# Characterization of a cDNA-clone encoding Nc-p43, a major *Neospora caninum* tachyzoite surface protein

A. HEMPHILL*, R. FELLEISEN, B. CONNOLLY, B. GOTTSTEIN, B. HENTRICH *and* N. MÜLLER

*Institute of Parasitology, University of Berne, Länggass-Strasse 122, CH-3012 Berne, Switzerland*

(*Received 26 March 1997; revised 27 May 1997; accepted 28 May 1997*)

SUMMARY

*Neospora caninum* is an apicomplexan parasite of veterinary importance which invades many different cell types and tissues. *N. caninum* tachyzoites proliferate intracellularly by endodyogeny. Eventually the massive proliferation of tachyzoites leads to host cell lysis and the newly formed parasites are released and invade neighbouring cells. Tachyzoite cell surface molecules could serve as ligands, mediating host cell adhesion and invasion. Nc-p43 is a recently identified *N. caninum* tachyzoite surface protein which is functionally involved in the processes leading to host cell invasion *in vitro*. Affinity-purified antibodies directed against Nc-p43 were used to screen a lambda gt22A-cDNA expression library constructed from *N. caninum* tachyzoites. The cDNA insert of one immunoreactive clone was subcloned and expressed in *E. coli* as a poly-histidine fusion protein. The identity of the resulting recombinant antigen termed recNc-p43 was confirmed by immunoblotting, immunofluorescence and electron microscopy using affinity-purified antibodies. The sequence of the cDNA insert encoding recNc-p43 was determined†. Analysis of the deduced amino acid sequence revealed that Nc-p43 exhibited similarity to SAG1 (p30) and SAG3 (p43), 2 major surface antigens of *Toxoplasma gondii* tachyzoites. These similarities were not reflected on the immunochemical level, since no cross-antigenicity between SAG1, SAG3 and Nc-p43 was observed.

Key words: *Neospora caninum*, Apicomplexa, invasion, dense granules, cDNA, cell surface molecule.

## INTRODUCTION

*Neospora caninum* is an apicomplexan parasite which causes neuromuscular disease and abortion in dogs, cattle and other animals (Dubey & Lindsay, 1996). *N. caninum* was established as an independent species by Dubey *et al*. (1988). For years, the parasite was misdiagnosed as *Toxoplasma gondii*, since the infective stages (tachyzoites) and the tissue cysts of these two parasites are morphologically very similar (Bjerkas & Presthus, 1988). Like *T. gondii*, *N. caninum* tissue cysts containing bradyzoites can persist in infected tissue for several years (Dubey & Lindsay, 1993). In infected animals, *N. caninum* tachyzoites cause cell death by intracellular multiplication and produce grossly visible necrotic lesions in a few days p.i.. Tachyzoites have been found in many different cell types. *In vitro* cultivation of these tachyzoites has been achieved in several well-established cell lines (Lindsay *et al*. 1993; Dubey & Lindsay, 1993), and parasites obtained from cell cultures retain their infectivity for animals (Dubey & Lindsay, 1996). Although carnivorous birds have been suspected as definitive hosts (Baker *et al*. 1995), the complete life-cycle of *N. caninum* remains to be elucidated.

Distinct differences between *Toxoplasma* and *Neospora* were demonstrated by immunohistochemistry using antisera directed against the corresponding parasites, and by serological assays (Bjerkås & Presthus, 1988; Bjerkås, Jenkins & Dubey, 1994; Bjoerkman *et al*. 1994). Differences have also been demonstrated at the genetic level. Sequence analysis of ribosomal RNA genes from similar coccidian parasites and of the p22 and p30 gene loci (Brindley *et al*. 1993; Ellis *et al*. 1994; Holmdahl *et al*. 1994; Marsh *et al*. 1995), and the finding that the *T. gondii*-specific B1-gene is not present in *N. caninum* (Burg *et al*. 1989; Müller *et al*. 1996) suggested clear phylogenetical differences between the two species. *N. caninum* is now placed into the family *Sarcocystidae* and is established as a sister group to *T. gondii* in the phylum Apicomplexa (Ellis *et al*. 1994).

The surface proteins of all obligatory intracellular parasites are prime candidates for mediating adhesion and host cell invasion. Earlier investigations have shown that the physical interaction between *N. caninum* tachyzoites and host cell surface membranes is mediated by a receptor–ligand system which is most likely based on protein–protein interactions (Hemphill, Gottstein & Kaufmann, 1996). Recently, a major *N. caninum* tachyzoite surface protein

* Corresponding author. Tel: +31-6312384. Fax: +31 6312622. E-mail: hemphill@ipa.unibe.ch

† The sequence information presented in this manuscript was submitted to GenBank data base under accession number U93870.

 
https://doi.org/10.1017/S0031182097001650 Published online by Cambridge University Press

named Nc-p43 was identified, and by using affinity-purified antibodies it was shown that no immunologically related proteins exist in *T. gondii* tachyzoites (Hemphill & Gottstein, 1996). Nc-p43 was demonstrated to be functionally involved in the adhesion and invasion process *in vitro* (Hemphill, 1996). This paper describes the isolation and characterization of a cDNA clone coding for Nc-p43, and demonstrates that, at the molecular, but not at the immunochemical, level, this *N. caninum* surface protein is related to SAG1 (p30) and SAG2 (p43), 2 major surface antigens of *T. gondii* tachyzoites.

## MATERIALS AND METHODS

Unless otherwise stated, all reagents and tissue culture media were purchased from Sigma (St Louis, MO, USA).

### *Vero cell culture*

Cultures of Vero cells were maintained in 10–12 ml of RPMI-1640 medium (Gibco-BRL, Basel, Switzerland) supplemented with 7% foetal calf serum (FCS), 2 mM glutamine, 50 U/ml penicillin, and 50 $\mu$g/ml streptomycin at 37 °C/5% $CO_2$ in T-25 tissue culture flasks. Cultures were trypsinized at least once a week.

### *Maintenance and purification of parasites*

*N. caninum* tachyzoites of the Nc-1 isolate (Dubey *et al*. 1988), and *T. gondii* tachyzoites of the RH strain (Sabin, 1941) were maintained in Vero-cell monolayers at 37 °C/5% $CO_2$ in RPMI-medium containing 2 mM glutamine, 50 U/ml penicillin, 50 $\mu$g/ml streptomycin, and 2–7% Ig-free horse serum (Gibco-BRL, Zürich, Switzerland). Parasites were harvested from their feeder cell cultures as previously described (Hemphill, 1996).

