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Research article

New comprehension of the apicoplast of *Sarcocystis* by transmission electron tomography

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Background information. Apicomplexan parasites (like *Plasmodium*, *Toxoplasma*, *Eimeria* and *Sarcocystis*) contain a distinctive organelle, the apicoplast, acquired by a secondary endosymbiotic process analogous to chloroplasts and mitochondria. The apicoplast is essential for long-term survival of the parasite. This prokaryotic origin implies that molecular and metabolic processes in the apicoplast differ from those of the eukaryotic host cells and therefore offer options for specific chemotherapeutic treatment. We studied the apicoplast in high-pressure frozen and freeze-substituted cysts of *Sarcocystis* sp. from roe deer (*Capreolus capreolus*) to get better insight in apicoplast morphology.

Results and conclusions. We observed that the apicoplast contains four continuous membranes. The two inner membranes have a circular shape with a constant distance from each other and large-sized protein complexes are located between them. The two outer membranes have irregular shapes. The periplastid membrane also contains large-sized protein complexes, while the outer membrane displays protuberances into the parasite cytoplasm. In addition, it is closely associated with the endoplasmic reticulum by 'contact sites'.

Introduction

Sarcocystis is a worldwide-distributed apicomplexan parasite. It is found in many domestic and wildlife species, including humans (Levine, 1973). It has an obligatory heteroxenous life cycle with herbivores as intermediate and carnivores as definitive hosts. Omnivores, such as humans, serve as both intermediate and definitive hosts. Typically the apicomplexan parasites, including *Plasmodium*, *Toxoplasma*, *Eimeria* and *Sarcocystis*, possess a distinctive organelle, the apicoplast. This organelle is essential for long-term parasite survival (Fichera and Roos, 1997; He et al., 2001a).

It is commonly accepted that the apicoplast was acquired by secondary endosymbiosis (Gibbs, 1978; Delwiche and Palmer, 1997; McFadden, 1999, 2001), an event in which a non-photosynthetic eukaryote initially engulfed a cyanobacteria-like prokaryotic cell followed by subsequent engulfment of this alga by the apicomplexan ancestor (for reviews, see: Cavalier-Smith, 1999; Van Dooren et al., 2001).

The apicoplast is indispensable for survival of the parasite but its exact role is still unclear. The plastid is known to play a role in lipid metabolism by hosting the mevalonate-independent isoprenoid biosynthesis and fatty acid type II biosynthesis (McFadden and Waller, 1997; Waller et al., 1998; Jomaa et al., 1999; Wilson, 2002). Apicoplast malfunctioning or complete absence of the plastid is not instantly lethal to apicomplexan parasites; rather, it causes a 'delayed-death' phenomenon instead. These

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Abbreviations used: ER, endoplasmic reticulum; Toc 159, translocon of the outer chloroplast envelope-159.

parasites remain viable for a while and continue replicating but are unable to successfully re-invade another host cell and die soon thereafter (Fichera et al., 1995; Fichera and Roos, 1997; He et al., 2001a).

The apicoplast has its own genome (35 kb DNA-like circles; McFadden et al., 1996; Wilson et al., 1996), but most of the apicoplast proteome is encoded in the parasite nuclear genome. The products of these parasite genes are post-translationally modified and targeted to the organelle by a bipartite N-terminal leader sequence, which is proteolytically cleaved (Nielsen et al., 1997).

The prokaryotic nature of the apicoplast opens great potential for new drug development for chemotherapeutic treatment of severe infectious diseases like malaria, toxoplasmosis and coccidiosis in both human and livestock. For efficient drug targeting, it is important to have accurate knowledge of the interaction between the apicoplast and the parasite cell, e.g. what are the pathways for importing polypeptides into the plastid. In the literature, however, there are contradicting reports on the ultrastructure of the apicoplasts (Hopkins et al., 1999; Köhler, 2005). The information concerning the ultrastructure of the apicoplast is incomplete and based on thin (sometimes serial) sections from chemically fixed material. It is, however, known that chemical fixation destabilizes membranous structures (Ebersold et al., 1981; Dubochet et al., 1983; Szczesny et al., 1996; Murk et al., 2003) and during dehydration and resin embedding most of the lipids are lost. Only preparation methods based on cryofixation are able to preserve membranous structures properly. The first choice in terms of optimal preservation would be imaging of the cysts in the frozen-hydrated state (Dubochet et al., 1988); however, the cysts are too large to be analysed by cryoelectron tomography (Medalia et al., 2002). Furthermore, artefact-free thick cryosections justifying tomographic investigations cannot be produced (Al-Amoudi et al., 2005). The next best option is freeze substitution, a hybrid method of cryofixation and resin embedding. It was demonstrated that with freeze substitution and resin embedding most of the lipids can be preserved to a high degree (Verkleij et al., 1985; Weibull and Christiansson, 1986; Humbel and Schwarz, 1989) and that cellular organelles do not change their morphology (Ebersold et al., 1981; Murk et al., 2003).

