Molecular characterization of *Neospora caninum* MAG1, a dense granule protein secreted into the parasitophorous vacuole, and associated with the cyst wall and the cyst matrix

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SUMMARY

In *Neospora caninum* and *Toxoplasma gondii*, the parasitophorous vacuole (PV) is synthesized at the time of infection. During tachyzoite-to-bradyzoite stage conversion, the PV is later transformed into a tissue cyst that allows parasites to survive in their host for extended periods of time. We report on the characterization of NcMAG1, the *N. caninum* orthologue of *T. gondii* MAG1 (matrix antigen 1; TgMAG1). The 456 amino acid predicted NcMAG1 protein is 54% identical to TgMAG1. By immunoblotting, a rabbit antiserum raised against recombinant NcMAG1 detected a major product of ~67 kDa in extracts of *N. caninum* tachyzoite-infected Vero cells, which was stained more prominently in extracts of infected Vero cells treated to induce *in vitro* bradyzoite conversion. Immunofluorescence and TEM localized the protein mainly within the cyst wall and the cyst matrix. In both tachyzoites and bradyzoites, NcMAG1 was associated with the parasite dense granules. Comparison between NcMAG1 and TgMAG1 amino acid sequences revealed that the C-terminal conserved regions exhibit 66% identity, while the N-terminal variable regions exhibit only 32% identity. Antibodies against NcMAG1-conserved region cross-reacted with the orthologuous protein in *T. gondii* but those against the variable region did not. This indicates that the variable region possesses unique antigenic characteristics.

Key words: *Neospora caninum*, cyst matrix, cyst wall, parasitophorous vacuole, matrix antigen 1 (MAG1).

INTRODUCTION

Neospora caninum is an intracellular apicomplexan parasite that is closely related to *Toxoplasma gondii*, and represents a major cause of abortion in cattle and neuromuscular disease in dogs worldwide (Dubey *et al*. 2006; Hemphill *et al*. 2006). Transplacental transmission is regarded as the major source of infection. The life cycle of *N. caninum* consists of 3 principal stages: (1) tachyzoites, which rapidly replicate within the parasitophorous vacuole (PV), and disseminate in the host during the acute phase, (2) bradyzoites, slowly dividing parasites, which are enclosed within a tissue cyst that develops particularly in the brain during the chronic phase of the infection (Dubey *et al*. 2006), and (3) oocysts which represent the product of a sexual process taking place within the intestine of the definitive hosts (dog and coyote), and upon fecal shedding, sporulate and form sporozoites (McAllister *et al*. 1998; Gondim *et al*. 2004). There is evidence that tachyzoites differentiate into bradyzoites as a reaction to the immune response occurring in immune-competent hosts (Dubey *et al*. 2006; Hemphill *et al*. 2006).

Intracellularly, all invasive stages of *N. caninum* are located within a parasitophorous vacuole (PV), separated from the host cell cytoplasm through the parasitophorous vacuole membrane (PVM). The PVM is derived from the host cell cytoplasmic membrane during host cell invasion, and subsequently undergoes important modifications. While proteins secreted by the parasite are incorporated into the PVM, those of the host cell are selectively eliminated and the PV becomes resistant to fusion with lysosomes (Beyer *et al*. 2002). The PV of *T. gondii* transforms into a tissue cyst very early after parasite penetration into brain cells, and the PVM progressively matures into a cyst wall that protects bradyzoites from the immunological and physiological responses on part of the host (Beyer *et al*., 2002). Intact tissue cysts containing bradyzoites of both *N. caninum* and *T. gondii* can persist in the host, usually without being harmful or causing an

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inflammatory response (Dubey *et al*. 1988; Innes *et al*. 2002).

The proteins involved in tachyzoite-bradyzoite interconversion could represent important targets for vaccination or chemotherapy in order to prevent or reduce the development of intra-cerebral cysts. Stage-specifically expressed proteins in *T. gondii* and *N. caninum* have been identified (McAllister *et al*. 1996; Fuchs *et al*. 1998; Ferguson, 2004; Risco-Castillo *et al*. 2007; Aguado-Martinez *et al*. 2009), while others are expressed in both stages, but differ in their localization (Vonlaufen *et al*. 2004). In this context, *T. gondii* MAG1 was first reported as a bradyzoite-specific antigen localized in the cyst matrix (Parmley *et al*. 1994). Subsequently, the protein was also detected in the parasitophorous vacuoles containing intracellular tachyzoites and in tachyzoite lysates (Ferguson and Parmley, 2002). Besides its importance as a marker for cerebral infection with *T. gondii* in AIDS patients (Contini *et al*. 2002; Pfrepper *et al*. 2005), TgMAG1 represents a potentially useful tool for the immunodiagnosis of patients with toxoplasmosis (Di Cristina *et al*. 2004; Pfrepper *et al*. 2005). In addition, TgMAG1 has been considered as a promising candidate in vaccination trials (Parmley *et al*. 2002; Nielsen *et al*. 2006). We have recently shown that intraperitoneal vaccination of mice with recombinant NcMAG1 expressed in *E. coli* confers 50% protection against experimental *N. caninum* infection in a murine acute disease model (Debache *et al*. 2009). In this study, we report on the molecular characterization, localization and antigenic properties of NcMAG1.

MATERIALS AND METHODS

Parasite cultures

Parasites were cultured as previously described (Barber *et al*. 1995; Hemphill and Gottstein, 1996). Briefly, *N. caninum* NC-Liverpool or *T. gondii* ME49 tachyzoites were maintained in Vero cells at 37 °C under 5% CO_2 in RPMI 1640 medium containing 10% FCS (Gibco-Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. *N. caninum* grown in keratinocytes were cultured as previously described (Vonlaufen *et al*. 2002).

Tachyzoite-to-bradyzoite stage conversion was performed *in vitro* by 10 days treatment of NC-Liverpool-infected Vero cells with 17 μM sodium nitroprusside (SNP; Sigma, St Louis, MO, USA), using a procedure adapted from Vonlaufen *et al*. (2004) and by 4 days treatment of *T. gondii*-infected Vero cells with 100 μM SNP as previously described (Bohne *et al*. 1994; Weiss *et al*. 1998).

For purification, parasites were mechanically released from infected cells by passing through a 25G, 5/8 needle, washed in ice-cold medium, and run on PD-10 columns (GE Healthcare, Piscataway, NJ, USA) as described by Hemphill *et al*. (1996).

Oligonucleotides

N. caninum MAG1-specific primers were designed based on the ApiDots (http://www.cbil.upenn.edu/apidots/; (Li *et al*. 2003, 2004)) DT.92484705 transcript. All primers were purchased from MWG (Ebersberg, Germany).

RNA isolation and first strand cDNA synthesis

Total RNA was isolated from 3×10^6 purified *N. caninum* Liverpool tachyzoites using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First strand cDNA synthesis reactions were performed for 1 h at 42 °C in a 20 μl reaction mixture containing 2 μg of total RNA, 1× first-strand buffer (Invitrogen), 0·5 mM of each dNTP (Endotell, Allschwil, Switzerland), 10 mM dithiothreitol, 20 U of RNasin RNase inhibitor (Promega, Madison, WI, USA), 200 U of SuperScript II reverse transcriptase (Invitrogen). Primers (20 pmol) were either MAG1-R1, 5′-CTGTCCCTTACACCTACACT-3′, for the 5′-RACE (Rapid Amplification of cDNA Ends) and to generate the recombinant NcMAG1 protein, or T-primer 3, 5′-AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$ VN-3′ (V = A, C or G; N = any nucleotide), for the 3′-RACE (Matz *et al*. 1999).

RACE procedures

A manual hot-start PCR (Chou *et al*. 1992) was used in order to reduce background amplifications in both RACE reactions.

