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Identification of ribosomal phosphoprotein P0 of *Neospora caninum* as a potential common vaccine candidate for the control of both neosporosis and toxoplasmosis☆

Houshuang Zhang[a], Eung-goo Lee[a], Min Liao[a], Muller K.A. Compaore[a], Guohong Zhang[a], Osamu Kawase[a], Kozo Fujisaki[a], Chihiro Sugimoto[b], Yoshifumi Nishikawa[a], Xuenan Xuan[a,*]

[a] *National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan*
[b] *Research Center for Zoonosis Control, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan*

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Abstract

The characterization of the cross-reactive antigens of two closely related apicomplexan parasites, *Neospora caninum* and *Toxoplasma gondii*, is important to elucidate the common mechanisms of parasite–host interactions. In this context, a gene encoding *N. caninum* ribosomal phosphoprotein P0 (NcP0) was identified by immunoscreening of a *N. caninum* tachyzoite cDNA expression library with antisera from mice immunized with *T. gondii* tachyzoites. The NcP0 was encoded by a gene with open reading frame of 936 bp, which encoded a protein of 311 amino acids. The NcP0 gene existed as a single copy in the genome and was interrupted by a 432 bp intron. The NcP0 showed 94.5% amino acid identity to *T. gondii* P0 (TgP0). Anti-recombinant NcP0 (rNcP0) sera recognized a native parasite protein with a molecular mass of 34 kDa in Western blot analysis. Immunofluorescence analysis showed that the NcP0 was localized to the surface of *N. caninum* tachyzoites. A purified anti-rNcP0 IgG antibody inhibited the growth of *N. caninum* and *T. gondii in vitro* in a concentration-dependent manner. These results indicate that P0 is a cross-reactive antigen between *N. caninum* and *T. gondii* and a potential common vaccine candidate to control both parasites.

Keywords: *Neospora caninum*; *Toxoplasma gondii*; Ribosomal phosphoprotein P0; Cross-reactive

1. Introduction

Neospora caninum is an important pathogen known to cause abortion in cattle; in addition, it causes neuromuscular disease in dogs and other animals [1–3]. In phylogenetic analysis, *N. caninum* is closely related to *Toxoplasma gondii* in the phylum Apicomplexa [4,5]. It is known that there are highly similar biological and morphological characteristics between *N. caninum* and *T. gondii*. Both neosporosis and toxoplasmosis have been regarded as economically important diseases, as they have considerable impact on the livestock industry.

Antigenic cross-reactivity between *N. caninum* and *T. gondii* has been reported with immunoblotting and the enzyme-linked immunosorbent assay (ELISA) using crude tachyzoite extracts as the antigen [6–9] and with an immunohistochemical test using anti-*T. gondii* polyclonal and monoclonal antibodies (mAbs) [10,11]. Previously, we identified four proteins, protein disulfide isomerase (PDI), heat-shock protein 70 (HSP70), ribosomal protein 1 (RP1), and apical membrane antigen 1 (AMA1), as cross-reactive antigens between *N. caninum* and *T. gondii* [12,13]. These results suggested that the conserved antigens of the two parasites could be useful in the development of vaccines or drugs for controlling the diseases caused by the two parasites.

All three ribosomal proteins (P0, P1, and P2) form a pentameric complex, P0 $(P1\text{-}P2)_2$, which possesses a conserved

Abbreviations: P0, ribosomal phosphoprotein P0; PAGE, polyacrylamide gel electrophoresis; IFA, immunofluorescence analysis

☆ *Note:* The nucleotide sequence data reported in this paper are available in the DDBJ, GenBank, and EMBL databases under the accession number AB284186

* Corresponding author. Tel.: +81 155 49 5648; fax: +81 155 49 5643.
E-mail address: gen@obihiro.ac.jp (X. Xuan).

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carboxyl-terminal 22-amino-acid domain. The complex constitutes the stalk region at the GTPase center of the eukaryotic large ribosomal subunit [14–16]. This pentamer is an important structural element involved in the translocation step of protein synthesis [17], and these ribosomal proteins are phylogenetically well-conserved across eukaryotic organisms.

Ribosomal phosphoprotein P0 (P0) is a neutral protein that is found in all eukaryotes [14] and is highly conserved [18]. The P0 protein has a molecular mass between 34 and 38 kDa in all eukaryotes [19] and plays the most crucial role. Recently, P0 homologues were identified from apicomplexan parasites, such as *Plasmodium* spp., *Babesia* spp., and *T. gondii*, as immunogenic proteins [20–24].

