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Identification of a major surface protein on *Neospora caninum* tachyzoites

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Abstract *Neospora caninum* is a recently identified coccidian parasite that is closely related to *Toxoplasma gondii*. Molecules associated with the surface of *N. caninum* tachyzoites are likely to be involved in the process of adhesion and invasion of host cells. They probably also participate in the interaction of the parasite with the immune system, and they could play an important role in the pathogenesis of the parasite. To identify such surface molecules, we performed subcellular fractionation studies of isolated *N. caninum* tachyzoites. Employing the nonionic detergent Triton-X-114, we prepared a membrane fraction. Immunoblot analysis of this fraction using polyclonal antisera directed against tachyzoites of *N. caninum* and *T. gondii* resulted in the identification of a protein of approximately 43 kDa (Nc-p43). This molecule was present in two isolates of *Neospora* (Nc-1 and Liverpool) but was absent in *Toxoplasma* (RH-strain) tachyzoites. Further immunofluorescence and immunogold transmission electron microscopy (TEM) studies using affinity-purified anti-Nc-p43 antibodies demonstrated the presence of this molecule on the surface of *N. caninum* tachyzoites.

Introduction

Neospora caninum is an obligatory intracellular parasite that was originally identified in tissues of paralyzed dogs (Bjerkås and Presthus 1988; Dubey et al. 1988). 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 and Lindsay 1993). The only two stages of the (largely unknown) life cycle of *Neospora* discovered to date are dividing tachyzoites and tissue cysts.

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Prior to 1988, *N. caninum* was misdiagnosed as *Toxoplasma gondii* due to its close structural similarity (Dubey 1992). However, due to distinct ultrastructural differences such as the thickness of the cyst wall and the number of rhoptries it had been possible to distinguish these two species from each other (Lindsay et al. 1993). Other criteria were found by immunohistochemistry using antisera directed against the corresponding parasites and by serological assays (Bjerkås and Presthus 1988; Bjerkås et al. 1994; Bjoerkman et al. 1994). Differences between *Neospora* and *Toxoplasma* were also demonstrated on the genetic level. Sequence analysis of nss-rRNA genes, the p22 and p30 gene loci (Brindley et al. 1993; Ellis et al. 1994; Holmdahl et al. 1994), the finding that the *T. gondii*-specific B1 gene is not present in *N. caninum* (Burg et al. 1989), and the sequence analysis and comparison of ribosomal DNA from bovine *Neospora* with that of similar coccidial parasites (Marsh et al. 1995) have suggested clear phylogenetic differences between *Neospora* spp. and *T. gondii*.

In vitro cultivation of tachyzoites has been achieved using several well-established cell lines (Lindsay and Dubey 1989; Dubey and Lindsay 1993). Infected bovine monocytes, bovine pulmonary artery endothelial cells, and cultured human foreskin fibroblast cells were used to compare the ultrastructural features of *N. caninum* and *T. gondii* (Lindsay et al. 1993). In vitro cultivation has also recently provided detailed information on the process of adhesion to and invasion of bovine aorta endothelial cells by *N. caninum* tachyzoites (Hemphill et al. 1996). Chemical and enzymatic modifications of both endothelial and parasite cell surfaces revealed that the initial contact between *N. caninum* and endothelial cells, as well as the invasion process itself, was a receptor-mediated event. The substantial nature of this receptor-ligand system was found to be based on protein-protein interactions rather than on protein-carbohydrate interactions (Hemphill et al. 1996). Thus, in analogy to *T. gondii* tachyzoites, parasite surface proteins of *N. caninum* play a crucial role during adhesion and penetration of the host cell membrane.

Detailed studies on various aspects of host-parasite interactions have been carried out on *T. gondii* (Werk 1985; Bonhomme et al. 1992; Joiner and Dubremetz 1993; Kasper and Mineo 1994). The close relationship between *T. gondii* and *N. caninum* suggested that they would employ similar mechanisms and would use similar surface molecules to encounter their host cells. On the other hand, the distinct antigenic properties of these two species indicated a different surface-molecule composition (Dubey and Lindsay 1993). This is confirmed by the present report, which describes the identification of a novel protein in *N. caninum* tachyzoites with a molecular weight of 43 kDa (named Nc-p43). We demonstrate that Nc-p43 is associated with the cell surface of *N. caninum* tachyzoites and that no immunologically related counterpart for this protein exists in *T. gondii*.

