



Neospora caninum: Cloning and expression of a gene coding for cytokine-inducing profilin

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ABSTRACT

Profilins are actin-binding proteins that in *Toxoplasma gondii* stimulate innate immunity in mice by binding Toll-like receptors (TLR) on dendritic cells (DC) leading to release of inflammatory cytokines, primarily IL-12 and IFN- γ . The purpose of the present study was to characterize *Neospora caninum* profilin, termed NcProfilin. Recombinant NcProfilin was purified by affinity chromatography, and used to prepare specific antisera to allow characterization of native NcProfilin antigen in *N. caninum* tachyzoites. By immunoblotting, recombinant NcProfilin is 22 kDa, and is similar in size to the respective 22 kDa native protein. Immunofluorescence and immunoelectron microscopy localized native NcProfilin to the apical end of *N. caninum* tachyzoites. Incubation of recombinant NcProfilin with spleen cells from BALB/c mice induced release of IFN- γ . Also, injection of BALB/c mice with purified rNcProfilin elicited a strong IFN- γ and IL-12 responses at 6 and 24 h after injection indicating that NcProfilin may be an important protein in regulation of cytokine responses to *N. caninum*.

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1. Introduction

Neospora caninum is a protozoan that is a major cause of reproductive failure in dairy cattle worldwide (Dubey et al., 2007). Fetal infection occurs following one of two events: (1) an active primary infection of dams after they ingest *N. caninum* oocysts or (2) a reactivated infection in dams that harbor the *N. caninum* tissue cysts. Passage of *N. caninum* tachyzoites from mother to fetus after parasite recrudescence is termed “endogenous transplacental transmission” and is thought to represent the major route of infection (Trees and Williams, 2005). Substantial evidence in mice and cattle indicate that immunity to experimental *N. caninum* tachyzoite infection involves Th1-type responses, and is dependent on the release of cytokines such as IL-12 and IFN- γ (for review see Nishikawa et al., 2002; Innes et al., 2005). Also, a number of investigators have elicited protective immunity against *N. caninum* tachyzoite challenge by vaccination of mice with native and recombinant *N. caninum* proteins, such as NcSRS2, NcROP2, NcGRA7 (Nishikawa et al., 2001; Lundén et al., 2002; Cannas et al., 2003; Liddell et al., 2003; Jenkins et al., 2004a; Haldorson et al., 2005; Pinitkiatisakul et al., 2007; Ribeiro et al., 2009). Although a few studies have found protective immunity concomitant with a Th2 response (Haldorson

et al., 2005; Debache et al., 2009), most reports indicate that protection is associated with antigen-specific Th1-type responses, and release of cytokines IL-12 and IFN- γ (Staska et al., 2005; Debache et al., 2009). Vaccination of sheep with extracts of *N. caninum* tachyzoite native protein prior to or during pregnancy has yielded significant levels of protection against experimental neosporosis (O’Handley et al., 2003; Jenkins et al., 2004b). Studies in ruminants and mice indicate that the degree of immunity is dependent on the type of adjuvant used, possibly reflecting the particular immune response elicited (Lundén et al., 2002; Jenkins et al., 2004a; Pinitkiatisakul et al., 2005; Ribeiro et al., 2009). Recent studies in *Toxoplasma gondii*, a sister group to *N. caninum*, have found that a highly conserved protein, termed profilin, binds to TLR11, and activates dendritic cells (DC) to release IL-12, which is involved in production of IFN- γ and in differentiation of naive T lymphocytes to Th1 phenotype (Yarovinsky et al., 2005, 2006; reviewed in Lauw et al., 2005; Yarovinsky and Sher, 2006). The importance of IL-12 in resistance to *T. gondii* and *N. caninum* infection has been demonstrated by challenge studies in IL-12 $^{-/-}$ mice and in mice after *in vivo* IL-12 neutralization (Lauw et al., 2005; Yarovinsky and Sher, 2006). Also, mice deficient in TLR11 were incapable of responding to TgProfilin or to whole *T. gondii* tachyzoite antigen, and were highly susceptible to *T. gondii* challenge infection (Lauw et al., 2005; Yarovinsky and Sher, 2006). The purpose of the present study was to characterize *N. caninum* profilin, and determine whether it can stimulate release of inflammatory cytokines.

