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Characterization of Glycoside Hydrolase Family 5 Proteins in *Schizosaccharomyces pombe*▽†

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In yeast, enzymes with β-glucanase activity are thought to be necessary in morphogenetic events that require controlled hydrolysis of the cell wall. Comparison of the sequence of the *Saccharomyces cerevisiae* exo-β(1,3)-glucanase Exg1 with the *Schizosaccharomyces pombe* genome allowed the identification of three genes that were named *exg1*$^+$ (locus SPBC1105.05), *exg2*$^+$ (SPAC12B10.11), and *exg3*$^+$ (SPBC2D10.05). The three proteins have different localizations: Exg1 is secreted to the periplasmic space, Exg2 is a membrane protein, and Exg3 is a cytoplasmic protein. Characterization of the biochemical activity of the proteins indicated that Exg1 and Exg3 are active only against β(1,6)-glucans while no activity was detected for Exg2. Interestingly, Exg1 cleaves the glucans with an endohydrolytic mode of action. *exg1*$^+$ showed periodic expression during the cell cycle, with a maximum coinciding with the septation process, and its expression was dependent on the transcription factor Sep1. The Exg1 protein localizes to the septum region in a pattern that was different from that of the endo-β(1,3)-glucanase Eng1. Overexpression of Exg2 resulted in an increase in cell wall material at the poles and in the septum, but the putative catalytic activity of the protein was not required for this effect.

Schizosaccharomyces pombe cells, like other yeasts, are surrounded by a rigid cell wall that provides mechanical strength and protection from environmental stresses. The cell wall determines cellular morphology during the different stages of the life cycle, and it is continuously remodeled during the cell cycle to allow cellular growth. Extracellular cues that trigger shape changes, such as nutrient deprivation or exposure to mating factors, also result in cell wall remodeling. During vegetative growth, *S. pombe* cells are rod shaped and grow by tip elongation, first at the old end and then at both ends. Subsequently, cell wall deposition occurs during septation, and the two new ends are thus sealed off (39).

The *S. pombe* cell wall is composed of mannoproteins, α-glucan and β-glucan (7, 32). Recent studies have shown that *S. pombe* cell walls also contain up to 15% of a branched β(1,3)-β(1,6)-glucan, which has been termed diglucan (31). β(1,3)-Glucan is a major structural component of the fungal cell wall, and it forms a fibrillary network that is thought to be responsible for the mechanical strength of the cell wall. These components are assembled in different layers that can be visualized by electron microscopy (26, 45). There is an outer layer enriched in glycoproteins and an inner layer of carbohydrates. *In situ* localization studies have indicated that β(1,6)-branched β(1,3)-glucan is localized all over the cell wall and throughout the septum; β(1,6)-glucan appears in the same layer and in the secondary septum, whereas linear β(1,3)-glucan is present only at the primary septum (23).

Synthesis of the cell wall is a complex process that requires the participation of different enzymes, some of which have been identified. The biosynthesis of β(1,3)-glucan is carried out by the β(1,3)-glucan synthase complex, whose catalytic subunit in *S. pombe* is encoded by the *bgs* genes. *S. pombe* contains four proteins of this family; Cps1/Bgs1, Bgs3, and Bgs4 are essential for cell viability during vegetative growth (14, 15, 29, 33), while Bgs2 performs an essential role during spore wall formation (27, 34). In yeast and filamentous fungi, the β(1,3)-glucan chains synthesized by the glucan synthase complex are extruded to the periplasmic space in a vectorial process (6). It has been postulated that the nascent β(1,3)-glucan chains should be cross-linked to other components of the cell wall by the action of glycoside hydrolases (GH) and transglycosidases. However, *in vivo* evidence of such a mechanism has been shown only for the *Saccharomyces cerevisiae* Crh1 and Crh2 proteins, which are involved in the cross-linking of β(1,3)-glucan to chitin (8, 9). Other proteins involved in cell wall assembly are the β(1,3)-glucanosyl-transferases of glycoside hydrolase family 72 (GH72), which *in vitro* are able to elongate β(1,3)-glucan oligosaccharides (42, 47). Four genes encoding proteins of this family (*gas1*$^+$, *gas2*$^+$, *gas4*$^+$, and *gas5*$^+$) are present in the *S. pombe* genome, performing specific functions at different moments of the life cycle (21).

Other proteins that may be involved in the modification of the cell wall β(1,3)-glucans are the exo-β(1,3)-glucanases from the GH5 family. Three proteins of this family have been characterized in *S. cerevisiae*. Exg1 of *S. cerevisiae* (ScExg1) is a polypeptide whose differential glycosylation accounts for the two main extracellular exo-β(1,3)-glucanases detected in cul-

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TABLE 1. Yeast strains used in this study

Strain	Genotype[a]	Source or reference
h20	*h⁻ leu1-32*	Lab stock
h123	*h⁻ ura4-Δ18*	Lab stock
PPG148	*h⁻ ura4-Δ18 cdc25-22*	Lab stock
LE1	*h⁻ leu1-32 exg1::kanMX4*	This work
LE2	*h⁻ ura4-Δ18 exg1::kanMX4*	This work
LE3	*h⁻ leu1-32 exg2::kanMX4*	This work
LE4	*h⁻ ura4-Δ18 exg2::kanMX4*	This work
LE5	*h⁻ leu1-32 exg3::kanMX4*	This work
LE 6	*h⁻ ura4-Δ18 exg3::kanMX4*	This work
LE 14	*h⁻ ura4-Δ18 exg1-HA::kanMX4*	This work
LE 15	*h⁻ ura4-Δ18 exg2-HA::kanMX4*	This work
LE 16	*h⁻ ura4-Δ18 exg3-HA::kanMX4*	This work
LE19	*h⁻ leu1-32 exg1-GFP::kanMX4*	This work
LE20	*h⁻ ura4-Δ18 exg1-GFP::kanMX4*	This work
LE21	*h⁻ leu1-32 exg1-GFP::kanMX4*	This work
LE22	*h⁻ ura4-Δ18 exg1-GFP::kanMX4*	This work
LE23	*h⁻ leu1-32 exg3-GFP::kanMX4*	This work
LE24	*h⁻ ura4-Δ18 exg3-GFP::kanMX4*	This work
LE48	*h? ura4-Δ18 exg1::kanMX exg2::kanMX4*	This work
LE50	*h⁻ exg1::kanMX4 exg3::ura4⁺*	This work
LE52	*h⁻ exg2::kanMX4 exg3::ura4⁺*	This work
LE54	*h? exg1::kanMX4 exg2::kanMX4 exg3::ura4⁺*	This work
YAB79	*h⁻ eng1::kanMX4 exg1::ura4⁺*	This work
YAB156	*h? exg1-GFP::kanMX4 eng1-RFP::kanMX4 ura4-Δ18 ade6-M210 leu1-32*	This work
LE25	*h⁻ ura4-Δ18 ace2::kanMX4 ura4⁺*	35
A131	*h⁺ sep1::ura4 ura4-Δ18 leu 1-32*	M. Sipizcki

[a] *h*?, uncertain mating type.

TABLE 2. Oligonucleotides used in this study

Name	Sequence
305	AAAGGATCCGCTGCACCATCCTCCTTCC
306	TAACTCGAGATCGATTTATATGTCCCTTCAA
424	TAACTCGAGATTAAAATGCTCTCTTTTACATCGG
425B	TAAGGATCCACCTGTGGCTGAGTGAAAACCTA
426	TAACTCGAGTAAAATATGAGCAATCTTTTAGAA
427B	TAAGGATCCATCGATTTATATGTCCCTTCAA
428	TAAGTCGACATTACGATGGGATTGAATAAACAA
429B	TAAGGATCCTAACGTCAATAAATACTACTCCTT
640	ACGATTATAGGACAGTGGAGCCTTGCGGAT
641	CGCAAGGCTCCACTGTCCTATAATCGTCGG
643	AATGCCACTCAATGGAGTTAC
644	TAATTACCATCACATATGAAATTCAGATTG
645	CCATCTGAATTTCATATGTGATGGTAATTA
646	CCTTCGGATTCGTCAGCGTGT
647	CTAACGTCACAATGATGGATCGG
648	AAGAGCCTTCTTGTCTAAAAGATTGCTCAT
649	ATGAGCAATCTTTTAGACAAGAAGGCTCTT
650	GGTTCCATTGAAAGCCATCCACC
651	AGCGTGAGGAATAATGATAGTAATAAGAAG
652	CTTCTTATTACTATCATTATTCCTCACGCT
653	ATGTCTCGGGTATTGGGTGG
654	GAAGAAATTTGGTGCGTTAAGTGCACCGTA
655	GGTGCACTTAACGCACCAAATTTCTTCGTT
657	CTCGTTCAAAGGAGGAGCGTGAGGAATAAT
658	ATTATTCCTCACGCTCCTCCTTTGAACGAG
1010	GTACGGTCGACTTCCCCATATGGGTAGCAGCCACC
1011	TCTACGAGCTCAATGATGGAATCATTTTACAAAG

