Molecular Mechanisms of Yeast Cell Wall Glucan Remodeling

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Yeast cell wall remodeling is controlled by the equilibrium between glycoside hydrolases, glycosyltransferases, and transglycosylases. Family 72 glycoside hydrolases (GH72) are ubiquitous in fungal organisms and are known to possess significant transglycosylase activity, producing elongated (1–3) glucan chains. However, the molecular mechanisms that control the balance between hydrolysis and transglycosylation in these enzymes are not understood. Here we present the first crystal structure of a glucan transglycosylase, Saccharomyces cerevisiae Gas2 (ScGas2), revealing a multidomain fold, with a (βα)8 catalytic core and a separate glucan binding domain with an elongated, conserved glucan binding groove. Structures of ScGas2 complexes with different β-glucan substrate/product oligosaccharides provide "snapshots" of substrate binding and hydrolysis/transglycosylation giving the first insights into the mechanisms these enzymes employ to drive (1–3) glucan elongation. Together with mutagenesis and analysis of reaction products, the structures suggest a "base occlusion" mechanism through which these enzymes protect the covalent protein-enzyme intermediate from a water nucleophile, thus controlling the balance between hydrolysis and transglycosylation and driving the elongation of (1–3) glucan chains in the yeast cell wall.

The cell wall of fungal organisms is a dynamic structure, providing protection against hostile environments, yet also harboring many hydrolytic and toxic molecules required for the fungus to invade its ecological niche (1). Polysaccharides account for over 90% of the cell wall. The central skeletal component of the cell wall common to the vast majority of fungal species is a branched core of (1,3) glucan, linked to chitin via a (1,4) linkage (1). Interchain (1,6) glucosidic linkages account for 3 and 4% of the total glucan linkages in Saccharomyces cerevisiae and Aspergillus fumigatus, respectively (2–4). This core is embedded in a complex of amorphous proteins and/or polysaccharide whose composition is highly species-dependent. The core (1,3) glucan is subjected to continuous synthetic elaboration, degradation, and remodeling by a large arsenal of enzymes, whose activities must be appropriately balanced to provide the cell wall with adequate elasticity to allow growth, budding, or branching and yet sufficient strength to guard against cell lysis (1).

Glucan synthase is a protein complex located at the plasma membrane, synthesizing (1,3) glucan from UDP-glucose (65–90% of the total glucan). In cell wall remodeling, glycoside hydrolases and glycosyltransferases/transglycosylases play a crucial role (1, 5). Pure glycoside hydrolases degrade glucans mainly to regulate the plasticity of the cell wall under different circumstances, such as cell division, cell separation, and sporulation (5), whereas glycoside hydrolases with significant transglycosylase activity are capable of forming new glycosidic bonds between oligosaccharides, generating longer or branched polymers. Previous studies have shown that several proteins anchored to the plasma membrane by a glycosylphosphatidylinositol (GPI)3 anchor have transglycosylase activities (6–10). Among them are the Gas (in S. cerevisiae)/Gel (in A. fumigatus) proteins that belong to the GH72 family in the CAZy data base (11). For laminarioligosaccharides with >10 sugars, these enzymes are able to cleave a (1–3) bond and transfer the newly formed reducing end (the "donor") to the nonreducing end of another oligosaccharide (the "acceptor") (6, 12, 13). This transferase reaction generates a new (1,3) linkage, resulting in the elongation of (1,3) glucan chains, offering a mechanism for the synthesis of longer glucan chains as alternative to, or in synergy with, glucan synthase. The Gas/Gel proteins consist of a signal sequence, a catalytic core, and either a cysteine-rich domain (classified as a carbohydrate-binding module, CBM43) (11) or a Ser-Thr-rich motif, followed by a GPI anchor (Fig. 1A). Based on the presence or absence of the C-terminal cysteine-rich domain, the family is subdivided into GH72+ (with a CBM43 domain) and GH72- (without a CBM43 domain) (14). The genome of S. cerevisiae contains five proteins (Gas1–Gas5), two of which (Gas1 and Gas2) belong to the GH72+ subfamily. With the exception of Gas3, transglycosylase activity has been reported for all these enzymes (14, 15). A. fumigatus contains seven genes (gel1–gel7), with only Gel1p, Gel2p (both GH72-), and Gel4p (GH72+) being expressed during mycelial growth in

The abbreviations used are: GPI, glycosylphosphatidylinositol; CTD, C-terminal domain; PDB, Protein Data Bank; r.m.s.d., root mean square deviation.