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2. Materials and methods

2.1. Cloning and expression of *NcProfilin* gene sequence

The cDNA coding for *NcProfilin* was amplified by RT-PCR of *N. caninum* tachyzoite total RNA using primers *NcPro-F*: 5'-AGGAATT CCGATGTCGGACTGGGATCCCGTT-3' and *NcPro-R*: 5'-GGCTCGAGTT AATAGCCAGACTG-3' which correspond to the respective ATG start site and TAA stop codon of the *NcProfilin* gene sequence (GenBank Accession No. BK006901). The RT-PCR amplification product was purified using a PCR isolation kit (Qiagen, Valencia, CA), digested with *EcoRI* and *XhoI* using standard procedures, and subjected to agarose gel electrophoresis. *NcProfilin* cDNA was excised from the gel after EtBr staining, purified using a Gel Purification kit (Qiagen), ethanol precipitated, and then ligated to *EcoRI*- and *XhoI*-digested pET28c plasmid expression vector (Novagen, Madison, WI). The ligation mixtures were introduced into *Escherichia coli* DH5 α using standard transformation procedures (Hanahan, 1983). Plasmid DNA was prepared from *E. coli* harboring recombinant pET28c-*NcProfilin* using a plasmid DNA mini-kit (Qiagen), and the *NcProfilin* sequence and reading frame was confirmed by DNA sequencing using pET28-specific primers. For recombinant protein expression studies, pET28c-*NcProfilin* plasmid was introduced into *E. coli* BL21 cells (Novagen) using standard transformation procedures (Hanahan, 1983). In preliminary optimization studies, the greatest recombinant *NcProfilin* production occurred by culturing the *E. coli* BL21 transformants in LB broth at 37 °C until OD₆₀₀ = 1.0, followed by induction with 1 mM IPTG for 4 h. After induction, the cultures were harvested by centrifugation, and protein extracted in native binding buffer (NBB, Invitrogen, Carlsbad, CA) containing PMSF by 3 freeze-thaws between a dry ice-ethanol bath and a 37 °C water bath. The *E. coli* protein extract was treated with 1 U/ml RNase and DNase for 30 min at room temperature and pelleted by centrifugation at 5000g for 30 min, and the supernatant and was subjected to NiNTA affinity chromatography to purify recombinant *NcProfilin* protein using procedures recommended by the manufacturer (Qiagen).

2.2. Characterization of native and recombinant *NcProfilin* by immunoblotting

Antisera specific for recombinant *NcProfilin* were prepared by a commercial company (Pacific Immunology, Ramona, CA) by immunizing 2 New Zealand White rabbits with 250 μ g NiNTA-purified recombinant *NcProfilin*/injection. *NcProfilin* was emulsified in Freund's Complete Adjuvant for the primary immunization (day 1) and in Freund's Incomplete Adjuvant for 3 subsequent booster immunizations (days 21, 42, 63). Venous blood was collected before the primary immunization, at each booster injection, and 3 weeks after the final immunization, and processed for antisera using standard procedures.

Neospora caninum NC-1 tachyzoites were harvested from cultures, passed through a 21 gauge needle to break open host cells, purified by centrifugation using Percoll gradient for 30 min at 2000g, and pelleted by centrifugation for 5 min at 2000g. The pelleted tachyzoites were washed once with PBS, suspended in protein extraction buffer (10 mM Tris-HCl [pH 7.3], 1 mM MgCl₂) containing PMSF, and extracted by freeze-thawing, sonication, and treatment with RNase and DNase.

Recombinant and native *N. caninum* protein were treated with sample buffer (Laemmli, 1970), heated for 1 min at 95 °C, and fractionated by SDS-polyacrylamide gel electrophoresis, followed by transblotting to Immobilon membrane (Millipore, Billerica, MA) in a semi-dry transblotter apparatus (Invitrogen, Carlsbad, CA). After transfer, the membranes were treated with SuperBloc (Thermo Scientific, Rockford, IL) containing 0.05% Tween 20 to

block non-specific immunoglobulin binding in subsequent steps. After blocking, the membranes were incubated with anti-recombinant *NcProfilin* sera or pre-immune sera (1:500 dilution) for 2–4 h at room temperature on a laboratory shaker, followed by 1 h incubation with peroxidase-conjugated goat anti-rabbit IgG biotinylated (10 η g/ml) (Thermo Scientific). All antibodies were diluted in PBS containing 0.05% Tween 20 (PBS-TW), and removed after each step by 3 washes with PBS-TW. Binding of *NcProfilin* antibodies was assessed by a final incubation with Luminol reagent (Super Signal West Dura Extended Substrate, Thermo Scientific).