### *Preparation of membrane protein-enriched fractions of* N. caninum *tachyzoites employing Triton X–114*

Fractions enriched for membrane proteins of *N. caninum* tachyzoites of the Nc-1-isolate ($5 \times 10^7$–$10^8$ parasites/ml) were prepared as previously described (Bouvier, Etges & Bordier, 1985; Hemphill & Gottstein, 1996). In brief, purified parasites were resuspended in 3 ml of PBS, and phenylmethylsulfonyl fluoride (PMSF) was added to a concentration of 0·2 mM. After a 5 min incubation on ice, the cell suspension was adjusted to 0·75% Triton X–114, and parasites were extracted for 10 min on ice. The preparation was centrifuged (30 min, 10000 ***g***, 4 °C), and the supernatant was collected. The Triton X-114 supernatant was then incubated at 30 °C for 3 min, cooled on ice for 10 min, and centrifuged as above in order to remove possible aggregates. The supernatant was collected and was again incubated at 30 °C for 3 min. The detergent and hydrophilic phases were separated by a further centrifugation step (3 min, 1000 ***g***, 24 °C). The supernatant was removed, and the detergent phase was brought to 100 $\mu$l with PBS. This final fraction containing potential cell surface membrane proteins was further processed for SDS–PAGE by methanol/chloroform extraction as described (Wessel & Fluegge, 1984).

### *SDS–PAGE, immunoblotting and affinity purification of antibodies*

Samples of total and Triton X–114 extracts corresponding to the same number of *N. caninum* and *T. gondii* tachyzoites, as well as *E. coli* extracts expressing recombinant Nc-p43 (recNc-p43, see below), were separated by SDS–PAGE and electrophoretically transferred to nitrocellulose filters. Blocking of unspecific binding sites was carried out for 4 h at 24 °C in PBS containing 3% BSA (bovine serum albumin) and 0·3% Tween–20. The antiserum directed against *N. caninum* was applied at a dilution of 1:2000 in PBS/0·3% BSA/0·3% Tween–20 overnight at 4 °C. The filters were then washed 3 times in PBS/0·3% Tween, and the bound antibodies were visualized using horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin antibodies (Promega, Madison, WI, USA) according to the instructions provided by the manufacturers. As a control, nitrocellulose filters containing *E. coli* lysates expressing recNc-p43 were also incubated with the anti-rabbit-HRP conjugate alone in order to exclude that the conjugate itself would react with the recombinant protein.

Specific affinity-purified antibodies directed against Nc-p43 or against recombinant Nc-p43 (anti-recNc-p43 antibodies) were prepared as described earlier (Hemphill & Gottstein, 1996; Hemphill, 1996). Briefly, the bands corresponding to these 2 proteins were cut from nitrocellulose filters following SDS–PAGE and Western blotting. After blocking of unspecific binding sites in PBS/3% BSA/0·3% Tween–20, the anti-*N. caninum* antiserum was applied at a dilution of 1:10 in PBS/0·3% BSA/0·3% Tween–20 overnight at 4 °C. After washing the strip in PBS/0·3% Tween–20 three times for 10 min each, the bound antibodies were eluted in 900 $\mu$l low pH buffer (50 mM Tris, 50 mM glycine, pH 2·6) for 5 min on ice with occasional vortexing. Then the strip was removed, and 100 $\mu$l of 1 M Tris base was immediately added. The eluted antibody fraction was centrifuged (10000 ***g***, 20 min), in order to remove small nitrocellulose particles with bound antigen, and BSA was added to the supernatant to a final concentration of 0·1%. Affinity-purified antibodies were aliquoted and stored at

https://doi.org/10.1017/S0031182097001650 Published online by Cambridge University Press

−20 °C. They were used for immunoblotting at dilutions between 1:100 and 1:200.

### *Construction and screening of a* N. caninum *tachyzoite cCDNA library*

Messenger RNA was prepared from freshly purified tachyzoites employing the Trizol™ reagent and the message maker™ kit according to the instructions provided by the manufacturer (Gibco-BRL, Basel, Switzerland). Double-stranded cDNA was synthesized from 5 μg of mRNA and ligated into the lambda-gt22A vector employing the SuperScript™ Lambda System (Gibco-BRL, Basel, Switzerland). For *in vitro* packaging the Gigapack Gold II packaging extracts from Stratagene were used. The primary library was amplified on *E. coli* strain Y1090r- (Stratagene). Immunoscreening was performed using the affinity-purified anti-Nc-p43 antibodies and secondary antibodies conjugated to HRP (see above). Plaques which reacted positively were isolated and subjected to several rounds of rescreening until a pure plaque population was obtained. The phage clone expressing the Nc-p43 cDNA insert was finally amplified.

### *Analysis of the Nc-p43 cDNA insert*

The length of the Nc-p43 cDNA insert was determined by PCR-amplification in a thermal cycler (Perkin Elmer Cetus, Rotkreuz, Switzerland). Primers were derived from the 5′-flanking sequence on the sense strand (Primer 1218A: 5′-GCG GAT CCG GTG GCG ACG ACT CCT GGA GCC CG-3′) and the 3′-flanking sequence on the anti-sense strand (Primer 1222K: 5′-GCG GTA CCT TGA CAC CAG ACC AAC TGG TAA TG-3′) of the lambda gt22A vector. The primers were purchased from Gibco-BRL (Basel, Switzerland). PCR-products were electrophoretically analysed on 1 % agarose gels (Sambrook *et al*. 1989). PCR products were purified using the Wizard™ PCR-purification kit (Promega, WI, USA). The cDNA sequence of the insert was obtained by direct sequencing of purified PCR products by an automated sequencing service provided at the Institute of Clinical Pharmacology, University of Bern. Sequences were processed using the GCG-computer program set for VAX/VMS computers (Devereux, Haeberli & Smithies, 1984).

Using a 5′-*Sal* I site (provided by the phage vector lambda gt22A0 and a 3′-*Kpn* I site (introduced via the primer 1222K), the PCR-amplified Nc-p43 cDNA fragment was inserted in-frame into the *Xho* 1/ *Kpn* I digested expression vector pTRC-HisC (Promega, Zürich, Switzerland). This was possible since these 2 enzymes generate ligatable DNA ends. The cloned sequence was expressed in *E. coli* strain XL-1-blue under the control of the IPTG-inducible Ptac promoter as a C-terminal fusion to a peptide containing a poly-histidine (6 his) stretch. Bacteria expressing the recombinant protein (recNc-p43) were harvested by centrifugation, the pellet was solubilized in sample buffer and processed for SDS–PAGE and Western blotting as described above.

