

A new thrombospondin-related anonymous protein homologue in *Neospora caninum* (NcMIC2-like1)

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SUMMARY

Neospora caninum is an Apicomplexan protozoan that has the dog as a definitive host and cattle (among other animals) as intermediate hosts. It causes encephalopathy in dogs and abortion in cows, with significant loss in worldwide livestock. As any Apicomplexan, the parasite invades the cells using proteins contained in the phylum-specific organelles, like the micronemes, rhoptries and dense granules. The aim of this study was the characterization of a homologue (denominated NcMIC2-like1) of *N. caninum* thrombospondin-related anonymous protein (NcMIC2), a micronemal protein previously shown to be involved in the attachment and connection with the intracellular motor responsible for the active process of invasion. A polyclonal antiserum raised against the recombinant NcMIC2-like1 functional core (thrombospondin and integrin domains) recognized the native form of NcMIC2-like1, inhibited the *in vitro* invasion process and localized NcMIC2-like1 at the apical complex of the parasite by confocal immunofluorescence, indicating its micronemal localization. The new molecule, NcMIC2-like1, has features that differentiates it from NcMIC2 in a substantial way to be considered a homologue†.

Key words: *Neospora caninum*, Apicomplexa, thrombospondin-related anonymous protein, microneme, invasion assay, TRAP, MIC2-like1, MIC2.

INTRODUCTION

Neospora caninum is an Apicomplexan protozoan that was isolated in 1988 by Dubey and collaborators when investigating the origin of a severe neuromuscular disorder in dogs. The disease was first described in dogs in 1984, in sheep in 1987 and was correlated to economical losses in cattle in 1988 (Dubey *et al.* 1988). Due to morphological and biochemical similarities between *N. caninum* and *Toxoplasma gondii*, and the fact that both life cycles involve 2 types of host with similar kinetics, the first cases of neosporosis could have been misdiagnosed as toxoplasmosis (Hemphill *et al.* 2006). *N. caninum* has been detected in definitive and/or intermediate hosts in almost all continents (Dubey *et al.* 2007), and it is now known that dogs and coyotes are definitive hosts and cattle are economically the most relevant intermediate hosts.

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† Nucleotide sequence data reported in this paper are available in the GenBank™ under the Accession number HQ011383.

As all other Apicomplexa, *N. caninum* is an obligatory intracellular parasite and invades the host cells by a conserved and extremely successful mechanism. The host cell invasion process occurs via a parasite-driven mechanism known to be an active invasion (Cesbron-Delauw *et al.* 2008), requiring the use of its own actin/myosin motor-system (Daher and Soldati-Favre, 2009). The multi-factorial process of invasion involves receptors related to the recognition, gliding and invasion. Receptors are constituted by a set of proteins released by micronemes, rhoptries and dense granules. Micronemes are organelles containing a concentrated mixture of adhesive proteins released to enable parasite invasion (Friedrich *et al.* 2010). They possess modules homologous to adhesive domains of higher eukaryotes that have been demonstrated to act in the direct binding to host cells (DiCristina *et al.* 2000). These motifs are present in one or multiple copies in micronemal proteins and a large number of combinations of these blocks have been described, making each micronemal protein structurally unique, which increases their invasion capacity (Soldati *et al.* 2001).

Among Apicomplexans, a highly conserved family of microneme proteins is the thrombospondin-related anonymous protein (TRAP). A range of different denominations have been described, there are members of TRAP in *Plasmodium* sporozoite

(TRAP) and ookinete (CTRP), *T. gondii* (TgMIC2) (Sultan *et al.* 1997; Wan *et al.* 1997; Dessens *et al.* 1999; Menard, 2000; Huynh *et al.* 2004), *B. bovis* (BbTRAP, Gaffar *et al.* 2004a), *Eimeria* (EtMIC1, Tomley *et al.* 2001), *N. caninum* (NcMIC2, Lovett *et al.* 2000) and *Cryptosporidium parvum* (CpMIC 1, Putignani *et al.* 2008). All members of the TRAP family in Apicomplexa are type I transmembrane proteins which have a variable number of 2 adhesive extracellular domains: (i) integrin I domain or von Willebrand factor (vWFA) A domain and (ii) thrombospondin type I (TSR or TSP-1), followed by a transmembrane domain and a cytoplasmic tail (Starnes *et al.* 2006).

In *N. caninum*, 5 micronemal proteins have been described, NcMIC1, NcMIC2, NcMIC3, NcMIC4 and NcMIC10 (Sonda *et al.* 2000; Lovett *et al.* 2000; Hoff *et al.* 2001; Naguleswaran *et al.* 2002; Keller *et al.* 2002). Preliminary analysis of ESTs from *N. caninum* indicated the presence of a second potential protein TRAP, besides the previously described NcMIC2 (Lovett *et al.* 2000), which we designated as NcMIC2-like1. The presence of a second TRAP in *N. caninum* represents a unique fact for coccidia, including *Eimeria*, *Toxoplasma*, *Sarcocystis* and *Cryptosporidium*. Although the whole genome of *T. gondii* has been sequenced (Aurrecochea *et al.* 2010), several investigations have unsuccessfully searched for other members of TRAP in *T. gondii*. Therefore, we decided to investigate NcMIC2-like1.

