The apicomplexan inner membrane complex

Maya Kono[1], Dhaneswar Prusty[2], John Parkinson[3], Tim W. Gilberger[1,2]

[1]Department of Molecular Parasitology, Bernhard-Nocht-Institute for Tropical Medicine, Hamburg, Germany, [2]Department of Pathology and Molecular Medicine, M.G. DeGroote Institute for Infectious Disease Research, McMaster University, Hamilton, Canada, [3]Departments of Biochemistry and Molecular Genetics, Hospital for Sick Children, University of Toronto, Toronto, Canada

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1. ABSTRACT

Dinoflagellates, apicomplexans and ciliates are members of the monophyletic supergroup of Alveolata. The protists of this phylogenetic cluster have adapted to various ecological niches and lifestyles. Dinoflagellates and cilates can be found in any aquatic environment, whereas the phylum Apicomplexa solely comprises intracellular parasites. Despite their diversity all alveolates are united by the presence of membranous vesicles, so called alveoli, located beneath the plasma membrane. In addition to strengthening the cytoskeleton, these vesicles appear to possess taxon-specific functionality. In dinoflagellates and ciliates the alveoli predominantly play a structural role and can function as calcium stores. However, for the Apicomplexa, the alveolar vesicles -here jointly called the inner membrane complex (IMC)- are additionally involved in invasion of the host cell and are important scaffold elements during cytokinesis. Recent studies shed light on the architecture of the apicomplexan IMC and the number and diversity of its constituent proteins. This plethora of proteins and their varying evolutionary origin underlines the versatility of the IMC as a result of the adaption to a parasitic lifestyle.

2.INTRODUCTION

Apicomplexan parasites such as *Plasmodium* are a major threat to global health. Recent years have seen progress in our understanding of the molecular basis of their complex life cycle and their host-cell interactions but to date, no effective vaccine against *Plasmodium* infection is available and multi-drug resistance is spreading. It is estimated that the infection with the malaria parasite causes up to 1,2 million deaths annually (1).

Plasmodium falciparum, the causative agent of the most severe from of malaria belongs to the phylum of Apicomplexa, a large group of obligate parasitic protists united by a set of specific organelles. Collectively with ciliates and dinoflagellates the Apicomplexa form the supergroup of Alveolata. This monophyletic group comprises extreme diverse unicellular organisms like the red tide causing algae *Karenia brevis* (dinoflagellate), *Paramecium* and *Tetrahymena* (ciliates) and the parasitic apicomplexans with the most prominent genera *Plasmodium* (causative agent of malaria) or *Toxoplasma* (responsible for toxoplasmosis). Due to their diverse lifestyle and morphology, the concept of the Alveolata has