ture supernatants, while Exg2 is a highly glycosylated minor exo-β(1,3)-glucanase with a C-terminal glycosylphosphatidylinositol (GPI) anchor site (12, 13, 43, 57). The third protein, Ssg1/Spr1, is a sporulation-specific glucanase (52). These enzymes are exo-β(1,3)-glucanases, but they also usually act on β(1,6)-linkages, although with less efficiency. It has been proposed that within the cell wall, these proteins would catalyze a transglycosylation rather than a hydrolytic reaction since transferase activity has also been demonstrated for the *S. cerevisiae* and *Candida albicans* Exg1 proteins (54, 55). Here, we report the characterization of the three GH5 family proteins present in *S. pombe*, which were named Exg1, Exg2, and Exg3. They were present in different compartments: the cell wall, membrane, and cytoplasm, respectively. Two of the proteins were active against β(1,6)-glucans but not against β(1,3)-glucans. Unexpectedly, enzymatic assays with purified Exg1 indicated that the protein has an endohydrolytic mode of action.

MATERIALS AND METHODS

Strains, growth conditions, and genetic manipulations. The *S. pombe* strains used in this study are listed in Table 1. Yeast cells were grown on YES (yeast extract with supplements) medium or Edinburgh minimal medium (EMM) with appropriate supplements (41). For overexpression experiments using the *nmt1⁺* promoter, cells were grown in EMM containing 20 μg/ml thiamine up to logarithmic phase. Then, the cells were harvested, washed five times with EMM, and inoculated in fresh medium (with or without thiamine) at an optical density at 595 nm (OD_{595}) of 0.05 to 0.1. Synchronization of strains carrying the thermosensitive *cdc25-22* mutation was achieved by growing the cells at the permissive temperature (25°C) to early log phase (OD_{595} of 0.5) and then shifting the cultures to 37°C for 4 h. Cells were released from arrest by transfer to 25°C, and samples were taken every 20 min.

Plasmids and DNA manipulations. The oligonucleotides used for different DNA manipulations are shown in Table 2. Construction of plasmid pED138 carrying the *exg1⁺* coding sequence under the control of the *nmt1⁺* promoter was achieved by PCR amplification of the coding sequence using oligonucleotides 424 and 425B (which introduced XhoI and BamHI sites) and cloning of the resulting fragment between the XhoI and BamHI sites of plasmid pJCR-3XL (40). A similar approach was used to construct plasmids pML2 (pJCR-3XL carrying *exg2⁺*) and pED139 (pJCR-3XL carrying *exg3⁺*) by using oligonucleotide pairs 426-427B and 428-429B, respectively.

To construct the plasmids carrying the different mutated versions of *exg2⁺*, first the *exg2⁺* coding sequence and flanking regions were amplified with oligonucleotides 305 and 306. Chromosomal HindIII and SacI sites were used to clone the gene with its promoter and terminator (from −473 to +278) at the same sites of vector pUC19, generating plasmid pEX22. Then, an NdeI site was introduced before the stop codon by recombinant PCR using oligonucleotides 643, 644, 645, and 646, yielding plasmid pED162. The NdeI site was used to clone the c-*myc* epitope obtained from plasmid pGEMMH (16), producing plasmid pED168′. Finally, a SalI-SacI fragment containing the *exg2-myc* region was cloned in plasmid pML2, generating plasmid pED172. The different mutations were generated by amplifying specific DNA fragments carrying the desired mutations by recombinant PCR and cloning the fragments in plasmid pED168′ or pED172. Thus, the following constructs were generated with the indicated oligonucleotides: *exg2*-A1-*myc* (where *exg2* harbors the mutation *E338A*, designated A1) with oligonucleotides 646, 653, 654, and 655; *exg2*-A2-*myc* (where *exg2* harbors the mutation *E349Q*, designated A2) with oligonucleotides 646, 655, 640, and 641; *exg2*-ΔN-*myc* (where is *exg2*-ΔN is a mutant lacking the cytoplasmic tail) with oligonucleotides 647, 648, 649, and 650; *exg2*-ΔTM-*myc* (where *exg2*-ΔTM is an *exg2* mutant lacking the transmembrane [TM] domain) with oligonucleotides 647, 650, 651, and 652; *exg2*-Δout-*myc* (where *exg2*-Δout lacks the spacer domain that separates the transmembrane and catalytic domains) with oligonucleotides 647, 657, 658, and 650; and *exg2*-ΔGH5-*myc* (where *exg2*-ΔGH5 lacks the catalytic domain) with oligonucleotides 1010 and 1011. The amplified fragments carrying the *exg2*-A1, *exg2*-A2 and *exg2*-ΔGH5 mutations were first cloned in plasmid pED168′, and then the complete *exg2* coding sequence fused to c-*myc* was cloned in plasmid pML2 using SalI-SacI sites, generating plasmids pED188 (*exg2*-A1-*myc*), pED193 (*exg2*-A2-*myc*), pED195 (*exg2*-A1A2-*myc*, where *exg2* harbors both the A1 and A2 mutations), and pED215 (*exg2*-ΔGH5-*myc*). For *exg2*-Δout, *exg2*-ΔN and *exg2*-ΔTM, the amplified fragments carrying the mutations were cloned in plasmid pED172 using different restriction sites, yielding plasmids pED177 (*exg2*-Δout-*myc*), pED178 (*exg2*-ΔN-*myc*), and pED179 (*exg2*-ΔTM-*myc*). In all cases, the presence of the desired mutations was confirmed by sequencing the amplified fragments.

To construct plasmid pED173, carrying *exg2*-green fluorescent protein (GFP) under the control of the *nmt1*$^+$ promoter (41X), the NdeI site of plasmid pED162 was used to clone the GFP coding sequence obtained from plasmid pGEM-EGFP (16), producing plasmid pED166. The *exg2*$^+$ coding region obtained from plasmid pML2 as a XhoI-BamHI fragment was cloned under the control of the *nmt1*$^+$ promoter present in plasmid pJCR-41XU (40) to create plasmid pED169. Then, a SalI-SacI fragment from plasmid pED166 containing the C-terminal region of *exg2*$^+$ and the GFP was used to replace the same region of plasmid pED169, generating plasmid pED173.

To construct null mutants lacking the *exg1*$^+$ (locus SPBC1105.05), *exg2*$^+$ (SPAC12B10.11), or *exg3*$^+$ (SPBC2D10.05) genes, the entire coding sequences were replaced by the *ura4*$^+$ or *kanMX4* cassette. The deletion cassettes were constructed using recombinant PCR. For this purpose, DNA fragments of 300 to 500 bp corresponding to the 5′ and 3′ flanking regions of each gene were PCR amplified using specific oligonucleotide pairs, and the resulting fragments were then fused, by recombinant PCR, to the *kanMX4* cassette, which confers resistance to the G418 antibiotic (4) or to the *ura4*$^+$ gene. The C-terminally tagged strains carrying *exg1-HA* (where HA is hemagglutinin), e*xg2-HA*, *exg3-HA*, *exg1-GFP*, e*xg2-GFP*, or *exg3-GFP* were constructed by direct chromosome integration of PCR fragments generated using plasmid pFA6a-3HA-kanMX6 or pFA6a-GFP-kanMX6 as a template and specific oligonucleotides (4). The amplified fragments contained the HA or GFP coding regions fused in frame to the last codon of the gene and the *kanMX6* cassette to select for transformants. Correct integration of the DNA fragment was verified by PCR or Southern blotting.

RNA isolation and Northern blot analyses. Cells (1.3×10^9) were collected at different time intervals after release from the restrictive temperature (37°C) or from different mutant strains, and total RNA was prepared using the method described by Percival-Smith and Segall (46). For Northern blot analyses, 12.5 μg of RNA was used. The DNA probes used to detect the different transcripts were DNA fragments (400 to 500 nucleotides [nt]) obtained by PCR amplification with specific oligonucleotides. For *act1*$^+$, a 1.1-kb fragment containing the whole coding region obtained by PCR was used.