2.3. Immunofluorescence staining (IF) of *N. caninum* tachyzoites

Neospora caninum tachyzoites were harvested from cell culture as described above, suspended to 10⁶ parasites/ml in PBS, pipetted onto individual wells of multi-well glass slides (10⁴ tachyzoites/well, Erie Scientific Co., Portsmouth, NH), and allowed to air-dry. After drying, the slides were either left untreated or were immersed for 5 min in cold methanol. All wells containing either non-fixed or methanol-fixed tachyzoites were treated with PBS-NFDM for 30 min at room temperature in a humidified chamber, gently rinsed with PBS, air-dried, and then incubated for 2 h at room temperature with a 1:1000 dilution of rabbit anti-*NcProfilin* sera or control sera (pre-immune sera or antisera to a non-*N. caninum* polyHis recombinant protein). The wells were gently rinsed 3 times with PBS, allowed to air dry, and then incubated for 1 h at room temperature with a 1:100 dilution of FITC-anti-rabbit IgG (Sigma). The wells were gently rinsed 3 times with PBS, allowed to air dry, overlaid with several drops of Vectashield mounting medium (Vector Laboratories, Burlingame, CA) followed by a coverslip, and then examined under epifluorescence microscopy.

2.4. Immunoelectron microscopic (IEM) staining of *N. caninum* tachyzoites

Neospora caninum tachyzoites were harvested from cell culture as described above and pelleted by centrifugation for 2 min at 5000g. The tachyzoite pellet was briefly mixed, and then suspended in 100 μ l fixative consisting of 3% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M cacodylate buffer. After a 5 min fixation, the tachyzoites were transferred to a 1.5-ml microcentrifuge tube, pelleted by centrifugation for 5 min at 5000g, and gently washed twice with cacodylate buffer, and then briefly mixed to form a dispersed pellet in the bottom of the tube. The tachyzoite mixture was then dehydrated in a graded ethanol series, infiltrated overnight with LR White hard-grade acrylic resin (London Resin Company, London, UK), and cured at 55 °C for 24 h. Thin sections (90 nm thickness) were obtained using a Diatome diamond knife on a Reichert/AO Ultracut microtome and collected on 200-mesh Formvar-coated nickel grids. The grids were floated for 5 min with the tissue section facing down on drops of PBS containing 0.1 M glycine and 1% bovine serum albumin, followed by 5 min on drops containing PBS-TW-NFDM. Grids were incubated tissue side down for 2 h at room temperature on drops of PBS-TW containing a 1:1000 dilution of rabbit anti-*NcProfilin* sera or control sera (pre-immune sera or antisera to a non-*N. caninum* polyHis recombinant protein). The grids were rinsed 3 times with PBS-TW, incubated for 1 h at room temperature on PBS containing a 1:100 dilution of gold-labeled-anti-rabbit IgG (Sigma), washed 2 times with PBS-TW, once with PBS, once with H₂O, air-dried, stained with 5% uranyl acetate for 30 min, and examined with a Hitachi H7000 electron microscope.

2.5. Cytokine response of spleen cells to recombinant *NcProfilin*

Mice were euthanized by CO₂ asphyxiation followed by cervical dislocation, and spleen and femur were removed for the prepara-

