Reduced expression of the O-mannosyltransferase 2 (AfPmt2) leads to deficient cell wall and abnormal polarity in Aspergillus fumigatus

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Received on October 15, 2009; revised on December 23, 2009; accepted on December 25, 2009

Protein O-mannosyltransferases (PMTs) initiate O-mannosylation of secretory proteins, which are of fundamental importance in eukaryotes. The human fungal pathogen Aspergillus fumigatus possesses three genes encoding for PMTs, namely, Afpmt1, Afpmt2 and Afpmt4. We have previously shown that lack of Afpmt1 leads to a temperature-sensitive phenotype featured with severe defects in hyphal growth, conidiation, cell wall integrity and morphology at elevated temperatures. In this study, a conditional mutant P2 was constructed by replacing the native promoter of the Afpmt2 with the Aspergillus nidulans alcA promoter. Reduced expression of the Afpmt2 gene led to a lagged germination, retarded hyphal growth, reduced conidiation and defect in cell wall integrity; however, no temperature-sensitive growth was observed. Further analysis revealed that reduced expression of the Afpmt2 caused a failure of the actin re-arrangement. Our results suggest that Afpmt2 gene was required for growth and played a role distinct from that of the Afpmt1 in A. fumigatus.

Keywords: Aspergillus fumigatus/cell wall integrity/O-mannosylation/O-mannosyltransferase/polarity

Introduction

The protein O-mannosylation is initiated by a family of protein O-mannosyltransferases (PMTs) that are evolutionarily conserved from yeast to humans (Strahl-Bolsinger et al. 1999; Willer et al. 2003). In fungi, proteins are O-mannosylated at serine or threonine residues during import into the endoplasmic reticulum. The O-glycans in yeast are short, typically containing one to two mannosyl residues. In mammalian cells, the inner O-linked mannosyl linkage is elongated with the first addition of N-acetylglucosamine and then various sugars (Endo 1999). Mammalian homologues of PMT proteins have been described (Jurado et al. 1999; Willer et al. 2002), and recently protein O-mannosyltransferase activity of human POMT proteins has been verified (Manya et al. 2004).

The genetics of fungal O-glycosylation has been studied in the yeasts Saccharomyces cerevisiae, Candida albicans, Schizosaccharomyces pombe and Cryptococcus neoformans (Strahl-Bolsinger et al. 1999; Ernst and Prill 2001; Willer et al. 2005; Olson et al. 2006). In S. cerevisiae, a total of seven PMT family members (ScPmt1–7p) are present (Gentzsch and Tanner 1996; Romero et al. 2003), which fall into three major groups of homology: (i) Pmt1/5/7, (ii) Pmt2/3/6 and (iii) Pmt4. Specific protein substrates that are O-glycosylated by ScPmt1p, ScPmt2p or ScPmt4p have been described (Strahl-Bolsinger et al. 1993; Gentzsch and Tanner 1997; Ecker et al. 2003). Protein O-mannosyltransferase activity has been demonstrated in vivo for ScPmt1–4p and ScPmt6p (Strahl-Bolsinger et al. 1999). Single pmt1 mutants fail to grow in anaerobic conditions on some media (Bourdineaud et al. 1998). The pmt1,2,3-triple mutants grow only in osmotically stabilized medium, whereas the pmt1,2,4- and pmt2,3,4–triple mutants are not viable in any conditions, indicating that PMT protein activity is essential in S. cerevisiae, although individual genes are dispensable (Gentzsch and Tanner 1996).

In S. pombe, only one member of each PMT subfamily is present, namely, oma1+, oma2+ and oma4+. They all act as protein O-mannosyltransferases in vivo. Deletion of oma2+, as well as simultaneous deletion of oma1+ and oma4+, is lethal. Characterization of the viable S. pombe oma1D and oma4D single mutants showed that a lack of O-mannosylation results in abnormal cell wall and septum formation, therefore severely affecting cell morphology and cell–cell separation (Willer et al. 2005). More recently, the Pmt4p in C. neoformans is shown to be essential for morphogenesis and virulence (Olson et al. 2006).

