Identification and partial characterization of a 36 kDa surface protein on *Neospora caninum* tachyzoites

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SUMMARY

Neospora caninum, the causative agent of neosporosis, is a recently identified apicomplexan parasite which is structurally and biologically closely related to, but antigenically distinct from, *Toxoplasma gondii*. Molecules associated with the surfaces of *N. caninum* tachyzoites are likely to participate in the host cell entry process, could be involved in the interaction of the parasite with the immune system, and they could influence the pathogenesis of neosporosis. Isolated *N. caninum* tachyzoites were extracted with the non-ionic detergent Triton X–114 and were further analysed using a polyclonal anti-*N. caninum* antiserum. Immunoblots revealed several reactive bands, 1 of which represented a glycoprotein of approximately 36 kDa (Nc-p36). This molecule was present in 2 isolates of *Neospora* (NC-1 and Liverpool), but was absent in *Toxoplasma* (RH-strain) tachyzoites. Immunofluorescence and pre-embedding immunogold transmission electron microscopy employing affinity-purified anti-Nc-p36 antibodies showed that the Nc-p36 is a cell surface-associated protein. Immunogold on-section labelling of LR-White-embedded parasites, fixed prior and at defined time-points after host cell entry, demonstrated the presence of this molecule on the surface as well as within the dense granules of *N. caninum* tachyzoites.

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

INTRODUCTION

Neospora caninum is an obligatory intracellular parasite which was originally identified in tissues of paralysed dogs (Dubey *et al*. 1988; Bjerkas & Presthus, 1988). Prior to 1988, *N. caninum* was, due to its close structural similarity, misdiagnosed as *Toxoplasma gondii* (Dubey & Lindsay, 1993). A distinction between these two parasites is sometimes possible on the level of electron microscopy because of slight ultrastructural differences such as the thickness of the cyst wall and the number of rhoptries (Lindsay *et al*. 1993). The first and most useful assay to distinguish *Neospora* from *Toxoplasma* infections is the immunofluorescence antibody-binding test first described by Dubey *et al*. (1988). Other serological assays were developed which allowed discrimination between respective infections (Björkman *et al*. 1994; Baszler *et al*. 1996). Phylogenetical differences between *Neospora* and *Toxoplasma* were demonstrated by sequence analysis of rRNA genes, 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). In cattle, *N. caninum* is now regarded as a major cause of neonatal mortality and abortion in certain geographical areas (Barr *et al*. 1994). Other structurally and antigenically similar parasites have been reported from sheep, goats and horses (Dubey & Lindsay, 1993). The only 2 stages of the (largely unknown) life-cycle of *Neospora* discovered to date are dividing tachyzoites and tissue cysts. The former can be cultivated *in vitro* using methods previously established for the cultivation of *T. gondii* tachyzoites (Lindsay *et al*. 1993).

A common feature of all parasites of the genus *Apicomplexa* is the apical complex, a structure at the posterior end of the cells which defines the polarity of the zoites. The apical complex contains a set of secretory organelles named micronemes, rhoptries and dense granules. Molecules released from these organelles are likely to participate in host cell adhesion and invasion by directly associating with the host- or parasite-cell surface membranes. In addition they are involved in the generation of a parasitophorous vacuole (PV), and subsequent modification of the parasitophorous vacuole membrane (PVM; Joiner & Dubremetz, 1993; Cesbron-Delauw, 1994; Dubremetz & McKerrow, 1995; Smith, 1995; Galinski & Barnwell, 1996; Sam-Yellowe, 1996). Detailed studies on the various aspects of host–parasite interactions during infection with apicomplexan parasites have been carried out on *T. gondii* (reviewed by Werk, 1985; Joiner & Dubremetz, 1993; Kasper & Mineo, 1994). The close relationship between *T. gondii* and *N. caninum* suggested that they would employ similar mechanisms, and would use similar surface

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molecules, in order to encounter their host cells. *In vitro* cultivation of *N. caninum* tachyzoites in bovine endothelial cells showed that the initial contact between parasites and host cell monolayers is a receptor-mediated event, based rather on protein–protein than on protein–carbohydrate interactions (Hemphill, Gottstein & Kaufmann, 1996). A major *N. caninum* tachyzoite surface protein, Nc-p43, was identified (Hemphill & Gottstein, 1996) which was subsequently shown to be most likely functionally involved in the adhesion and invasion process (Hemphill, 1996). However, antibodies directed against Nc-p43 failed to detect antigenically related proteins in *T. gondii* tachyzoites (Hemphill & Gottstein, 1996). Indeed, distinct antigenic properties of these 2 species had already earlier indicated a different surface molecule composition (Dubey & Lindsay, 1993). This is confirmed by the present study which reports on the identification of a surface protein in *N. caninum* tachyzoites with a molecular weight of 36 kDa (named Nc-p36). In this paper it is shown that Nc-p36 is associated with the cell surface and with the dense granules of *N. caninum* tachyzoites. In order to further define the functional significance of this protein, the distribution of Nc-p36 at different time-points during and after host cell entry was investigated.

