# Alveolins, a New Family of Cortical Proteins that Define the Protist Infrakingdom Alveolata

Sven B. Gould,\* Wai-Hong Tham,† Alan F. Cowman,† Geoffrey I. McFadden,\* and Ross F. Waller\*

\*School of Botany, University of Melbourne, Victoria, Australia; and †Walter & Eliza Hall Institute of Medical Research, Melbourne, Victoria, Australia

Alveolates are a recently recognized group of unicellular eukaryotes that unites disparate protists including apicomplexan parasites (which cause malaria and toxoplasmosis), dinoflagellate algae (which cause red tides and are symbionts in many corals), and ciliates (which are microscopic predators and common rumen symbionts). Gene sequence trees provide robust support for the alveolate alliance, but beyond the common presence of membranous sacs (alveoli) subtending the plasma membrane, the group has no unifying morphological feature. We describe a family of proteins, alveolins, associated with these membranous sacs in apicomplexa, dinoflagellates, and ciliates. Alveolins contain numerous simple peptide repeats and are encoded by multigene families. We generated antibodies against a peptide motif common to all alveolins and identified a range of apparently abundant proteins in apicomplexa, dinoflagellates, and ciliates, and ciliates. Immunolocalization reveals that alveolins are associated exclusively with the cortical regions of apicomplexa, dinoflagellates, and ciliates where the alveolar sacs occur. Alveolins are the first molecular nexus between the unifying structures that defines this eukaryotic group. They provide an excellent opportunity to explore the exceptional compartment that was apparently the key to a remarkable diversification of unique protists that occupy a wide array of lifestyle niches.

## Introduction

Eukaryotic microbiology is in flux. Traditional systematic schemes based largely on light microscopy have had to be revised and rerevised with the advent of electron microscopy then phylogenetic inference from gene sequence data. Protist classification schemes of the 19th and 20th centuries have been almost completely dismantled and a new system of supergroups, which seeks to incorporate all available information, installed. Thus, opisthokonts now unite fungi and animals; rhizaria assembles the foraminifera, the cercozoan filose amoebae, and most of the traditional radiolaria; excavata includes the kinetoplastids, euglenids, diplomonads, and oxymonads; amoebozoa includes the traditional amoebae and the slime molds; archaeplastida unites the red algae, green algae and plants, and the glaucophyte algae; and the chromalveolates embraces the cryptophytes, haptophytes, heterokontophytes, chrysophytes, oomycetes, dinoflagellates, apicomplexa, and ciliates. Some of these alliances were recognized before the advent of electron microscopy and gene phylogenies, but many are novel and still controversial (Cavalier-Smith 1993; Baldauf et al. 2000; Simpson and Roger 2002; Adl et al. 2005, 2007; Keeling et al. 2005; Burki et al. 2007).

The group known as the "alveolates" (which unites apicomplexan parasites, dinoflagellate algae, and the ciliates) is a recently recognized alliance of diverse protists with very different morphologies and lifestyles. The alveolates are tremendously important protists in health, environment, and agriculture. The apicomplexa includes major human and livestock parasites that cause malaria, toxoplasmosis, and coccidiosis (Adl et al. 2005). Malaria kills millions of people annually (Sachs and Hotez 2006), and coccidiosis is the single most important infection in the poultry industry with an

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E-mail: r.waller@unimelb.edu.au

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estimated economic burden of 1.4 billion dollars to poultry farmers (Williams 1999). Dinoflagellates are key marine phytoplankton contributing to ocean primary productivity and carbon cycling. Dinoflagellate symbionts (zooxanthellae) of corals and other invertebrates are vital for healthy coral reefs providing the animal hosts with carbon and energy in food-depleted tropical waters (Little et al. 2004). Dinoflagellates are also harmful algae causing red tides, ciguatera poisoning, and large kills of fish stocks. Ciliates are major environmental micropredators (Johansson et al. 2004), important members of livestock rumen flora (Williams and Coleman 1988), and the cause of the human diarrheal disease balantidiosis (Schuster and Visvesvara 2004).

Due to their extreme diversity, the concept of an alveolate group was slow to emerge. Taylor (1974) alluded to a possible affinity between dinoflagellates and ciliates in 1974 but never formalized an alliance. Subsequently, Cavalier-Smith (1987) allied the dinoflagellates and the apicomplexan parasites as branch Miozoa, and this grouping along with ciliates was later confirmed with the advent of rRNA phylogeny by Wolters (1991). In the same year (1991), Cavalier-Smith (1991) recognized the importance of alveoli, a system of membranous sacs subtending the plasma membrane, as a unifying morphological character and linked apicomplexa and dinoflagellates with ciliates to create infrakingdom Alveolata. The group Alveolata was initially treated with caution, but as more and more sequence data emerged, phylogenies continued to ally the 3 types of protists into a robust clade and alveolates entered the lexicon of protistology and features in all modern schemes despite the paucity of obvious similarities among members beyond alveoli (Baldauf et al. 2000; Simpson and Roger 2002; Keeling et al. 2005; Burki et al. 2007). Recent taxonomic schemes continue to retain this group as either phylum Alveolata (Hausmann et al. 2003) or infrakingdom Alveolata (Adl et al. 2005, 2007).