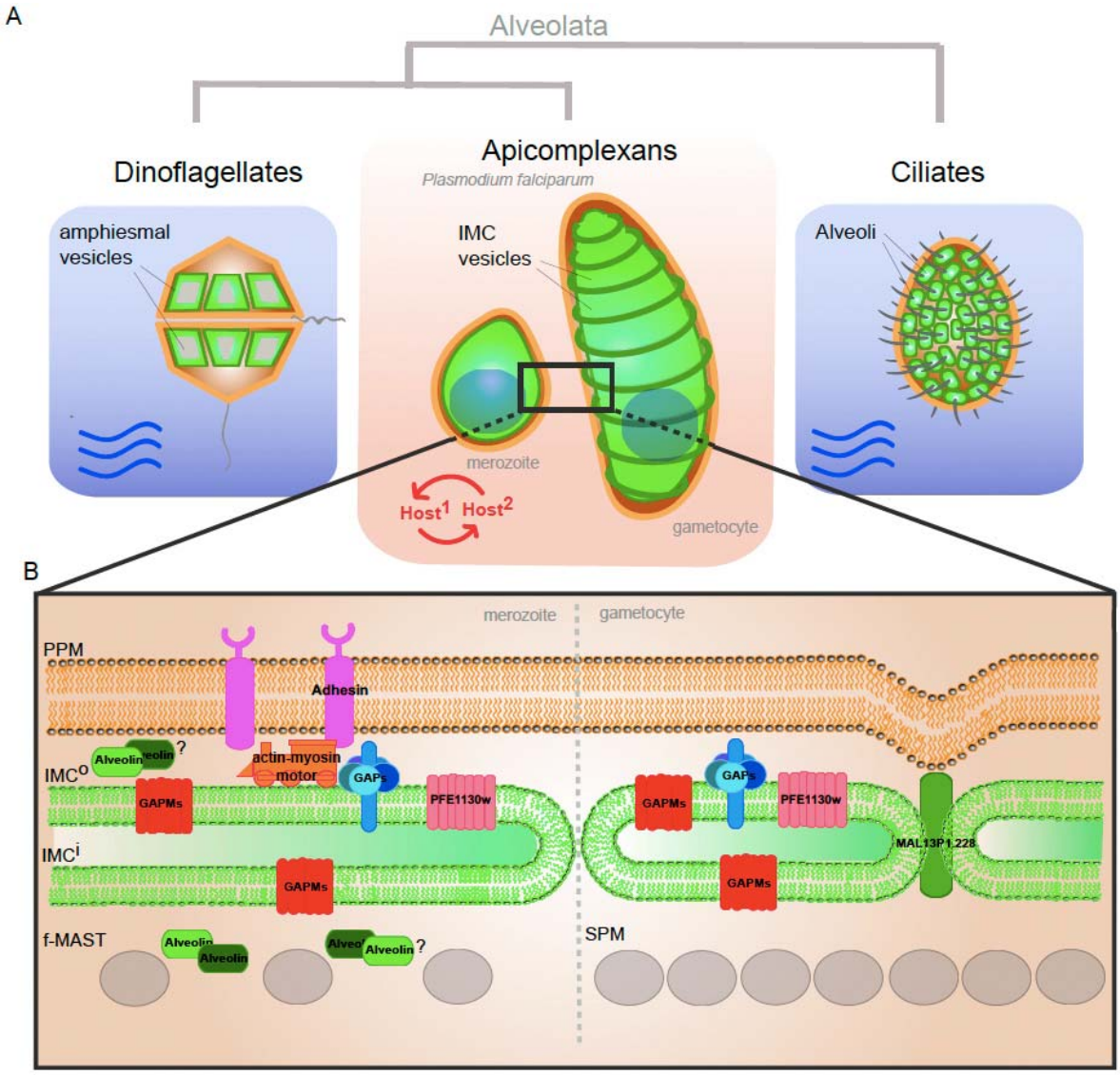

Figure 1. Schematic representation of the arrangement of alveolar vesicles in dinoflagellates, apicomplexans and ciliates. (A) The apicomplexans, in contrast to the aquatic dinoflagellates and ciliates (blue background), are intracellular parasites. Here they are represented by two different stages of the P. falciparum lifecycle: the asexual merozoite and an immature gametocyte. While in merozoites the IMC appears to build up by only one vesicle, in gametocytes several vesicles are sewed together. (B) Molecular details of the pellicle in P. falciparum in both the asexual and the immature stage of this organism. The actin-myosin motor ("glideosome") includes actin, MyoA as well as MTIP and is connected to adaptor proteins (GAPs, glideosome associated proteins). MAL13P1.228 is the only protein known so far that emerges as a marker for individual borders of IMC vesicles in gametocytes. GAPMs: A family 6 transmembrane domains proteins. PFE1130w possesses 7 transmembrane domains. SPM: subpellicular microtubules; f-MAST: P. falciparum merozoite assemblage of supbellicular microtubules. The membrane association of alveolins in Plasmodium with either the inner (IMCi) or outer membrane (IMCo) or both membranes has to be elucidated.

for some time been the subject of controversy, but emerging (18S rRNA) sequence data as well as the presence of cortical vesicles established the monophyletic origin of the three groups (2). These alveolar vesicles are the most prominent, unifying morphological feature of this group. The physiological and morphological diversity of the alveolate organisms is reflected in the astonishing manifoldness of the alveolar vesicles that were adapted to the specific needs of the respective lifestyles. Emerging phylogenetic as well as experimental data is starting to reveal the molecular composition of the alveolar vesicles. Their protein components are structurally diverse and their level of conservation within the alveolate clades differs greatly. This article reviews established data with the most recent insights into this membranous structure with a focus on *Plasmodium spp*. and *Toxoplasma gondii*.