In the multicellular eukaryotes, human, mouse and Drosophila genes with significant homology to PMTs have been cloned (Martin-Blanco and Garcia-Bellido 1996; Jurado et al. 1999; Willer et al. 2002). In comparison with S. cerevisiae, the PMT family is less redundant in higher eukaryotes. In Drosophila, only two PMT family members are present (rotated abdomen and twisted) (Martin-Blanco and Garcia-Bellido 1996; Willer et al. 2002). The same is true for mouse and humans (POMT1 and POMT2) (Jurado et al. 1999; Willer et al. 2002). Mutations in human POMT1, a homologue of the yeast Pmt4, cause Walker–Warburg Syndrome (WWS), which is characterized by severe congenital muscular dystrophy, neurological defects and by structural abnormalities of the eye (Beltran-Valero de Bernabe et al. 2002). Targeted deletion of Pomp1 in mouse results in embryonic lethality due to the defects in the formation of Reichert’s membrane, the first
basement membrane to form in the embryo (Willer et al. 2004). Mutations of the Drosophila PMT homologues alter muscle structures and the alignment of adult cuticle (Martin-Blanco and Garcia-Bellido 1996; Ichimiya et al. 2004). Taken together, pmt mutants from different species revealed that protein O-mannosylation is of fundamental importance in uni- and multicellular eukaryotes.

Previously, we have identified three genes responsible for O-mannosylation in human opportunistic fungal pathogen Aspergillus fumigatus, namely, Afpmt1, Afpmt2 and Afpmt4 (Zhou et al. 2007). Lack of AfPmt1 results in temperature-sensitive phenotypes, including a defect in growth and cell wall integrity, thereby affecting cell morphology, conidia formation and germination. In a mouse model, the Afpmt1 was not required for the virulence of A. fumigatus under the experimental conditions. Here, we report functional analysis of the Afpmt2 in A. fumigatus. We demonstrate that AfPmt2p, an O-mannosyltransferase belonging to the PMT2 subfamily, plays a different role from the AfPmt1 and is essential for polarized growth in A. fumigatus.

Results

Construction of the conditional inactivation mutant

We first tried to construct a deletion mutant by replacing the Afpmt2 gene with a pyrG gene, but we failed to obtain such mutant. Therefore, a conditional inactivation mutant was constructed by replacing the native promoter of Afpmt2 gene with the P_{alcA}, a strictly regulated promoter that can be induced by ethanol, glycerol or threonine and repressed completely on Yeast Extract Peptone Dextrose (YEPD) medium (Waring et al. 1989; Romero et al. 2003). To this end, plasmid pALpmt2 that contains the pyr-4 gene as a fungal selectable marker and P_{alcA}, fused to a 3′ truncated version of the Afpmt2 gene, was employed in transformation of A. fumigatus CEA17 to generate a strain carrying the P_{alcA}-pmt2 fusion gene by homologous recombination. Seven transformants were obtained. Only one transformant, namely P2 mutant, was confirmed to be correct. Polymerase chain reaction (PCR) analysis revealed that a 1217-bp fragment of pyr-4 and a 2929-bp fragment of P_{alcA}-pmt2 could be amplified from the genomic DNA of strain P2, while no such fragment was amplified from the wild-type strain (Figure 1A). Southern blotting analysis of the PstI-digested genomic DNA of strain P2 confirmed correct integration. A 5270-bp fragment containing pyr-4 and P_{alcA} was detected, while no such fragment was found in the wild type (Figure 1B). These results demonstrated that the promoter of the Afpmt2 gene was replaced by the P_{alcA} in strain P2.

Strain P2 grew normally on solid minimal medium (MM) containing 0.1 M glycerol, 0.1 M ethanol or 0.1 M threonine (MMT). Although the growth of strain P2 was inhibited when grown on MM containing 1–3% glucose and significantly retarded on YEPD or complete medium (CM) (Figure 2), it appeared that glucose, YEPD or CM could not completely suppress the P_{alcA}. By measuring the transfer of [3H]mannose from Dol-P-[3H]mannose to the synthetic acceptor peptide Ac-YATAV-
NH₂, PMT activity for strain P2 was determined. As summarized in Table 1, PMT activity of strain P2 grown in MMT at 37°C for 36 h was 72% of that of the wild type, while its activity was inhibited by 80% or 81% when strain P2 was cultured in YEPD or CM. Previously, we showed that 40% of PMT activity was detected in the ΔAf pmt1 mutant grown in YG liquid medium (1% yeast extract and 2% glucose, pH 8.0) at 30°C for 24 h, suggesting this remaining activity is contributed by AfPmt2 and probably AfPmt4 as well (Zhou et al. 2007). In this study, we also measured PMT activity in the ΔAf pmt1 mutant grown in CM at 37°C for 36 h; as compared with the wild type, 29% of PMT activity was detected in the ΔAf pmt1. The difference in the remaining activity for the ΔAf pmt1 mutant between this study (29%) and a previous one (40%) is due to different culture conditions. Theoretically, under the culture condition used in this study, strain P2 should retain at least 71% of its PMT activity even if the Af pmt2 is completely inhibited in CM medium. However, only 19% of PMT activity was detected in strain P2 grown in CM. Although the mechanism is not clear, these observations indicate that suppression of the P alcA led to a remarkable reduction of PMT activity and inhibition of hyphal growth, suggesting that Af pmt2 gene is required for growth in A. fumigatus.