For 5′-RACE, the first strand cDNA primed with MAG1-R1 primer was ultrafiltrated 3 times against water on a Microcon YM-100 filter unit (Millipore, Billerica, MA, USA). The cDNA in the whole retentate was dA-tailed for 20 min at 37 °C in 50 μl of 1× terminal deoxynucleotidyl transferase (TdT) buffer (Roche, Basel, Switzerland) using 25 U of TdT (Roche) and 0·2 mM dATP (Endotell). The reaction was terminated by heating at 95 °C for 5 min. Two rounds of amplification were performed to generate the 5′-RACE product. The first round was carried out in a 50 μl reaction volume containing 5 μl (1/10th) of the (dA)-tailed product, 5 pmol of each T-primer 3 and MAG1-R2 (5′-GCATTACCAACTTCGTCCTC-3′) primers, 0·2 mM of each dNTP and 2·5 U of *Pfu* DNA polymerase (Promega) in the supplied reaction buffer. Ten PCR cycles were performed with an annealing temperature of 55 °C. The second round of amplification was carried out in 50 μl containing 20 μl of the first step

amplification reaction, 15 pmol of Heel-carrier (5′-CTAATACGACTCACTATAGGGCAAGCAG-TGGTATCAACGCAGAGT-3′) and MAG1-R2 primers, 0·2 mM of each dNTP, and 2·5 U of *Pfu* DNA polymerase (Promega) in the supplied reaction buffer. PCR was performed for 30 cycles with an annealing temperature of 58 °C.

The 3′-RACE reaction was carried out in a 100 μl reaction mixture containing 2 μl (1/10th) of the first strand cDNA prepared with T-primer 3, 4 pmol of Heel-carrier primer, 20 pmol of each MAG1-F (5′-TGAACAACCCTATGAACAAACAGACGC-3′), and Heel-specific (5′-CTAATACGACTCA-CTATAGGGC-3′) primers, 0·2 mM of each dNTP and 2·5 U of *Pfu* DNA polymerase in the supplied reaction buffer. PCR was performed for 35 cycles with an annealing temperature of 55 °C.

Sequencing and sequence analyses

RACE products were cloned into pCR Blunt II TOPO (Invitrogen) and sequenced using a primer walking approach. All expression constructs were verified by sequencing of the insert. Sequencing reactions were carried out using BigDye v3.1 fluorescent dye terminators and run on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Raw sequencing data were assembled and edited with the Staden package (Bonfield *et al*. 1995). Sequence data reported in this paper are available in the GenBank database under the Accession number EF580924.

Homology searches were done using BLAST (blastp) program (http://www.ncbi.nlm.nih.gov/blast/; (Altschul *et al*. 1990)) and the Conserved Domain Database CD-Search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) with default settings. Proteins were aligned on the Muscle server (http://www.drive5.com/muscle/ (Edgar, 2004)), minimally edited, and formatted with GeneDoc (Nicholas *et al*. 1997). Residue grouping and shading was according to the MM5 reduced amino acid alphabet of Melo and Marti-Renom (2006). Potential signal peptide cleavage sites were identified with SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/ (Bendtsen *et al*. 2004)) and potential transmembrane regions were checked with the ProtScale tool on the Expasy server (http://expasy.org/tools/protscale.html).

Expression of recombinant N. caninum *MAG1 proteins*

A portion of NcMAG1, thereafter referred to as recNcMAG1, encoding aa 31-394 (numbering according to the precursor) was amplified by RT-PCR using MAG1-*Bam*HI-F (5′-GGATCCCAAAGG-GTGCCTCGCTACCC-3′) and MAG1-*Sma*I-R (5′-CCCGGGTTATTCCTCCACTATTTCGT-CCGC-3′) primers; *Bam*HI and *Sma*I restriction sites underlined. The PCR product was cloned into pCR blunt II TOPO (Invitrogen) and verified by sequencing. The *Bam*HI/*Sma*I MAG1 insert was then subcloned into the $(His)_6$-tag pQE-30 expression vector (Qiagen, Hilden, Germany), resulting in pQE-30-NcMAG1. We also expressed portions of the conserved (C) and variable (V) regions within NcMAG1 (Fig. 2). In order to monitor their expression and to facilitate their purification, the two NcMAG1 C and V regions were expressed as $(His)_6$-GFP (green fluorescent protein) fusion proteins in *E. coli*. The portion of NcMAG1 central to the C region (aa 241–386, numbering according to the precursor) was amplified using MAG1-C-*Xma*I-F (5′-CTGCGTCCCGGGGGACCGACGGTT-TCGACTCG-3′) and MAG1-C-*Pst*I-R (5′-CT-GCGTCTGCAGGGTCCCCACGAATTGTCT-CG-3′) primers; *Xma*I and *Pst*I restriction sites underlined. The portion of NcMAG1 central to the V region (aa 31–162) was amplified using MAG1-V-*Xma*I-F (5′-CTGCGTCCCGGGCAAAGGGT-GCCTCGCTACCC-3′) and MAG1-V-*Pst*I-R (5′-CTGC GTCTGCAGCGACGTGGAAAGT-GGTAGCG-3′) primers; *Xma*I and *Pst*I restriction sites underlined. The pQE-30-NcMAG1 vector was used as template for PCR amplifications. Restricted PCR products were inserted into the *Xma*I/*Pst*I-cut pQE-GFP vector (C.G. and F.A., unpublished; sequence available upon request), resulting in pQE-GFP-MAG1-C and pQE-GFP-MAG1-V.

Expression vectors were used to transform *E. coli* BL21 (Novagen-EMD Biosciences, Madison, WI) harbouring the pREP4 repressor plasmid (Qiagen). To express the recombinant proteins, 1 l of 2× YT medium (per litre: 16 g bacto tryptone, 10 g bacto yeast extract, and 5 g NaCl), pre-warmed to 37 °C and supplemented with carbenicillin and kanamycin (Sigma; 100 and 25 mg/ml, respectively), was inoculated with 10 ml of an overnight starter culture grown in the same medium. When cultures reached an OD of 0·5 at $\lambda = 600$ nm, isopropyl β-D-1-thiogalactopyranoside (Sigma) was added to 1 mM and expression was carried on for 3 h at 37 °C. Soluble recNcMAG1 and GFP-MAG1-V proteins were purified by nickel chelate chromatography on Protino Ni-IDA columns (Macherey-Nagel, Düren, Germany), as recommended by the manufacturer. Due to its extreme sensitivity to proteolysis after bacterial lysis, GFP-MAG1-C could not be purified by chromatography. It was therefore run on a large format SDS-PAGE gel after boiling over-expressing bacteria in SDS sample buffer. The whole gel was transferred onto a nitrocellulose membrane and the major band was excised and used for the purification of anti-GFP-MAG1-C antibodies.

Production and purification of antibodies

Antisera were obtained after immunization of 2 female white New Zealand rabbits, with 150 μg of recNcMAG1 antigen per injection, using a standard 10-week immunization protocol (Institut für Labortierkunde, Zurich, Switzerland). Antibodies specific to either the whole recNcMAG1, the C or the V region were affinity-purified using recNcMAG1, GFP-MAG1-C or GFP-MAG1-V bound to nitrocellulose membranes, respectively (Robinson *et al*. 1988).

Preparation of protein extracts

For preparation of total protein extracts from infected or uninfected Vero cells, cultures in a T-75 culture flask were washed twice with PBS (137 mM NaCl, 2·7 mM KCl, 4·3 mM Na_2HPO_4, 1·47 mM KH_2PO_4, pH 7·4) and proteins were extracted with 1 ml of ice-cold RIPA buffer (Pierce-Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10 μl/ml Halt protease inhibitor cocktail (Pierce-Thermo Fisher Scientific). Protein extracts were sonicated, clarified by centrifugation, and the protein concentration in the supernatant was measured using the DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA). For SDS-PAGE, samples of protein extracts from infected cultures were adjusted to contain the required parasite number using data obtained by quantitative real-time PCR.

Protein extracts from free parasites were prepared using 100 μl of RIPA buffer per 5×10^6 purified parasites.

