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Identification and characterization of a microneme protein (NcMIC6) in *Neospora caninum*

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Abstract *Neospora caninum*, an Apicomplexa parasite, is the causative agent of neosporosis. As described for other members of Apicomplexa, microneme proteins (MICs) play a key role in attachment and invasion of host cells by *N. caninum*. Herein we identified *N. caninum* microneme protein 6 (NcMIC6) that is orthologous to *Toxoplasma gondii* microneme protein 6 (TgMIC6). The open reading frame of the NcMIC6 gene is 984 bp and encodes a 327 amino acid peptide. Sequence analysis showed that NcMIC6 included a signal peptide, a transmembrane region, three epidermal growth factor-like (EGF) domains, and two low complexity regions. Antibodies raised against recombinant NcMIC6 recognized an approximately 35-kDa native MIC6 protein in Western blots of *N. caninum* tachyzoites. Immunofluorescence analysis showed that NcMIC6 had a polar labeling pattern, which was consistent with localization of micronemes in the apical region. Pulse invasion assays showed that NcMIC6 translocated from the apical tip to the posterior end of the parasites. Secretion assays demonstrated that NcMIC6 was released into the supernatants. Importantly, it was clearly revealed by co-immunoprecipitation that NcMIC6 formed a complex with other two soluble microneme proteins (NcMIC1 and NcMIC4). In conclusion, identification and characterization of the novel microneme protein NcMIC6 may contribute to understanding how this protein functions during the parasite motility and host cell invasion.

Introduction

Neospora caninum is an obligate intracellular parasite belonging to phylum Apicomplexa. This parasite has a worldwide distribution and causes neosporosis in many species, including dogs, cattle, sheep, goats, horses, and deer (Dubey and Lindsay 1996; Dubey et al. 2007). Neosporosis is a major cause of congenital deficiency and abortion in cattle and causes neuromuscular disease in the intermediate and definitive host. Previous reports showed that 12–42 % of aborted fetuses from dairy cattle were infected with *N. caninum* (Dubey 2003). Due to its high prevalence and association with abortion in cattle, neosporosis represents a serious threat to the dairy and livestock industries and can result in significant economic losses (Dubey et al. 2007). Additionally, although humans are not regarded as intermediate hosts of *N. caninum*, serological testing has indicated that humans might be susceptible to *N. caninum* infection (Tranas et al. 1999). Considering the adverse effects in animal husbandry and the potential harm to humans, there is an urgent need for increased understanding of *N. caninum* to develop effective measures and to prevent infection and transmission.

For all members of the phylum Apicomplexa, the key step in infection is host cell invasion. Invasion propelled by the parasite is an active multi-step and rapid process that leads to the formation of a parasitophorous vacuole (PV) where the parasite survives and proliferates. There are three secretory organelles (micronemes, rhoptries, and dense granules) that play a key role in cell invasion by Apicomplexa. Micronemes are small vesicles that cluster in the apical portion of the zoite and rapidly secrete a large number of proteins when initial contact is made between the parasite and the host cell. Microneme proteins (MICs) act as

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major cellular adhesion factors for host cells and participate in parasite recognition, reorientation, and entry. *Toxoplasma gondii*, an opportunistic pathogen, has been studied for many years and thus serves as a model organism in phylum Apicomplexa. At least 20 MICs have been well characterized from this parasite. MICs are defined as having the following characteristics based on *T. gondii* researches: First, most soluble and transmembrane (TM) MICs possess one or more adhesive domains responsible for protein–protein or protein–carbohydrate interactions (Brecht et al. 2001; Lourenco et al. 2001); second, several TM MICs function as "escorters" and assist in trafficking of soluble proteins to the correct micronemal target (Reiss et al. 2001; Meissner et al. 2002); third, some TM MICs depend on interactions with soluble proteins in which they tend to accumulate in the early compartments of the secretory pathway when they form into incorrect complexes (Reiss et al. 2001; Huynh et al. 2003); and finally, many MICs cleave their pro-peptides by proteolysis and mature in the Golgi or post-Golgi compartment (Harper et al. 2006; El Hajj et al. 2008). Although numerous reports have described the *T. gondii* MICs (TgMICs), less information is available for *N. caninum* MICs (NcMICs). To date, six different NcMICs have been characterized and reported. NcMIC2, the first microneme protein identified in *N. caninum*, is homologous to TgMIC2 and belongs to the thrombospondin-related anonymous protein family (Lovett et al. 2000). The second microneme protein identified is NcMIC3, which binds both the surface of the parasite and the surface of the host cell via its epidermal growth factor-like (EGF) domains (Naguleswaran et al. 2001). The third identified microneme protein, NcMIC10, does not contain an adhesive domain, similar to TgMIC10 described by Hoff et al. (2001). It can be used as an excellent marker for the diagnosis of neosporosis (Yin et al. 2012). Both the fourth and fifth microneme proteins, NcMIC1 and NcMIC4, were reported by Keller et al. (2002, 2004). NcMIC1 binds to sulfated glycosaminoglycans on host cells (Keller et al. 2002), while NcMIC4 possesses unique lactose-binding properties (Keller et al. 2004). The last identified is *N. caninum* apical membrane antigen 1 (NcAMA1), an antigen that is cross-reactive between *N. caninum* and *T. gondii* and plays a key role in parasite invasion (Zhang et al. 2007).

