NOTE

\(\beta\)(1-3)Glucanosyltransferase Gel4p Is Essential for \textit{Aspergillus fumigatus}\n
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Received 3 May 2010/Accepted 6 June 2010

The \(\beta\)(1-3)glucanosyltransferase \textit{GEL} family of \textit{Aspergillus fumigatus} contains 7 genes, among which only 3 are expressed during mycelial growth. The role of the \textit{GEL4} gene was investigated in this study. Like the other Gelps, it encodes a glycosylphosphatidylinositol (GPI)-anchored protein. In contrast to the other \(\beta\)(1-3)glucanosyltransferases analyzed to date, it is essential for this fungal species.

\(\beta\)(1-3)Glucan is the main component of the fungal cell wall (11). In fungi, \(\beta\)(1-3)glucans are synthesized by a plasma membrane-bound glucan synthase complex. Neosynthesized glucans are then extruded into the periplasmic space (2, 3, 9), where they become branched and covalently linked to other cell wall components, resulting in the formation of three-dimensional rigid structures. In the search of transglycosidase in the filamentous fungus \textit{Aspergillus fumigatus}, \(\beta\)(1-3)glucanosyltransferases were identified and classified as a unique family (GH72) in the Carbohydrate-Active enZymes database (http://www.cazy.org/). These enzymes cleave the \(\beta\)(1-3) bond of a \(\beta\)(1-3)glucan oligosaccharide with at least 10 glucose units and transfer the newly formed reducing end (>5 glucose units) to the nonreducing end of another \(\beta\)(1-3)glucan oligosaccharide, resulting in the elongation of the \(\beta\)(1-3)glucans. This reaction can proceed \textit{in vitro} until the neosynthesized \(\beta\)(1-3)glucan becomes insoluble. Initially demonstrated biochemically, the requirement for long-chain \(\beta\)(1-3)glucan oligosaccharide has now been confirmed by the analysis of the first crystal structure obtained in this transglycosidase family (7, 8). First discovered in \textit{Aspergillus fumigatus} and named Gelp for glucan elongase, this activity has been found in all fungal species investigated to date and could be assigned to orthologous proteins, such as Gasp or Phrp, that were known to be involved in cell wall integrity but were endowed with an unknown biochemical function (12, 13, 14).

Three members of the \textit{A. fumigatus} \textit{GEL} family are expressed during mycelial growth. In \textit{A. fumigatus}, the \textit{GEL} family contained 7 open reading frames (ORFs) named \textit{GEL1} (AFUA_2G01170), \textit{GEL2} (AFUA_6G11390), \textit{GEL3} (AFUA_2G12850), \textit{GEL4} (AFUA_2G05340), \textit{GEL5} (AFUA_8G02130), \textit{GEL6} (AFUA_3G13200), and \textit{GEL7} (AFUA_6G12410). Gel2p to Gel7p showed 37, 24, 28, 26, 29, and 26% identity with \textit{A. fumigatus} Gel1p (AfGel1p), respectively. Among them, only \textit{GEL1}, \textit{GEL2}, and \textit{GEL4} were expressed constitutively during mycelial growth. Quantitative real-time PCR (qRT-PCR) assays using RNA extracted at different times of culture and amplified as described previously (10) showed that among those 3 genes,
was the most expressed at all times of growth (Fig. 1). GEL1 and GEL2 were previously characterized. Disruption of GEL1 did not result in a phenotype, whereas a gel2 mutant and the double mutant gel1 gel2 exhibited slower growth, associated with a decrease of β(1-3)glucan content, an increase of chitin, and abnormal conidiogenesis (14). The function of GEL4 is reported here.

GEL4 is an essential gene in A. fumigatus. To delete GEL4, a deletion cassette containing the hygromycin resistance marker inserted inside the GEL4 ORF was constructed (Fig. 2A). The resulting fusion PCR product was used for transformation of protoplasts of the A. fumigatus akuB<sup>AKu80</sup> strain (4). In spite of repeated attempts, no mutant was recovered by classical gene replacement experiments, suggesting that this gene was essential. The heterokaryon rescue technique recently used to demonstrate gene essentiality in A. fumigatus (10, 15) was applied here. The presence of both wild-type and mutant alleles in heterokaryons was verified using Southern blotting, genomic DNA was digested with EcoRI and hybridized with probe A. DNA isolated from the wild-type strain showed one band of 6.3 kb corresponding to a copy of the wild-type allele, whereas the DNA isolated from the heterokaryon showed the wild-type allele and the mutated allele at 4.1 kb (see panel A).

