Characterization of an MPLP6, a gene coding for a yeast phase specific, antigenic mannoprotein in Penicillium marneffei

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A gene encoding an antigenic mannoprotein of Penicillium marneffei, MPLP6, was isolated by an antibody screening approach and characterized. The polypeptide chain containing deduced 220 amino acids has a predicted molecular mass of 24 kDa. It has high similarity to Mp1p, the first mannoprotein antigen isolated from P. marneffei. The polypeptide sequence presents the property of cell wall mannoproteins by containing a putative N-terminal signal peptide and potential O-linked glycosylation sites. However, absence of a GPI-anchored signal sequence suggested that this protein is secreted. The MPLP6 transcript was present specifically in the pathogenic yeast form. The transcript was completely absent in the mold phase and conidia. The fusion protein produced in E. coli was Western immunoblotted with P. marneffei-infected human sera and 95% of the patients’ sera were positive in the assay. None of the sera obtained from patients with aspergillosis, tuberculosis, histoplasmosis or cryptococcosis tested positive. These results suggest that Mplp6 can be used as a marker in a serodiagnostic assay.

Keywords Penicillium marneffei, MPLP6, mannoprotein, antigen

Introduction

Penicillium marneffei is the only dimorphic species of the genus Penicillium. The growth of P. marneffei can be divided into two phases, i.e., its natural growth phase (saprobic mold at 25°C) and infective stage (a parasitic yeast at 37°C). Dimorphism is an adaptive mechanism whereby the fungus is a saprobe in nature but is able to adjust to the 37°C host body temperature. During in vitro growth, phase transitions are triggered when the incubation temperature is shifted between 25°C and 37°C [1–4]. At 25–30°C, P marneffei grows as a mold and produces a soluble red pigment that diffuses throughout the medium. At 37°C, the fungus develops as yeast-like cells which divide by fission and appear as cells with centrally located transverse septa, a feature that allows differentiation of P. marneffei from other dimorphic fungi, especially Histoplasma capsulatum. Endemic areas of this fungus are in Southeast Asian countries including southern China and the Manipur state of India [5–7]. Moreover, the incidence of infection in AIDS patients in Africa who have never traveled to the endemic areas underscores the potential for this fungus to continue to emerge in unexpected geographic locations [8].

The incidence of infection caused by P. marneffei has greatly increased in immunocompromised individuals, particularly those with AIDS. In Thailand, penicilliosis marneffei has emerged as the third most common cause of opportunistic infections after tuberculosis and cryptococcosis [9]. In the absence of immune reconstitution with antiretroviral therapy, relapse rates are very high and patients must take prophylactic antifungals indefinitely [10]. The itraconazole prophylaxis can be discontinued when CD4+ T-cell counts of patients are consistently greater than 100 cells/μl [11]. However, infection due to P. marneffei is still emerging as a health problem among AIDS patients in Southeast Asia and visitors as long as the pandemic of AIDS exists.

The standard diagnostic method, cultivation in vitro, to detect P. marneffei infection has an inherent disadvantage in that it has a low sensitivity and is time-consuming. Therefore, alternative immunodiagnostics methods are useful since immune response to microbes occurs early during infection. Development of an accurate serodiagnostic test requires identification of specific antigens.
In our previous study, a cDNA library from the pathogenic yeast form of *P. marneffei* was constructed using a lambda ZipLox expression system (Invitrogen) [12]. The cDNA library was induced for expression. The expressed proteins were probed with *P. marneffei*-infected sera to isolate clones encoded for protein antigens. Several antigen encoding genes were isolated, partially sequenced, and analyzed. One of them encoded for a protein that had high similarity to a previously described Mp1 mannoprotein antigen of *P. marneffei* [13]. This gene of interest was named MPLP6 in the genome sequencing project (unpublished data). In this study, we report on the characterization of the MPLP6 and the potential usage of its encoded protein in the serodiagnosis of penicilliosis marneffei.