Growth phenotypes led by reduced expression of the Af pmt2

It has been shown that S. cerevisiae pmt1,4, pmt2,3 and pmt2,4 double mutants, as well as pmt1,2,3 triple and pmt1,3,4 triple mutants, are temperature sensitive; the pmt2,3 and pmt2,4 double mutants, as well as pmt1,2,3 triple mutants, are osmolabile (Gentzsch and Tanner 1996). Also, a temperature-sensitive growth phenotype has been reported in the A. fumigatus ΔAf pmt1 mutant (Zhou et al. 2007). When we tested the growth of strain P2 grown at 37°C or 50°C, strain P2 did not show any temperature sensitivity in growth rate on MMT medium (Figure 3A). However, a retarded growth was observed when strain P2 was grown on CM medium at 37°C, while only a slightly retarded growth of strain P2 was observed at 50°C as compared with the wild type (Figure 3B). These results demonstrated that, unlike the temperature-sensitive phenotype associated with the ΔAf pmt1 mutant, reduced

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**Table 1. In vitro O-mannosyltransferase activity of strain P2**

<table>
<thead>
<tr>
<th>Strain</th>
<th>MMT</th>
<th>YEPD</th>
<th>CM</th>
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<tbody>
<tr>
<td></td>
<td>Activity (cpm/mg/h)</td>
<td>Relative activity (%)</td>
<td>Activity (cpm/mg/h)</td>
</tr>
<tr>
<td>Wild type</td>
<td>12,170 ± 2437</td>
<td>100</td>
<td>40,961 ± 2625</td>
</tr>
<tr>
<td>Δpmt1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>P2</td>
<td>8803 ± 510</td>
<td>72</td>
<td>9005 ± 3073</td>
</tr>
</tbody>
</table>

The strains were cultured in liquid MMT, YEPD or CM at 37°C for 36 h. Membrane protein fraction (25 μg) from the wild type and P2 strain were incubated in the in vitro mannosyltransferase assay following the transfer of [1H]mannose from Dol-P-[1H]Man to the peptide Ac-YATAV-NH₂ (see Materials and methods). The assay was repeated at least twice. Average values of a typical experiment are shown.
expression of the Afpmt2 did not cause temperature-sensitive growth.

To evaluate the effect of down-regulation of the Afpmt2 on strain P2, we further investigated the phenotypes of strain P2 in CM medium. The growth of strain P2 was inhibited in CM (Figure 3C). Total RNAs were prepared from mycelia cultured in liquid CM at 37°C for 48 h, and transcription levels of the Afpmt2 in strain P2 and the wild type were examined by real-time quantitative PCR as described under Materials and methods.

Table 2. Quantitative real-time RT-PCR analysis of strain P2 under suppression condition

<table>
<thead>
<tr>
<th>Strain</th>
<th>ΔCt (pmt2-TBP)</th>
<th>Δ△Ct (ΔCt\text{sample} − ΔCt\text{wild})</th>
<th>2^{−Δ△Ct}</th>
</tr>
</thead>
<tbody>
<tr>
<td>YJ-407</td>
<td>1.2424</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>P2</td>
<td>−0.264</td>
<td>−1.5064</td>
<td>0.351988</td>
</tr>
</tbody>
</table>

Total RNAs were prepared from mycelia cultured in liquid CM at 37°C for 48 h, and transcription levels of the Afpmt2 in strain P2 and the wild type were examined by real-time quantitative PCR as described under Materials and methods.

that reduced expression of the Afpmt2 led to a defect in cell wall integrity.