In the present study, we describe the initial molecular characterization of NcMIC6, a transmembrane MIC similar to TgMIC6. We investigate the subcellular localization of NcMIC6 and the effects of temperature and reagents on NcMIC6 secretion.

Materials and methods

Ethics statement

All experiments with animals in this study were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of China. All experimental procedures were approved by the Institutional Animal Care and Use Committee of China Agricultural University (Beijing Laboratory Animal employee certificate ID 18049).

Sequence analysis

The gene sequence and amino acid sequence of *N. caninum* NcMIC6 were obtained from ToxoDB (http://toxodb.org/toxo/; Gene ID: NCLIV_061760), which were orthologous to TgMIC6. To analyze the amino acid sequence of NcMIC6, we used online servers: (1) The signal peptide and its cleavage sites were predicted by SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/); (2) potential transmembrane regions of NcMIC6 were analyzed by TMHMM (http://www.cbs.dtu.dk/services/TMHMM/); and (3) predicted topological structural information of NcMIC6 was analyzed using SMART (http://smart.embl-heidelberg.de).

Parasite culture and preparation

Tachyzoites of the Nc-1 strain were propagated on Vero cells grown in Dulbecco's modified Eagle's medium (DMEM) plus L-glutamine, 10 % fetal bovine serum (FBS), penicillin (50 U/ml), and streptomycin (50 μg/ml) at 37 °C in a 5 % CO_2 environment. Parasites were purified and harvested as described previously (Zhang et al. 2007). Freshly egressed tachyzoites were purified from contaminating host cell debris by syringing three times with a 27-gauge needle at 4 °C, filtering through a 5-μm pore filter (Millipore, USA), washing twice with cold phosphate-buffered saline (PBS), and finally centrifuging for 10 min at 3,000×*g* at 4 °C.

Cloning and sequencing of the NcMIC6 gene

The genomic DNA was extracted from purified Nc-1 tachyzoites (~1×10^7) using QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions and then used as the template for NcMIC6 cloning. The full length NcMIC6 gene was generated by PCR using specific primers P1 (5′-ATGTGGCTCTTCCGGAACTG-3′) and P2 (5′-TTAATCCCATGTTTTGCTATCC-3′). The PCR product was purified on a 1 % agarose gel and cloned into the pEASY-simple T1 cloning vector (TransGene, China). The sequence was determined by a commercial sequencing service (Invitrogen, Beijing).

Recombinant NcMIC6 expression, purification, identification, and polyclonal antiserum production

To obtain proteins for antibody generation, the DNA fragment of NcMIC6 lacking the signal peptide and transmembrane

region was PCR-amplified using primers P3 (5′-CGGGAT CCGAAGGGTTTCTGTGGCTACAG-3′) and P4 (5′-CCGCTCGAGAGCAGATCCTTTACTTTCTTC-3′) with the insertion of *Bam*HI and *Xho*I restriction enzyme sites (underlined). The amplified product was digested with *Bam*HI and *Xho*I, ligated into a *Bam*HI/*Xho*I-digested pET-28a pro-karyotic expression vector (Novagen, USA), and then trans-formed into *Escherichia coli* for expression. Recombinant protein NcMIC6 (rNcMIC6) expression and purification were performed using HisTrap FF purification columns (Novagen, Germany) according to the manufacturer's instructions. The purified protein was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immu-noblotting with anti-*N. caninum* polyclonal sera. To produce polyclonal antibodies against NcMIC6, 6–8-week-old female BALB/c mice were immunized with 100 μg purified rNcMIC6 in an equal volume of Freund's complete adjuvant (Sigma, USA), followed by two successive boosts at 2-week intervals of 50 μg purified rNcMIC6 in an equal volume of Freund's incomplete adjuvant (Sigma, USA).

SDS-PAGE and Western blotting

Purified Nc-1 tachyzoites (2×10^7) were suspended in cold PBS with 1 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma, USA), sonicated in an ice bath, and then boiled in SDS sample buffer with β-mercaptoethanol for 10 min. Pro-teins were separated in 12 % polyacrylamide gels. Following electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA) by semi-dry electrophoretic transfer in Tris-glycine buffer (pH 8.3). The membranes were blocked with PBS containing 5 % (*w/v*) non-fat dry milk and incubated with primary antibody solution for 1 h at 37 °C. After washing with PBS containing 0.05 % Tween-20, the membranes were incubated with secondary an-tibody conjugated to horseradish peroxidase for 1 h. After further washing, the labeled proteins were visualized with ECL chemiluminescence reagents (CoWin, China).