Microscopy techniques. For light microscopy, cells were fixed in 3.7% formaldehyde and stained with aniline blue or calcofluor white. Samples were viewed using a Leica DMRXA microscope equipped for Nomarski optics and epifluorescence and photographed with a Photometrics Sensys charge-coupled-device (CCD) camera. For transmission electron microscopy (TEM), cells were stained with potassium permanganate and examined on a Zeiss EM 902 transmission electron microscope.

Immunoblotting and protein methods. Total cell extracts of *S. pombe* were prepared by breaking the cells with glass beads in radioimmunoprecipitation assay (RIPA) buffer (10 mM sodium phosphate, pH 7, 1% Triton X-100, 0.1% SDS, 2 mM EDTA, 150 mM NaCl). Cell extracts were centrifuged at 10,000 × *g* for 10 min to separate a pellet from the supernatant. Culture supernatants were concentrated 10 times using Amicon units. The conditions used to examine whether Exg2 is a membrane protein have been described previously (28). The lysates were spun at maximal speed in a microcentrifuge for 30 min at 4°C. Supernatants were recovered, and the pellets were resuspended in SDS-PAGE loading buffer. For immunoblotting, 50 μg of protein extract was resolved by SDS-PAGE on 8% gels. Protein transfer, blotting, and enhanced chemiluminescence (ECL) detection were performed using standard procedures. Mouse monoclonal anti-myc antibodies (clone 9E10; Roche) were used.

Expression in *Pichia pastoris*. To purify Exg1, the catalytic domain lacking the signal sequence (amino acids 28 to 407) was cloned into the EcoRI-XbaI sites of plasmid pPICZαA (Invitrogen). The resulting plasmid (pED204), containing the *exg1* gene under the control of the *AOX1* promoter, and a C-terminal fusion to the c-*myc* epitope and His_6 was introduced into strain KM71H (*arg4 aox1*::*ARG4*). Production of the fusion protein was accomplished according to the protocol described by the supplier (Invitrogen). Exg1-His_6 was purified using His-Trap columns (Amersham Pharmacia).

Assay for β-glucanase activity. β-Glucanase activity was assayed as previously described (5). The substrates used to test activity were laminarin [β(1,3)-glucan], pustulan [β(1,6)-glucan], scleroglucan [β(1,3)-glucan with one β(1,6)-glucose side chain every three residues], or lichenan [mixed linkage β(1,3)-β(1,4) glucan]. Determination of the reducing sugars released in the reactions was performed by the methods of Somogyi (53) and Nelson (44). One unit of activity was defined as the amount of enzyme that catalyzed the release of reducing sugar groups equivalent to 1 μmol of glucose per hour, and specific activity was expressed as units per milligram of protein. For activity against *p*-nitrophenyl-β-D-galactopyranoside (pNPG), the amount of *p*-nitrophenol released was determined by measuring optical density at 420 nm. One unit of enzyme catalyzed the release of 1 μmol of *p*-nitrophenol per h under the reaction conditions used. The degree of polymerization (DP) of oligosaccharides released from laminarin or pustulan by Exg1 was determined by high-performance anion-exchange (HPAE) chromatography using a pulsed electrochemical detector (PED) and an anion-exchange column (Carbo-PAC PA1; 4.6 mm by 250 mm; Dionex) under the following conditions: flow rate of 1 ml/min; buffer A, 50 mM NaOH; buffer B, 500 mM sodium acetate in 50 mM NaOH; gradient, 0 to 2 min with 98% buffer A and 2% buffer B (isocratic); 2 to 15 min with 75% buffer A and 25% buffer B (linear); 15 to 45 min with 60% buffer A and 40% buffer B (linear).

Labeling and fractionation of cell wall polysaccharides. Labeling and fractionation of cell wall polysaccharides were performed as described previously (3). Cultures of *S. pombe* cells that had been incubated in the presence or absence of thiamine for 18 h were diluted in medium supplemented with D-[U-^{14}C]glucose (3 μCi/ml) and incubated for 4 additional hours. Total glucose incorporation was monitored by measuring radioactivity in trichloroacetic acid-insoluble material. Cells were harvested and broken with glass beads. Cell walls were purified by repeated washing and differential centrifugation (once with 1 mM EDTA, twice with 2 M NaCl, and twice with 1 mM EDTA) at 1,000 × *g* for 5 min. Finally, they were heated at 100°C for 5 min. Aliquots of the cell walls were incubated with 100 μg of Zymolyase 100T (Seikagaku Kogyo Co.) in 50 mM citrate-phosphate buffer (pH 5.6) or with 100 U of Quantazyme (Qbio) in 50 mM Tris, pH 7.5, for 36 h at 30°C. After incubation, samples were centrifuged and washed with the same buffer. One milliliter of 10% trichloroacetic acid was added to the pellets, and their radioactivity levels were measured. The supernatants from the Zymolyase 100T reaction were considered β-glucan plus galactomannan, and the pellet was considered α-glucan. The supernatants from the Quantazyme reactions were considered β(1,3)-glucan, and the pellet was considered α-glucan plus galactomannan. All determinations were carried out in duplicate.

RESULTS

***S. pombe* contains three proteins belonging to glycoside hydrolase family 5.** Family 5 glycoside hydrolases (GH5) comprise a group of enzymes that cleave different glucan polymers [β(1,3)- and β(1,6)-glucans, cellulose, lichenan, or xylan] that are widespread in fungi and bacteria. *S. cerevisiae* contains four different polypeptides belonging to this family (Exg1, Exg2, Ssg1, and YBR056w). Comparison of the ScExg1 sequence with the *S. pombe* genome database revealed the presence of three proteins belonging to this family, *exg1*$^+$ (locus SPBC1105.05), *exg2*$^+$ (SPAC12B10.11), and *exg3*$^+$ (SPBC2D10.05). The *S. pombe* Exg1, Exg2, and Exg3 proteins show 40.5%, 25%, and 21% overall identity to ScExg1, respectively.

***exg1*$^+$ expression peaks during the septation process.** Northern blot analysis in asynchronous cultures indicated that the three open reading frames (ORFs) were expressed during the vegetative cycle (data not shown). When RNAs obtained from a synchronized *cdc25-22* mutant strain were analyzed, a periodic cell cycle variation was found for *exg1*$^+$ (Fig. 1A), with the maximum accumulation of mRNA occurring during the septation process, suggesting that the product of this gene might exert its function during the last stages of the cell cycle, namely, septum assembly or cell separation. No significant variations that correlated with cell cycle progression were observed for the other two genes, *exg2*$^+$ and *exg3*$^+$. These results are in good agreement with the results obtained in a large-scale analysis (50). To study whether the periodic expression of *exg1*$^+$ was dependent on the transcription factors Ace2 or Sep1, Northern analyses were performed to compare the expression in wild-type, *ace2*Δ, and *sep1*Δ mutants (2, 50, 58). The results revealed that the expression of *exg1*$^+$ was clearly reduced in the *sep1*Δ mutant (Fig. 1B), suggesting that this transcription factor is required for its expression. As expected for genes that do not show fluctuations during the cell cycle, no significant differences were seen in the expression of *exg2*$^+$ and *exg3*$^+$.

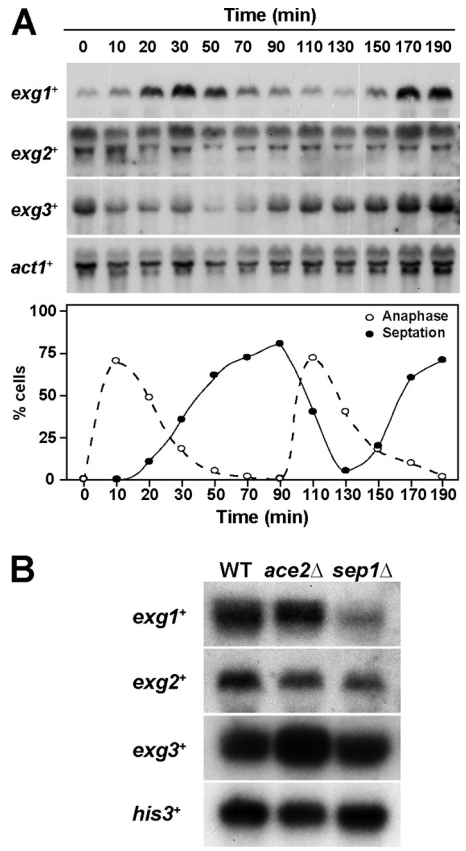

FIG. 1. Transcription pattern of *exg* genes. (A) Expression during the cell cycle. Synchrony was induced by arrest-release of a *cdc25-22* mutant, and samples were taken at the indicated time points after the release for RNA extraction. RNA was hybridized with specific probes for *exg1*$^+$, *exg2*$^+$, *exg3*$^+$, or *act1*$^+$. The graph represents the anaphase index or septation index at each time point. In this experiment, the peak of septum formation occurred at 70 to 90 min. (B) Dependence on the Ace2 and Sep1 transcription factors. RNA from wild-type, *ace2*Δ, and *sep1*Δ mutants was extracted, transferred to nitrocellulose membranes, and probed with a specific probes for *exg1*$^+$, *exg2*$^+$, or *exg3*$^+$ or *his3*$^+$ as a control.

