Elsevier Editorial System(tm) for Molecular & Biochemical Parasitology

Manuscript Draft

Manuscript Number:

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

Article Type: Research Paper

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

Corresponding Author: Dr Xuenan Xuan, DVM, PhD

Corresponding Author's Institution: Obihiro University of Agriculture and Veterinary Medicine

First Author: Houshuang Zhang

Order of Authors: Houshuang Zhang; Eung-goo Lee; Min Liao; Muller K.A. Compaore; Guohong Zhang; Osamu Kawase; Kozo Fujisaki; Chihiro Sugimoto; Yoshifumi Nishikawa; Xuenan Xuan, DVM, PhD

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 an intron with 432 bp. 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 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.

Cover Letter

Dear Sir:

We send the paper entitled "Identification of ribosomal phosphoprotein P0 of *Neospora caninum* as a potential common vaccine candidate for the control of both neosporosis and toxoplasmosis" that we would like to submit for publication in the *Molecular and Biochemical Parasitology*.

In this report, we identified ribosomal phosphoprotein P0 is a cross-reactive antigen between *N. caninum* and *T. gondii*, and we also evaluated the anti-rNcP0 IgG antibody inhibited the growth of *N. caninum* and *T. gondii in vitro*.

We, Houshuang Zhang, Eung-goo Lee, Min Liao, Muller K.A. Compaore, Guohong Zhang, Osamu Kawase, Kozo Fujisaki, Chihiro Sugimoto, Yoshifumi Nishikawa, and Xuenan Xuan, are the authors of this above manuscript.

We, Houshuang Zhang, Eung-goo Lee, Min Liao, Muller K.A. Compaore, Guohong Zhang, Osamu Kawase, Kozo Fujisaki, Chihiro Sugimoto, Yoshifumi Nishikawa, and Xuenan Xuan, acknowledge that we have read the above manuscript and accept responsibility for its contents. We confirm this manuscript has not been published previously and if accepted in the *Molecular and Biochemical Parasitology* will not be published elsewhere without the approval of the Editor-in-Chief. We also confirm there are no financial or other relationships that might lead to a conflict of interest. Your consideration of this paper is greatly appreciated.

Corresponding author

Dr. Xuenan Xuan

National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan

Tel. : +81-155-49-5648

Fax: +81-155-49-5643

E-mail: gen@obihiro.ac.jp

Title

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

Authors

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, *]

Addresses

[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

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).

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 an intron with 432 bp. 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 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.

Key words

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 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 *B. 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℃ in a 5% CO_2 air environment. For the purification of 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 1,500 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℃ 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 two 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 *EcoR*I and *Sal*I sites (underlined), P1 (5'-CTGAATTC CAAGATGGCAGGATCCAAGGGCAAA-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 purified from 20 ml of pooled rabbit anti-rNcP0 sera through Protein A Chromatography Columns (Bio-Rad Laboratories, USA) according to the manufacturer's instructions. The purity of IgG was determined by SDS-PAGE. The quantity of IgG was determined with a BCA protein assay kit (PIERCE, USA), and the binding activity of IgG was tested by ELISA.

2.7. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional electrophoresis (2-DE), immunoblotting, and protein identification

For the determination of the native NcP0, the *N. caninum* lysates and *T. gondii* lysates were separated on 12% SDS-PAGE under reducing conditions as described by Liao et al. [12]. 2-DE, immunoblotting, and protein identification by mass spectrometry were performed as described by Lee et al. [32].

2.8. Immunofluorescence analysis (IFA)

IFA of NcP0 in both intracellular and extracellular *N. caninum* tachyzoites was performed as described previously [12]. Fluorescent signals were visualized using a laser microdissection system (TCS NT, Leica, Germany).

2.9. In vitro *assay for the inhibitory effects of rNcP0 antibody on the growth of the* N. caninum *and* T. gondii *parasites*

Fluorescence-based quantification of the growth inhibition by both *N. caninum* and

T. gondii expressing the green fluorescent protein (GFP) *in vitro* was carried out as described previously [33,34]. Vero cell monolayers were grown in 12-well tissue culture plates. The anti-rNcP0 and anti-GST IgG antibodies were sterilized by filtration through a 0.20-μm filter (Pall, USA). The tachyzoites (2.5×10^5) were resuspended in 500 μl of MEM containing 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, and 0.1 mg/ml of anti-rNcP0 or anti-GST IgG antibodies and incubated for 1 hr at 37℃/5% CO_2 before the Vero cells were infected. Parallel wells without the antibodies were used as controls. Thereafter, the mixtures were added to the monolayers of Vero cells. After 48 hrs of culture, the percentage of fluorescent parasite-infected cells was determined using a flow cytometer (EPICS DIVISION, USA), and 5×10^3 cells were counted in one well sample. The percentage of invaded tachyzoites was calculated by the following formula: [1–the percentage of fluorescent parasites in the tested IgG treatment wells/the percentage of fluorescent parasites in control wells]×100%. The assays were repeated in five individual experiments.