MATERIALS AND METHODS

If not 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, Zürich, Switzerland) supplemented with 7% foetal calf serum (FCS), 2 mM glutamine, 50 U/ml penicillin and 50 μg/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), the Liverpool isolate (Barber, Trees & Owen, 1993; Barber *et al*. 1996), 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 μg/ml streptomycin, and 2–10% Ig-free horse serum (HS). Parasites were harvested from their feeder cell cultures as previously described (Hemphill *et al*. 1996). The preparation (containing tachyzoites and host cell debris) was washed twice in cold PBS. The final pellet was resuspended in 2 ml of cold PBS and passed through a PD-10™ column filled with Sephadex G25M (Pharmacia), previously equilibrated with PBS. The eluted, purified, parasites were centrifuged at 4 °C and were resuspended in cold PBS. The viability of parasites was monitored by exclusion of Trypan blue staining (0·25% in PBS).

Subcellular fractionation of N. caninum *tachyzoites employing Triton X–114*

For the subcellular fractionation of tachyzoites the procedure described by Hemphill & Gottstein (1996) was followed, employing the non-ionic detergent Triton X–114 for the biochemical separation of membrane proteins. Briefly, 5×10^7–10^8 *N. caninum* tachyzoites/ml were resuspended in 3 ml of PBS containing 0·2 mM phenylmethylsulfonyl fluoride (PMSF). After a 5 min incubation on ice, the cell suspension was adjusted to 0·75% Triton X–114, and, after gentle vortexing, the parasites were extracted for 10 min on ice. They were centrifuged (30 min, 10000 ***g***, 4 °C), the supernatant fraction was collected, and the pellet was discarded. 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 fraction was collected again and 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 water-soluble supernatant was removed, and the detergent phase, potentially containing hydrophobic membrane-associated proteins, was then processed for SDS–PAGE by methanol/chloroform extraction (Wessel & Fluegge, 1984).

Preparation and affinity-purification of antibodies

A polyclonal rabbit anti-*N. caninum* (NC-1-isolate) antiserum (Hemphill, 1996) and affinity-purified anti-Nc-p36 antibodies were used. Affinity purification of antibodies was performed as previously described (Hemphill & Gottstein, 1996). Briefly, the band corresponding to Nc-p36 was cut out from nitrocellulose filters following SDS–PAGE and Western blotting of Triton X–114 extracts. After blocking of unspecific binding sites in PBS/3% BSA (bovine serum albumin)/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 μl of 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 μl of 1 M Tris base was immediately added. The eluted antibody fraction was centrifuged

(10000 ***g***, 20 min), in order to remove small nitro cellulose particles with bound antigen, and BSA was added to the supernatant fraction to a final concentration of 0·1 %. Affinity-purified antibodies were aliquoted and stored at −20 °C. They were used for immunoblotting at a dilution of 1:50.

SDS–PAGE, immunoblotting and carbohydrate detection of nitrocellulose-bound proteins

Protein extracts of the various subcellular fractions (cell lysates of *N. caninum* Nc-1 and Liverpool isolates, of *T. gondii* total cell extracts, and hydrophobic phases of Triton X–114 extracts) were processed for SDS–PAGE as previously described (Hemphill & Gottstein, 1996). Samples corresponding to the same number of parasites were separated by SDS–PAGE and transferred to nitrocellulose filters (Towbin, Staehelin & Gordon, 1979). In some experiments proteins were chemically deglycosylated by treating the filters with 40 mM $NaIO_4$ in 50 mM sodium acetate buffer, pH 4·5, for 30 min at room temperature in the dark (McDonald, McCrossan & Petry, 1995). Blocking of unspecific binding sites was carried out for 4 h at 24 °C in PBS containing 3 % BSA 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. Affinity-purified anti-Nc-p36 antibodies were diluted 1:50. The filters were then washed 3 times in PBS/0·3 % Tween, and the bound antibodies were visualized using peroxidase-conjugated anti-rabbit immunoglobulin antibodies (Promega, Zürich, Switzerland) according to the instructions provided by the manufacturers.