Quantitation of parasites

To determine the number of parasites in infected Vero cells, a quantitative real-time PCR (LightCycler, Roche Diagnostics, Basel, Switzerland) was used. DNA was purified from *Neospora*- or *Toxoplasma*-infected cells using the High Pure PCR Purification kit (Roche Diagnostics) according to the manufacturer's recommendations. DNA concentrations were measured by Hoechst 33258 (Sigma) fluorimetry (Ausubel *et al*. 1997) on a Synergy HT plate reader (Biotek Instruments, Winooski, VT, USA). Experimental procedures described by Müller *et al*. (2002) for *Neospora* and Reischl *et al*. (2003) for *Toxoplasma* were followed, using 200 ng DNA per sample. Free parasites were directly quantified by counting using a Neubauer chamber.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot

Protein samples were mixed with reducing, denaturing sample buffer, boiled for 5 min and protein separation was conducted by SDS-PAGE. Proteins were transferred onto PVDF membranes (Bio-Rad) for 1 h at 100 V using a wet transfer apparatus (Bio-Rad). Membranes were blocked by incubation in a blocking buffer containing 5% non-fat milk in TBST (20 mM Tris, 150 mM NaCl, 0·1% (v/v) Tween 20) and then incubated for 2 h at room temperature or overnight at 4 °C with affinity-purified anti-NcMAG1 antibodies at a dilution of 1 : 3000. Antibodies against the NcMAG1-conserved region and against the NcMAG1-variable region were applied at a dilution of 1 : 400. Detection was performed with a donkey anti-rabbit alkaline phosphatase-conjugated secondary antibody (Pierce-Thermo Fisher Scientific) at a dilution of 1 : 10 000 and NBT / BCIP (nitro blue tetrazolium/bromochloroindolyl phosphate; Sigma) (Ausubel *et al*. 1997).

Immunofluorescence (IF) analysis

IF staining was performed on purified parasites or on parasite-infected Vero cells grown on poly-L lysine-coated glass cover-slips. For intracellular bradyzoites, cover-slips were seeded (10^5 infected cells per well) on the sixth day following addition of SNP to the culture medium and the treatment carried on until day 9 post-infection. Purified parasites were fixed using 4% paraformaldehyde in PBS (20 min at RT). Cover-slips were rinsed 3 times in PBS, immersed into pre-cooled (−20 °C) methanol/acetone (v:v), and incubated at −20 °C for 30 min. The cover-slips were washed 3 times with PBS, 5 min each, and incubated in blocking buffer (PBS/3% BSA) for 2 h. The following primary antibodies, diluted in PBS/0·3% BSA, were applied: (a) affinity-purified polyclonal rabbit anti-NcMAG1 antibody at a 1 : 2500 dilution, (b) rabbit anti *N. caninum* antiserum (Hemphill *et al*. 1996) at a 1 : 2500 dilution, (c) CC2, a rat mAb reacting with a *T. gondii* cyst wall antigen (Gross *et al*. 1995), diluted at 1 : 300, and (d) rabbit anti-BAG1 polyclonal antibody, directed against a bradyzoite-specific antigen (McAllister *et al*. 1996), diluted at 1 : 300. Affinity-purified antibodies against NcMAG1-conserved and NcMAG1-variable regions were used at 1 : 200 to assess their cross-reactivity on *T. gondii*.

Incubations with primary antibodies were performed for 1 h, followed by 3 washes in PBS for 5 min each. Cells were then incubated for 45 min with the appropriate Alexa Fluor (AF)-labelled secondary antibodies (Invitrogen) at a dilution of 1 : 3000 (AF-488-, AF-546) or 1 : 300 (AF-350) in PBS/0·3% BSA. For multiple detection, antibodies were applied sequentially, without a given order. Finally, the preparations were washed in PBS 3 times for 5 min each. Cover-slips with infected cells were incubated for 2 min in PBS containing DAPI at 1 μg/ml to stain nuclei, rinsed briefly in PBS, and mounted in ProLong Gold antifade reagent (Invitrogen).

Images were acquired on an Axioskop 2 microscope equipped with an AxioCam CCD camera (Carl Zeiss, Oberkochen, Germany) or on a Nikon Eclipse 80i microscope (Kanagawa, Japan) equipped with a QImaging cooled CCD camera (Retiga 2000R Fast 1394; Surrey, BC, Canada). Images were processed with ImageJ 1.38 software (http://rsb.info.nih.gov/ij/; (Abramoff *et al*. 2004)).

Immunogold-labelling and transmission electron microscopy (*TEM*)

LR-White embedding and on-section labelling of *N. caninum* cultures were performed essentially as previously described (Hemphill and Croft, 1997; Vonlaufen *et al*. 2002, 2004). Sections were loaded onto formvar-carbon coated grids and non-specific binding sites were blocked for 2 h in PBS/1% BSA. They were then incubated in affinity-purified anti-NcMAG1 antibodies, diluted 1 : 100 in PBS/0·1% BSA for 1 h. After washing in 5 changes of PBS (2 min each), the goat anti-rabbit antibody conjugated to 10 nm diameter gold particles (Amersham) was applied at a dilution of 1 : 5 in PBS/0·1% BSA. After extensive washing in PBS, grids were air-dried and stained with lead citrate and uranyl acetate. Specimens were viewed on a Philips 400 TEM (Philips Electronics, Eindhoven, The Netherlands) operating at 80 kV.

RESULTS

Cloning and analysis of a cDNA encoding Neospora caninum *MAG1*

A sequence homology search against *N. caninum* expressed sequence tags (ESTs) in the ApiDots database (Li *et al*. 2003, 2004) using *T. gondii* MAG1 cDNA (Parmley *et al*. 1994) resulted in a single hit, a 2886 NT assembly of 21 ESTs: DT.92484705. This consensus sequence possesses an open reading frame (ORF) potentially encoding a protein sharing over 50% identity with *T. gondii* MAG1. To confirm the DT.92484705 data, we amplified *NcMAG1* cDNA by RACE, using a first strand cDNA prepared from *N. caninum* Liverpool tachyzoites as a template. The 5′-RACE reaction produced a single amplicon of ~1·5 kb, consistent with the sequence of the five 5′-RACE clones that we analysed. On the contrary, agarose gel electrophoresis of 3′-RACE products revealed a more complex banding pattern, with discrete fragments ranging from 0·6 to over 1·5 kb (data not shown). After bulk cloning and sequencing of twelve 3′-RACE products, we found that differences between clones were limited to their 3′-end, immediately preceding the poly(A) tail, suggesting an alternative polyadenylation site usage.

The unique 5′- and the longest 3′-RACE products, overlapping by 345 NT, were used to build a 2288 NT *NcMAG1* cDNA sequence (GenBank Accession no. EF580924; Fig 1). The consensus cDNA possesses a 1371 nucleotide ORF (including the TGA stop codon), potentially encoding a 456 amino acid protein identical to that predicted to be encoded by the DT. 92484705 transcript. The putative ATG initiation codon is part of a sequence (AGCA-CAATGG) matching 8 positions of the previously described *T. gondii* translation initiation consensus sequence (gNCAAaATGg) (Seeber, 1997). An additional, out-of-frame, ATG triplet was found closer to the *NcMAG1* cDNA 5′-end (NT 52–54); however, in a context apparently less favourable for translation initiation.

The predicted 5′-UTR is 128 NT long, 76 NT shorter than in the ApiDots transcript. The twelve 3′-RACE clones sequenced had a 3′-UTR ranging from 304 to 789 NT and a poly(A) tail, but electrophoretic analysis of 3′-RACE products suggested that polyadenylation of the *NcMAG1* transcript could occur even further downstream, consistent with the longer 3′-UTR present on the DT.92484705 transcript. We did not observe any consensus eukaryotic polyadenylation signal (AATAAA) downstream of the coding sequence. However, performing a less stringent search, 6 out of the 12 analysed transcripts appeared to be terminated in close vicinity (within the generally accepted 10–30 NT window) to upstream sequences differing at most by 1 mismatch from the consensus AATAAA hexamer. An imperfect 84/90 NT direct repeat was identified (NT 1934–2017 and 2019–2108) in the 3′-UTR (Fig. 1).

The predicted NcMAG1 precursor is 54% identical to TgMAG1 (Fig. 2) but does not share significant similarity to any other protein in GenBank nor in the Conserved Domain Database (CDD v2.17, October 2009). A typical signal peptide with a predicted signal peptidase cleavage site between Gly_{30} and Gln_{31} was identified by SignalP 3.0 with the hidden Markov models and the neural networks prediction methods (Bendtsen *et al*. 2004). The position of the predicted cleavage site was conserved in TgMAG1, but only with the hidden Markov models method (Fig. 2). The predicted MWs of NcMAG1 are 52·9 and 50·5 kDa, for the precursor and the mature protein, respectively.