NcPro	MSDWDPVVKEWLVDTGYCCAGGIANAEDGVVFAAAADDDGWSKLYKEDHEEDTIGEDGN	60
	MSDWDPVVKEWLVDTGYCCAGGIANAEDGVVFAAAADDDGWSKLYK+DHEEDTIGEDGN	
TgPro	MSDWDPVVKEWLVDTGYCCAGGIANAEDGVVFAAAADDDGWSKLYKDDHEEDTIGEDGN	60
NcPro	VNGKVTVNEASTIKA ^{AVDDGSAPNGVWIGGQKYKVV} RPEKGF ^{EYNDCTFDITMCARSKGG}	120
	GKV++NEASTIKA ^{AVDDGSAPNGVWIGGQKYKVV} RPEKGF ^{EYNDCTFDITMCARSKGG}	
TgPro	ACGKVSINEASTIKA ^{AVDDGSAPNGVWIGGQKYKVV} RPEKGF ^{EYNDCTFDITMCARSKGG}	120
NcPro	AHLIKTPNGSIVIALYDEEKEQDKGNSR ^T SALAF ^{AEYLHQSGY}	163
	AHLIKTPNGSIVIALYDEEKEQDKGNSR ^T SALAF ^{AEYLHQSGY}	
TgPro	AHLIKTPNGSIVIALYDEEKEQDKGNSR ^T SALAF ^{AEYLHQSGY}	163

Fig. 1. Alignment of predicted amino acid sequence of cDNA coding for mature *Neospora caninum* NcProfilin protein (NcPro) with predicted amino acid sequence of *Toxoplasma gondii* profilin (TgPro, XP002370171). Underlined amino acids represent potential N-linked glycosylation site.

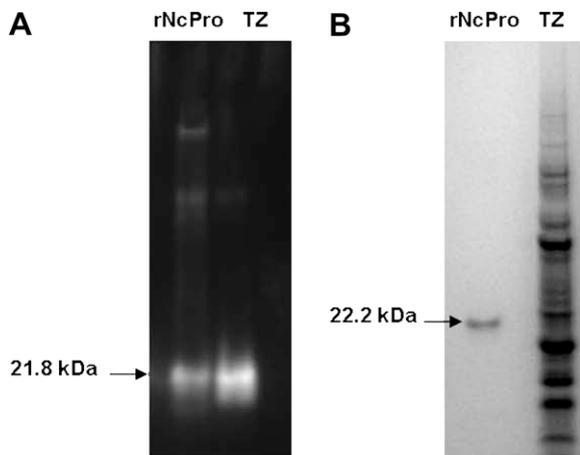


Fig. 2. Size estimation of recombinant and respective native *Neospora caninum* profilin (NcProfilin) proteins by immunostaining a translot (A) of SDS-PAGE fractionated *N. caninum* tachyzoite (TZ) and recombinant NcProfilin (rNcPro) with antisera specific for rNcPro antigen. Coomassie stain image (B) of SDS-PAGE fractionated native and recombinant protein shows purity of recombinant NcPro. Mr. sizes indicated by arrows are expressed in kDa, and estimated by electrophoresing Mr. size standards.

tion of spleen cells and bone marrow cells. Splenic lymphocytes were prepared by forcing the whole spleen through a metal mesh using a syringe plunger and collecting cells into a Petri dish containing RPMI-1640-EDTA. The cell suspension was pipetted up and down to break up cell clumps and underlain with the Histopaque-1088 (Sigma). Following centrifugation at 400g for 30 min at 4 °C, cells at the interface were collected and washed 3 times with RPMI-1640-EDTA and suspended with complete

medium (RPMI-1640 supplemented with 10% FBS, 2.5 mM GLN, 5 µg/ml gentamicin). Spleen cells were seeded in 24-well plates at 2×10^6 cells per well in 1 ml complete medium and duplicate wells were treated with increasing concentrations of recombinant NcProfilin. As a control, protein extracts were made from *E. coli* harboring non-recombinant pTrcHis plasmid, adsorbed to NiNTA agarose, and eluates were collected using a procedure identical to the NiNTA purification of recombinant NcProfilin. Plates were incubated at 37 °C in the presence of 5% CO₂ and 95% air for 48 h and supernatants were collected, centrifuged and stored at –20 °C until assayed for IFN-γ. The spleen cell IFN-γ responses to rNcProfilin and non-recombinant protein were analyzed by non-linear regression (Systat Software, San Jose, CA).