MATERIALS AND METHODS

Tissue culture and parasite purification

Vero cell cultures were maintained in RPMI-1640 medium (LGC Biotechnology) supplemented with 5% fetal calf serum (Cultilab), 2.05 mM glutamine, 50 U of penicillin/streptomycin at 37 °C and 5% CO₂ in T-25 cm² or 75 cm² tissue-culture flasks. Cultures were trypsinized at least once a week. *N. caninum* tachyzoites of the Nc-1 isolate were maintained in Vero-cell monolayers and the excess Vero cell debris was removed by exclusion chromatography in Sephadex G-25 (PD-10 columns, GE). The recovered parasites were counted in a Neubauer chamber after adequate dilution and the average recovery on 25 cm² culture was 1 × 10⁷ tachyzoites, yielding around 95% tachyzoite purification.

Determination of the full length NcMIC2-like1 cDNA sequence

The RNA ligase-mediated rapid amplification (RACE) of 5' (primer Race 5'; CGACTGGAGCAGAGGACACTGA) and 3' (primer Racer 3'; GCTGTCAACGATACGCTACGTAACG) cDNA ends (RLM-RACE, Gene Racer kit Invitrogen) was initially used, based on information of partial

ESTs of NcMIC2-like1 available by that time (e.g. CF273823, CF261023, CF598400). The RACE PCR generated approximately 99.7% of the NcMIC2-like1 protein sequence. The non-annotated genomic database from *N. caninum* (www.sanger.ac.uk/cgi-bin/blast/submitblast/n_caninum) allowed the analysis of contigs flanking the NcMIC2-like1 gene region. Based on the Sanger nomenclature, the NcMIC2-like1 sequence obtained by that time was at the contig 630, which was flanked by contig 8340. Therefore, one forward primer (GGGAGATTTGAGAAGAAGCGAGA) located at the contig 8340, and a reverse primer at the contig 630 (TTTGGCGACGGGAATTACTATGG) were designed. After several PCRs and sequencing, the analyses indicated a contig between 8340 and 630, contig 1212, and it was possible to conclude that the complete NcMIC2-like1 gene sequence was located at the 1212 and 630 contigs, which were recently all merged into a single contig 1051 (http://www.sanger.ac.uk/cgi-bin/blast/getseq?id=n01DT_Cy929480J7997P.1;db=yeastpub/NEOS.contigs.fasta;acc=Contig1051).

Expression of recombinant NcMIC2-like1 in *E. coli* and generation of polyclonal antiserum

The NcMIC2-like1 open reading frame without the putative signal peptide was amplified by PCR from bases 138 (numbered from the first base of the initiation codon) to 1260 using the forward *Bam*HI primer 5' CCC**GGATCCC**CAGGCAGCGTCAAATGAA 3' and the reverse *Hind*III 5' CCC**AAGCTTA**GATCCTGACCGACTTCT 3' (restriction sites are bold underlined). For expression, *E. coli* strain BL21 was transformed with pET28/NcMIC2-like1, and cultures incubated with or without 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Bacterial pellets were sonicated first in 10 mM Tris buffer (pH 7.0) followed by a sonication in 8 M urea, run on 10% SDS-PAGE minigels and silver stained to confirm expression. Histidine-tagged purified recombinant pET28/NcMIC2-like1 protein (150–200 μg/shot) and tachyzoite protein extract (250–300 μg/shot), quantified by the Bradford method, were used to immunize 1 rabbit each (Kawasaki *et al.* 2007). The animals were immunized 3 times (3-week intervals) firstly with incomplete Freund's adjuvant and subsequently with complete Freund's adjuvant, generating a polyclonal antiserum against the fragment of NcMIC2-like1 (anti-NcMIC2-like1) and against tachyzoite protein extract.

Neospora caninum tachyzoite protein extract and control

The *N. caninum* tachyzoite protein extract and Vero protein extract (control) were obtained by lysis of the tachyzoites (2 × 10⁸ per 0.5 ml) or the Vero cells

(5×10^6 per 0.5 ml) in 7 M urea, 2 M thiourea, and 4% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate). The Vero control extracts (secreted and total protein) were made because we have previously observed that polyclonal antiserum cross-reacts with the cell source of the parasite (Gaffar *et al.* 2004a,b).

Secreted fraction from Neospora caninum and control

Freshly purified tachyzoites (5×10^7) in 1 ml of RPMI were incubated for 10 min at 37 °C and subsequently in ice for 10 min, followed by mild centrifugation (3000 g, 10 min, 4 °C) to remove the tachyzoites. The supernatant was then centrifuged for 10 min at higher speed (10000 g, 4 °C) to remove residual insoluble components before TCA/acetone precipitation. Equal parts of RPMI protein content were incubated with 30% trichloroacetic acid (TCA), 50 mM dithiothreitol (DTT) in acetone for 40 min at -20 °C and centrifuged for 15 min, 10000 g at 4 °C. The supernatant was discarded and the pellet was then washed with 10 mM DTT in acetone and centrifuged for 5 min, 10000 g at 4 °C. The final pellet was dried at room temperature and dissolved in 7 M urea, 2 M thiourea and 4% CHAPS. The same procedure was performed with Vero cells as a control.

