SWP5, a Spore Wall Protein, Interacts with Polar Tube Proteins in the Parasitic Microsporidian Nosema bombycis

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Microsporidia are a group of eukaryotic intracellular parasites that infect almost all vertebrates and invertebrates. The microsporidian invasion process involves the extrusion of a unique polar tube into host cells. Both the spore wall and the polar tube play an important role in microsporidian pathogenesis. So far, five spore wall proteins (SWP1, SWP2, Enp1, Enp2, and EcCDA) from Encephalitozoon intestinalis and Encephalitozoon cuniculi and five spore wall proteins (SWP32, SWP30, SWP26, SWP25, and NbSWP5) from the silkworm pathogen Nosema bombycis have been identified. Here we report the identification and characterization of a spore wall protein (SWP5) with a molecular mass of 20.3 kDa in N. bombycis. This protein has low sequence similarity to other eukaryotic proteins. Immunolocalization analysis showed SWP5 localized to the exospore and the region of the polar tube in mature spores. Immunoprecipitation, mass spectrometry, and immunofluorescence analyses revealed that SWP5 interacts with the polar tube proteins PTP2 and PTP3. Anti-SWP5 serum pretreatment of mature spores significantly decreased their polar tube extrusion rate. Taken together, our results show that SWP5 is a spore wall protein localized to the spore wall and that it interacts with the polar tube, may play an important role in supporting the structural integrity of the spore wall, and potentially modulates the course of infection of N. bombycis.

Microsporidia are a group of obligate intracellular, spore-forming, fungus-like, unicellular eukaryotic animal pathogens with an extensive host range, including almost all vertebrates and invertebrates (1, 9, 34, 35, 39). More than 160 genera and 1,300 species have been reported (11), of which 14 species from 8 genera have been isolated from humans, and several of them are important sources of opportunistic infections in AIDS patients (7). They are also important pests in fisheries and shrimp farms and in sericulture (2, 12, 43). Microsporidia derived from fungi (14, 20, 21, 36, 41), but they lack mitochondria. Some species contain a mitosome, thought to be a relic of the mitochondria (15, 19, 42).

Both the spore wall and the polar tube play an important role during microsporidian infection. The dense and rigid spore wall protects the microsporidian, helping it to resist various pressures from the environment (47). The spore wall consists of an electron-dense outer layer, the exospore, which is principally proteinaceous, and an electron-lucent inner endospore layer, which contains chitin and proteins (2, 10, 20, 24, 27, 28). To date, five proteins have been identified from Encephalitozoon species. Two exospore proteins, SWP1 and SWP2, were identified from Encephalitozoon cuniculi and Encephalitozoon intestinalis (4, 16). Three endosporosome proteins, Enp1, Enp2, and the chitin deacetylase-like protein EcCDA, were found in E. cuniculi (4, 6, 16, 26, 47). Enp1 was thought to be an adherence ligand allowing the parasite to attach to host cells and potentially modulate infection (17, 32, 33). It is well known that microsporidian spores display an original invasion mechanism involving the polar tube. The long hollow polar tube is divided into an anterior straight portion and a posterior coiled region. The former is attached to the inside of the anterior end of the spore by an anchoring disc. The posterior coiled region forms from 4 to ~30 coils around the sporoplasm in the spore, depending on the species (43, 46). Upon appropriate environmental stimulation, the polar tube can suddenly extrude and penetrate the plasma membrane of the host cell, and then the sporoplasm is transferred into the cytoplasm of the host cell, where the spores develop and complete the life cycle (40, 43, 46). The polar tube is composed of electron-dense and -lucent concentric layers in cross section (8, 10, 24, 31, 37). Three previously identified major polar tube proteins (PTP1, PTP2, and PTP3) interact with each other and form the major protein components of the polar tube (5).