Table 1. General overview over the IMC composition and the associated structures in *Toxoplasma* and *Plasmodium spp*

Parasite stage	Lenght of cell	Number of IMC vesicles	SPM	SPN
Toxoplasma gondii tachyzoites	8µm	numerous	22	√
Plasmodium falciparum sporozoites	12-15µm	1	15-16	√
merozoites	1µm	1	2-3	ND
ookinetes	10-13µm	1	ca. 60	ND
gametocytes	3-12µm	9-15	numerous	ND

SPM = subpellicular microtubules; SPN= subpellicular network; ND = not determined

3. ALVEOLI, AMPHIESMAL VESICLES AND THE INNER MEMBRANE COMPLEX: SYNAPOMORPHIC CHARACTERS OF THE ALVEOLATE SUPERGROUP

The homologous alveolar structures that are made up by flattened vesicles underlying the plasma membrane are called "alveoli" in ciliates, whereas they are known as "amphiesmal vesicles" in dinoflagellates or "Inner Membrane Complex" in apicomplexans (Figure 1A) (2-5).

In ciliates the outer membrane of the alveolar vesicles is connected to the plasma membrane by short bridge-like structures while the inner membrane facing the cytosol is covered by a fibrous layer called the epiplasm (6). Extrusive organelles like trichocysts or mucocysts and the basal bodies of the cilia lie embedded between the single alveoli that in some rare cases may contain plates of glycoprotein, which can be calcified (7). Major proteins of these so called alveolar plates include plateins, cytoskeletal proteins that represent a subclass of articulins (8,9). In some ciliates the alveoli extend longitudinally (*Tetrahymena)* while in others they are short and pillow-shaped (*Paramecium*). These different arrangements accommodate the specific physiological needs of the different species. In *Paramecium* the alveoli not only stabilize the ejectile structures but were also shown to be involved in calcium storage, trichocyst exocytosis, ciliae beating and cytoskeletal dynamics during division (10,11).

The amphiesmal vesicles of dinoflagellates can contain plates of a non-cellulosic glucan (12,13) that define them as thecate or armoured. The cellulosic plates are delimited by sutures and usually fit tightly together collectively forming the theca of the cell, which imposes specific shapes upon these forms. The arrangement of the thecal plates within the vesicles (termed tabulation) has also been used for purposes of taxonomic classification. In so-called naked or athecate dinoflagellates the membranous vesicles appear to be without plate-like structures and simply play a general structural role (5,7).

The architecture of the homologous structure of the Apicomplexa - the Inner Membrane Complex or IMC- has been most extensively studied in *Eimeria* and *Toxoplasma spp*. In these genera all motile stages (called "zoites") possess a conoid (contrary to *Plasmodium* species), a special cytoskeletal organ in the apical tip of the cell that extends and retracts as the parasite moves (14). These zoites are characterized by a single, cone-shaped IMC vesicle at the very apex of the cell above numerous IMC cisternae. All IMC plates are interconnected building a coherent network of vesicles. Interestingly, unlike the coccidians, the IMC architecture in *Plasmodium* appears to vary even between different stages of the lifecycle. Whereas the IMC of the non-invasive gametocyte consists of several vesicular structures comparable to *Toxoplasma* (15-18) (Figure 1), the IMC of merozoites appears to consist of only a single vesicle (17,19,20). The latter is also true for *Plasmodium* ookinetes, the motile zygotes of the malarial organism that penetrate the mosquito midgut as well as for sporozoites, the infective forms of the parasite that are transferred from salivary glands to humans when the mosquito feeds (21,22).

4. IMC ARCHITECTURE IN APICOMPLEXANS

Studies applying freeze-fracture electron microscopy to *Toxoplasma* tachyzoites as well as several *Plasmodium* stages have revealed detailed insights into the overall organization of the IMC and its connection with components of the cytoskeleton (15,22,23,25). The general architecture of the IMC and the associated structures in *T. gondii* and the different stages of *P. falciparum* are summarized in Table 1. The IMC of tachyzoites is composed of a single apical as well as several flattened vesicles or plates connected at sutures creating an alveolar patchwork that itself is sandwiched between the plasma membrane and 22 subpellicular microtubules (SPM) (16,25,38). As opposed to the microtubules, which span only two-thirds of the parasite, the IMC covers the entire cell except the very basal and apical pole as well as the micropore. Freeze fracture analysis of the pellicle shows that the four faces of the IMC membranes have a distinct content and distribution of intermembranous particles (IMPs) (16,26,27). The IMPs associated with the cytoplasmic face of the inner IMC membrane (Figure 1B) are not arranged randomly but appear organized in 22 parallel, longitudinal double rows that spiral in a manner corresponding to the underlying microtubules (27,28). This lead to the suggestion, that the IMPs of the IMC represent the protein bridges between IMC and the microtubule-associated proteins (MAPs) of the SPM (16). The double rows of particles are interspersed with a number of single rows and have a vertical spacing of approximately 30 nm, a pattern that is continuous throughout all IMC vesicles. Cryofracture analysis of the pellicle of *Plasmodium*