Considering that glycoproteins are involved in the synthesis and organization of the fungal cell wall, we also analyzed the cell wall contents, including mannoproteins, glucans, and chitin. As compared with those of the wild type, the contents of mannoprotein and α-glucan in the mycelial cell wall of the mutant grown at 37°C were increased by 41% and 54%, respectively. When strain P2 was grown on CM at 50°C, the contents of mannoprotein and α-glucan in the mycelial cell wall were increased by 24% and 51%, respectively. While the contents of β-glucan and chitin in strain P2 were similar to those of the wild type at both temperatures. These results indicate that Afpmt2 is involved in synthesis of cell wall mannoprotein and α-glucan in A. fumigatus. Interestingly, as compared with the wild type, although the cell wall protein in strain P2 was increased by 41% at 37°C or 24% at 50°C, mannose residues released from these proteins were reduced by about 40% at either 37°C or 50°C; moreover, the amount of mannose in strain P2 at 37°C was similar to that at 50°C (Table 3), suggesting that mannosylation in strain P2 might not be temperature sensitive.

Morphogenesis of strain P2 under Afpmt2-suppression condition

Generally, filamentous fungus initiates its life cycle from conidial germination and terminates it with conidiation. When the conidia break dormancy, nuclear division is accompanied...
by a series of ordered morphological events including the switch from isotropic to polar growth, the emergence of the second germ tube from the conidia and septation. In *A. fumigatus*, it has been shown that the switch from isotropic to polar growth precedes the first mitosis during early stage of germination. The earliest emergence of second germ tube from the conidia occurs after the third mitotic division, and the first septation usually occurs in germ tube that has undergone four rounds of mitosis (Momany and Taylor 2000).

As shown in Figure 5, after 6–7 h of incubation at 37°C in CM medium, all wild-type conidiospores had undergone the switch from isotropic to polar growth. These germinated cells contained two or four nuclei. In contrast, 60% of the mutant conidia grew isotropically at 12 h, which is about 6 h later than the wild type; however, these isotropically growing cells had undergone several rounds of mitotic division, suggesting an uncoupling of the link between mitosis and morphogenesis.

After incubation at 37°C for 15 h, 76% of the mutant cells initiated their polarized growth, in which 66% of the mutant cells showed morphological abnormalities, including a high frequency of apical branching (39%), random budding (9%) and multinucleation (18%).

When strain P2 was cultured on CM at 50°C, a delayed polarity establishment and morphological abnormalities were also observed. In addition, most germ tubes formed ballooned hyphal tips, especially after 15 h of incubation (Figure 6).

Furthermore, we also investigated the germination of strain P2 in YEPD medium. In liquid YEPD medium, all wild-type conidiospores had undergone the morphogenetic switch after 8–9 h of incubation at 37°C, while conidiospores of strain P2 were clumped together after 9 h of incubation at 37°C; at this time, only 57% had undergone two rounds of mitotic division, and 8% formed the first germ tube. After incubation for 15 h, about 41% of conidia had four rounds of mitosis, and 546

**Fig. 5.** Delayed polarity establishment and uncoupling of nuclear division from the morphogenetic switch. Freshly harvested conidia (10^7) were poured into a Petri dish containing glass coverslips and incubated in 10 mL complete liquid medium at 37°C for the time indicated in each experiment. The coverslips with adherent germinated conidial cells were removed, fixed and stained with Calcofluor white and DAPI, as described in Materials and methods. Typical photographs are shown. Bar, 10 μm. c, conidium; s, septum; g, germ tube.
69% germinated the first germ tube (Figure 7). When the conidiospores of strain P2 were transferred into MMT after 9 h of incubation in CM at 37°C, further incubation in MMT at 37°C for 4 h enabled an occurrence of the first germ tube in 57% of the mutant spores, and the second germ tube and the first septation was found in 18% and 29% of the mutant spores, respectively. These results suggest that expression of the \( \text{AfPmt2} \) induced by MMT can restore the phenotype led by the reduced expression of the \( \text{AfPmt2} \).

Taken together, our results show that \( \text{AfPmt2} \) is required for the polarized growth of \( \text{A. fumigatus} \). Reduced expression of the \( \text{AfPmt2} \) led to an uncoupling of the link between mitosis and polarity establishment, as well as abnormalities of polarized growth and septation.

Fig. 6. Delayed polarity establishment and abnormally ballooned regions of strain P2 at 50°C. Freshly harvested conidia \( (10^7) \) were poured into a Petri dish containing glass coverslips and incubated in 10 mL complete liquid medium at 50°C for the time indicated in each experiment. The coverslips with adherent germinated conidial cells were removed, fixed and stained with Calcofluor white and DAPI, as described in Materials and methods. Typical photographs are shown. Bar, 10 µm. g, germ tube; t, hyphal tip; s, septum.

