this concentration (19), whereas the 49-kDa transferase did not exceed 65% hydrolysis below this concentration with rG_{11} as substrate (Fig. 4). The *Sac*BGT1 was originally described as an exo-β-glucanase (18) and subsequently purified by a different method and described by the same group as an endo-β-glucanase (35). Their assay was done at a low concentration of laminarin (150 μg ml^{-1} or less than 40 μM) with analysis of products by thin layer chromatography (35), a method with which only hydrolysis products are detectable. This may explain their description of a β-glucanase as opposed to a transferase.

The transferase reactions of β-(1–3)-glucanosyltransferases are more easily understood in terms of the reaction mechanism involving a carbonium-ion intermediate (32, 36), which is formed from the substrate (donor) as a nonreducing end side fragment and stabilized in the active site of the enzyme by the reaction of the two catalytic residues (36). Glycosyl transfer would take place when the carboniumion intermediate is attacked by an intact substrate molecule (acceptor). The reason for the high degree of transfer of transferases compared that of hydrolases in general, may be due to differences in the physicochemical environment at the active site. A site-directed mutagenesis study with *Saccharomycopsis* α-amylase in which a tryptophan residue in substrate-binding subsite 3 was replaced

by a leucine residue resulted in an increase in transferase activity (37). The authors suggested that the tryptophan residue might play an important role not only to hold the substrate but also to quickly release the hydrolysis products from the substrate binding pocket. The replacement by a leucine residue may result in inefficient release of the hydrolysis products, thereby promoting the formation of a carbonium ion intermediate and resulting in increased transfer (37). In addition, many of the residues proposed to be involved in substrate binding and catalysis are conserved between cyclodextrin glucanotransferase and α-amylases, enzymes that are considered to be of the α-amylase family because of their primary and tertiary structural similarities. However, four aromatic residues are present at the active center of a *Bacillus* sp. cyclodextrin glucanotransferase, but not in α-amylases (30).

A transferase activity similar to that of the 49-kDa transferase but acting on xyloglucan has been found associated with the cell wall of a number of different plants (38–40). Nishitani and Tominaga (40) purified an endo-xyloglucan transferase from the cell wall of *Vigna angularis*, a bean plant, which catalyzes endo-type splitting of a xyloglucan molecule, followed by transfer of the newly generated reducing end to the nonreducing end of another xyloglucan molecule. Xyloglucan is the major component of the plant cell wall-matrix polymers and functions to interconnect the crystalline cellulose microfibrils in the wall through extensive hydrogen bonding (41, 42). Endoxyloglucan transferase has been proposed to be directly responsible for cleaving and rejoining the intermicrofibrillar xyloglucan chains, thereby allowing cell wall expansion during plant growth and the integration of newly synthesized xyloglucan chains into the architecture of the wall (39, 40). Analogous roles can be envisaged for BGT2, except that it would act on β-(1–3)-glucan. To allow expansion of the fungal wall, pre-existing glucan chains must be cleaved and new wall polymers continuously inserted, particularly where most of the wall expands in a zone of highly active growth, such as the extreme tip in filamentous fungi (43) or sites of branch formation in hyphae or bud and germ-tube formation in yeast.

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