Alveoli consist of single-membrane flattened sacs probably derived from the endomembrane system. Although they vary enormously in shape and arrangement among the different members of the alveolates, they always

subtend the plasma membrane, in many cases, creating the appearance of 3 membranes around an alveolate cell. In apicomplexan parasites, alveoli have been termed inner membrane complex (IMC), but we endorse the term alveoli recognizing these structures as homologues. The alveoli of apicomplexa are vital to the parasite's motility and invasion of host cells and hence of key medical significance (Gaskins et al. 2004; Sibley 2004; Soldati et al. 2004). Apicomplexan alveoli are most conspicuous in the motile stages that seek out and invade host cells. A system of actin/myosin generates gliding motility by transposing transmembrane proteins (which have external attachments to the substrate) through the cell membrane by bearing against the alveolar (inner membrane) complex (Gaskins et al. 2004; Sibley 2004; Soldati et al. 2004). In dinoflagellates, no such gliding motility is known, and the dinoflagellate alveoli (referred to as amphiesmal vesicles by phycologists) can contain cellulosic armored plates. Unarmored (naked) dinoflagellates also possess alveoli, but they contain no detectable cellulose and their function is unknown (Dodge 1987). In ciliates, the alveoli are part of the complex cortical epiplasm comprising ejectile structures known as extrusomes, the basal bodies of the cilia, and an intricate cortex of cytoskeletal elements (Hausmann and Bradbury 1996).

Recently several protein components from the alveoli of apicomplexan parasites have been identified in studies seeking to understand the role of alveoli in host cell invasion. Several of these proteins share repetitive domains of high sequence similarity suggestive of a common origin, and homologues have been identified in several diverse apicomplexan groups (Mann and Beckers 2001; Mann et al. 2002; Gubbels et al. 2004). Following the apicomplexan nomenclature, these proteins were previously referred to as IMC proteins, but we will call them alveolins (see supplementary table S1, Supplementary Material online, for gene nomenclature and Discussion for justification of a new name). Little is known about the functions of apicomplexan alveolins, and their exact location within the alveolar zone is not yet clear.

Given the ubiquity of alveolins in apicomplexan parasites and their association with the alveoli, we decided to search for homologues in dinoflagellates and ciliates where alveoli also occur. Our goal was to find proteins associated with the sole conspicuous morphological feature that unites the infrakingdom Alveolata. Recently, a complete genome became available for the ciliates *Tetrahymena* (Eisen et al. 2006) and Paramecium (Aury et al. 2006), and a substantial number of expressed sequence tags (EST or cDNAs) sequences are available from dinoflagellates (Bachvaroff et al. 2004; Hackett et al. 2004; Patron et al. 2006), creating an opportunity to identify shared proteins with roles in alveolar activities. Here, we describe the identification of numerous alveolins in apicomplexa, dinoflagellates, and ciliates. We use antibodies to demonstrate that these alveolins are expressed in all Alveolata, and we localize the proteins to the subplasmalemmal region in close association with alveolar membranes.

## **Material and Methods**

Cultures, EST Libraries, and cDNA Sequencing

Karlodinium veneficum (Dinoflagellate) was cultured at 16 °C and a 12-h light/dark cycle in sterile, filtered seawater (Queenscliff, Victoria, Australia) supplemented with f/2 AlgaBoost (AusAqua, Wallaroo, Australia). As an EST resource of K. veneficum, we used the library generated previously by Patron et al. (2006), and alveolin cDNAs were recovered from bacterial stocks and fully sequenced. The EST sequences from Oxyrrhis marina were provided by Dr Patrick Keeling (Canadian Institute for Advanced Research, Canada). The parasites Toxoplasma gondii (Apicomplexa) and *Plasmodium falciparum* (Apicomplexa) were cultured as described previously (Striepen et al. 1998; Trager and Jensen 1976). Paramecium caudatum (Ciliata), and P. caudatum growth medium containing Chilomonas sp. (Cryptophyta) as a food source, were obtained from Southern Biological (Victoria, Australia) and cells grown in petri dishes at room temperature under low light conditions. New K. veneficum and O. marina sequences were deposited in EMBL Webin (AM931978-AM931982).

## Antibodies

The 2 peptide epitopes EKVIEVPQTQVMEKV (Alv1.1) and EVVRQVPRPQTVELT (Alv1.2) were synthesized by Invitrogen (Invitrogen, Carlsbad, CA) and used for rabbit immunization to generate  $\alpha$ Alv1.1 and  $\alpha$ Alv1.2. Preimmune sera and 10-week bleeds were tested for their reactivity to Western blots of denatured protein extracts from *K. veneficum*, *T. gondii*, and *P. falciparum* 3D7 using a dilution of 1:2,000 and 1:500. The preimmune sera were also tested in equivalent concentrations to the 10-week bleeds in the immunofluorescent assays and immunogold labeling for transmission electron microscope (TEM) analysis, which resulted in no labeling (data not shown).

#### Immunofluorescence Assays

*Karlodinium veneficum* and *P. caudatum* cells were fixed in their growth medium using a standard 4% paraformaldehyde-fixing protocol. Triton X-100– permeabilized cells were incubated with the primary antibodies in phosphate-buffered saline (PBS) for 60 min and secondary antibody ( $\alpha$ -rabbit Alexa-Fluor 488 and 633, Invitrogen) for 30–60 min. The 4',6-diamidino-2-phenylindole was added at a final concentration of 1 µg/ml and washed out after 10–12 min of staining with 2 times 2 volumes of PBS. *Toxoplasma* was fixed as described by Striepen et al. (2001). Images were taken on a Leica TCS2 confocal laser scanning microscope (Wetzlar, Germany) and processed with Leica LCS Lite and Photoshop CS3 for 3-dimensional (3D) image calculation and assembly, respectively.