2.10. Statistical analysis

The group treated with anti-rNcP0 antibodies and the group treated with anti-GST antibodies were statistically analyzed by the Student's *t*-test.

3. Results

3.1. Identification and characterization of the NcP0 gene

The *N. caninum* cDNA expression library was immunoscreened with anti-*T. gondii* tachyzoite serum, and a total of 5 positive clones encoding NcP0 were obtained. The

full-length cDNA of NcP0 was 1,379 bp (GenBank accession number **AB284186**), including a single open reading frame of 936 bp, encoding a polypeptide of 311 amino acid residues with a molecular mass of 33,861 Da and a theoretical pI of 5.28, as calculated with MacVector software (Oxford Molecular Group, Inc.). The amino acid sequence of NcP0 did not show a signal sequence (SignalP3.0) or a transmembrane domain (TMHMM2.0).

To evaluate the phylogenetic relationship between NcP0 and P0 proteins from other apicomplexan parasites, the deduced amino acid sequence of NcP0 (AB284186) was aligned with P0 amino acid sequences from three *Plasmodium* species (*P. falciparum* (AE014840), *P. berghei* (AY099370), *P. yoelii* (XM721013)), *Cryptospodium parvum* (AAEE01000008), *Theileria parva* (AAGK01000001), *Eimeria tenella* (AF353516), *B. bovis* (AF498365), *B. gibsoni* (AB266721), *B. microti* (AB267377), and *T. gondii* (AF390866) (Fig. 1A and B). The result revealed the highest amino acid sequence homology between NcP0 and TgP0 (94.5% identity) and the lowest homology between NcP0 and *T. parva* P0 (58.5%), as calculated using DNASTAR software (NetWell Corporation, Japan) (Fig. 1B).

In Southern blot analysis, the NcP0 cDNA probe hybridized to two bands with restriction enzymes that cut once within the gene (Fig. 1C, lane 1) and a single band with enzymes that do not cut within the gene (Fig. 1C, lanes 2 and 3). This result indicated that the NcP0 gene existed as a single copy in the genome.

The complete sequence of the PCR-amplified genomic DNA containing the NcP0 gene was acquired by primer walking, cloned into a pGEM-T vector (Promega, USA), and subjected to DNA-sequencing analysis. The genomic sequence revealed an intron of

432 bp flanked by exons of 345 and 591 bp.

3.2. Characterization of the NcP0 protein

In order to characterize the antigenicity of NcP0, rNcP0 was expressed in *E. coli* as a GST fusion protein. The rNcP0 was reactive with the sera from either mice or dogs experimentally infected with live *N. caninum* tachyzoites (data not shown).

To determine the native NcP0, we analyzed the protein profile of tachyzoite lysates via 2-DE and immunoblot analysis with the mouse anti-rNcP0 sera. The NcP0 was clearly recognized as one spot in an immunoblot image (Fig. 2B), and the corresponding protein spot was subsequently analyzed with MALDI-TOF mass spectrometry. The PMF of the spot (Fig. 2A) was successfully acquired and highly matched to NcP0 with a sequence coverage of 45.3%. The analysis indicated that the protein spot had an approximate molecular weight of 34 kDa and an isoelectric point (*PI*) of 5.53, as calculated with 2D Expression software (nonlinear dynamics). The matched peptide mass and corresponding amino acid sequences of peptide fragments are shown in Fig. 2C. The mouse anti-GST sera were used as the control to probe the *N. caninum* lysates, but no reacted bands or spots were observed (data not shown).

In immunoblot analysis, the mouse anti-rNcP0 sera recognized a specific protein band in the lysates that migrated at approximately 34 kDa. In addition, the mouse anti-rNcP0 sera cross-reacted with a specific protein band in the lysates of *T. gondii* that migrated at the same position (Fig. 3A).

To examine the localization of NcP0 on parasites, the mouse anti-rNcP0 serum was used to perform the IFA with the intracellular and extracellular parasites. The specific

fluorescence was distributed on the surface of both intracellular and extracellular tachyzoites under non-permeabilized conditions (Fig. 3B d-f, g-i). On the other hand, the specific fluorescence distributed on the inside the parasites under permeabilized conditions (Fig. 3B a-c). In addition, a similar distribution of TgP0 on both extracellular and intracellular *T. gondii* parasites was observed by IFA using mouse anti-rNcP0 sera (data not shown).

