Toxoplasma gondii: further studies on the subpellicular network

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The association of the pellicle with cytoskeletal elements in Toxoplasma gondii allows this parasite to maintain its mechanical integrity and makes possible its gliding motility and cell invasion. The inner membrane complex (IMC) resembles the flattened membrane sacs observed in free-living protozoa and these sacs have been found to associate with cytoskeletal proteins such as articulins. We used immunofluorescence microscopy to characterise the presence and distribution of plateins, a sub-family of articulins, in T. gondii tachyzoites. A dispersed labelling of the whole protozoan body was observed. Electron microscopy of detergent-extracted cells revealed the presence of a network of 10 nm filaments distributed throughout the parasite. These filaments were labelled with anti-platein antibodies. Screening the sequenced T. gondii genome, we obtained the sequence of an IMC predicted protein with 25% identity and 42% similarity to the platein isoform alpha 1 present in Euplotes aediculatus, but with 42% identity and 55% similarity to that found in Euglena gracilis, suggesting strong resemblance to articulins.

Key words: Toxoplasma gondii - cytoskeleton - articulin - platein - subpellicular network

Toxoplasma gondii is an important opportunistic pathogen that can lead to serious complications in congenitally infected newborns and in HIV-positive individuals and other immunocompromised patients (Israelski & Remington 1993, Luft et al. 1993, Wong & Remington 1994).

In order to survive in different challenging environments, such as the digestive tract and the circulatory system of hosts (i.e., both intra and extra-cellular milieu), T. gondii must maintain its structural integrity, a property greatly dependent upon the organism’s pellicle and cytoskeletal components. The association of the plasma membrane with a system of flattened vesicles composes the pellicle. Connected with this structure, there are 22 subpellicular microtubules running down two-thirds of the parasite from its apical pole (De Souza et al. 2009). A network of interwoven filaments called the subpellicular network extends along the parasite’s entire cell body (Mann & Beckers 2001). This network is resistant to detergent extraction and is also connected to the pellicle, suggesting that it functions as a part of the membrane skeleton in T. gondii.

Membrane skeletons are commonly found in free-living protozoa, maintaining the cell shape and providing mechanical stability. Examples of such cytoskeletal proteins are the tetrins, described in Tetrahymena (Honts & Williams 1990, Brimmer & Weber 2000), the epiplasmins, identified in Paramecium (Nahon et al. 1993, Coffe et al. 1996, Pomel et al. 2006) and other protists (Huttenlauch et al. 1998, Bouchard et al. 2001), and the articulins, described in the cortex of the euglenoid Euglena gracilis (Marrs & Bouck 1992) and the ciliate Pseudomicrothorax dubius (Huttenlauch et al. 1998). Kloetzel et al. (2003) first described a sub-family of articulins, the plateins, which are the major structural components of a monolayer of flattened scales (the “alveolar plates”) located within membranous sacs (cortical alveoli) in the free-living protozoon Euplotes aediculatus. As the alveolar plates of these free-living protozoa constitute a skeletal system related to that described in Apicomplexa, we decided to use specific antibodies recognising these structures to determine if articulins are found in T. gondii. Our present results suggest that components of the subpellicular network have a common nature with cytoskeletal elements of free-living protozoa.

MATERIALS AND METHODS

Indirect immunofluorescence - T. gondii RH strain tachyzoites were harvested in MTSB (4 M glycerol, 100 mM PIPES, 1 mM EGTA, 5 mM MgCl₂), pH 6.8 and fixed in 2% paraformaldehyde in the same buffer. After fixation, the cells were rinsed in MTSB, allowed to adhere to previously poly-l-lysine-coated coverslips and permeabilised with 0.2% (v/v) Triton X-100 for 10 min. After washing, unspecific sites were quenched with 3% (w/v) bovine serum albumin solution for 1 h and the cells were incubated with undiluted primary antibody - mouse monoclonal mAB PL-5 (which recognises all platein isoforms) or 1/10 dilution of the rabbit polyclonal AP-2 (which recognises only alpha platein isoforms) (Kloetzel et al. 2003). After 1 h of incubation at RT, parasites were washed and incubated with the corresponding secondary antibodies (conjugated to AlexaFluor® 488; Molecular Probes) at RT for 1 h. Coverslips were observed in a Zeiss Axioplan microscope equipped with a fluorescein filter set. The images were acquired with a colour-chilled CCD camera (Hamamatsu, Japan).

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Immunoelectron microscopy of uranyl acetate stained preparations - Tachyzoites were incubated with MTSB containing 1% (v/v) Triton X-100, 1% (v/v) NP-40 for 20 min. Detergent-treated parasites were allowed to settle onto formvar-coated copper grids for 10 min, blocked with 3% bovine serum albumin and incubated with 0.01% Tween 20 for 1 h. Afterwards, grids were incubated in a humid chamber with mAB PL-5 or AP-2 at the same dilutions used for fluorescence microscopy for 1 h. Grids were then incubated with secondary antibody conjugated to 10 nm gold particles (BBInternational) for 1 h and fixed in 2.5% glutaraldehyde. For staining, parasites were incubated for 1 min with 1% (v/v) uranyl acetate. After washing in distilled water, the grids were observed in a Jeol 1,200 EX transmission electron microscope.

Sequence analysis - Predicted protein sequences were analysed using public databases at the BLAST program (http://www.ncbi.nlm.nih.gov) and the T. gondii genome project (http://toxodb.org). Peptide alignments were performed using ClustalW (European Bioinformatics Institute).