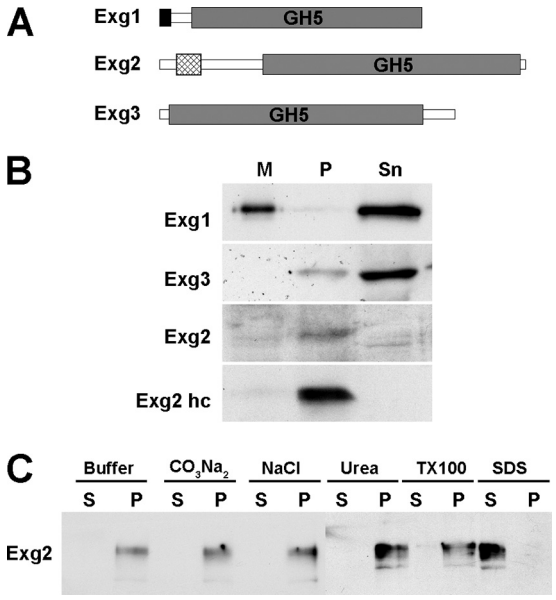

FIG. 2. Cellular fractionation of the Exg proteins. (A) Schematic representation of the characteristics of the Exg1, Exg2, and Exg3 proteins. The GH5 domain, common to the three proteins, is indicated. The black box represents a hydrophobic region with the characteristics of the signal secretion peptides, and the hatched box represents a putative transmembrane domain. (B) Cells expressing Exg1-HA, Exg2-HA, or Exg3-HA were grown in minimal medium to late log phase (OD_{595} of 1.5). Those carrying Exg2-myc under the control of the *nmt1* promoter (Exg2 hc) were grown in the absence of thiamine for 22 h. Cells were collected by centrifugation and broken with glass beads. Extracts were centrifuged at 10,000 rpm for 10 min to separate the cell wall and membranes (pellet [P]) from the cytoplasmic content (supernatant [Sn]). Concentrated culture medium (M) was also loaded in the gels. Proteins were fractionated using SDS-PAGE gels and immunoblotted using antibodies against the HA or c-*myc* epitopes. (C) Cells carrying Exg2-myc under the control of the P3X*nmt1* promoter were extracted after lysis in buffer containing 0.6 M NaCl, 0.1 M Na_2CO_3, 1.6 M urea, 4% Triton X-100, and 2% SDS. Soluble and insoluble proteins were separated by centrifugation at 13 000 × *g* for 30 min, as indicated by supernatant (S) and pellet (P), respectively.

The three β-glucanases have different cellular fates. Analysis of the sequence of the three Exg proteins revealed the presence of different features in addition to the GH5 domain. Thus, Exg1 contained an N-terminal signal peptide for secretion, with the likely cleavage site between positions 22 and 23 (AFS-YV). In contrast, Exg2 contained a predicted transmembrane region (amino acids 39 to 60), while Exg3 did not contain any sequence for extracellular localization (Fig. 2A). These observations suggested that the three proteins might have different cellular localizations: the cell wall, the plasma membrane, and the cytoplasm, respectively. To test these predictions, they were tagged with the HA epitope at their C termini, and the distribution of the proteins in culture supernatants, the membrane/cell wall fraction, and the cytoplasm was analyzed. The results confirmed that only Exg1 was present in culture supernatants, indicating that the protein was secreted to the exterior of the cell and then released to the surrounding medium (Fig. 2B). In contrast, Exg3 was found mainly in the cytoplasm (supernatant) of the cell extracts, with a minor fraction in the pellet. Although Exg2 was difficult to detect, it was present mainly in the pellet fraction. To analyze the localization of Exg2 with more detail, the gene was placed under the control of the *nmt1* promoter and tagged with the c-*myc* epitope. Overexpression of the gene confirmed that Exg2 was present in the membrane/cell wall fraction (Fig. 2B, Exg2 hc). Thus, these results confirm the predictions of the sequence analysis and indicate that Exg1 is a secreted protein, Exg2 is a possible membrane-associated protein, and Exg3 is a cytoplasmic protein.

To ascertain whether Exg2 was indeed an integral membrane protein, protein extracts were prepared under different conditions. Cells were extracted with buffer alone, buffer containing 0.6 M NaCl or 1.6 M urea (to solubilize peripheral membrane proteins), 0.1 M Na_2CO_3 (to solubilize intracellular vesicles), 4% Triton X-100 (a nonionic detergent that solubilizes most membrane proteins), or 2% SDS (an ionic detergent that solubilizes all membrane proteins), and extracts were separated into the pellet and supernatant fractions. With the exception of SDS treatment, Exg2 was found to be insoluble under all conditions tested and was detected in the pellet

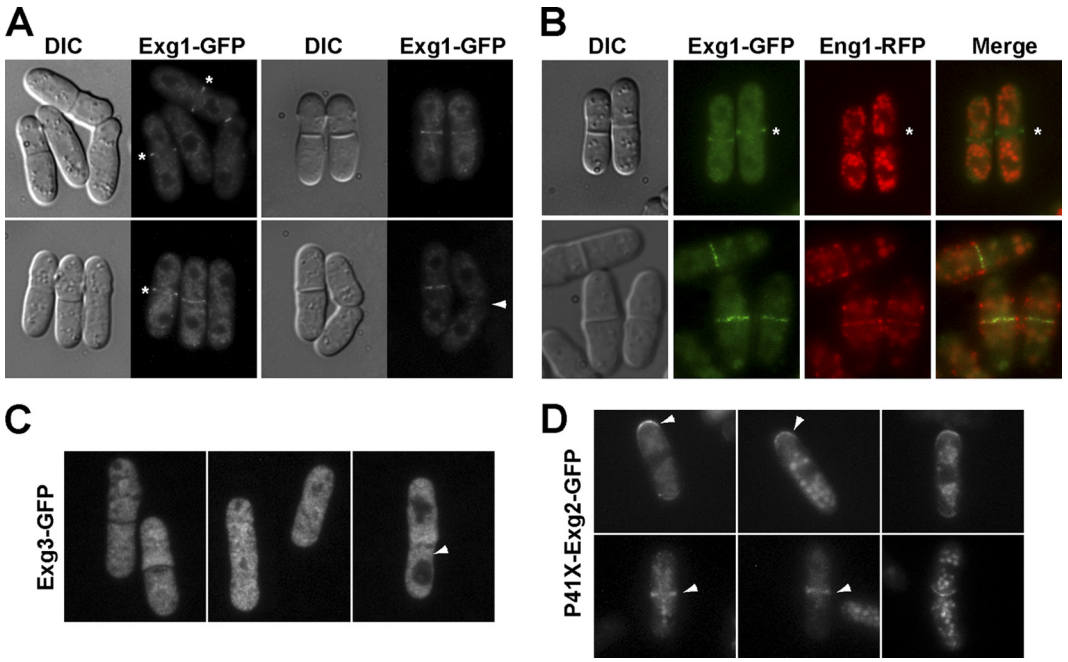

FIG. 3. Localization of Exg proteins. (A) Wild-type cells expressing *exg1-GFP* were grown to early log phase. Photographs of differential interference contrast microscopy (DIC) or Exg1-GFP are shown. (B) Exponentially growing wild-type cells expressing Exg1-GFP and Eng1-RFP were imaged for DIC, RFP, and GFP fluorescence. Overlay of the red and green channels is also shown in the merged images. (C) Wild-type cells expressing *exg3-GFP* were grown to early log phase, and live cells were used for microscopic observation. (D) Wild-type cells expressing *exg2-GFP* under the control of the P41X-*nmt1* promoter were grown in medium without thiamine for 18 h, and live cells were used for microscopic observation. Representative cells at different stages of the cell cycle are shown.

fraction (Fig. 2C). Thus, Exg2 is a novel integral membrane protein that is solubilized only by strong ionic detergents but not by nonionic detergents, as has been previously described for the Cps1/Bgs1 subunit of the β-glucan synthase (28).