### *Immunofluorescence*

All procedures were carried out at room temperature. Freshly purified *N. caninum* or *T. gondii* tachyzoites suspended in PBS at $10^7$ parasites/ml were applied to poly-L-lysine-coated (100 μg/ml) glass cover-slips. After 10 min, the cover-slips were rinsed 3 times in PBS, and placed into fixation buffer (PBS/3 % paraformaldehyde/0·05 % glutaraldehyde, pH 7·2) for 15 min. The cover-slips were then rinsed extensively in PBS and were subsequently incubated in blocking solution (PBS/1 % BSA/50 mM glycine) for 30 min. The affinity-purified anti-recNc-p43 antibody was applied at a dilution of 1:1 in blocking solution for 30 min, followed by 3 buffer rinses. The second FITC-conjugated goat anti-rabbit antibody was used at 1:100 in blocking solution. As a negative control, parasites were also incubated with an affinity-purified rabbit anti-betagalactosidase antibody (Hemphill, 1996). Specimens were then washed in PBS (5 × 5 min). Finally, the preparations were briefly rinsed in distilled water, and embedded in a mixture of glycerol/gelvatol containing 1,4-diazobicyclo(2.2.2)octan (Merck, Milan, Italy) as an anti-fading reagent (Hemphill *et al*. 1996). Results were obtained by inspection of specimens on a Leitz Laborlux S fluorescence microscope.

### *LR-White-embedding and on-section labelling of* N. caninum*-infected Vero cell cultures*

Vero cells were grown in tissue culture flasks to 80–90 % confluency, and were incubated with purified parasites in growth medium as described (Hemphill, 1996). The medium was removed, and cells were fixed in PBS containing 3 % paraformaldehyde and 0·05 % glutaraldehyde for 30 min at 24 °C. The preparations were washed 3 times in PBS, and were removed from the surface of the tissue culture flask using a rubber policeman. They were kept in PBS/50 mM glycine for 1 h at 4 °C, and were washed extensively in PBS by several rounds of centrifugation. Specimens were then dehydrated using a graded series of ethanol (50–70–90–100 %) at −20 °C, 5 min each, and were embedded in LR-White resin at −15 °C, with 4 changes of fresh resin over a period of 3 days. The resin was polymerized at 58 °C for 24 h. Sections were cut using a Reichert & Jung ultramicrotome, and were picked up onto 200 mesh formvar-carbon-coated nickel grids

https://doi.org/10.1017/S0031182097001650 Published online by Cambridge University Press

(PLANO GmbH, Marburg, Germany). Loaded grids were stored at 4 °C for a maximum of 48 h. Prior to antibody labelling of sections, EM grids were incubated in EM-blocking buffer (PBS/0·5 % BSA/50 mM glycine) overnight at 4 °C. All subsequent steps were performed at room temperature. Sections were rinsed in PBS, and incubated either in affinity-purified anti-Nc-p43 or in anti-recNc-p43 antibodies diluted 1 : 1 in EM blocking buffer for 1 h. After washing in 5 changes of PBS, 2 min each, the goat anti-rabbit antibody conjugated to 10 nm gold particles (purchased from Amersham, Zürich, Switzerland) was applied at a dilution of 1 : 5 in PBS/0·5 % BSA for 45 min. After 6 washes in PBS, 5 min each, the specimens were shortly rinsed in distilled water, and were air-dried. Finally, grids were stained with lead citrate and uranyl acetate (Hemphill & Croft, 1997) and were subsequently viewed on a Phillips 300 transmission electron-microscope operating at 60 kV.

## RESULTS

A *N. caninum* tachyzoite cDNA expression library was constructed in the lambda gt22A phage system. A total of $5 \times 10^4$ plaques was screened with an affinity-purified anti-Nc-p43 antibody (Hemphill, 1996), and 1 phage clone was isolated by repeated screening. As demonstrated by PCR amplification, this clone contained a cDNA insert of about 1·5 kb. The cDNA insert was subcloned into the pTRC-HisC expression vector and was expressed in *E. coli* XL-1-blue (Fig. 1). After SDS-PAGE of the respective *E. coli* lysates, the poly-histidine fusion protein was not visible on Coomassie brilliant blue-stained gels (Fig. 1A). However, on subsequent Western blots, the recombinant protein (recNc-p43) could be clearly detected using the affinity-purified anti-Nc-p43 antibody originally used for screening of the expression library (Fig. 1B). The apparent molecular weight of rec-Nc-p43 was approximately 43 kDa. An additional reactive band of about 35 kDa could be observed on all Western blots of *E. coli* XL-1-blue lysates (Fig. 1B), but this band originated from the anti-rabbit-HRP conjugate.

Further experiments clearly showed that most likely the cDNA-insert isolated from the gt22A expression library did indeed code for a recombinant antigen which cross-reacts with Nc-p43. The anti-*N. caninum* antiserum was affinity-purified on recNc-p43 using the same procedure as for affinity purification of the original anti-Nc-p43 antibodies. On immunoblots, these affinity-purified anti-recNc-p43 antibodies were shown to exhibit the same immunolabelling pattern as anti-Nc-p43 antibodies (Fig. 1B, C). (i) They stained a distinct 43 kDa band in *N. caninum* tachyzoite extracts, (ii) they labelled the recombinant 43 kDa protein in *E. coli* XL-1-blue extracts, and (iii) no staining could be observed in *T. gondii* tachyzoite extracts. In addition, immunofluorescence surface staining of *N. caninum* and *T. gondii* tachyzoites showed that the epitopes corresponding to recNc-p43 were abundant on the *N. caninum*, but not on the *T. gondii* tachyzoite cell surface (Fig. 2). The cell surface association of the respective epitopes was confirmed by electron microscopical inspection of LR-White embedded parasites immunogold-labelled with affinity-purified anti-recNc-p43 antibodies: gold particles were abundant on the surface of both extracellular (Fig. 3A) and intracellular (Fig. 3B) *N. caninum* tachyzoites. In addition to the surface staining, distinct compartmentalized, intracellular labelling, namely of the dense granules at both the posterior and the anterior end of the tachyzoites, could also be demonstrated.