Immunofluorescence assay (IFA)

For analysis of intracellular parasites, human foreskin fibro-blast (HFF) cells were grown on glass coverslips and infected with freshly harvested Nc-1 tachyzoites for 16–24 h. The coverslips were fixed with 4 % (*v/v*) paraformaldehyde (PAF) for 30 min, permeabilized with 0.25 % Triton X-100 in PBS for 20 min, and then blocked with 3 % BSA in PBS for 20 min at room temperature. The samples were incubated with primary antibodies diluted 1:100 in 1 % BSA-PBS for 1 h at 37 °C. After extensive washing in PBS, secondary antibodies conjugated to fluorescein isothiocyanate (FITC, Proteintech, USA) or Texas Red (Proteintech, USA) diluted 1:100 in 1 % BSA-PBS were added for 1 h at 37 °C. Finally, the parasites

were examined under a laser confocal scanning microscope (Leica TCS SP5 II, Germany) and all images were processed by employing the LAS AF Lite software (Leica, Germany).

To examine the distribution of NcMIC6 during invasion, HFF cells grown on sterilized glass coverslips were pulse infected as described previously (Carruthers and Sibley 1997). Purified Nc-1 tachyzoites were spotted onto glass cov-erslips and incubated for 15 min at 4 °C. Then, the coverslips were incubated for 45 min at 37 °C, fixed with 4 % (*v/v*) PAF at different invasion times, and blocked with 3 % BSA in PBS for 20 min at room temperature. The samples were incubated with rabbit anti-*N. caninum* polyclonal sera diluted 1:100 in 1 % BSA-PBS for 1 h at 37 °C. After extensive washing in PBS, the coverslips were permeabilized for 20 min with PBS containing 0.002 % saponin and then incubated with mouse anti-rNcMIC6 polyclonal sera (diluted 1:100). The secondary antibodies conjugated to FITC or Texas Red (diluted 1:100) were added for 1 h at 37 °C. Confocal images were collected with a Leica laser scanning microscope, and all images were processed by employing the LAS AF Lite software.

Co-immunoprecipitation

The co-immunoprecipitation procedure was performed as described previously (Reiss et al. 2001). Briefly, 50 μl of polyclonal antisera was added to protein A-coated sepharose beads in 1 ml PBS, incubated for 2 h at 4 °C under agitation, and then washed three times in cold PBS. Freshly released tachyzoites (2×10^8) were harvested, washed three times in PBS, and lysed in 1 ml RIPA buffer (150 mM Tris pH 8.0, 150 mM NaCl, 0.5 % sodium deoxycholate, 1 % Triton X-100, and 0.1 % SDS) in the presence of 1 mM PMSF. After centrifugation at 12,000 rpm for 20 min at 4 °C, the parasite lysates were incubated with antibody-coated beads overnight at 4 °C under agitation. Lysates incubated in the absence of antibodies and antibodies incubated in the absence of lysates were included as controls. Finally, the beads were collected by centrifugation and washed three times in 1 ml cold PBS, and 30 μl of 2× loading buffer with β-mercaptoethanol was added before boiling and loading onto SDS-PAGE.

Secretion assay

The secretion assay was performed according to an online protocol (http://www.sibleylab.wustl.edu/pdf/ToxoSecretion_assay.pdf). Briefly, purified Nc-1 tachyzoites (2×10^8) suspended in 100 μl Hank's balanced salt solution (HBSS) were transferred to a microfuge tube, 1 μl of 100 % ethanol was added, and the mixtures were incubated at various tem-peratures in a water bath for 10 min. The effects of three additional components (400 nM calcium ionophore A23187, 5 % FBS, and 20 mM NH$_4$Cl) on NcMIC6 secretion were also assessed by adding them to the HBSS. After removal of the

tachyzoites by centrifugation (2,000 rpm, 10 min, 4 °C), the supernatants were processed for SDS-PAGE followed by Western blotting.

Results

Genetics and bioinformatics of NcMIC6

Using specific primers based on the putative NcMIC6 gene sequence predicted by ToxoDB, a 984-bp PCR product was amplified. Sequencing showed that the nucleotide sequence amplified from the Nc-1 strain perfectly matched the putative NcMIC6 gene predicted online. The target gene had 69 % nucleotide similarity with TgMIC6, so we designated it NcMIC6. The NcMIC6 gene encoded a precursor protein of 327 amino acids (aa) with a predicted molecular mass of 34.1 kDa. The overall amino acid sequence similarity was high, with 48 % identity shared between NcMIC6 and TgMIC6. Using SignalP 4.1, we predicted that the N-terminal portion of NcMIC6 contained a signal peptide of 14 aa, which was shorter than the signal peptide found on TgMIC6 (24 aa). Cleavage of the signal peptide sequence would yield a mature protein of 313 aa with a theoretical molecular mass of 32.5 kDa. TMHMM showed that the only putative transmembrane domain of NcMIC6 was 265–287 aa and was 86 % identical to the same region in TgMIC6. Using SMART, we identified three EGF domains located at 38–79 aa, 97–138 aa, and 147–191 aa and two low complexity regions located at 215–229 aa and 250–286 aa (Fig. 1).