3.3. Anti-rNcP0 antibodies inhibited the growth of both N. caninum *and* T. gondii *tachyzoites* in vitro

Both the *N. caninum* and *T. gondii* tachyzoites expressing GFP were grown in Vero cells in the presence of a purified anti-rNcP0 IgG antibody. The anti-rNcP0 IgG antibody inhibited the growth of *N. caninum*, with an inhibition rate of 52.5±3.6% at 1 mg/ml IgG, whereas anti-GST antibodies did not have a significant effect on growth inhibition (8.8±2.6% inhibition) (Fig. 4A). In addition, the anti-rNcP0 IgG cross-inhibited the growth of *T. gondii*, with an inhibition rate of 46.1±3.3% at 1 mg/ml IgG, whereas anti-GST antibodies did not have a significant effect on growth inhibition (7.7±2.8% inhibition) (Fig. 4B). The growth inhibitory effect against both parasites was dose-dependent.

4. Discussion

In the present study, we identified P0 as a cross-reactive antigen between *N. caninum* and *T. gondii*. Phylogenetic analysis of the deduced amino acid sequence of the NcP0 with the amino acid sequences of P0 proteins from other apicomplexan parasites,

Plasmodium spp, *C. parvum*, *T. parva*, *E. tenella*, *Babesia* spp, and *T. gondii*, demonstrated that the NcP0 was closer to TgP0 than those of other apicomplexan parasites (Fig. 1B). In addition, the genomic analysis indicated that the NcP0 gene existed as a single copy in the genome of *N. caninum* and was interrupted by an intron, similarly to TgP0 [24]. These results suggested that *N. caninum* had a closer evolutionary relationship with *T. gondii* than others.

The extent of the high homology of the apicomplexan P0 proteins (*Plasmodium*, *Cryptospodium*, *Theileria*, *Eimeria*, *Babesia*, *Toxoplasma*, and *Neospora*) and the highly conserved acidic C-terminal end region including ××DDMGF×LFD motif [35] implies that they may fulfill a similar function in the host-parasite interaction process (Fig. 1A). Recent studies have demonstrated that BALB/c mice immunized with recombinant *Trypanosoma cruzi* ribosomal P2β protein develop a strong and specific antibody response against its 13 residue-long C-terminal epitope (××DDMGF×LFD motif) [36]. This result indicates that the conserved acidic C-terminal end motif probably plays a crucial role in the process of anti-rNcP0 IgG antibody on parasite growth inhibition.

The localization of the P0 was predominantly intracellular, and fluorescent staining was evident at the surface. P0 shows surface localization in protozoan parasites [19,24] as well as other species, including yeast and mammalian cells [37]. However, the function of surface-localized P0 is yet to be elucidated. How P0 protein gets transported on the surface remains a question, as the amino-acid sequence of P0 shows neither a signal sequence nor a transmembrane domain. Recent studies have revealed that the protein domain of the hypothetical integral membrane protein, YFL034W, showed strong interaction with P0 genetically, providing a possible explanation regarding the surface translocation of P0 [38].

An antigenic cross-reactive assay and cross-growth inhibitory assay indicated that P0 is a cross-reactive antigen between *N. caninum* and *T. gondii.* Anti-rNcP0 antibodies inhibited the growth of both parasites. The growth inhibitory effect was dose-dependent and seems to act at the invasion step. The surface translocation of NcP0 and inhibition of tachyzoite growth by anti-rNcP0 antibodies suggest that NcP0 plays an important role in the *N. caninum* invasion of host cells. In addition, rNcP0 was strongly reactive to sera from animals experimentally infected with either *N. caninum* or *T. gondii* (data not shown). This result indicated that NcP0 might be an immunodominant antigen in both the *N. caninum* and *T. gondii* infection courses. A previous study has demonstrated that mice infected with *N. caninum* could induce cross-protective immunity against lethal *T. gondii* challenge infection [39]. Further research will be required to clarify whether NcP0 is responsible for the cross-protective immunity against *T. gondii* infection. Consequently, further evaluation of the usefulness of NcP0 as a potential common vaccine against both neosporosis and toxoplasmosis will also be required.

This research was supported by a grant from The 21st Century COE Program (A-1) and a Grant-in-Aid for Scientific Research, both from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

[1] Anderson ML, Palmer CW, Thurmond MC, et al. Evaluation of abortions in cattle attributable to neosporosis in selected dairy herds in California. J Am Vet Med

Assoc 1995;207:1206–10.