Therefore we chose to combine high-pressure freezing, freeze substitution, resin embedding and electron tomography to elucidate the cellular ultrastructure of the parasites and to develop models of the apicoplast and to establish a hypothesis on the import of proteins.

Results

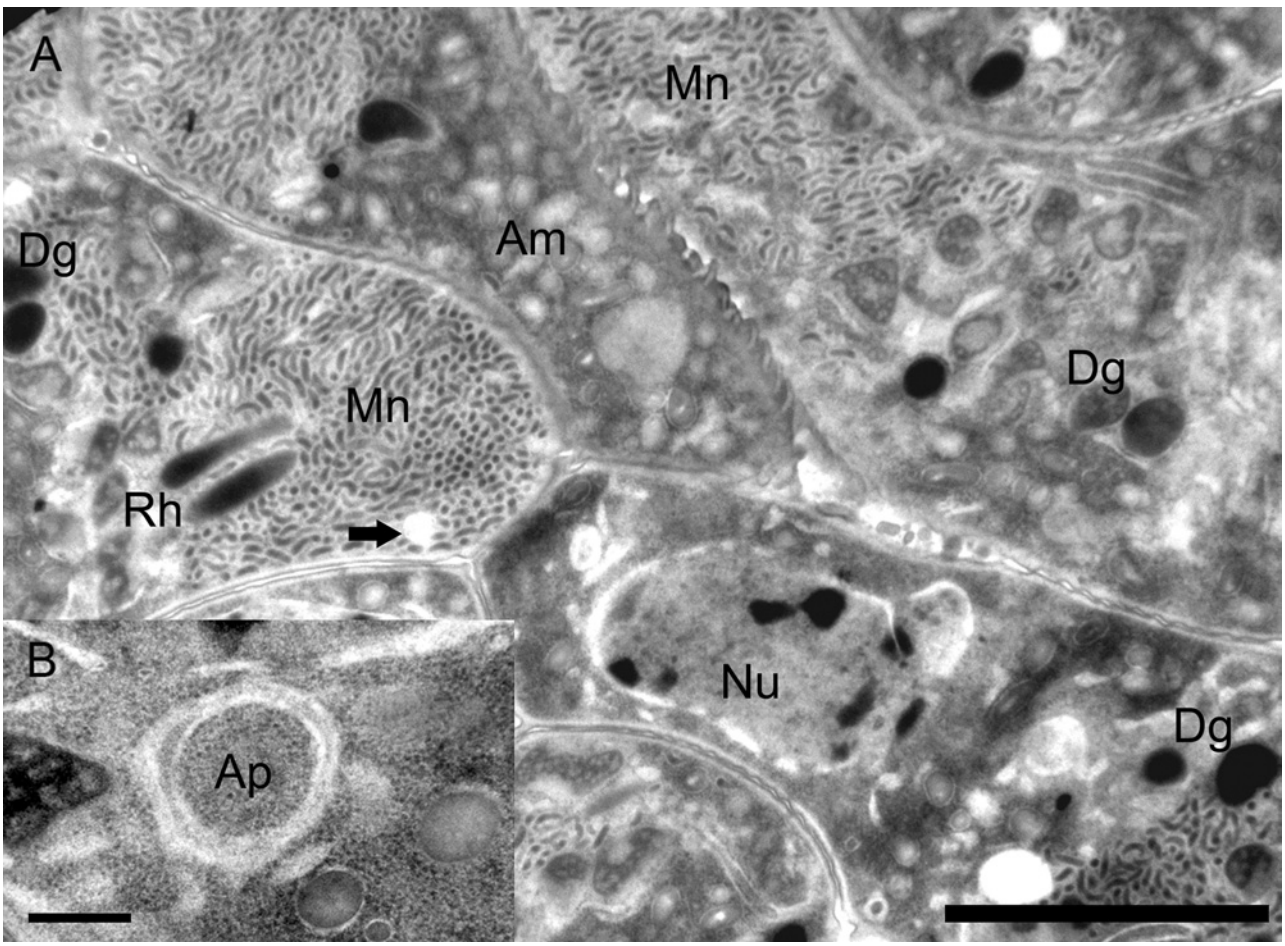
The ultrastructural preservation of the cysts was very good (Figure 1A) and no intracellular ice crystal formation of detectable size was observed. At low magnification, the plasma membranes of the individual parasites can be clearly distinguished. Internal parasite structures like the nucleus (Nu), dense granules (Dg), micronemes (Mn) and rhoptries (Rh) can be clearly seen. It is even possible to distinguish a density gradient in the amylopectin granules (Am). At higher magnification (Figure 1B), the membranes of the apicoplast (Ap) are visible. Both the cytoplasm of the parasite and the lumen of the apicoplast have a homogeneous protein distribution pattern, which is typical for a well-preserved ultrastructure.

