

Identification and Characterization of *Neospora caninum* Cyclophilin That Elicits Gamma Interferon Production

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Gamma interferon (IFN- γ) response is essential to the development of a host protective immunity in response to infections by intracellular parasites. Neosporosis, an infection caused by the intracellular protozoan parasite *Neospora caninum*, is fatal when there is a complete lack of IFN- γ in the infected host. However, the mechanism by which IFN- γ is elicited by the invading parasite is unclear. This study has identified a microbial protein in the *N. caninum* tachyzoite *N. caninum* cyclophilin (NcCyP) as a major component of the parasite responsible for the induction of IFN- γ production by bovine peripheral blood mononuclear cells (PBMC) and antigen-specific CD4⁺ T cells. NcCyP has high sequence homology (86%) with *Toxoplasma gondii* 18-kDa CyP with a calculated molecular mass of 19.4 kDa. NcCyP is a secretory protein with a predicted signal peptide of 17 amino acids. Abundant NcCyP was detected in whole-cell *N. caninum* tachyzoite lysate antigen (NcAg) and *N. caninum* tachyzoite culture supernatant. In *N. caninum* tachyzoite culture supernatant, three NcCyP bands of 19, 22, and 24 kDa were identified. NcAg stimulated high levels of IFN- γ production by PBMC and CD4⁺ T cells. The IFN- γ -inducing effect of NcAg was blocked by cyclosporine, a specific ligand for CyP, in a dose-dependent manner. Furthermore, cyclosporine abolished IFN- γ production by PBMC from naive cows as well as PBMC and CD4⁺ T cells from infected/immunized cows. These results indicate that the *N. caninum* tachyzoite naturally produces a potent IFN- γ -inducing protein, NcCyP, which may be important for parasite survival as well as host protection.

Gamma interferon (IFN- γ) production in response to intracellular microbial exposure is critical to the development of a host protective immunity (25). During neosporosis (7), a disease caused by the intracellular protozoan parasite *Neospora caninum*, IFN- γ induced by *N. caninum* tachyzoites plays a pivotal role in control of the acute phase of the disease (2, 18, 20). It has been clearly shown that the complete lack of IFN- γ in the IFN- γ ^{-/-} mouse model renders the host highly susceptible to infection (often fatal) by *N. caninum* (21). Continued production of *N. caninum*-elicited IFN- γ production during infection will lead to a chronic state of neosporosis in cows with no obvious acute symptoms of the disease. However, infection by environmental *N. caninum* oocysts or reactivation of chronic infection, leading to the release of tissue bradyzoites from the tissue cyst in infected animals, will elicit high levels of IFN- γ , which may be an important cause for bovine abortion (13, 15). Presently, it remains unknown how host IFN- γ production is regulated by the invading parasite.

Cyclophilins (CyPs) are ubiquitous cytosolic proteins and have been described in prokaryotic as well as eukaryotic organisms (9). CyP was discovered for its peptidyl-prolyl *cis-trans* isomerase (PPIase) activity and its high binding affinity to cyclosporine, an immunosuppressant drug commonly used to prevent graft rejection (8–10, 19, 27). The PPIase activity is considered to mediate protein folding and function as chaperons (14). The cyclosporine binding proteins, CyPs, and other

similar immunosuppressive drug binding proteins have also been named immunophilin (9). Cyclosporine binding has been shown to block the effect of the PPIase activity of CyP. A large number of cyclosporine binding proteins have been reported belonging to the CyP family, most of which have been shown to function as mediators of intra- and intercellular communications (5). In particular, *Toxoplasma gondii* 18-kDa CyP (*T. gondii* C-18) has been recently shown to act as a molecular mimic to bind the cysteine-cysteine chemokine receptor CCR5 on murine dendritic cells and stimulate interleukin-12 (IL-12) production (1). The production of IL-12 by murine dendritic cells was blocked by cyclosporine *in vitro* and *in vivo*, indicating the role of CyP in regulating IL-12 (1). In an attempt to search for *N. caninum* immunodominant antigens using bovine CD4⁺-T-cell lines, the present study has identified *N. caninum* CyP (NcCyP) using mass spectrometry from *N. caninum* tachyzoite lysate that was associated with T-cell antigenic stimulatory activity (30). NcCyP has the highest protein sequence homology with *T. gondii* C-18. The *N. caninum* tachyzoite lysate antigen (NcAg) induced high levels of IFN- γ production by peripheral blood mononuclear cells (PBMC) from naive and exposed/infected cows as well as CD4⁺ T cells established from immunized/challenged cows. Abundant NcCyP was detected in *N. caninum* tachyzoite whole-cell lysate or tachyzoite culture supernatant. The CyP ligand cyclosporine was able to block IFN- γ production by bovine PBMC or CD4⁺ T cells in response to NcAg stimulation, suggesting that the ability of NcAg to elicit IFN- γ production may be due largely to NcCyP. NcCyP may play an important role in the development of host protective immunity as well as in the induction of abortion when high levels of IFN- γ are elicited. Our results indicate that the study of NcCyP may be crucial to the understanding of host

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protective immunity to *N. caninum* infection and will facilitate the development of a subunit vaccine against neosporosis.