[2] Dubey JP, Lindsay DS. A review of *Neospora caninum* and neosporosis. Vet Parasitol 1996;67:1–59.

[3] Dubey JP. Recent advances in *Neospora* and neosporosis. Vet Parasitol 1999;84: 349–67.

[4] Ellis JT, Luton K, Baverstock PR, Brindley PJ, Nimmo KA, Johnson AM. The phylogeny of *Neospora caninum*. Mol Biochem Parasitol 1994;64:303–11.

[5] Mugridge NB, Morrison DA, Heckeroth AR, Johnson AM, Tenter AM. Phylogenetic analysis based on full-length large subunit ribosomal RNA gene sequence comparison reveals that *Neospora caninum* is more closely related to *Hammondia heydorni* than to *Toxoplasma gondii*. Int J Parasitol 1999;29:1545–56.

[6] Harkins D, Clements DN, Maley S, et al. Western blot analysis of the IgG responses of ruminants infected with *Neospora caninum* and with *Toxoplasma gondii*. J Comp Pathol 1998;119:45–55.

[7] Lee EG, Kim JH, Shin YS, et al. Application of proteomics for comparison of proteome of *Neospora caninum* and *Toxoplasma gondii* tachyzoites. J Chromatogr B: Analyt Technol Biomed Life Sci 2005;815:305–14.

[8] Nishikawa Y, Claveria FG, Fujisaki K, Nagasawa H. Studies on serological cross-reaction of *Neospora caninum* with *Toxoplasma gondii* and Hammondia heydorni. J Vet Med Sci 2002;64:161–4.

[9] Pare J, Hietala SK, Thurmond MC. An enzyme-linked immunosorbent assay (ELISA) for serological diagnosis of *Neospora* sp. infection in cattle. J Vet Diagn

Invest 1995;7:352–9.

[10] McAllister MM, Parmley SF, Weiss LM, Welch VJ, McGuire AM. An immunohistochemical method for detecting bradyzoite antigen (BAG5) in *Toxoplasma gondii*-infected tissues cross-reacts with a *Neospora caninum* Bradyzoite antigen. J Parasitol 1996;82:354–5.

[11] Sundermann CA, Estridge BH, Branton MS, Bridgman CR, Lindsay DS. Immunohischemical diagnosis of *Toxoplasma gondii*: Potential for cross-reactivity with *Neospora caninum*. J Parasitol 1997;83:440–3.

[12] Liao M, Xuan X, Huang X, et al. Identification and characterization of cross-reactive antigens from *Neospora caninum* and *Toxoplasma gondii*. Parasitology 2005;130:481–8.

[13] Zhang HS, Compaore KA, Lee EG et al. Apical membrane antigen 1 is a cross-reactive antigen between *Neospora caninum* and *Toxoplasma gondii*, and the anti-NcAMA1 antibody inhibits host cell invasion by both parasites. Mol Biochem Parasitol 2006;(article in press).

[14] Rich BE, Steitz JA. Human acidic ribosomal phosphoproteins P0, P1 and P2: analysis of cDNA clones, in vitro synthesis and assembly. Mol Cell Biol 1987;7:4065–74.

[15] Uchiumi T, Wahha AJ, Traut, RR. Topography and stoichiometry of acidic proteins in large ribosomal subunits from Artemia salina as determined by cross linking. Proc Natl Acad Sci 1987;84:5580–4.

[16] Uchiumi T, Kominami R. Direct evidence for interaction of the conserved GTPase domain within 28S RNA with mammalian ribosomal acidic phosphoproteins and

L12. J Biol Chem 1992;267:19179–85.

[17] Liljas A. Comparative biochemistry and biophysics of ribosomal proteins. Int Rev Cytol 1991;124:103–36.

[18] Aruna K, Chakraborty T, Rao PN, Santos C, Ballesta JP, Sharma S. Functional complementation of yeast ribosomal P0 protein with *Plasmodium falciparum* P0. Gene 2005;357:9–17.

[19] Goswami A, Chatterjee S, Sharma S. Cloning of a ribosomal phosphoprotein P0 gene homologue from *Plasmodium falciparum*. Mol Biochem Parasitol 1996;82:117–20.

[20] Goswami A, Singh S, Redkar VD, Sharma S. Characterization of P0, a ribosomal phosphoprotein of *Plasmodium falciparum*. Antibody against amino-terminal domain inhibits parasite growth. J Biol Chem 1997;272:12138–43.

[21] Rajeshwari K, Patel K, Nambeesan S, et al. The P domain of the P0 protein of *Plasmodium falciparum* protects against challenge with malaria parasites. Infect Immun 2004;72:5515–21.