Fig. 7. Conidial germination of strain P2 in YEPD at 37°C. Freshly harvested conidia \( (10^6) \) were poured into a Petri dish containing glass coverslips and incubated in 10 mL complete liquid medium at 37°C for the time indicated in each experiment. The coverslips with adherent germinated conidial cells were removed, fixed and stained with Calcofluor white and DAPI, as described in Materials and methods. Typical photographs are shown. Bar, 10 µm.
When strains were grown on solid CM at 37°C for 36 h or 50°C for 48 h, the hyphae differentiated into conidiophore vesicles and phialides. In comparison with the wild type, conidiospores of strain P2 had a normal morphology at 37°C or 50°C; however, the number of conidiospores produced by strain P2 was reduced by 75% and 60% at 37°C and 50°C, respectively (Table 4). These results suggested that reduced expression of the Afpm2 led to a reduced conidiation.

Table 4. Number of conidia formed by strain P2 at various temperatures

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Number of conidia (10⁷)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>YJ-407</td>
</tr>
<tr>
<td>37°C</td>
<td>57.5 ± 4.8</td>
</tr>
<tr>
<td>50°C</td>
<td>7.8 ± 1.4</td>
</tr>
</tbody>
</table>

Conidia (10⁵) were spread on CM plates and incubated at 37°C or 50°C for 36 h. The conidia were then harvested and counted by microscopy. A triplet of each strain was counted. The same experiment was repeated twice. The values shown are mean ± SD.

When strains were grown on solid CM at 37°C for 36 h or 50°C for 48 h, the hyphae differentiated into conidiophore vesicles and phialides. In comparison with the wild type, conidiospores of strain P2 had a normal morphology at 37°C or 50°C; however, the number of conidiospores produced by strain P2 was reduced by 75% and 60% at 37°C and 50°C, respectively (Table 4). These results suggested that reduced expression of the Afpm2 led to a reduced conidiation.

**Actin cytoskeleton in strain P2 under Afpm2-suppression condition**

Since actin cytoskeleton is required for cell polarization and polarized hyphal growth in filamentous fungi (Heath et al. 2000), we monitored the actin cytoskeleton of strain P2 by staining the actin with anti-actin antibody. When the wild type was cultured on CM at 37°C (Figure 8A and B) or 50°C (Figure 8E and F), the actin patches accumulated in the expanding tip of germ tube in a polarized manner. However, the actin patches were distributed randomly in strain P2 (Figure 8C, D, G and H). These results strongly suggest that abnormalities of polarity associated with the reduced expression of the Afpm2 is due to failure of the actin re-arrangement.

**Discussion**

*A. fumigatus* is one of the most ubiquitous of airborne saprophytic fungi, which has been shown to be an opportunistic pathogen causing pneumonia and other fatal invasive infec-

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**Fig. 8.** The actin cytoskeleton phenotype resulting from the repression of the Afpm2. A 10-mL volume of complete liquid medium was inoculated with 10⁷ freshly harvested conidia, poured into a Petri dish containing glass coverslips and incubated at 37°C (A – D) or 50°C (E – H) for 7 – 24 h. At the specified times, coverslips with adhering germinated conidial cells were removed and fixed as described under Materials and methods. The coverslips were incubated with mouse anti-actin antibody, stained with Alexa Fluor® 488 goat anti-mouse IgG antibody (Invitrogen) and examined under a Leica TCS SP2 microscope. A, B, E and F, wild type; C, D, G and H, strain P2. t, hyphal tip.
Reduced expression of the O-mannosyltransferase 2 (AfPmt2)

The yeast cell remodels its rigid structure to accommodate cell expansion during vegetative proliferation, mating-pheromone-induced morphogenesis and nutrient-driven filamentation through cell wall integrity (CWI) signaling pathway. The CWI signaling pathway comprises a family of cell surface sensors coupled to a small G-protein called Rho1p, which activates the CWI MAPK cascade via protein kinase C (Pkc1p) and allows the specific activation of genes involved in cell wall remodeling. In addition, activated Rho1p also activates organization of the actin cytoskeleton and secretory vesicle targeting to the growth site (Levin 2005). Also, a family of cell surface sensors has been implicated in detecting and transmitting cell wall status to Rho1p (Levin 2005). These include Wsc1 (Hcs77/Slg1) (Gray et al. 1997; Verna et al. 1997; Jacoby et al. 1998), Wsc2 and Wsc3 (Verna et al. 1997; Ketela et al. 1999; Rajavel et al. 1999). It has also been reported that extensive O-glycosylation of Wsc2 and Wsc3 (Verna et al. 1997; Ketela et al. 1999), suggesting an important role of the CWI signaling pathway to up-regulate the synthesis of mannoprotein and α-glucan and thus regulate polarized growth in A. fumigatus.