Nosema bombycis, a silkworm (Bombyx mori) parasite, was first described in 1857 (25). It is the etiological agent of the deadly protozoan disease pébrine in the silkworm and inflicts severe worldwide economic losses in regions where sericulture is practiced, such as China, India, and other regions of the world (2). Recently, using proteomics-based approaches, 14 hypothetical spore wall proteins were predicted for N. bombycis (44). Two exospore proteins (SWP32 and SWP26) and two endospore proteins (SWP30 and SWP25) were identified (23, 44, 45). In addition, three spore wall proteins (71, 48, and 30 kDa) and three polar tube proteins (NhPTP1, NhPTP2, and NhPTP3) were also studied (38). Recently, the exospore protein NbSWP5 was proved to protect spores from phagocytic uptake (30). In this study, the expression and localization of SWP5 were investigated by reverse transcription-PCR (RT-PCR), Southern blotting, indirect immunofluorescence assay (IFA), and immunoelectron microscopy (IEM). The interaction between SWP5 and polar tube proteins was also explored using immunoprecipitation, mass spectroscopy (MS), immunofluorescence, and germination analyses.
MATERIALS AND METHODS

Microsporidian spore preparation and purification. N. bombycis isolate CQ1 (isolate 102059) was obtained from the China Veterinary Culture Collection Center. It was originally isolated from infected silkworms in Chongqing, China. Spores were isolated from the silkworms and purified by discontinuous Percoll gradient centrifugation as previously described (44).

Bioinformatic analysis. In our previous studies, 14 hypothetical spore wall proteins, including the NbHSWP5 protein (GenBank accession number EF683105) used in this study, were identified by proteomic-based approaches. To further identify and functionally characterize NbHSWP5, protein motif prediction was carried out using ExPaSy proteomic tools (http://www.expasy.org/tools/). The signal peptide and post-translational modification sites were predicted using online tools (http://www.cbs.dtu.dk/services/) (18). Glycosylphosphatidylinositol (GPI) anchor prediction was performed using the online program big-PI Predictor (http://mendel.imp.univie.ac.at/sat/gpi/gpi_server.html). Subcellular localization was predicted using the PSORT server (http://psort.hgc.jf). Sequence similarity was analyzed by BLAST analysis of our local database. Amino acid sequence alignments were generated with the ClustalW program.

Gene expression pattern analysis. The midguts of infected silkworms were prepared at 1, 3, 5, and 7 days postinfection (first- to fifth-instar larvae). Total RNA was extracted using TRizol reagent (Invitrogen). DNA was removed by using an RNase-free DNase set, followed by phenol-chloroform extraction and ethanol precipitation. Corresponding cDNA was synthesized by reverse transcription using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega). The SWPS5 gene was amplified using the specific primer pair 5'-CGAGTTCATAACATCTAACCAATA-3' and 5'-TGGACGCCATTAGACAAG-3'. Amino acid sequence alignments were generated with the ClustalW program.

Gene cloning and recombinant protein expression and purification. The SWPS5 gene (GenBank accession number EF683105) was cloned using the forward primer 5'-CCCCAGATCTAAAAGAAATAGAATTGCCG G-3' and the reverse primer 5'-ACGGGTTGCAATTTATTTATCCGAGGTGCAG-3', containing a Sall restriction site (GGTCCG). PCR products were analyzed in a 1% agarose gel and purified with a gel extraction minikit (Watson, China). Purified products were cloned into the PMD19-T vector (Takara BioTech, Japan) and transformed into competent cells of Escherichia coli strain JM109. Positive clones were confirmed by colony PCR and sequenced by Invitrogen BioTech (Shanghai, China). The amplified fragment and the expression vector p-Cold I were digested with EcoRI and SalI, ligated, and transformed into E. coli Rosetta Gami cells. A single positive colony was induced for 48 h at 16°C with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and then harvested by boiling for 10 min in SDS extraction buffer. Western blotting was performed using a histidine tag-specific antibody (Sigma-Aldrich, St. Louis, MO) to confirm the expression of the recombinant protein. A Ni-nitrilotriacetic acid (Ni-NTA) superflow cartridge was used to purify the recombinant protein according to the manufacturer's instructions (Qiagen, Germany).