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§ The atomic coordinates and structure factors (codes 2w61, 2w62, and 2w63) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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Candida albicans

Scgas4

Sc

/H9252

Tris, pH 7.0, on an AKTA

resulted in slower growth, abnor-

Sc

and double

sites) using the forward primer, 5′-GAAATTTTT-

TGATTTCTATAAAGGATACATACAAATGCAGCAT-

TATTC-3′, and the reverse primer, 5′-CCTTTCTTTCTA-

CGGATGAGCTTGGATTTTGAATAGCGCATG-3′.

The resulting plasmid, pPICZnAgas2 (Ser26–Ser525) N498D/N510D, referred to here as the wild type, was used as template for introducing the following single amino acid changes by site-
directed mutagenesis as follows: Q62A, Y107F, Y107Q, D132N, N175A, E176Q, Y244F, Y244Q, E275Q, Y307Q, F404A, and Y474A, such that each of the resulting 12 plasmids carried the indicated mutation in addition to the previously introduced asparagine to aspartic acid mutations at positions 498 and 510. Site-directed mutagenesis was carried out following the QuikChange protocol (Stratagene), using the KOD HotStart DNA polymerase (Novagen). All plasmids were verified by sequencing (DNA Sequencing Service, College of Life Sciences, University of Dundee, Scotland, UK).

All plasmids were isolated from Escherichia coli strain DH5α, linearized with Pmel, and used to transform P. pastoris strain into X-33 following the LiCl method (Invitrogen) or using the Pichia EasyComp™ transformation kit (Invitrogen). Transformants were selected on YPD plates (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose) containing 100 µg/ml Zeocin (Invitrogen). Batch cultures were performed in a 100-ml volume of BMGY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate (pH 6.0), 1.34% (w/v) yeast nitrogen base, and 1% (v/v) glycerol). 50 ml were used to grow 500 ml of BMGY medium overnight at 30 °C, and expression was induced by methanol (1%, v/v) for 72 h at room temperature in a shaking incubator (270 rpm). Yeast cells were harvested by centrifugation at 3480 × g for 30 min. The supernatants containing soluble ScGas2 were filtered to 0.2 µm, concentrated to 50 ml using a Vivaflow 200 cassette (10,000 M̅, weight cutoff, PES membrane; Vivasience), and dialyzed against water.

The samples were then loaded onto a 2 × 5-ml HiTrap Q FF column (Amersham Biosciences) that had been equilibrated with 10 column volumes of 25 mM Tris, pH 7.0, on an AKTA purifier system. Following loading, the column was washed with 10 column volumes of 25 mM Tris, pH 7.0. The protein was eluted with a salt gradient (0–500 mM NaCl) over 20 column volumes, collecting 2-ml fractions. The fractions containing the proteins were then pooled and concentrated to 5 ml using Vivaspin 10,000 M̅, weight cutoff. Subsequently, gel filtration was carried out using a Superdex 75 XK26/60 column in 25 mM Tris, 150 mM NaCl, pH 7.0. The concentrated ScGas2 proteins were used for both kinetic analysis and crystallization trials.