The nucleotide sequence of the cDNA fragment encoding recNc-p43 was determined. An open reading frame extended over the first 867 nucleotides, with a coding capacity of 285 amino acids. Thus, the calculated molecular weight of recNc-p43 was 35·6 kDa (with 31·6 kDa comprised of *Neospora* protein and 4 kDa originating from the vector). In order to establish a possible relationship to already published sequences, the deduced amino acid sequence of this open reading frame was compared to the SWISS-PROT sequence data base (release 34). The highest score of homology was found with the amino acid sequences of SAG1 (p30) and SAG3 (p43), 2 major surface proteins of *T. gondii* tachyzoites (Fig. 4) (Kasper *et al.* 1992; Cesbron-Delauw *et al.* 1994). Alignment of all 3 sequences demonstrated that the 285 amino acid sequence of recNc-p43 showed homologies to the C-terminal domains of SAG1 and SAG3 of *T. gondii*, while the sequence was beginning approximately 60–70 amino acids from the N-terminus of SAG3 (Fig. 4). This demonstrated that the 5′-end corresponding to the N-terminal domain of Nc-p43 was missing. In relation to SAG1, the recNc-p43 sequence exhibited an overall similarity of 43 % with 30 % identities. With respect to SAG3, the overall similarity is higher (44 %), but identities were only found within 23 % of the 285 amino acids.

Although the overall similarities of the 3 sequences were relatively low, some amino acid motifs indicated in Fig. 4 seemed to be well conserved, such as the sequence GATLTI (at positions 300–305), and the position of cysteine residues (at positions 133, 191, 201, 221, 247, 274 and 321). Another sequence motif, namely QYCSG (positions 261–265 and 264–268 in SAG3 and SAG1, respectively, see Fig. 4) was present only in the *Toxoplasma* surface proteins SAG1 and SAG3, but not in recNc-p43. Analysis of the amino acid sequence of recNc-p43 also demonstrated the presence of 1 potential *N*-glycosylation site at position 121 (NES), and 7 potential myristylation sites (see Fig. 4).

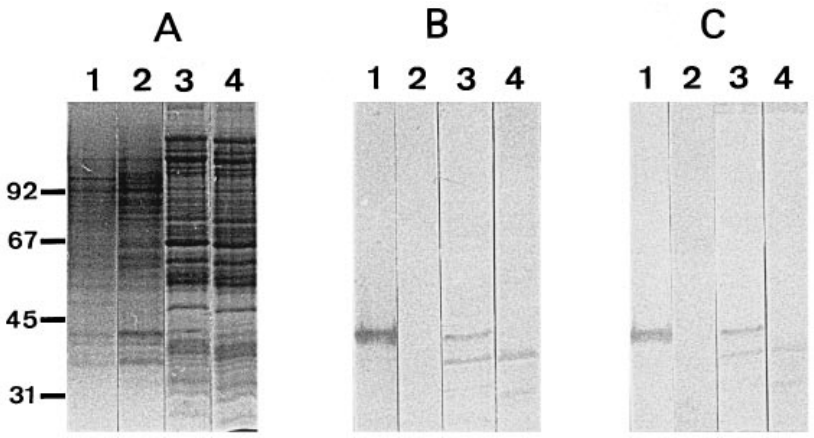


Fig. 1. Comparison of the reactivity of the antibodies affinity purified on the parasite-derived Nc-p43 band (B) and of antibodies affinity-purified on the expressed recombinant protein recNc-p43 (C). (A) Coomassie Brilliant blue stained 10% SDS–PAGE of *Neospora caninum* tachyzoite extracts (Lane 1), *Toxoplasma gondii* tachyzoite extracts (Lane 2), *E. coli* strain XL-1-blue expressing recNc-p43 (Lane 3), *E. coli* strain XL-1-blue harbouring the pTRC-HisC plasmid without the insert (Lane 4). (B) and (C) are the corresponding Western blots labelled with affinity-purified anti-Nc-p43 and anti-recNc-p43 antibodies, respectively. Molecular weight markers (kDa) are indicated.

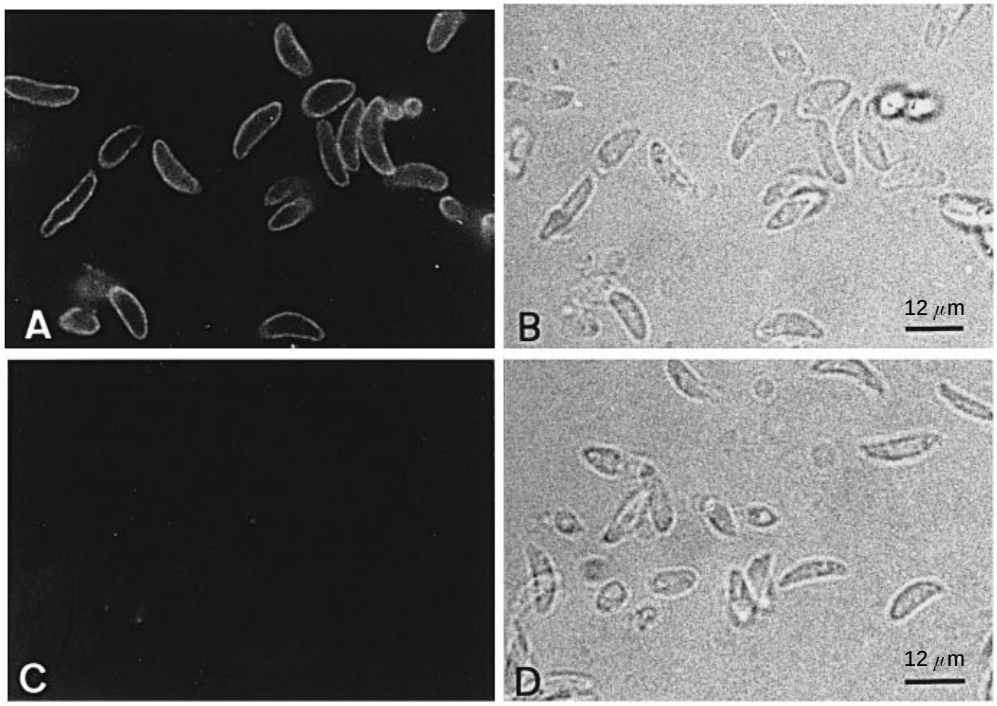


Fig. 2. Immunofluorescent staining of isolated *Neospora caninum* and *Toxoplasma gondii* tachyzoites with affinity-purified anti-recNc-p43 antibodies. Immunofluorescent cell surface staining of *N. caninum* tachyzoites (A), and of *T. gondii* tachyzoites (C) with affinity-purified anti-recNc-p43 antibodies. (B) and (D) are the corresponding phase-contrast images. Notice the absence of labelling on the *T. gondii* surface.

## DISCUSSION

In recent years, the importance of cell surface-associated molecules of intracellular parasites with respect to adhesion and invasion of host cells has been demonstrated (reviewed by Dubremetz & McKerrow, 1995; Manuël, 1996). Parasite surface components have also been shown to be important factors during the intracellular development of parasites (Russel, 1995; Turco, 1995), and in the presentation of antigens as these pathogens are internalized by antigen-presenting cells such as macrophages (Harding, 1995). Receptor–ligand systems responsible for mediating the direct physical contact between (glyco)proteins, glycolipids and lectin-binding sites have been identified in *Trypanosoma*, *Plasmodium* and *Toxoplasma* (Dubremetz & McKerrow, 1995; Galinski & Barnwell, 1996).