[22] Terkawi MA, Jia H, Zhou J, et al. *Babesia gibsoni* ribosomal phosphoprotein P0 induces cross-protective immunity against *B. microti* infection in mice. Vaccine 2006;(article in press).

[23] Ahn HJ, Kim S, Nam HW. Molecular cloning of ribosomal P protein in *Toxoplasma gondii* and the availability to detect antibody against recombinant protein in toxoplasmosis patients. Kor J Parasitol 2003;41:89–96.

[24] Sehgal A, Kumar N, Carruthers VB, Sharma S. Translocation of ribosomal protein P0 onto the *Toxoplasma gondii* tachyzoite surface. Int J Parasitol

2003;33:1589–94.

[25] Chatterjee S, Singh S, Sohoni R, et al. Characterization of domains of the phosphoriboprotein P0 of *Plasmodium falciparum*. Mol Biochem Parasitol 2000;107:143–54.

[26] Chatterjee S, Singh S, Sohoni R, et al. Antibodies against ribosomal phosphoprotein P0 of *Plasmodium falciparum* protect mice against challenge with *Plasmodium yoelii*. Infect Immun 2000;68:4312–8.

[27] Iborra S, Soto M, Carrion J, et al. The *Leishmania infantum* acidic ribosomal protein P0 administered as a DNA vaccine confers protective immunity to *Leishmania major* infection in BALB/c mice. Infect Immun 2003;71:6562–72.

[28] Iborra S, Carrion J, Anderson C, Alonso C, Sacks D, Soto M. Vaccination with the *Leishmania infantum* acidic ribosomal P0 protein plus CpG oligodeoxynucleotides induces protection against Cutaneous Leishmaniasis in C57BL/6 mice but does not prevent progressive disease in BALB/c mice. Infect Immun 2005;73:5842–52.

[29] Sambrook J, Russell DW. Molecular Cloning: a Laboratory Manual. 3rd ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press 2001.

[30] Kimbitaa EN, Xuan X, Huang X, et al. Serodiagnosis of *Toxoplasma gondii* infection in cats by enzyme-linked immunosorbent assay using recombinant SAG1. Vet Parasitol 2001;102:35–44.

[31] Zhou J, Fukumoto S, Jia H, et al. Characterization of the *Babesia gibsoni* P18 as a homologue of thrombospondin related adhesive protein. Mol Biochem Parasitol 2006;148:190–8.

[32] Lee EG, Na BK, Bae YA, et al. Identification of immunodominant

excretory-secretory cysteine proteases of adult *Paragonimus westermani* by proteome analysis. Proteomics 2006;6:1290–300.

[33] Kaneko Y, Takashima Y, Xuan X, et al. Natural IgM antibodies in sera from various animals but not the cat kill *Toxoplasma gondii* by activating the classical complement pathway. Parasitology 2004;128:123–9.

[34] Nishikawa Y, Xuan X, Makala L, Vielemeyer O, Joiner K, Nagasawa H. Characterization of *Toxoplasma gondii* engineered to express mouse interferon-gamma. Int J Parasitol 2003;33:1525–35.

[35] Bergami PL, Scaglione J, Levin MJ. Antibodies against the carboxyl-terminal end of the *Trypanosoma cruzi* ribosomal P proteins are pathogenic. 2001;15:2602–12.

[36] Bergami PL, Gomez KA, Levy GV, Grippo V, Baldi A, Levin MJ. The beta1 adrenergic effects of antibodies against the C-terminal end of the ribosomal P2beta protein of *Trypanosoma cruzi* associate with a specific pattern of epitope recognition. Clin Exp Immunol 2005;142:140–7.

[37] Singh S, Sehgal A, Goswami A, Waghmare S, Chakrabarty T, Sharma S. Surface expression of the conserved ribosomal protein P0 on parasite and other cells. Mol Biochem Parasitol 2002;119:121–4.

[38] Aruna K, Chakraborty T, Nambeesan S, et al. Identification of a hypothetical membrane protein interactor of ribosomal phosphoprotein P0. J Biosci 2004;29:33–43.

[39] Kasper LH, Khan IA. Antigen-specific $CD8^+$ T cells protect against lethal toxoplasmosis in mice infected with *Neospora caninum*. Infect Immun 1998;66:1554–60.

