The Role of NbTMP1, a Surface Protein of Sporoplasm, in Nosema Bombycis Infection

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Abstract

Background: *Nosema bombycis* is a unicellular eukaryotic pathogen of the silkworm, *Bombyx mori*, and a hazard to the silkworm industry. Because of its long incubation period and horizontal and vertical transmission, it is a quarantine pathogen in sericulture. The microsporidian life cycle includes a dormant extracellular phase and intracellular proliferation phase. The proliferation period is the most active period of the microsporidian. This period lacks spore wall protection and may be the most susceptible stage for control.

Results: The *N. bombycis* protein (NBO_76g0014) was identified as a transmembrane protein and named NbTMP1. It is not homologous with proteins from other microsporidia and species. NbTMP1 has a transmembrane region of 23 amino acids at the N-terminus. Indirect immunofluorescence analysis (IFA) results suggest that NbTMP1 is secreted on the plasma membrane as the spores develop. Western blot and qRT-PCR analysis showed that *NbTMP1* expressed in all development stages of *N. bombycis* in infected cells and in the silkworm midgut. Down-regulation of *NbTMP1* expression resulted in significant inhibition of *N. bombycis* proliferation.

Conclusions: We confirmed that NbTMP1 is a membrane protein of *N. bombycis.* Reduction of the transcription level of *NbTMP1* significantly inhibited *N. bombycis* proliferation, and this protein may be a target for the selective breeding of *N. bombycis* resistant silkworm strains.

1. Introduction

Microsporidia are obligate intracellular parasitic eukaryotes with a wide range of hosts. They infect almost all vertebrates and invertebrates, including humans (1–3). Microsporidia have a unique ultrastructure and life cycle (3). They were originally considered to be primitive organisms such as protozoans or protists (4). However, microsporidia are now considered to be related to fungi, or they may be a sister branch of fungi, which has lost many genes and undergone genome compression. This could be due to their adaptation to intracellular parasitism (5–8). Lack of mitochondria is evidence that microsporidia are ancient eukaryotes (9). Because of the loss of many metabolic pathways, microsporidia must obtain nutrients from the host and export the cytotoxic compound through the cell membrane (10–12). Microsporidia have a distinctive mechanism for infecting host cells. The polar tube is ejected when microsporidia are stimulated by suitable conditions, and the infective sporoplasm is transferred into the host cells through the polar tube prior to proliferation (13). The sporoplasm can adhere to host cells and enter the host cells by phagocytosis (14). The membrane proteins of the sporoplasm then interact with the cytoplasma content of host cell.

Nosema bombycis, the first microsporidian recognized by Nageli in 1857, causes silkworm pébrine disease by vertical and horizontal transmission (15). The prevention and control of *N. bombycis* is an important focus of sericulture research. In this study, we identified a transmembrane protein (NBO_76g0014) unique to *N. bombycis*, designated as NbTMP1. We cloned and expressed recombinant

protein NbTMP1 and prepared monoclonal antibodies. We also characterized the subcellular localization of NbTMP1 in *N. bombycis*, and explored its function in *N. bombycis* proliferation.

2. Materials And Methods

2.1 Preparation of *N. bombycis* and cell cultivation

Mature spores of *N. bombycis* CQ1, from the China Veterinary Culture Collection Center (CVCC No. 102059), were isolated from infected silkworm pupae and purified by Percoll density gradient centrifugation (21,000 g, 40 min) (16). Sf9-III cells in Sf-900™ III SFM were purchased from Thermo Fisher Scientific (Santa Clara, CA, USA)and cultured in Sf-900™ III SFM medium at 28 °C.