MATERIALS AND METHODS

Animals. Holstein dairy cows were immunized subcutaneously with killed, whole-cell *N. caninum* tachyzoite lysate in ImmunoMax SR (Zonagen, Woodlands, TX) and challenged intravenously with culture-derived live *N. caninum* (NC-1 strain) tachyzoites (6). The cows were bled from a jugular vein weekly using Vacutainers containing EDTA as anticoagulant. The cows were maintained at the U.S. Department of Agriculture (USDA) Dairy facility at Beltsville, MD. Animal use and care were approved by the USDA/Agricultural Research Service Beltsville Agricultural Research Center Animal Use and Care Committee.

Reagents. Cyclosporine (99% pure) was purchased from LC Laboratories (Woburn, MA). Bovine antiserum against NcAg was collected from cows that were previously immunized with whole-cell NcAg and challenged with live *N. caninum* (29). Rabbit antiserum against *N. caninum* tachyzoites was generated by injecting rabbits with live *N. caninum* tachyzoites (16). Rabbit antiserum against *T. gondii* cyclophilin was a generous gift from Alan Sher (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) (1). A bovine IFN- γ enzyme-linked immunosorbent assay kit (BOVIGAM) was purchased from Pfizer Animal Health (Omaha, NE).

***T. gondii* tachyzoite propagation and lysate preparation.** *T. gondii* tachyzoites were obtained from the peritoneal cavities of mice inoculated intraperitoneally with tachyzoites of the RH strain of *T. gondii*. Tachyzoites were passed through a 27-gauge needle, filtered with a 3- μ m-pore-size filter, and washed twice with phosphate-buffered saline (PBS; pH 7.4). *T. gondii* tachyzoites were then subjected to three freezing-thawing cycles and centrifuged at $10,000 \times g$ at 4°C for 15 min. Protein concentration of the supernatant was determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, Illinois) and stored at -70°C until use.

***N. caninum* tachyzoite propagation and NcAg preparation.** *N. caninum* tachyzoites and NcAg were prepared as described elsewhere with modifications (3, 29). *N. caninum* (NC-1 isolate) tachyzoites were cultured on bovine monocytes (17) in RPMI 1640 medium supplemented with 2% fetal calf serum and 50 μ g/ml gentamicin at 37°C in a 95% air and 5% CO₂ atmosphere. *N. caninum* tachyzoite-infected host cells were collected, passed through 20- and 27-gauge needles, and pelleted by centrifugation at $1,000 \times g$ for 20 min at 4°C. Tachyzoites were then purified by centrifugation over a 40% Percoll gradient at $2,000 \times g$ for 30 min at 4°C. The tachyzoite pellet was then resuspended in PBS (pH 7.4) and washed three times. The resulting pellet was resuspended in 25 mM Tris (pH 8.0) and subjected to three 15-s pulses of sonication on ice at maximal frequency using a sonicator (Virsonic Cell Disrupter; The Virtis Company, New York, NY) in the presence of a protease inhibitor cocktail mixture (Boehringer Mannheim Cooperation, Indianapolis, Indiana). Host cell protein extract was similarly prepared from the host cell line. Protein concentration was determined with the bicinchoninic acid protein assay kit (Pierce, Rockford, Illinois). NcAg and the host cell protein extract control were stored at -25°C until use.

Western blotting, silver staining, and Coomassie blue staining. NcAg was separated on 4 to 12% gradient NuPAGE gels (Invitrogen, Carlsbad, California), followed by silver and Coomassie blue staining and Western blotting. After electrophoresis, proteins were either visualized directly with silver and Coomassie blue staining (Invitrogen) or transferred to a polyvinylidene difluoride membrane (Millipore) using an electrotransblotter (Invitrogen). For Western blotting, the membrane was blocked with Superblock solution with 0.05% Tween 20 (Pierce) for 2 h or overnight and washed once with washing buffer (PBS with 0.05% Tween 20). The blot was incubated with rabbit antiserum (1:1,000) or bovine antiserum (1:500) for 1 h and washed six times with washing buffer, followed by incubation with goat anti-rabbit immunoglobulin G-horseradish peroxidase (Pierce) or goat anti-bovine immunoglobulin G-horseradish peroxidase (KPL, Gaithersburg, MD) for 1 h. Following three washes in washing buffers, the blot was developed by adding the chemiluminescence substrate (Pierce). Benchmark (unstained standards; Invitrogen) was used for silver staining, and MagicMark (prestained standards; Invitrogen) was used for Western blotting. Silver and Coomassie blue stainings were performed according to the manufacturer's instructions (Invitrogen). The images were captured using Epi Chemi II Darkroom (UVP Laboratory Products, Upland, California).