**Materials and methods**

**Fungal strain and culture conditions**

*P. marneffei* CBS119456 strain was obtained from a hemoculture of an AIDS patient in 1999. The fungus was grown on malt extract agar for 7 days at 25–28°C. A conidial suspension was prepared by scraping the surface of a mold colony in a sterile normal saline solution and the resulting suspension was then filtered through sterile glass wool to collect the conidia. Approximately 10⁷ conidia per ml were inoculated into 50-ml Sabouraud’s dextrose broth and incubated at 37 °C. The cultures were collected at time points of 6, 12, 24, 48, and 72 h after the change in the incubation temperature.

In a study of MPLP6 gene expression during phase transition, the conidia were cultured in Sabouraud’s dextrose broth for 3 days at 25°C with shaking at 150 rpm to produce the mycelia or yeast cells, respectively.

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To study the effect of heat shock stress on different cell types of *P. marneffei*, 3 day-old mold, yeast cells, and conidia were heated to 39°C for 30 min. In another stress-induced condition involving oxidative stress, all cell types were treated with H₂O₂ at concentrations of 1 mM and 2 mM for 30 min.

**Isolation and characterization of MPLP6**

An MPLP6 gene was isolated by antibody screening of an expression of the cDNA library [12]. The NCBI BLAST programs (http://www.ncbi.nlm.nih.gov) were used to search for nucleotide and protein sequence similarities. The programs of ‘proteomics and sequence analysis tools’ (http://www.expasy.org/) were used to predict the potential glycosylation sites and to deduce amino acid sequences from nucleotide sequences. Alignment of the deduced amino acid sequence of *P. marneffei* was performed via the ClustalW program (http://www.ebi.ac.uk/clustalw).

**Accession number**

The sequence of the MPLP6 gene was submitted to the GenBank database under the accession number EF637041.

**Total RNA isolation**

Total RNA was isolated from *P. marneffei* cells with an RNasy Mini Kit (Qiagen GmbH, Germany). Briefly, the fungal cells were harvested by centrifugation, approximately 0.2–1 g wet weight of each sample was resuspended with 600 µl of RLT buffer (Qiagen) and 0.6 g acid-washed glass beads were added. The fungal cells were disrupted in a Bead beater (Biospec, Bartlesville, OK, USA) at 5000 rpm for 30 sec each, 5–6 times, with interval cooling in an ice bath. The contaminated DNA was eliminated by on-column DNase digestion. The quality of RNA was visualized and assessed by electrophoresis on a denaturing 1% agarose gel (NorthernMax-Gly system, Ambion, Austin, USA).

**Reverse transcription-polymerase chain reaction (RT-PCR)**

The RNA samples from *P. marneffei* were subjected to RT-PCR assay using the One-Step RT-PCR kit (Qiagen). The reaction mixture consisted of 50 ng of the DNase-treated total RNA, 0.4 µM of each primer (forward primer sequence 5’-AGCTCTACGAGCAACTATAAGCAG-3’ and reverse primer sequence 5’-CCTAGTTAGCAGACTAGCAGCATCAG-3’), 3 mM MgCl₂, 200 µM dNTP mix, and 1 U of *Taq* DNA polymerase in a total volume of 25 µl. The expected product size was 722 bp. The internal control was a 630-bp PCR product of 18S rRNA gene (primer RRF1 [5’-ATCTAAATCCCTTAAAGGAACAA-3’] and RRH1 [5’-GGGCTGTACCCCTATGAGTCG-3’]) [14]. The initial reverse transcription step was performed at 50°C for 30 min. Subsequently, 25 cycles of PCR amplification were performed at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. The normal PCR without reverse transcriptase enzyme was performed to examine the presence of DNA contamination in RNA samples. The RT-PCR products were electrophoresed and band intensity was analyzed using a GelDoc1000 (Bio-RAD, Hercules, CA, USA). Relative expression level was calculated from the ratio of band intensities between the MPLP6 and 18S rRNA control.