2.2 *NbTMP1* sequence analysis and ORF amplification

NbTMP1(GenBank Accession No. EOB13409.1), highly expressed in the early stages of infection, was screened from the transcriptome data of *Bombyx mori* infected with *N. bombycis*. The amino acid sequence of NbTMP1 was submitted to the signal 5.1 Server (http://www.cbs.dtu.dk/services/SignalP/) and TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/) for signal peptide and transmembrane domain predictions. The molecular weight and isoelectric point (PI) were predicted using ExPASy server (http://web.expasy.org/compute_pi/). Protein function domain was predicted using SMART (http://smart.embl-heidelberg.de/). BLAST (https://blast.ncbi.nlm.nih.gov/) was used to analyze the multiple sequence alignment. We cloned the extramembranous region of *NbTMP1* using the forward primer 5'-GGATCCATGTTCAAGTCTAGTGATGA-3' containing a *Bam*H I restriction site, and the reverse primer 5'-GTCGACCTTATCATTTTCATTATTTCCC-3' containing a *Sal* I restriction site. Complementary DNA (cDNA) was obtained by reverse transcription of RNA from the Sf9-III cells (Thermo Fisher, Santa Clara, CA, USA) infected with *N. bombycis* and used as the PCR template. The PCR products were purified with the Gel Extraction Kit (OMEGA, Norcross, GA, USA), integrated into pET-28 vector, then the vectors were transformed into competent DH5α *E.coli* strain cells. The positive pET-28-*NbTMP1* recombinant vectors were sequenced by Sangon (Shanghai, China).

2.3 Recombinant protein expression, monoclonal antibody preparation, and immunoblotting

The identified pET-28-*NbTMP1* vector was transformed into *E. coli* Rosetta for expression. The recombinant bacteria were induced for 4 h at 37 °C with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in LB medium. The target protein was purified with Nickel chelating affinity chromatography (Roche, Basel, Switzerland). All animal experiments were approved by Laboratory Animals Ethics Review Committee of Southwest University (Chongqing, China) under Permit Number: AERCSWU2017-7. The mice were maintained according to the recommendations of the committee, and food and water were provided ad libitum. Female BALB/c mice (6- to 8-week-old) were injected subcutaneously four times with rNbTMP1 (100 μg/mouse) mixed with Freund's complete/incomplete adjuvant (1:1; Sigma, St. Louis, MO, USA). The injection interval was 7 d. Monoclonal antibodies (mAbs) were produced and screened as

previously described (17). The mAb IgG subtypes were detected using the Mouse Monoclonal Antibody Isotyping Kit (Roche, Switzerland) according to manufacturer recommendations.

For immunoblotting, the proteins of mature spores and infected cells were prepared by the glass bead breaking method (18), isolated by SDS-PAGE, and transferred to PDVF membranes (Roche, Basel, Switzerland). After blocking in 5% skim milk diluted in TBST (150 mM NaCl, 20 mM Tris-HCl, 0.05% Tween-20), the membrane was incubated for 1 h in anti-NbTMP1 ascites (1:1000). After washing three times, the membrane was incubated with goat anti-mouse IgG (1:5000; Sigma, St. Louis, MO, USA) conjugated with HRP-label. Finally, the protein bands were visualized with ECL Plus Western Blotting Detection Reagents (Bio-Rad, Richmond, CA, USA).

2.4 Silkworm midgut paraffin section

Newly molted fifth instar silkworm larvae were starved for 1 d and fed with mulberry leaf coated with spores(1×10^7 spores per larva). The midguts of infected silkworms were collected at 4 d post-infection (dpi) and stored at -80 °C. After washing with PBS (137 mM NaCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, and 2.7 mM KCl) for three times, each time for 5 min, the midguts were fixed with 4% paraformaldehyde for 24 h. The sections were prepared as previously described (19). After deparaffination, the sections were boiled in sodium citrate buffer solution for 10 min and then used for immunolocalization.