One dimensional (1D) electrophoresis and 1D Western blotting (WB)

The purified NcMIC2-like1 recombinant fragment, tachyzoite protein extract, secreted fraction and respective Vero controls were separated by 10% SDS-PAGE and transferred to PVDF membrane (ImmobilonTM-P, Millipore). The blot was blocked with 0.8% porcine gelatin (Sigma-Aldrich) diluted in phosphate-buffered saline (PBS) containing 0.5% Tween (PBST) for 1 h at 37 °C. Rabbit antisera were diluted in PBST containing 0.2% porcine gelatin and incubated overnight at 4 °C. The blot was washed with PBST and incubated with anti-rabbit-immunoglobulin conjugated to HRP (ZyMed-Invitrogen) for 1 h at 37 °C. After washing with PBST, the blot was developed with an enhanced chemiluminescent substrate for the detection of HRP (SuperSignal West Pico Chemiluminescent Substrate, Pierce). Images of the gels and blots were acquired with LabScan v5.0 software on a flatbed scanner (Image Scanner, GE).

Two dimensional (2D) electrophoresis and 2D Western blotting

Tachyzoite protein extract (50 µg) and secreted fraction (~ 25 µg) were dissolved in a rehydration solution containing 7 M urea, 2 M thiourea, 4% CHAPS,

2% carrier ampholyte mixture (pH 3–11 NL in Immobiline Drystrip gels, GE), 20 mM dithiothreitol (DTT) and supplemented with protease inhibitors (Protease Inhibitor cocktail, Sigma-Aldrich). The samples were loaded on 7 cm IPG strips (pH 3–11 NL), rehydrated and focused in an automated overnight run (IPGPhorTM) using 10–14 h of rehydration, and a step voltage focusing procedure (20 min 100 V, 30 min 300 V, 1.2 h 1000 V, 15 min 5000 V, followed by 5000 V until a total of 5 Kvh was reached). Image acquisition was performed as described for one-dimensional gels/blots.

Induction assays for microneme secretion

Freshly purified tachyzoites were washed once with RPMI, pelleted (3000 g, 3 min, room temperature) and re-suspended in RPMI (2×10^8 ml). To induce secretion, 50 µl of tachyzoite suspension were added to 50 µl of RPMI containing doubled drug concentrations (EtOH 1%, DMSO 1%, ionomycin 400 nM or A23187 400 nM) and incubated for 2 min, 37 °C. To inhibit secretion, tachyzoites were pre-treated with 100 µM BAPTA-AM at 18 °C, 10 min, then added to the drug solution and incubated for 2 min at 37 °C. The supernatant was collected by sequential centrifugation (3000 g, 10 min followed by 10000 g, 10 min), applied in a 12.5% SDS-PAGE and analysed by Western blot. The inadvertent lysis of tachyzoites was evaluated by determination of β-galactosidase activity as described by Howe *et al.* (1997) and Carruthers and Sibley (1999).

Confocal immunofluorescence microscopy

For immunofluorescence, 5-day cultures of *N. caninum* from polylysine-coated glass coverslips were fixed in 3% paraformaldehyde in PBS. After 1 h, the coverslips were rinsed in PBS, permeabilized with PBS/0.2% Triton-X for 30 min and placed into blocking buffer solution (PBS/3% BSA/50 mM glycine) for 16 h at 4 °C. Samples were rinsed in PBS and incubated with serum against recombinant NcMIC2-like1 (1:100) for 1 h, followed by 3 washes in PBS. The secondary antibody was a goat anti-rabbit-fluorescein isothiocyanate (FITC) conjugate diluted at 1:100 and nuclear staining was performed with propidium iodide (0.5 µM). After extensive washing, the slides were embedded in glycerol and were observed on a confocal microscope (Leica TCS SP4 Laser Scanning Confocal Microscope, Leica Microsystems, Heidelberg, Germany). The images were captured by a 100× objective in oil immersion. Eight images of 0.6 µm from each channel were captured, grouped and processed by the program Image J 1.41 (National Institutes of Health, USA).