## Electron Microscopy

All cells were fixed in their growth medium with 3% paraformaldehyde and 1% glutaraldehyde and pelleted cells embedded in 1% low-melting agarose. The agarose block was then dehydrated in 5 steps of 25, 50, 70, and twice 100% ethanol for 30 min each step and subsequently embedded in Lowicryl White Resin (ProSciTech, Thuringowa, Australia) through a 5-step series of increasing amounts of

Lowicryl white resin from 25% to 100%. In each step, the agarose blocks were incubated for 30 min, except the final one, which was incubated for 5 h or overnight. After overnight polymerization at 58 °C, sections of 90 nm were cut on a Leica Ultracut R and collected on gold grids. Sections were blocked with 0.8% bovine serum albumin and 0.01% Tween 80 in PBS for 1 h prior to  $\alpha$ Alv1.1 and  $\alpha$ Alv1.2 antibody incubation at a concentration of 1:50 to 1:1,000 for 2 h and subsequent secondary 10- or 18-nm colloidal gold antibodies (Jackson ImmunoResearch, Baltimore, MD) at a concentration of 1:20 overnight. Immunolabelled sections were contrasted with 2% uranyl acetate for 2–4 min followed by 5% lead citrate for 10–30 s according to the sections' requirements.

## Results

Alveolins, a Family of Proteins Common to All Members of Alveolata

Apicomplexan alveolin proteins from Toxoplasma (TgALV1, 3, 4) and Plasmodium (PfALV1) contain multiple repeat domains of high sequence similarity and, for *Toxoplasma* at least, are known to localiz in close proximity of the alveoli (Mann et al. 2002; Gubbels et al. 2004; Khater et al. 2004). The repetitive region is variable (e.g., 2 repeats within 69 amino acids in TgALV1 or 13 repeats within 202 amino acids in PfALV2) and is flanked by unique N- and Cterminal peptide sequences, resulting in considerable size variation among alveolins. Nonetheless, these alveolins constitute a family of related, but diverse apicomplexan proteins, all of which are implicated in alveolar function. We used these proteins to search for similar protein sequences in a range of available genomic databases from other members of the Alveolata, including dinoflagellates, ciliates, and other apicomplexa. Strong sequence matches were found in all alveolate groups for which broad genomic data exists. At least 2 putative alveolins were found in each organism, but up to 50 unique genes were recovered from some taxa.

We uncovered the largest diversity of alveolins in the apicomplexan parasites. In addition to the previously described *Tg*ALV1-4 and *Pf*ALV1, we identified a further 8 alveolins in *T. gondii* and a total of 7 in *P. falciparum* (see supplementary table S1, Supplementary Material online). Recently, 8 rodent malaria (*Plasmodium yoelii*) alveolin genes, termed *Py*IMC1a-h by Khater et al. (2004), were also described. The cattle parasite *Theileria parva* encodes 5 alveolins, and the human parasite *Cryptosporidium parvum* possesses 6 alveolins (supplementary table S1, Supplementary Material online).

No genome sequencing projects have yet been undertaken for dinoflagellates because their nuclear genomes (up to 2,20,000 Mb) are the largest known in nature (Hackett and Bhattacharya 2006). Nevertheless, substantial dinoflagellate gene sequence data have been generated by EST sequencing programs, and we identified numerous strong matches to apicomplexan alveolins from these EST collections. In an EST database of the dinoflagellate *K. veneficum* (formerly *Karlodinium micrum*), approximately 1.2% of the 16,548 sequenced mRNAs (aligning into about

11,000 unique sequences clusters) encode proteins showing the highest sequence identity to the TgALV8 from T. gondii (52.m01590; e value  $4 \times 10^{-14}$ ) and *Pf*ALV2 from *P. falciparum* (Pfc0185w; e value  $4.3 \times 10^{-13}$ ). Careful comparison of the mRNA sequences, including their untranslated regions, allowed us to identify at least 50 different genes encoding alveolin proteins in K. veneficum. This large family of genes is quite homogeneous with overall amino acid sequence identity of about 95% among the different genes; most of the variation is confined to the first and last 70 amino acids. The *K. veneficum* alveolins are also relatively uniform in size: 533-557 amino acids long or 58-60 kDa in mass (supplementary table S1, Supplementary Material online). Alveolin-encoding sequences were also detected for Karenia brevis and Amphidinium carterae (supplementary table S1, Supplementary Material online), but we found far fewer genes, possibly due to the less extensive collections of sequence data from these 2 dinoflagellates. In the plastidlacking dinoflagellate O. marina, we identified several alveolin-encoding ESTs, of which 7 clustered into 2 contigs, encoding 2 different alveolin proteins of 454 and 259 amino acid lengths (51 and 30 kDa in mass) (supplementary table S1, Supplementary Material online).

Searches of the 2 ciliate genomes of *Paramecium* tetraurelia and *Tetrahymena thermophila* with the apicomplexan TgALV1 sequence as a query retrieved alveolinencoding sequences with e values between  $4 \times 10^{-04}$  and  $4 \times 10^{-11}$ . Three alveolins were identified for *P. tet*raurelia and 2 for *T. thermophila* (supplementary table S1, Supplementary Material online). Whereas *PtALV1* and *PtALV2* are very similar having predicted masses of approximately 84 and 82 kDa, respectively, *PtALV3* is considerably smaller being 47 kDa. The 2 alveolins *TtALV1* and *TtALV2* of *T. thermophila* are divergent in mass (200 vs. 66 kDa) and sequence (supplementary table S1, Supplementary Material online).