Polycystal antibody production and immunoblotting. Antibody production and immunoblotting protocols to detect the SWPS5 recombinant protein have been described previously (23, 44, 45). Briefly, five mice were immunized intradermally (at the base of the tail) with phosphate-buffered saline (PBS) (one mouse as a negative control) or recombinant protein (four mice) mixed with Freund's adjuvant (1:1; Sigma) every week. One week after the third injection, the mice were bled and their sera collected.

For immunoblotting, N. bombycis proteins were extracted as previously described (26), with minor modifications. Briefly, spore (10^6 spores/ml) germination was induced in 0.1 mol/liter K_2CO_3 at 28°C for 30 min, and then the sample was boiled for 10 min in SDS extraction buffer. Total protein was collected by centrifugation at 14,000 x g for 10 min. Proteins were separated by SDS-PAGE on 12% polyacrylamide gels, transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore), and blocked under standard conditions. Negative-control serum and anti-SWP5 serum (1:100 dilution) were used as primary antibodies. The secondary antibody, a goat anti-mouse IgG-IgM antiserum (Sigma) labeled with peroxidase, was detected by adding the substrate diamino benzidine tetrahydrochloride (DAB).

IFA. Purified spores were fixed in 80% acetone, permeabilized with 70% ethanol containing 0.5% Triton X-100 for 10 min at room temperature, incubated with polyclonal antisef or negative serum dilutions with PBS-bovine serum albumin (BSA) at 37°C for 60 min, and finally washed with PBS. The secondary fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (1:100; Sigma) was used to detect the bound primary antibodies. DNA was stained with DAPI (4',6-diamidino-2-phenylindole) for 20 min. Spores were examined with an Olympus BX50 fluorescence microscope.

To analyze the interaction between SWPS5 and the polar tube, germination of purified mature spores was induced in 0.1 mol/liter K_2CO_3 at 28°C for 3 h. The extruded polar tubes were incubated with anti-SWP5 mouse polyclonal antibody (1:200 dilution) at 37°C for 2 h and then washed with PBS. Control samples were incubated with PBS without the anti-SWP5 serum. After the initial incubation, the polar tubes were incubated with FITC-conjugated goat anti-mouse IgG (1:100 dilution) at 37°C for 60 min. To investigate the effect of germination on SWPS5, the spores were initially incubated with the anti-SWP5 mouse polyclonal antibody and then induced to germinate with K_2CO_3. Finally, the spores were incubated with FITC-conjugated goat anti-mouse IgG antibody (1:100 dilution) at 37°C for 60 min. After being washed with PBS, the samples were stained with DAPI (1:64 dilution) for 20 min. Spores were examined with an Olympus BX50 fluorescence microscope.

IEM analysis. Purified spores were fixed with 3% formaldehyde and 1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), rinsed with PBS, dehydrated with a graded ethanol series, and finally permeabilized in K4M resin at ~20°C. Samples were embedded and photopolymerized in K4M resin. Ultrathin sections were placed on nickel grids and immunostained. After blocking, samples were incubated with primary SWPS5 antisera or negative serum, followed by gold-conjugated anti-mouse IgG (Sigma). The grids were then rinsed, dried, examined, and photographed with a Hitachi H-7500 transmission electron microscope.

Immunoprecipitation and LC-MS/MS analysis. Total protein was prepared by disrupting purified spores in a nondenaturing lysis buffer containing a protease inhibitor (phenylmethylsulfonyl fluoride [PMSF]), vortexed with 0.5 M acetic acid, and centrifuged. In brief, the immunoprecipitation procedure was as follows. The total protein and the anti-SWP5 polyclonal antibody were incubated overnight at 4°C, transferred to a tube with washed protein A beads, and again incubated overnight at 4°C. The beads were centrifuged and washed with 1× immunoprecipitation buffer followed by 0.1× immunoprecipitation buffer. After separation by SDS-PAGE, the different protein bands were excised, destained, digested, and analyzed by liquid chromatography-tandem MS (LC-MS/MS) as previously described (23, 44, 45).