Enzymology—To test for β(1,3) glucanotransferase/hydrolase activity, the purified proteins (10 µg) were incubated with the linear reduced laminarioligosaccharides rG5, rG7, and rG19 at a concentration of 4 mM in 50 mM sodium acetate buffer, pH 5.5, at 37 °C. Aliquots of 2.5 µl were withdrawn at different times (0, 1, and 3 h and overnight), supplemented with 47.5 µl of 50 mM NaOH, and then analyzed by high performance anion-exchange chromatography through a CarboPAC-

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification—The DNA se-

quence encoding amino acid residues 26–525 of the S. cere-

visiae ScGas2 (Swiss-Prot, locus ScGas2_YEAST; Swiss-

Prot accession number Q60135; GenElD, 851056), defined as

scgas2, was obtained by PCR from S. cerevisiae strain W303

genomic DNA, using the forward primer, 5′-ATCTCGAGAA-

AAGAGGGCTGAAAGCTCGTACTGTTGGAAA-

AAACCCCTG, containing a recognition sequence for Xhol (underlined) and a KEX2 cleavage signal (italic), and the reverse primer, 5′-GCTCTAGAATCTGATTGGAATTTACGTT-

TCAAACTTTCC-3′, containing a recognition sequence for XbaI (underlined), and cloned into the pSC-B vector (Stratagene). Following digestion with Xhol and XbaI, the cloned sequence was subcloned into the Pichia pastoris protein expression and secretion vector pPICZaA (Invitrogen), resulting in the expression plasmid pPICZaAAsgas2 (Ser26–Ser525). Subsequently, the
codons for the asparagine residues at positions 498 and 510 in ScGas2 were simultaneously changed by site-directed mutagenesis to aspartic acid codons (removing N-linked glycosylation sites) using the forward primer, 5′-GAAATTTTT-

TGATTTCTATAAAGGATACATACAAATGCAGCAT-

TATTC-3′, and the reverse primer, 5′-CCTTTCTTTCTA-

CGGATGAGCTTGGATTTTGAATAGCGCATG-3′.
PA1 column (Dionex 4.6 mm inner diameter × 250 mm), as described by Hartland et al. (21).

Crystallization and Data Collection—ScGas2 was spin-concentrated to 21 mg/ml. Crystals were grown by sitting drop experiments at 20 °C through mixing 1 μl of protein with an equal volume of a reservoir solution (20% 1,4-butanediol, 5% acetone, 0.1 M sodium acetate, pH 4.5). Under these conditions, crystals appeared within 3–7 days. They were cryoprotected with 0.1 M sodium acetate, 30% 1,4 butanediol, 5% acetone, pH 4.5, and flash-cooled prior to data collection at 100 K. (NH₄)₂IrCl₆ derivative, as well as oligosaccharide complexes, were generated by soaking apo-crystals in cryoprotectant supplemented with heavy atom salt (10–100 mM), laminaranetaose (200 mM), or laminariheptaose (100 mM), respectively, for 10–20 min prior to data collection. Data for heavy atom derivative was collected at beamline ID23-2 (ESRF, Grenoble, France). Insights into fungal transglycosylation...
Table 1. Coordinates and structure factors have been deposited in the Protein Data Bank. The structure has several disordered regions as follows: one in the poorly conserved loop between β3 and α1, one covering a short stretch of the interdomain region between α9 and α10, and one (absent in three of the present four structures) preceding α11. In addition, the C terminus of ScGas2 (from at least Ser507, but in some cases as early as Leu483) is completely disordered. Although density for the α13 helix could be observed in all three structures, its quality was too poor for it to be modeled in the oligosaccharide complexes (Fig. 1B).

RESULTS AND DISCUSSION

Structure of ScGas2 Reveals a Two-domain Fold—A truncated form of ScGas2 (amino acids 26–525, excluding the signal/GPI-anchor sequences, Fig. 1A) was expressed as a secreted protein in P. pastoris and purified by ion-exchange and gel filtration chromatography. The structure of ScGas2 was solved by SIRAS with an iridium derivative, complexes with laminariolipidoligosaccharides and a structure of a E176Q mutant were solved by molecular replacement, and all structures were refined to 1.6–2.1 Å with Rfree <0.22 (Table 1).