Material 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) supplemented with 7% fetal calf serum (FCS), 2 m*M* glutamine, 50 U penicillin/ml, and 50 µg/ streptomycin/ml 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

Neospora caninum tachyzoites of the Nc-1 (Dubey et al. 1988) and Liverpool isolates (Barber et al. 1993) and *Toxoplasma 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 m*M* glutamine, 50 U penicillin/ml, 50 µg streptomycin/ml, and 7% FCS. Parasites were harvested from their feeder-cell cultures as previously described for *N. caninum* (Hemphill et al. 1996). The preparation (containing tachyzoites and host cell debris) was washed twice in cold phosphate buffered saline (PBS). The final pellet was resuspended in 2 ml of cold PBS and passed through a PD-10 column filled with Sephadex G25 M (Pharmacia) that had previously been equilibrated with PBS. The eluted, purified parasites were centrifuged at 4°C and resuspended in cold PBS. The viability of parasites was monitored by exclusion of trypan blue staining (0.25% in PBS).

Antisera

Polyclonal rabbit anti-*N. caninum* (Nc-1-isolate; Hemphill et al. 1996) and anti-*T. gondii* (RH-strain) antisera were used in this study. Prior to immunization of rabbits, samples of preimmune sera were taken so as to confirm the absence of antibodies directed against *T. gondii* and *N. caninum* tachyzoites by indirect immunofluorescence (Seefeldt et al. 1989; Trees et al. 1991). The serologically negative rabbits were inoculated intravenously with 10^7 freshly purified tachyzoites. These procedures were repeated 2 times at intervals of 10 days before the sera were taken.

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

N. caninum tachyzoites of the Nc-1-isolate (5×10^7–5×10^8/ml) were resuspended in 3 ml of PBS, and phenylmethylsulfonyl fluoride (PMSF) was added to 0.2 m*M*. After 5 min of incubation on ice, the cell suspension was adjusted to 0.75% Triton-X114, and after gentle vortexing the parasites were extracted for 10 min on ice. The preparation was centrifuged (30 min; 10,000 *g*; 4°C), and the supernatant was collected, whereas the pellet containing the (per definition) "cytoskeletal" preparation 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 described above to remove possible aggregates. The supernatant was collected and incubated at 30°C for 3 min again. The detergent and hydrophilic phases were separated by a further centrifugation step (3 min; 1,000 *g*; 24°C). The supernatant was removed, and the detergent phase was brought to 100 µl with PBS. This final fraction, containing potential cell-surface membrane proteins, was then processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by methanol/chloroform extraction as described elsewhere (Wessel and Fluegge 1984).

SDS-PAGE, immunoblotting, and chemical deglycosylation of nitrocellulose-bound proteins

Protein extracts of the various subcellular fractions (cell lysates of *N. caninum* Nc-1 and Liverpool isolates, *T. gondii* total cell extracts, cytoskeletal preparations, and hydrophilic and hydrophobic phases of Triton-X-114 extracts) were processed for SDS-PAGE as previously described (Hemphill et al. 1992). Samples corresponding to the same number of parasites were separated by SDS-PAGE and transferred to nitrocellulose filters (Towbin et al. 1979). In some experiments, nitrocellulose filters containing bound proteins were treated with 40 m*M* $NaIO_4$ in 50 m*M* sodium acetate buffer (pH 4.5; McDonald et al. 1995). Blocking of unspecific binding sites was carried out for 4 h at 24°C in PBS containing 3% bovine serum albumin (BSA) and 0.3% Tween-20. The antisera directed against *N. caninum* and and *T. gondii* were applied at a dilution of 1:2,000 in PBS/0.3% BSA/0.3% Tween-20 overnight at 4°C. The filters were then washed three times in PBS/0.3% Tween-20, and the bound antibodies were visualized using peroxidase-conjugated anti-rabbit immunoglobulin antibodies (Promega) according to the instructions provided by the manufacturers.

Affinity purification of specific antibodies against Nc-p43

Affinity purification of specific antibodies directed against the most immunodominant band from a Triton-X-114 extract was performed essentially as described by Mueller et al. (1992). Briefly, the region corresponding to Nc-p43 was cut out from the nitrocellulose following SDS-PAGE and Western blotting of Triton X-114 extracts. In some cases the strip was treated with $NaIO_4$ as described above. After blocking of unspecific binding sites, the anti-*N. caninum* antiserum was applied at a dilution of 1:20 in PBS/0.3% BSA/0.3% Tween-20 overnight at 4°C. After washing of 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 m*M* TRIS, 50 m*M* glycine, 0.05% Tween-20, 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 (10,000 *g*, 20 min) to remove small nitrocellulose particles, and BSA was added 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 1:100.

Both the nitrocellulose strip and the antiserum were usually reused for a second round of affinity purification. After washing of the strip in PBS/0.3% Tween-20 and subsequent blocking for 4 h in PBS/3% BSA/0.3% Tween-20, the procedure described above

was repeated. The anti-Nc-p43 antibodies isolated from this second round of purification proved to be suitable for immunoblots at a dilution of 1:50.