BALB/c mice ($n = 4$) were administered purified recombinant NcProfilin (1 µg/g body weight) by intraperitoneal injection. Venous blood was collected at 6 and 24 h-post injection, and processed for sera for testing for IFN-γ and IL-12 as described below. As a control for stimulation by *E. coli* protein that may have co-purified with recombinant NcProfilin, a group of mice were injected with NiNTA-purified non-recombinant *E. coli* proteins. The volume of purified non-recombinant was identical to that used in the recombinant NcProfilin injection. In addition, an equal number of mice were immunized with PBS alone. Mean IFN-γ or IL-12 responses were compared between rNcProfilin and control (NR, PBS) controls at both time-points using Student's *t*-test (Systat Software), with significant differences indicated at $P < 0.05$.

2.6. Cytokine ELISA and reagents

IFN-γ and IL-12 ELISA kits were purchased from eBioscience (San Diego, CA). CpG (ODN1826, Invitrogen) was used as a positive control. Unless otherwise indicated, all cell culture supplies

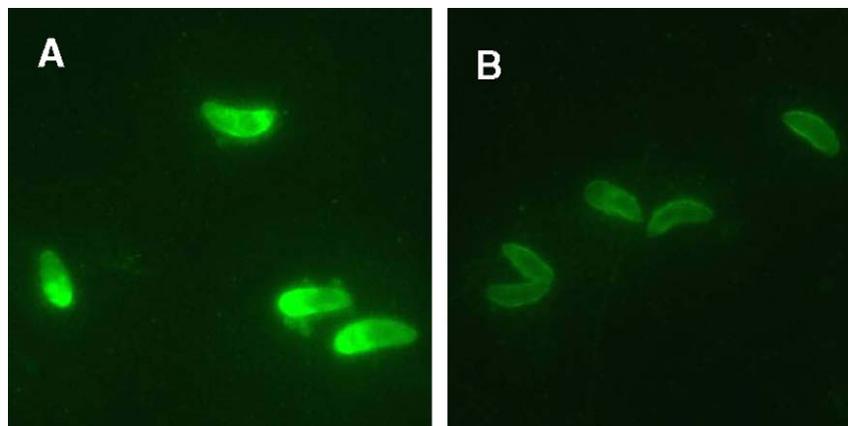


Fig. 3. Indirect immunofluorescence staining of *Neospora caninum* tachyzoites with antisera specific for recombinant NcProfilin. (A) Non-methanol fixed (dried) *N. caninum* tachyzoites. (B) Methanol-fixed *N. caninum* tachyzoites.

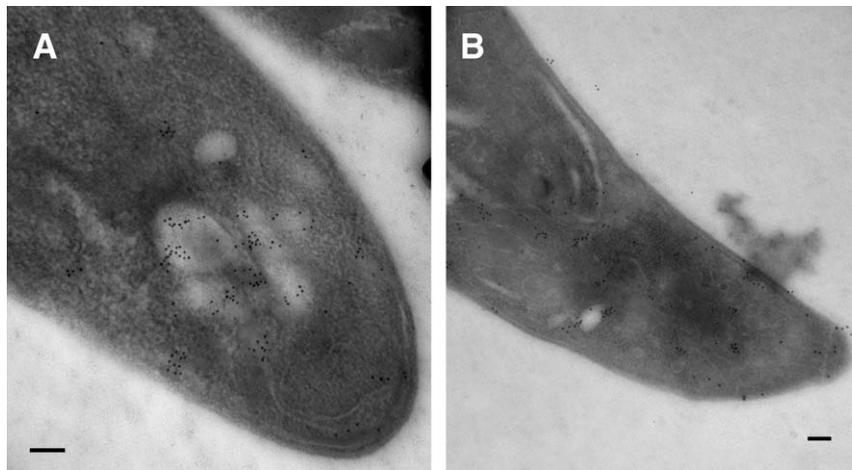


Fig. 4. Immunoelectron microscopic staining of *Neospora caninum* tachyzoites with antisera specific for recombinant NcProfilin. Note concentration of NcProfilin in apical end of *N. caninum* (A) and release of NcProfilin from tachyzoite (B). Bar = 100 nm.

including RPMI 1640, fetal bovine serum, glutamine, HEPES, and gentamicin were obtained from Hyclone or Mediatech. Con A was obtained from Sigma–Aldrich. Recombinant murine GM-CSF was purchased from ProSpec-Tany TechnoGene (Rehovot, Israel).