Detection of NcMIC6 protein in *N. caninum* tachyzoites

To investigate native NcMIC6, a recombinant version of NcMIC6 protein (rNcMIC6, 15–264 aa) was expressed as a His$_6$-tagged fusion protein in *E. coli*, purified by

Fig. 1 The polypeptide sequence of NcMIC6 aligned with homologous protein TgMIC6. The signal peptide is marked in *italics*. EGF domains are *underlined*. The transmembrane region is indicated in *bold font*. The low complexity regions are indicated with *asterisks*

Ni^{2+}-affinity chromatography, and used to immunize animals for polyclonal antisera production. Purified rNcMIC6 had a molecular weight of approximately 38 kDa as determined by SDS-PAGE. The resulting Western blotting analysis using anti-rNcMIC6 polyclonal sera and Nc-1 tachyzoite lysates showed that two bands (35 and 40 kDa) of NcMIC6 were recognized (Fig. 2a). The difference between the two bands may be due to the cleavage of the first EGF domain akin to TgMIC6 (Meissner et al. 2002). The observed molecular weights are slightly higher than the theoretical molecular weights, possibly due to post-translational modification of the protein. Additionally, anti-rNcMIC6 polyclonal sera failed to detect TgMIC6, indicating that the MIC6 proteins do not share common immunogenicity between *N. caninum* and *T. gondii*.

Subcellular localization of NcMIC6

The homology between NcMIC6 and TgMIC6 suggested that NcMIC6 was a microneme protein of *N. caninum*. To confirm that NcMIC6 is located in the microneme and determine its localization in parasites, double immunofluorescence employing anti-rNcMIC6 antibodies and polyclonal sera directed against NcMIC1 or *N. caninum* was carried out on infected HFF (Fig. 2b). In the resulting images, NcMIC6 was found predominately at the apical tip of the intracellular tachyzoites and co-localized with NcMIC1. After 18 h post-invasion, the tachyzoites were replicating in the PV, and NcMIC6 seemed to be more pronounced and strictly located

in the parasite's apical region, consistent with microneme localization during tachyzoite replication.

A previous study showed the distribution of NcMIC3 in other compartments of tachyzoites (Naguleswaran et al. 2002). To determine whether this phenomenon could occur with NcMIC6 in tachyzoites of *N. caninum*, we purified tachyzoites from cultures without any cell debris and observed the distribution of NcMIC6 at different time points prior to and following host cell invasion by IFA (Fig. 3). Following purification at 4 °C, tachyzoites were incubated at 37 °C for investigation of the potential appearance of NcMIC6 on the parasite surface. Anti-rNcMIC6 immunofluorescence staining of tachyzoites fixed in 4 % paraformaldehyde after purification (0 min) revealed that no labeling was visible. After incubation at 37 °C for 5 to 10 min, NcMIC6, which exhibited punctate staining at the apical region of the parasites, was slightly visible. Subsequently (after 10–25 min), NcMIC6 was still accumulated at the anterior of the parasites, with a higher density of labeling. At later time points (30–45 min), when tachyzoites of *N. caninum* nearly completed invasion, a fraction of NcMIC6 clustered near the posterior region of the parasites. This retrograde movement of NcMIC6 is consistent with NcMIC3 observed by Naguleswaran et al. (2001). Collectively, these results show that NcMIC6 is targeted to the micronemes and can translocate from the apical tip of the tachyzoite to its posterior region.

NcMIC6 secretion

To investigate whether the NcMIC6 could secrete into the supernatants of medium, freshly egressed tachyzoites were
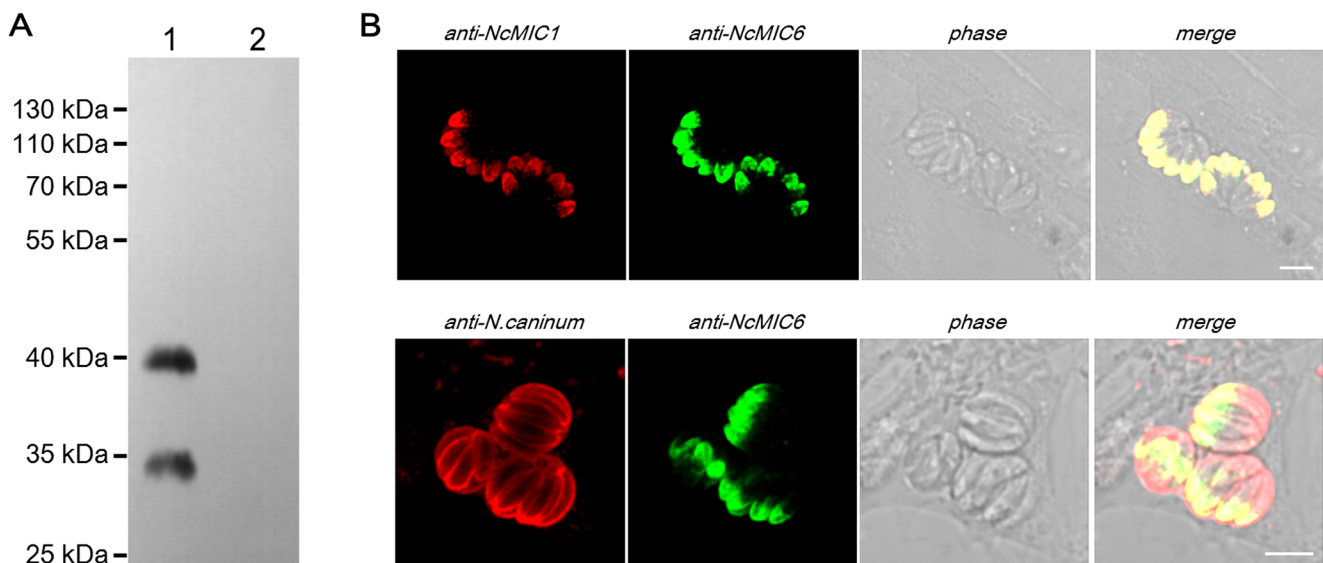