Materials and methods

Strains and growth conditions

A. fumigatus strain YJ-407 (CGMCC0386) was maintained on potato glucose (2%) agar slants (Zhou et al. 2007). A. fumigatus strain CEA17 (Weidner et al. 1998) was propagated at 37°C on YGA (0.5% yeast extract, 2% glucose, 1.5% Bacto-agar) with addition of 5 mM uridine and uracil (d’Enfert 1996). To induce the A. nidulans promoter, A. fumigatus was grown on MM (Armitt et al. 1976) with 0.1 M glycerol, 0.1 M threonine or 0.1 M ethanol as carbon sources. YEPD (2% (w/v) yeast extract, 2% (w/v) glucose and 0.1% (w/v) peptone) medium and CM (Cove 1966) were utilized to repress the A. nidulans promoter completely and partially, respectively.

Strains were grown in liquid CM at 37°C, with shaking at 250 rpm. At the specified culture time point, mycelia were harvested, washed with distilled water, frozen in liquid N2 and then ground using a mortar and pestle. The powder was stored at −70°C for DNA, RNA and protein extraction.

Conidia were prepared by growing A. fumigatus strains on solid CM with uridine and uracil for 48 h at 37°C. The spores were collected, washed twice with 0.1% Tween 20 in physiological saline and resuspended in 0.1% Tween 20 in saline, and the concentration of spores was confirmed by hemocytometer counting and viable counting. Vectors and plasmids were propagated in Escherichia coli DH5α (Bethesda Research Laboratories, Bethesda, MD 20877).

Isolation of the AfPmt2 gene from A. fumigatus

Based on genomic sequence of the AfPmt2 gene (http://www.tigr.org/db/af1/af1.html), the forward primer P1 (5′-ATGGTCCATCAACTACCAGTG-3′) and the reverse primer P2 (5′-TCAATTCGCAATCCGCAACCG-3′) were designed for cloning the AfPmt2 cDNA by PCR. The PCR products were subcloned into pGEM-T easy Vector (Promega, Madison, WI 53711), named T-pmt2, and sequenced, and the position of the intron was determined by comparing the cDNA with the genomic sequence.

ditions in immuno-compromised population, particularly among patients undergoing cytotoxic chemotherapy or bone marrow transplantation (Latgé 1999). There has been a dramatic increase in severe and usually fatal invasive aspergillosis caused by the yeast A. fumigatus (Zimeii and Soubani 2007). Therefore, the investigation of virulence factors and potential chemo-therapeutical targets of A. fumigatus are of clinical interests.

The initial O-mannosylation catalyzed by PMTs is commonly found in Aspergillus, as it is in yeasts (Goto 2007). However, in comparison to yeasts, very little is known about the synthesis of O-glycans in Aspergillus. Recently, it has been revealed that Aspergillus nidulans possesses PmtA, PmtBp and PmtCp that belong to the PMT1, PMT2 and PMT4 subfamilies, respectively. Each of the A. nidulans PMTs appears to function independently, in which the pmtA is required for the formation of a normal cell wall (Oka et al. 2004; Oka et al. 2005). Similarly, A. fumigatus also possesses AfPmt1, AfPmt2 and AfPmt4 (Zhou et al. 2007). We previously showed that deletion of the AfPmt1 in A. fumigatus leads to temperature-sensitive phenotypes (Zhou et al. 2007), which is different from the one in S. cerevisiae (Gentzsch and Tanner 1996) and suggests a distinct function from the yeast Pmt1.

To further understand the function of the A. fumigatus PMTs, we analyzed the function of the AfPmt2 in this study. Although we failed to obtain any deletion mutant, we could not conclude that AfPmt2 gene was essential for viability since we were not able to verify this possibility due to promoter leakage. By constructing a conditional mutant, we showed that reduced expression of the AfPmt2 led to a retarded growth, cell wall defect, abnormal polarity and reduced conidiation; however, unlike our previous finding with the AfPmt1, no temperature-sensitive growth was found. Our observations suggest that AfPmt2 is required for cell wall synthesis and morphogenesis in A. fumigatus, and its function is distinct from that of the AfPmt1.

Reduced expression of the O-mannosyltransferase 2 (AfPmt2) cell wall stress sensor molecule and its O-mannosylation remain to be investigated, it is reasonable to postulate that reduced expression of the AfPmt2 leads to an activation of the CWI signaling pathway to up-regulate the synthesis of mannoprotein and α-glucan and thus regulate polarized growth in A. fumigatus.