Cellular localization of Exg proteins. To determine the *in vivo* localization of the three Exg proteins, the GFP was inserted in frame before the stop codon of each gene. The localization of the three proteins was monitored in live cells, but the fluorescence from only Exg1-GFP and Exg3-GFP was detectable. In good agreement with the peak of transcription during cytokinesis observed for *exg1*$^+$, the protein was found mainly at the center of the cells by the time the septum was starting to be visible by differential interference contrast (DIC). Exg1 first started to accumulate as a ring surrounding the septa during the initial steps of their assembly (Fig. 3A, asterisks). At later stages, when the septum was clearly visible by DIC, Exg1 was found spanning the whole septum, and it had disappeared by the time the cells were separating (Fig. 3A, arrowhead). Staining of the cells with calcofluor white or aniline blue to visualize the dynamics of septum assembly more clearly resulted in a loss of the GFP fluorescence.

We have previously shown that the *S. pombe* endo-β(1,3)-glucanase Eng1 has a localization similar to that of Exg1 (35). To determine whether the two hydrolases colocalized, we studied the localization of both proteins in the same cell using a strain that simultaneously expressed Exg1-GFP and Eng1-red fluorescent protein (RFP). Exg1-GFP was seen in the septum before the Eng1-RFP fluorescence could be detected (Fig. 3B, asterisks), consistent with the fact that *exg1*$^+$ expression is dependent on Sep1 while that of *eng1*$^+$ requires Ace2. Only when the septum was clearly apparent by DIC did Eng1-RFP start to accumulate in the region, indicating that Eng1 secretion occurs after septum assembly has been completed. Even though Eng1 and Exg1 were present in the septum region, the distributions were different, and there was no clear colocalization of either protein. The fluorescence of Exg1-GFP was generally wider and less uniform than that of Eng1-RFP, suggesting that Exg1 localization might not be restricted to the primary septum but also spread to the secondary septum. This is in contrast with the perfect colocalization found for the two endoglucanases involved in cell separation, Eng1 and Agn1 (38).

In the case of Exg3, we found that this was a cytoplasmic protein, consistent with the fractionation experiments, since the fluorescence was detected as a diffuse cytoplasmic staining excluded from the nucleus although no specific pattern or accumulations could be seen (Fig. 3C). Exg2 could not be visualized when the Exg2-GFP protein was expressed from its own promoter, suggesting that the gene is transcribed at low levels. When Exg2-GFP was mildly overexpressed using the weak P41X-*nmt1*$^+$ promoter, the localization of the protein could be determined. Exg2 was localized at the sites of cell growth, that is, the poles of the cell and the septum (Fig. 3D, arrowheads). Thus, these results are in good agreement with the fractionation experiments and indicate that the three GH5 proteins have different cellular localizations.

Exg1 and Exg3 are β(1,6)-glucanases. The observed amino acid sequence similarity between *S. pombe* Exg proteins was an indication that they might also be β(1,3)-glucanases. However, since *S. pombe* lacks any detectable exo-β(1,3)-glucanase ac-

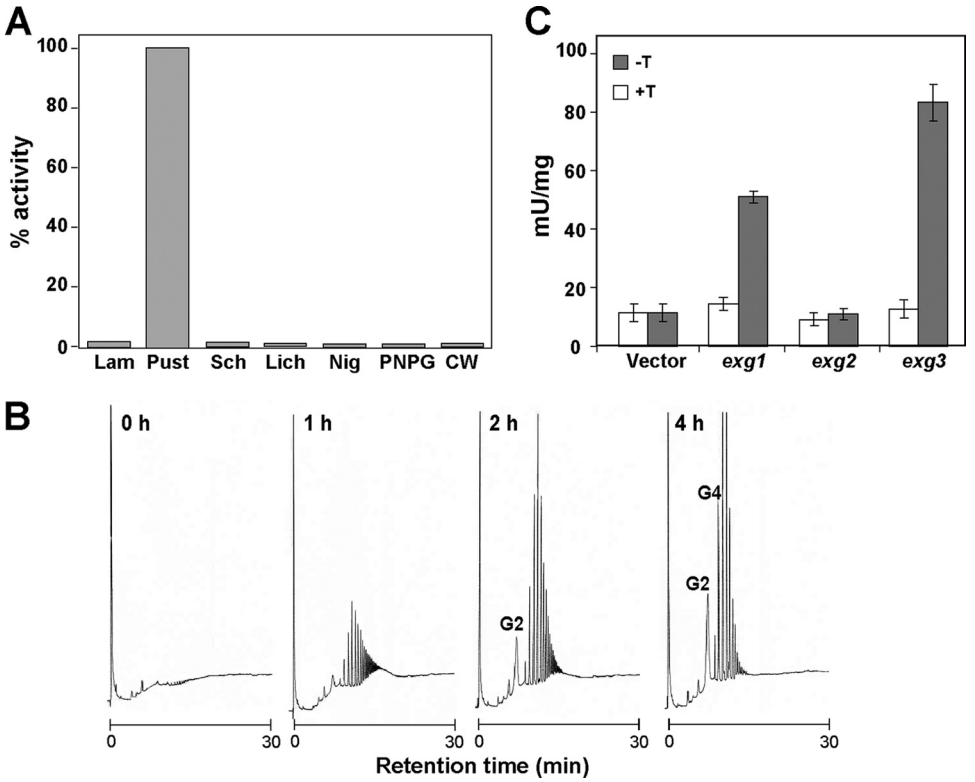

FIG. 4. Exg proteins have activity against β(1,6)-glucans. (A) β-Glucanase activity of purified Exg1 against laminarin [β(1,3)-glucan], pustulan [β(1,6)-glucan], schleroglucan [β(1,3)-glucan with β(1,6) ramifications], lichenan [β(1,3)-(1,4)-glucan], nigeran [α(1,3)-glucan], pNPG, or *S. pombe* cell wall (CW). Five micrograms of protein was incubated with the substrates for 24 h before the concentration of reducing sugars released was assayed. Activity is shown as a percentage of the maximum activity detected for pustulan. (B) HPAE-PED chromatographic analysis of oligosaccharides released by Exg1. The reaction was conducted in acetate buffer at 37°C using soluble pustulan as a substrate for the indicated times (h) before the products were analyzed by HPAE-PED chromatography. G_2, gentobiose, G_4, gentotetraose. (C) Enzymatic activity against pustulan [β(1,6)-glucan] of cells overexpressing *exg1*$^+$, *exg2*$^+$, or *exg3*$^+$. Wild-type cells transformed with plasmids pED138 (carrying P3X*nmt1-exg1*), pML2 (P3X*nmt1-exg2*), pED139 (P3X*nmt1-exg3*), or vector alone (pJCR-3XL) were grown for 22 h in the presence (white bars) or absence (black bars) of thiamine (T) to induce the expression of the genes. Cell extracts were prepared and incubated in the presence of pustulan for different amounts of time. Activity is presented as mU/mg. The result is the mean of two independent assays. Error bars indicate the standard deviation.

tivity (48), two possibilities can be envisioned. First, it is possible that the proteins might be related only in sequence but lack any enzymatic activity. Alternatively, they might have different substrate specificities since it has been reported that the *S. cerevisiae* and *C. albicans* Exg1 proteins are active against not only β(1,3)-glucan but also β(1,6)-glucans (54, 55). To check whether the *S. pombe* Exg proteins indeed contained glucanase activity, Exg1 was purified from *P. pastoris* cells expressing an Exg1-His_6-myc construct. The enzymatic activity of the purified protein was assayed using laminarin [a β(1,3) polymer], pustulan [a β(1,6) polymer], scleroglucan [a β(1,3)-glucan with β(1,6) branches], lichenan [a mixed β(1,3)-β(1,4)-glucan], nigeran [an insoluble α(1,3) polymer], or the synthetic compound *p*-nitrophenyl-β-D-glucopyranoside (pNPG) as a substrate. As shown in Fig. 4A, activity was detected only when pustulan was used as a substrate, indicating that Exg1 was a glucanase specific for β(1,6) linkages, in contrast to the *S. cerevisiae* and *C. albicans* proteins, which are able to cleave both β(1,3) and β(1,6) polymers. In addition, no activity was detected against pNPG, suggesting that the mechanism of action was not exohydrolytic.