In order to identify carbohydrate moieties on nitrocellulose-bound proteins, the GlycoTrack™ Carbohydrate Detection Kit (Oxford GlycoSystems, Oxford, England) was employed. Briefly, Triton X–114 extracts were separated by SDS–PAGE and transferred to nitrocellulose as described above. Glycoproteins were oxidized with sodium periodate, and subsequently reacted with biotin-hydrazide. The biotinylated glycoproteins were then detected by incubation with a streptavidin–alkaline phosphatase conjugate followed by a colour reaction.

Immunofluorescence

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, cover-slips were rinsed 3 times in PBS, and were placed into fixation buffer (PBS/3 % paraformaldehyde/0·05 % glutaraldehyde, pH 7·2) for 10 min. Subsequently, the parasites were permeabilized by immersion into 100 % methanol at −20 °C for 10 min. The preparations were then rinsed in PBS and were subsequently incubated in blocking solution (PBS/1 % BSA/50 mM glycine) for 30 min. The affinity-purified anti-Nc-p36 antibodies were applied at a 1:1 dilution in blocking solution for 30 min, followed by 3 buffer rinses. The bound primary antibodies were then detected by a goat anti-rabbit antibody conjugated to fluorescein isothiocyanate (FITC) (The Binding Site). Specimens were then rinsed in PBS (5 × 5 min), briefly rinsed in distilled water, and were embedded in a mixture of glycerol/gelvatol containing, 1,4-diazobicyclo(2.2.2)octan (Merck, Germany) as an anti-fading reagent (Hemphill, Affolter & Seebeck, 1992). The preparations were inspected on a Leitz Laborlux S fluorescence microscope.

Immunogold labelling and transmission electron microscopy (EM)

Pre-embedding labelling of N. caninum *tachyzoites.* Purified *N. caninum* and *T. gondii* tachyzoites (5×10^7/ml) were fixed in suspension using the same fixation buffer as for immunofluorescence (see above). After blocking unspecific binding sites and 3 additional washes in blocking buffer, the first antibody layer (diluted as above) was applied for 60 min. Parasites were then washed in PBS (3 × 5 min), and the second layer (goat anti-rabbit conjugated to 10 nm gold particles, Amersham Zürich) was applied at a dilution of 1:5 in blocking buffer for 45 min. Gold conjugates were centrifuged at 4800 ***g*** for 10 min to remove possible aggregates (Hemphill & Croft, 1997). After 3 washes in PBS, the labelled parasites were fixed in 2 % glutaraldehyde diluted in 100 mM sodium phosphate buffer, pH 7·2, for 4 h at 4 °C. The parasites were then washed in phosphate buffer, post-fixed in 2 % OsO_4, dehydrated in a graded series of ethanol and embedded in Epon 812 (Fluka Chemicals, Switzerland) as described (Hemphill *et al.* 1996). The preparations were finally polymerized at 65 °C for 24–48 h. Thin sections were cut with a diamond knife using a Reichert and Jung ultramicrotome, and grids were stained with lead citrate and uranyl acetate (Hemphill & Croft, 1997).

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). After different time-points (0, 30, 60 min, 4, 8, 24, 48 and 72 h) 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 × 5 min in PBS, and were removed from the surface of the tissue culture flask using a

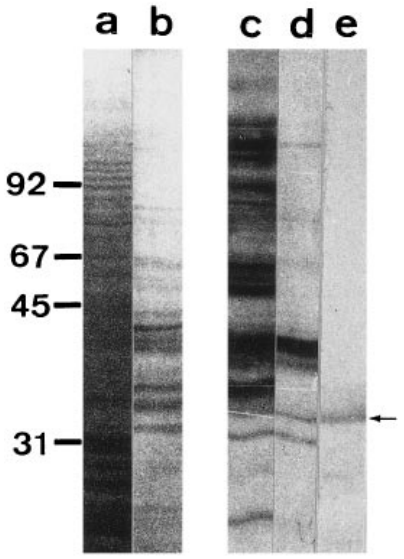

Fig. 1. SDS–PAGE and Western blots of *Neospora caninum* tachyzoites. Lanes a and b show a silver-stained 8–15% SDS–PAGE of a whole *N. caninum* tachyzoite extract (a) and the detergent-phase of a Triton X–114 extract (b). Lanes c and d are the corresponding immunoblots stained with the polyclonal anti-*N. caninum* antiserum. Lane e shows an immunoblot of a *N. caninum* tachyzoite extract labelled with the affinity-purified anti-Nc-p36 antibody. The position of Nc-p36 is indicated by the arrow.