3. Results

3.1. Cloning and expression of NcProfilin cDNA sequence

An *N. caninum* sequence (GenBank Accession No. CF943178) with homology to *T. gondii* profilin was identified by BLAST-N searching of an *N. caninum* EST database (GenBank) with the TgProfilin gene sequence (GenBank Accession No. XP002370171). BLAST-X analysis revealed a 96% identity and 98% similarity between NcProfilin and TgProfilin amino acid sequences (Fig. 1). The coding region (492 bp) containing the putative ATG start site (nt 62–64) and the TAA termination codon (nt 551–553) was cloned by PCR amplification into an expression vector for production of His-tagged recombinant fusion protein (termed rNcProfilin). SDS–PAGE analysis of rNcProfilin revealed a highly expressed 21.8 kDa protein (~50% total *E. coli* protein), whose expression was optimal after IPTG induction at 37 °C for 4 h (data not shown). This size estimate is similar to the expected size based on the predicted NcProfilin amino acid sequence (17.6 kDa + 3.6 kDa (His tag) = 21.2 kDa). Immunostaining SDS–PAGE-transblots containing affinity-purified rNcProfilin with rNcProfilin-specific sera identified a 22.4 kDa protein, which is similar in size to rNcProfilin observed after Coomassie blue staining (Fig. 2).

3.2. Immunofluorescence and immunoelectron microscopy

By immunofluorescence staining with anti-recombinant protein sera, NcProfilin localized to the apical end of *N. caninum* tachyzoites (Fig. 3A). Pre-treatment of *N. caninum* tachyzoites with methanol altered the staining pattern, such that a surface or sub-surface location was observed (Fig. 3B). Immunoelectron microscopy confirmed the localization of NcProfilin to the apical end of *N. caninum* tachyzoites (Fig. 4A and B). On many tachyzoites, external labeling was also observed suggesting that NcProfilin was released or excreted during the fixation and embedding procedure (Fig. 4B).

3.3. NcProfilin-induced cytokine secretion

Affinity-purified rNcProfilin elicited a dose-dependent IFN- γ response in spleen cells isolated from BALB/c mice (Fig. 5). The

IFN- γ response curve displayed a significant curve–linear relationship between NcProfilin concentration and release of IFN ($r^2 = 0.99$, $P < 0.05$). The IFN response to non-recombinant *E. coli* protein was much less than the IFN response to rNcProfilin, and showed no significant relationship ($P > 0.05$) between concentration and IFN levels (Fig. 5).

Injection of mice with recombinant NcProfilin elicited strong IFN- γ and IL-12 responses at 6 h post-injection, reflecting a significant increase ($P < 0.05$) over the response to non-recombinant protein and PBS alone (Fig. 6). Serum cytokine levels decreased appreciably by 24 h post-injection, but remained significantly ($P < 0.05$) higher than non-recombinant and PBS controls (Fig. 6).

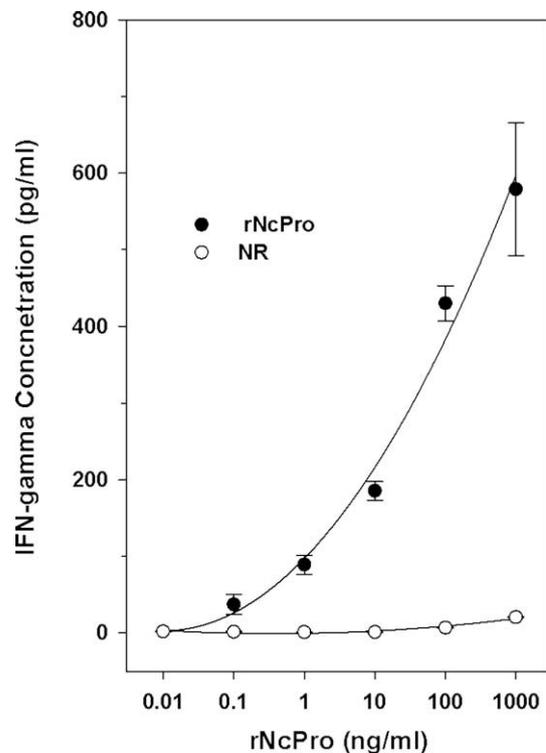


Fig. 5. Dose response of interferon-gamma (IFN- γ) release by BALB/c mouse spleen cells to various concentrations of affinity-purified recombinant NcProfilin (rNcPro) and non-recombinant (NR) control protein.