All alveolins share a core of repeated sequence motifs, flanked on either side by unique and nonrepetitive sequences (fig. 1). The repeated sequences can vary in length and the number of intervening amino acids, but typically harbor a core subrepeat being EKIVEVP or very similar (fig. 1). The sequences between the core subrepeat also show similarities and are made up mainly by the same amino acids as the core subrepeat itself. Often they show a second subrepeat (e.g., EVVR or VPV). In some cases, the intervening stretches comprise as few as 4 amino acids; in other cases, 20 amino acids separate 2 repeats. The iterated repeat element of negative, positive, hydrophobic, hydrophobic, negative, hydrophobic, Pro, and X (fig. 1) seems to be restricted to the alveolate proteins. We could not identify any proteins with the same repetitive element in nonalveolates, except for 2 fungal sequences from Cryptococcus neoformans (XP568826) and Coprinopsis cinerea okayama (EAU89423).

Distribution of the alveolin genes in the genome was examined by either data mining (apicomplexan and ciliate complete genomes) or polymerase chain reaction (PCR) analyses for dinoflagellates. Using inverse-PCR techniques, we identified the majority of alveolin genes in the dinoflagellate genome to be arranged in tail-to-head repetitive arrays, although the size of these arrays is yet to be determined (data not shown). Interestingly, the same holds true



FIG. 1.—Schematic representation of the alveolin family and the localization of the characteristic repeat motif. Alveolins are present in all 3 major alveolate groups, namely the dinoflagellates, apicomplexa, and ciliates. The proteins are characterized by a repetitive peptide with the consensus sequence EKIVEVP. The localization of this repeat is shown schematically for selected Alveolins by a red box and their detailed position for 3 sequences, 1 of each group, on the right. Arrows indicate putative posttranslational modification sites. *Kv*, *Karlodinium veneficum*; *Om*, *Oxyrrhis marina*; *Pf*, *Plasmodium falciparum*; *Tg*, *Toxoplasma gondii*; *Tt*, *Tetrahymena thermophila*; *Pt*, *Paramecium tetraurelia*.

for some alveolin genes encoded in the genomes of T. gondii, where, for example, TgAlveolin1 and 4 or TgAlveolin10 and 11 are found adjacent to each other in the genome but in a head-to-head manner. A similar head-to-head arrangement is evident in *P*. falciparum (*PfAlveolin1* and 2) and *C*. parvum (*CpALV1* and 2).

# Expression of Alveolin Proteins in Alveolata

To explore the expression and localization of alveolins in the different alveolates, we raised antibodies against the common repeat motif. We designed 1 epitope (Alv1.1) to represent the core repeat as well as a second (Alv1.2) to represent the region between the core repeats. These epitopes EKVIEVPQTQVMEKV and EVVRQVPRPQT-VELT, respectively are based upon the *Kv*ALV1 sequence. The 2 synthetic peptides were used to immunize rabbits generating antisera aAlv1.1 and aAlv1.2. Western blot analyses of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)-separated protein extracts from T. gondii, P. falciparum, and K. veneficum were undertaken to test these antisera and examine alveolin expression. Preimmune sera did not recognize any major bands in host cells, T. gondii, P. falciparum, or K. veneficum (fig. 2) (only a minor band,  $\sim 84$  kDa, reacted in the *P. falciparum* lane with  $\alpha TgAlv3$  preimmune serum, and this band was discounted in the immune reaction). To establish that our antisera were detecting genuine alveolins, we probed *T. gondii* and *P. falciparum* proteins with a previously generated antibody specific for *Tg*Alv3 (previously IMC3; Gubbels et al. 2004) alongside identical blots probed with our antiserum ( $\alpha$ Alv1.1). Neither antibody reacted strongly with proteins from the host cells (fig. 2). The *Tg*Alv3specific antisera strongly decorated 2 major bands (60 and 72 kDa) and a lesser band (82 kDa) in *T. gondii* and 1 major band (66 kDa) in *P. falciparum* (fig. 2). Our antibody  $\alpha$ Alv1.1, generated to the core repeat domain, recognized the same major bands in both taxa, consistent with reactivity to alveolins (fig. 2). Minor bands were also detected with both antisera (fig. 2).

Western blot analyses of *K. veneficum* using  $\alpha$ Alv1.1 identify several antigenic bands, with 3 major bands of apparent mass 64, 68, and 73 kDa (fig. 2). Using the second antiserum  $\alpha$ Alv1.2, generated against sequence between the core repeats, the same 3 bands were identified (fig. 2). These near identical profiles using the 2 different antisera imply specific reactivity to alveloin proteins, despite the bands appearing slightly larger than the predicted masses of the KvALVs (58–60 kDa) based on EST sequence alone. Thus,  $\alpha$ Alv1.1 demonstrates reactivity to alveolins across a broad range of taxa. The observation of multiple bands identified by  $\alpha$ Alv1.1 (and  $\alpha T_g$ Alv3) in all taxa (fig. 2) is also



FIG. 2.—A universal alveolin antibody identifies multiple expressed proteins. Western blot analysis of SDS-PAGE–separated proteins from the 2 apicomplexan parasites *Toxoplasma gondii* and *Plasmodium falciparum* and the dinoflagellate *Karlodinium veneficum* with antisera generated against the universal alveolin repeat detect several specific proteins not recognized by the preimmune sera. Labeling with an antibody raised against the *TgALV3* (formerly *TgIMC3*) confirms that the alveolin antisera are detecting known alveolins (highlighted with matching symbols: diamond, star, and circle). In *Karlodinium*, at least 3 distinctive bands of around 70 kDa are recognized, congruent with the numerous isoforms detected in dinoflagellates. Molecular weight is indicated on the left of the blots in kilodaltons. HFF, human foreskin fibroblasts; RBC, red blood cell.

consistent with the multigene families that occur in *T. gondii*, *P. falciparum*, and *K. veneficum* (fig. 1 and supplementary table S1, Supplementary Material online).