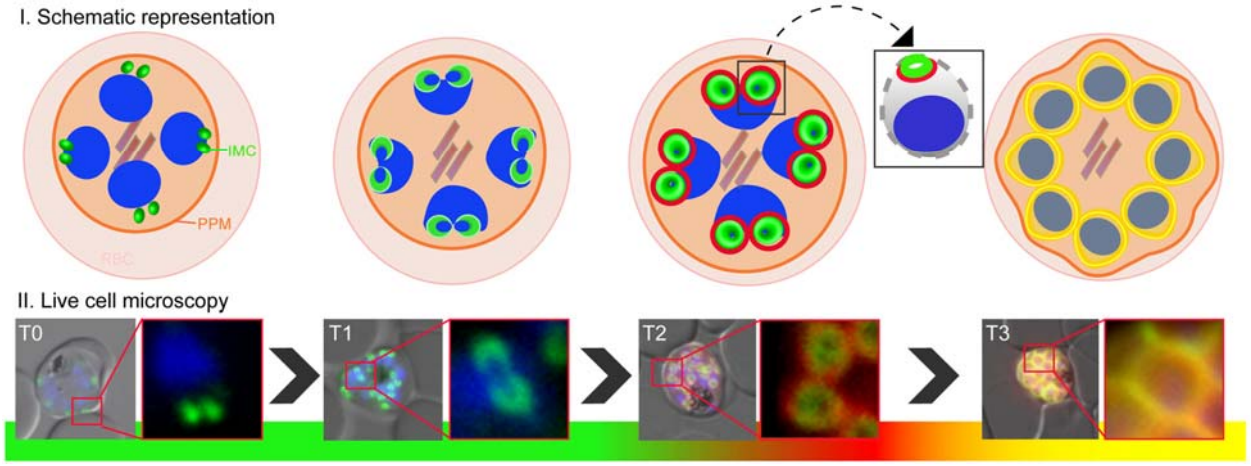

Figure 2. IMC biogenesis during blood stage proliferation in P. falciparum. Schematic representation and live cell microscopy of the IMC dynamics during merozoite development (T0-T3). Two IMC proteins with distinct phenotypes where either tagged with GFP (the group A protein MAL13P1.130, green) or mCherry (the group B protein PF13_0039, red). T0: nascent IMC compartments visible as two dots in young schizonts. T1: IMC forms cramp-like structures. T2: IMC is enlarged and group B protein (red) emerges at the proximal rim; square: highlighting the spatial arrangement of group A and B proteins. T3: The IMC represented by group A and B proteins fully surrounds the mature merozoite.

gallinaceum ookinetes revealed a similar association of IMC and microtubules as well as the presence of a unique set of large pores in the alveolar membranes (22). Here the cytoplasmic face of the inner IMC membrane is decorated by numerous IMPs of which the majority is organized in paired rows comparable to the previously described patterns. However they display a wider spacing of 90 nm (22). In addition to the IMC core structure, the pellicle of *Toxoplasma* is characterized by a meshwork of interwoven filaments with a diameter of 8-10 nm that lies between the IMC and microtubules. The pattern of this so-called subpellicular network (SPN) resembles the IMP arrangement and creates a membrane basket enclosing the entire parasite. Studies suggest it to be flexible and detergent-sensitive in forming daughter parasites when the nascent cell still changes in size and form, while it becomes more rigid and detergent-resistant as the parasite matures (28-30). Thus the SPN and the IMC are believed to be the major sources for maintaining cell integrity and shape rather than the tubulin-based cytoskeletal SPM (28). Cryoelectron tomography analysis revealed the existence of a dense layer underlying the IMC of *Plasmodium berghei* sporozoites and suggested it to be the analogous structure of the *Toxoplasma* SPN (31). It is characterized by 27 nm long linker molecules that exhibit the same 32 nm periodicity described for the IMP arrangement of the *Toxoplasma* IMC. The linkers connect the IMC and SPM and are arranged at a mean angle of 30° pointing in either the apical or basal direction, thus conferring stability as well as flexibility to the cytoskeleton. They are distributed over the entire cytoplasmic face of the IMC – including the area that is free of microtubules - where they couple apicoplast, mitochondrion, nucleus and ER to the IMC (31). This association of IMC and microtubules as well as organelles might be important for an agile parasite stage such as the *Plasmodium* sporozoite. To date no comparative ultrastructural investigation of the *Plasmodium* merozoite or gametocyte has been published confirming the presence of a SPN in these stages. However, it is well established that the very small merozoite is characterized by 2-3 SPMs, collectively termed f-MAST, that are located underneath the only IMC vesicle (32). In contrast the immature gametocytes of *P. falciparum* contain an entire row of SPMs underlying the pellicular membranes (15). This stage possesses between 10-15 IMC cisternae that are connected at transverse sutures (15,17) (Figure 1). Theses sutures are specific for this parasite stage and seem to connect the IMC and the plasma membrane, thus differ from the single one reported for the sporozoite (21).