Immunofluorescence

All procedures were carried out at room temperature. Freshly purified *N. caninum* or *T. gondii* tachyzoites suspended in PBS at 10^7 parasites/ml were applied to polylysine-coated (100 µg/ml) glass coverslips. After 10 min the coverslips were rinsed three times in PBS and were placed in fixation buffer (PBS/3% paraformaldehyde/0.05% glutaraldehyde, pH 7.2) for 15 min. The coverslips were then rinsed extensively in PBS and were subsequently incubated in blocking solution (PBS/1% BSA/50 m*M* glycin) for 30 min. The anti-*N. caninum* and anti-*T. gondii* antisera were applied at a dilution of 1:400 and the affinity-purified anti-Nc-p43 antibody was applied at 1:1 in blocking solution for 30 min, followed by three buffer rinses. The second antibody layer (goat anti-rabbit conjugated to Texas red; Becton Dickinson Immunocytometry Systems) was applied at a 1:100 dilution in blocking buffer. In the case of double-labeling experiments, the third antibody was a monoclonal antibody directed against alpha-tubulin (kindly provided by Tom Seebeck, University of Bern) and a fourth layer comprised fluorescein isothiocyanate (FITC)- conjugated goat anti-mouse antibody (Cappel). Specimens were then rinsed in PBS (five times for 5 min). In some experiments the coverslips were subsequently stained with the DNA-specific dye Hoechst 33258 (25 µg/ml in PBS) for 2 min. Finally, the preparations were briefly rinsed in distilled water and were embedded in a mixture of glycerol/gelvatol containing 1,4-diazobicyclo(2,2,2)octane (Merck) as an antifading reagent (Hemphill et al. 1992). Results were obtained by inspection of specimens on a Leitz Laborlux S fluorescence microscope.

Immunogold labeling and transmission electron microscopy

Purified *N. caninum* and *T. gondii* tachyzoites (5×10^7/ml) were fixed in suspension employing the same fixation buffer used for immunofluorescence studies. After blocking of unspecific binding sites and three additional washes in blocking buffer, the first antibody layers (diluted as described above) were applied for 60 min. Parasites were then washed in PBS (three times for 5 min), and the second layer (goat anti-rabbit antibody conjugated to 10-nm gold particles; Amersham, Zuerich) was applied at a dilution of 1:5 in blocking buffer for 45 min. Gold conjugates were centrifuged at 4,800 *g* for 10 min to remove possible aggregates (Hemphill et al. 1991). After three washes in PBS, the labeled parasites were fixed in 2% glutaraldehyde diluted in 100 m*M* sodium phosphate buffer (pH 7.2) for 4 h at 4°C. The parasites were then washed in phosphate buffer, postfixed in 2% OsO_4, dehydrated in a graded series of ethanol, and embedded in Epon 812 (Fluka Chemicals, Switzerland) as previously 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 (Smith and Croft 1991).

Results

Characterization of anti-*Neospora caninum* and anti-*Toxoplasma gondii* antisera

Polyclonal antisera were raised in rabbits immunized with purified, intact *N. caninum* (Nc-1-isolate) and *T. gondii* tachyzoites, respectively. These antisera were first characterized by SDS-PAGE and immunoblotting with regard to their reactivity with cell extracts derived from these two parasites. Figure 1 illustrates the differences found in the reactivities of the two antisera. The anti-*N. caninum* antiserum recognized a high number of proteins in *N. caninum* (Nc-1) extracts, but the affinity for epitopes in *T. gondii* cell lysates was mainly restricted to distinct bands of 48, 43, and 40 kDa (Fig. 1B). The anti-*T. gondii* antiserum, however, reacted very intensely with many bands in *T. gondii* extracts, one of which presumably was P30, the major surface antigen, but labeling of bands in *N. caninum* extracts was almost absent (see Fig. 1C).