It is very likely that *N. caninum* tachyzoites are no exception to this rule, and that host cell entry is possibly mediated by a receptor–ligand system where

https://doi.org/10.1017/S0031182097001650 Published online by Cambridge University Press

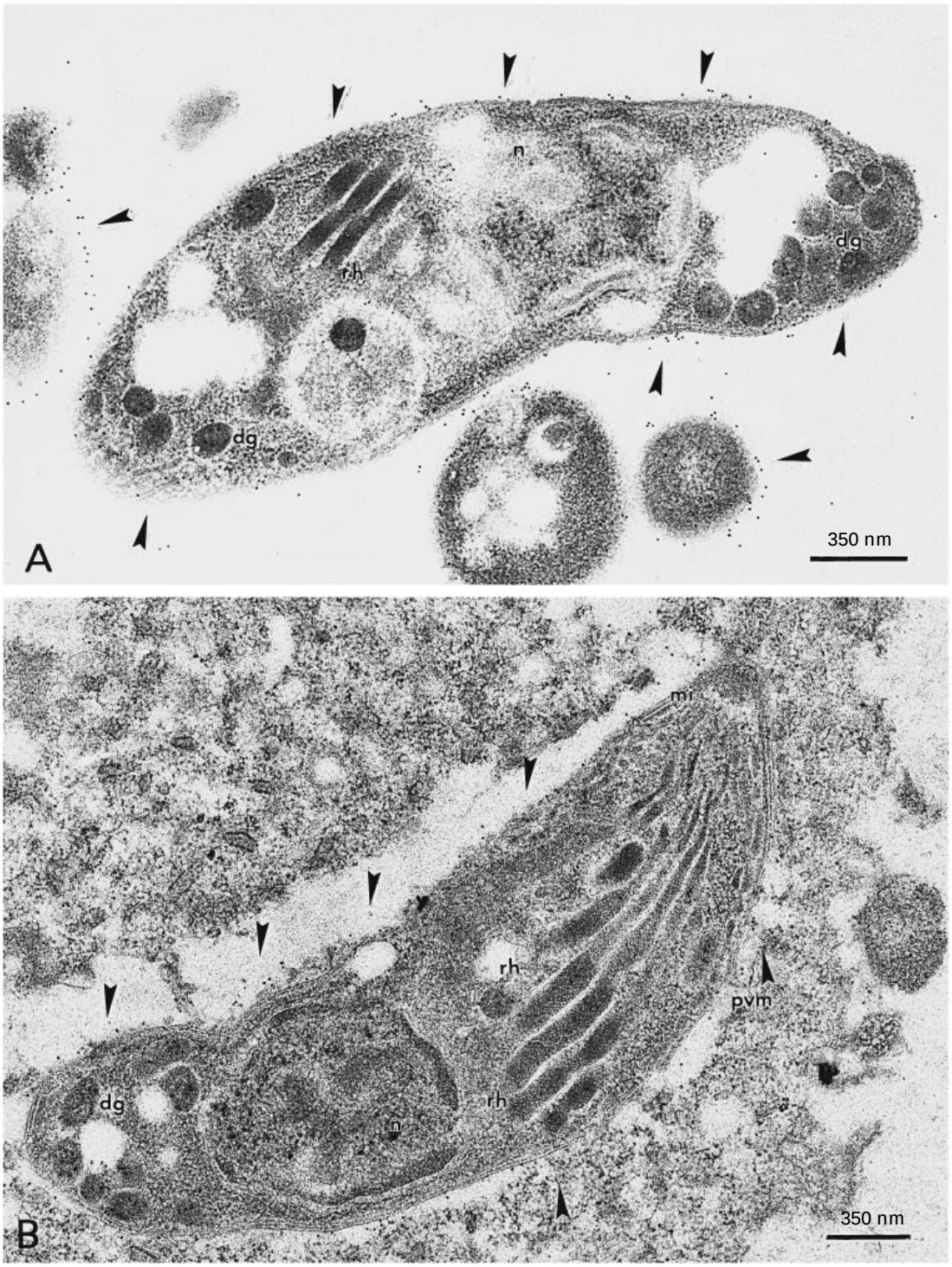


Fig. 3. Immunogold labelling of LR-White-embedded *Neospora caninum* tachyzoites using affinity-purified anti-recNc-p43 antibodies. LR-White sections of extracellular (A) and intracellular (B) *N. caninum* tachyzoites reveal that epitopes corresponding to recNc-p43 are found on the surface of the parasite (arrowheads) and within dense granules (dg). Note the only marginal staining of the rhoptries (rh), and the absence of labelling of any other part of the parasite including the micronemes (m), the nucleus (n) and the parasitophorous vacuole membrane (pvm). Gold particles were also absent from any part of the host cell.

1 (or several) parasite surface molecule(s) interact with 1 (or several) host cell surface receptor(s) (Hemphill *et al*. 1996). These events are most likely based on protein–protein interactions, while carbohydrates apparently do not play a major role (Hemphill *et al*. 1996). Keeping this in mind, our initial investigations focused on the identification of cell surface-associated proteins in *N. caninum* tachyzoites, and their putative role during the host cell adhesion and entry process. One of the proteins identified was Nc-p43 (Hemphill & Gottstein, 1996), and antibodies directed against this *N. caninum*

https://doi.org/10.1017/S0031182097001650 Published online by Cambridge University Press