In summary, for the first time, the physiological function of the Pmt2 in A. fumigatus was investigated in this study. Our results demonstrated that AfPmt2 was required for growth in A. fumigatus. Reduced expression of the AfPmt2 led to a retarded growth, defect in cell wall integrity, reduced conidia formation, and abnormal polarity; however, it appeared that AfPmt2 activity did not affect heat response in A. fumigatus. These results suggest that the function of the AfPmt2 is distinct from that of the AfPmt1. Obviously, it will be helpful for understanding the functional mechanism of the human WWS since the mutations in POMT1, a homologue of the AfPmt4, are featured with a failure in polarized growth, such as neuronal-migration defects (Beltran-Valero de Bernabe et al. 2002).
Construction of the conditional inactivation mutant

Plasmid pAL3 was employed to construct the A. nidulans alcA promoter (P_{alcA}) and the Neurospora crassa pyr-4 gene as a fungal selectable marker (Waring et al. 1989) was employed to construct a suitable vector allowing the replacement of the native promoter of the AlcA gene from pAL3 was used as a probe. Labeling was confirmed (PCR and Southern blot analysis).

For PCR analysis, two pairs of primers (P1 and P2, P3 and P2) were employed with the following cycling conditions: 94°C for 30 s, 53°C for 30 s and 72°C for 3 min. Primers P5 (5′-AACGCAATACACACAGCCGAAC-3′) and P6 (5′-CTGCCAGCGTCCCGG-3′) were used to amplify the pyr-4 gene with the conditions: 94°C for 30 s, 56°C for 30 s and 72°C for 1 min. For Southern blotting, genomic DNA was digested with PstI, separated by electrophoresis and transferred to a nylon membrane (Roche Applied Science, Indianapolis, IN). The 1.2-kb HindIII internal fragment of the N. crassa pyr-4 gene from pAL3 was used as a probe. Labeling and visualization were performed using the DIG DNA labeling and detection kit (Roche Applied Science, Indianapolis, IN), according to the manufacturer’s instructions.

Assay of protein O-mannosyltransferase activity

The membranes of A. fumigatus were prepared from 36-h-old mycelial cultures grown on a shaker (200 rpm) at 37°C in different medium. The fungal material was filtered, washed with water extensively and homogenized in liquid nitrogen. The homogenate was resuspended in 10 mL of 50 mM Tris–HCl (pH 7.4) containing 15 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride and 9 mM mercaptoethanol. Cell debris was removed by centrifugation at 12,000 × g, 4°C for 10 min. The membranes in the supernatant were collected by centrifugation at 70,000 × g for 1 h and solubilized in 50 mM Tris–HCl (pH 7.4) containing 5 mM MgCl₂, 6 mM β-mercaptoethanol, 1.2% Chaps and 20% glycerol.

The in vitro peptide assay for protein O-mannosyltransferase activity was performed as described by Weston et al. (1993) with several modifications. The assay was optimized using 0.02 μCi of Dol-P-[³H]mannose (40 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO) and acceptor peptide Ac-YATAV-NH₂ (final concentration of 3.5 mM). The assay was performed in 1.5 mL Eppendorf tubes. Dol-P-[³H]mannose (0.02 μCi) together with 2 μg 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphocholine, 5 μl of 20% Chaps and 10 μl of 0.2 M HEPES (pH 7.5) were added to each tube and sonicated in a water bath for 30 min. The assay was started by the addition of peptide and 25–50 μg of membrane proteins in a total volume of 50 μl and incubated at 30°C for 30 min. The reaction was stopped by the addition of 1 mL of chloroform/methanol (3:2) followed by 200 μl of water. The amount of [³H]mannosylated peptide was measured by a liquid scintillation counter. For each sample, the assay was repeated at least twice.

Phenotypic analyses of the mutant

Spores (1×10⁵) were inoculated into 100 mL liquid CM medium. After incubation at 37°C with shaking (300 rpm), three aliquots of 1 mL culture liquid were taken for each strain at intervals, dried and weighed. The mean weight was used to plot the growth kinetics. The experiment was repeated three times.

To test the sensitivities of the mutant to antifungal reagents, conidiospores were spotted on CM plates in the presence of 20 μg Calcofluor white mL⁻¹, 20 μg Congo red mL⁻¹ or 50 μg SDS mL⁻¹. After incubation at 37°C for 24 h, the plates were taken out and photographed.