Fig. 2 Western blotting analysis and subcellular localization of NcMIC6. **a** Western blotting analysis of lysates from *N. caninum* (*lane 1*) and *T. gondii* (*lane 2*) using anti-rNcMIC6 polyclonal sera. **b**

Immunofluorescence analysis of intracellular Nc-1 tachyzoites reveals that NcMIC6 is located at the apical region of tachyzoites. *Bar*, 5 μm

Fig. 3 NcMIC6 translocates from the apical tip of the tachyzoite to the posterior end (arrowhead). NcMIC6 was detected using anti-rNcMIC6 polyclonal sera followed by FITC-conjugated secondary anti-mouse antibody. *N. caninum* was stained with anti-*N. caninum* polyclonal sera followed by Texas Red-conjugated secondary anti-rabbit antibody
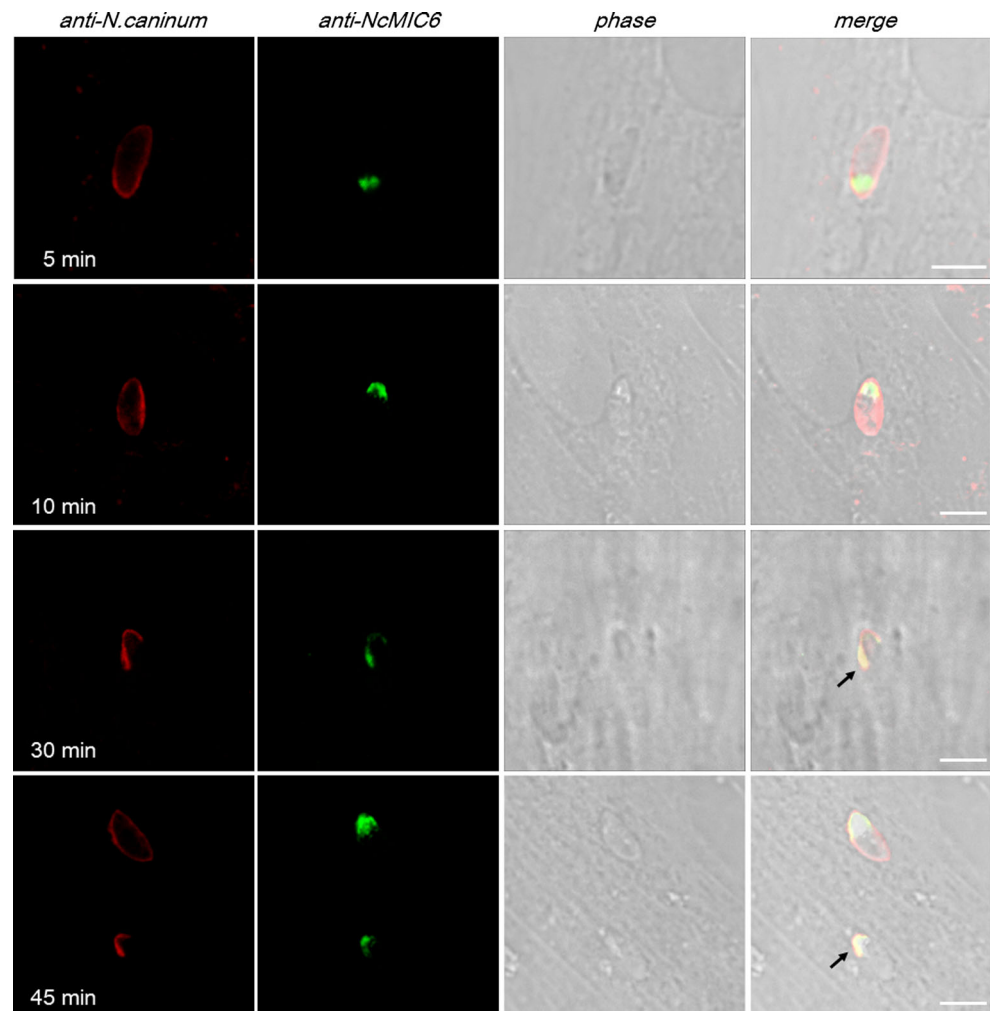
incubated in the medium at different temperatures for 30 min. The supernatants were collected following incubation and assessed for the presence of NcMIC6 by Western blotting using anti-rNcMIC6 polyclonal sera. As shown in Fig. 4a, NcMIC6 was detected in supernatants in comparison with soluble protein NcMIC4.