In total, more than 20 double tilt tomograms were recorded and analysed. In the approx. 6 nm thin slices of the tomograms, we clearly identified four continuous membranes surrounding the plastid (Figure 2).

The two innermost membranes, thought to be derived from a primary symbiont, are very regular and oval-shaped with a constant distance of 4–6 nm from each other (Figure 2A). They have no protuberances or other deformations. Patches of lighter mass densities between the two inner membranes interrupt the profile of the two inner membranes in a regular pattern. The patches seem to form discs of a regular size and shape that are approx. 15–20 nm thick and 60–80 nm in diameter.

The third membrane is thought to be a remnant of a plasma membrane of the eukaryotic endosymbiont. It is known as the 'periplastid membrane' (Cavalier-Smith, 1999). The space between the second and third membranes, the periplastid space, generally is 10–15 nm in thickness; however, sometimes the periplastid membrane seems to touch the second inner membrane (Figure 2A) but they can become more distant, forming a kind of pocket (Figures 2B–2D). This membrane also contains patches similar to those in the inner membranes (Figure 2A). These patches do not seem to bridge membranes.

Figure 1 | Structural preservation of the morphology of the *Sarcocystis*
(**A**) Low-magnification electron micrograph of the part of a cyst containing the cystozoites of *Sarcocystis* after high-pressure freezing and freeze substitution to illustrate the well-preserved morphology. The section is 80 nm thick. Black arrow indicates very light amylopectin granule. Scale bar, 2 μm. Nu, nucleus; Mn, micronemes; Dg, dense granules; Rh, rhoptries; Am, amylopectin granules. (**B**) Detail of a typical apicoplast in *Sarcocystis* sp. Note the homogeneous distribution of the proteins in the apicoplast lumen and the parasite cytoplasm. Ap, apicoplast. Scale bar, 300 nm.



The outermost membrane is believed to be derived from the endomembrane system of the heterotrophic protists themselves (Douglas, 1998). In some areas, the membrane forms prominent protuberances into the cytoplasm (Figure 2A). Apart from the protuberances, it is equidistant to the third membrane. In the reconstructed tomograms, these protuberances have diverse shape and size (Figures 2A and 2B). Some apicoplasts have a bilobed appearance, also of different shapes and sizes (Figures 2C and 2D).

An important observation in the tomograms was the close proximity of the apicoplast to the ER (endoplasmic reticulum), visible as tubules dotted with ribosomes (Figures 2B, 2D and 3A). At some places, the ER touches the forth membrane of the apicoplast (Figures 2D and 3A), most probably forming 'contact points' between the ER and the outermost membrane. The size, shape, number and spatial distribution of the contact points between the outermost membrane and the ER varied.

The models presented give an overview of the results observed in the present study. The close proximity of the ER to the apicoplast is illustrated in Figure 3(B). It can be clearly demonstrated that the four

Figure 2 | Structure of the apicoplast and variation in size and shape of the protuberances

(**A**–**D**) Representative tomographic slices, of a thickness of approx. 6 nm, of the three-dimensional double-tilt reconstruction of four different apicoplasts. The images elucidate most of the features of the apicoplast revealed by tomography: firstly, the four continuous membranes surrounding the apicoplast (**A**). Secondly, the protuberances (**A**, **B**, star) from the outermost membrane into the parasite cytoplasm. Thirdly, the protein complexes spanning the two innermost membranes (black arrowhead) and in the periplastid membrane (white arrowhead). Fourthly, the variability of the periplastid space (black arrow). Fifthly, the close proximity of the apicoplast to the ER (white arrow), visible as tubules dotted with ribosomes. In (**C**, **D**), the apicoplasts have a bilobed appearance. This feature might indicate some dynamics of *Sarocystis* even in a so-called dormant state in the cyst, maybe a dividing apicoplast. Scale bar, 150 nm.