For examination of conidial germination, 10 mL of complete liquid medium was inoculated with 10⁷ freshly harvested conidia, poured into a Petri dish containing a glass coverslip and incubated at 37°C or 50°C. At the specified times, the coverslips with adhering germinated conidial cells were removed and fixed in fixative solution (4% formaldehyde, 50 mM phosphate buffer, pH 7.0, and 0.2% Triton X-100) for 30 min. Coverslips were then washed with phosphate-buffered saline (PBS), incubated for 15 min with 1 mg 4′,6-diamidino-2-phenylindole (DAPI) mL⁻¹ (Sigma, Perth, Australia) washed with PBS, then incubated for 5 min with a 10 mg mL⁻¹ solution of fluorescent brighter 28 (Sigma, Perth, Australia) and washed again, and germinated conidial cells were photographed using a microscope.

Quantitative real-time reverse transcription-PCR analysis

Total RNAs from the spores cultured at specific time were extracted with 1 mL of Trizol reagent (Invitrogen, Carlsbad, CA). The cdNA synthesis was performed with 5 μg RNA using SuperScript-First-Strand Synthesis System (Promega, Madison, WI). Primers P7 (5′-GAGCAGCATGTGGAGCTT-3′) and P8 (5′-GAGACTGTCCTGCAGTCC-3′) were used to amplify an 80-bp fragment of the Afpmt2, and primers TBP-5′ (5′-CCACCTGAAACATGGT-3′) and TBP-3′ (5′-TACTTCGATTTCCGCAGTT-3′) were used for an 80-bp of fragment TBP. To exclude contamination of cdNA preparations with genomic DNA, primers were designed to amplify regions containing one intron in the gene (Bustin 2000; Bustin 2002). The PCR reaction components were 5 μL cdNA, 12.5 μL SYBR GreenER qPCR SuperMix (Invitrogen, Carlsbad, CA) and 0.6 μM of each pair of primers. Thermal cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 60 s. Samples isolated from different strains and different times were tested in triplicate.

Chemical analysis of the cell wall

Conidia were inoculated into 100 mL complete liquid medium at a concentration of 10⁶ conidia mL⁻¹ and incubated at 37°C with shaking (200 rpm) for 24 h. The mycelium was harvested, washed with deionized water and frozen at −80°C. The cell wall components were isolated and assayed as previously described (Fang et al. 2009). Three independent samples were used as for cell wall analysis, and the experiment was repeated twice.
Immunofluorescence microscopy
A 10-mL volume of liquid CM was inoculated with $10^7$ freshly harvested conidia, poured into a Petri dish containing glass coverslips and incubated at $37^\circ$C. At the specified times, coverslips with adhering germinated conidial cells were removed and fixed. Briefly, coverslips were transferred to 3.7% formaldehyde in PBS and incubated for 30 min at room temperature, then washed twice. For cell wall digestion, coverslips were overlaid for 40 min at room temperature with a solution of 10 mg Novozym 234 per milliliter PBS containing 2% bovine serum albumin (BSA) (Esnault et al. 1999). After three washes, coverslips were immersed in absolute ethanol at $-20^\circ$C for 10 min. After being washed several times in PBS, the coverslips were incubated for 1 h at room temperature with mouse anti-actin C4 monoclonal (MP Biomedicals, Solon, OH) at a 1:400 dilution in PBS–BSA containing 0.5% Nonidet P-40. After four times of 10-min washes with PBS, coverslips were then stained for 1 h in the dark with Alexa Fluor® 488 goat anti-mouse IgG antibody (Invitrogen) at a 1:400 dilution in PBS–BSA. Finally, the coverslips were washed for 10 min with PBS, mounted on glass slides in 25% glycerol and examined under a Leica TCS SP2 microscope.

Funding
This project was supported by the State “863” High-tech Project (2007AA02Z164) and the National Natural Science Foundation of China (30770485 and 30621005) to C. Jin.

Acknowledgements
Genomic sequence data for A. fumigatus were obtained from the Institute for Genomic Research website at http://www.tigr.org/tdb/e2k1/afu1/.

Abbreviations
BSA, bovine serum albumin; CM, complete medium; CWI cell wall integrity; MM, minimal medium; MMT, MM medium containing 0.1 M glycerol, 0.1 M ethanol or 0.1 M threonine; PBS, phosphate-buffered saline; PMTs, protein O-mannosyltransferases; TBP, TATA Box-binding Protein; WWS, Walker–Warburg Syndrome; YEPD, Yeast Extract Peptone Dextrose; YG, Yeast extract Glucose medium; YGA, Yeast extract Glucose Agar medium.

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