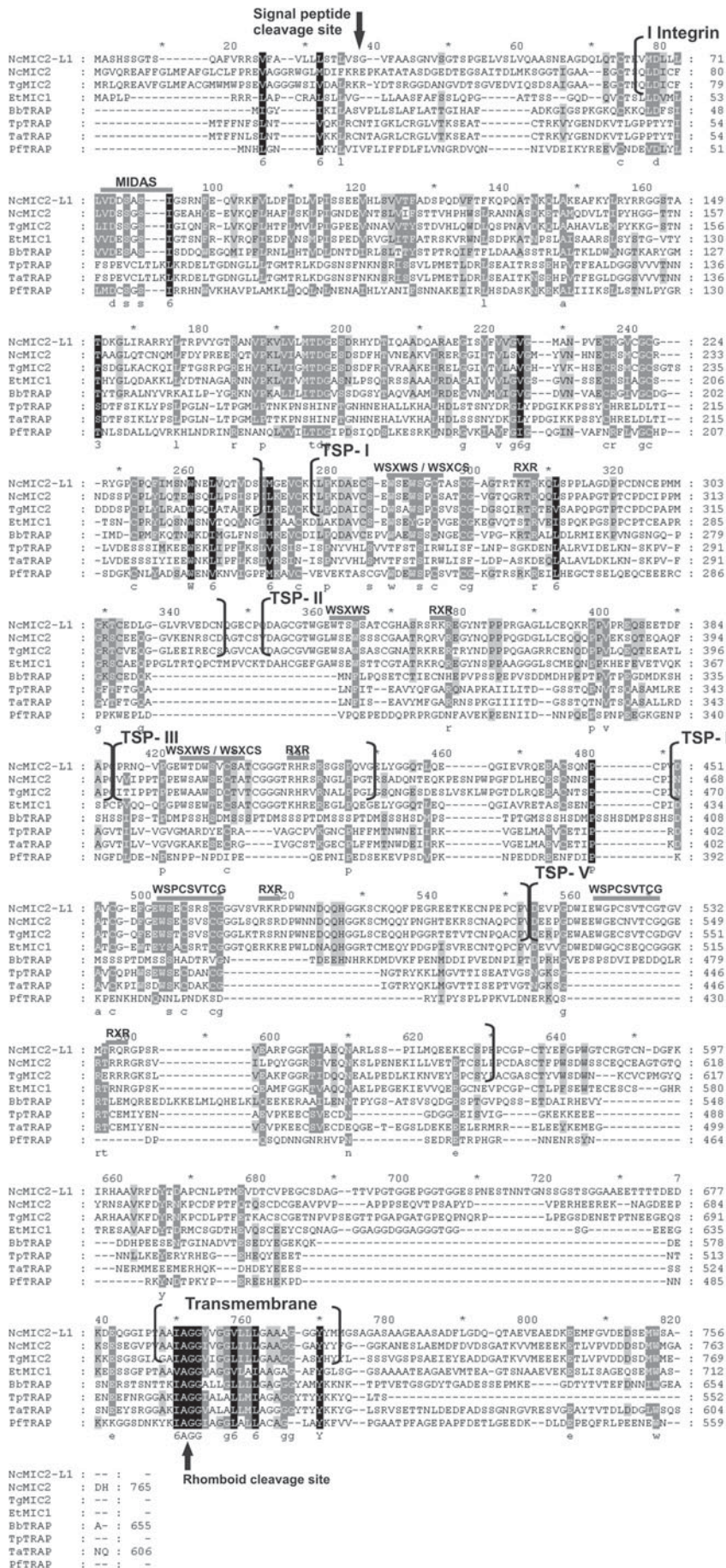


Fig. 1. For legend see opposite page.

Invasion assays

Invasion of Vero cells by *N. caninum* tachyzoites was performed with Vero cell confluent monolayers grown in 24-well flat-bottomed tissue-culture plates (Test Plate, TPP) or 8-well (Lab-Tek Chamber Slide, Nunc) for counting, either by real-time PCR or microscopically, respectively. Freshly purified *N. caninum* tachyzoites (3×10^5 for real-time or 2×10^5 for microscopical assay) were resuspended in 100 μ l of RPMI1640 with different sera (all diluted 1/50) and incubated for 1 h at 37 °C/5%CO₂. The following sera were used: pre-immune (obtained before the first immunization with recombinant NcMIC2-like1), against the recombinant NcMIC2-like1 and against tachyzoite protein extract. As controls, rabbit sera against recombinant *Trypanosoma cruzi* alpha dihydroorotate dehydrogenase enzyme expressed in the same vector pET28 (DH α DH) and no sera (RPMI1640 incubation) were used. These mixtures were added to cell monolayers and allowed to invade for 1 h at 37 °C/5%CO₂ and subsequently washed with RPMI1640. For the real-time PCR, samples were transferred to microcentrifugation tubes, and DNA was purified according to manufacturer's instructions (Wizard SV Genomic DNA Purification System, Promega). Quantification of the parasites was determined by real time PCR using the primers Nc 5, previously described (Collantes-Fernandez *et al.* 2002; Naguleswaran *et al.* 2003; Ghalmi *et al.* 2008) but the forward primer was linked to FAM fluorophore Lux (Invitrogen). The experiment was carried out in a Mastercycler ep Realplex (Eppendorf) and the results analysed in the RealPlex (Eppendorf) software. As external standards, DNA corresponding to 1 to 10⁴ parasites was used for the standard curve and the samples were calculated by interpolation from this standard curve. In the microscopical assay, monolayers were fixed with methanol for 12 h and stained with Giemsa for 10 min. For optical microscopy (Leica), 200 cells and tachyzoites detected inside these cells were counted. For both assays the final percentage of invading tachyzoites was calculated as the difference between the average value obtained by the control without serum and the value in each sample divided by the average value obtained by control without serum multiplied by 100. The invasion assays calculated by microscopic counting was performed in duplicate in 3 independent experiments and the invasion measured