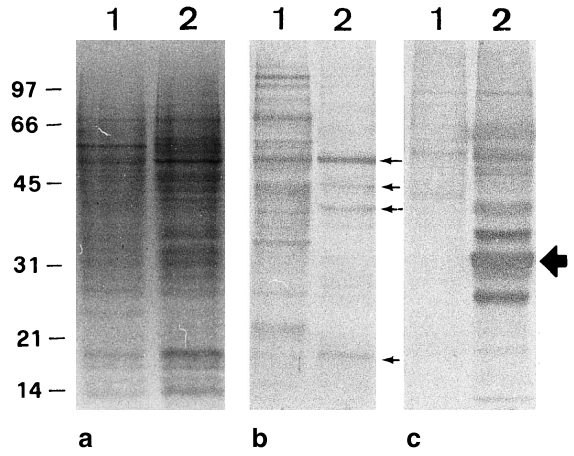

Fig. 1a–c Coomassie-stained SDS-PAGE of total cell extracts (**a**) and corresponding immunoblots labeled with anti-*Neospora caninum* (**b**) and anti-*Toxoplasma gondii* (**c**) antisera (*Lane 1 N. caninum* tachyzoites, *lane 2 T. gondii* tachyzoites). Note the relative specificity of the two antisera with respect to the species they were raised against. The *small arrows* indicate cross-reacting bands in *T. gondii* extracts, and the *large arrow* shows the position of P30, the major *T. gondii* surface antigen

To find out whether these two antisera would bind to epitopes present on the surface of tachyzoites, parasites were fixed in a mixture of 3% paraformaldehyde/0.05% glutaraldehyde in PBS (conditions that are recommended for preserving the integrity of the cell surface) and immunofluorescence staining was performed. Immunofluorescence labeling demonstrated a clear specificity of these two antisera toward the species against which they were raised, at least with respect to cell-surface-exposed epitopes (Fig. 2a–d). In addition, prefixed *N. caninum* (Nc-1) tachyzoites were immunogold-labeled using the anti-*N. caninum* antiserum and were subsequently embedded in Epon 812, and thin sections were inspected by TEM. Dense labeling was seen distributed uniformly all over the parasite cell surface (Fig. 2e). Gold particles were absent in control experiments using preimmune serum as the first antibody layer, and immunogold labeling of *T. gondii* tachyzoites with anti-*N. caninum* antiserum also failed to result in binding of gold particles to the *Toxoplasma* cell surface (data not shown). These initial experiments demonstrated that the antiserum directed against *N. caninum* (Nc-1) tachyzoites represents a valuable tool for the further identification of specific cell-surface components of this parasite.