The structure of ScGas2 is composed of two interacting domains as follows, a (βα)s catalytic domain, abundantly found in other carbohydrate active enzymes; and a C-terminal cysteine-rich domain of the CBM43 family (Fig. 1). The (β/α)s domain deviates from the canonical topology by kinks in the sixth and seventh α-helix (α6/α6’ and α7/α7’), Fig. 1A). Similar to many other (β/α)s barrel proteins, the first strand of the barrel (β3) is preceded by a two-stranded antiparallel β-sheet, which seals the “bottom” of the barrel. A second short two-stranded sheet (β11/β12) is inserted into the last βα loop (31), placing it on the opposite side of the barrel and within 20 Å of the active site (Fig. 1).
Not surprisingly, a DALI (32) search with the ScGas2 catalytic domain yields over 300 proteins with significant structural similarity; among the most similar structures are numerous glucosidases. The two crystal structures with the highest Z scores are domain 3 from human β-glucuronidase (PDB ID 1BHG (33)) and Cellvibrio mixtus mannosidase 5A (PDB ID 1UUQ (34)), which superimpose with r.m.s.d. of 2.7 Å and 3.0 Å for ≈260 C-α atoms, respectively.

The ScGas2 catalytic domain contains three disulfide bridges, one between Cys159 and Cys112 connecting α1 and α2, and another between Cys231 from the fifth αβ loop and Cys367 from the interdomain loop. It is noteworthy that both these disulfides occur in the vicinity of disordered loops, and it is possible that they help to limit flexibility. The third disulfide of the catalytic domain is formed by Cys247 and Cys367 from the sixth and seventh αβ loops (preceding α6 and α8, respectively). Both these loops are involved in forming the acceptor saccharide-binding site, and it is possible that the disulfide bridge helps to correctly position them.

When a sequence alignment of GH72 enzymes (Fig. 1A) is interpreted in the context of the ScGas2 structure (Fig. 1B), it is clear that most of the sequence conservation locates to the catalytic core, whereas the C-terminal cysteine-rich CBM43 domain of ScGas2 is less conserved. In particular the active site of ScGas2 is highly conserved (Fig. 1B), from the −2 to the +2 site, suggesting that ScGas2 may be a good representative of the GH72 family for further mechanistic studies.

CBM43 Domain Contains a Conserved Cysteine Structure and Exposed Aromatic Residues—Based on its sequence, the C-terminal domain of ScGas2 (Fig. 1) has been assigned to the CBM43 family of carbohydrate-binding modules (11). It assumes a predominantly α-helical structure; a core formed by four α-helixes is augmented by two small antiparallel two-stranded β-sheets (Fig. 1C). Although the amino acid sequence of the catalytic domain is reasonably well conserved among GH72 family members (32–61% identity), there is considerably more variation in the C-terminal domain, where, even among GH72+ subfamily members, sequence identity can drop below 20% (Fig. 1A). The few amino acids that are completely conserved include a number of hydrophobic residues and eight cysteines, which, in the ScGas2 structure, form four disulfide bridges (Cys390–Cys442;
Cys\textsubscript{399}–Cys\textsubscript{466}, Cys\textsubscript{419}–Cys\textsubscript{424}; and Cys\textsubscript{457}–Cys\textsubscript{489}, Fig. 1, A–C), in agreement with a recent mass spectrometry-based assignment (35). The C-terminal \( \approx \)40 residues of the expressed protein, which in the full-length protein would lead up to the GPI anchor, are (mostly) disordered in our structures; it is likely that the poorly conserved stretch between \( \alpha \)15 and the GPI anchor site functions as a flexible tether.