2.5 Immunofluorescence analysis (IFA)

The samples were fixed with 4% paraformaldehyde and washed three times with PBS. Then the samples were permeabilized with 0.5% Triton X-100 for 30 min and then blocked with PBS-bovine serum albumin for 1 h at room temperature. Next, the samples were incubated with the primary antibodies including anti-NbTMP1 ascites (mouse) and antibody of Nbβ-tubulin (rabbit), which was used to label meronts of *N. bombycis* (20). After washing three times with PBS, Alexa 488 conjugate Goat anti-Mouse IgG and Alexa 568 conjugate Goat anti-Rabbit IgG (Thermo Fisher, Santa Clara, CA, USA) were used to detect the bound primary antibodies, and 4', 6-diamidino-2-phenylindole (DAPI) (Thermo Fisher, Santa Clara, CA, USA) was used to label the nucleus. The results were observed by a confocal laser scanning microscope (Olympus, Japan).

2.6 Immunoelectron microscopy (IEM) analysis

Infected silkworm midguts were used for IEM. The 70-nm ultrathin sections were prepared as previously described (19) and placed on nickel grids. After blocking with PBS-bovine serum albumin, the grids were incubated with anti-NbTMP1 ascites diluted 1:30 or negative serum at room temperature for 1 h. Then, the grids were incubated with gold conjugated anti-mouse IgG (Sigma) diluted to 1:100. After staining with uranium and lead, the sections were examined and photographed with a JEM-1400 Plus TEM transmission electron microscope (JEOL, Japan).

2.7 RNAi of *NbTMP1*

A 331 bp interferential fragment was selected by BLOCK-iT™ RNAi Designer (http://rnaidesigner.thermofisher.com/rnaiexpress/design.do). The fragment was amplified by F-RI-NbTMP1-T7 5'-TAATACGACTCACTATAGGGAGATGTCTCTAGGGCAGATGC-3'and R-RI-NbTMP1-T7 5'-TAATACGACTCACTATAGGGAGATCCAGTACGTGTTGCCTG-3' that was used as the template for synthesizing dsRNA. RiboMAX™ Large Scale RNA Production System-T7 (Promega, Madison, WI, USA) was used to obtain the dsRNA. The dsRNA of *EGFP* was used as a negative control and was synthesized as described above (17). After the Sf9-III cells were cultured in 12-well plates overnight, 2 μg dsRNA of *NbTMP1* or *EGFP* was transfected into the cells. After 4 h, the spores were germinated with 0.1 M KOH and added to the Sf9-III cells (spores: cell = 5:1). Samples of infected cells were collected at 1, 3, and 5 dpi and immediately stored in PBS or TRIzol (Invitrogen, Carlsbad, CA, USA) at -80 °C.

2.8 Real-time quantitative PCR analysis

A DNA Extraction Kit (Omega, Norcross, GA, USA) was used to extract the gDNA of the infected cells. Total RNA Kit II (Omega, Norcross, GA, USA) and EvoScript Universal cDNA Master (Roche, Switzerland) were used to prepare the cDNA. Quantitative PCR was amplified by F-q-Nb TMP1 5'-CCTATCTCTAAAGACGGT-3' and R-q-Nb TMP1 5'-CTTTTTCTATTTTGGCAGCA-3' primers, and reference gene primers F-q-SSU 5'-CTGGGGATAGTATGATCGCAAGA-3' and R-q-SSU 5'-CACAGCATCCATTGGAAACG-3'. The transcription levels were calculated by the $2^{-\triangle\triangle t}$ values method with three replicates. GraphPad Prism v6.01 was used to conduct the multiple t tests.

Nbβ-tubulin copy number was used to count the *N. bombycis*. The gDNA of infected cells samples were analyzed by qPCR. The reaction systems were conducted using the primers: Nbβ-tubulin-qF 5'-AGAACCAGGAACAATGGACG-3' and Nbβ-tubulin-qR 5'-AGCCCAATTATTACCAGCACC-3'. The standard template used was described in previous research (17). The standard curve covered six orders of magnitude (1.3×10^2–10^7).