We did not identify a hydrophobic region that could represent an internal transmembrane domain. The predicted NcMAG1 and TgMAG1 mature proteins share an overall 53% identity but sequence conservation is not homogeneously distributed along the proteins. The C-terminal region (aa189–456) is much more conserved than the N-terminal region (aa 31–188), with 66% and 32% sequence identity, respectively (Fig. 2).

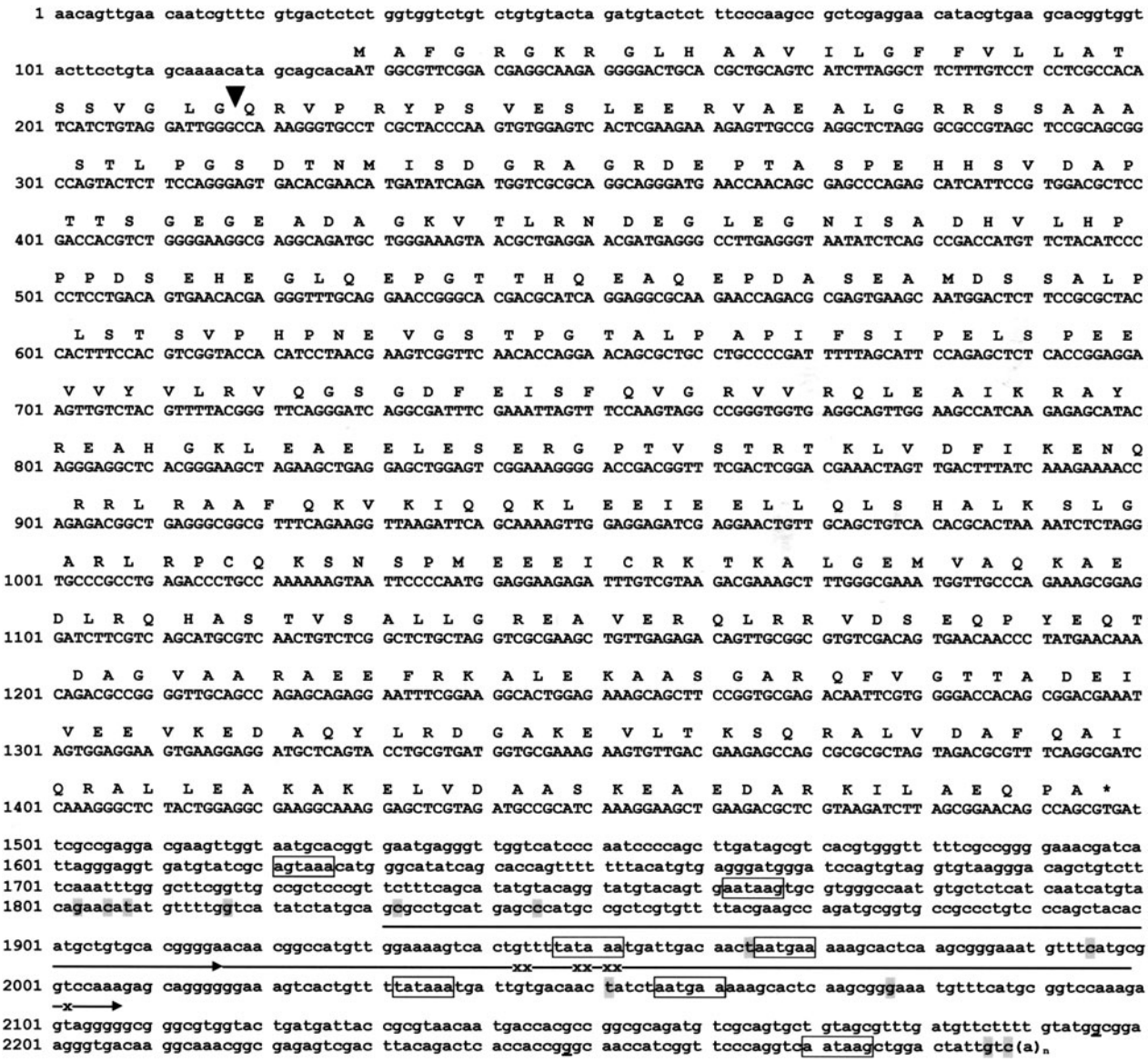

Fig. 1. The cDNA encoding NcMAG1. The largest cDNA clone (GenBank Accession no. EF580924, bottom) is represented together with the deduced MAG1 precursor protein (GenBank Accession no. ABQ52425, top). The predicted signal peptide cleavage site is indicated by a filled arrowhead. The 5′- and 3′-UTRs are in lowercase with the two nucleotides differing from the ApiDots DT.92484705 transcript underlined. Nucleotide residues at which polyadenylation was observed in other cloned *NcMAG1* cDNAs are shaded in grey. Sequences differing at most by 1 nucleotide from the consensus polyadenylation signal (AATAAA) are boxed. Tandem repeat units (NT 1931–2016 and 2017–2107) present in the 3′-UTR are shown as horizontal arrows above the nucleotide sequence. Mismatches and gaps created to align the two repeat units are displayed as 'x'.

Expression and immunolocalization of NcMAG1 in N. caninum *tachyzoites and bradyzoites*

A recombinant protein (recNcMAG1), comprising aa 31–394, was expressed as a $(His)_6$-tagged fusion protein in *E. coli* and used to immunize rabbits. All intermediate and final bleeds had a high titre against the purified recombinant protein as determined by Western blot and ELISA (data not shown). Sera from the third bleed were affinity-purified against recNcMAG1 and used for all further experiments.

In Western blots of protein extracts from purified extracellular *N. caninum*, anti-NcMAG1 antibodies detected a ~67 kDa band. The band was more prominent in lysates from SNP-treated cultures (containing predominantly bradyzoites) than in tachyzoite lysates (Fig. 3, lanes 1 and 2). Similarly, the protein was much more abundant in lysates of SNP-treated *Neospora*-infected Vero cells compared to untreated, infected cells (Fig. 3, lanes 3 and 4). The antibodies did not show any reactivity with uninfected Vero cell lysates (Fig. 3, lanes 5 and 6).

Affinity-purified antibodies were then used to localize NcMAG1 by IF and TEM. IF detection of MAG1 in extracellular parasites showed that the protein was distributed in a punctuated pattern