Structural homology searches of this domain with DALI (32) yielded no significant hits. As CBM43 domains are most commonly associated with \( \beta \)(1,3) glucan-processing domains from CAZy families GH17/72, it is possible that they would possess \( \beta \)(1,3) glucan binding activity. It has been shown that carbohydrate binding to CBM domains is generally effected by surface-exposed tyrosine, tryptophan, and phenylalanine residues (36). The ScGas2 cysteine-rich domain contains six such surface aromatic amino acids: Phe\textsubscript{404}, Tyr\textsubscript{417}, Tyr\textsubscript{474}, Phe\textsubscript{493}, Tyr\textsubscript{501}, and Tyr\textsubscript{506} (Fig. 1C), which could play a role binding to \( \beta \)(1,3) glucan, although none of them are conserved between different GH72\textsuperscript{+} family members (Fig. 1A). To test this hypothesis, Phe\textsuperscript{404} and Tyr\textsuperscript{474} were mutated to alanines. Only the F404A mutant showed a small difference in transglycosylation/hydrolysis activity of a G19 laminarioligosaccharide compared with the wild type enzyme (Table 2). Earlier studies also showed that the presence or absence of the CBM43 domain in the GH72 enzymes does not appear to significantly affect transglycosylation activity (13, 14).

So far, only two CBM43 proteins, both olive pollen allergens, have been biochemically characterized in some detail. The olive pollen-derived Ole e 10 (an isolated CBM43 domain) and the GH17 family member Ole e 9 (with a C-terminal CBM43 domain) have been shown to possess the ability to bind \( \beta \)-1,3 glucan structures (37, 38). Although alignment between the olive pollen CBM43 domains and that of ScGas2...
shows poor sequence conservation (identity of \( \approx 17\% \)), six of the GH72 \( \beta \)-cysteines appear to be conserved. Very recently, an NMR structure of the C-terminal domain (CTD) of \( \text{Ole e} \) has become available (39). A superposition with the \( \text{ScGas2} \) cysteine-rich domain is relatively poor, giving an r.m.s.d. of 2.7 Å (with only 64 out of the 101 possible equivalenced C-\( \alpha \) atoms; Fig. 1C). Although the two CBM43 structures share the “core” formed by \( \alpha1, \alpha12, \beta13, \text{ and } \beta16 \) (ScGas2 numbering), the \( \text{Ole e} \) CTD lacks the small \( \beta \)-sheet (\( \beta14-\beta15 \)) of ScGas2 as well as the \( \alpha10 \) and \( \alpha13 \) helices. Of the six equivalent cysteine residues, only four participate in structurally conserved disulfide bridges, whereas the other two form a third disulfide in the \( \text{Ole e} \) CTD but participate in two separate disulfide bridges in ScGas2 (Fig. 1C). Altogether, the two available structures of CBM43 domains suggest that GH17-associated “plant” and GH72-linked “fungal” CBM43 domains, although sharing some structural motifs, are overall not similar enough that their functional equivalence can be assumed. Some of the ScGas2 structural elements absent from the \( \text{Ole e} \) CTD participate in interactions with the catalytic domain (see below), and their absence from the plant protein may indicate differences in the interaction between the CBM and the two classes of catalytic domains. It is noteworthy that the part of the CBM43 domains incorporating the shared features is closest to the ScGas2 active site, whereas the dissimilar side faces away from it (Fig. 1B). It is thus possible that the CBMs of ScGas2 and \( \text{Ole e} \) bind glucans on that side of the domain, using similar binding sites. \( \text{Ole e} \) exposes a cluster of four surface aromatic residues on this side, most of which are conserved in \( \text{Ole e} \) (39). Only one of these residues is conserved between \( \text{Ole e} \) and ScGas2 (Tyr\( ^{417} \) in ScGas2 numbering).

One of the characteristics of CBM motifs/modules is that they are frequently separate domains and indeed can occur as individual proteins (40). In contrast, the cysteine-rich domain of ScGas2 shares extensive contacts with the catalytic core, incorporating hydrophobic interactions as well as seven strong direct hydrogen bonds and burying \( \approx 2650 \) Å\(^2\), compatible with a stable domain interface (41). The catalytic subunit contributes residues from around the N termini of helices \( \alpha3 \) and \( \alpha4 \) as well as the loop preceding \( \alpha10 \) to the interface, whereas the CBM uses residues from the N-terminal ends of \( \alpha10 \) and \( \alpha12 \) and, most importantly, the small \( \beta \)-sheet formed by \( \beta14 \) and \( \beta15 \) that is absent from the \( \text{Ole e} \) structure.