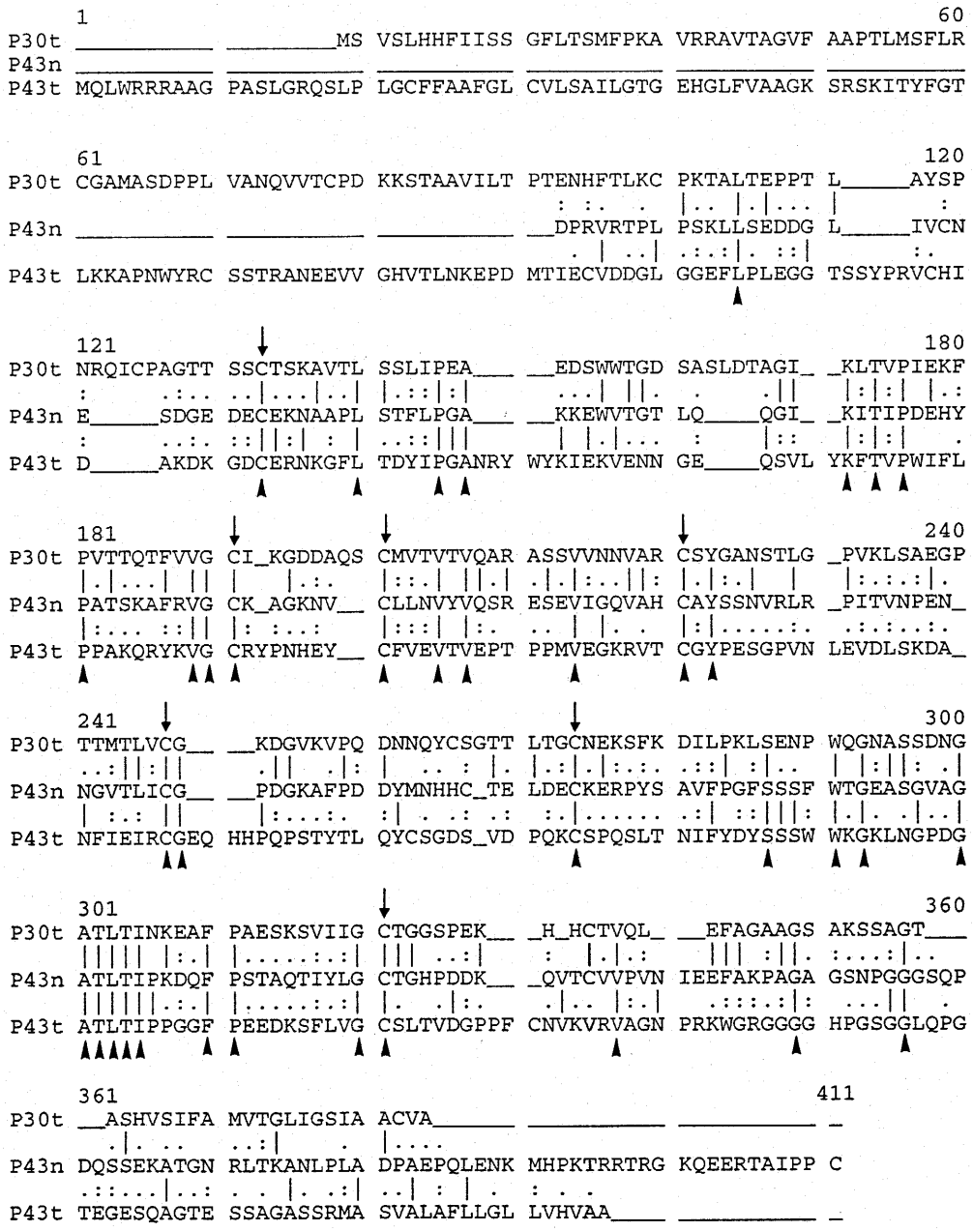

Fig. 4. Comparison of the amino acid sequences of SAG1 of *Toxoplasma gondii* (p30t) and SAG3 of *Toxoplasma gondii* (p43t) with the sequence corresponding to recNc-p43 (p43n). The sequences were aligned using the 'gap' program of the GCG software package (Devereux *et al*. 1984). The vertical lines between amino acids mark identities. Double dots are indicative of higher degrees of similarities, and single dots indicate low similarities between amino acids as defined by the GCG software package. Horizontal lines between amino acids indicate gaps introduced for optimal alignment. Those amino acids common to all 3 sequences are marked with arrowheads, conserved cysteine residues are indicated by arrows. Numbers refer to the sequence of SAG3 (p43) from *T. gondii* tachyzoites.

tachyzoite surface protein were capable of blocking adhesion and invasion of Vero cell monolayers *in vitro* (Hemphill, 1996).

Specific antibodies directed against Nc-p43 were used for the immunosccreening of a *N. caninum* lambda gt22A-cDNA expression library, and led to the identification of a single positive cDNA clone. The corresponding cDNA insert was expressed in *E. coli*, and the identity of the resulting recombinant protein, named recNc-p43, with its native counterpart was confirmed by immunoblotting and immunocytochemistry. On Western blots, affinity-purified recNc-p43 antibodies and the original anti-Nc-p43 antibodies revealed an identical staining pattern of *E. coli* lysates and of *N. caninum* tachyzoite extracts, and a complete absence of labelling of *T. gondii* tachyzoite extracts. However, the calculated molecular weight of recNc-p43 of 35·6 kDa (31·6 kDa originating from the parasite-derived sequence and 4 kDa from the vector-derived sequence) does exhibit a significant discrepancy to what is observed by SDS–PAGE. Similar results for

https://doi.org/10.1017/S0031182097001650 Published online by Cambridge University Press

both antibodies were also obtained by immunofluorescent surface labelling of *N. caninum* and of *T. gondii* tachyzoites. These experiments confirmed previous findings which had shown that no immunologically related counterpart to Nc-p43 could be detected in *T. gondii* tachyzoites (Hemphill & Gottstein, 1996). These findings thus indicate an immunodiagnostic potential for recombinant Nc-p43, the evaluation of which is currently being carried out in our laboratory.

Previous studies employing affinity-purified anti-Nc-p43 antibodies had shown that reactive epitopes were abundant on the surface of *N. caninum* tachyzoites, as well as within 2 types of secretory organelles, namely dense granules and rhoptries (Hemphill, 1996). In this study, affinity-purified anti-recNc-p43 were used to determine the localization of the respective epitopes by on-section labelling of *N. caninum* tachyzoites previously embedded in LR-White resin. These immunogold labelling experiments demonstrated that anti-recNc-p43 antibodies labelled not only the surface of the parasites, but also the dense granules, while staining of the parasite rhoptries was marginal or absent. Thus, the immunogold labelling achieved with antibodies affinity purified on the recombinant Nc-p43 did only partly correlate with the staining pattern obtained with the original anti-Nc-p43 antibodies (Hemphill, 1996). The reasons for the observed discrepancy are presently unknown, but several possibilities could account for this. (i) The recombinant protein is lacking the N-terminal domain and epitopes within this domain could be causing the rhoptry specific labelling, (ii) bacterially expressed proteins lack post-translational modification which could occur in eukaryotic cells, and this lack of post-translational modifications could result in the differential staining, and (iii) the rhoptry staining found with the original affinity-purified anti-Nc-p43 antibody could have resulted from another population of proteins of similar molecular weight, but different isoelectric point, epitopes of which are not represented on the recombinant expressed recNc-p43. All 3 possibilities should be taken into account and they are currently being investigated.