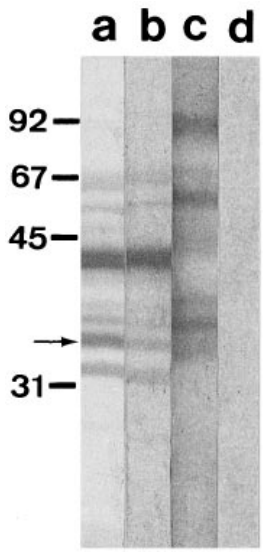

Fig. 2. Glycoprotein detection in *Neospora caninum* Triton X–114 extracts. The reactivity of the anti-*N. caninum* antiserum is demonstrated prior (lane a) and after chemical deglycosylation (lane b) of nitrocellulose-bound proteins using sodium periodate oxidation following 10% SDS–PAGE and Western blotting. Lane c demonstrates the presence of carbohydrate residues on Triton X–114 detergent phase-proteins using sodium periodate oxidation and subsequent incubation with biotin hydrazide followed by streptavidin–alkaline phosphatase. Lane d is a control incubated without sodium periodate treatment. The position of Nc-p36 is indicated by the arrow.

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 and Jung ultramicrotome, and were picked up onto 200 mesh formvar-carbon-coated nickel grids (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 in affinity-purified anti-Nc-p36 antibodies diluted 1:1 in EM blocking buffer for 1 h. After washing in 5 changes of PBS for 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

Characterization of antibodies and epitopes

The reactivity of a polyclonal rabbit antiserum directed against whole *N. caninum* tachyzoites is shown in Fig. 1. As visualized on immunoblots of parasites separated by SDS–PAGE, whole tachyzoite extract immunolabelling yielded a complex immune-reactive banding pattern (Fig. 1, lane c). By extraction of tachyzoites with the non-ionic detergent Triton X–114 and subsequent phase separation, resulting in a fraction potentially enriched for membrane proteins (Fig. 1, lane d), a banding pattern was obtained where the individual reactive bands could be more easily separated. The most immunodominant protein in this detergent fraction migrated at a relative molecular weight of 43 kDa. However, 2 additional, albeit less intensely stained bands of 33 and 36 kDa could also be seen (Fig. 1, lane d). These 2 polypeptides were also clearly visible on nitrocellulose filters which were stained with Ponceau-red after transfer of SDS–PAGE-separated proteins, and this allowed the reactive protein band of 36 kDa to be cut out. This nitrocellulose strip was then used for the affinity purification of the respective immunoglobulins out of the complex anti-*N. caninum*

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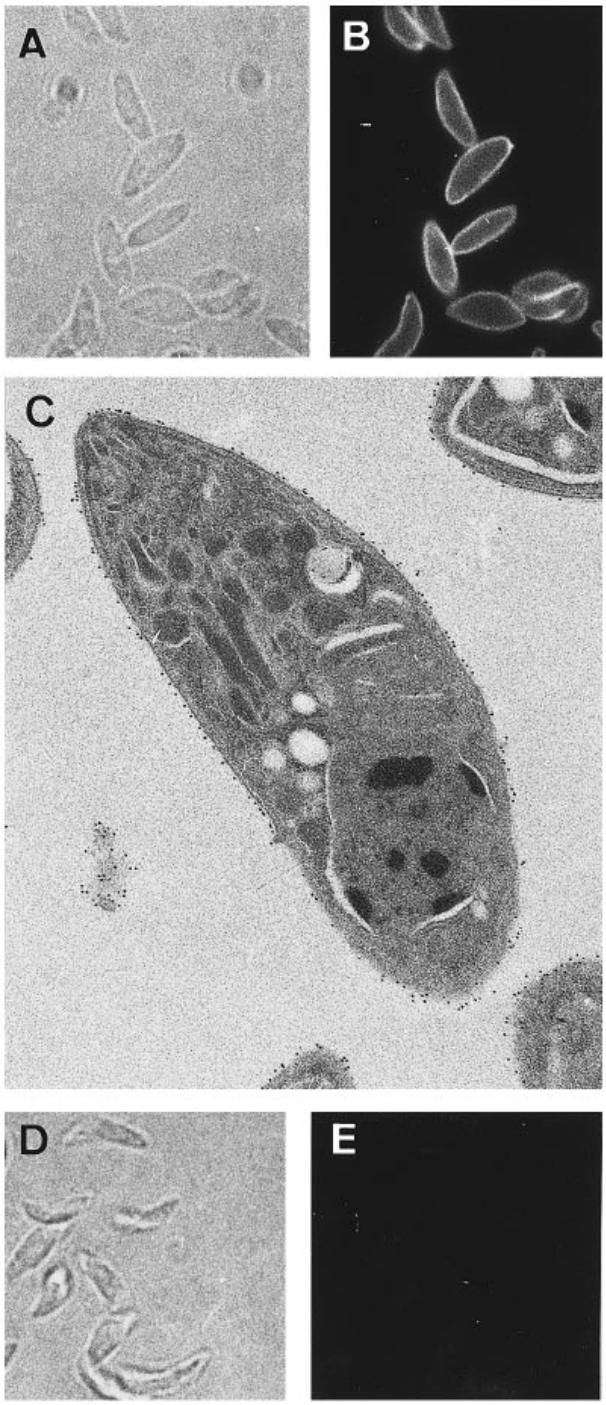