Figure legends

Fig. 1. Characterization of the NcP0 gene. (A) Multiple-sequence alignment of P0 proteins from *N. caninum* (Nc), *T. gondii* (Tg), *C. parvum* (Cp), *P. falciparum* (Pf), *T. parva* (Tp), *E. tenella* (Et), and *B. bovis* (Bb). Strictly conserved residues are indicated with a black background. (B) Phylogenetic tree of P0 proteins from *N. caninum* (Nc), *T. gondii* (Tg), *B. bovis* (Bb), *B. gibsoni* (Bg), *B. microti* (Bm), *Plasmodium* species P0 (*P. falciparum* (Pf), *P. berghei* (Pb), *P. yoelii* (Py)), *C. parvum*(Cp), *T. parva* (Tp), and *E. tenella* (Et). The sequence identities were calculated using DNASTAR software. (C) Southern blot analysis. The *N. caninum* genomic DNA was digested with *Xba*I (lane 1), *Sac*I (lane 2), and *Hind*III (lane 3); the separated DNA fragments were hybridized with the NcP0 cDNA probe.

Fig. 2. Characterization of the NcP0 protein. (A) Two-dimensional electrophoresis of *N. caninum* tachyzoite lysates under reducing conditions. Proteins were resolved by isoelectric focusing (pH 3~10) in the first dimension followed by SDS-PAGE in the second dimension on a 10% gradient gel. A spot corresponding to the NcP0 is indicated. (B) Western blot analysis of NcP0. The arrow indicates specific spot recognized by the antiserum against rNcP0. (C) MALDI-TOF mass analysis of the NcP0. The blue lines and letters represent the matched peptide mass and corresponding amino acid sequence of peptide fragments.

Fig. 3. Western blot analysis and immunofluorescence microscopy analysis. (A) Western blot analysis of *N. caninum* tachyzoite lysates and *T. gondii* tachyzoite lysates. The blots were probed with anti-rNcP0 antibodies after 1D SDS-PAGE. Lane 1, Vero cell lysates; lane 2, *N. caninum* lysates; lane 3, *T. gondii* lysates. (B) The extracellular and intracellular tachyzoites were stained with anti-rNcP0 antibodies followed by Alexa Fluor 488-conjugated goat anti-mouse IgG. Panels a–c, permeabilized extracellular tachyzoites; panels d–f, non-permeabilized extracellular tachyzoites; panels g–i, permeabilized Vero cells infected with tachyzoites treated with anti-rNcP0 sera. Bars indicate 10 μm.

Fig. 4. *In vitro* inhibition of growth. (A) *In vitro* inhibition assay for *N. caninum* growth by IgG of anti-rNcP0 antibodies. The value for the invasion of *N. caninum* tachyzoites in the absence of antibodies was considered to be a 100% value. Values for the host-cell infection of *N. caninum* tachyzoites after preincubation with anti-GST IgG antibodies (▪) and anti-rNcP0 IgG antibodies (▲). (B) *In vitro* cross-inhibition assay for *T. gondii* growth by anti-rNcP0 IgG antibodies. Values for the host-cell infection of *T. gondii* tachyzoites after preincubation with anti-GST IgG antibodies (▪) and anti-rNcP0 IgG antibodies (▲). The results are representative of five individual experiments, and the error bars indicate standard deviations. (*) The differences between the treatment with anti-GST antibodies and anti-rNcP0 antibodies at the same IgG concentration were significant ($P< 0.05$).