The mature *Toxoplasma* tachyzoite is further characterized by a sub-compartimentalization of the IMC accentuated by the division into apical, central and basal regions representing discrete sections of the overall same structure. The different members of the so-called "IMC-subcompartment-proteins" (ISPs) are localized to either one of the three IMC sub-domains and are also differentially targeted to their specific destinations (33,34). Of further interest is their localization to the cortical part of the IMC at the periphery and not the SPN underlying the IMC vesicles (33,34). There are indications that a similar partitioning of the IMC occurs in *P. falciparum* merozoites showing at least a distinction of the basal and the apical regions of the compartment with the tendency of certain proteins to localize to either one (35). Since the IMC in this zoite is presumably made up by only one vesicle, it remains to be determined if

there is an exclusivity associated with protein targeting and how this might be mediated.

5. ORIGIN AND PROTEIN COMPOSITION OF THE IMC IN APICOMPLEXANS

With the sequencing of multiple genomes from different apicomplexans as well as ciliates and dinoflagellates, comparative studies are beginning to reveal considerable diversity in the composition of the IMC. The emerging complement of structurally distinct proteins associated with the IMC of *Toxoplasma* and *Plasmodium* is summarized in Figure 2. A recent study of 17 IMC proteins suggests that the evolution of the IMC involved the recruitment of several ancestral eukaryotic proteins (17), together with the innovation of alveolate-specific genes that occurred early in the Alveolata lineage (similar to what has been observed for the alveolin gene family, see below (36)). The acquisition of these apparently novel genes during the emergence of the Alveolata presumably facilitated the construction of a new biological system that conferred a significant lifestyle advantage over their predecessors. Intriguingly, taxon-specific innovations of known IMC proteins within the Apicomplexa appear limited; there is only a single *Plasmodium*-specific component of the IMC (MAL13P1.228, Figure 1), while other lineages display few gene family expansion and/or loss events (17). On the other hand, with the functions of a large proportion of apicomplexan genes still unaccounted for (37), there is every possibility that additional components of the IMC remain to be discovered, that may also include lineage-specific innovations. Nevertheless, these findings suggest that the IMC is comprised of a core set of conserved proteins with functions that are likely preserved across the entire phylum. A limited number of taxon-specific proteins recruited to the IMC may then provide additional physiological roles to this structure, facilitating the adaptation of the IMC to genera specific niches. The observed evolutionary diversity of the IMC protein repertoire thus represents the spectrum of functionalities provided by this structure, from providing basic structural integrity to all Alveolates to the machinery that allows endoparasites to invade host cells. This functional specialization is exemplified by a group of well-characterized IMC proteins that form components of the so-called glideosome, the motor complex that drives the locomotion of all invasive stages (39-41). This sophisticated actin-myosin machinery powers the motility needed for transmigration, gliding, invasion and egress. The “glideosome associated proteins” GAP50 and GAP45 are associated with MyoA and the “myosin light chain” (MLC1 or myosin A tail-interacting protein (MTIP) (35,38-41). They are presumably also linked to 6-transmembrane domain proteins (GAPMs) that are hypothesized to span the lumen of the IMC vesicles thus stabilizing and flattening them (42,43). Additional glideosome-associated proteins like GAP40, GAP70 and the “essential light chain 1” protein (ELC1) have been recently identified (41,44). GAP40 is an apicomplexan specific polytopic protein, whose exact role during invasion remains to be determined, while GAP70 is a coccidian-specific homologue of the apicomplexan GAP45. This protein differs from GAP45 in the length of its coiled-coiled domain and its restriction to the apical cap IMC vesicle (41).