Fig. 3. Immunolocalization of Nc-p36 on isolated *Neospora caninum* and *Toxoplasma gondii* tachyzoites. *N. caninum* tachyzoites were labelled with the affinity-purified anti-Nc-p36 antibodies (A and B). Pre-embedding immunogold labelling confirms the presence of Nc-p36 on the tachyzoite cell surface (C). The absence of cross-reactive epitopes in *T. gondii* tachyzoites is demonstrated in D and E.

antiserum. On immunoblots of tachyzoite cell extracts these affinity-purified antibodies proved to be uniquely directed against their respective antigen of 36 kDa (Fig. 1, lane e). Based on its apparent molecular weight as determined by SDS–PAGE, this protein was named Nc-p36.

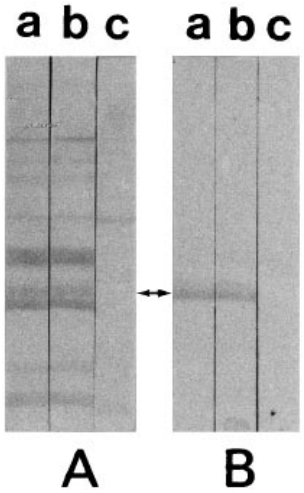

Fig. 4. *Neospora caninum* tachyzoites of the Nc-1 isolate (lane a) and Liverpool isolate (lane b) as well as *Toxoplasma gondii* tachyzoites (lane c) were separated by 10% SDS–PAGE, blotted onto nitrocellulose, and stained with the polyclonal anti-*N. caninum* antiserum (A) and affinity-purified anti-Nc-p36 antibodies (B).

Chemical deglycosylation of nitrocellulose-bound proteins using sodium periodate was performed. Most bands remained unaffected by this treatment, including the 43 kDa and the 33 kDa bands (Fig. 2, lanes a, b). However, periodate oxidation resulted in a clear decrease in the binding activity of antibodies directed against Nc-p36. In order to directly visualize the presence of glycoproteins in the Triton X–114 detergent-phase fraction, extracts were separated by SDS–PAGE and transferred to nitrocellulose, and the separated glycoproteins were detected using a specific carbohydrate detection kit. Several of the proteins in this fraction were indeed glycosylated, including Nc-p36, while no carbohydrate staining could be found neither on the 43 kDa nor the 33 kDa polypeptide bands (Fig. 2, lane c).

Immunolocalization of Nc-p36

As isolated *N. caninum* tachyzoites were processed for immunofluorescence staining using affinity-purified anti-Nc-p36 antibodies, in all tachyzoites, a bright, evenly distributed fluorescent labelling all over the cells could be observed (Fig. 3A–B). The presence of Nc-p36 on the surface of *N. caninum* tachyzoites was then demonstrated at the electron microscopical level by employing pre-embedding labelling: *N. caninum* tachyzoites were incubated with affinity-purified anti-Nc-p36 antibodies prior to fixation, dehydration and embedding in Epon 812 resin (Fig. 3C). On-section labelling of LR-White-embedded tachyzoites confirmed that Nc-p36 was indeed a surface protein. However, affinity-purified anti-Nc-p36 antibodies also labelled epitopes localized within the parasite dense granules (Fig. 5A–D). Identical results were obtained when tachyzoites of