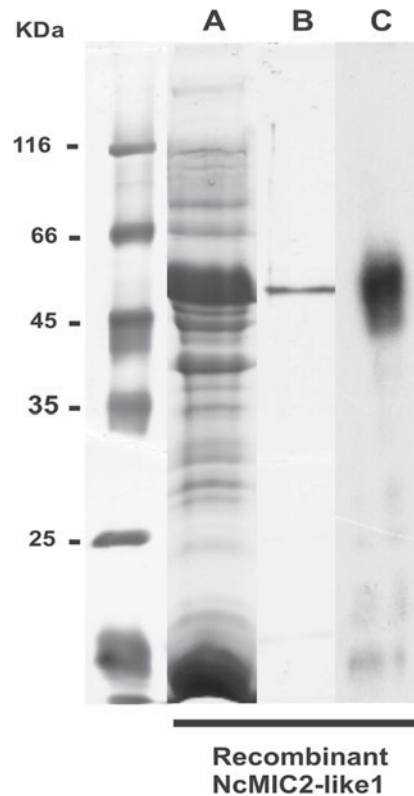


Fig. 2. Recombinant NcMIC2-like1 expression, purification and detection of recombinant. (A) Plasmid pET 28/NcMIC2-like1 was expressed as an insoluble fragment of 52 KDa in *E. coli* BL21. (B) Ni-Sepharose purified recombinant NcMIC2-like1. (C) 1D Western blot, 1 μ g of purified NcMIC2-like1 detected with anti-NcMIC2-like1 serum (1/600 000, conjugate 1/600 000). Molecular weights are indicated on the left.

by real-time PCR was done in triplicate (with duplicates of them for real-time, $n=6$) in 3 independent assays.

Statistical analyses

The results of invasion assays were expressed as mean \pm standard deviation and analysis of variance (ANOVA) was employed for comparison among different experimental groups. Values among the different samples were compared by the test of Bonferroni, considering statistical significance to be less than 5%. The analyses were performed by the statistical program Graph Pad PRISM 4.0 (Graph Pad, USA).

Fig. 1. Alignment of TRAP proteins from Apicomplexan parasites. MIC2-like1 from *Neospora caninum* was aligned with the homologues *N. caninum* MIC2 (AF061273), *Toxoplasma gondii* MIC2 (AAB63303.1), *Eimeria tenella* MIC1 (AAD03350), *Babesia bovis* TRAP (AAS58046), *Theileria parva* TRAP (EAN31658), *Theileria annulata* TRAP (CAJ20069) and *Plasmodium falciparum* TRAP (AAA29777). Brackets indicate the domains, such as I Integrin, 5 Thrombospondins (TSP-s) and the transmembrane region. Arrows indicate the cleavage sites of signal peptide and rhomboids. Grey bars indicate the known regions of each domain related to interaction.

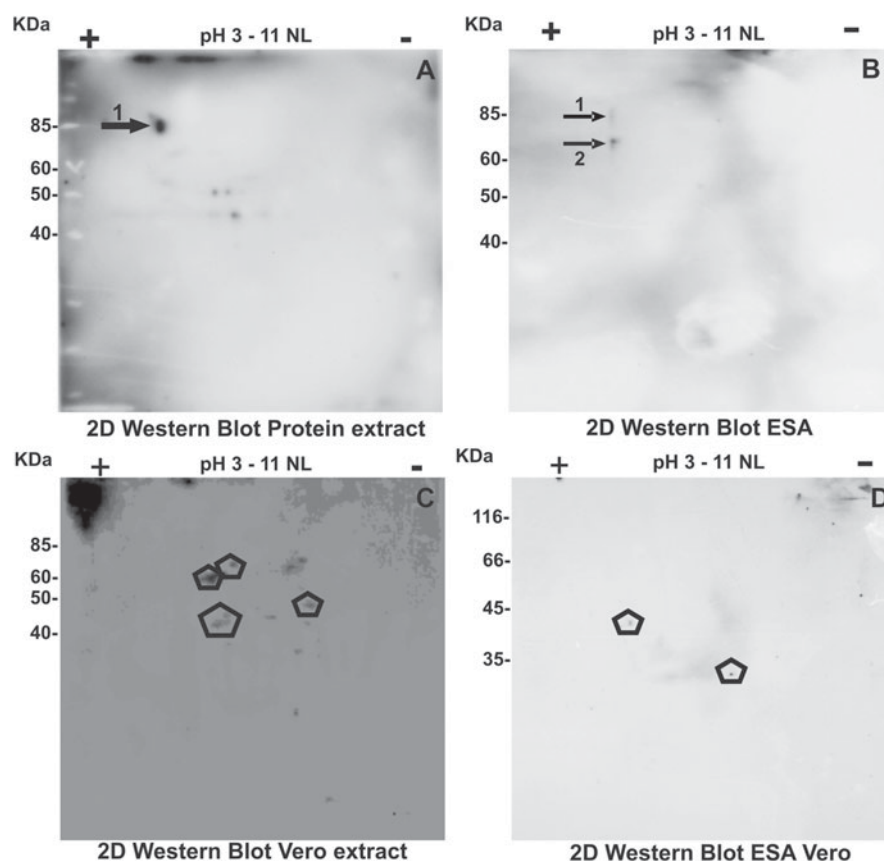


Fig. 3. Detection of native NcMIC2-like1 by 2D Western blot with anti-NcMIC2-like1 serum on *Neospora caninum* (Nc) tachyzoite protein extract and secreted fraction and on respective Vero controls. (A) Anti-NcMIC2-like1 serum on tachyzoite protein extract. (B) Anti-NcMIC2-like1 serum on Nc secreted fraction. (C) Anti-NcMIC2-like1 serum on Vero protein extract. (D) Anti-NcMIC2-like1 serum on Vero secreted fraction. Arrow 1 represents putative complete form of NcMIC2-like1, arrow 2 represents putative processed form of NcMIC2-like1 and trapezoidal forms represent Vero proteins. The 2D Western blot was made on IPG 13 cm strips with pH 3-11NL and 12.5% gel. The dilution of anti-NcMIC2-like1 was 1/8000 and conjugate 1/80000 on tachyzoite protein extract and 1/4000 (antiserum) and 1/40000 (conjugate) on secreted fraction.