Previous studies indicated that the secretion of micronemes can be stimulated by treating apicomplexan parasites with different reagents (Carruthers and Sibley 1999). To assess whether the secretion of NcMIC6 is similarly stimulated, we examined four reagents (e.g., ethanol, calcium ionophore A23187, 5 % FBS, and NH₄Cl) for their ability to induce secretion. As shown in Fig. 4b, NcMIC6 was discharged into the supernatant following incubation with any of the reagents. It is noteworthy that the band at ~25 kDa detected in the supernatants by Western blotting was different from the two bands detected in parasite lysates, indicating that NcMIC6 undergoes a proteolytic processing event upon secretion. Taken together, our results show that NcMIC6 can secrete into the

supernatants, and its secretion can be induced with different reagents.

NcMIC6 physically interacts with NcMIC1 and NcMIC4

Previous research showed that transmembrane microneme protein TgMIC6 interacted with TgMIC1 and TgMIC4, and its absence caused complete mistargeting of the two soluble microneme proteins TgMIC1 and TgMIC4 to the microneme (Reiss et al. 2001). Due to the high amino acid similarity between NcMIC6 and TgMIC6 and the close genetic relationship between *N. caninum* and *T. gondii*, we speculated that a complex composed of NcMIC1, NcMIC4, and NcMIC6 might exist in *N. caninum*. To confirm this hypothesis, *N. caninum* tachyzoites were lysed under mild conditions and then subjected to immunoprecipitation with different anti-NcMIC polyclonal sera. Western blotting analysis revealed that both NcMIC1 and NcMIC4 coprecipitated with anti-rNcMIC6 polyclonal sera. Similarly, anti-rNcMIC1 polyclonal sera coprecipitated NcMIC4 and NcMIC6, and
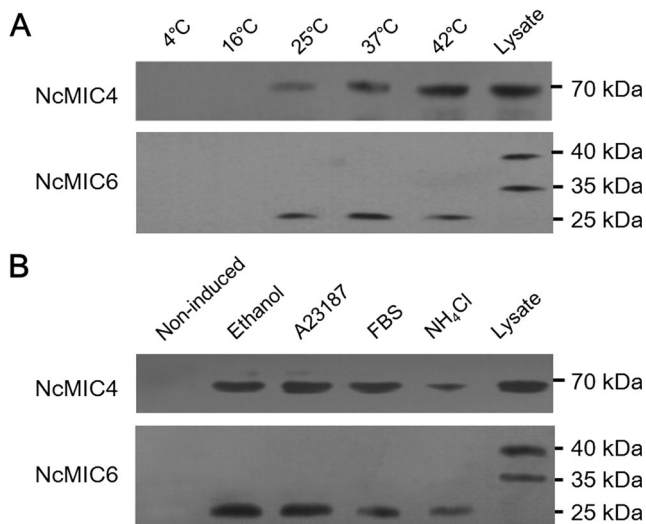
Fig. 4 Effects of temperature and reagents on NcMIC6 secretion. **a** Western blotting analysis shows that NcMIC6 secrete into the supernatants compared to the characterized microneme protein NcMIC4. **b** Western blotting with anti-rNcMIC6 antibodies shows that NcMIC6 is discharged into the supernatant after the addition of reagents followed by incubation for 10 min at 37 °C

polyclonal anti-rNcMIC4 co-immunoprecipitated NcMIC1 and NcMIC6 from tachyzoite lysates (Fig. 5). Collectively, these data indicate that NcMIC1, NcMIC4, and NcMIC6 form a complex in *N. caninum* tachyzoites.

Discussion

Micronemes, which populate the apical portion of apicomplexan parasites, are the smallest of three secretory organelles that secrete proteins during the invasive stages of apicomplexan parasites. The multistep process of active invasion of host cells, which seems to be conserved among the apicomplexan, is preceded by apical tip initial contact with the host cell surface and coincides with the secretion of micronemes (Carruthers and Sibley 1997; Sonda et al. 2000; Carruthers and Boothroyd 2007). Next, microneme proteins distribute over the surface of the parasites and are involved in the interaction between the parasites and the host cells (Hehl et al. 2000; Tyler et al. 2011). Additionally, microneme proteins have been demonstrated to be necessary for parasite gliding motility (Sultan et al. 1997). Thus, micronemes and their contents are essential for effective motility, migration, adhesion, and host cell invasion. Although numerous microneme proteins in several members of the apicomplexan were identified, only six microneme proteins of *N. caninum* have been reported. Herein, we reported the identification and partial characterization of a microneme protein termed NcMIC6. Identification of the NcMIC6 gene, which is orthologous to TgMIC6 (TGGT1_218520), was accomplished by searching the gene sequence in ToxoDB. The

NcMIC6 protein shares approximately 47 % sequence identity with TgMIC6, and its predicted characterizations using online software (e.g., signal peptide, transmembrane region, EGF domains, and low complexity region) are similar to TgMIC6. These results indicate that NcMIC6 may have functions similar to TgMIC6.