3. Results

3.1 Sequence characteristics and immunoblot analysis of NbTMP1

Sequence analysis showed that NbTMP1 was a unique protein in *N. bombycis*. *NbTMP1* contained a complete ORF 732 bp in length and encoded a polypeptide of 243 amino acids with a transmembrane domain. The protein has a calculated molecular weight of 27.59 kDa and a theoretical pI value of 9.92. It does not have a signal peptide and typical functional domains. The recombinant NbTMP1 protein was expressed as soluble protein of about 30 kDa (Fig. 1A) and was purified to prepare the monoclonal antibody F12.

Western blot analysis indicated a unique positive band was detected by F12 ascites in mature spores and the infected cell proteins, respectively (Fig. 1B). The band is about 35 kDa, which is greater than the

molecular weight of NbTMP1. This may be due to post-translational modification, such as phosphorylation and glycosylation of native NbTMP1. Bioinformatics analysis showed that there are 2 glycosylation sites and 20 phosphorylation sites. Since the recombinant protein lacks a transmembrane domain, the molecular mass of the rNbTMP1 and the native protein differed. The Mouse Monoclonal Antibody Isotyping Kit (Roche, Switzerland) was used to identify the subtypes of monoclonal antibody F12. The test strip demonstrated that the subtypes of F12 were IgG1-κ (Fig. 1C).

3.2 Subcellular localization of NbTMP1

To study the subcellular localization of NbTMP1, IFA was performed with the F12 ascites. Sporoplasm was obtained by germination of mature spores by 0.1 M KOH. The IFA indicated that NbTMP1 is localized on the plasma membrane of the sporoplasm (Fig. 2). We used the Nbβ-tubulin antibody to label meronts and NbTMP1 co-located with Nbβ-tubulin in the proliferation stage (Fig. 3). We verified the subcellular localization of NbTMP1 in the infected tissues by paraffin sections. The fluorescence signal of NbTMP1 was consistent with the contour of mature spores (Fig. 4A), but there was no fluorescence signal in mature spores that had not been sliced (Fig. 4B). Antibody cannot penetrate the spore wall of *N. bombycis*, which also implies that NbTMP1 is located on the plasma membrane. IEM analysis demonstrated that NbTMP1 is located on the membrane of *N. bombycis* (Fig. 5). This result indicated that NbTMP1 may be secreted by the plasma membrane as the spores develop.

3.3 Transcriptional profile of *NbTMP1* in infected cells and midguts

After *N. bombycis* infected the Sf9-III cells, the expression of *NbTMP1* increased from the 1st day to the 6th d.p.i. The drop in the expression on the 4th d.p.i may due to the formation of mature spores (Fig. 6A). In the midguts of infected silkworm, *NbTMP1* had a low expression level in the pre-infection phase, and the expression continued to increase after the 4th d.p.i (Fig. 6B). On the 4th d.p.i, *N. bombycis* began a new round of host infection, and the transcript profile of *NbTMP1* indicated that it may play a role in spore infection.

3.4 RNAi of *NbTMP1* inhibited *N. bombycis*' proliferation

The effects of RNAi were analyzed using qPCR. After adding the dsRNA, the transcription level of *NbTMP1* was significantly down-regulated in the experimental groups (NbTMP1-dsRNA) (Fig. 7A). Since *Nbβ-tubulin* is a keeping and conserved gene of *N. bombycis*, its copy number was used to reflect the number of *N. bombycis* in the two groups (17). The qPCR results showed that *N. bombycis* began to proliferate from 1 to 5 d.p.i in the mock groups (EGFP-dsRNA). However, the pathogen load was remarkably lower in the experimental groups (Fig. 7B). These results suggest that NbTMP1 plays an important role in the proliferation of *N. bombycis*.