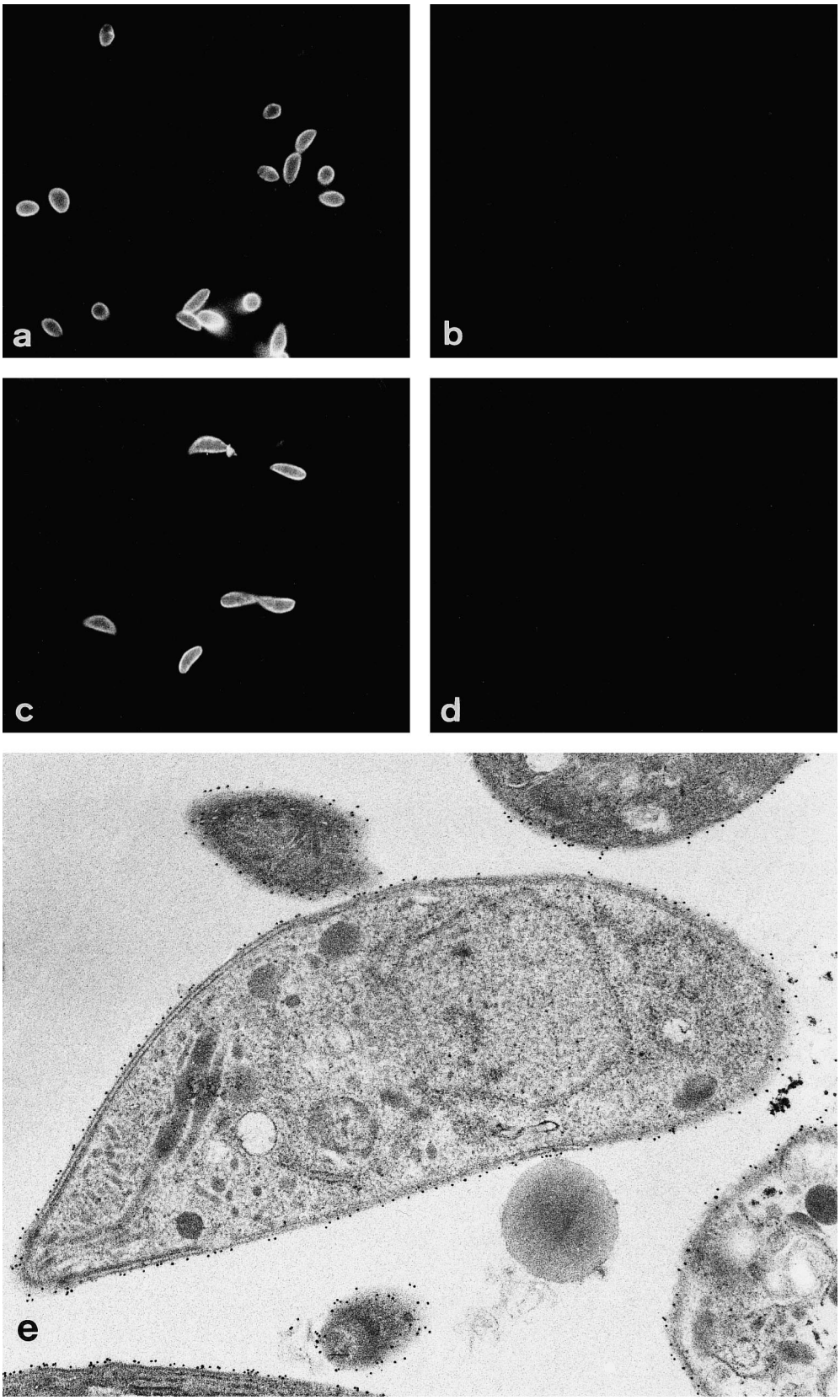

Fig. 2a–e Cell-surface labeling of *N. caninum* (**a, d**) and *T. gondii* (**b, c**) tachyzoites as obtained using antisera directed against these two parasites. **a, b** Tachyzoites labeled with anti-*N. caninum* antiserum. **c, d** Tachyzoites labeled with anti-*T. gondii* antiserum. Note the absence of fluorescent staining in **b** and **d**. **e** Immunogold surface labeling of *N. caninum* tachyzoites as obtained using anti-*N. caninum* antiserum. Note the uniform, dense labeling of the tachyzoite surface

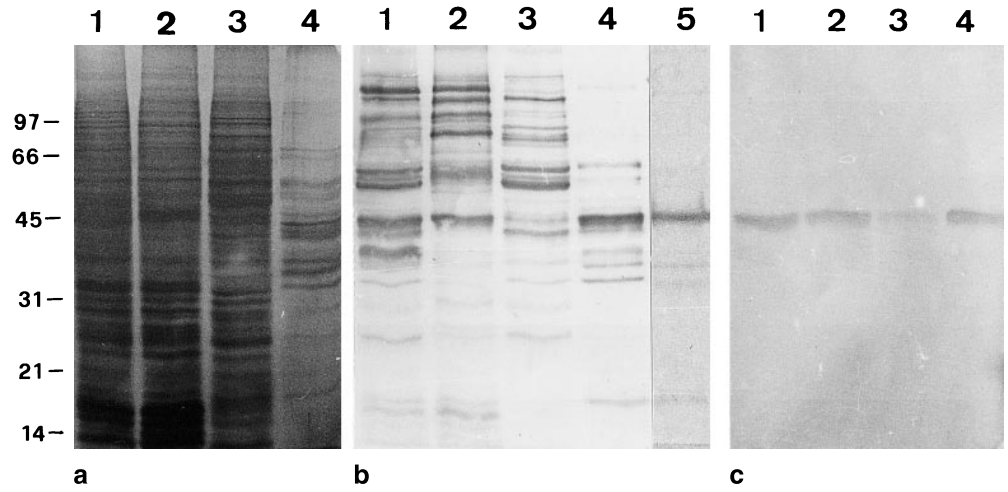

Fig. 3a–c Subcellular fractionation of *N. caninum* tachyzoites. A Silver-stained SDS-PAGE. Amounts corresponding to the same number of tachyzoites were loaded (*1* Total cell extract, *2* Triton X-114-insoluble fraction, *3* Triton X-114-soluble hydrophilic phase, *4* Triton X-114-soluble apolar phase). **b** Corresponding immunoblots labeled with anti-*N. caninum* antiserum (*Lanes 1–4* As defined in **a**, *lane 5* immunoblot of the Triton X-114-soluble apolar fraction after chemical deglycosylation with sodium periodate). **C** Immunoblots corresponding to those shown in **a** as obtained using affinity-purified anti-Nc-p43 antibody. Note the absence of Nc-p43 in the hydrophilic Triton-X-114 fraction