The fact that anti-Nc-p43 antibodies inhibited parasite adhesion and entry into host cells (Hemphill, 1996) suggested that Nc-p43 plays a similar role during host cell invasion as proposed for SAG1 (p30) and SAG3 (p43), 2 major surface proteins of *T. gondii* tachyzoites (Kasper & Mineo, 1994; Cesbron-Delauw *et al*. 1994). However, the localization of these 2 proteins is restricted to the surface of *T. gondii* tachyzoites (Smith, 1995). Our results indicated that Nc-p43 would be accumulated and stored within the dense granules, and would be subsequently released onto the cell surface, in a way similar to that described for the 21 and 28·5 kDa antigens secreted from *T. gondii* tachyzoites (Charif *et al*. 1990). Dense granules are found at both the anterior and posterior end of *N. caninum* tachyzoites. These organelles resemble the secretory vesicles of mammalian cells and, by analogy, are probably formed by budding from the Golgi apparatus (Cesbron-Delauw, 1994). In apicomplexan parasites, molecules secreted from dense granules are targeted to different sites: (i) either onto the parasite surface, enabling the zoite to physically interact and subsequently invade its host cell, or (ii) dense granule proteins are secreted into the parasitophorous vacuole (PV) during or after host cell penetration, and are targeted to the parasitophorous vacuole network (PVN) and/or the parasitophorous vacuole membrane (PVM, Cesbron-Delauw, 1994; Kasper & Mineo, 1994; Dubremetz & McKerrow, 1995; Galinski & Barnwell, 1996). On-section labelling of LR-White-embedded Vero cell cultures infected with *N. caninum* tachyzoites suggested that the former pathway, namely storage within dense granules and subsequent release onto the tachyzoite cell surface membrane, could account for Nc-p43.

The affinity-purified anti-Nc-p43 antibodies had been used earlier to functionally characterize Nc-p43 as a possible parasite ligand, mediating host cell attachment and invasion (Hemphill, 1996). Thus Nc-p43 was postulated to be functionally related to SAG1, the major surface antigen 1 (or p30) of *T. gondii* (Kasper & Mineo, 1994). The cDNA insert encoding recNc-p43 was sequenced, and alignment of the deduced amino acid sequence with the corresponding sequence of SAG1 (Kasper *et al*. 1992) indicated a certain degree of overall similarity (43% with 30% identity) between these two proteins. In addition, overall similarity (44%) and an identity of 23% could also be demonstrated with respect to the major surface antigen 3 of *T. gondii* tachyzoites (Cesbron-Delauw *et al*. 1994). This was not predictable, since (i) no DNA sequence homologies could be found between the coding regions of the recNc-p43 cDNA insert (not shown), SAG1 and SAG3 (Kasper *et al*. 1992; Cesbron-Delauw *et al*. 1994); (ii) no cross-antigenicity has ever been observed between Nc-p43, SAG1 and SAG3, neither by monoclonal nor polyclonal antibodies (Cesbron-Delauw *et al*. 1994; Hemphill & Gottstein, 1996) and (iii) SAG1- and SAG3-deduced amino acid sequences contain numerous cysteine residues which are believed to be involved in maintaining a reduction-sensitive conformation. Thus SAG1 and SAG3 both migrate more slowly in SDS–PAGE under reducing conditions (Cesbron-Delauw *et al*. 1994; Tomavo, 1996), while the migration behaviour of Nc-p43 is identical when SDS–PAGE is carried out under reducing and non-reducing conditions (Hemphill, 1996). The role of highly conserved sequences such as the GATLTI motif is not known, but since all 3 proteins appear to

be involved in host cell invasion, these conserved amino acids could play a functional role in the processes leading to host cell adhesion and invasion.

More work remains to be done before Nc-p43 is fully characterized on the genetic level, and further information is required on the degree and the functional importance of post-translational modifications of this molecule, its expression in different stages of the parasite, and its role in the host–parasite relationship. However, this study has demonstrated that, although the surface molecules of *N. caninum* and *T. gondii* exhibit distinct antigenic and biochemical differences, their functional relationship is reflected in terms of their localization and the distinct similarities in their amino acid sequence.

We gratefully acknowledge Tony Wyler and Giovanni Rossi (Institute for Zoology and Institute for Veterinary Pathology, respectively) for allowing the use of their electron microscopy facilities. We thank J. P. Dubey (United States Department of Agriculture, Beltsville, USA) and J. Holmdahl (University of Uppsala, Sweden) for their gifts of parasites. Many thanks to Katrin Ingold (Institute of Parasitology, University of Bern) for providing some of the SDS gels used in this study, and to Vreni Zimmerman for excellent technical help. Banu Yürüker is also gratefully acknowledged. This work was financed through a grant of the Swiss National Science Foundation (project grant No. 31.46846.96), the 'Stiftung zur Foerderung der wissenschaftlichen Forschung der Universitaet Bern', and the COST-820 grant (No. C96.0068) provided by the 'Bundesamt für Bildung und Wissenschaft', Switzerland.

## REFERENCES

BAKER, D. G., MORISHITA, T. Y., BROOKS, D. L., SHEN, S. K., LINDSAY, D. S. & DUBEY, J. P. (1995). Experimental oral inoculations in birds to evaluate potential definitive host of *Neospora caninum*. *Journal of Parasitology* **81**, 783–785.

BJERKAS, I. & PRESTHUS, J. (1988). Immuno-histochemical and ultrastructural characteristics of a cyst-forming sporozoon associated with encephalomyelitis and myositis in dogs. *Acta Pathologica Microbiologica Immunologica Scandinavica* **96**, 445–454.

BJERKAS, I., JENKINS, M. C. & DUBEY, J. P. (1994). Identification and characterization of *Neospora caninum* tachyzoite antigens useful for diagnosis of neosporosis. *Clinical and Diagnostic Laboratory Immunology* **1**, 214–221.

BJOERKMAN, C., LUNDEN, A., HOLMDAHL, J., BARBER, J., TREES, A. J. & UGGLA, A. (1994). *Neospora caninum* in dogs: detection of antibodies by ELISA using an iscom antigen. *Parasite Immunology* **16**, 643–648.

BOUVIER, J., ETGES, R. & BORDIER, C. (1985). Identification and purification of membrane and soluble forms of the major surface protein of *Leishmania* promastigotes. *Journal of Biological Chemistry* **260**, 15504–15509.

BRINDLEY, P. J., GAZZINELLI, R. T., DENKERS, E. Y., DAVIS, S. W., DUBEY, J. P., BELFORT, R., MARTINS, M.-C., SILVEIRA, C., JAMRA, L., WATERS, A. P. & SHER, A. (1993). Differentiation of *Toxoplasma gondii* from closely related coccidia by riboprint analysis and a surface antigen gene polymerase chain reaction. *American Journal of Tropical Medicine and Hygiene* **48**, 447–456.