4. Discussion

Membrane protein is rarely studied in microsporidia. In *Trachipleistophora hominis*, plasma membrane-located purine nucleotide transport proteins (NTTs) are key components for stealing ATP from hosts (11). EhSSP1 was identified as *Encephalitozoon hellem* sporoplasm surface protein 1, which bound to human foreskin fibroblasts and was associated with the final step of invasion on the invasion synapse (21). NbTMP1 was predicted to have a transmembrane domain, suggesting that it may locate on the membrane of the spore. The IFA and IEM results proved that NbTMP1 localized on the membrane of sporoplasm and spores. The expression profiles in vitro and in vivo, as well as the western blot results, showed NbTMP1 is present in all stages of the *N. bombycis* life cycle. Although we showed that down-regulation of NbTMP1 expression can inhibit the proliferation of *N. bombycis*, the specific mechanism remains unknown.

There are two main hypotheses about how microsporidia infect host cells. One is that the microsporidia eject a polar tube, penetrate the membrane of new host cells, and deliver the contents into the host cell cytoplasm. The other hypothesis is that the microsporidia gain access to the host cell by phagocytosis, and then the polar tube is used to escape the phagosome and to infect the host cytoplasm (13). Sporoplasm is the earliest stage of microsporidia infection of host cells. The mechanism used by microsporidia to transmit genetic information after infecting a host cell is unclear. However, surface proteins, such as NbAQP, NbSWP9, and NbSWP7, can play important roles in the process of spore germination and infection (22, 23). NbTMP1 is located on the plasma membrane of the sporoplasm, suggesting that it may be involved in the proliferation of spores in cells.

Understanding the protein functions of *N. bombycis* has been hampered by the lack of stable and reliable gene manipulation methods. RNAi has been used in fungi, including *Heterosporis saurida* and *Nosema ceranae* to study protein functions (24, 25). *N. bombycis* possesses all of the genes required for RNA silencing and in vivo function (26). A stable RNAi strategy was previously established by an in vitro transcription system and lipofection in Sf9-III infection (27). RNAi has been successfully used in anti-BmNPV resistant silkworm construction (28). RNAi can also be used as a strategy for breeding *N. bombycis*-resistant silkworms, and NbTMP1 is a potential target of RNAi. In addition, single-chain antibodies are also a strategy for breeding *N. bombycis*-resistant silkworms. In *Anopheles*, transposon-mediated transformation was used to generate m1C3, m4B7, and m2A10 single-chain antibodies (scFvs), and the transgenic mosquitoes expressing the scFv gene had significantly lower infection levels of *Plasmodium falciparum* (29, 30). The transgenic Sf9-III cells, which express single-chain antibodies of SWP12 of *N. bombycis*, effectively inhibit the proliferation of *N. bombycis* in cells (17). In this study, we obtained the monoclonal antibody (F12) of NbTMP1, and plan to evaluate the performance of the single-chain antibody for breeding of *N. bombycis*-resistant silkworms.

5. Conclusions

We identified a novel membrane protein in the microsporidium *N. bombycis*. NbTMP1 was localized on the plasma membrane of the sporoplasm, and its proliferation was significantly inhibited by RNAi. This protein is a potential target for construction of *N. bombycis*-resistant silkworms.

Abbreviations

IFA: indirect immunofluorescence analysis; IEM: immunoelectron microscopy; Sf9-III cells: ovarian cell line of *Spodoptera frugiperda*; RNAi: RNA interference; qPCR: real-time quantitative PCR.

Declarations

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Authors' contributions

SZ, YH and HH performed the exeriments, analyzed the data. SZ and CL drafted the manuscript and performed manuscript preparation. SZ, HH, BY and NZ supervised and performed material collection, and all the other laboratory experiments. SZ, JW, GP, CL and ZZ conceived the idea and coordinated the project. All authors read and approved the final manuscript.

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Availability of data and materials

Data supporting the conclusions of this article are included within the article. All data are fully available without restriction upon reasonable request.

Ethics approval and consent to participate

All animal procedures were approved by Laboratory Animals Ethics Review Committee of Southwest University (Chongqing, China) under Permit Number: AERCSWU2017-7.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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