Analysis of *N. caninum* tachyzoite proteins by mass spectrometry. Coomassie blue-stained proteins migrating at approximately 18 kDa on 4 to 12% NuPAGE gels were excised and submitted for protein sequencing by mass spectrometry (Mass Spectrometry Lab, Center for Functional Genomics, University at Albany, Rensselaer, NY). Coomassie blue-stained gel slices containing the proteins of

interest were subjected to in situ alkylation of cysteines followed by in-gel tryptic digestion. The digested mixture was injected onto an electrospray liquid chromatography time of flight II tandem mass spectrometer (Microarrays; Waters). The amino acid sequence data generated in this study were searched against all gene and protein databases for matches using Mascot (Matrix Science, United Kingdom).

IFN- γ enzyme-linked immunosorbent assay. Bovine IFN- γ concentrations in culture supernatants were determined with the BOVIGAM bovine IFN- γ test (Pfizer Animal Health, Omaha, NE) according to the manufacturer's instructions. Bovine recombinant IFN- γ standard was purchased from SEROTEC (Raleigh, NC). IFN- γ levels in supernatants were calculated based on the standard curve.

Bovine CD4⁺-T-cell lines. NcAg-specific CD4⁺-T-cell lines were established as described previously (28). Briefly, NcAg-specific T-cell lines were established from the PBMC of *N. caninum*-infected cows. All cell lines were maintained by weekly stimulation with NcAg and antigen-presenting cells (APCs). After the first week in culture and weekly thereafter, all cell lines were subcultured with irradiated autologous PBMC as a source of APCs. T cells were analyzed for phenotype by flow cytometry and tested for antigen-dependent proliferation 7 days following the last antigen stimulation. The phenotypes of T-cell lines were analyzed for surface expression of CD3, CD4, and the delta chain of the γ/δ T-cell receptor by using bovine-specific monoclonal antibodies (Washington State University Monoclonal Center, Pullman, WA) in a flow cytometer (Beckman Coulter) as described previously (28). Stained cells were resuspended in 2% formaldehyde in PBS and stored at 4°C until analyzed. Flow cytometric data are expressed as percentages of total CD3⁺ T cells.

Lymphocyte proliferation assay. The proliferation assay was performed as described elsewhere (28). T cells (3×10^4 cells per well) and APCs (2×10^5 cells per well) were cocultured in triplicate wells with complete medium, NcAg (1 μ g/ml), or host cell protein (1 μ g/ml) in a total volume of 100 μ l complete medium per well using 96-well plates. Cells were radiolabeled for the last 4 to 18 h of culture with 0.25 μ Ci/well [³H]thymidine (PerkinElmer), harvested using a semiautomated cell harvester (Tomtec, Orange, CT), and counted with a liquid scintillation counter (Wallace 1450 Microbeta Trilux liquid scintillation & luminescence counter; PerkinElmer). Data are expressed as mean counts per minute.

Culture of PBMC and CD4⁺ T cells in the presence of NcAg and cyclosporine. PBMC (2×10^6 cells per well) and CD4⁺ T cells (1×10^6 cells per well) plus APCs (1×10^6 cells per well) in 48-well plates (Costar) were cultured with complete medium alone, NcAg (1 μ g/ml) alone, or NcAg (1 μ g/ml) in the presence of increasing concentrations (0.1 to 1,000 nM) of cyclosporine in 10-fold serial dilutions. Cells were incubated for 48 h at 37°C in a 5% CO₂ and 95% air atmosphere. Supernatants were collected and centrifuged at $1,000 \times g$ for 15 min at 4°C and stored at -25°C until analyzed for IFN- γ concentration. Cyclosporine was solubilized in 100% ethanol at 100 mM and stored at -25°C until used. Final concentration of ethanol in culture was less than 0.001% (vol/vol).

Nucleotide sequence accession number. The protein sequence data reported in this paper will appear in the Swiss-Prot and TrEMBL data banks under accession number P84343.

RESULTS

Immunodominant antigens were recognized with bovine anti-NcAg sera or rabbit anti-*N. caninum* tachyzoite sera. In our laboratory previously, a band of proteins was identified by high-pressure liquid chromatography to be associated with CD4⁺-T-cell stimulatory activity (30). In the present study, Western blotting revealed that immunodominant antigens migrate at 17 to 19 kDa on 4 to 12% NuPAGE gels, which was recognized by both sera from NcAg-immunized/challenged dairy cows (Fig. 1A, lane 1) and *N. caninum* tachyzoite-infected rabbits (Fig. 1B, lane 1). The 17- to 19-kDa proteins were also visualized by both silver (Fig. 1C) and Coomassie blue (Fig. 1D) staining. This protein band was excised from the Coomassie blue-stained gel and submitted for mass spectrometric analysis.