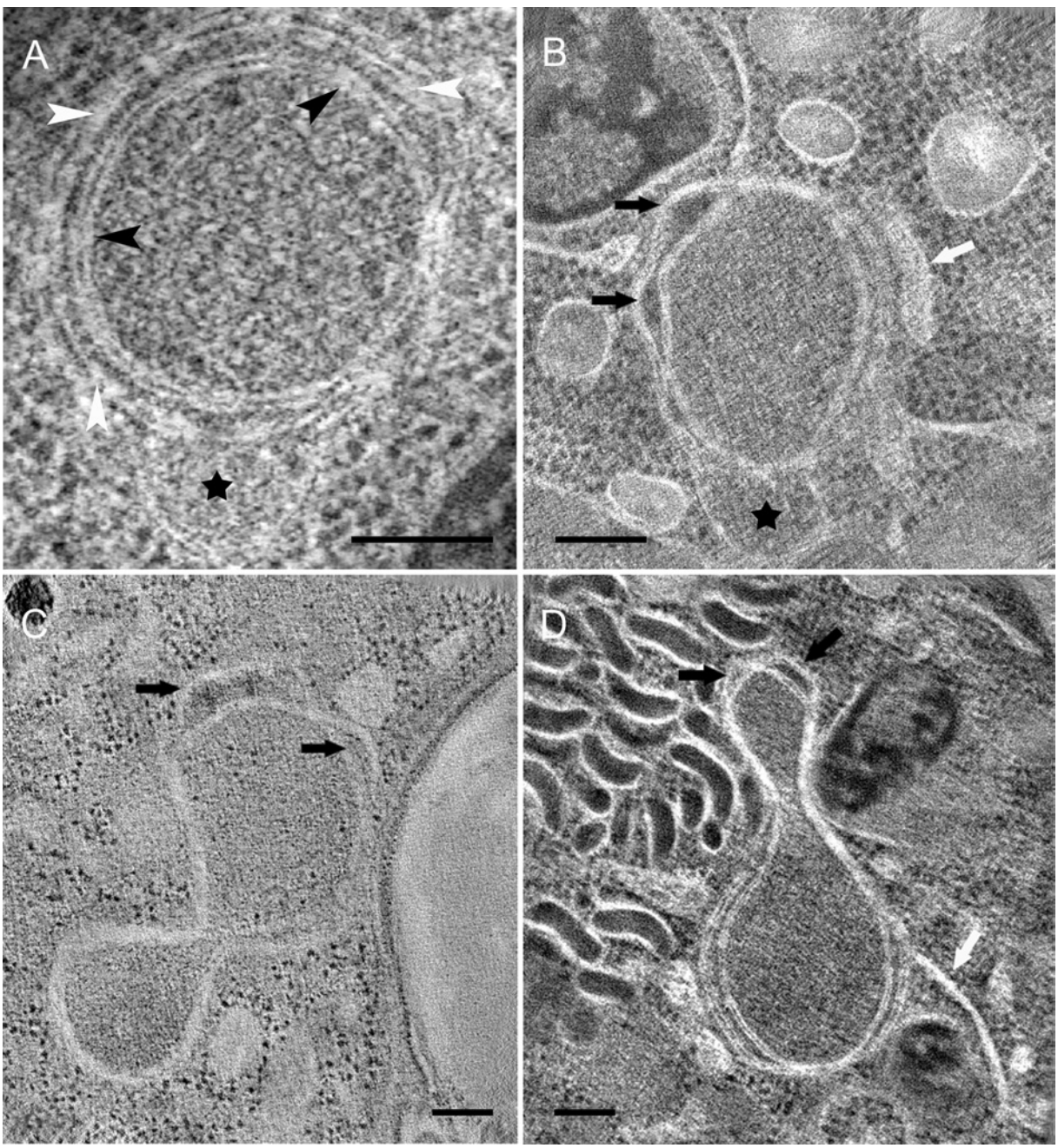
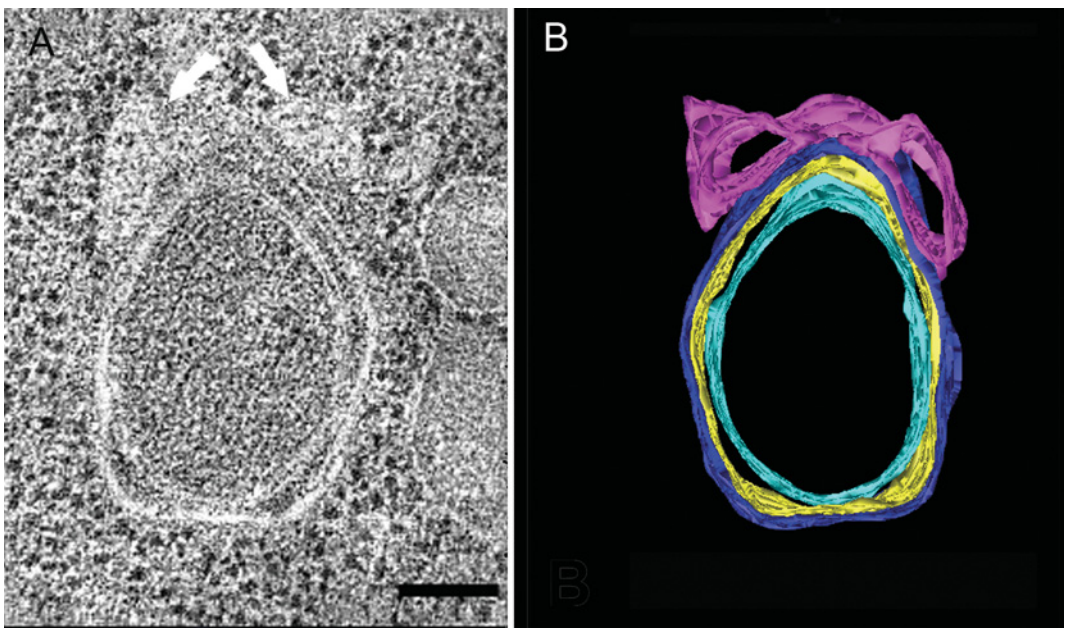