**Production of the N-terminal histidine-tagged Mplp6 recombinant protein**

The N-terminal histidine-tagged Mplp6 recombinant protein was produced in *E. coli*. An expression plasmid was prepared using Gateway technology (Invitrogen, Inc.).
To generate an entry clone, first the MPLP6 sequence was amplified from the cDNA clone. The sequence of forward primer was 5’-GGGACGAATTTGATACAAAAAAGCGGTTATGAAGTTCTACCTC-3’ and the sequence of reverse primer was 5’-GGGACCAGCTTTGTACAAAGAAAGCCTGGTACAGCTAGCAGATC-3’. Fifty nanograms of the template DNA were amplified in the reaction containing 0.4 μM of each primer, 200 μM of each dNTP, 3 mM MgCl₂, and 1 U pfu polymerase. The PCR condition was 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and final extension at 72°C for 10 min. The expected product of 725 bp was then purified and ligated to a pDONR221 vector in a reaction containing a BP clonase II enzyme to yield an entry vector, pENTR221-MPLP6. Aliquots of 2 μl from the BP recombination reaction were transformed into an OmniMax E. coli 2T-1 competent cell and plated on a kanamycin (50 μg/ml)-LB plate. The entry vector was then prepared from a transformant using a DNeasy Mini Kit (Qiagen) and used in subsequent reaction to generate an expression vector.

To generate an expression vector, a LR reaction was performed. The pENTR221-MPLP6 entry vector was used to transfer the MPLP6 gene into a pDestTM17 destination vector which yielded a pEXP17-MPLP6 expression vector. An equal amount of 150 ng of pENTR221-MPLP6 entry vector and pDestTM17 vector was incubated in the presence of LR clonase II enzyme mix overnight at room temperature. Proteinase K solution (2 μg in 1 μl) was added and the mixture was incubated at 37°C for 15 min to inactivate the clonase enzyme. The expression vector was transformed into an OmniMax E. coli 2T-1 competent cell, and the transformants were selected on LB plate containing 100 μg/ml of ampicillin. Then, the pEXP17-MPLP6 vector was isolated from the transformant. In order to verify fidelity and orientation of the cloned insert, the gene sequence was confirmed by automated DNA sequence analysis (ABI PRISM dye terminator cycle sequencing ready reaction kit, Perkin-Elmer).

The pEXP17-MPLP6 expression vector was used to transform E. coli BL21 strain for protein expression. Expression of the recombinant protein in the selected transformant was induced by adding IPTG to a final concentration of 0.2 μM at a cell optical density (at 600 nm) of 0.5. After growth for an additional 2 h, bacteria were collected, suspended in 10 ml of lysis buffer (50 mM sodium phosphate, 1 mM phenylmethylsulfonyl fluoride, pH 7.0), and sonicated for 10 min (1-min sonications with a 1-min pause) in an ice bath using a Branson Sonicator (model S250) at a power of 80 W. The lysate was centrifuged, and the pellet was resuspended in 10 ml binding buffer (6 M urea, 20 mM imidazole, 0.5 M NaCl, 0.1 M sodium phosphate, pH 7.4) and centrifuged (12,000 g at 4°C for 15 min). The supernatant was collected, filtered through a 0.2 μm membrane, and used to load the affinity column (HisTrap® HP kit, Amersham Pharmacia Biotech). The fusion protein was eluted with an elution buffer (8 M urea, 300 mM imidazole, 0.1 M sodium phosphate, 0.5 M NaCl) and collected as 1-ml fractions. Aliquots were used for SDS-PAGE and Western blot assay. Refolding of the recombinant proteins was performed by elimination of urea. For this purpose, pooled fractions were dialyzed for 12 h at 4°C against decreasing concentrations of urea (4 M, 2 M, or no urea) and 0.1 M sodium phosphate, pH 7.4.