To further confirm these results and to investigate the kinetics and degradation pattern of different substrates, high-performance liquid chromatography (HPLC) was used to analyze the reaction products. Soluble pustulan was used as substrate for the assay. After 2 h of incubation, Exg1 had released small oligosaccharides (DP of 2 to 30). The progressive degradation resulted in the formation of numerous reducing β(1,6)-oligosaccharides of various size (Fig. 4B). Gentobiose to gentohexose (Fig. 4B, G_2 to G_6) were the main reaction products after 4 h of incubation. The production of small amounts of glucose and large amounts of oligosaccharides of different sizes proved that Exg1 degraded linear β(1,6)-glucan substrates with an endolytic mode of action. No degradation of laminari-oligosaccharides [β(1,3)-glucans] such as laminarin, laminari-hexaose, or octaose was observed (data not shown), confirming that β(1,3)-glucans are not a substrate for Exg1.

Having determined that Exg1 was a β(1,6)-glucanase, we tested whether Exg2 and Exg3 possessed similar activities. To this end, extracts from cells overexpressing each of the three genes under the control of the thiamine-repressible *nmt1*$^+$ promoter were prepared and assayed using pustulan as a substrate. The results indicated that overexpression of *exg1*$^+$ and

exg3$^+$ produced a 3-fold and 6-fold increase in activity, respectively, in comparison with the activity found in cells grown in the presence of thiamine (promoter repressed) or in wild-type cells, confirming that the two proteins are β(1,6)-glucanases (Fig. 4C). However, no activity could be detected in cells overexpressing *exg2*$^+$.

Deletion of *exg* genes presents no apparent phenotype. To determine the biological role of the three Exg proteins during the life cycle of the fission yeast, mutant strains lacking each of the three genes were constructed. The strains were viable, and no growth defects were observed in different media (YES medium or minimal medium) or at different temperatures (25 to 37°C). Growth on plates supplemented with cell wall-disturbing agents, such as calcofluor white or Congo red was also tested, and no significant differences with the wild-type strain were detected. This could be due to a redundant function of the three Exg proteins. However, similar results were obtained when the triple mutant *exg1*Δ *exg2*Δ *exg3*Δ was tested, indicating that these proteins do not perform an essential role in cell wall construction or that the cells have additional mechanisms to compensate for the absence of Exg proteins. Since Exg1 is expressed during the cell cycle slightly before the endo-β(1,3)-glucanase Eng1 and since it also localizes to the septum region during cell division, the double mutant *exg1*Δ *eng1*Δ was constructed to analyze whether Exg1 plays a minor role during cell separation. The phenotype of *exg1*Δ *eng1*Δ cells was almost indistinguishable from that of *eng1*Δ cells.

Overexpression of *exg2*$^+$ causes alterations in the cell wall. The effect of overexpression of the three *exg*$^+$ genes was also assessed. Strains containing the genes under the control of the strong version of the *nmt1*$^+$ promoter were constructed and grown in the absence of thiamine. Overexpression of *exg1*$^+$ and *exg3*$^+$ produced a moderate defect in the growth of the strains, but the microscopic appearance of the cells was similar to that of wild-type cells (data not shown). In contrast, cells overexpressing *exg2*$^+$ had a severe growth defect, and they stopped growing at 10 to 12 h after induction (Fig. 5A). To determine the nature of the defect, the morphology of cells was analyzed. By 12 h, the cells had an abnormal morphology and had become rounded and irregularly shaped. Also, large amounts of abnormal material had accumulated at the poles of the cell. In an effort to determine the identity of the accumulated material, cells overexpressing *exg2*$^+$ were stained with aniline blue, a dye that preferentially binds to β(1,3)-glucans and is used to stain the cell wall and septum in *S. pombe* (25). The material stained well with aniline blue (Fig. 5B), indicating that it was cell wall and that at least a portion of it was comprised of β-glucans. The excess of cell wall accumulated specifically at the poles and septum of the cells.

The previous observations were confirmed when the cells were observed by electron microscopy (Fig. 6A to F). In comparison with the normal appearance of the wall of wild-type cells (Fig. 6A and E), the wall of cells overexpressing *exg2*$^+$ clearly contained extra material that accumulated at the poles of the cell and the separation septum (Fig. 6B to D and F). Interestingly, the excess material was incorporated into the cell wall and had a similar appearance to the rest of the cell wall.

Cells overexpressing *exg2*$^+$ accumulate α- and β-glucans. To identify the nature of the material that accumulated in cells overexpressing *exg2*$^+$ more precisely, cell wall constituents were isolated and characterized after growing the cells in the presence of [U-^{14}C]glucose. Incorporation of radioactive glucose into the wall of cells grown in the presence of thiamine was similar to that of wild-type cells, but it increased considerably in the absence of thiamine (from 34 to 47% of total glucose incorporated) (Fig. 6G). A dramatic increase in the amount of α(1,3)- and β(1,3)-glucan was detected under conditions of *exg2*$^+$ overexpression (Fig. 6H), but the β/α-glucan ratios were similar in the three strains, indicating a simultaneous increase in both glucan polymers. Additionally, the amount of galactomannan was not significantly affected, leading to an alteration in the ratio of glucan to galactomannan (around two times more glucan than galactomannan). These results suggest that strains overexpressing *exg2*$^+$ have lost the ability to properly coordinate glucan polymer synthesis with cell growth and that glucan synthases produce an excess of cell wall material.

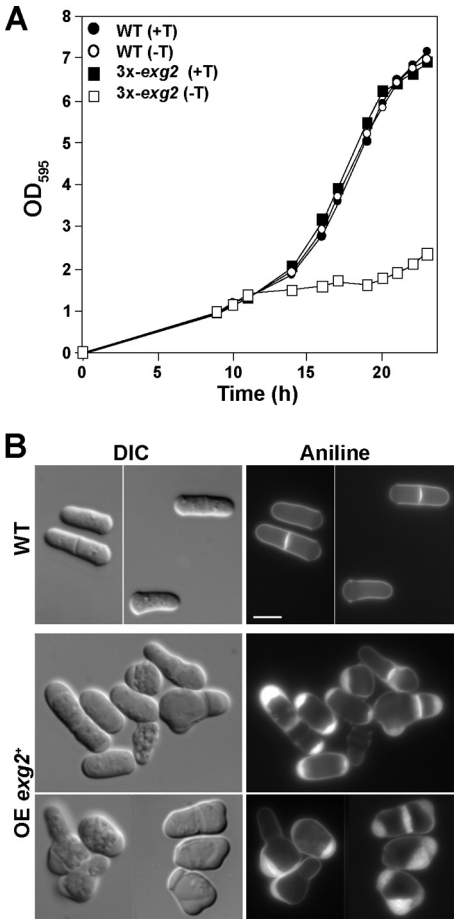

FIG. 5. Overexpression of *exg2*$^+$ produces severe defects in cell growth. (A) Growth of wild-type (WT) strains carrying P3X*nmt1-exg2* (3X-*exg2*) or vector alone grown in the presence (+) or absence (−) of thiamine (T). Cells overexpressing *exg2*$^+$ cease growth after 10 to 12 h of induction. (B) Microscopic appearance of wild-type and cells overexpressing *exg2*$^+$ (OE *exg2*$^+$). Cells that had been growing in the absence of thiamine for 22 h were stained with aniline blue, a fluorochrome that preferentially binds β(1,3)-glucans. Photographs of DIC microscopy or aniline blue-stained cells are shown. Bar, 5 μm.