Figure 3 | The ER is in close association with the apicoplast

(**A**) Tomogram reconstruction slide illustrating the close proximity of the ER (white arrows) to the outermost membrane of the apicoplast. Scale bar, 150 nm. (**B**) Model of a part of the ER (violet) showing the close proximity to the outer membrane (dark blue). The periplastid inner membrane (light blue) and the periplastid outer membrane (in yellow) enclose the periplastid space.



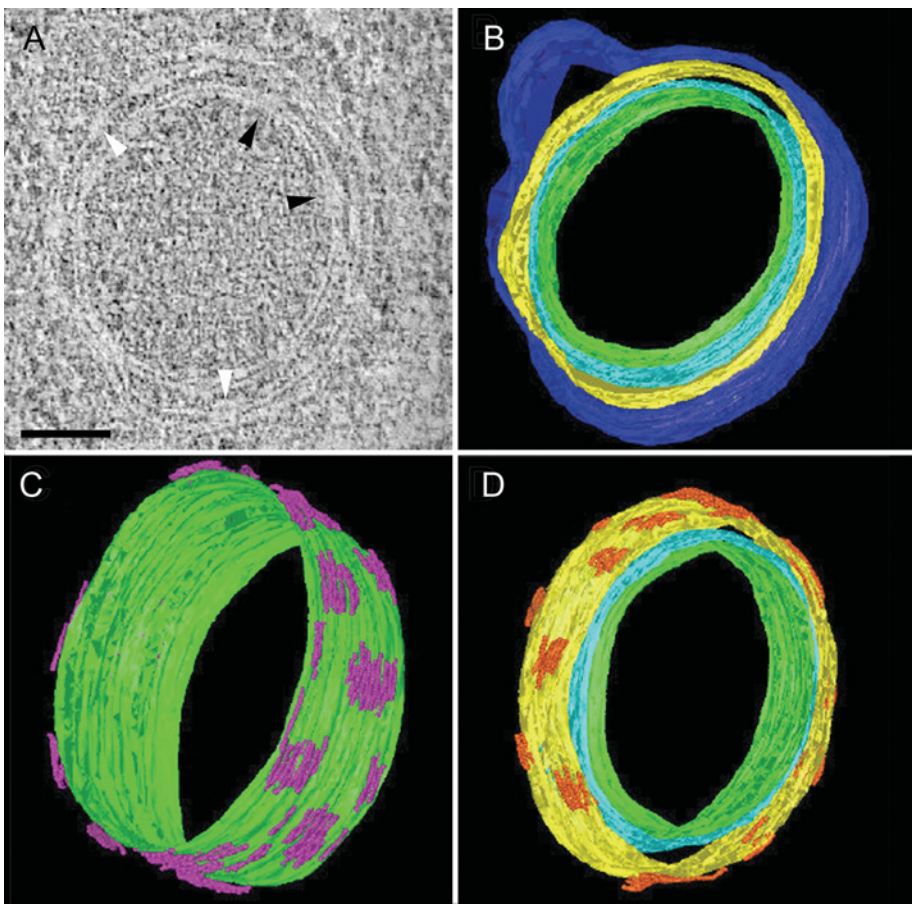
membranes are individual structures and each continuous in three dimensions (Figure 4). Though sometimes touching each other, they do not cross over. The equidistance of the two inner membranes and the variable distance of the two outer membranes are evident. The protuberances of the fourth membrane sometimes are large and extend deep into the parasite cytoplasm (Figures 2 and 4). Patches of lighter mass densities are bridging the two innermost membranes. There are also patches of lighter mass densities located on the periplastid membrane (Figures 2A and 4A).