BURG, J. L., GROVER, C. M., POULETTY, P. & BOOTHROYD, J. C. (1989). Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction. *Journal of Clinical Microbiology* **27**, 1787–1792.

CESBRON-DELAUW, M.-F. (1994). Dense granule organelles of *Toxoplasma gondii*: the role in the host–parasite relationship. *Parasitology Today* **10**, 239–246.

CESBRON-DELAUW, M.-F., TOMAVO, S., BEAUCHAMPS, P., FOURMAUX, M.-P., CAMUS, D., CAPRON, A. & DUBREMETZ, J.-F. (1994). Similarities between the primary structures of two distinct major surface proteins of *Toxoplasma gondii*. *Journal of Biological Chemistry* **269**, 16217–16222.

CHARIF, H., DARCY, F., TORPIER, G., CESBRON-DELAUW, M.-F. & CAPRON, A. (1990). *Toxoplasma gondii*: characterization and localization of antigens secreted from tachyzoites. *Experimental Parasitology* **71**, 114–124.

DEVEREUX, J. R., HAEBERLI, P. & SMITHIES, O. (1984). A comprehensive set of sequence analysis programs for the Vax. *Nucleic Acids Research* **12**, 387–395.

DUBEY, J. P., CARPENTER, J. L., SPEER, A., TOPPER, M. J. & UGGLA, A. (1988). Newly recognized fatal protozoan disease of dogs. *Journal of the American Veterinary Medicine Association* **198**, 1269–1285.

DUBEY, J. P. & LINDSAY, D. S. (1993). Neosporosis. *Parasitology Today* **9**, 452–458.

DUBEY, J. P. & LINDSAY, D. S. (1996). A review of *Neospora caninum* and neosporosis. *Veterinary Parasitology* **67**, 1–59.

DUBREMETZ, J. F. & MCKERROW, J. H. (1995). Invasion mechanisms. In *Biochemistry and Molecular Biology of Parasites* (ed. Marr, J. J. & Müller, M.), pp. 307–322. Academic Press, New York.

ELLIS, J., LUTON, K., BAVERSTOCK, P. R., BRINDLEY, P. J., NIMMO, K. A. & JOHNSON, A. M. (1994). The phylogeny of *Neospora caninum*. *Molecular and Biochemical Parasitology* **64**, 303–311.

GALINSKI, M. R. & BARNWELL, J. W. (1996). *Plasmodium vivax*: merozoites, invasion of reticulocytes and considerations for malaria vaccine development. *Parasitology Today* **12**, 20–28.

HARDING, C. V. (1995). Phagocytic processing of antigens for presentation by MHC molecules. *Trends in Cell Biology* **5**, 105–109.

HEMPHILL, A. (1996). Subcellular localization and functional characterization of Nc-p43, a major *Neospora caninum* tachyzoite surface protein. *Infection and Immunity* **64**, 4279–4287.

HEMPHILL, A. & CROFT, S. L. (1997). Electronmicrosopy in parasitology. In *Analytical Parasitology* (ed Rogan, M.), pp. 227–268. Springer Verlag, Berlin, Germany.

HEMPHILL, A. & GOTTSTEIN, B. (1996). Identification of a major surface protein on *Neospora caninum* tachyzoites. *Parasitology Research* **82**, 497–504.

HEMPHILL, A., GOTTSTEIN, B. & KAUFMANN, H. (1996). Adhesion and invasion of bovine endothelial cells by *Neospora caninum*. *Parasitology* **112**, 183–197.

HOLMDAHL, O. J. M., MATTSON, J. G., UGGLA, A. & JOHANSSON, K. (1994). The phylogeny of *Neospora*

*caninum* and *Toxoplasma gondii* based on ribosomal RNA sequences. *Federation of the European Microbiology Society Microbiology Letters* **119**, 187–192.

KASPER, L. H., KHAN, I. A., ELY, K. H., BUELOW, R. & BOOTHROYD, J. C. (1992). Antigen-specific (p30) mouse CD8+ T cells are cytotoxic against *Toxoplasma gondii*-infected peritoneal macrophages. *Journal of Immunology* **148**, 1493–1498.

KASPER, L. H. & MINEO, J. R. (1994). Attachment and invasion of host cells by *Toxoplasma gondii*. *Parasitology Today* **10**, 184–188.

LINDSAY, D. S., SPEER, C. A., TOIVIO-KINNUCAN, M. A., DUBEY, J. P. & BLAGBURN, B. L. (1993). Use of infected cultured cells to compare ultrastructural features of *Neospora caninum* from dogs and *Toxoplasma gondii*. *American Journal of Veterinary Research* **54**, 103–106.

MARSH, A. E., BARR, B. C., SVERLOW, K., HO, M., DUBEY, J. P. & CONRAD, P. A. (1995). Sequence analysis and comparison of ribosomal DNA from bovine *Neospora* to similar coccidial parasites. *Journal of Parasitology* **81**, 530–535.

MAUËL, J. (1996). Intracellular survival of protozoan parasites with special reference to *Leishmania* spp., *Toxoplasma gondii* and *Trypanosoma cruzi*. *Advances in Parasitology* **38**, 1–35.

MÜLLER, N., ZIMMERMAN, V., HENTRICH, B. & GOTTSTEIN, B. (1996). Diagnosis of *Neospora caninum* and *Toxoplasma gondii* infection by PCR and DNA hybridization immunoassay. *Journal of Clinical Microbiology* **34**, 2850–2852.

RUSSELL, D. G. (1995). *Mycobacterium* and *Leishmania*: stowaways in the endosomal network. *Trends in Cell Biology* **5**, 125–128.

SABIN, A. (1941). Toxoplasmic encephalitis in children. *Journal of the American Medical Association* **116**, 801–814.

SMITH, J. E. (1995). A ubiquitous intracellular parasite: the cellular biology of *Toxoplasma gondii*. *International Journal for Parasitology* **25**, 1301–1309.

TOMAVO, S. (1996). Surface proteins of *Toxoplasma gondii*. *Current Topics in Microbiology and Immunology* **219**, 45–54.

TURCO, S. J. (1995). Surface constituents of kinetoplastid parasites. In *Biochemistry and Molecular Biology of Parasites* (ed. Marr, J. J. & Müller, M.), pp. 172–202. Academic Press, New York.

WESSEL, D. & FLUEGGE, U. I. (1984). A method for the quantitative recovery of protein in dilute solution in the presence of detergent. *Analytical Biochemistry* **138**, 141–143.