The P0 has been shown to be located on the surfaces of *Plasmodium* and *T. gondii* [19,24]. The P0 of the human malarial parasite, *P. falciparum* (PfP0), has been identified as a protective antigen, and specific antibodies raised to different domains of PfP0 could block the invasion of *P. falciparum* merozoites into red blood cells [25] and cross-protect the mice against *P. yoelii* challenge infection [26]. The *Leishmania infantum* P0 has been shown to confer protective immunity to *L. major* infection in mice [27,28]. Recently, the *B. gibsoni* P0 has been reported as a cross-protective vaccine candidate against a *Babesia microti* challenge infection in mice [22]. These results suggested that P0 might be a potentially universal candidate for a vaccine to control parasite infections.

In the present study, we identified the ribosomal protein P0 of *N. caninum* (NcP0) as a cross-reactive antigen between *N. caninum* and *T. gondii* and showed that the anti-rNcP0 antibody inhibited the growth of both parasites. NcP0 provided additional insights into the host–parasite interactions and might be useful as a candidate for the development of a novel common vaccine to control both diseases.

2. Materials and methods

2.1. Parasite culture and purification

N. caninum tachyzoites (Nc-1 strain) and *T. gondii* tachyzoites (RH strain) were maintained in African green monkey kidney (Vero) cells cultured in a minimum essential medium (MEM, Sigma, USA) supplemented with 8% heat-inactivated fetal bovine serum (FBS) and 50 μg/ml kanamycin at 37 °C in a 5% CO_2 air environment. For the purification of *N. caninum* and *T. gondii* tachyzoites, parasites and host cell debris were washed in cold phosphate-buffered saline (PBS), and the final pellet was resuspended in cold PBS and syringed three times with a 27-gauge needle. The parasites were then filtered through a 5.0 μm pore filter (Millipore, USA), washed twice with 10 ml of PBS, and pelleted at 1500 rpm for 10 min.

2.2. Preparation of parasite lysates

N. caninum and *T. gondii* tachyzoites were harvested and purified as described above. Parasites were then resuspended in PBS, disrupted three times by a freeze-thaw cycle in liquid nitrogen, and then sonicated in an ice slurry. The protein concentration of the lysates was determined with a BCA protein assay kit (PIERCE, USA), and the lysates were stored at −80 °C until use.

2.3. Production of anti-T. gondii tachyzoite sera

Six-week-old ddY mice (Clea, Japan) were immunized intraperitoneally with *T. gondii* tachyzoite (1×10^8/mouse) lysates in an equal volume of Freund's complete adjuvant (Sigma) for the first injection. Mice were immunized with *T. gondii* tachyzoite (0.5×10^8/mouse) lysates in Freund's incomplete adjuvant (Sigma) at 2 and 4 weeks post-primary injection. The sera were collected 2 weeks after the last immunization.

2.4. Cloning of the NcP0 gene

A cDNA expression library was constructed as described previously [12]. The plaques were transferred onto nitrocellulose membranes and screened with the antisera from mice immunized with *T. gondii* tachyzoite lysates. Positive clones were excised for isolation of the phagemid inserts as described in the manufacturer's instructions and sequenced with M13 forward and reverse primers using an automated sequencer (ABI PRISM 3100 Genetic Analyzer, USA). A full-length NcP0 cDNA including the noncoding 5′ end was obtained with a GeneRacer kit (Invitrogen, USA) according to the manufacturer's instructions, and the clone obtained was sequenced on both strands.

2.5. Southern blot analysis

Southern blot analysis was performed according to the standard procedure [29]. Genomic DNA was extracted from *N. caninum* tachyzoites. The DNA was digested overnight with *Xba*I, *Sac*I, and *Hind*III, separated through a 1.5% (w/v) agarose gel, and transferred onto a Hybond^{TM}-N$^+$ membrane (Amersham Pharmacia, USA) through capillary action. The membrane was probed with an NcP0 fragment labeled with an Alkphos direct labeling kit (Amersham Biosciences, UK).

2.6. Expression of the recombinant NcP0, production of anti-rNcP0 sera, and IgG purification

The cDNA fragment encoding the entire NcP0 was amplified by PCR using primers with the introduced *Eco*RI and *Sal*I sites (underlined), P1 (5′-CTGAATTC CAAGATGGCAGGATCCA-AGGGCAAA-3′) and P2 (5′-CTGTCGACTTAGTCGA AGAGAGAGAACCCCAT-3′). The product was inserted into *Escherichia coli* expression vector pGEX-4T-3 (Pharmacia Biotech, USA). The recombinant NcP0 (rNcP0) fused with a glutathione *S*-transferase (GST) tag was expressed in the *E. coli* BL21 strain according to the manufacturer's instructions. The denaturing and refolding of insoluble rNcP0 with urea and subsequent purification were performed as described previously [30]. Production of mouse anti-rNcP0 sera was performed as described previously [13]. Rabbit anti-rNcP0 sera were produced as described by Zhou et al. [31]. Total IgG was