Discussion

To study the role of the apicoplast for the parasite–host cell interaction, it is important to have reliable morphological information. The time-consuming conventional chemical fixation procedures are known for their rather poor preservation of the cellular ultrastructure. Especially membranous continuities are vulnerable to fixation artefacts (Ebersold et al., 1981; Dubochet et al., 1983; Szczesny et al., 1996; Murk et al., 2003). It is generally accepted that cryofixation methods provide better ultrastructural preservation (Steinbrecht and Zierold, 1987; Dubochet et al., 1988; Müller, 1992). The most favourable follow-up method would be cryosectioning and imaging in the frozen-hydrated stage (Dubochet et al., 1988). Whereas cryosectioning is working well for thin sections of approx. 100 nm, adequate thick sections of approx. 300 nm needed for electron tomography cannot be made (Al-Amoudi et al., 2005). Therefore the next best option is freeze substitution, which has proven to give a reliable view of the cellular ultrastructure (Van Harreveld et al., 1965; Steinbrecht, 1980; Ebersold et al., 1981; Humbel and Schwarz, 1989; Engfeldt et al., 1994; Kaneko and Walther, 1995). Furthermore, it could be shown by freeze substitution with acetone that only 5% of the lipids are extracted (Weibull et al., 1984). In addition, epoxy resins act as an additional fixative (Matsko and Müller, 2005). In summary, cryofixation followed by freeze substitution and embedding in

Figure 4 | Three-dimensional models of the apicoplast membranes and the protein complexes in the different apicoplast membranes

(**A**) A median slice of the apicoplast in *Sarcocystis* generated from a double-tilt tomographic reconstruction. The image shows the four continuous membranes, the protein complexes in the inner membrane (black arrowheads), the protein complexes in the outer membrane (white arrowheads) and a protuberance of the outer membrane into the cytoplasm of the parasite (asterisk). Scale bar, 100 nm. (**B**) Three-dimensional model of the four membranes surrounding the apicoplast: in green the innermost membrane, in light blue the second membrane, in yellow the periplastid membrane and in dark blue the outermost membrane are shown. In the upper left part of the image, a clear protuberance of the outer membrane can be observed. (**C**) The model illustrates the distribution and size of the protein complexes (violet) between the two inner membranes. For clarity, only the innermost membrane (in green) is shown. Note that the model is not scaled correctly with respect to models (**B**, **D**). (**D**) Distribution of the protein complexes (orange) in the third periplastid membrane. A Supplementary Video to accompany this Figure can be seen at http://www.biolcell.org/boc/098/boc0980535add.htm.



Epon is the best compromise to preserve the cellular architecture with high fidelity and to be able to cut thick sections for electron tomography.

In combination with improved ultrastructural preservation by cryofixation methods, transmission electron tomography can add valuable three-dimensional information. The laborious technique of serial thin sectioning (Hopkins et al., 1999) can be circumvented, thereby reducing material loss between individual sections and allowing the analysis of larger numbers and volumes of the apicoplast. The Z-resolution of transmission electron tomography is approx. 10 times higher (in the range of 5–8 nm) than in reconstructions of serial sections (50–80 nm section thickness). Combining two single-tilt tomograms into a double-tilt tomogram reduces the

loss of information caused by the missing wedge (Mastronarde, 1997).

By combining and applying these state-of-the-art methods, the present study gives new insights concerning the morphology of the apicoplast and interpretation of already existing data.

Most of the proteins in the apicoplast are encoded in the nuclear genome of the parasite, synthesized on the cytoplasmic ribosomes and post-translationally imported into the apicoplast (Waller et al., 1998; Roos et al., 1999). In order to get a better understanding of the import mechanism, it is relevant to know how many membranes enclose the lumen of the plastid and whether they are continuous. The number of membranes is also indicative of the endosymbiotic origin of the apicoplast. In our cryofixed and freeze-substituted material, we now clearly show that four membranes surround the apicoplast. This is in agreement with the commonly accepted secondary endosymbiosis theory (Delwiche and Palmer, 1997; McFadden and Waller, 1997; Douglas, 1998; Cavalier-Smith, 1999). The four membranes are continuous without any visible tightly adjoined intervals or flattened structures in contrast with previous and most recent reports (Hopkins et al., 1999; Köhler, 2005). In *Plasmodium* (Hopkins et al., 1999), there were three membranes found to enclose the apicoplast and in *Toxoplasma* (Köhler, 2005) two. This difference might be a species-specific phenomenon or a metabolically dependent fact. On the other hand, this alteration in the numbers of membranes surrounding the apicoplast is in contradiction with the theory of a secondary symbiosis (Cavalier-Smith, 1999).

The two inner membranes are regularly shaped, almost circular membranes with a constant distance of 4–6 nm from each other. The third and the fourth membranes are less regularly shaped. The outer membrane displayed various outside-directed protuberances. The diverse shapes, sizes and number suggest that these protuberances might be involved in dynamic processes (Figure 2). This suggests that vesicular transport could be one of the ways for protein trafficking between cytosolic compartments and the apicoplast. Some experimental support for this notion comes from apicoplast-deficient cells of *Toxoplasma gondii*, in which apicoplast-targeted green fluorescent protein has been observed in vesicles located in the apical region of the cell (He et al., 2001b). The bilobed appearance of the apicoplast (Figures 2C and 2D) further substantiates the idea that the cyst of *Sarcocystis*, referred to also as a dormant state, is actually metabolically active. It is tempting to speculate that the apicoplast is dividing.