The group of glideosome-associated proteins is complemented by a multi-gene family of proteins termed the alveolins (36). The first alveolin was initially identified in *T. gondii* as part of the SPN and has been named Inner Membrane Complex Protein 1 (TgIMC1) (28). Alveolins are characterized by short repetitive sequence domains (EKIVEVP) that are slightly variable (36) (Figure 4 VI). In *Toxoplasma* the alveolin group contains 14 members (Figure 2), which show very distinct spatio-temporal distribution patterns that are useful chronological markers during daughter cell development (45). Since alveolins are ubiquitous, spread across the Alveolata, this constantly expanding protein family was recognized as the molecular nexus of this phylum (36). In *Plasmodium berghei* serveral alveolins have been shown to be implicated or even essential for normal motility and morphogenesis of sporozoites as well as ookinetes (46-48).

Additionally, an unknown number of proteins associated with the IMC falls into neither the glideosome- nor Alveolin-category. One highly intriguing protein is the “membrane occupation and recognition nexus protein1” (MORN1), a conserved IMC protein that is involved in assembly of the basal complex and the cytokinesis process where it serves as a linker between the IMC and the cytoskeleton (49-51). The ISP family is another example of this third category. These apicomplexan specific proteins share a high content of charged residues as well as N-terminal myristoylation and palmitoylation motifs. Each of the four ISPs could be exclusively localized to a specific part of the Toxoplasma IMC defining sub-compartiments (33,34). TgHsp20 is a small chaperone-like protein that is also an IMC member. It is associated with the outer leaflet of the IMC facing the plasma membrane and reveals a stripe-like organization. It has been speculated that it may possess a role in protecting and modulating the IMC membrane properties (52) and a recent publication showed that this protein is critically involved in locomotion as well as infection during the liver phase (53). Further information about the protein composition of the IMC was delivered by a pellicle proteome study of the ciliate Tetrahymena thermophila (54). Gould and coworkers identified pellicular proteins that share domains with a strong amino acid bias towards charged residues. Theses so called CRMPs (Charged Repeat Motif Proteins) seem to be conserved throughout the Alveolata and significantly enlarge the molecular network of this monophyletic group.