**Western blot analysis to detect anti-Mlp6 antibodies in patients’ sera**

In the Western blot assay, 20 serum samples from patients with culture-confirmed *P. marneffei* infections were obtained from Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand [15]. They were tested to contain immunoreactivity to crude protein antigens of *P. marneffei*. Serum samples used to assess the specificity of Mlp6 were obtained from patients with other opportunistic infections, including aspergillosis (*n* = 13), cryptococcosis (*n* = 8), histoplasmosis (*n* = 7) and tuberculosis (*n* = 2). Aspergillosis cases were identified by either galactomannan test or cultivation methods. Eight aspergillosis sera were obtained from patients in Thailand, only one sample was culture-confirmed positive for *A. fumigatus* infection. Five aspergillosis sera were obtained from the USA and they all tested positive to the galactomannan antigen (MiraVista diagnostics, Indianapolis, USA). Among histoplasmosis sera, two of them were obtained from Thailand (endemic region for *P. marneffei*) and five from the USA. Normal healthy sera (*n* = 5) were used as a negative control. All sera were diluted to dilution 1:10 in a blocking solution (5% skim milk in PBS) and incubated with a membrane containing *E. coli* BL21 lysate at 4°C for overnight to remove anti-*E. coli* antibodies.

Ten micrograms of the recombinant protein were electrophoresed on SDS-PAGE and subsequently electroblotted onto a nitrocellulose membrane. The membrane was cut into 4-mm strips and each strip was incubated with a 1:100 dilution of the individual serum at room temperature for 1 h. After washing with PBS-0.05% Tween20, the blot was incubated with 1:2,000 dilution of horseradish peroxidase-conjugated goat anti-human IgG (Pierce Biotechnology, Belgium). The antigen-antibody complex was color-developed in 4-chloro-1-naphthol substrate solution (0.3 mg/ml 4-chloro-1-naphthol and 0.03% *H₂O₂* in 50 mM Tris, pH 7.6) for 10–15 min at room temperature.

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Results

Characterization of the MPLP6 gene and its encoded protein

The MPLP6 gene was isolated by antibody screening from the cDNA library of our previous study [12]. The MPLP6 sequence of 878 nucleotides was obtained and analyzed. The length of the entire ORF was 663 nucleotides. The open reading frame was delimited by start (ATG) and stop (TAG) codons (Fig. 1). The translated polypeptide of 220 amino acids had a calculated molecular mass of 24 kDa and a pI of 6.51. The Mplp6 is similar to the Mp1 of *P. marneffei* and Afmp2p of *A. fumigatus*. Both proteins contained antigenic property, yet their functions were not known. The Mplp6 protein is novel as no match was found when the attempt was made to determine its possible function by searching for conserved domains via PROSITE (http://www.expasy.ch/prosite/) and pfam (http://pfam.sanger.ac.uk/) databases. It contained the signal sequence at the first 17 amino acids, 11 potential O-glycosylation sites (S/T sequences), but no GPI anchoring signal sequence was detected (Figs. 1 and 2A). The sequence analysis suggests that Mplp6 is a secreted mannoprotein.

Comparison between MPLP6 and MP1 of *P. marneffei*

MPLP6 and MP1 are paralogous genes in *P. marneffei* belonging to a novel mannoprotein superfamily [16]. There are approximately 14 proteins in this family in the genome of *P. marneffei*. We found high similarity of the MPLP6 to two halves of the MP1 at both the nucleotide and amino acid levels (Fig. 2B). At the nucleotide level, the first half of the MP1 (encodes for amino acids 1–187) has 46% identity to an entire sequence of MPLP6, and the second half of the MP1 gene (encodes for amino acids 197–377)

Fig. 1  The nucleotide sequence and deduced amino acid sequence of MPLP6. Shaded nucleotides represent the start and stop codon of the ORF. Signal peptide is shown in bold letters. Asterisks indicate the potential sites for O-glycosylation.

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has 44% identity to the whole sequence of *MPLP6* (data not shown). This result may imply a duplication event of *MPI* gene in the *P. marneffei* genome.