Germination assays. Mature spores were preincubated with PBS or with mouse negative serum or SWPS5 antisera (1:100 or 1:200 dilution, respectively) for 2 h at 28°C and then centrifuged and washed with PBS. Spore germination was induced in 0.1 mol/liter K_2CO_3 for 2 h at 28°C, and spores were then fixed with 80% acetone and stained with DAPI. The
Spores were examined with an Olympus BX50 fluorescence microscope and counted (>20 random fields/data point).

Statistical analysis. Two-tailed Student’s *t* test was performed to test the significance of differences, using SPSS software (V12.0; SPSS). Differences were considered significant if the *P* value was <0.001. Data were collected from three independent experiments.

Nucleotide sequence accession numbers. Genes encoding SWP5 (another copy), PTP2, and PTP3 were deposited in GenBank under sequence accession numbers HQ881497, HQ881498, and JF739554, respectively.

RESULTS

Identification and characterization of SWP5. Fourteen hypothetical spore wall proteins, including NbHSWP5, were identified in our previous study (44). NbSWP5 was identified as an exospore protein in a recent report (30). In this report, we renamed the protein SWP5. Bioinformatic analysis showed that SWP5 has a calculated molecular mass of 20.3 kDa and a theoretical pI of 4.54 (Fig. 1). A bacterial Ig-like domain 1 was predicted, and a 23-amino-acid signal peptide was also predicted, with a cleavage site between amino acid residues A23 and K24, but no transmembrane domain or GPI anchor sequence signal was predicted. In addition, potential posttranslational modification sites were identified in SWP5, including phosphorylation sites, O-glycosylation sites (T43, T105, T180, S169, S179, S181, and S186), and sites of glycation of ε-amino groups of lysines (K52, K70, K86, K100, K125, K160, K174, and K188). Similarity analysis showed that SWP5 shares low sequence identity with proteins of any other eukaryotes, including *E. cuniculi*, *Encephalitozoon bieneusi*, and *Nosema locustae*, except for a protein in *Nosema ceranae* (GenBank accession no. XP_002996352.1; 25% identity).

Distribution of the SWP5 gene in the *N. bombycis* genome. Genome sequence analysis showed that SWP5 has another copy (GenBank accession number HQ881497) (Fig. 1). Southern blotting revealed two chromosome regions with strongly hybridized signals in the *N. bombycis* genome (Fig. 2A), in agreement with the

![FIG 2](image2.png) Chromosome localization and gene expression analysis. (A) Lane 1, *N. bombycis* chromosomal DNA separated by pulsed-field gel electrophoresis (PFGE). In lane 2, the arrow indicates the SWP5 probe strongly hybridized to two chromosome regions. (B) RT-PCR was performed using cDNAs from the midguts of infected silkworms (first- to fifth-instar larvae) at 1, 3, 5, and 7 days postinfection. The *tubulin* gene was used as an internal control.

![FIG 3](image3.png) Western blot analysis. Spore and coat proteins were extracted and analyzed by SDS-PAGE. Western blotting was performed using anti-SWP5 serum. Lanes 1 and 3, total proteins of mature spores; lane 2, spore coat proteins; lane 5, immunoblotting reactivity of normal mouse serum as a negative control; lane M, protein marker (Fermentas).
results of bioinformatic analysis showing that the SWP5 gene has another homologous gene distributed in the *N. bombycis* genome.

**Gene expression pattern at different developmental stages.** To survey the expression pattern of SWP5 at different development stages, RT-PCR was performed using cDNAs from the midguts of silkworms at 1, 3, 5, and 7 days postinfection. The results showed that SWP5 was expressed at a low level at 1 day postinfection and then expressed significantly at 3, 5, and 7 days postinfection compared with the level at 1 day postinfection.