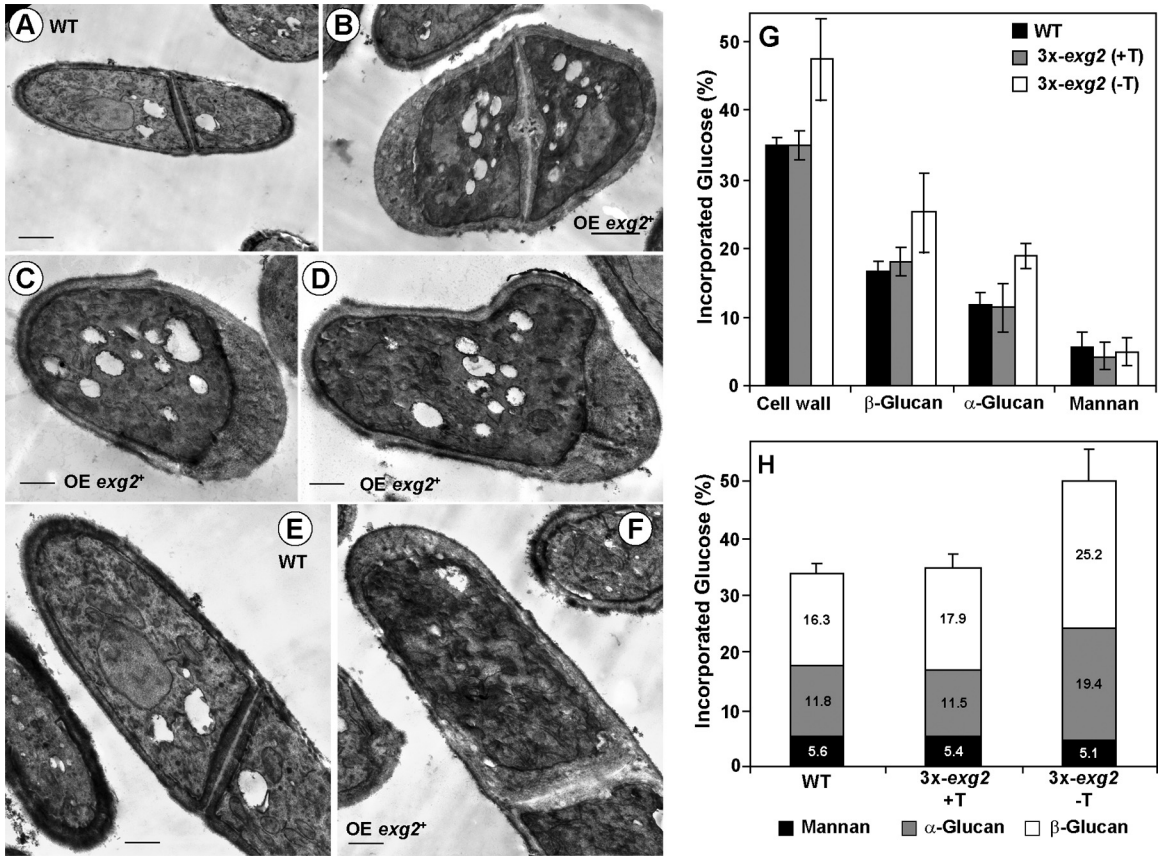

FIG. 6. Effects of *exg2*$^+$ overexpression. Wild-type (WT) cells containing vector or plasmid pML2 (overexpressing [OE] *exg2*$^+$), as indicated, were grown for 22 h in the absence of thiamine and prepared for transmission electron microscopy. Images of panels A to D show a general view of the cells, while panels E and F show details of the septa. Bars, 0.6 μm (C to E) and 1.1 μm (A and B). (G and H) Composition of the cell wall in strains overexpressing *exg2*$^+$. The relative levels of [^{14}C]glucose radioactivity incorporated into each cell wall polysaccharide in a 4-h labeling are shown for the wild-type strain (h20) containing vector (pJCR-3XL) or plasmid pML2 grown in the presence (+T) or absence (−T) of thiamine. Values are the means of three independent experiments with duplicate samples. Standard deviations are shown.

The N-terminal region and the catalytic domain of Exg2 are required for cell wall accumulation. To investigate the reason for increased cell wall accumulation in cells overexpressing *exg2*$^+$, versions of the Exg2 protein in which different domains were deleted were constructed. When the *exg2* sequence was analyzed using topology prediction programs such as TopPred (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html) or TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/), the N terminus was predicted to be cytoplasmic while the catalytic domain would be extracellular. Thus, mutant proteins lacking the putative cytoplasmic tail of the protein (Exg2-ΔN), the transmembrane domain (Exg2-ΔTM), the spacer domain that separates the transmembrane and catalytic domains (Exg2-Δout), or the catalytic domain (Exg2-ΔGH5) were generated (Fig. 7A). One possibility for explaining these results is that an excess of enzymatic activity could weaken the cell wall, and as a consequence the synthesis of glucans would be induced. To test this possibility, we also created proteins containing single amino acid changes in the two glutamic acid residues that form part of the catalytic center of GH5 proteins, which are also conserved in *exg2*$^+$. These mutations involved replacing E338 with Ala (E338A, yielding Exg2-A1), the replacement of E439 with Gln (E349Q, yielding Exg2-A2), and the double mutant (E338A-E349Q, yielding Exg2-A1A2). As described for other members of this family (11, 30), these mutations should completely eliminate the catalytic activity of the enzyme, if present. All the constructs were cloned under the control of the strong version of the *nmt1*$^+$ promoter and introduced into the wild-type strain to test their effects.

Overexpression of Exg2-ΔN resulted in cells that had an abnormal morphology (the cells were rounder than wild-type cells) but did not exhibit a large accumulation of cell wall material (Fig. 7B). Overexpression of Exg2-ΔTM resulted in almost wild-type cells, but since the protein levels were significantly lower than those found in WT cells (Fig. 7C), the absence of phenotype could be due to the small amount of protein. The same reason—reduced protein levels—could account for the fact that the overexpression of Exg2-Δout resulted in a modest phenotype, with a minor accumulation of glucans in the septum region. When the construct lacking the catalytic domain (Exg2-ΔGH5) was overexpressed, the cells showed a wild-type morphology, indicating that this region of the protein is required for the activation of glucan synthesis. However, the putative catalytic activity of the enzyme was not required for this effect since cells carrying the double mutant Exg2-A1A2 (and also the two single mutants Exg2-A1 and

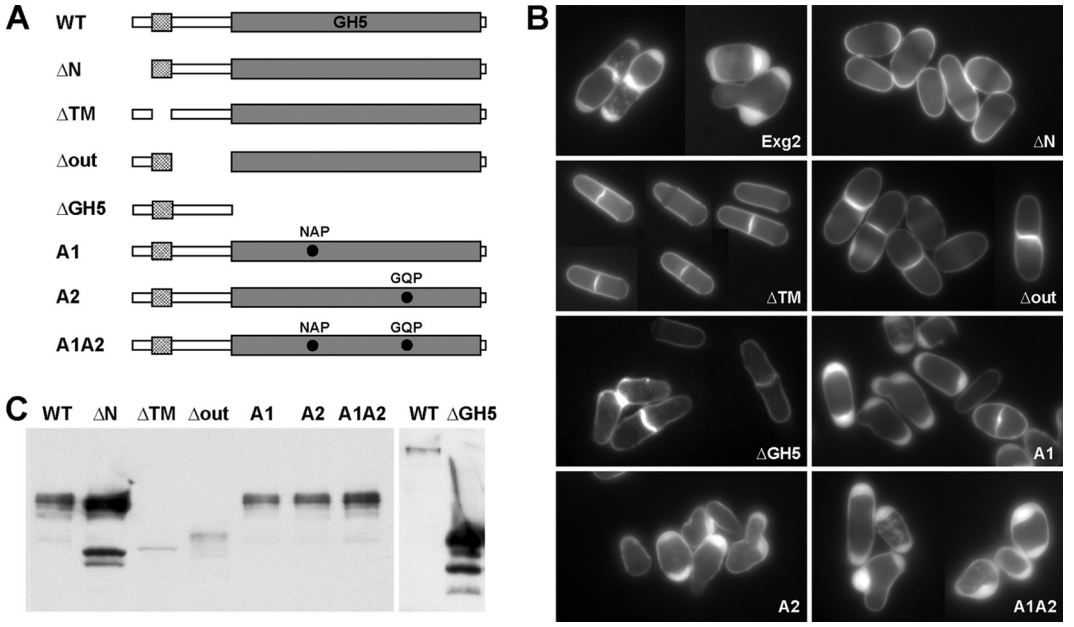

FIG. 7. Overexpression of mutant forms of *exg2*$^+$. (A) Schematic representation of Exg2 and different mutant versions generated. The gray box represents the GH5 domain, and the hatched box represents the putative transmembrane domain. The different constructs were cloned under the control of the *nmt1* promoter and contained the c-*myc* epitope at the C terminus. (B) Representative images of cells carrying Exg2 (pED172), Exg2-ΔN (pED178), Exg2-ΔTM (pED179), Exg2-Δout (pED177), Exg2-A1 (pED188), Exg2-A2 (pED193), Exg2-A1A2 (pED195), and Exg2-ΔGH5 (pED215) grown for 22 h in the absence of thiamine. Cells were stained with aniline blue. (C) Exg2 protein levels. Protein extracts from the same strains were prepared, separated by SDS-PAGE (12% for Exg2-ΔGH5 and 8% for the other constructs), transferred to nitrocellulose membranes, and probed with anti-myc antibodies.