Subcellular fractionation of *N. caninum* tachyzoites

We applied a subcellular fractionation approach employing extraction and phase separation in the nonionic detergent Triton X-114. Phase separation at elevated temperature (30°C) separated hydrophilic Triton X-114 soluble proteins from the hydrophobic components constituting potential integral membrane proteins. The different steps of the subcellular fractionation of *N. caninum* tachyzoites of the Nc-1 isolate are summarized in Fig. 3. Fig. 3B demonstrates in immunoblots of SDS-PAGE-separated extracts the reactivity of the whole-cell-lysate, the cytoskeletal fraction, and the hydrophilic and hydrophobic phases of Triton X-114 extracts with the anti-*N. caninum* antiserum. The most immunodominant protein in the apolar phase of Triton X-114 extracts migrated at a molecular weight of approximately 43 kDa (Fig. 3B, lane 4). To determine whether the epitopes recognized by the polyclonal anti-*N. caninum* antiserum in the apolar Triton X-114 fraction were of proteinous or carbohydrate nature, deglycosylation of nitrocellulose-bound proteins was achieved by sodium periodate treatment. Whereas chemical deglycosylation profoundly diminished antibody-binding activity to several nitrocellulose-bound polypeptides, binding of antibodies to the 43-kDa protein remained largely unaffected (Fig. 3B, lane 5). This suggested that the epitopes, or at least a large portion of them, on this most immunodominant molecule were not constituted of carbohydrates. This protein was subsequently named Nc-p43.

Nc-p43 was also clearly visible on nitrocellulose filters that had been stained with Ponceau red after transfer of SDS-PAGE-separated proteins. Affinity purification of anti-*Neospora* antiserum on this particular band was perfomed to obtain an immunoglobulin fraction that was specific for Nc-p43. The specificity of these affinity-purified antibodies with respect to other proteins in all subcellular fractions of *N. caninum* (Nc-1) is demonstrated in Fig. 3C.

Nc-p43 is a cell-surface-associated protein in *N. caninum* but not in *T. gondii*

Affinity-purified anti-Nc-p43 antibodies were used to demonstrate the location of this molecule on *N. caninum* tachyzoites of the Nc-1 isolate. Double-immunofluorescence experiments were performed. Purified tachyzoites were fixed as described above and were incubated with affinity-purified anti-Nc-p43 antibodies and a second antibody conjugated to Texas red. Staining of the *N. caninum* tachyzoite surface was evident (Fig. 4a). Identical results were obtained when tachyzoites of the *N. caninum* Liverpool isolate were immunolabeled with affinity-purified anti-Nc-p43 antibodies (data not shown). For verification that the fixation procedure employed really did reflect labeling of the cell surface and not of internal proteins, the same preparations were subsequently incubated with a monoclonal anti-tubulin antibody and a goat anti-mouse antibody conjugated to FITC. Only those cells that remained unlabeled with the monoclonal antibody reflected true cell-surface staining (Fig. 4b). In addition, *T. gondii* tachyzoites were fixed identically and were processed for fluorescence surface staining as described above (Fig. 4d, e). The complete absence of immunolabel from *T. gondii* tachyzoites suggested that Nc-p43 was a *Neospora*-specific molecule. Preembedding immunogold surface labeling of *N. caninum* tachyzoites using anti-Nc-p43 antibodies was performed to show conclusively the presence of this protein on the tachyzoite surface. Gold particles were distributed along the entire surface of the tachyzoites (Fig. 4e), demonstrating