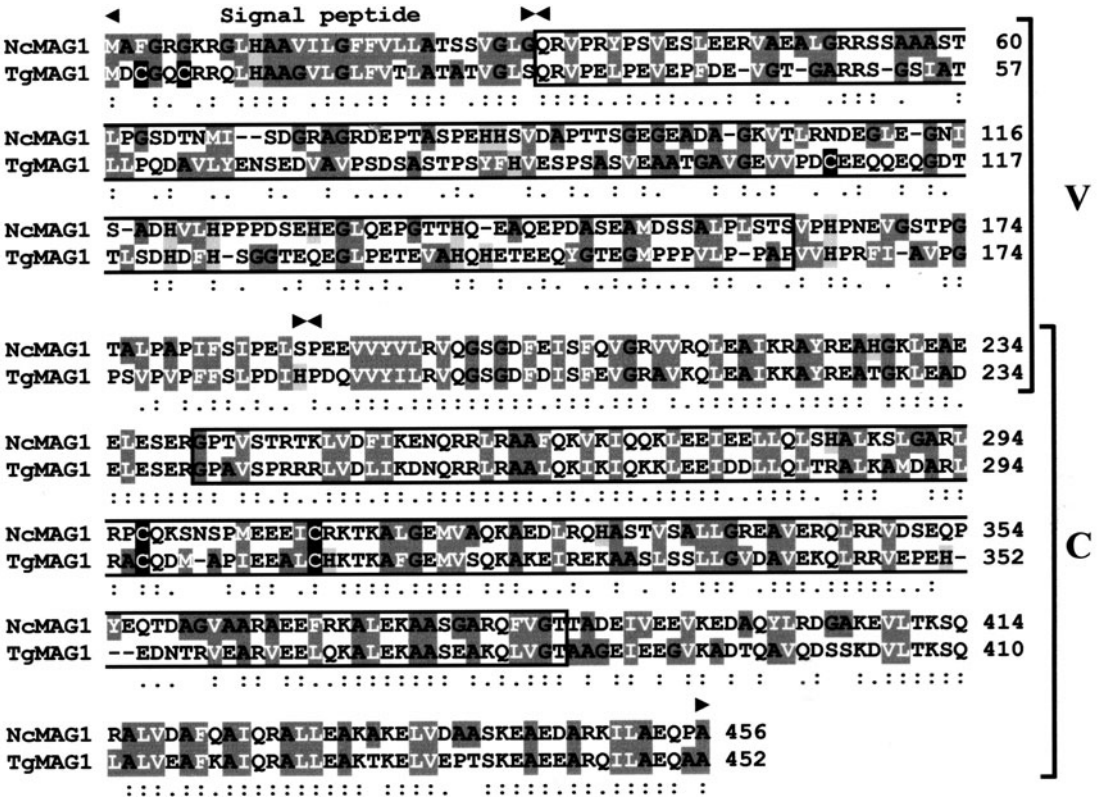

Fig. 2. Protein sequence alignment of deduced MAG1 precursors. *Neospora caninum* (Nc; GenBank Accession no. ABQ52425, this work) and *Toxoplasma gondii* (Tg; GenBank Accession no. AAC46484) MAG1 predicted protein sequences (single letter code) were aligned with MUSCLE and shaded according to the MM5 reduced, 5-letter amino acid alphabet. Residue grouping is as follows: [C]; [FILMVWY]; [AG]; [H]; [DEKNPQRST]. Identical residues are indicated by colons, similar residues are marked by dots. The putative signal peptide was identified with SignalP 3.0. Boundaries between the predicted signal peptide, the conserved (C) C-terminal region (66% identity, aa 189–456) and the less conserved (V) N-terminal region of NcMAG1 (32% identity, aa 31–188) are indicated by filled arrowheads. Antibodies were raised against a large portion of a recombinant NcMAG1 (aa 31–394, not shown). Amino acid stretches within the V and C regions, which were used for affinity-purification of antibodies specific to these regions are boxed.

within the cytoplasm. These punctuations were more apparent in bradyzoites (Fig. 4A). Staining of intracellular tachyzoites detected the protein mostly within the lumen of the parasitophorous vacuole (PV); the PV periphery was also faintly labelled. In contrast, after triggering tachyzoite-to-bradyzoite conversion with SNP, a more pronounced staining of the cyst wall and the cyst matrix was observed (Fig. 4B and C). The efficiency of the *in vitro* stage conversion using SNP treatment was confirmed using the bradyzoite-specific marker BAG1 on *Neospora*-infected cells (Fig. 4B) and on purified parasites (Fig. 4D). In a double-labelling experiment with anti-recNcMAG1 and mAbCC2, a monoclonal antibody directed against a cyst-specific antigen, both antigens appeared to largely co-localize (Fig. 4C)

By immunogold-TEM, performed on keratinocytes infected with *N. caninum* tachyzoites, minor labelling was observed in the intra-parasitic space, the lumen of the PV, and within the dense granule organelles of the tachyzoites (Fig. 5A). Staining of cell cultures undergoing SNP-treatment and hence tachyzoite-to-bradyzoite stage conversion (Fig. 5B–D) exhibited a much more pronounced immunogold staining. Gold particles were abundant within the cyst matrix and, in many cysts, also on the cyst wall. In addition, dense granule (DG) organelles were intensely labelled (Fig. 5B–D), indicating an increased expression of NcMAG1 during stage conversion, or an accumulation of the protein due to reduced degradation.

Antibodies against the NcMAG1 conserved region, but not the variable region, cross-react with TgMAG1

Since NcMAG1 and TgMAG1 are orthologous proteins, we investigated the potential antibody-cross-reactivity of these antigens. Immunoblot and immunofluorescence using affinity-purified antibodies against the recombinant NcMAG1 resulted in a weak, but distinct, cross-reactivity with *T. gondii*, no matter whether cultures had been treated with 100 μM SNP or not (data not shown). We thus determined, which region (conserved or variable) could

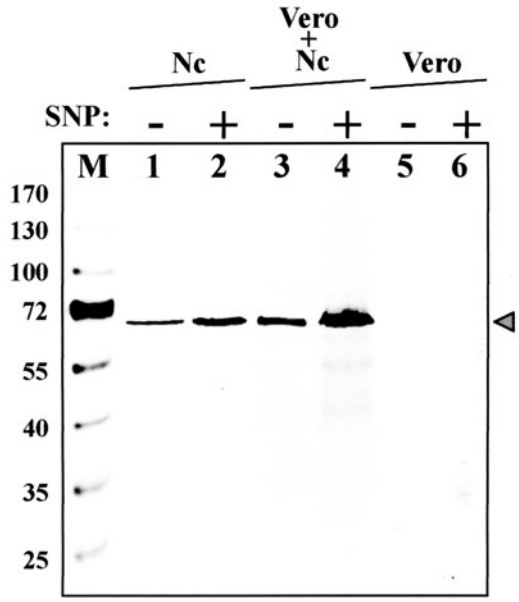

Fig. 3. Western blot analysis of NcMAG1 expression in *Neospora caninum*. Affinity-purified antibodies raised against a recombinant NcMAG1 were used to detect the protein in lysates obtained from free parasites (Nc), *Neospora*-infected Vero cells (Vero + Nc), and uninfected Vero cells (Vero). Cultures were either left non-treated (−) or treated for 9 days with SNP (+). Lanes 1 and 2: lysates from 2×10^5 free *N. caninum*; lanes 3 and 4: *N. caninum*-infected Vero cells lysates containing 5×10^4 parasites. Total protein concentrations in control lanes 5 and 6 were adjusted to fit those in lanes 3 and 4, respectively. M: pre-stained protein marker; apparent MWs in kDa are indicated. The open arrowhead indicates the position of the 67 kDa NcMAG1 band.

be responsible for this cross-reactivity. Western blots were performed, using extracts of non-treated or SNP-treated *Neospora*- and *Toxoplasma*-infected cells. In order to enhance the detection of any weak cross-reactivity, the samples loaded from *Toxoplasma*-infected cells contained 5 times more parasites than those loaded from *Neospora*-infected cells (non-treated cells: 10^5 *Neospora* and 5×10^5 *Toxoplasma*; SNP-treated cells: 10^4 *Neospora* and 5×10^4 *Toxoplasma*). Even though the *Toxoplasma* samples were more concentrated in terms of parasite numbers, the 65 kDa TgMAG1 was only faintly detected by the antibodies affinity-purified on the conserved region of NcMAG1 in both non-treated and SNP-treated cultures (Fig. 6A, upper panel, lanes 3 and 7). In contrast, no cross-reactivity in extracts containing *Toxoplasma* was observed when antibodies affinity-purified on the variable region were used (Fig. 6A, lower panel, lanes 3 and 7). Note that the 67 kDa NcMAG1 was recognized by both affinity-purified antibodies in *Neospora*-infected cells (Fig. 6A, lanes 1 and 5). These results were largely confirmed by IF performed on infected cells after treatment with SNP (Fig. 6B) or without treatment (not shown). Antibodies directed against the variable region of NcMAG1 failed to detect *T. gondii* in spite of an exposure time 20 times longer than what was used for the detection of *Neospora*.