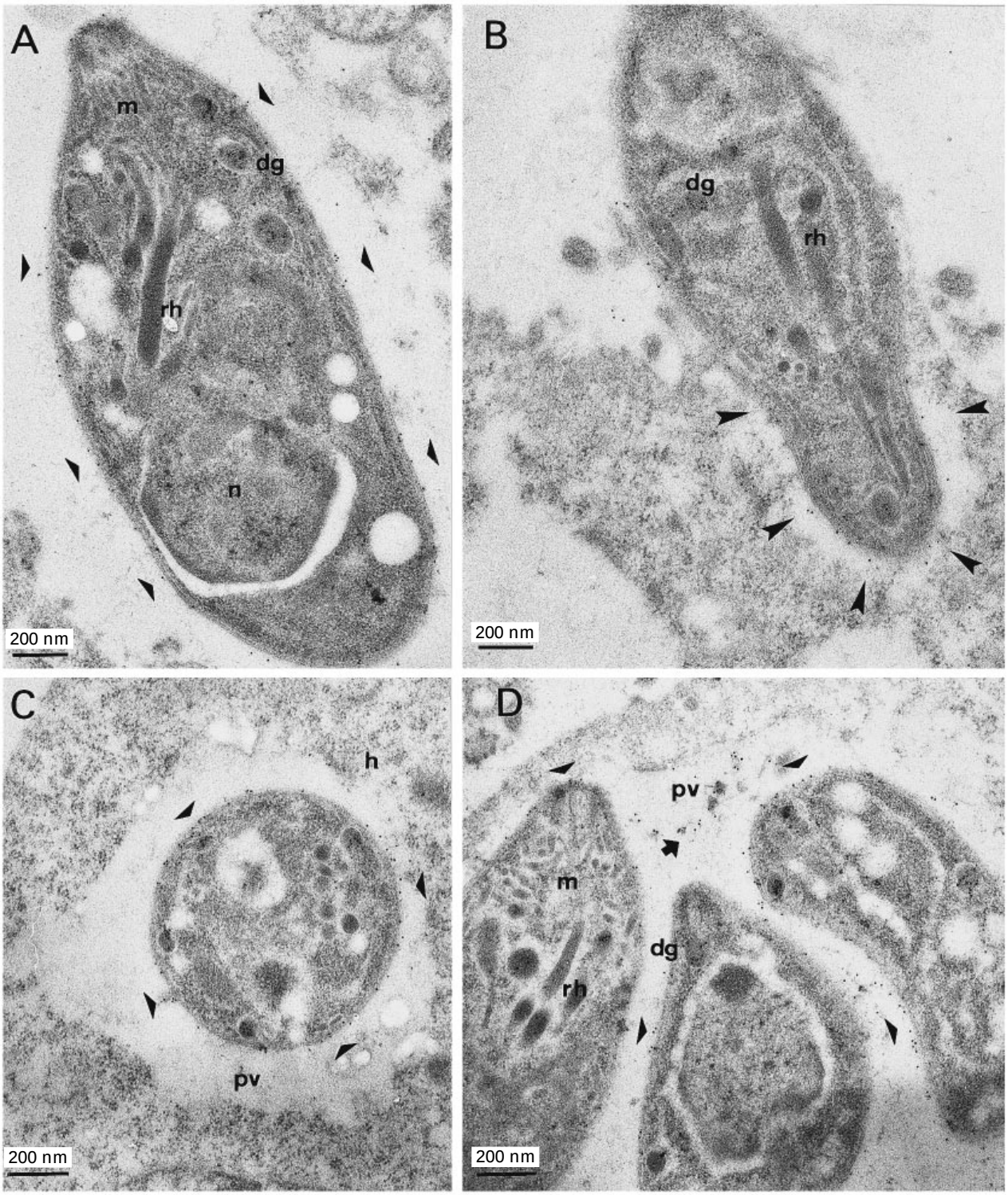

Fig. 5. Immunogold localization of Nc-p36 in LR-White-embedded *Neospora caninum* tachyzoites fixed prior to, and at defined time-points after, infection of Vero cells. (A) Extracellular tachyzoite. Note the labelling on the tachyzoite surface (flattened arrows) and the gold particles within the dense granules (dg). Labelling is absent from rhoptries (rh) and micronemes (m). n = nucleus. (B) *N. caninum* tachyzoite fixed at the time-point of host cell invasion (20 min after the addition of tachyzoites to Vero cells). Note the presence of gold particles at the immediate site of host- and parasite cell surface interaction during the invasive process (arrowheads). (C) Intracellular tachyzoite at 4 h after invasion. Note the abundance of Nc-p36 on the parasite cell surface. Gold particles are virtually absent from the lumen of the parasitophorous vacuole (pv). h = host cell cytoplasm. (D) Larger pseudocyst fixed 48 h post-invasion. Nc-p36 is still present within dense granules (dg), and on the tachyzoite cell surface (flattened arrowheads). Note the additional labelling of the intravacuolar network (bold arrows) within the lumen of the parasitophorous vacuole (pv). Rhoptries (rh) and micronemes (m) remain unlabelled.

the *N. caninum* Liverpool isolate (Barber *et al*. 1996) were immunolabelled with these antibodies (data not shown). In addition, *T. gondii* RH-strain tachyzoites were fixed and permeabilized, and were identically processed for immunofluorescence staining (Fig. 3 D–E). No labelling was detected on these closely related parasites. The presence of Nc-p36 in cell lysates of *N. caninum* (Nc-1 and Liverpool isolates), and the absence of an immunologically related protein in *T. gondii* (RH-strain) tachyzoites were further confirmed by immunoblotting (Fig. 4).