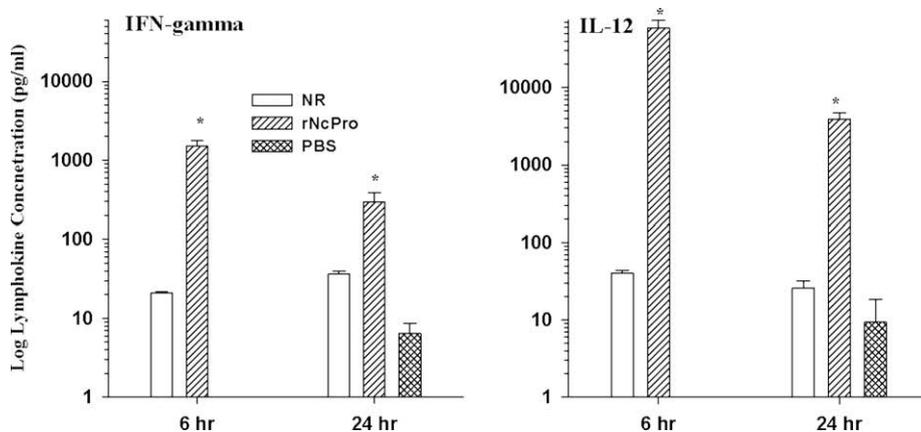


Fig. 6. Systemic interferon-gamma (IFN- γ) and interleukin 12 (IL-12) responses in BALB/c mice at 6 and 24 h-post-injection with recombinant NcProfilin (rNcPro) or control non-recombinant *Escherichia coli* protein (NR) or PBS alone (PBS). Asterisks indicated significant differences between cytokine responses to rNcPro and controls (NR and PBS).

4. Discussion

The present study describes the cloning and expression of a profilin-like molecule from *N. caninum* tachyzoites. Antisera prepared against purified recombinant NcProfilin recognized a ~22 kDa *N. caninum* tachyzoite protein in a SDS-PAGE/immunoblotting assay. While this size is similar to that described for TgProfilin (Yarovinsky et al., 2005; Plattner et al., 2008) and other profilins (Witke, 2004), it is 4–5 kDa greater than expected based on the amino acid composition of the protein. This difference may have been due to post-translational modification of native NcProfilin. Although no O-glycosylation sites appear to be present, a putative N-glycosylation site exists at amino acids 128–130 (NGS) as predicted using ExPasy software (<http://www.expasy.ch/tools/>).

NcProfilin appears to stimulate the release of IFN- γ in cultured spleen cells, and is capable of eliciting systemic IFN- γ and IL-12 responses after injection into BALB/c mice, similar to IL-12 responses observed in mice injected with TgProfilin (Yarovinsky et al., 2005; Plattner et al., 2008). As discussed by several authors (Yarovinsky et al., 2006; Yarovinsky and Sher, 2006; Plattner et al., 2008; Denkers and Striepen, 2008), it remains to be determined how *T. gondii* profilin, a cytosolic protein that interacts with actin, binds to TLR on dendritic cells. The presence of NcProfilin at the apical end of *N. caninum* tachyzoites may indicate that, similar to other rhoptry and microneme proteins, at least some of this protein is released during host cell invasion. The immuno-fluorescence and immunoelectron microscopy staining pattern indicates that there are two forms of NcProfilin in *N. caninum* tachyzoites. One form appears to reside in the apical end of the parasite, and is released from tachyzoites after methanol treatment, while a second form remains associated with the parasite membrane. Our attempts to co-localize NcProfilin to actin in *N. caninum* tachyzoites using phalloidin or antibodies to eukaryotic actin were unsuccessful (unpublished observations). However, this inability to localize actin may relate to the nature of actin in *N. caninum*, as observed in *Plasmodium falciparum* (Schüler et al., 2005; Kursula et al., 2008) and *T. gondii* (Sahoo et al., 2006). Further studies are required to determine if native NcProfilin is released from *N. caninum* tachyzoites during host cell penetration, as are microneme and rhoptry proteins in *T. gondii*, and inducing similar cytokine responses in vivo (Carruthers, 1999; Dubremetz, 2007; Soldati-Favre, 2008).

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