DISCUSSION

The apicomplexan EST database (Li *et al.* 2003, 2004) allowed retrieval of the *NcMAG1* sequence and amplification of the corresponding cDNA by RACE. The 3′-ends of the *NcMAG1* transcripts were highly heterogeneous in size, strongly suggesting that polyadenylation occurs at multiple sites. Interestingly, a number of *NcMAG1* mRNA properties were reminiscent of *T. gondii* dihydrofolate reductase (DHFR) mRNA. Common features include the heterogeneity of the 3′-end lengths, the absence of a consensus AATAAA polyadenylation signal downstream of the coding sequence, and the presence of a direct repeat in the 3′-UTR (Matrajt *et al.* 2004). Half of the *NcMAG1* transcripts sequenced were polyadenylated within 10–30 NT downstream of sequences differing at most by 1 NT from the consensus AATAAA hexamer. This suggests that apicomplexan parasites, similarly to mammals, may use, in addition to the consensus AATAAA, some of its single NT sequence variants. In the other half of the transcripts that we analysed, there was no evident sequence reminiscent of a consensus hexamer within 10–30 NT upstream of the polyadenylation site, suggesting that, similar to that observed in yeast, a variety of other positioning elements might also be used (Guo and Sherman, 1995). In the *DHFR* transcript, the direct repeat in the 3′-UTR was found to exert a slight stimulatory effect on chloramphenicol acetyltransferase (CAT) expression in a CAT reporter assay (Matrajt *et al.* 2004). In this context, it would be of interest to assess whether the 84/90 NT direct repeat present in *NcMAG1* 3′-UTR would produce a similar effect.

The deduced amino acid sequences of *T. gondii* and *N. caninum* MAG1 share 54% identity and each protein contains a single 30 aa hydrophobic domain, which is located at the N-terminus and represents a predicted signal peptide. Since TgMAG1 and NcMAG1 do not exhibit any significant similarity with other proteins, it remains rather difficult to postulate a biological function for these proteins at present. Given their relative abundance in the tissue cysts of *T. gondii* and *N. caninum*, they could possibly exert a scaffolding role. In addition, the rather conserved C-terminal region of MAG1 in the two closely related parasites suggests that this part of the protein could be involved in common functional activities.

To date, information concerning the composition of *N. caninum* tissue cysts is limited, since they have not been really amenable to experimental procedures. A protocol based on the SNP treatment described by Vonlaufen *et al.* (2004) was used to produce *N. caninum* tissue cysts *in vitro*. In this way,

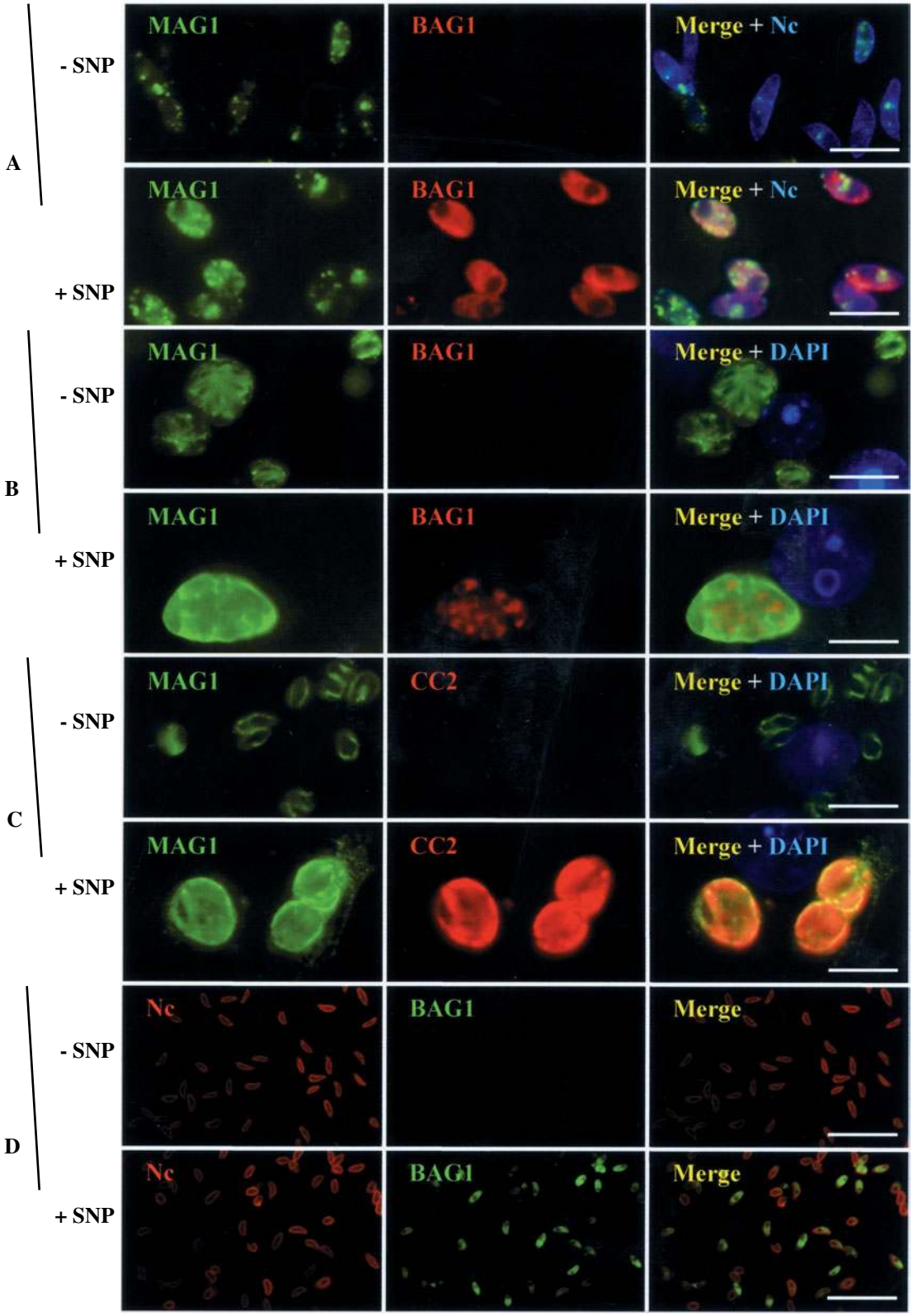

Fig. 4. Immunofluorescence analysis of NcMAG1 expression. *Neospora caninum* were cultured in non-treated (−SNP) or in SNP-treated Vero cells (+SNP), and analysed by indirect IF, either within infected host cells (B-C) or after purification from host cells (A and D). The primary antibodies were: anti-NcMAG1 affinity-purified polyclonal antibodies (MAG1), a polyclonal antibody directed against the bradyzoite-specific marker BAG1 (BAG1), the monoclonal antibody CC2 directed against a cyst-specific antigen (CC2), and an antiserum directed against *N. caninum* crude extract (Nc). The following secondary Alexa Fluor (AF)-labelled antibodies were used: AF-350 (blue), AF-488 (green) and AF-568 (red). An overlay image with DAPI staining of nuclei (B-C, blue) or with AF-350 staining of whole parasites (A, blue) is presented on the right panel. Scale bars = 5, 10 and 20 μm in panels (A), (B-C), and (D), respectively.