#### Immunolocalization of Alveolins to Alveoli

Immunofluorescence assays (IFAs) using our  $\alpha$ alveolin sera on the dinoflagellate K. veneficum, gametocytes of the apicomplexan parasite P. falciparum, and the ciliate P. caudatum show accumulated alveolins at the cortical region, which corresponds to the localization of the alveoli (fig. 3). Confocal scanning microscopy and 3D reconstruction of individual K. veneficum cells reveal an even localization of the protein around the cells in a thin layer in the region of the plasma membrane (fig. 4). This layer is maintained during cell division, and there seems to be slightly less protein in the region of cell cleavage (fig. 4C and E). Immunogold localization of the dinoflagellate alveolins revealed labeling exclusively in the narrow supra-alveolar space between the plasma membrane and the alveoli (fig. 4G-I). No labeling was observed on the cytoplasmic alveoli face, and we did not observe any labeling between the individual alveoli (fig. 41). The swollen appearance of the K. veneficum alveoli is likely a fixation artifact because no such dilation occurs when we fixed the dinoflagellate with glutaraldehyde and  $OsO_4$  (data not shown).

Immunofluorescent localization of alveolins in tachyzoites of *T. gondii* produced virtually the same pattern of localization as described previously for the TgALV1 by Mann and Beckers (2001). Both antisera detect a thin, near continuous layer at the periphery of the cell (fig. 5). The alveolins are clearly associated with the alveoli of newly forming daughter cells developing within their mother cell during endodyogeny (fig. 5*F*). Concurrent labeling of the plasma membrane with antisera to surface antigen 1 (Hartati et al. 2006) in the dividing cell clearly demonstrates that some alveolins are remote from the plasma membrane during this division process (fig. 5E-H). In contrast to the situation in the dinoflagellate *K. veneficum* immunogold analysis, the alveolins in *T. gondii* localizes these proteins to the cytoplasmic alveoli face (fig. 5J-L). This corresponds to the localization of the TgALV1 in the network between the subpellicular microtubules described by Mann and Beckers (2001) on isolated pellicles. In *T. gondii* tachyzoites, the alveolin distribution is punctate and not evenly distributed around the cortex (fig. 5*B*, *K*, and *L*).

IFAs on the ciliate P. caudatum again revealed accumulation of alveolins in the cortical region of the cell, but with a more complex waffle-patterned distribution not seen in the apicomplexa and dinoflagellates (fig. 6). The waffle pattern extends over the whole cell, including the invaginated region of the gullet. A glancing section of the ciliate shows the sidewalls of the waffle pattern as the profile turns through about 90° (fig. 6C). The waffle pattern of alveolins is consistent with the complex architecture of the ciliate pellicle, which consists of numerous square to rectangular units approximately 1 µm across (Allen 1971). A single cilium arises from the center of each square-shaped depression, which is surrounded by 4 ridges (fig. 6D-G). A pair of alveoli is associated with each cilium, and the alveoli extend up into the ridges where they adjoin an alveolus from the neighboring depression (fig. 6G).

Immunogold localization of alveolins in *P. caudatum* provided a higher resolution view of the waffle pattern distribution (fig. 6D–F). In glancing sections, cutting transversely through the cilia, a square lattice pattern of alveolins surrounds each cilium (fig. 6D). In oblique sections not perfectly perpendicular to the surface, the 3D nature of the lattice pattern extending upward into the ridges creating each ciliary depression is evident (fig. 6E). In sections perpendicular to the surface and cutting the cilium



FIG. 3.—Alveolins localize to the cortical regions of alveolate cells. Immunofluorescence detection of alveolin proteins in the dinoflagellate *Karlodinium veneficum* (A–D), gametocytes of the apicomplexan parasite *Plasmodium falciparum* (E–H), and the ciliate *Paramecium caudatum* (I–L). The 4',6-diamidino-2-phenylindole fluorescence depicts the DNA, and optical sections through the cell midregion localize alveolins to the periphery of the cells where alveoli occur. Scale bars represent 10 µm.

longitudinally, the box-like nature of the alveolin waffle pattern is clear, with alveolin occupying the space between 2 adjoining alveoli extending up into the ridge surrounding each cilium (fig. 6F and G). The distribution of the alveolins corresponds to the location of a granulofibrillar material previously reported to fill the peaks of these ridges (Allen 1971).

# Discussion

We have identified a family of proteins with a unique repeating motif that occurs in all alveolates. Antisera generated against the repeat motif common to all alveolins identify multiple bands from SDS-PAGE–separated proteins consistent with the expression of multiple members of the gene family found in each organism. Localization of alveolins by imuunofluorescence and immunogold microscopy demonstrates that this group of proteins is exclusively located to the cortical regions and are associated with alveoli, the common feature of alveolate protists. Alveolins are the first known component of this important structural synapomorphy for a eukaryotic group and are a muchneeded key to identifying more alveolar components and unraveling the function of alveoli.