In the present study, sequencing analysis of NcMIC6 using online services shows that NcMIC6 has an N-terminal signal peptide and a C-terminal transmembrane region, suggesting that it is routed to micronemes via the endoplasmic reticulum and that it is an integral membrane protein. The EGF domain is an evolutionarily conserved protein domain that is found in a large number of mostly animal proteins. It comprises 30–40 aa residues and typically contains six cysteine residues that form three disulfide bonds. EGF domains have been found in many apicomplexan MICs. The role of EGF domain is not yet fully understood, but one of its significant features is that it is presented in secreted proteins or in extracellular portions of transmembrane proteins involved in mediating adhesive properties (Davis 1990). It is noteworthy that NcMIC6 contains three continuous EGF domains, indicating that it might have the potential ability to adhere to other parasite proteins or ligands of the host cell. Additionally, the EGF domain appears to play a central role in immune responses. A recombinant protein containing the EGF domains from *Plasmodium yoelii* meorozoite surface protein-1 (MSP-1) was used as a subunit vaccine to immunize mice, resulting in the protection of the immunized mice against a lethal challenge by the same parasite strain (Ling et al. 1997). Therefore, a recombinant protein containing the EGF domains from NcMIC6 might be a potential vaccine candidate against neosporosis, but the investigation of its immune effects is necessary in future studies.

Apicomplexan microneme secretion is regulated by cytoplasmic calcium levels and can be triggered by the use of calcium ionophores, ethanol, or acetaldehyde or by increases in temperature (Carruthers et al. 1999). When apicomplexan parasites contact host cell surfaces, their cytoplasmic calcium levels significantly increase (Vieira and Moreno 2000). This phenomenon suggests that parasite calcium is critical for the process of attachment and invasion. In contrast, elevating or reducing the cytoplasmic calcium levels of host cells has no effect on parasites invasion. In our studies, four reagents that could affect cytoplasmic calcium levels were used to investigate the secretion of NcMIC6. Calcium ionophores and ethanol have been shown to transiently increase intracellular calcium levels of *T. gondii* and induce microneme secretion (Carruthers and Sibley 1999). FBS is able to stimulate microneme secretion of *Eimeria tenella* sporozoites (Bumstead and Tomley 2000). NH_4Cl is a weak base that transiently raises intracellular pH and calcium levels in *T. gondii* by alkalization of acidocalcisomes (Moreno and Zhong 1996). Previous studies have shown that TgMIC6
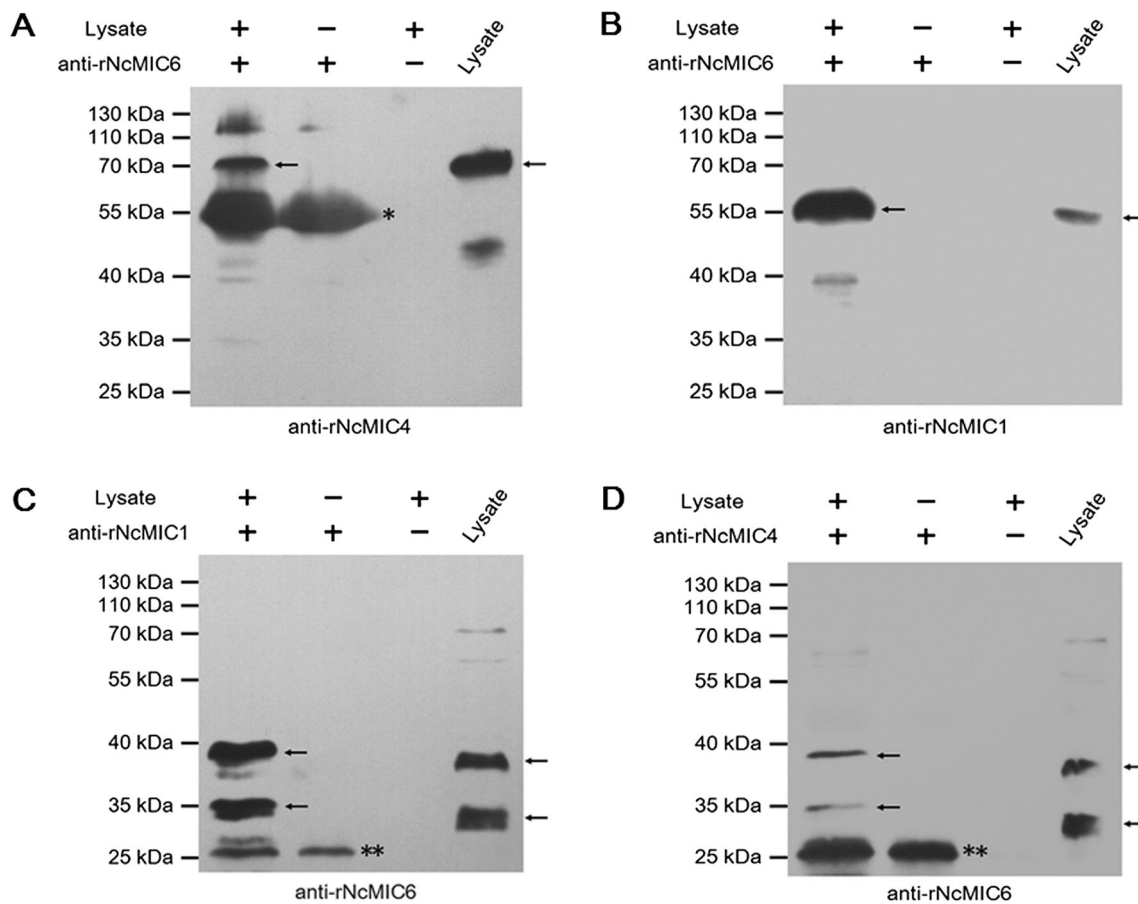
Fig. 5 NcMIC1, NcMIC4, and NcMIC6 physically interact. **a** Co-immunoprecipitation of a complex containing NcMIC4 from Nc-1 tachyzoite lysates with anti-rNcMIC6 polyclonal sera. NcMIC4 is indicated by an *arrow*. Heavy chain of anti-rNcMIC6 antibody is indicated with *asterisk*. **b** The co-immunoprecipitates were shown to contain NcMIC1 by Western blotting analysis using anti-rNcMIC1 polyclonal sera. NcMIC1 is indicated by *arrow*. **c** Co-immunoprecipitation of NcMIC1 from Nc-1 tachyzoite lysates with anti-rNcMIC6 polyclonal sera. **d** Co-immunoprecipitation of NcMIC4 with anti-rNcMIC6 poly-clonal sera. Light chain of anti-rNcMIC1 or anti-rNcMIC4 antibody is indicated with two *asterisks*. Controls included co-immunoprecipitation in the absence of lysate or in the absence of polyclonal sera