Exg2-A2) were almost identical to those overexpressing the full-length protein (Fig. 7B). Thus, these results indicate that the N-terminal region and the GH5 domains are required to induce abnormal glucan synthesis but that the catalytic activity, if present, is not essential.

DISCUSSION

Cell wall growth and extension represent a delicate balance between the hydrolysis of the existing cell wall and the synthesis of new wall. A considerable body of evidence suggests that fungal β(1,3)-glucanases play key roles in morphogenetic processes during development and differentiation. Since β-glucans are major components of fungal and yeast cell walls, it seems likely that β-glucanases would play a crucial role in this process, partially hydrolyzing localized areas and enabling the insertion of new cell wall material, without disturbing the overall integrity of the cell (1). The complement of glucan-modifying enzymes present in *S. cerevisiae* is very complex, and a large number of proteins have been characterized, but much less is known about the role of hydrolases and glucan-remodeling enzymes during the life cycle of fission yeasts. Only a few glycoside hydrolases have been studied in fission yeast, such as the endo-α(1,3)-glucanases Agn1 and Agn2, the endo-β(1,3)-glucanases Eng1 and Eng2, and the glycanosyl-transferases from family GH72 (18, 19, 21, 22, 35–38). In this study, we have characterized the three genes belonging to family GH5 that are present in the *S. pombe* genome. They were identified by comparison of the *S. cerevisiae* Exg1 protein with the fission yeast genomic sequences and were named *exg1*$^+$ (SPBC1105.05), *exg2*$^+$ (SPAC12B10.11), and *exg3*$^+$ (SPBC2D10.05).

Family GH5 is a large and diverse group of hydrolases with different substrate specificities, such as exo-β(1,3)-glucanases, endo-β(1,4)-glucanases (cellulases), endo-β(1,6)-glucanases, endo-β(1,4)-xylanases, or β(1,3)-mannanases (Carbohydrate Active Enzymes database [http://www.cazy.org/]). In many cases, hydrolysis of glycoside bonds takes place via a general acid catalysis mechanism, which requires two acidic residues, one acting as an acid/base catalyst (proton donor) and the other acting as a nucleophile (17). There is a low degree of conservation of the primary sequence of these proteins, but all of them contain a conserved fold consisting of a $(\beta/\alpha)_8$ barrel. Despite the considerable sequence divergence, all of the proteins share the signature [LIV]-[LIVMFYWGA](2)-[DNEQG]-[LIVMGST]-{SENR}-N-E-[PV]-[RHDNSTLIVFY] as well as eight invariant residues that are involved in the catalysis and the recognition of the glycosyl group attacked during cleavage. Comparison of the three *S. pombe* proteins to GH5 proteins revealed that the signature and the eight invariant residues were conserved, suggesting that they are new members of this family.

The *S. pombe* proteins share sequence homology to *S. cerevisiae* Exg1 and *C. albicans* Xog1 (CaXog1), which are exo-β(1,3)-glucanases that also act on β(1,6) linkages (10, 43, 57). However, since it has been reported that *S. pombe* lacks any detectable exo-β(1,3)-glucanase activity (48), the biochemical activity and substrate specificity of the three *S. pombe* proteins were analyzed in strains overexpressing each of them. In con-

trast to ScExg1 and CaXog1, the *S. pombe* Exg1 and Exg3 were highly specific for β(1,6)-glucans (pustulan), being unable to degrade linear or branched β(1,3)-glucans. However, no enzymatic activity was detected for Exg2 in different assays or using different substrates. Therefore, it is possible that Exg2 may have diverged from other GH5 members, losing its catalytic activity during evolution. Alternatively, Exg2 might act on a substrate different from the substrates used in the assay, or it could catalyze a transglycosidase reaction that cannot be detected with the assay used. Indeed, transglycosidase activity has been reported for ScExg1 and CaXog1 (54, 55). Analysis of the reaction products released by Exg1 on β(1,6)-glucans by HPLC revealed that the enzyme had endolytic activity since the main products were gento-oligosaccharides (DP of 2 to 6). Therefore, the mode of action of *S. pombe* Exg1 is similar to that previously suggested for the BGN16.2 glucanase from the filamentous fungus *Trichoderma harzianum* (20), and it is different from that found in ScExg1 or CaXog1, which have nonspecific β(1,3)-glucanase, β(1,6)-glucanase, and β-glucosidase activities, with an exolytic mode of action (10, 43, 51). Multiple sequence alignment of yeast and fungal GH5 proteins indicated that the *S. pombe* proteins cluster in three different branches, but none of them associated with the branch that contains β(1,6)-glucanases such as *T. harzianum* BGN16.2 (see B9VQ16_TRIHA in Fig. S1 in the supplemental material).

Although no phenotypes have been detected for *exg1*Δ mutants, based on the fact that Exg1 was secreted and that it localized to the cell wall, it should be involved in the metabolism of cell wall β(1,6)-glucans *in vivo*. Similarly, the physiological role of ScExg1 has not been clearly established, but it has been proposed that *in vivo* it would be involved in the metabolism of β(1,6)-glucans since its deletion results in an increase in killer toxin sensitivity, while its overexpression produces resistance (24). Furthermore, overproduction or deletion of the gene leads to detectable *in vivo* alterations in the cell wall β(1,6)-glucan content, suggesting that its function in the cell wall could be related to the metabolism of β(1,6)-glucan. This protein localized to the septum region in a pattern that was different from that found for Eng1, the endo-β(1,3)-glucanase responsible for primary septum degradation (35). Interestingly, the β(1,6)-branched β(1,3)-glucan spans the whole thickness of the septum, with a tendency to become more concentrated in the primary septum, while β(1,6)-glucan is close to the cell membrane, labeling only the secondary septum (23). Thus, it is possible that Exg1 could play a minor role during cell separation, acting as an endo-β(1,6)-glucanase required for the hydrolysis the β(1,6)-glucans of the secondary septum.

Exg2 protein is different from the other *S. pombe* GH5 proteins in that it contains a putative transmembrane region before the catalytic domain. Sequence alignment indicates that Exg2 clusters in a branch containing a group of proteins of fungal origin (*Aspergillus fumigatus*, *Aspergillus nidulans*, *Aspergillus terreus*, *Aspergillus clavatus*, *Neurospora crassa*, or *Magnaporthe grisea*), all of which contain a putative transmembrane domain before the catalytic domain (see Fig. S1 in the supplemental material). However, the extent of the putative cytoplasmic region is longer in the fungal proteins than in *S. pombe* Exg2 although no function has been described for any of them. We have demonstrated that Exg2 is indeed an integral membrane protein that fractionates in the detergent-resistant membrane fraction, a biochemical test for lipid-raft association (56), and that its overexpression produces abnormal cell wall deposition. Interestingly, it has recently been reported that overexpression of a catalytically inactive form of Gas3 is toxic for *gas1*Δ mutants in *S. cerevisiae* (49), and it was proposed that hyperaccumulation of Gas3 might produce a physical disturbance of the cell wall structure. Thus, it is possible that the defect in *exg2*$^+$ overexpression in *S. pombe* might be due to a similar cause. Alternatively, it is possible that Exg2 might activate cell wall synthesis through an unknown mechanism. Unfortunately, attempts to identify proteins that might interact with the Exg2 cytoplasmic tail by two-hybrid screenings were negative (data not shown). Further analysis will be necessary to analyze this effect in more detail.

Finally, Exg3 is a β(1,6)-glucanase that localizes to the cytoplasm. The function of cytoplasmic glucanases is not currently known although they are present in different yeast organisms. Indeed, Exg3 clusters in a tree branch in which all the members lack a putative signal secretion sequence (see Fig. S1 in the supplemental material). Also, *S. pombe* contains cytoplasmic glucanases from other families, such as the endo-α(1,3)-glucanase Agn2 (family GH71) and the endo-β(1,3)-glucanase Eng2 (family GH81). It has been shown that these two proteins are required to hydrolyze the cell wall of asci, allowing the dehiscence of spores and their dispersal (19, 22). Thus, cytoplasmic glucanases might perform their function at specific moments of the life cycle.

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