RESULTS AND DISCUSSION

Uranyl acetate negative staining of detergent-extracted tachyzoites of T. gondii provided a full visualisation of the cytoskeleton arrangement (Fig. 1A). The interwoven filaments of the subpellicular network, approximately 10 nm in diameter, extended homogeneously along the entire length of the parasite. The subpellicular microtubules, which originate from the conoid, do not reach the posterior portion of the protozoan. The thin filaments observed appeared to surround the subpellicular microtubules in the anterior region of the protozoan (Fig. 1A). They ended in a well-delimited circular structure, which ranged from 300-650 nm in diameter (Fig. 1A), localised at the posterior tip. This structure, recently characterised as a basal complex, contains proteins such as TgMORN1, TgCentrin 2 and a Dynein light chain (Hu 2008). Apparently, the subpellicular network provides the shape of the parasite. In daughter parasites, however, this structure is loose and yet susceptible to detergent extraction, to be expanded and remodelled during growth of the parasites (Mann et al. 2002). This unstable structure changes into the rigid and stable structure in mature parasites, as observed in our detergent extraction preparations.

Fluorescence microscopic analysis using the PL-5 antibody, which recognises all platein isoforms and AP-2, which recognises the alpha-platein isoform only, showed a dispersed labelling of the tachyzoites (Fig. 2B, D). The labelling pattern was similar to that found by Mann and Beckers (2001), using antibodies that recognise two proteins [Tg-inner membrane complex (IMC) 1 and 2] isolated from the protozoan cytoskeleton. No labelling was observed in negative controls when we omitted the primary antibodies (data not shown). The concentrations of the antibodies were the same as those used by Kloetzel et al. (2003) in E. aediculatus. Although the concentrations of antibodies were high, the labelling was specific in both EM and IF preparations. The localisation of platein-like proteins using immunoelectron microscopy of detergent-extracted samples with the same antibodies showed a labelling in the elements which form the subpellicular network, as can be seen in Fig. 1B. This labelling pattern also resembles that observed using antibodies recognising actin (Patron et al. 2005), indicating that both microfilaments and articulins are associated with the subpellicular network.

Plateins are N-terminal signal polypeptides (Kloetzel et al. 2003). Their canonical hydrophobic signals direct the plateins within the membrane-limited alveolar sacs, where they are assembled into plates. T. gondii subpellicular network elements do not form plates (M Attias, W De Souza 2009, unpublished observations), unlike those observed in E. aediculatus; however, these structures are in close contact with a group of flattened cisternae, the IMC that may be a specialised region of the endoplasmic reticulum (De Melo & De Souza 1997).
In the first description of the subpellicular network, the authors proposed the interaction of this structure with subpellicular microtubules and these structures that were in contact with the cytoplasmic face of the IMC, forming the *T. gondii* membrane-skeleton (Mann & Beckers 2001). Patron et al. (2005) have proposed that the subpellicular network may also be in contact with the plasma membrane face of the IMC and may take part in the organism’s gliding machinery, as *T. gondii* actin has been found to be associated with this structure. The subpellicular network could thus anchor *Toxoplasma*’s cortical myosin in the IMC membrane, thereby providing mechanical support to the parasite, maintaining its shape during movement as well as during the invasion of host cells. This model is in accordance with the observations made by Khater et al. (2004) of *Plasmodium* sporozoites lacking the membrane skeleton protein PbIMC1, a structural orthologue of the TgIMC1. These mutant parasites demonstrated reduced motility, infectivity and resistance to osmotic shock relative to wild-type organisms. However, in our negative staining preparations, we could not establish any connection of the filaments to the IMC in *T. gondii*.

Using the BLAST program and the *T. gondii* genome resource (ToxoDB), we obtained a protein sequence (access ID 42.m03481) similar to the alpha-1 platein isoform (25% identity and 42% similarity) (Fig. 3), predicting a function for this sequence. The same protein sequence was obtained when blasting with alpha-2 isoform. Using the beta/gamma platein isoform, we obtained another sequence; however, this sequence had low similarity and was thus not considered further. We used the program SignalP to determine whether the sequences obtained also presented an N-terminal signal sequence, a hallmark characteristic of plateins. No signal sequence was identified, however, indicating that the sequence identi-
fied (42.m03481) did not correspond to a secreted protein and therefore it could not be considered a platein. Interestingly, the sequence obtained has 42% identity and 55% similarity with *E. gracilis* articulin, suggesting that the sequence is more closely related to articulins than plateins (Fig. 4). Since plateins are a sub-family of articulins, they share common sequences, explaining why we observed a labelling with the antibodies for platein isoforms. The blast score obtained with *E. gracilis* was higher than that obtained by Mann and Beckers (2001), comparing TgIMC1 and *E.* articulin (37% identity and 45% similarity). This particular *T. gondii* sequence shares only a weak identity (26%) with the TgIMC1 sequence obtained by Mann and Beckers (2001), even though the encoded proteins appear to occupy a common location within the protozoan. This suggests that we have detected an additional member of the IMC family of genes in this parasite, expressing a protein with a resemblance to articulins.

Overall, our present results, in comparison with the findings of others, led to two important conclusions. First, they support the general pattern that cytoskeletal elements in protozoa seem to be comprised of multiple forms of related proteins; this is exemplified by the multiple fibre-forming tetrins found in *Tetrahymena* (Brimmer & Weber 2000, Bouchard et al. 2001) and the platein variants found together in the alveolar plates of *Euglena* (Kloetzel et al. 2003). Secondly, our findings suggest that subpellicular network filaments in *Toxoplasma* share several common features with cytoskeletal elements of free-living protozoa. We thus infer that articulins and the IMC proteins of Apicomplexa share a common ancestry. 

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**REFERENCES**