**Heterologous expression and immunoblot analysis of recombinant SWP5.** To characterize SWP5, recombinant SWP5 was expressed in *E. coli* and purified using a Ni-NTA superflow cartridge. An anti-SWP5 polyclonal antibody was successfully generated in mice. Western blot analysis indicated that a unique positive band (expected size, 20 kDa) was detected in the spore total proteins and the spore coat proteins (Fig. 3). These data suggested that the SWP5 protein localizes in the walls of mature spores.

**Immunolocalization of SWP5.** Subcellular localization analysis predicted SWP5 to be an extracellular protein. To confirm its cellular location, SWP5 was analyzed by IFA and IEM using the specific polyclonal antibody. In a previous study, surface antigens of mature spores could be removed by SDS (10). In this study, spores were incubated with the SWP5 polyclonal antibody and displayed a bright green fluorescence signal (Fig. 4B), while no green fluorescence signal was observed in those treated with SDS (Fig. 4C) or in the negative control (Fig. 4A). The results suggested that SWP5 was localized on the exospore. By immunogold labeling and transmission electron microscopy, the gold particles were distributed mainly on the exospore and in the polar tube region of spores. No gold particles were detected on the chitin-rich layer between the exospore and the plasma membrane (Fig. 5A). No gold particles were detected in the negative-control samples (Fig. 5B).

**Immunoprecipitation and mass spectrometry analysis of protein interaction.** Total proteins were extracted from mature spores and immunoprecipitated with the polyclonal antibodies against SWP5. The immunoprecipitated proteins were then subjected to mass spectrometry analysis. The results showed that SWP5 interacted with several proteins, indicating its role in the life cycle of *N. bombycis*.
spores by use of acid-washed glass beads. Two target proteins potentially interacting with SWP5 were identified by immunoprecipitation. As shown in Fig. 6, two bands were identified, with apparent molecular masses of 31 and 150 kDa, which were significantly different from the bands in the control samples (Fig. 6, lane 3). The SWP5 protein band was displayed clearly (Fig. 6, lane 2). Subsequently, both bands were excised and analyzed by LC-MS/MS. Sequence analysis was performed against the *N. bombycis* protein database (unpublished data), and two genes were identified. One encodes PTP2, a 278-amino-acid protein with a calculated molecular mass of 31.4 kDa and a pI of 9.69. The other encodes PTP3, a 1,370-amino-acid protein with a calculated molecular mass of 150.3 kDa and a pI of 6.74 (Table 1). Taken together, immunoprecipitation and mass spectrometry analyses showed that SWP5 probably interacts with PTP2 and PTP3.

**Analysis of interaction with the polar tube.** To confirm the results described above, spores were induced to germinate in a K$_2$CO$_3$ solution. The polar tubes were incubated with anti-SWP5 serum, which was then detected by FITC-conjugated goat anti-mouse IgG. Interestingly, bright green fluorescence signals were detected (Fig. 7A2 and A3). This result indicated that the anti-SWP5 serum could interact with the polar tubes extruded from the *N. bombycis* spores. No fluorescence signal was obtained on the polar tubes in the control (data not shown).

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**FIG 5** Immunogold electron microscopy analysis. The anti-SWP5 serum and secondary antibodies conjugated with 10-nm colloidal gold were used. (A) Mature *N. bombycis* spore, with gold particles localized mainly to the exospore and the polar tube region, not at the chitin-rich layer. The inset shows an enlarged section of the image. (B) Negative control. Arrowheads mark colloidal gold particles. Bar, 0.2 μm. En, endospore; Ex, exospore; N, nucleus; PT, polar tube.