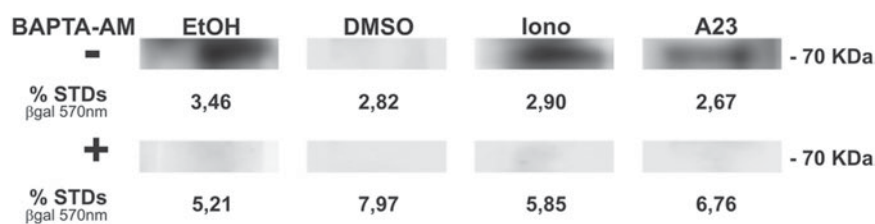


Fig. 4. Calcium-dependent secretion of NcMIC2-like1 secreted from *Neospora caninum* tachyzoites. Parasites were pre-treated with 100 μ M BAPTA-A (+) or not (-), and then incubated with ethanol (EtOH, 1%), DMSO (1%), ionomycin (Iono, 400 nM) or A23187 (A23, 400 nM) and the supernatants were detected by anti-NcMIC2-like1 (1/2000, conjugate 1/20000). Beta-galactosidase activity at 570 nm was used as a control for inadvertent parasite lysis, where serial loadings of tachyzoite whole cell lysates were employed for obtaining a curve of the percentage secretion standards (% STDs). Supernatants of the treatments were compared to the standards and the values are displayed below the Western blot.

RESULTS

Analysis of the NcMIC2-like1 amino acid sequence

Full-length NcMIC2-like1 protein was composed of 765 amino acids and the mature protein (without signal peptide) had a predicted molecular weight of 78.0 kDa and a pI of 4.55. Like its homologue

NcMIC2 and TgMIC2, NcMIC2-like1 was constituted of a signal peptide, an integrin domain (I domain), 5 thrombospondin-1 (TSP-1) domains, a transmembrane region and a cytoplasmic tail. The NcMIC2-like1 deduced amino acid sequence aligned to NcMIC2 and other Apicomplexa TRAP homologues are shown in Fig. 1. NcMIC2-like1 was 39%

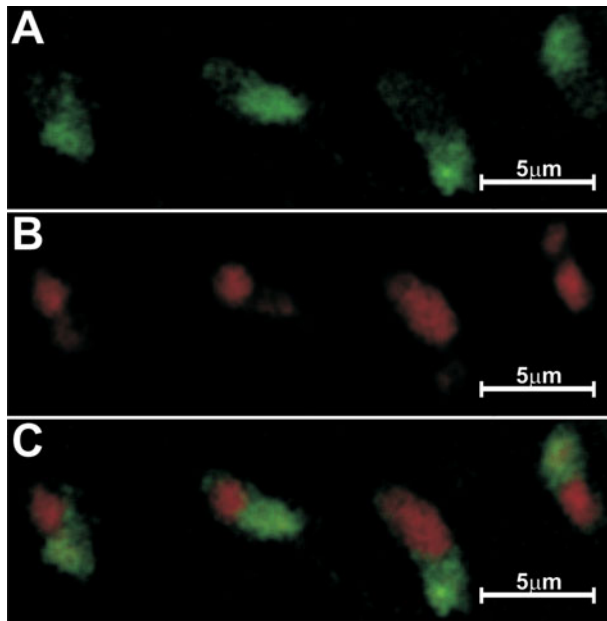


Fig. 5. Immunofluorescent localization of NcMIC2-like1 on *Neospora caninum* tachyzoites. Tachyzoites were labelled with rabbit anti-NcMIC2-like1 and visualized with goat anti-rabbit FITC (A). The nucleus was visualized with propidium iodide (B) and the merged image is indicated in (C). Scale bar = 5 μ m.

identical (53% similar) to NcMIC2 and 38% identical (52% similar) to TgMIC2. The signal peptide had a cleavage site predicted between amino acids 33 and 34, whereas the putative transmembrane spanning helix was found between residues 687 and 756, near the C-terminus of NcMIC2-like1, which was towards residues 757–756. The adhesive domain (residues 68–247) has homology with several mammalian integrin proteins and was similar to the I domains of other Apicomplexan TRAP homologues. Five repeats, similar to the thrombospondin type 1 motif, were distributed along residues 262 up to 627 constituting the second putative adhesive domain.

Expression of the recombinant NcMIC2-like1 protein

The expressed recombinant NcMIC2-like1 protein was auto-induced, and under silver staining, detected as a prominent 52 kDa band (Fig. 2, lane A) which was successfully purified (Fig. 2, lane B).