**Binding Mode of a Transglycosylation Acceptor Substrate—** Recent landmark studies toward the structure and mechanism of a plant xylosyltransferase PttXET16A has revealed how a large, fully ordered, oligosaccharide is bound to the acceptor site, tightly interacting with the catalytic base (Fig. 2), whereas elegant kinetic studies have demonstrated a remarkably long lived covalent enzyme-donor intermediate (42, 43).

To allow trapping of an ScGas2-acceptor transglycosylation complex, we sought to identify the minimal (and therefore most soluble) \( \beta \)-glucan-derived laminarioligosaccharide that would not undergo hydrolysis, yet still serves as an acceptor substrate given a suitable donor in a transglycosylation reaction. Hydrolysis experiments show that laminariolipentaose is not hydrolyzed by ScGas2 (Fig. 3A), although a previous study of the \( A. \text{fuigatus} \) Gell ScGas2 orthologue showed that laminariolipentaose was the smallest laminarioligosaccharide acceptor used by the enzyme (21). Surprisingly, soaking experiments with ScGas2 revealed not only an ordered laminariolipentaose bound to the acceptor site, but also a (nonconvalently bound) laminariolipentaose bound in the donor site (Fig. 2). The active site is defined by two catalytic residues, Glu\( ^{176} \) and Glu\( ^{275} \), previously identified by mutagenesis (13), and three tyrosines, Tyr\( ^{107}, \text{ Tyr}^{244}, \text{ and } \text{Tyr}^{250} \). These residues are all conserved in the GH72 family, as well as several other residues lining the binding groove (Fig. 1A and Fig. 2). Together, the two laminariolipentaose molecules appear to occupy the \(-5 \) to \(+1 \) to \(+5 \) binding sites, without any evidence of bond formation between the \(-1/+1 \) sugars, but with the 1-O-1 and 1-O-3 hydroxyls only 4.3 Å apart. Thus, this arrangement may represent the position of transglycosylation reactants, with the \(-5 \) to \(+1 \) sugars representing the donor site, and the \(+1 \) to \(+5 \) sugars representing the acceptor site.

The functions of the two catalytic residues, Glu\( ^{176} \) and Glu\( ^{275} \), can be inferred from the ScGas2-laminariolipentaose complex (Fig. 2). Glu\( ^{176} \), the catalytic acid/base, hydrogen bonds O-3 of the +1 sugar in the acceptor site, occupying a position equivalent to the catalytic base (Glu\( ^{89} \)) in the PttXET16A structure (Fig. 2) (44). Glu\( ^{275} \), on the opposite side of the binding groove, approaches the anomic carbon of the \(-1 \) sugar to within 4 Å under a geometry compatible with nucleophilic attack. Mutation of either of these glutamates to glutamine abrogates catalytic activity (Table 2 and Fig. 3). GH families can be classified as inverting or retaining enzymes based on the distance between the two catalytic residues, with inverting enzymes giving a distance of 10 ± 2 Å on average, although retaining enzymes have the two residues located \(-5.5 \) Å apart (45). In the ScGas2 crystal structure, Glu\( ^{176} \) and Glu\( ^{275} \) are 5.1 Å apart, suggesting the active site structure is compatible with a retaining mechanism, in agreement with previously published product analysis of ScGas2 (21). Given the sequence conservation of these residues, this will extend to all GH72 members. In addition to the two glutamates, there are three conserved tyrosines lining the active site. Two of these (Tyr\( ^{107} / \text{ Tyr}^{244} \)) interact with Glu\( ^{275} \), positioning it for nucleophilic attack (Fig. 2). Mutation of these residues to phenylalanines shows small effects on hydrolysis (Table 2 and Fig. 3). A plethora of further interactions between protein and substrate is found in the elongated binding groove, involving both hydrogen bonds and stacking interactions with aromatic residues (Fig. 2). For instance, two conserved residues, Tyr\( ^{107} \) and Pro\( ^{136} \), are involved in hydrophobic stacking interactions with sugars in the donor site, whereas the conserved Arg\( ^{142} \) gives the donor site a groove-like character (Fig. 2). Residues Asn\( ^{175} \) and