Identification of *N. caninum* cyclophilin by mass spectrometry. A total of 11 distinctive genes and proteins were matched to the peptide sequences identified in the 17- to 19-kDa *N.*

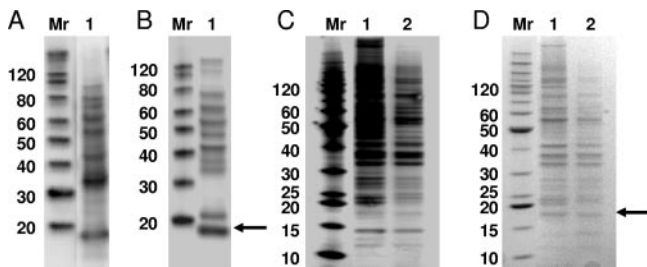


FIG. 1. Western blot analyses of NcAg using bovine anti-NcAg serum (A) or rabbit anti-*N. caninum* tachyzoite serum (B) and one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis separation of whole-cell NcAg or *N. caninum* tachyzoite culture supernatant and visualization by silver staining (C) and Coomassie blue staining (D). Twenty μ g of total protein per lane for Western blotting and Coomassie blue staining or 5 μ g of total protein per lane for silver staining was loaded. Lanes 1 of panels A and B and lanes 2 of panels C and D, whole-cell NcAg; lanes 1 of panels C and D, supernatant of *N. caninum* tachyzoite cultured in PBS for 1 h at 37°C. Mr, molecular weight markers, MagicMark for panels A and B, Benchmark for panels C and D. Arrows indicate the immunodominant protein(s) at an apparent molecular mass of ~18 kDa recognized by both bovine and rabbit sera against *N. caninum* tachyzoite proteins or live *N. caninum* tachyzoites (A and B) or the protein band excised for mass spectrometric analysis (D).

caninum proteins by mass spectrometry (Table 1). Since the objective of the present study was to identify and characterize *N. caninum* proteins that regulate host IFN- γ production, we then focused our effort on the *N. caninum* CyP (Tables 1 and 2) whose homolog from *T. gondii* was shown to modulate the production of IFN- γ (1). Five nonoverlapping peptide sequences were obtained from the mass spectrometric analysis (Table 2), which were matched perfectly to the deduced protein sequences of nine identical, but different in length, CyP expressed sequence tag (EST) cDNA sequences (Table 3) specific for *N. caninum* tachyzoites in the databases (USDA—Washington University *Neospora* EST Project, GenBank). This *N. caninum* protein sequence is similar (86%) to *T. gondii* PPIase or CyP (12) (Fig. 2A). The NcCyP sequence has a total of 178 amino acids and contained a predicted signal peptide of

TABLE 1. *N. caninum* genes and proteins that matched peptides identified within the 18- to 19-kDa-molecular-mass range of *N. caninum* proteins by mass spectrometric analysis^a

Gene accession no.	Protein
CF371177	GRA1
BF716434	Unknown
CF422590	Peptidyl-prolyl <i>cis-trans</i> isomerase
BF824629	Unknown
BM132349	CG8415 protein
BF249328	Putative nucleoside-diphosphate kinase
N61052	Histone H2A.Z
CD537951	40S ribosomal protein S19
BF249206	Unknown
BF716647	60S ribosomal protein L32
AI180389	Unknown

^a This table lists the significant hits of Mascot search results (Matrix Science, United Kingdom) from all EST databases. The EST databases were used because the *N. caninum* genome has not been sequenced yet and most of the *N. caninum* sequences available now are ESTs. None of these sequences have been published, but references can be found in GenBank.

TABLE 2. Peptides identified by mass spectrometry and matched to *N. caninum* cyclophilin cDNA sequences^a

Sequence no. ^b	Peptide amino acid sequence	Corresponding amino acid positions
1	KAFMDIEIDGESAGR	24–38
2	NFIGLFDKYK	53–63
3	VIADFMIOGGDFENHNGTGGHSIYG PRFEDENFTLK	69–105
4	GVISMANAGPNTNGSQFFITTVK	108–131
5	ITNSDWPTVQAI EALGSSGGRPSKI	144–169

^a These peptides cover 61% (109 of 178 amino acids) of the entire NcCyP sequence. The protein sequence data reported in this paper will appear in the Swiss-Prot and TrEMBL data banks under accession number P84343.

^b Peptides are numbered from the amino terminus to the carboxyl terminus of the NcCyP sequence.

17 amino acids (SignalP 3.0 server; Technical University of Denmark) (Fig. 2B). NcCyP has theoretical molecular masses and pIs of 19.4 kDa and 6.2 with the signal peptide and 17.6 kDa and 6.0 without the signal sequence, respectively. It was predicted that NcCyP has three potential N-linked glycosylation sites (NetNGlyc 1.0 server; Technical University of Denmark). Unlike NcCyP, *T. gondii* CyP has only two potential N-linked glycosylation sites. The NcCyP sequence also has three protein kinase C phosphorylation sites, three myristylation sites, and one RGD sequence (Fig. 2B). The cyclophilin type peptidyl-prolyl *cis-trans* isomerase signature sequence was mapped to amino acid positions 62 through 79 (PPSearch, PROSITE, Database of Protein Families and Domains, EMBL-European Bioinformatics Institute) (Fig. 2B).