The alveolin genes identified by us thus far comprise varying numbers of repeats and variable interepeat spacers and encode proteins ranging in molecular mass from 21 (TpAlv2) up to 200 kDa (TtAlv1). Although the primary structure of the repeat motif varies slightly, the amino acid properties of the motif are conserved. Thus, the alveolins comprise numerous iterations of negative, positive, hydrophobic, hydrophobic, negative, hydrophobic, Pro, and X. The strong conservation of this motif and the multiple iterations in a range of proteins are possibly indicative of a structural role for alveolins, but any firm conclusions



FIG. 4.—Immunolocalization of the alveolins to the supra-alveolar space in the dinoflagellate *Karlodinium veneficum*. (A and C) 3D stacked projections of IFAs against alveolins (green) costained for DNA with 4',6-diamidino-2-phenylindole (blue) with corresponding bright field images (B and D). The alveolin protein is uniformly distributed over the interphase cell surface (A-C) but is less concentrated in the region between 2 dividing cells (C). (E and F) Single optical section of a dividing dinoflagellate. (G–I) Immunogold localization by TEM shows the alveolin proteins to be exclusively located in the supra-alveolar space (arrows) between the plasma membrane and the alveoli (Alv). ec, extracellular space; Pl, plastid; Nu, nucleus. Scale bar for IFA images represents 10 µm and for TEM images 0.5 µm.

await further data. The alveolin core of repeats is flanked by variable N- and C-termini. Again, the function of these elements is unknown. No targeting elements are identifiable from primary structure, and the proteins are predicted to be cytosolic. Select alveolins of apicomplexa contain Nterminal acylation motifs and possible prenylation motifs, but no experimental evidence of such posttranslational modifications is yet available (see below).

The alveolates are a large and remarkably diverse group. The dinoflagellates include photoautotrophs, mixotrophs, predators, and parasites. Members of the dinoflagellate genus Symbiodinium enter into mutualistic symbioses with a range of invertebrates, especially corals, and provide fixed carbon for their animal hosts (Little et al. 2004). Other dinoflagellates poison and eat fish (Drgon et al. 2005), parasitize invertebrates (Skovgaard et al. 2005), and cause harmful red tides (Naar et al. 2007). The apicomplexan parasites infect most types of animals and are responsible for the loss of millions of human lives every year and severe disruption of livestock industries (Sachs and Hotez 2006). The ciliates are among the largest (up to 2 mm) and most complex unicellular organisms and exhibit remarkable abilities in prey capture as well as forming symbiotic and parasitic relationships with animals (Hausmann and Bradbury 1996). Ciliates also exhibit unique, dimorphic nuclei not known from any other protist (Hausmann and Bradbury 1996). The alveolar sacs subtending the plasma membrane are the 1 unifying morphological feature of this disparate group (Cavalier-Smith 1993), but their importance in the origin and diversification of alveolates has not yet been realized due to a lack of knowledge about alveolar components.

We have shown that homologues of proteins initially described from the alveolar complex of apicomplexan parasites also occur in dinoflagellates and ciliates extending the distribution of the alveolins to the infrakingdom Alveolata. We also found alveolin genes in *Perkinsus marinus* (http://www.tigr.org/), a group of parasites related to dinoflagellates, further confirming the ubiquity of alveolins in alveolates. We predict that alveolins will also occur in alveolates such as colpodellids and the recently identified *Chromera*, a photosynthetic apicomplexa with alveoli (Moore et al. 2008). As a universal feature of infrakingdom Alveolata, we have adopted the new protein name, alveolin, to reflect the commonality of these proteins throughout Alveolata.

The function of alveolin proteins has so far not been adequately examined but they are best characterized from apicomplexan parasites where they were first discovered.  $T_gALV1$  is a 70-kDa protein thought to be a major subunit of a network of 10-nm filaments situated on the cytosolic



FIG. 5.-Immunolocalization of alveolins beneath the alveoli in the apicomplexan Toxoplasma gondii. (A-H) IFAs of tachyzoites using antisera to the plasma membrane marker surface antigen 1 (red) and alveolins (green) and DNA stained with 4',6-diamidino-2-phenylindole (blue). Alveolins form a punctate distribution at the cell periphery (A-D). During formation of 2 daughter cells within the mother cell (endoyogeny), the localization of the alveolins to the newly forming alveolar complexes of the daughter cells remote to the mother cell plasma membrane is apparent (F-H). (I) TEM section of T. gondii tachyzoite showing closely appressed membrane pairs of alveoli beneath the plasma membrane, and (J-L) immunogold localization of alveolins in tachyzoites showing punctate accumulations of alveolins on the cytoplasmic side of the alveoli (J-L) and not in the supra-alveolar space. Images (J-L) are non-Osmium treated cells with negative-stained membranes (white), whereas image (1) shows an Osmium-fixed cell for ultrastructural purposes, with membranes appearing black.

side of the alveoli (Mann and Beckers 2001). Mutations induced in TgALV1 result in cells with reduced mechanical stability, indicating a key role for  $T_gALV1$  in maintaining cell shape (Mann et al. 2002). Coiled-coil domains are proposed to facilitate aggregation of  $T_gALV1$  units into a higher order structure-perhaps the 10-nm filaments of the epiplasm. TgALV1 might be acylated at the N-terminus based on the presence of consensus palmitoylation and or myristoylation motifs, but no direct evidence of this is yet in hand (Mann and Beckers 2001). TgALV1 may also be prenylated at the C-terminus, which could mediate attachment to the membrane of the alveoli. C-terminal truncation, consistent with prenylation, occurs, but a prenyl group has not yet been identified (Mann and Beckers 2001). Processing of this protein seems to be linked to the detergent resistance of the subpellicular network and