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**FIGURE 3. High pressure liquid chromatography analysis of \( \beta(1,3) \) glucanosyltransferase/hydrolase products.** A, comparison of wild type ScGas2 kinetics against laminaripentaose and laminariheptaose, identifying laminaritetrose and laminarinriose as the main two degradation products of hydrolyzed laminariheptaose. B, product analysis from the incubation of the recombinant wild type ScGas2 and the following single mutant enzymes, Y107F, Y244Q, E275Q, and Y307Q, with 4 mu reduced G19 samples taken at the indicated time points.
Insights into Fungal Transglycosylation

The base occlusion hypothesis also suggests that if interactions between the +1 sugar and the protein are disrupted, this might shift the balance between hydrolysis and transglycosylation away from the latter. We attempted this by studying the effects on hydrolysis and transglycosylation of a mutant (Y244F) of a conserved tyrosine lining the +1 subsite (Figs. 2 and 3). Strikingly, although hydrolysis is only moderately affected, a 10-fold reduction in transglycosylation is observed.

Concluding Remarks—Cell wall remodeling is an essential process in fungal organisms. Several enzymes with transglycosylation activities have been proposed to be involved in this process, but only the GH72 enzymes have been shown in vitro to transglycosylate the main cell wall carbohydrate polymer, β(1,3) glucan (13, 14, 21). Genetic data in different organisms show the involvement of these enzymes in virulence, morphology, and growth, in some cases supporting an essential function in sporation (12, 15, 17–20, 46).

Enzymes displaying significant transglycosylation are essentially glycoside hydrolases that have developed mechanisms to protect the (covalent) reaction intermediate from nucleophilic attack by a water molecule, although these mechanisms are not understood. The first crystal structure of a GH72 transglycosylase enzyme described here shows two interacting domains and a wide, conserved, and solvent-exposed active site. This includes the first crystal structure of an ordered CBM43 domain, which, although defining the CBM43 fold, does not immediately offer clues as to how it might contribute to glucan binding/hydrolysis/transglycosylation, as either truncation of the domain, or mutation of exposed aromatic residues does not have large effects on activity on (short) substrates.

Although recent work on xylosyltranferases has defined acceptor–protein interactions and has demonstrated that the donor–enzyme intermediate, generated after the initial nucleophilic attack, is long lived, this did not yet explain how transglycosylases might protect the intermediate from nucleophilic attack by water. The data described here provide the first insights into the mechanisms that may control the balance between hydrolysis and transglycosylation in a transglycosylase. The substrate–product trapped complexes, together with the mutagenesis data, suggest that product binding in the acceptor subsite might offer a method for occluding the catalytic base, and therefore prevent activation of an incoming water molecule. Compatible with this hypothesis, a substrate that yields a product that does not occupy the +1 site (leaving the catalytic base accessible to solvent), exclusively shows hydrolysis.

The fungal cell wall has been thought to be a treasure trove for novel drug targets to combat the increasing occurrence of invasive fungal infections. Indeed, the most recently developed anti-fungals of the echinocandin class target glucan synthase (1, 47), and there are efforts to develop inhibitors of fungal chitinases with anti-fungal activity (48, 49). Significant genetic validation now exists for the GH72 enzymes; they appear to be essential for proper development and morphogenesis of the fungal cell. Given the significant degree of sequence conservation in the GH72 family, it may be possible to develop chemical tools/probes that would inhibit all of the members of this mul-
tigene family in a single organism. The work here provides a useful scaffold for the future development and/or evaluation of such molecules.

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