**FIG 6** Protein-protein interaction analysis. *N. bombycis* total protein was extracted and immunoprecipitated using anti-SWP5 serum. Lane M, protein marker (Fermentas); lane 1, total protein of mature spores; lane 2, spores treated with SWP5 antiserum; lane 3, negative control treated with mouse serum. The inset shows an enlarged section of the image. The arrows mark the SWP5, PTP2, and PTP3 bands.
or on the surface of the spore coat. These results imply that SWP5 might be released from the spore wall accompanied by the extruding polar tube (Fig. 7B1 to B3).

Effect of anti-SWP5 serum on spore germination. Purified spores were induced to germinate by use of 0.1 mol/liter K₂CO₃ (Fig. 8). A significant difference was found between the samples incubated with anti-SWP5 serum, mouse negative serum, and PBS treatment. These results indicate that the extruding polar tube (Fig. 7B1 to B3).

Importantly, the results showed that SWP5 interacted with PTP2 and PTP3 (Table 1; data not shown). In previous studies, SWP5 was localized to the exospore and the polar tube in our previous studies (23, 44, 45). A novel exospore protein (NbSWP5) was identified and could protect spores from phagocytic uptake by cultured insect cells (30).

In this study, immunolocalization analysis showed that SWP5 is a spore wall protein distributed at the region of the polar tube (Fig. 5). Importantly, the results showed that SWP5 interacted with PTP2 and PTP3 (Table 1; Fig. 6 and 7). These data indicate that the polar tube interacts with the spore wall in N. bombycis (data not shown). In previous studies, E. cuniculi spores were shown to interact with host cells through the exospore protein Enp1 to modulate the invasion process (17, 32, 33). The interaction between the mannosylation of PTP1 and some unknown host cell mannose-binding molecule was important to the infection of Encephalitozoon hellem (48). In this study, immunolocalization analysis showed that SWP5 is a spore wall protein distributed at the region of the polar tube (Fig. 5). Importantly, the results showed that SWP5 interacted with PTP2 and PTP3 (Table 1; Fig. 6 and 7). These data indicate that the polar tube interacts with the spore wall in N. bombycis. Some photographic evidence showed that the polar tube is always enclosed by the sporoplasm and adheres to the inner spore wall by an undefined interaction. This adherence may be important for keeping the stability and order of the cytoplasmic organelles. Once the long polar tube is separated from the spore wall in N. cera (data not shown).

### DISCUSSION

The microsporidian spore wall consists of an electron-dense exospore containing primary proteins, an electron-lucent endospore composed of chitin and proteins, and an inner plasma membrane (3, 9, 22, 43, 46). To date, five spore wall proteins have been identified in the mammalian microsporidia E. intestinalis and E. cuniculi: the exospore proteins SWP1 and SWP2 and the endospore proteins Enp1, Enp2, and EcCDA (4, 6, 16, 26, 47). In the insect microsporidian N. bombycis, four spore wall proteins (SWP32, -30, -26, and -25) have been identified in our previous studies (23, 44, 45). A novel exospore protein (NbSWP5) was identified and could protect spores from phagocytic uptake by cultured insect cells (30). In the present study, SWP5 was localized to the exospore and the polar tube in N. bombycis (Fig. 4 and 5). Meanwhile, the SWP5 protein sequence has no known functional domains and low sequence similarity with proteins in other eukaryotes, except for one in N. ceranae. SWP5 also shows no sequence similarity with other known spore wall proteins in N. bombycis (data not shown).

In previous studies, purification of anti-swsp5 serum was used to germinate spores by use of 0.1 mol/liter K₂CO₃ (Fig. 8). A significant difference was found between the samples incubated with anti-SWP5 serum, mouse negative serum, and PBS treatment. These results indicate that the extruding polar tube (Fig. 7B1 to B3).