Multiple sequence alignment revealed that NcCyP has the highest homology (86%) with *T. gondii* CyP (C-18) (Fig. 2C). The NcCyP protein sequence was also compared to those of other species, including both prokaryotic and eukaryotic organisms (Fig. 2C). Surprisingly, in addition to *T. gondii* CyP, NcCyP is more similar (62%) to the CyP of the zebra fish (*Brachydanio rerio*) than to CyPs from other species (Fig. 2C). The sequence homology between the NcCyP and CyPs of other selected species including *Homo sapiens* (human), *Mus musculus* (house mouse), *Theileria parva*, *Gallus gallus* (chicken), *Bos taurus* (cattle), *Sus scrofa domestica* (domestic pig), *Dro-*

TABLE 3. EST cDNA sequences in GenBank that matched the *N. caninum* peptide sequences identified in the present study^a

Accession no.	EST cDNA length (bp)	Coding sequence ^b
CF273924	466	Partial
CF274554	491	Partial
CF275431	497	Partial
CF421713	525	Partial
CF421891	485	Partial
CF422590	621	Complete
CF422613	496	Partial
CF775429	590	Partial
CF940514	551	Partial

^a Sequences listed in this table were generated by Cole et al. in the USDA—Washington University *Neospora* EST Project, Washington University School of Medicine, St. Louis, MO, and obtained from the GenBank database (unpublished data). The EST sequence CF422590 was used to infer the rest of the amino acid sequence of NcCyP.

^b Coding sequence for NcCyP.

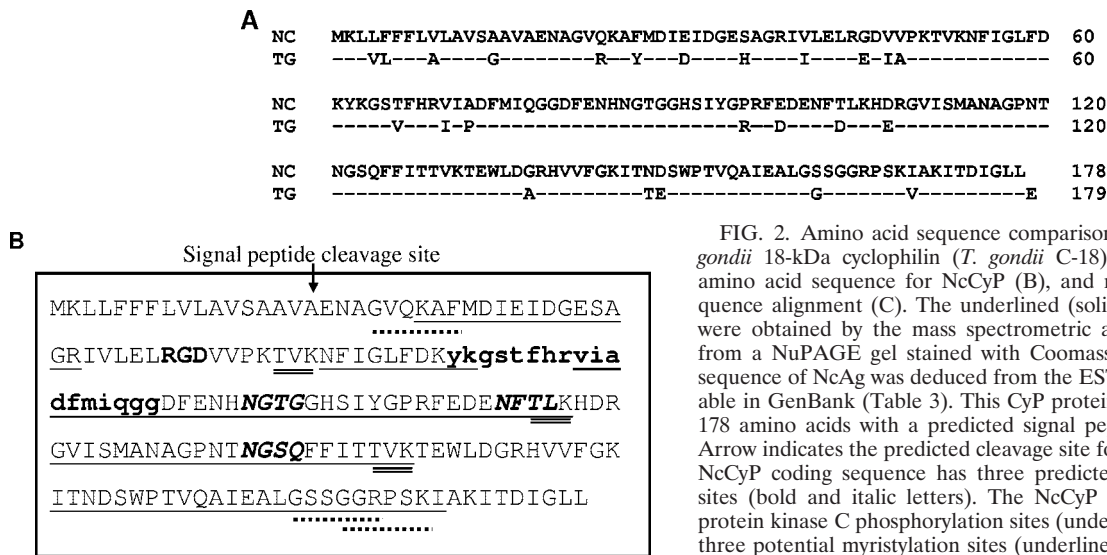


FIG. 2. Amino acid sequence comparison between NcCyP and *T. gondii* 18-kDa cyclophilin (*T. gondii* C-18) (A), annotation of the amino acid sequence for NcCyP (B), and multiple CyP protein sequence alignment (C). The underlined (solid single lines) sequences were obtained by the mass spectrometric analysis of NcAg excised from a NuPAGE gel stained with Coomassie blue. The rest of the sequence of NcAg was deduced from the EST cDNA sequences available in GenBank (Table 3). This CyP protein sequence has a total of 178 amino acids with a predicted signal peptide of 17 amino acids. Arrow indicates the predicted cleavage site for the signal peptide. The NcCyP coding sequence has three predicted N-linked glycosylation sites (bold and italic letters). The NcCyP sequence also has three protein kinase C phosphorylation sites (underlined with double lines), three potential myristylation sites (underlined with dotted lines), and one RGD sequence (bold uppercase letters). The cyclophilin type peptidyl-prolyl *cis-trans* isomerase (PPIase) signature sequence is indicated in bold lowercase. Dashes in Fig. 2B indicate identical amino acids between the *N. caninum* and *T. gondii* CyP sequences. NcCyP is 1 amino acid shorter at the N terminus than that in *T. gondii* C-18. In Fig. 2C, multiple alignment of CyPs among species *Brachydanio rerio* (zebra fish), *Homo sapiens* (human), *Mus musculus* (house mouse), *Theileria parva*, *Gallus gallus* (chicken), *Bos taurus* (cow), *Sus scrofa domestica* (domestic pig), *Drosophila melanogaster* (fruit fly), *Felis catus* (cat), *Lycopersicon esculentum* (tomato), *Trypanosoma cruzi*, and *Plasmodium yoelii yoelii* is shown. -, a gap; *, a single, fully conserved residue; :, one of these strong groups is fully conserved (STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, AND FYW); .., one of these weaker groups is fully conserved (CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIK, AND HFY) (ClustalX Multiple Sequence Alignment Program, version 1.83, 2003).