Figure1

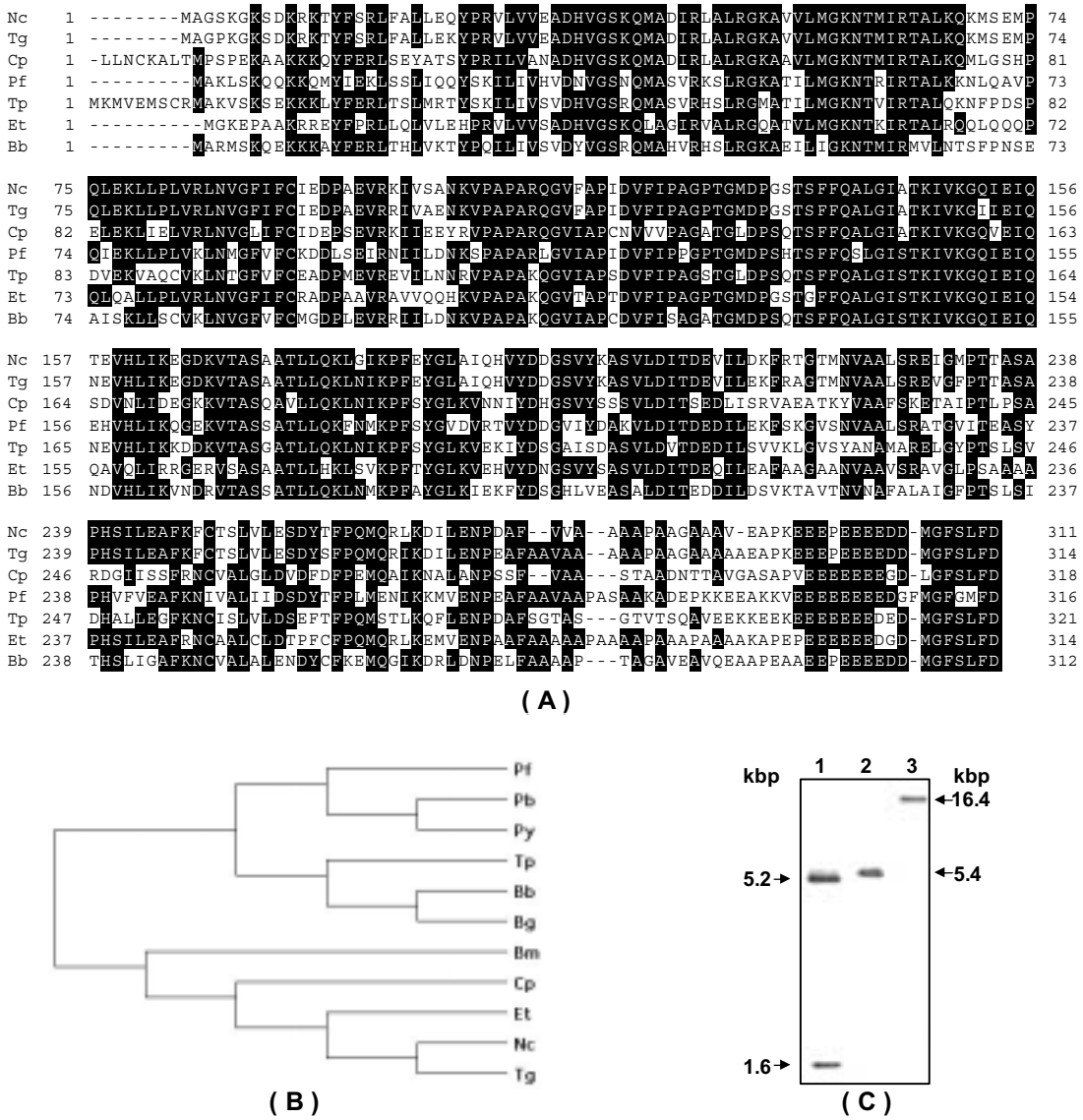


Fig. 1.