*Mp1p* and *Mplp6* polypeptides also share several structural features including N-terminus signal peptide (SP) which instructs the transport of the protein to the cell surface, a serine/threonine-rich region which probably serves as sites of extensive O-glycosylation and directs the protein to be exposed to the environment [17]. Unlike *Mp1p*, loss of the GPI anchoring sequence in *Mplp6* suggests that the protein cannot be located at the cell wall. We assumed that *Mplp6* is a secreted protein based on this sequence analysis.

**MPLP6 is expressed only in the pathogenic yeast phase**

The expression of the *MPLP6* was investigated in different phases of *P. marneffei* (mold, conidia, and yeast). We found that *MPLP6* is a yeast phase specific gene. As shown in Fig. 3, the *MPLP6* transcript was absent in the mycelial phase, suggesting that the function of *Mplp6* is not necessary in this form. The transcript was also not detected in conidia, suggesting that its function is not important to germination. However, the large amount of transcript found in the yeast phase suggested that *Mplp6* may play a key role in this pathogenic form.
MPLP6 encodes a yeast phase specific, antigenic mannoprotein of *P. marneffei*

To determine the role of Mplp6 during phase transition, the expression level was investigated during conversion from the mycelia to yeast phase. The *MPLP6* transcript was detected at 6, 12, 24, 48, and 72 h after a temperature shift from 25 °C to 37 °C (Fig. 4). We could detect the expression of *MPLP6* at 6 h after the temperature shift. At 24 h, the expression level was increased and reached a constant level until 72 h. This result indicated that Mplp6 may be involved in the growth of the yeast phase of *P. marneffei*.

Expression of the *MPLP6* was determined under heat and oxidative stress conditions. The expression level did not increase with the introduction of heat or hydrogen peroxide treatments to the yeast cells. Additionally, these stress conditions could not induce the expression of *MPLP6* in the mold phase (Fig. 4). The *MPLP6* transcripts were absent in conidia under normal and stress conditions (data not shown). Altogether, these results indicated that Mplp6 is a yeast phase specific protein that may play a role in the growth and development, but not in the stress response, of the yeast phase.

**Western immunoblot assay**

We performed a Western immunoblot assay to evaluate the recombinant protein Mplp6 value in serodiagnosis. Twenty sera of culture-confirmed *P. marneffei*-infected patients were used to determine the quality of this protein for the detection of specific antibodies. These sera possessed immunoreactivity to *P. marneffei*’s antigen. Figure 5 (lanes 1–20) shows that 19 out of 20 sera from *P. marneffei*-infected patients (95%) gave positive reactivity to the Mplp6 protein. This result implies the high capability of the Mplp6 antigen to elicit antibody response in patients during *P. marneffei* infection.

To tentatively determine the specificity of Mplp6, sera obtained from patients infected with other common opportunistic infections were used, i.e., aspergillosis (*n* = 13), tuberculosis (*n* = 2), cryptococcosis (*n* = 8) and histoplasmosis (*n* = 7). Five serum samples from normal healthy subjects were used as a negative control. All sera tested negative in Western blot assay (data not shown) suggesting that Mplp6 is a specific antigen and a useful diagnostic marker.

**Discussion**

The genome of *P. marneffei* contains approximately 14 homologous genes belonging to a mannoprotein superfamily [16] which have rapid evolutionary rates. *MPLP6* is a homologue which is highly similar to *MP1* and interestingly, *MP1* seems to be a duplicate molecule of *MPLP6*. We also noticed that the characteristic of expression was the same in *MPLP6* and *MP1*. Whether or not they have relative function, they were expressed only in the yeast...
pathogenic form (data not shown). Interestingly, an insertion of bases inside the MP1 genes that cause a premature stop did not affect viability or pathogenicity of P. marneffei, as they were recovered from infected patients [16]. As shown in a study from Woo et al. [16], there were variations in the sequence of MP1 among clinical isolates which indicated that MP1 is dispensable. In contrast, no polymorphism has been detected in the sequence of MPLP6 among 10 isolates. Lack of polymorphism implies the high conservation of this gene in P. marneffei genome and thus may suggest a more important function. Further study by making a null mutation of MPLP6 may help to elucidate the function of this mannoprotein.