The distribution of Nc-p36 prior, during and after host cell entry

The distribution of Nc-p36 was studied by performing immunogold labelling on sections of LR-White-embedded specimens which had been fixed at defined time-points after infection of Vero cell cultures with *N. caninum* tachyzoites. During host cell entry, Nc-p36 was found to be localized at the immediate site of physical interaction of the parasite- and host-cell surface membranes (Fig. 5 B). During subsequent development of the PV and proliferation of tachyzoites Nc-p36 always remained associated with the surface and the dense granules, but was always absent from any other part of the parasites. No labelling could be found either within the lumen of the PV or on the PVM. No other part of the host cell was labelled. However, at later time-points, meaning 48–72 h post-invasion, gold particles were also found associated with the intravacuolar tubular network, but remained largely absent from the PVM at all times (Fig. 5 C–D).

DISCUSSION

This paper describes the identification, subcellular localization and chemical nature of a cell surface-associated protein of *N. caninum* tachyzoites. Antibodies specifically directed against this 36 kDa protein were obtained by affinity purification of immunoglobulins out of a complex anti-*N. caninum* antiserum following SDS–PAGE and Western blotting onto nitrocellulose filters. This approach had previously proven to be suitable for many applications where specific antibodies were needed (reviewed by Mueller & Felleisen, 1995), and has also recently led to the identification and characterization of Nc-p43, a major 43 kDa *N. caninum* tachyzoite surface protein (Hemphill & Gottstein, 1996; Hemphill, 1996). In immunoblots, the affinity-purified anti-Nc-p36 antibodies were solely reacting with their respective 36 kDa polypeptide. Thus, based on its apparent molecular weight, the protein was named Nc-p36.

Both the previously identified Nc-p43 as well as Nc-p36 reacted strongly with a polyclonal rabbit antiserum directed against whole *N. caninum* tachyzoites. In order to get further information on the nature of Nc-p36-associated epitopes which are recognized by the anti-*N. caninum* antiserum, SDS–PAGE-separated and nitrocellulose-bound Triton X–114-extracted proteins were chemically deglycosylated by incubation in sodium periodate at low pH. For antibodies directed against Nc-p36 this treatment resulted in a clearly visible decrease in antibody reactivity, indicating that most likely a large portion of the immunoglobulins which bound to Nc-p36 were reacting with epitopes of carbohydrate nature. In contrast, antibodies directed against Nc-p43 and those directed against the 33 kDa polypeptide were mostly directed against protein epitopes. Specific carbohydrate staining of Triton X–114-extracts following SDS–PAGE and transfer to nitrocellulose revealed that Nc-p36 was indeed a glycoprotein (see Fig. 2, lane c). In contrast, no carbohydrate staining could be detected on Nc-p43 and the 33 kDa polypeptide, confirming the absence, or at least a very low content, of sugar residues on these two proteins.

Monospecific, but polyclonal, affinity-purified antibodies, like those used in this study, are ideal tools for the localization of their respective epitopes. Initial immunofluorescence experiments carried out on isolated, non-permeabilized *N. caninum* tachyzoites revealed that Nc-p36 was most likely expressed on the tachyzoite cell surface (Fig. 3 A and B). Cell surface staining was also achieved by immunogold TEM using a pre-embedding approach, where unfixed tachyzoites were incubated with anti-Nc-p36 antibodies prior to processing for electron microscopy. Immuno-electron microscopical analysis employing on-section labelling of LR-White-embedded parasites confirmed that Nc-p36 is indeed a tachyzoite surface protein. In parasites which were fixed during the host cell entry process, Nc-p36 was demonstrated to be localized directly at the site of physical contact of the parasite and host cell surface membranes. However, Nc-p36 was also found within electron-dense, vesicular organelles known as dense granules (Lindsay *et al*. 1993). The fact that a surface protein occurs, also within dense granules is not surprising. Similar immunocytochemical studies performed in our laboratory using an antibody directed against a bacterially expressed recombinant protein derived from a cDNA clone corresponding to the *N. caninum* tachyzoite surface protein Nc-p43, also showed that Nc-p43 occurs on the surface as well as within dense granules of *N. caninum* tachyzoites (Hemphill, 1997).