Detection of native NcMIC2-like1

The 52 kDa recombinant NcMIC2-like1 was recognized by anti-NcMIC2-like1 serum (Fig. 2, lane C) and no reaction was detected on the BL21 protein extract (data not shown). The NcMIC2-like1 protein was predicted to have a molecular weight of 78 kDa and PI of 4.55 whereas the processed form (without transmembrane and cytoplasmic tail) was predicted

to be 72 kDa and PI of 4.7. The native NcMIC2-like1 forms were detected both at *N. caninum* tachyzoite protein extract and at the secreted fraction with 78 kDa (Fig. 3A and B, arrow 1) and at the secreted fraction with 70 kDa (Fig. 3B, arrow 2). In both cases Vero spots were recognized by the antiserum (Fig. 3, C and D), but none of them coincided with the spots mentioned previously.

Calcium-dependent secretion of NcMIC2-like1

Similar to the pattern described for TgMIC2 (Carruthers and Sibley, 1999) and NcMIC2 (Lovett *et al.* 2000), NcMIC2-like1 was secreted (70 kDa) when incubated with ethanol and the calcium ionophores ionomycin and A23183, whilst the process was blocked by treatment with calcium chelator BAPTA-AM (Fig. 4), indicating that secretion of NcMIC2-like1 requires intracellular Ca^{2+} . The β -galactosidase activity (indicative of inadvertent lysis of tachyzoites) of the treated samples remained below 10% in comparison to the control with 100% of tachyzoite lysate.

Localization of NcMIC2-like1 to micronemes

NcMIC2-like1 was apically localized when compared to the nucleus (Fig. 5) and only diffuse fluorescence was detected when the serum was used on Vero cells as a control (data not shown), suggesting specific binding.

In vitro invasion assay

Tachyzoite invasion was inhibited when quantified by microscopical assay and real-time PCR (Fig. 6). Anti-NcMIC2-like1 serum inhibited the invasion process by 55.5% in the microscopical assay, while in real-time PCR inhibition was quantified to be 69.6%. Anti-tachyzoite protein extract inhibited invasion by 75.4% (microscopical assay) and 85.2% (real-time PCR). Pre-immune serum, the serum obtained before the first immunization with recombinant NcMIC2-like1, inhibited 11.5% (microscopical assay) and 20.2% (real-time). The vector control serum inhibited the invasion by 16.35% (microscopical assay) and 25.5% (real-time). All values of inhibition with anti-NcMIC2-like1 and anti-tachyzoite protein extract were significant ($P < 0.05$), in contrast to the vector control serum (anti- α DH α DH, $P > 0.05$). The results represent 3 independent experiments for the invasion assays and the real-time PCR reactions.

DISCUSSION

The fact that TgMIC2 (Wan *et al.* 1997; Barragan *et al.* 2005) and NcMIC2 (Lovett *et al.* 2000)

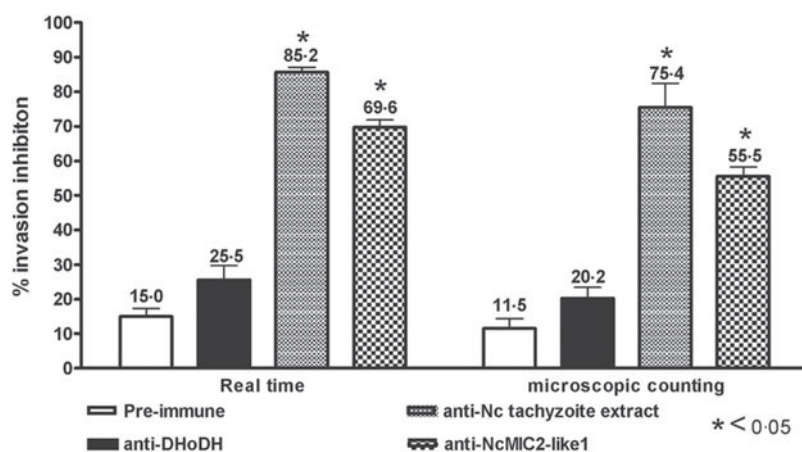


Fig. 6. *In vitro* invasion assay of *Neospora caninum* tachyzoites. Tachyzoites were incubated with different sera (1/50) for 1 h before invading Vero cells. The invading tachyzoites were evaluated by microscopical assay and real time PCR. The white columns represent serum from rabbit before the first immunization procedure (pre-immune), black columns anti-DHoDH (plasmid control), dotted columns anti-NcMIC2-like1 and grid columns represents anti-Nc tachyzoite extract. * $P < 0.05$ in comparison to pre-immune serum.

are closer homologues than NcMIC2 with NcMIC2-like1 led us to designate NcMIC2-like1 as a new thrombospondin anonymous related protein. The integrin region in NcMIC2-like1 is located from amino acids 64 to 208 while in NcMIC2 from 73 to 216 and in TgMIC2 from 72 to 215. This domain is described to act in cell adhesion, migration and in signalling functions (Jonhson *et al.* 2009) and there is a region known as MIDAS, which is a DXSXS sequence conserved in NcMIC2-like1 as well as in TgMIC2 and NcMIC2 (Lovett *et al.* 2000). This domain, first detected in mammals, is responsible for the coordinated binding with Mg^{2+} and Mn^{2+} , mediators of recognition interactions and cell adhesion (Heckmann *et al.* 2009). Mutations in this domain lead to the loss of ability of interaction of integrin of *T. gondii* with the host cell (Brossier and Sibley, 2005).