On the other hand, the clearly visible contact points between the ER and the outermost membrane suggest that ER–apicoplast protein transport could also occur directly. This suggestion takes into consideration that analogous contacts between the mitochondria and the ER were observed in rat liver cells (Mannella et al., 1998). Furthermore, physiological and structural interactions between mitochondria and the ER in terms of membrane and/or protein flux have been described previously (Pitts et al., 1999; Simmen et al., 2005).

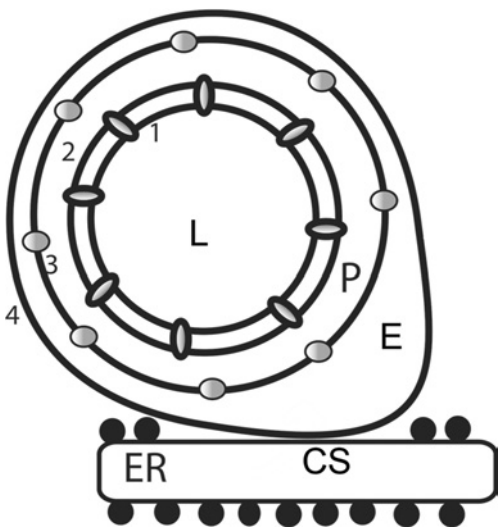
Gibbs hypothesized that the outermost membrane of plastids, bound by four membranes like the apicoplast, is derived from the ER (Gibbs, 1981). Although we found the ER in close proximity and with contact points to the apicoplast, we found no unequivocal proof for the continuity of the ER into the outer apicoplast membrane. Additionally, no ribosomes were present on the outermost apicoplast membrane. Therefore our results do not confirm the hypothesis that the outer membrane is a part of the ER.

The previously reported direct contact points between mitochondria and the apicoplast in *Plasmodium falciparum* (Van Dooren et al., 2005) could not be confirmed for *Sarcocystis* with our studies by electron microscopy.

The clearly visible mass densities, patches, between the two inner membranes and in the periplastid membrane most likely represent protein complexes. The regular distribution and the regular size of the patches favour the idea that these are real structures and not storage places or an accumulation of intermediates in transport. The exact nature and function of these protein complexes are not clear at the moment, but it is tempting to speculate that they might be involved in import of proteins and/or ions over the periplastid and the two inner membranes either by forming membrane pores or as transporter complexes. Our observations are in agreement with Cavalier-Smith's hypothesis (Cavalier-Smith, 1999), who favours the idea that transit peptide receptors are located in both the periplastid membrane and the second inner membrane of the apicoplast and that the same transit peptide is used serially to direct import across two successive membranes (the two inner membranes;

Figure 5 | Hypothetical model for the import of proteins encoded by the parasite nuclear genome into the lumen of the apicoplast

The schematic drawing represents a hypothetical model for the import of proteins encoded by the parasite nuclear genome into the lumen of the apicoplast. The model is based on the observed data in the present study and previously proposed models of protein import into plastids with three or four membranes (Cavalier-Smith, 1999; Van Dooren et al., 2001). It is hypothesized that there is a direct transport from the ER to the exoplastid space (E) via the contact sites (CS); from the exoplastid space (E), proteins could be translocated into the lumen via the protein complexes (patches), e.g. pores or transporters. 1, first inner membrane; 2, second inner membrane; 3, periplastid membrane; 4, outer membrane; L, lumen; P, periplastid space; E, exoplastid space; CS, contact site.



see Figure 5). In neither of the tomograms recorded, however, any evidence for the postulated periplastid vesicles was found.

It is known that the apicoplast targeting sequences are rich in asparagine residue, lysine residue and basic amino acids. This positively charged transit peptide is suggested to be electrophoretically pulled into the apicoplast lumen by a series of negatively charged transmembrane pores (Van Dooren et al., 2001; Foth et al., 2003). So far there are no clear reports of translocator components in the apicomplexan genome except for the hypothetical *Plasmodium* protein (GenBank® accession no. NP_705561; GI:23619599), which has some similarity to Toc 159 (translocon of the outer chloroplast envelope-159) (Schleiff et al., 2003; Soll and Schleiff, 2004) from pea with a leader sequence appropriate for plastid targeting (Nassoury and Morse, 2005). It is worthwhile to mention that the size of the published Toc 159 complex (height: 10–12.5 nm; diameter: 12–14 nm) is approximately one-fourth of the size of the mass densities observed in our tomograms (height: 15–20 nm; diameter: 60–80 nm).