sophila melanogaster (fruit fly), *Felis catus* (cat), *Lycopersicon esculentum* (tomato), *Trypanosoma cruzi*, and *Plasmodium yoelii yoelii* ranged from 52 to 57% at the protein level (Fig. 2C). Apparently, the differences in length of CyPs from different species are generated mostly from the amino termini of the CyP (Fig. 2C). For the selected sequences compared in this study, only 2 amino acid additions and/or deletions were observed at the carboxyl termini (Fig. 2C). NcCyP is 1 amino acid shorter than the *T. gondii* CyP at the carboxyl terminus. Overall, the cyclophilin type peptidyl-prolyl *cis-trans* isomerase signature sequence is conserved across selected species at 50% homology. The protein sequence homology between NcCyP and *T. gondii* CyP within the cyclophilin type peptidyl-prolyl *cis-trans* isomerase signature sequence is 83%, suggesting that NcCyP may also possess PPIase activity, which is being confirmed in our laboratory.

Detection of *N. caninum* CyP by rabbit antibodies against *T. gondii* CyP. It has previously been demonstrated in multiple studies that *N. caninum* and *T. gondii* are highly related organisms, which is reflected not only by their morphology but also by the high degrees of gene sequence homologies shared by these two organisms (4, 11, 15). Since *N. caninum* CyP and *T. gondii* CyP are highly similar at the protein level (Fig. 2B), a rabbit antiserum against *T. gondii* CyP (C-18) was used to detect CyP in lysates of *T. gondii* and *N. caninum* tachyzoites (Fig. 3A) and culture supernatant of *N. caninum* tachyzoites (Fig. 3B). *T. gondii* CyP with an apparent molecular mass of 19 kDa was detected in *T. gondii* tachyzoite lysate (Fig. 3A, lane 2), which is consistent with the molecular mass of *T. gondii* C-18 reported previously (1, 12). NcCyP was detectable in whole-cell NcAg with the apparent molecular masses of 19 and 24 kDa (Fig. 3A, lane 3). In addition to the 19- and 24-kDa bands, a minor band of 22 kDa was also detected in *N. caninum* tachyzoite culture supernatants obtained by incubating Percoll-purified *N. caninum* tachyzoites in PBS (pH 7.4) at 37°C for 1 h (Fig. 3B).

Dose-dependent cyclosporine inhibition of NcAg-induced IFN- γ production by bovine PBMC and T cells. *T. gondii* C-18

has been shown to stimulate dendritic cells to produce IL-12 (1), which in turn elicits the production of IFN- γ by T cells (24). The NcAg stimulation of IFN- γ production by PBMC and T cells and the inhibitory effect of cyclosporine on NcAg-elicited IFN- γ production by PBMC and T cells as an indirect indication of IL-12 production by dendritic cells are demonstrated in Fig. 4. The NcAg (1 μ g/ml) alone stimulated high levels of IFN- γ production by the PBMC of infected cows (Fig. 4). Cyclosporine exhibited a dose-dependent inhibitory effect on NcAg-stimulated IFN- γ production by the PBMC of *N. caninum*-exposed cows. A dose range of 100 to 1,000 nM for cyclosporine was effective to completely suppress the IFN- γ production stimulated by 1 μ g/ml of whole-cell NcAg (Fig. 4A and B).

Since CyP elicits IL-12 production by dendritic cells in an antigen-nonspecific manner (1), we have further determined the effect of CyP on IFN- γ production by PBMC from naive cows or antigen-specific T cells established from *N. caninum*-infected cows (Fig. 5A to D). PBMC isolated from the *N. caninum*-nonexposed cows used in this experiment did not proliferate in response to NcAg (data not shown). NcAg-specific T-cell lines composed of primarily the CD4⁺ T cells used for this experiment proliferated highly to NcAg stimulation (Fig. 5A and B). Again, NcAg alone stimulated high levels of IFN- γ production by both PBMC from naive cows and T cells