FIG. 2. (A to C) Deletion of GEL4 in A. fumigatus using the heterokaryon rescue method. (A) Strategy used to insert the hygromycin resistance gene (HPH) inside GEL4 (E, EcoRI site). (B and C) PCR and Southern blot analyses showing the presence of both wild-type and mutated alleles in the heterokaryon (H) and the absence of the mutated allele in the wild-type strain (WT). The PCR primer pair used in the heterokaryon and in the wild-type strain to show the presence of a wild-type allele is GEL4heteroc1 (CCAAAACAAATCATCAGCCCCAGCCCCAAATC)-GEL4heteroc2 (GTAGTCGGCAGCGGATGTGGGCG), and the pair used to show the presence of a deleted allele is GEL4heteroc1-HYGRO (CGACACGGCTCTCCGACCTGATGCGCTCTC). For Southern blotting, genomic DNA was digested with EcoRI and hybridized with probe A. DNA isolated from the wild-type strain showed one band of 6.3 kb corresponding to a copy of the wild-type allele, whereas the DNA isolated from the heterokaryon showed the wild-type allele and the mutated allele at 4.1 kb (see panel A). (D) Solubilization of membrane-bound Gel4p with GPI-PLC. Aqueous upper phase (marked “A”) and detergent lower phase (marked “D”) after Triton X-114 partitioning of GPI-PLC-treated (+) or control (−) membranes; immunolabeling with anti-Gel4p hyperimmune antiserum.
shown by PCR and Southern blotting, the primer pair GEL4heteroc1-GEL4heteroc2 amplified a DNA fragment corresponding to the wild-type allele, and the amplification obtained with primers GEL4heteroc1-HYGRO indicated the presence of the GEL4-deleted allele (Fig. 2B and C). Uninucleate conidia isolated from this heterokaryon were unable to germinate and grow on selective medium, even in the presence of 1.2 M sorbitol, indicating that GEL4 was essential for A. fumigatus.

**Gel4p is a GPI-anchored protein.** In membrane preparations, Gel4p with an apparent \( M_r \) of 70 was detected with an antiserum obtained from a rabbit immunized with the peptide C445DFDGKAQTKKGADA460 of Gel4p conjugated to keyhole limpet hemocyanin (KLH). A membrane fraction was treated with phosphatidylinositol (PI)-phospholipase C (PLC) of Bacillus thuringiensis (able to cleave the glycosylphosphatidylinositol [GPI] anchor) and submitted to a partitioning with Triton X-114, as described previously (12). Following this treatment, Gel4p was present in the aqueous phase, whereas it remained in the detergent phase in the absence of PI-PLC treatment (Fig. 2D). These data showed that Gel4p was bound to the membrane through a GPI anchor.

**Gel4p has \( \beta(1-3) \) glucanosyltransferase activity.** The sequence similarities of Gel4p with Gel1p and Gel2p, including the conservation of the two aspartic acid residues essential for the transglycosidase activity, FF(A/S)GNEV (E is the acid-base donor) and F(F/L)SE(Y/F)GCN (E is the nucleophilic residue) (13), suggested that Gel4p had \( \beta(1-3) \) glucanosyltransferase activity typical of the GH72 family (5, 13, 17). In order to analyze the enzymatic activity of Gel4p, a recombinant Gel4p (rGel4p; encompassing amino acids 20 to 488) was produced in Pichia pastoris by following a strategy used previously to produce rGel1p and rGel2p (14). Gel4p produced in P. pastoris had an apparent \( M_r \) of 70 and was glycosylated, as shown by a reduction in \( M_r \) to 52 after peptide N-glycosidase F (PNGase F) treatment (data not shown). The recombinant Gel4p was incubated with reduced laminarioligosaccharides of different sizes, and the products of the reaction were analyzed using a Dionex high-performance anion-exchange chromatography with pulsed-electrochemical detection (HPAEC-PED).
system with a CarboPac PA200 column, as described previously (14). As an example, Fig. 3 shows the results of the incubation of rGel4p with an oligosaccharide of 26 glucose residues (G26r). The major products obtained were in agreement with those obtained using the previously described two-step reaction scheme (7), as follows: the enzyme acted first as an endoglucanase, released short laminarioligosaccharides, and transferred the new reducing ends formed to another oligosaccharide, resulting in the elongation of the \( (1-3) \) glucan chain, until it became water insoluble (corresponding with the largest peak of the HPAEC profile shown in Fig. 3). Transfer activity was seen with other oligosaccharides of lower degrees of polymerization (rG11, rG13, or rG14). Degradation of the reaction products by an endo\( (1-3) \)glucanase (as described in reference 6) resulted in the release of glucose and laminaribiose, without that of branched oligosaccharide, confirming that the transfer product contained only linear \( (1-3) \)glucan chains. The analysis comparing rGel4p to rGel1p under the same experimental conditions showed that rGel4p had a higher avidity for the \( (1-3) \)glucan than rGel1p (Fig. 3).

A phylogenetic tree of the GH72 \( (1-3) \)glucanosyltransferase family showed that its members can be grouped into 3 subfamilies based on the presence or absence of CBM43 (1), S/T-rich domain, and GPI anchor. \( (1-3) \)glucanase proteins are shown in boldface. (B) Hypothetical representation of the in situ localization of a member of the GH72 \( ^* \) ST \( ^* \) subfamily, such as AfGel4p. CBM43 directs the catalytic site toward the \( (1-3) \)glucan chain. After being anchored, the catalytic domain is able to modify the oligosaccharide.
ence of these domains are not important for \(\beta(1-3)\)glucanoylsyltransferase activity (17), they may be essential to localize the proteins at the right places for its morphogenetic activity. O-glycosylated stalks elevate the \(\beta(1,3)\)glucan binding domain above the plasma membrane, where the protein is anchored with GPI, and place the catalytic site in the best position to act on the nascent \(\beta(1,3)\)glucan (Fig. 4B).

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