Disseminated P. marneffei infections are relatively easy to diagnose because of the characteristic skin lesions from which the fission yeast form may be readily isolated. The organism is also relatively easy to culture from clinical samples, and the characteristic red pigment produced by the mycelial form is also diagnostically useful [18]. However, there remain obvious advantages to be gained from the early diagnosis of infection prior to the disseminatum stage and appearance of dermatological involvement. Although several studies have identified a number of useful antigens that can be recognized in the patients’ sera [19–21], the serodiagnosis of P. marneffei has not been well established. In the past, the major obstacles involved the lack of information on specific antigens and the labor involved in preparing the antigens. However, recombinant protein technology has contributed to solve these problems.

Our preliminary study on specificity of Mlp6 antigen showed no cross reactivity with the serum samples derived from patients with aspergillosis, histoplasmosis, cryptococcosis and tuberculosis. While the Mlp6 antigen has similarity to an antigenic protein Mp2p of A. fumigatus, it did not react with the serum obtained from A. fumigatus-infected patient. In this study we had only one serum of a patient with an A. fumigatus-confirmed infection due to the limitation of infected cases and identification of Aspergillus species. However, considering that A. fumigatus is the most frequent causes of aspergillosis in Thailand [22], some of the other seven aspergillosis cases could probably have been caused by this fungus. Thus, it is possible that Mlp6 does not cross react with A. fumigatus antigens. If this antigen is applied in diagnostic test, we would be able to confirm the specificity with more species-identified sera in the future. Regarding the histoplasma antigens, we did not find cross reactivity of Mlp6 to any proteins of H. capsulatum. In our assay, we used histoplasmosis sera from Thailand and the USA to exclude the possibility of mix infection or previous exposure to P. marneffei antigens of histoplasmosis patients in Thailand. Our immunoblot results showed no reactivity with the sera collected from endemic or non-endemic regions. Sera from other common opportunistic infections in Thailand, i.e., cryptococcosis and tuberculosis, also showed no cross reactions. Our findings suggested high specificity of the Mlp6 antigen to P. marneffei.

This study reports the isolation and characterization of gene encoding for Mlp6, a second mannoprotein, after Mp1 that has been isolated from P. marneffei. The recombinant protein produced in this study is ripe for development of serodiagnostic tests in the near future. Western blot assay showed the feasibility of using Mlp6 to detect specific antibodies in P. marneffei-infected patients’ sera in that it showed 95% positive identification in the assay. However, this high percentage does not reflect all populations of AIDS patients. Patients with severe impaired immune status could give a negative result, resulting in lowering the sensitivity of the test. For this reason, the development of complementary tests that detect both the antigen and antibody, using Mlp6 antigen or combination with other specific antigens is recommended to confirm infections due to P. marneffei in AIDS patients. This is not a first report of the immunological property of fungal mannproteins. Several studies have identified mannproteins as the major antigens stimulating host antibody responses [23–32]. The presence of antibodies to Mlp6 in patients with P. marneffei infection suggests that it is a good candidate for serodiagnosis. The yeast phase specific property also makes this protein a possible drug target. More studies are required to further discover the possible roles of this protein in pathogenesis.

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Fig. 5 Western blot analysis of P. marneffei-infected patient’s sera. Individual sera obtained from P. marneffei-infected patients were reacted to the electrophoresed recombinant Mlp6 antigen (lane 1-20). A pooled P. marneffei-infected patient’s sera (n = 5) was used as a positive control (P). Negative control (N) used a strip containing E. coli proteins incubated with the pooled patient’s sera (those are in lane 1-20) to determine the presence of anti-E. coli antibodies.
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