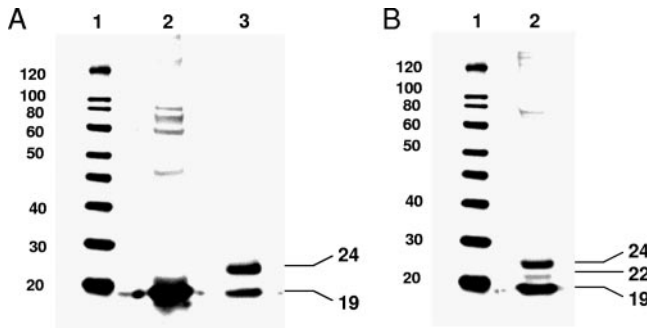


FIG. 3. Western blot analysis of NcCyP in lysates of *T. gondii* and *N. caninum* tachyzoites (A) and *N. caninum* tachyzoite culture supernatant (B) using a rabbit antibody against *T. gondii* C-18. Fifteen μ g total proteins was loaded per lane. Lanes 1, molecular weight markers (MagicMark); lane 2 (panel A), *T. gondii* tachyzoite lysate; lane 2 (panel B), *N. caninum* tachyzoite culture supernatant incubated in PBS at 37°C for 1 h; lane 3, whole-cell NcAg.

accountable for the induction of IL-12 production by dendritic cells in the mouse model (1). These results support the notion that the presence of IFN- γ -inducing factors, such as CyP, may be important for the control of acute neosporosis by eliciting high levels of IFN- γ , leading to the latent disease state which does not pose health problems unless animals become preg-

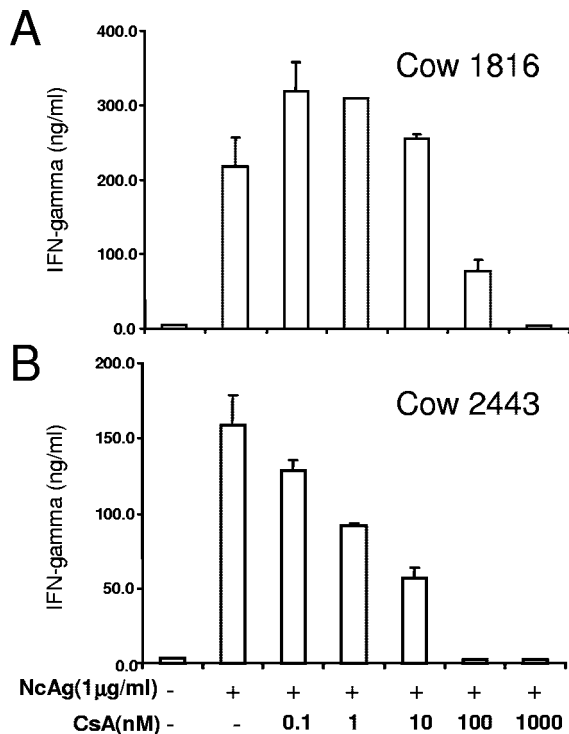


FIG. 4. Dose-dependent inhibition by cyclosporine (CsA) of NcAg-stimulated IFN- γ produced by bovine PBMC. Freshly isolated PBMC were cocultured for 48 h at 37°C with (+) or without (-) NcAg (1 μ g/ml) in the presence or absence (-) of increasing concentrations of cyclosporine (0.1 to 1,000 nM). Data represent results from two *N. caninum*-positive cows (with high anti-*N. caninum* tachyzoite antibody titers [data not shown]), cows 1816 (A) and 2443 (B), in two separate experiments. Values are means of ng/ml IFN- γ , and error bars represent standard errors of the means.