Figure2

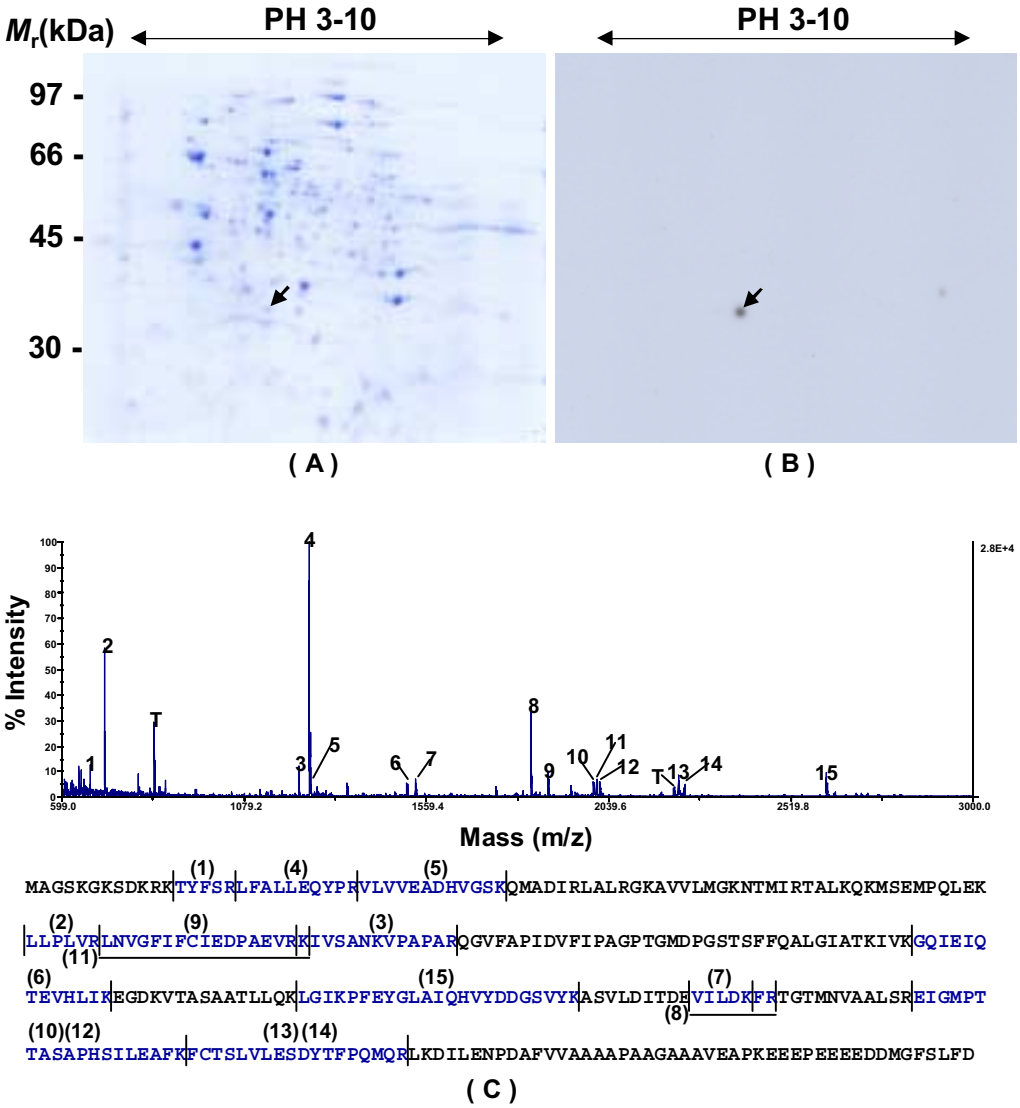
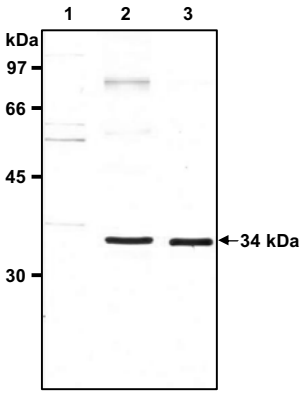
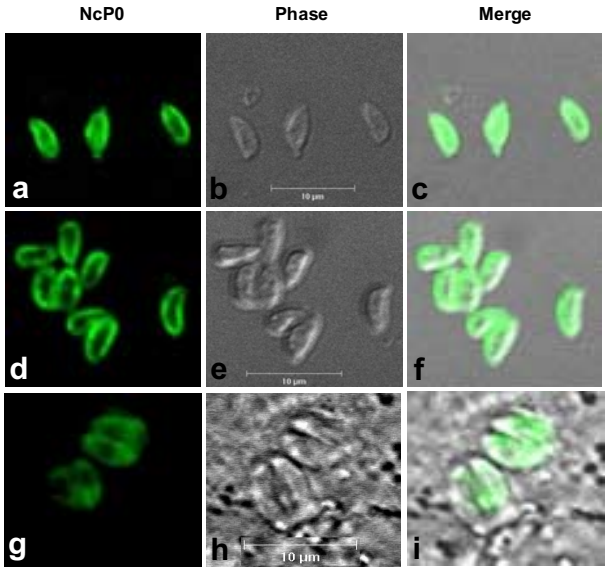


Fig. 2.

Figure3



(A)



(B)

Fig. 3.

Figure4

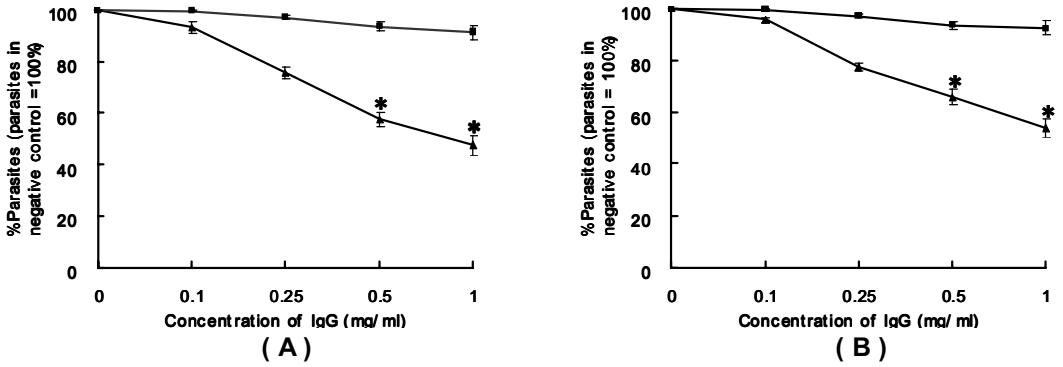


Fig. 4.