In NcMIC2-like1 there are 5 thrombospondins, similar to TgMIC2 and NcMIC2, which are responsible for the recognition of sulphate groups in proteoglycans (Soldati *et al.* 2001). The thrombospondin domains have several conserved motifs (WSPCSVTCG, RXR and WSXWS) with similar position among homologues TgMIC2, NcMIC2 and NcMIC2-like1 (Lovett *et al.* 2000).

In the transmembrane region, all homologues have a region of cleavage IAGG, site of rhomboid TgROM 5, essential for parasite release at the end of the invading process (Baum *et al.* 2008). The cytoplasmic tail of NcMIC2-like1 has 32% identity and 42% similarity with NcMIC2 and 27% and 46%, respectively, with TgMIC2. Compared to the cytoplasmic tail of TgMIC2, NcMIC2's one has 81% and 65% (Lovett *et al.* 2000). The cytoplasmic tails of these three proteins have various identical residues, possibly sharing a similar function in the interaction with aldolase, which is the linkage between the

actin/myosin motor system and the micronemal protein (Starnes *et al.* 2006). A conserved tyrosine in TgMIC2 (also present in NcMIC2-like1) is thought to be involved in the interaction with aldolase (Brossier and Sibley, 2005).

The recombinant NcMIC2-like1 was designed based on the integrin and thrombospondin domains, responsible for the attachment and gliding movement of parasite on host cell (Morahan *et al.* 2008). The expressed recombinant protein had a higher molecular weight (52 kDa) than predicted (42 kDa) when subjected to SDS-PAGE, a fact observed for most TRAP proteins, e.g. in *Plasmodium berghei*, *Plasmodium falciparum* (Robson *et al.* 1997), NcMIC2 (Lovett *et al.* 2000) and TgMIC2 (Achbarou *et al.* 1991). This phenomenon is more often observed for proteins with low or high isoelectric points (TRAPs have pI 4–5) and proteins containing a large number of prolines (NcMIC2-like1 have 48) which probably lead the protein to adopt a more extended conformation as observed, for example, in collagen and casein (Robson *et al.* 1997).

The native form of NcMIC2 was described with a molecular weight of 115 kDa and 95 kDa (Lovett *et al.* 2000), much higher than the predicted weight of 78 kDa. In contrast, in our work, the native form of NcMIC2-like1 was detected to be approximately 78 kDa and its secreted form 70 kDa, which represent values close to the predicted molecular weight for the whole molecule without signal peptide (78 kDa) and after cleavage in the transmembrane region (72 kDa). A wide range of molecular weights of NcMIC2 and NcMIC2-like1 could be explained by different assumptions, such as several sites of cleavage and different forms of post-translational modifications. Microneme proteins are found in the apical complex, stored in micronemes, which have an important

function for attachment and initiation of invading processes (Soldati *et al.* 2001; C  r  de *et al.* 2005; Ravindran and Boothroyd, 2008). Here we have demonstrated that NcMIC2-like1 is mainly located in the apical region of the tachyzoite, possibly in the micronemes, such as demonstrated for NcMIC2 (Lovett *et al.* 2000), TgMIC2 (Brossier and Sibley, 2005), TgMIC 3 (El-Hajj *et al.* 2008) and NcAMA 1 (Zhang *et al.* 2006). To corroborate with microneme localization, the secretion of NcMIC2-like1 was dependent on intracellular Ca²⁺. The discharge of microneme proteins after treatment with calcium ionophores and the blocking with calcium chelator BAPTA-AM has been described for *T. gondii* (Carruthers and Sibley, 1999; Kawase *et al.* 2010) and *N. caninum* (Naguleswaran *et al.* 2001; Lovett *et al.* 2000) and was related to regulation of the gliding movement of the parasite (Wetzel *et al.* 2004).

The methods of measurement of invasion assays in this work consisted of microscopical assay and real-time PCR. As described by Naguleswaran *et al.* (2003); these different assays showed similar outcomes. In this work, both methods provided very similar results for inhibition of cell invasion; however, real-time PCR had a lower variation and percentages of inhibition were slightly, but significantly, higher than the microscopical assay method. Due to its sensitivity and specificity, the method of real-time PCR has been used for quantification of samples from *Toxoplasma*, *Neospora* and *Plasmodium* (Bell and Ranford-Cartwright, 2002).

The fact that antiserum against NcMIC2-like1, as a single target, inhibited invasion at high levels (up to 69%), makes this molecule a potential candidate for inclusion in a multi-component vaccine. In *T. gondii*, selected motifs from crucial proteins for adherence/invasion of TgMIC2, MIC3 and SAG1 have been tested as a chimera in vaccinations (Jongert *et al.* 2008). The molecular characterization initiated here on NcMIC2-like1 opens the way for more detailed studies on major essential motifs related to cell invasion and on the effectiveness of this protein under *in vivo* immunization assays.

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