the release of the daughter cells from the Toxoplasma mother cell (Mann et al. 2002). In isolated pellicles, TgALV1 was found to be located between the individual subpellicular microtubles (Mann and Beckers 2001), similar to the interrupted distribution of alveolins seen by us in T. gondii. The homolog of TgALV1 from Plasmodium berghei (PbALV1) is also necessary for maintenance of cell shape as well as motility and infectivity, further emphasizing the medical importance of alveolins (Khater et al. 2004). *Tg*IMC2 is not an alveolin as it lacks the repetitive motif or sequence similarity to other alveolins, but is nevertheless a component of the filamentous network containing TgALV1 (Mann and Beckers 2001). It will be interesting to explore any interactions between  $T_g$ IMC2 and alveolins. TgALV3 was identified by random tagging of proteins followed by subcellular localization by fluorescence microscopy in T. gondii (Gubbels et al. 2004). TgALV3 colocalizes with  $T_gALV1$  and 2 and is highly conserved within the apicomplexa, although the termini are quite divergent (Gubbels et al. 2004). How TgALV3 localizes to the alveolar complex is not evident, but both reporter gene fusions and immune localization demonstrate clear alveolar complex labeling (Gubbels et al. 2004). TgALV4 is the most recently recognized alveolar protein and was discovered in a screen identifying about 200 proteins within the isolated conoid/apical complex (Hu et al. 2006).

# Alveolin-like Proteins

Although alveolins are apparently unique to infrakingdom Alveolata, similar proteins occur in unrelated organisms. Articulins are abundant proteins in the subplasmalemmal pellicle of the euglenoid Euglena gracilis. They are 80 and 86 kDa in size and implicated as being membrane cytoskeletal proteins associated with the plasma membrane and likely contribute to form the articulated pellicle diagnostic of euglenoids (Marrs and Bouck 1992). Articulins from Euglena are reminiscent of alveolins in that they comprise numerous iterations of a short motif, in this case, VPVPVeviV (Marrs and Bouck 1992), that bears a passing resemblance to the alveolin repeat element (EKIVEVP). Although both motifs feature an abundance of valine, proline, and glutamate, the articulin motif is distinct and lacks the basic charge diagnostic of alveolins. It is intriguing that 2 protein families, articulins and alveolins, with similar roles in protist cells of forming a subplasmalemmal superstructure have such similar repeat structures. Whether this is convergence or hints at some shared ancestry will probably require deduction of protein structures to resolve. The protist supergroup Euglenozoa, to which euglenoids and kinetoplastid parasites belong, is not known to be closely related to alveolates. It should also be noted though that euglenoids, and indeed the entire Euglenozoa, lack alveoli. Further, articulins are absent from other Euglenozoa such as the kinetoplastid parasites.

Ciliates possess protein-dubbed epiplasmins that may be related to articulins (Huttenlauch et al. 1998) and perhaps alveolins. Epiplasmins are located somewhere in the cortical region of the ciliates *P. tetraurelia* and *T. thermophila*, but the exact subcellular location is not known (Pomel et al. 2006). A low-complexity repeat structure,



Fig. 6.—Localization of the alveolins in the ciliate *Paramecium caudatum*. (A–C) IFAs of the ciliate with antialveolin (green) and DNA stained with 4',6-diamidino-2-phenylindole (blue). A waffle-like pattern of alveolins across the ciliate surface is evident in view of the surface (A) and the gullet (B). In optical sections glancing through the surface, the depth of the waffle-pattern is obvious (B and C). (D–F) Immunogold labeling shows the alveolins (green pseudocolor outlines the concentrations of gold particles) to be located beneath the plasma membrane where it forms waffle-shaped ridges. Sections glancing the surface and cutting the cilia in transverse section (D) show a series of square alveolin outlines around each cilium (Ci). In sections not quite perpendicular to the surface that section the cilium obliquely, the dilated alveolus on each side of the cilium is visible and the alveolins extend up into the ridges standing on each side of the cilium (E). The majority of alveolins is located in the protrusions that form the ridges in the zone where 2 adjacent alveoli (Alv) meet (F). A schematic 3D diagram (G) illustrates the localization of the alveolins (green) within the complex subpellicular region of the plasma membrane.

different to that of alveolins, is characteristic of epiplasmins (Huttenlauch et al. 1998; Pomel et al. 2006). Antibodies to epiplasmins cross-react with proteins from dinoflagellates (Huttenlauch et al. 1998), suggesting that this protein family extends beyond ciliates to other alveolates.

Another family of proteins, the plateins of the ciliate Euplotes aediculatus (Kloetzel et al. 2003), are also perhaps distant homologues of alveolins. The plateins contain multiple repeats of a motif (EVVPDV) reminiscent to that of articulins and alveolins. Again, it is difficult to assign homology between plateins and alveolins, or even articulins, on the basis of primary structure for such lowcomplexity polypeptides, and structural studies will be necessary to understand any evolutionary relatedness. Interestingly, the plateins have a novel subcellular location. Euplotide ciliates differ from other known ciliates in that their main cytoskeletal element is located within their cortical alveoli and not around it (Kloetzel et al. 2003). Consistent with this is the presence of signal peptides on the platein sequences and their translocation into the lumen of the ciliate alveoli, most likely by the cotranslational insertion into endoplasmic reticulum (ER) and subsequent vesicular transport to alveoli. We found no N-terminal signal peptides encoded by any of the alveolin genes, and our immunogold TEM studies invariably localized all the alveolins adjacent to the alveoli and never in the lumen. This suggests that plateins are adapted to a completely new function within the Euplotides, which are apparently unique in this respect. It will be interesting to search for alveolins in euplotide ciliates to determine if intra-alveolar plateins have replaced perialveolar alveolins. We found no plateins in *P. caudatum* or *T. thermophila* genomic data.