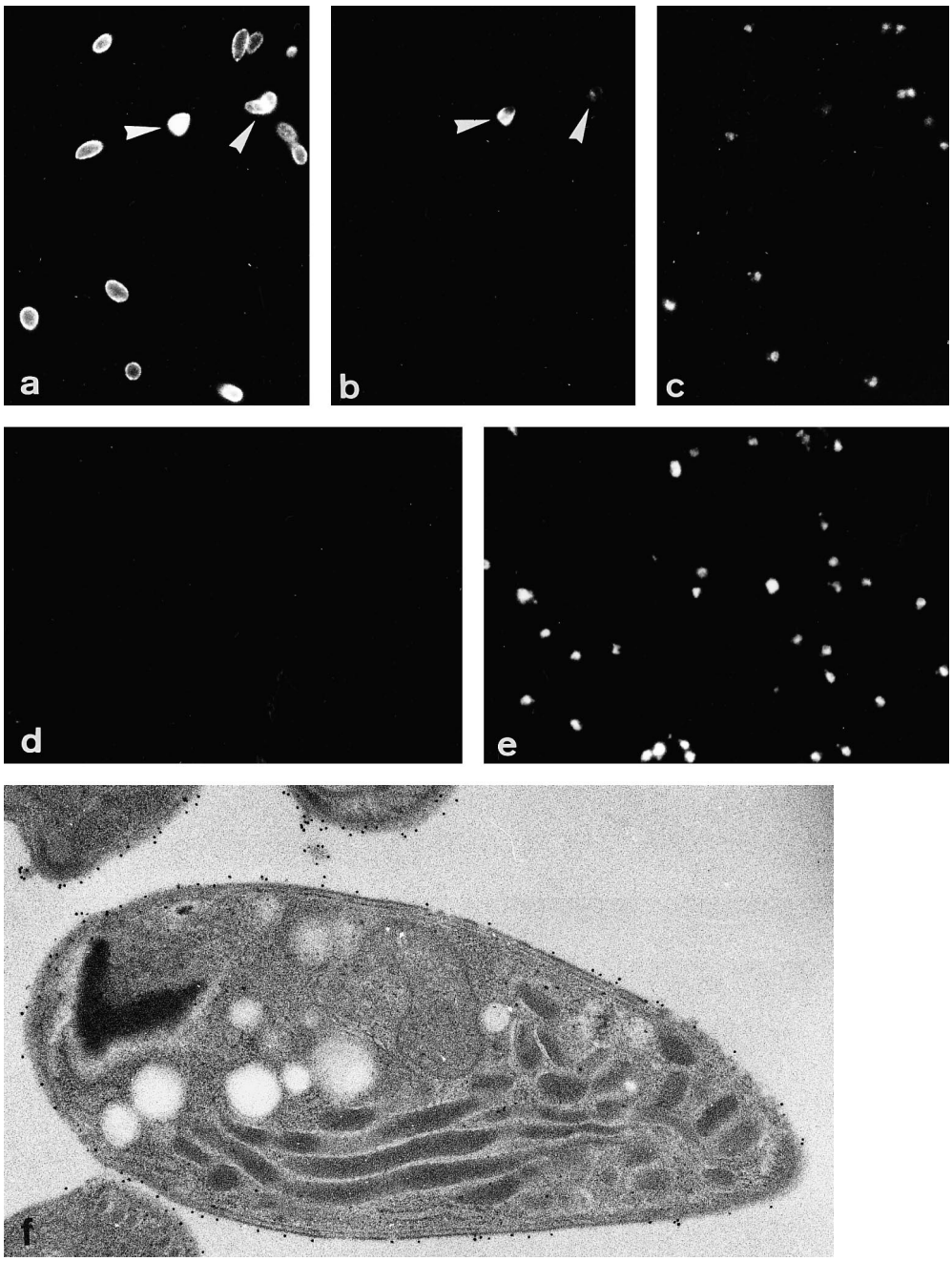

Fig. 4a–f Cell-surface labeling of *N. caninum* (**a–c, f**) and *T. gondii* (**d, e**) tachyzoites with affinity-purified anti-Nc-p43 antibody. *N. caninum* tachyzoites were surface-labeled with anti-Nc-p43 (**a**) and a monoclonal anti-tubulin antibody (**b**) to demonstrate those tachyzoites that were no longer intact (*arrowheads*). The nuclei of tachyzoites were labeled with Hoechst 33258 (**c**) to show that all parasites express the cell-surface protein Nc-p43. **d** *T. gondii* tachyzoites surface-labeled with affinity-purified anti-Nc-p43 antibody. **e** The same *T. gondii* stained with Hoechst 33258. **f** *N. caninum* tachyzoite immunogold-labeled with affinity-purified anti-Nc-p43 antibody and goat anti-rabbit antibody conjugated to 10-nm gold particles. Note the uniform distribution of gold particles on the tachyzoite surface

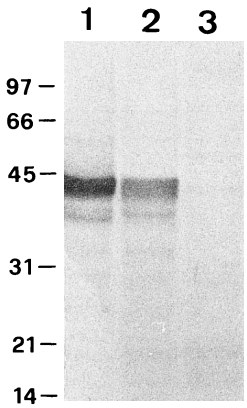

Fig. 5 Immunoblot labeled with affinity-purified anti-Nc-p43 antibody, demonstrating the presence of this molecule in an American isolate (Nc-1, *lane 1*) and a European isolate (Liverpool, *lane 2*). Note that Nc-p43 is absent from the *T. gondii* RH strain (*lane 3*)

that Nc-p43 was indeed a major cell-surface protein. The presence of Nc-p43 in cell lysates of *N. caninum* (Nc-1 and Liverpool isolates) and the absence of an immunologically related protein in *T. gondii* was further confirmed by immunoblotting employing the affinity-purified anti-Nc-p43 antibody (Fig. 5).

Discussion

Specific receptor-ligand interactions, usually mediated by cell-surface-associated molecules, are a prerequisite for microbial colonialization and pathogenesis. This is documented by the observation that surface molecules of

intracellular parasites play important roles with respect to the process of adhesion to and invasion of host cells, the intracellular development of these pathogens, and the presentation of antigens as these parasites are internalized by antigen-presenting cells such as macrophages. Thus, parasite molecules that are accessible to host immune or nonimmune components carry out key functions during the host-parasite interplay. To our knowledge, only very limited information is available on host-parasite interactions in neosporosis (Hemphill et al. 1996), and we therefore undertook a first step in this direction by investigating the composition of the cell surface of *Neospora caninum* tachyzoites.