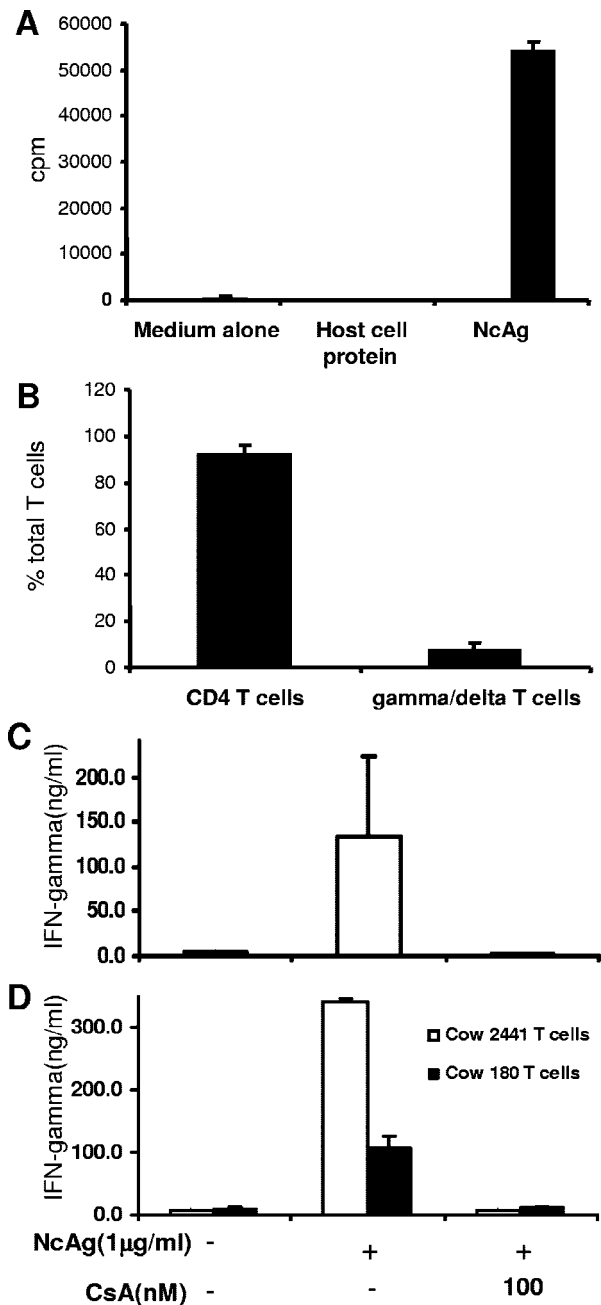


FIG. 5. Cyclosporine (CsA) suppression of NcAg-elicited IFN- γ production by PBMC from *N. caninum*-positive cows and bovine *N. caninum*-specific CD4⁺ T cells established from *N. caninum*-positive cows. Specific proliferation of the *N. caninum*-specific CD4⁺-T-cell lines in response to NcAg stimulation (A), phenotypes of these T cells (B), and CsA inhibition of NcAg-stimulated IFN- γ production by PBMC (C) and by CD4⁺-T-cell lines (D) of infected cows are shown. Values in panel A are means of counts per minute (cpm), in panel B are means of percent total T cells, and in panels C and D are means of ng/ml IFN- γ . Error bars represent standard errors of the means. Host cell proteins were prepared from the host cells used to propagate *N. caninum* tachyzoites in vitro.

nant (13). On the other hand, as speculated (1), production of CyP may be one of the survival strategies of the parasite, which intends not to kill the infected intermediate hosts.

NcCyP is highly similar to *T. gondii* C-18 in that they both

are secretory proteins containing a number of conserved functional motifs. One of the significant differences between *T. gondii* C-18 and NcCyP is that NcCyP is 1 amino acid residue shorter than *T. gondii* C-18 at the C terminus. However, it is unknown how such a change in NcCyP would affect its function. Since cyclosporine has effectively inhibited NcCyP function to upregulate IFN- γ , it may be reasonable to speculate that the amino acid residue at the C terminus is not critical to the IFN- γ -inducing effect of NcCyP or cyclosporine binding to CyP. In fact, most CyP sequences lack one to two of the amino acid residues at the C termini in comparison to *T. gondii* C-18, indicating that such a deletion may not have a significant effect on their PPIase and cyclosporine binding activities. The other difference between NcCyP and *T. gondii* C-18 is that NcCyP had an additional predicted N-linked glycosylation site (a total of three N-linked glycosylation sites) compared to *T. gondii* C-18 (a total of two N-linked glycosylation sites). This may explain the apparent molecular weight differences between NcCyP and *T. gondii* C-18 demonstrated in this study. The presence of an extra N-linked glycosylation site in NcCyP may explain the slight increase in molecular weight of NcCyP detected in Western blot analysis. The effect of such a glycosylation on NcCyP is unknown in comparison to *T. gondii* C-18. Recombinant *T. gondii* C-18 has been shown to have potent PPIase and IL-12-inducing activities (1), suggesting that additional N-linked glycosylation may have little effect on CyP function.

Our results indicated that cyclosporine inhibits NcAg-induced IFN- γ production in a dose-dependent manner without a noticeable effect on cell survival. Interestingly, cyclosporine was able to inhibit NcAg-elicited IFN- γ production by PBMC from naïve animals as well as PBMC and T cells derived from infected or immunized animals. For PBMC from naïve cows, the production of IFN- γ by naïve T cells and NK cells may rely largely on IL-12 induced by NcCyP in NcAg. Therefore, IFN- γ production may be abolished once the source for IL-12 is blocked. For PMBC and CD4⁺ T cells in the presence of APCs from infected or immunized cows, IFN- γ production by antigen-specific T cells may depend on both antigen-specific interactions between the APCs and CD4⁺ T cells and the IL-12 present at the antigen-priming stage. Apparently, as shown in this study, IL-12 production may be very critical for IFN- γ production by antigen-specific CD4⁺ T cells since the specific blockade of NcCyP, an IL-12-inducing microbial component, led to the elimination of IFN- γ . These results suggest that the lack of or reduced levels of NcCyP will influence the cytokine milieu at antigen priming and affect vaccination outcome. This further suggests that the lack of critical microbial regulatory components in vaccination regimes will have a major impact on vaccine efficacy. This may be particularly important for subunit vaccines in which only one or a few characterized proteins are included. The omission of important inflammatory cytokine-inducing microbial factors, in the case of intracellular pathogens, will explain why many of the subunit vaccines had no effect on protection or reduced efficacy (22, 23, 26). The study of such a potent microbe-derived IFN- γ inducer will facilitate our understanding of mechanisms by which the invading parasites manipulate host immune response for the purpose of long-term survival of the species. The knowledge obtained from these studies may be used to develop vaccines against

parasitic diseases by using the parasite's strategies that have evolved over millions of years.

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