Combining these observations (Figure 5), we hypothesize that apicoplast-specific proteins are transported into the space surrounded by the outermost membrane, either by vesicular transport and/or by direct import via ER contact points. This hypothesis is also based on previously proposed models on protein import into plastids with three or four membranes by Cavalier-Smith (1999) and is also in agreement with the later refined version of the same model by Van Dooren et al. (2001). It is suggested that protein complexes mediate translocation across the inner two membranes. This pore-like structure is proposed to be a specific protein complex, possibly a duplicate of the Toc apparatus (Van Dooren et al., 2000). A comparable import apparatus to the Tic–Toc system of plant chloroplasts is offered in the model for targeting host-encoded proteins into plastids of secondary endosymbiotic origin by McFadden (1999).

The protuberances are considered to indicate a dynamic process like vesicle formation. As already suggested by Van Dooren et al. (2001), secretory proteins lacking a transit peptide pass the apicoplast and are taken up by vesicles that bud from the outermost membrane, from where these vesicles carry proteins to another compartment of the secretory pathway (Van Dooren et al., 2001). It is also possible that these vesicles consist of cargo from the apicoplast itself. The validity of this suggestion has still to be proven by experimental data. In the model proposed here, it is hypothesized that there is a direct transport from the ER to the exoplastid space (Figure 5; 'E') via the contact sites (Figure 5; 'CS'). From there, they are directed into the apicoplast lumen passing through the pores formed by protein complexes in the periplastid membrane and in the two inner membranes. In the future, the hypothesis has to be tested with biochemical and molecular biological techniques in combination with specific immunolabelling studies.

Material and methods

Sample preparation

Sarcocystis cysts were isolated from samples of tongue muscle from naturally infected roe deer (*Capreolus capreolus*). Cysts were kept in 1 × PBS (pH 7.4) at 4°C for 2 days before further processing for electron microscopic examination. The cysts were transferred to specimen carriers containing 20% (w/v) BSA in M-9 buffer (22 mM KH_2PO_4, 19 mM NH_4Cl, 48 mM Na_2HPO_4 and 9 mM NaCl, pH 7.2) and cryofixed by high-pressure freezing (EM PACT2 + RTS; Leica Microsystems, Vienna, Austria) (Manninen et al., 2005) and freeze-substituted in a freeze-substitution medium consisting of anhydrous acetone, 1% osmium tetroxide and 0.1% uranyl acetate (McDonald and Müller-Reichert, 2002). The samples were kept at –90°C for 36 h, at –30°C for approx. 5 h and finally brought to room temperature (20°C) for 1 h in a freeze-substitution unit (AFS, Leica Microsystems). After removing osmium tetroxide and uranyl acetate with acetone, the samples were gradually infiltrated with Epon/Araldite resin (Mollenhauer, 1964). Cysts were embedded in thin layers of resin on microscope slides (Müller-Reichert et al., 2003).

Selected cysts were remounted on 'dummy' blocks for ultramicrotomy. Sections were cut using a Reichert Ultracut microtome (Leica Microsystems). Ultra-thin (~80 nm) and semi-thin (250–300 nm) sections were collected on Formvar–carbon-coated copper hexagonal 50 mesh grids and post-stained with 20% (w/v) uranyl acetate in 70% (v/v) methanol/water followed by Reynolds's lead citrate staining (Reynolds, 1963).

Electron tomography

Before recording electron microscopy projections of semi-thin sections (250–300 nm), 10 nm colloidal gold particles were applied on one surface of the sections to function as fiducial markers for subsequent image alignment. The specimens were placed in a high-tilt specimen holder (Fischione type 2020; Fischione Instruments, Pittsburgh, PA, U.S.A.) and datasets were recorded at 200 kV (Tecnai 20 LaB_6; FEI Company, Eindhoven, The Netherlands). Angular tilt range was from −65° to +65° with an increment of 1°. Images (1024 × 1024 square pixels) were recorded using a CCD (charge-coupled-device) camera (Temcam F214; TVIPS GmbH, Germany). The sections were pre-irradiated to avoid shrinking effects during recording (Luther, 1992). Automated data acquisition of the tilt series was carried out using Xplore 3D (FEI Company). For dual axis tomography (Penczek et al., 1995), the grids were manually rotated over 90°, and a second tilt series was acquired over the same tilting range. For image alignment, the colloidal gold particles were used as fiducial markers. Tomograms were computed for each tilt axis using the *R*-weighted back-projection algorithm and combined into one double-tilt tomogram with the IMOD software package (Kremer et al., 1996). No additional filtering of the raw images was applied. We recorded and reconstructed in total more than 20 double-tilt series of the apicoplast.

Modelling and analysis of tomographic data

Double-tilt tomograms were analysed and modelled using the IMOD software package (Kremer et al., 1996). Features of interest were contoured manually in serial optical slices extracted from the tomogram. The 'image slicer' window in IMOD was used to facilitate the recognition of membranous structures. Three-dimensional models were displayed and rotated to study its three-dimensional geometry.

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