This paper reports on the identification of a cell-surface-associated protein of 43 kDa in an American isolate (Nc-1; Dubey et al. 1988) and a European (Liverpool; Barber et al. 1993) isolate of *N. caninum*. An immunologically related protein is apparently not present in the *Toxoplasma gondii* RH strain. The *Neospora* surface protein Nc-p43 was identified using a polyclonal antiserum generated in rabbits by inoculation of live *N. caninum* tachyzoites into these animals (Hemphill et al. 1996). The original plan focused on the affinity purification of this antiserum on distinct bands of SDS-PAGE-separated total cell extracts and the use of affinity-purified antibodies for localization studies so as to determine the distribution of the corresponding epitopes on *N. caninum* tachyzoites (Mueller and Felleisen 1995). However, our antiserum generated a very complex banding pattern on immunoblots of *N. caninum* cell extracts (see Fig. 1). This confirmed the results previously obtained by other authors (Barta and Dubey 1992; Bjerkås et al. 1994; Bjoerkman et al. 1994), and it was therefore necessary to undertake subcellular fractionation so as to achieve antibody labeling of distinct, immunopositive bands (see Fig. 3).

Triton-X-114 extraction and subsequent phase separation has been employed by other investigators for the isolation of parasite cell-surface membrane-associated proteins (Bouvier et al. 1985). By immunoblot analysis of the Triton X-114 detergent-phase extracts of *N. caninum* with the polyclonal antiserum the number of immunoreactive proteins was significantly reduced, exhibiting a clearly discernible banding pattern (see Fig. 3). The most immunodominant band had a molecular weight of approximately 43 kDa. Antibodies affinity-purified on the 43-kDa band reacted strongly with cell-surface epitopes of *N. caninum* but did not react with those of *T. gondii* (see Fig. 4). This suggested the potential of this protein to represent a convenient tool for the discrimination of these two species by serological means. Respective investigations are currently being carried out in our laboratory.

Immunodominant proteins of *N. caninum* have also previously been identified by other groups working in the field. The most immunodominant antigens of *N. caninum* recognized by hyperimmunsera directed against this parasite have been described by Barta and Dubey (1992) as four antigens with approximate molecular weights of 16/17, 29, 37, and 46 kDa, respectively. Bjerkås et al. (1994) reported that the dominating *N. caninum* antigens recognized by immune sera from various species had apparent molecular weights of 17, 29, 30, and 37 kDa. However, immunoelectron microscopy studies employing the respective sera have shown that these antigens were located not in the surface membrane but in rhoptries, dense granules, micronemes, the tubular network, and the membrane of the parasitophorous vacuole (Barta and Dubey 1992; Bjerkås et al. 1994). Bjoerkman et al. (1994) characterized antigens that are currently used in an enzyme-linked immunosorbent assay (ELISA) for diagnosis of *Neospora* infections. This group of antigens comprised four proteins of 52–61 kDa, and three proteins of 31–36 kDa. As these antigens were isolated by ISCOM preparations and since ISCOM are formed by hydrophobic interactions (Araujo 1994), the isolation procedure selected for amphipatic molecules such as membrane proteins (Hoeglund et al. 1989), a membrane-associated location was suggested for these antigens as well.

Chemical deglycosylation of nitrocellulose-bound proteins from SDS-PAGE-separated Triton X-114 extracts significantly altered the staining intensity of several bands when these were probed with the polyclonal anti-*N. caninum* antiserum, but the reactivity of antibodies directed against the 43-kDa band remained largely unaffected (see Fig. 3). Our previous investigations (Hemphill et al. 1996) have shed some light on the role of carbohydrate residues on the *N. caninum* cell surface in relation to adhesion to and invasion of host cells. Sodium periodate treatment of *N. caninum* 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 host cells. In addition, preincubation of parasites with tunicamycin and treatment of parasites with several lectins were ineffective in achieving an inhibition of the adhesion or invasion process (Hemphill et al. 1996). In contrast, protease treatment of tachyzoites suggested that removal of proteins or protein fragments from the parasite surface altered the molecules responsible for mediating the interaction of *N. caninum* with the monolayer. Thus, carbohydrate residues were probably not important during the initial phases of the invasion process, but it is likely that the interaction between parasites and host cells involved specific proteinous molecules that would act as ligands, mediating host-cell recognition and invasion (Hemphill et al. 1996).

Nc-p43 could very likely be one of these proteins that participate in the adhesion and/or invasion process of *N. caninum* tachyzoites. However, this question needs further investigation employing specific antibodies directed against Nc-p43 with respect to their capacity to block the entry of the parasite into the intracellular compartment of its host.

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