Dense granules are found at both the anterior and the posterior end of *N. caninum* tachyzoites. The same accounts for *T. gondii* dense granules. 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 other 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 could account for Nc-p36. Our results indicated that Nc-p36 would be accumulated and stored within the dense granules, and would be subsequently released onto the cell surface, in a similar way as it had been described for the 21 and 28·5 kDa antigens secreted from *T. gondii* tachyzoites (Charif *et al.* 1990). However, at later time-points (48–72 h post-invasion), gold particles could also be demonstrated on the vacuolar tubular network. Whether, at these later time-points, Nc-p36 is actually a part of a, *per definition*, secreted fraction of *N. caninum* tachyzoites, or whether this molecule is released from the tachyzoite cell surface through some shedding mechanism, remains to be investigated.

Since most surface-associated molecules of eukaryotic cells are largely constituted of carbohydrate residues (Oebrink, 1993), it is not surprising that the same would account for Nc-p36. However, it is not yet clear whether carbohydrate residues are important for the actual adhesion and invasion process of *N. caninum* tachyzoites. Previous investigations (Hemphill *et al.* 1996) have studied the role of carbohydrate residues on the *N. caninum* cell surface in relation to adhesion and invasion of host cells. Mild sodium periodate treatment of tachyzoites which would remove potential carbohydrate residues from the parasite cell surface had no effect on the capacity of these parasites to adhere to, and invade, bovine endothelial monolayers. In addition, pre-incubation of parasites with tunicamycin, and treatment of parasites with several lectins, were ineffective in achieving any inhibition of the adhesion or invasion process (Hemphill *et al.* 1996). In the closely related species *T. gondii*, the role of carbohydrates and/or sugar binding proteins is also still unresolved: BSA–glucosamide, albumin-*N*-acetyl-D-glucosamine and albumin–galactose bound to extracellular *T. gondii* tachyzoites (Robert *et al.* 1991). EM studies with gold-labelled lectins and glycoproteins localized sugar residues on parasite rhoptries, but not on the surface and not within dense granules (DeCarvalho, Souto-Padron & DeSouza, 1991). Thus it was proposed that sugar-containing macromolecules would be released upon contact with the host cell membrane (Kasper & Mineo, 1994). However, further studies are required to determine the amount and type, and the functional significance of Nc-p36-glycosylation.

The affinity-purified anti-Nc-p36 antibodies used in this study did not exhibit any binding to *T. gondii* tachyzoites, suggesting that this protein, or at least the epitopes reacting with the corresponding antibodies, were not abundant in this closely related species. This was subsequently confirmed by immunoblotting of total tachyzoite extracts of 2 isolates of *N. caninum* (Nc-1 and Liverpool) and the *T. gondii* RH-strain. The absence of an immunologically related counterpart in *T. gondii* tachyzoites, was also previously reported for Nc-p43 (Hemphill & Gottstein, 1996). Those observations, and the findings presented in this paper, confirm the distinct differences between *T. gondii* and *N. caninum*. The use of Nc-p36 and Nc-p43 for potential differential immunodiagnosis of neosporosis and toxoplasmosis is currently being investigated.

Other authors (Bjerkas, Jenkins & Dubey, 1994; Barber *et al.* 1996) have previously described a 37 kDa protein in *N. caninum* tachyzoites as revealed by immunoblotting on non-reduced gels. Moreover it was shown that this protein was not recognized by anti-*Toxoplasma* antiserum, was immunodominant in convalescent bovine serum, and was present on the surface of the parasites. The fact that this 37 kDa protein exhibited increased solubility in detergent compared to aqueous buffer suggested that it was a membrane protein (Bjerkas *et al.* 1994). It is currently not known whether Nc-p36 and this 37 kDa protein are one and the same molecule.

In conclusion, this study reports on the identification and partial characterization of a cell surface-associated protein in the apicomplexan parasite *N. caninum*. Nc-p36 is a glycoprotein which is also abundant in the dense granules, suggesting that it could be integrated into the cell surface membrane via this type of secretory organelle. Upon host cell entry, Nc-p36 remains largely confined to the parasite, but is found on the intravacuolar tubular network at later time-points post-invasion. Thus, a functional role for this protein during host cell entry rather than during subsequent PVM maturation or parasite proliferation is proposed. Nc-p36 could very likely be one of these proteins which participate in the adhesion and/or invasion process of *N. caninum* tachyzoites. However, this question needs further investigation employing specific antibodies directed against Nc-p36 with respect to their capacity to block the entry of the parasite into the intracellular compartment of its host. Further studies at the molecular and biochemical levels will provide more insights on the structure and function of this protein, and its relationship to functionally related molecules found in other apicomplexan parasites.

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