Curiously, we identified 2 possible alveolins in 2 species of basidiomycete fungi *Cryptococcus* and *Coprinopsis*. The alveolin-like proteins from *C. neoformans* and *C. cinerea okayama* contain 18 and 4 repeats of the EKIVEVP motif, respectively, making them remarkably similar to alveolins. The encoded proteins also contain a chromosome segregation AT-Pase domain. The isolated presence of the 2 alveolin-like genes and their absence from other basidiomycetes such as *Ustilago* or the ascomycete *Saccharomyces* is suggestive of lateral gene transfer. It will be interesting to learn what function the alveolin-like proteins have in *Cryptococcus and Coprinopsis*. The glaucocystophyte algae are a group for which we identified no alveolins, or alveolin-like proteins, although genomic data remain limited for this group to date. Similar to the alveoli, however, a membrane-bound cortical compartment described as a lacunae system with underlying microtubules, does occur beneath the plasma membrane of these cells (Kies 1989). It is unknown whether these membranous structures are homologous to the alveoli of the Alveolata or if they are functionally equivalent. Glaucocystophytes are not known to be closely related to alveolates, and thus, if these are homologous structures, it would suggest that alveoli were relatively ancient eukaryotic structures that have been lost in many lineages. Evidence of the presence or absence of alveolins in glaucocystophytes might help to resolve this question.

#### Alveolin Diversity and Stage-Specific Expression

Our antisera directed against the definitive motif of alveolins allowed us to identify and localize multiple members of this protein family, and all are associated with the cortical regions of the cell in very close proximity to the alveoli. Our identification of an additional 8 members of the alveolin family in T. gondii raises the question of why apicomplexan parasites have such a diverse array of alveolins. During their lifecycle, apicomplexan parasites go through multiple different stages and a range of cell shapes, which include motile and nonmotile forms, in different hosts. The plethora of alveolins might be necessary to fulfill the diverse tasks demanded of the cytoskeleton in each individual life stage. The data assembled thus far point to different alveolins being expressed at different times and perhaps for different purposes. We detected expression of several alveolin genes at different levels. Other studies observed that *Pb*ALV1 is not detectable in gametocytes from P. berghei (Khater et al. 2004); however, our results on gametocytes from *P. falciparum* show at least 1 alveolin protein to be expressed and again located to the cortical region of the cell. Unraveling the expression and localization profiles of the different members will require protein-specific tools.

Some of the apicomplexan alveolins share a conserved double-cysteine motif within the N- and/or C-termini, which was reported to be relevant for cleavage of the proteins during daughter cell formation in *T. gondii* (Mann et al. 2002). This CC motif is present in both termini of *Pf*ALV1 and *Tg*ALV1 but only at the N-terminus of *Pf*ALV4, *Tg*ALV4, and *Tp*ALV2 (fig. 1, arrows). We have not identified a CC motif within dinoflagellate or ciliate alveolins, perhaps suggesting that the motif has a role in either the unique motility of apicomplexa or their unusual modes of cytokinesis.

# What is the Function of Alveolins?

When Cavalier-Smith (1991) created the infrakingdom Alveolata, he proposed that alveoli were probably an adaptation that allowed unicells to become extraordinarily large, and the alveolates—particularly the ciliates and the gregarines of Apicomplexa—are indeed remarkably large protists. Cavalier-Smith (1991) concluded that the development of an internal wall-like system of alveoli that could intermesh with cytoskeletal elements was critical to the development of large cells still capable of phagotrophy, a process precluded by rigid external walls such as occur in fungi and plants. We have shown that alveolins are a major component of this internal superstructure in all alveolates, and our working hypothesis is that alveolins are structural proteins with roles in generating rigidity. Support for our hypothesis comes from the fact that alveolins are associated with the isolated cytoskeleton from T. gondii (Mann and Beckers 2001) and that alveolin gene knockouts result in the collapse of apicomplexan cell stability (Mann et al. 2002; Khater et al. 2004). Alveolins are probably a component of the 10-nm fibrils in the T. gondii alveolar complex (Mann and Beckers 2001), and in the ciliate *P. caudatum*, we have shown that alveolins colocalize with the "granulofibrillar" material reported by Allen (1971) in the complex ciliate cortex. The iterative repeat modules of alveolins, combined with unique termini, are also consistent with an ability to generate higher order structure, perhaps even fibrils, though structural studies are necessary to confirm this. Alveolins almost certainly interact with other components of the alveolar complex. In the apicomplexan parasite *Toxoplasma*, for instance, the subpellicular microtubules are located on the cytosolic side of the alveoli (Mann and Beckers 2001; Bergholtz et al. 2005), which is on the same side as the alveolin proteins. These microtubules show a distinct 32-nm periodicity along their length, which corresponds to the periodicity of intramembranous particles located within the alveoli membranes. Hence, these intramembranous particles might associate with microtubules (Morrissette et al. 1997) and are also putative alveolin interaction partners. By contrast, in the dinoflagellate Karlodinium, the pellicular microtubules run between the plasma membrane and the alveoli (Bergholtz et al. 2005), and we found the alveolins in the same zone, again suggesting some association between alveolins and cortical microtubules. Recombinant expression of alveolins for structural studies and pull-down experiments to identify interaction partners are underway in our laboratory, and we hope to answer some of these questions with future work. Alveolins are the first identified component of the alveolar complex that defines a eukaryotic group. They now provide a beachhead from which to explore this unique cellular innovation that was apparently the key to a remarkable diversification of protists that now occupy a wide array of lifestyle niches.

## **Supplementary Material**

Supplementary table S1 is available at *Molecular Biology and Evolution* online (http://www.mbe. oxfordjournals.org/).

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