was N-terminally cleaved during its transport to microneme and then removed its C-terminal cytoplasmic tail before secretion (Meissner et al. 2002). In *T. gondii*, TgMIC6 N-terminal cleave site (QLS*ETP) between the first and second EGF domain resembles the pro-peptide cleave site (QLS*TFL) of TgM2AP (Meissner et al. 2002; Harper et al. 2006), suggesting that cleave sites (LS) of these proteins are conserved and might be cleaved by the same protease. By comparing the amino acid sequences of NcMIC6 and TgMIC6, we speculated that the cleavage occurred in the similar site (VLS*SSK) between the first EGF domain and the second EGF domain in NcMIC6. Further experiments will be necessary to confirm this possibility.

In *T. gondii*, some soluble and transmembrane microneme proteins assemble into protein complexes that perform functions in concert during tachyzoite invasion. To date, four types of complexes have been identified in the parasite (e.g., TgMIC1/MIC4/MIC6, TgMIC2/M2AP, TgMIC3/MIC8, and TgAMA1/RON2/RON4/RON5/RON8) (Rabenau et al. 2001;

Reiss et al. 2001; Meissner et al. 2002; Alexander et al. 2005). The selective participation of each of the four complexes in the attachment and invasion process have been investigated by generating conventional or conditional knockouts of the gene encoding components of the complexes (Sheiner et al. 2010). Our finding showed that NcMIC6 and TgMIC6 shared similar properties in amino acid sequences and co-immunoprecipitation profiles, confirming that the association of NcMIC1, NcMIC4, and NcMIC6 formed a stable complex. In *T. gondii*, the absence of TgMIC6 resulted in that TgMIC1 and TgMIC4 failed to accumulate in the micronemes (Reiss et al. 2001). The complete set of nuclear magnetic resonance assignments for the second EGF domain from MIC6 and its re-assignment in complex with the galectin-like domain from MIC1 have been reported (Saouros et al. 2008; Sawmynaden et al. 2008). Although the detailed function of NcMIC6 in *N. caninum* remains unexplained, at least one potential clue to its role suggests that NcMIC6 may function as an escorter, which is similar to the function of TgMIC6 (Reiss et al. 2001).

To study this function of NcMIC6, we constructed a NcMIC6 overexpression vector and electrotransfected it into Nc-1 tachyzoites. Unfortunately, no clones of Nc-1 stably over-expressing NcMIC6 were chosen. Thus, an important area for future investigations will be genetic strategies such as gene knockout to dissect the role of NcMIC6 in the life cycle of *N. caninum*.

In conclusion, in the present study, we identified and initially characterized a protein from *N. caninum* termed NcMIC6. The results of this study show that this protein is a transmembrane microneme protein that localizes to micronemes of *N. caninum* tachyzoites. This protein possesses three EGF domains that may be involved in mediating adhesive properties. Further molecular and functional characterizations of NcMIC6 in *N. caninum* are necessary, and its potential immunological role during the course of infection requires future investigation.

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