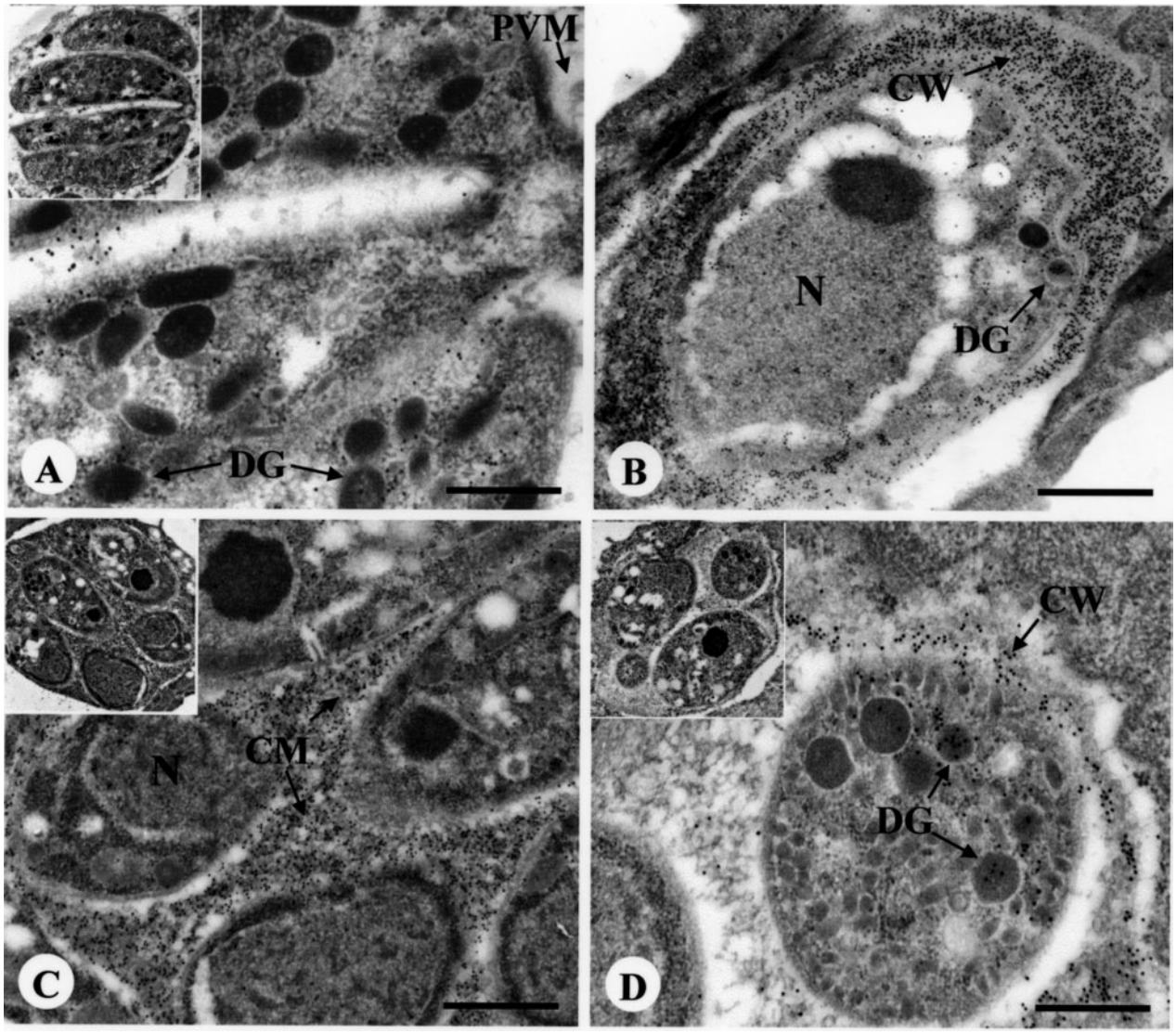

Fig. 5. Immunolocalization of NcMAG1 by immunogold EM. TEM on *Neospora caninum*-infected mouse epidermal keratinocytes using affinity-purified anti-NcMAG1 primary antibodies. (A) Parasitophorous vacuole containing tachyzoites. Note the relatively marginal staining of the tachyzoite dense granules (DG) and the vacuolar space between the parasites. PVM, Parasitophorous vacuole membrane. (B–D) Cyst-like vacuoles obtained after SNP-treatment. Cysts containing either a single parasite (B) or several parasites (C and D). Note the massive immunogold labelling within the cyst matrix (CM), the dense granules (DG) and the cyst wall (CW). N depicts the nucleus, which remains virtually unlabelled. Scale bars = 0·25μm in (A) and (D) and 0·4μm in (B) and (C). Inserts in (A), (C) and (D) represent the corresponding low-magnification views.

we could achieve up to 70% tachyzoite-to-bradyzoite conversion, as demonstrated by staining with antibodies directed against the bradyzoite-specific BAG1 marker (McAllister *et al*. 1996). The high percentage of stage conversion we achieved corroborates the validity of the comparison of NcMAG1 expression levels between tachyzoites and bradyzoites.

Immunoblotting of lysates from purified parasites of either tachyzoite or bradyzoite-converted cultures showed a similar 67 kDa band reacting with anti-NcMAG1 antibodies. This sharply contrasted with the predicted MW of the mature NcMAG1 (50·5 kDa). A difference between the predicted and the apparent MW of the protein was also observed in TgMAG1 (Parmley *et al*. 1994). This difference could, at least partially, be explained by post-translational modifications such as extensive glycosylations and/or phosphorylations. However, since the recombinant NcMAG1 expressed in *E. coli* also displayed an aberrant apparent MW in SDS-PAGE (65 kDa instead of the predicted 41 kDa), it is very likely that the main clue to the electrophoretic mobility shift lies within the protein sequence itself. The 67 kDa NcMAG1 product was more prominent in purified bradyzoites than in tachyzoites, indicating an increase in expression and/or a decrease of the protein turnover (degradation) resulting in a net