### TABLE 1 LC-MS/MS analysis of polar tube protein tryptic peptides of N. bombycis

| GenBank accession no. | pI/molecular mass (kDa) | % Coverage | m/z | Overlap/total | Charge | Molecular mass (MH⁺) (Da) | Peptide sequence | Difference (MH⁺) (Da) |
|-----------------------|------------------------|------------|-----|---------------|--------|-------------------------|-----------------|---------------------|
| PTP2                  | HQ881498               | 9.69/31.4  | 56.12| 1,172.7       | 2/22   | 1,841.0974              | AKEAYFVNAIGEVSTK | 1.29640            |
|                       |                        |            |      | 1,032.4       | 15/20  | 1,280.4488              | AVCNIQYINGK     | -2.73520           |
|                       |                        |            |      | 2,170.0       | 23/28  | 1,641.8630              | EAYFNIAIGEVSTK  | 0.56030            |
|                       |                        |            |      | 211.5         | 17/26  | 1,611.7350              | HIPANNNPAEQR    | -1.04170           |
|                       |                        |            |      | 777.4         | 19/24  | 1,552.8689              | IM*LAANIQYHFIK  | 1.20290            |
|                       |                        |            |      | 1,175.0       | 17/24  | 1,536.8650              | ILAANIQYHFIK    | 1.29550            |
|                       |                        |            |      | 809.0         | 18/28  | 1,739.9082              | KIIPANNNPAEQR   | 1.25520            |
|                       |                        |            |      | 1,249.5       | 26/52  | 1,681.0418              | KIM*LAANIQYHFIK | -0.47120           |
|                       |                        |            |      | 1,214.4       | 31/72  | 2,221.4069              | LLNELKNEPEYTVTGEENK | 2.29490          |
|                       |                        |            |      | 1,846.5       | 36/92  | 2,809.1207              | LLNELKNEPEYTVTGEENKVFK | 0.55470       |
|                       |                        |            |      | 646.9         | 18/24  | 1,510.5416              | NEPEYTVTGEENK   | 0.48860            |
|                       |                        |            |      | 975.2         | 22/34  | 2,098.2550              | NEPEYTVTGEENKVFK | 0.80340            |
|                       |                        |            |      | 1,251.4       | 24/42  | 2,467.6700              | NYDFVLNISNTTVSEPGEK | 0.11100         |
|                       |                        |            |      | 674.9         | 18/44  | 2,595.8429              | NYDFVLNISNTTVSEPGEK | -0.61110        |
|                       |                        |            |      | 397.6         | 18/30  | 1,746.8912              | STPTQATGPTINNECK | 0.56610            |
|                       |                        |            |      | 733.3         | 15/24  | 1,469.6919              | AQQQMLPAVVDPR   | 0.32090            |
|                       |                        |            |      | 1,252.4       | 18/24  | 1,453.6925              | AQQQMLPAVVDPR   | 0.36150            |
|                       |                        |            |      | 1,527.6       | 16/22  | 1,408.6217              | KAVCNIQYINGK    | 0.48770            |
|                       |                        |            |      | 824.9         | 15/18  | 1,059.1586              | QAQIAIETAR      | 0.23160            |
| PTP3                  | JF739554               | 6.73/150.3 | 4.56 | 897.4         | 17/30  | 1,800.9430              | TFDIEESVNVEAYVK | 0.01280            |
|                       |                        |            |      | 771.7         | 16/28  | 1,712.8382              | YPTVADVEEFSLVVR | -0.36580           |
|                       |                        |            |      | 947.2         | 19/34  | 2,015.2330              | ALADSLGMEEDFIQFAR | 0.58210         |
|                       |                        |            |      | 297.1         | 15/20  | 1,272.3901              | AVEAQLDEIQR     | -0.66390           |
|                       |                        |            |      | 1,221.8       | 22/42  | 2,099.2482              | SDQVJAPNGGNTSALSQATAPR | -0.27280        |
|                       |                        |            |      | 1,059.7       | 13/16  | 902.9755               | VAAENATAR       | 0.15650            |
|                       |                        |            |      | 535.7         | 13/24  | 91.467.1600             | VGLSITDFNTMLR   | -1.03300           |

* a, methionine oxidation.
tered, the spore probably becomes inactive. However, the mechanism is unclear at present. The evidence showing SWP5 interacting with the polar tube probably explains the phenomenon of the polar tube adhering to the spore wall in microsporidia, but further data are needed to confirm this possibility.

