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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 CGGATGTCGGACTGGGATCCCGTT-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* DH5α 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 OD_{600} = 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 5000*g* 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 μg NiNTA-purified recombinant NcProfilin/injection. NcProfilin was emulsified in Freunds Complete Adjuvant for the primary immunization (day 1) and in Freunds 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 2000*g*, and pelleted by centrifugation for 5 min at 2000*g*. The pelleted tachyzoites were washed once with PBS, suspended in protein extraction buffer (10 mM Tris–HCl [pH 7.3], 1 mM $MgCl_2$) 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 (Laemmeli, 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 ηg/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 10^6 parasites/ml in PBS, pipetted onto individual wells of multi-well glass slides (10^4 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 5000*g*. The tachyzoite pellet was briefly mixed, and then suspended in 100 μl 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 5000*g*, 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 ηm 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 H_2O, 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 CO_2 asphyxiation followed by cervical dislocation, and spleen and femur were removed for the prepara-