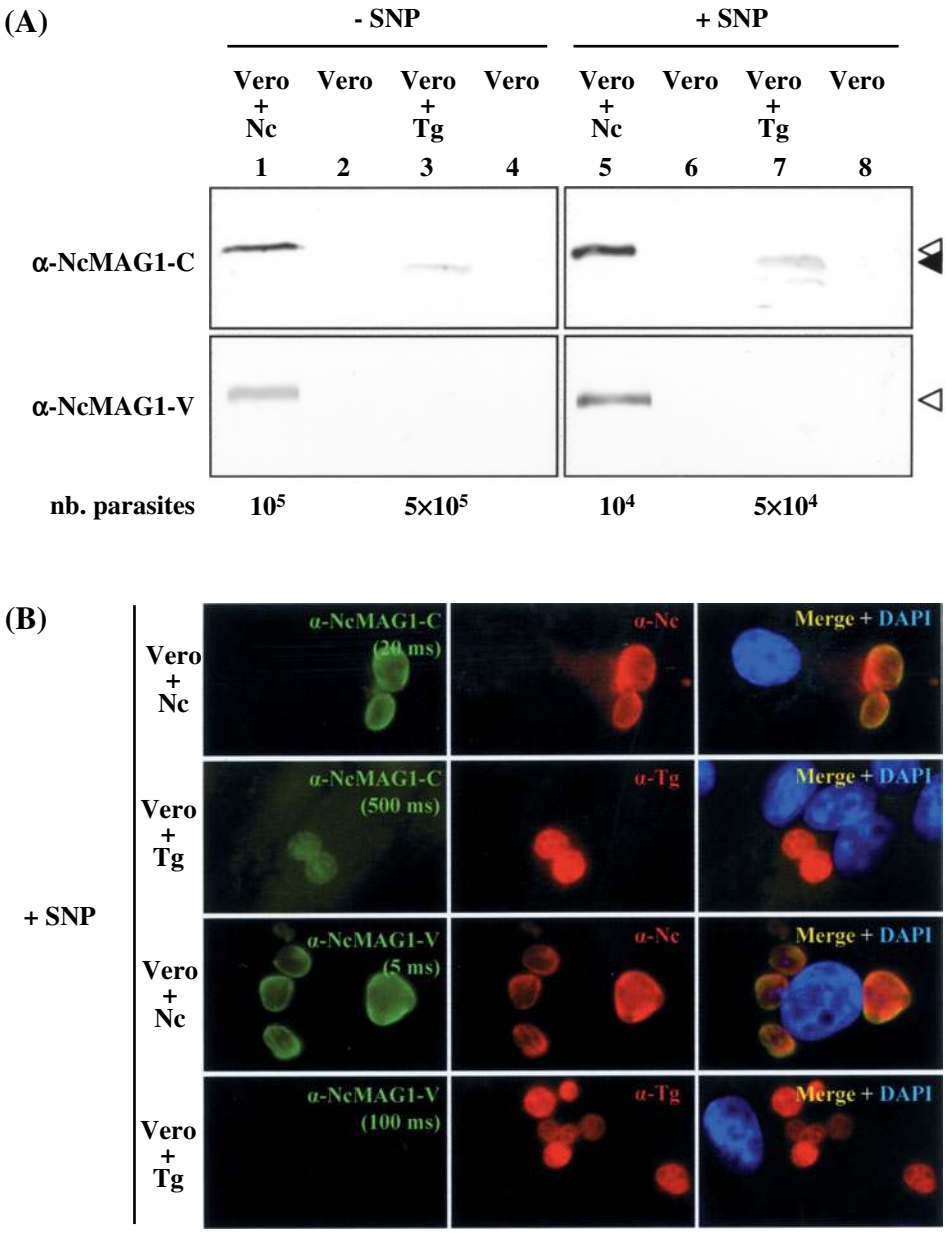

Fig. 6. Cross-reactivity of antibodies directed to the conserved or the variable region of NcMAG1. Polyclonal anti-NcMAG1 antibodies were affinity-purified on recombinant proteins corresponding to either the conserved (C) or the variable (V) region of NcMAG1. The cross-reactivity of the resulting antibodies (α-NcMAG1-C and α-NcMAG1-V) was assessed on *Neospora caninum*- (Vero + Nc) and *Toxoplasma gondii*-infected (Vero + Tg) Vero cells. (A) Western blot analysis. Infected cell cultures were either left non-treated (−SNP) or treated with SNP to trigger tachyzoite to bradyzoite conversion (+SNP). The number of parasites present in infected cell lysates is indicated. Total protein concentrations in uninfected control extracts (Vero; even lanes), were adjusted to match those in the corresponding infected cell extracts (odd lanes). The open and filled arrowheads indicate the positions of the 67 kDa NcMAG1 and the 65 kDa TgMAG1 bands, respectively. (B) Immunofluorescence analysis. *N. caninum* or *T. gondii* were cultured in SNP-treated Vero cells and analysed by indirect IF using α-NcMAG1-C or α-NcMAG1-V (green) and an antiserum directed against either *N. caninum* (α-Nc) or *T. gondii* (α-Tg) crude extracts (red). Overlay images with DAPI staining of nuclei (blue) are presented on the right. Green channel photographs were not enhanced post-acquisition; the respective exposure times are indicated.

accumulation of NcMAG1 in the developing cyst matrix and wall.

The higher steady-state level of NcMAG1 in bradyzoites was also confirmed by IF analysis. Similarly to TgMAG1 (Ferguson and Parmley, 2002), NcMAG1 was highly abundant within tissue cysts. The increase of NcMAG1 staining during stage conversion was paralleled by that of BAG1 within the parasite cytoplasm. The staining obtained with mAb CC2 and anti-NcMAG1 antibody also simultaneously increased during stage conversion, and the two antigens appeared to largely co-localize. The

antigen detected by mAb CC2 in cysts has not yet been identified, but it is nevertheless unlikely that the co-localization we observed was due to the binding of mAb CC2 to MAG1. Indeed, mAb CC2 recognizes a 115 kDa glycosylated cyst antigen (Gross *et al*. 1995), a MW, which does not fit with the apparent MW of neither NcMAG1 (~67 kDa) nor TgMAG1 (65 kDa) in bradyzoites. Therefore, NcMAG1 represents a reliable marker for the detection of tachyzoite-to-bradyzoite stage conversion.

Immunogold TEM confirmed the IF results and revealed the presence of NcMAG1 within the cyst wall and the cyst matrix, which was much more pronounced compared to the labelling of the PV of tachyzoite-infected cells. Importantly, the ultrastructural study revealed that NcMAG1 was also localized within DG. Taken together, our results suggest that NcMAG1 is targeted to the DG and subsequently released into the parasitophorous vacuole in a process that is upregulated during tachyzoite-bradyzoite stage conversion and cyst formation.

The fact that MAG1 is targeted to the cyst wall and the cyst matrix following secretion from the DG organelles was not surprising since the incorporation of DG proteins into the cyst wall during cyst biogenesis has already been reported for other proteins such as NcGRA1, NcGRA2 and NcGRA7, as well as for the antigen recognized by mAb CC2, (Fuchs *et al*. 1998; Vonlaufen *et al*. 2002, 2004). With the exception of a few DG proteins, for which the function is known (such as NTPases, cyclophilins and serine protease inhibitors) the majority of these GRA proteins, including *T. gondii* GRA1-10 and *N. caninum* GRA1, 2, 3, 6, and 7, do not exhibit sequence similarities with other known proteins (Adjogble *et al*. 2004; Ahn *et al*. 2005; Mercier *et al*. 2005). Moreover, their function remains largely unknown, except for TgGRA7, which participates in sequestering host cell lysosomes into the vacuolar space by acting like a garrote. However, this process does apparently not occur in *N. caninum* (Coppens *et al*. 2006).

GRA proteins share a few common features, including a signal peptide that appears to be sufficient to target the proteins to DG organelles and, being ubiquitous (Mercier *et al*. 2005) was predicted in the NcMAG1 primary sequence. An additional characteristic of GRA proteins that was also observed in MAG1 is the intriguing difference between the MW predicted from the protein primary sequence and the higher MW observed on SDS-PAGE gels (Mercier *et al*. 2005). A trans-membrane domain present in most (TgGRA3-10), but not all GRA proteins (Ahn *et al*. 2005; Mercier *et al*. 2005) was, however, not found in NcMAG1.

Interestingly, even though MAG1 is more abundant in bradyzoites and accumulates into cysts that define the chronic stage of infection, it was described as an antigen detected promptly after infection with *T. gondii*. Di Cristina *et al*. (2004) showed that a fragment of MAG1 (aa 28–126, therefore within the region we refer to as 'variable') is highly immunogenic and triggers a B-cell response in 73% of healthy individuals. The immune response against MAG1 is rapid, appearing as early as 1 month after infection (Di Cristina *et al*. 2004). Recombinant TgMAG1 was also tested, with encouraging results, in order to improve current serodiagnostic methods of acute toxoplasmosis during pregnancy (Pfrepper *et al*. 2005; Holec *et al*. 2007).

Two studies have so far reported that an efficient, although partial, protection against challenge with *T. gondii* cysts could be obtained, as evidenced by a marked reduction in the number of cerebral cysts following immunization with TgMAG1 alone (Parmley *et al*. 2002) or combined with a bradyzoite-specific antigen (Nielsen *et al*. 2006). In the first study, Parmley *et al*. demonstrated that vaccination with the 79-aa C-terminal part of TgMAG1 significantly increased the survival rate of animals and reduced the number of cerebral cysts by as much as about 3-fold. In the second study, Nielsen *et al*. observed an effective protection after DNA vaccination with a mixture of 2 plasmid vectors encoding TgMAG1 and the bradyzoite-specific TgBAG1. A preferential induction of antibodies of the IgG2a subclass directed against the immunizing antigens was also observed (Nielsen *et al*. 2006). Altogether, these results indicate that MAG1 could be a useful antigen for diagnosis, as well as a promising candidate for vaccination trials in *T. gondii*.

Recently, the use of NcMAG1 as a vaccine was assessed in an acute disease mouse model for neosporosis (Debache *et al*. 2009). In this experiment, mice were vaccinated either intraperitoneally or intranasally and, following challenge infection with *N. caninum* tachyzoites, a partial (50%) protection was observed for the intraperitoneally vaccinated animals, while only 10% of animals survived the challenge following intranasal vaccination. However, application of NcMAG1 as a vaccine will make more sense in the context of re-activation of neosporosis in chronically infected animals harbouring tissue cysts, or the challenge should ideally be performed by oral administration of tissue cysts. This procedure appeared to be difficult to carry out since, at present, only a limited number of studies have demonstrated the possibility to produce *N. caninum* cysts in immunosuppressed mice (McGuire *et al*. 1997; Rettigner *et al*. 2004). Thus, a more suitable experimental model than the mouse, such animals possessing the ability to develop cerebral tissue cysts, should preferentially be used.

Even though the MAG1 primary sequences in *T. gondii* and *N. caninum* share a high degree (66%) of identity at their C-termini, only limited, but still clear, antibody cross-reactivity was observed in

Toxoplasma when anti-NcMAG1 antibodies affinity-purified on the relatively conserved C-terminal region were tested. No cross-reactivity was found when antibodies affinity-purified on the variable region were assessed. Both affinity-purified antibodies reacted with *N. caninum* tachyzoites and bradyzoites. This indicates that only the variable but not the conserved region of the protein could be an ideal tool for distinguishing between *Toxoplasma* and *Neospora* species, and corresponding investigations are currently being carried out to investigate the serodiagnostic potential of the NcMAG1 variable region.

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