For the microsporidia, PTP1, PTP2, and PTP3 are the major proteins present in the polar tube. In this study, SWP5 was localized to the region of the polar tube (Fig. 5) and interacted with PTP2 and PTP3 (Fig. 6). More interestingly, the anti-SWP5 serum could bind to the polar tube (Fig. 7). These data suggest that the polar tube may be composed of the new protein SWP5. In general, the spore wall and the polar tube are two independent organelles in microsporidia. SWP5 was localized to the spore wall but also to the polar tube. We hypothesize that the polar tube is an organelle in the spore wall and participates in integrated cell wall formation. The hollow polar tube is probably a result of a portion of the cell wall becoming embedded in the spore during evolution.

Successful microsporidian invasion occurs through extrusion of the unique polar tube. Preincubation or continuous culture of *E. cuniculi* or *N. bombycis* with an antibody against the exospore protein resulted in a reduction of the germination rate and reproduction *in vitro* (13, 49). To explain the reduction of the germination rate, some hypotheses have been made: the exospore protein antibody may limit or exacerbate the spore extrusion of the coiled polar filament (49), or the exospore protein antibody may target neutralization-sensitive epitopes in *N. bombycis* spores (49). As is well known, some cell surface proteins sense extracellular stimuli as part of a mechanism to protect the cell or as receptors to mediate invasion in pathogens (15, 28). The spore wall surface protein may be involved in the initiation of polar tube extrusion during the mi-

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**FIG 7** Immunofluorescence analysis of SWP5 interaction with the polar tube. (A1 to A3) Purified mature spores were induced to germinate with K₂CO₃. The extruded polar tubes were treated with anti-SWP5 mouse polyclonal antibody and then incubated with FITC-conjugated goat anti-mouse IgG (Sigma) secondary antibody. (B1 to B3) The spores were incubated with anti-SWP5 mouse polyclonal antibody, germinated in K₂CO₃ solution, and finally incubated with FITC-conjugated goat anti-mouse IgG (Sigma) secondary antibody. (A1 and B1) Germinated spore stained with DAPI. Arrowheads mark a nongerminated spore with bright green fluorescence in panel B2 but no green fluorescence signal in panels B1 and B3. Panels A3 and B3 are merged images. The anti-SWP5 serum was used at a 1:100 dilution. The FITC-conjugated IgG was used at a 1:64 dilution. Magnification for all images, ×1,000. Bar, 3 μm.

**FIG 8** Effect of SWP5-specific antibody on spore germination. Mature spores were incubated with anti-SWP5 serum and mouse negative serum for 2 h at 28°C, and then germination was induced with 0.1 mol/liter K₂CO₃ for 2 h at 28°C. After DAPI staining, spores were examined and counted, and the germination rate was determined. Statistically significant differences are indicated with asterisks (*P < 0.001). Bars represent standard deviations for three independent replicates.
microsporidian infection process (41, 42). In this study, SWP5 localized to the region of the polar tube (Fig. 4 and 5) and interacted with PTP2 and PTP3 (Table 1; Fig. 6 and 7). Anti-SWP5 serum resulted in a reduction of the germination rate (Fig. 8). Taking these data together, we speculate that SWP5 is a surface receptor responsible for alkaline (K₂CO₃) stimulation signals, their transduction into the cell interior, and disruption of the interaction between the polar tube and the spore wall and of release of the polar tube.

In conclusion, this is the first report to explore the interaction between the spore wall protein SWP5 and the polar tube in the microsporidian N. bombycis. It provides important data revealing the mechanism of polar tube adherence to the spore wall. Identification of protein-protein interactions between spore proteins and the polar tube would be helpful in understanding the invasion mechanism of microsporidia.

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