6. APICOMPLEXAN IMC BIOGENESIS DURING DAUGHTER CELL DEVELOPMENT

Although the mode of cytokinesis of *Toxoplasma* (endodyogeny) and *Plasmodium*

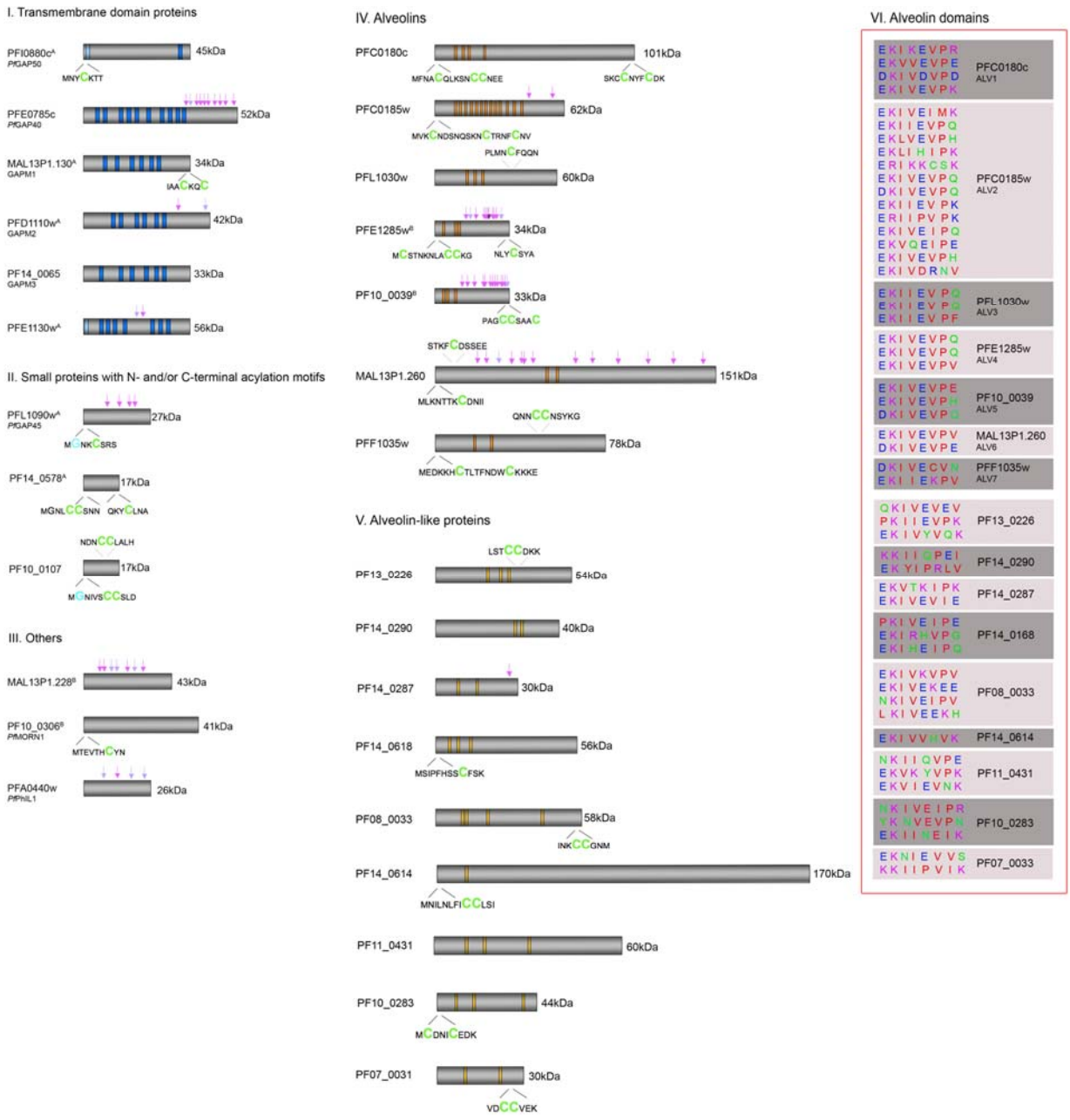

Figure 3. Structural characteristics and (putative) co- and posttranslational modifications of IMC proteins. I. Transmembrane proteins, II. Small, acylated proteins, III. Others, IV. Alveolins, V. Alveolin like proteins and VI. Primary sequence of the individual alveolin repeats. Rough positions of the alveolin repeats are highlighted in orange or in case of the alveolin-like proteins in yellow. Light blue: signal peptide, dark blue: transmembrane domains. Pink arrows: approximate position of corresponding serine phosphorylation sites; light purple arrow: approximate position of corresponding threonin phosphorylation site (threshold score ≤ 19) (58). Partial primary sequence is given for those proteins with myristoylation (G, predicted by Myristoylator) (78) and palmitoylation motifs (C, predicted by CSS.PALM 3.0. with medium threshold) (79). A or B in superscript above accession numbers indicates the affiliation to either group according to Figure 2 (16).

(schizogony) differs, the biogenesis of the IMC appears to be resemblant in both organisms. Aspects of the very early events of daughter cell development were recently reviewed for *T. gondii* (55) but in *Plasmodium spp*. still need to be further resolved. The formation of the IMC belongs to the initial processes next to centrosome development (17,56-59). Moreover, the IMC vesicles are believed to be Golgi-derived, thus the early establishment of the secretory system and IMC seem reasonably linked (35,60,61). Two members of Rab11-GTPase family (Rab11A and B) have been implicated in IMC

maturation and vesicle-shuttling between the Golgi and the nascent IMC in *Toxoplasma* (62,63). While the pellicle assembly in *Toxoplasma* has been analyzed in detail and reviewed elsewhere (55,61), comparable data in *Plasmodium* is only just starting to emerge (17,21). A more comprehensive study was recently published using a subset of 8 structurally distinct IMC proteins from *P. falciparum,* which revealed two distinct spatiotemporal localization patterns, termed group A and group B (17) (Figure 3). Group A comprises the classical glideosome components like GAP45, GAP50, all known IMC transmembrane proteins as well as PF14_0578. Since these proteins are associated with the IMC throughout schizogony, they are helpful markers following the IMC biogenesis from initiation of the compartment until full maturity. They first appear at the apical poles of young schizonts during the first nuclear division. Co-localisation with a centrosome marker revealed the intimate association of both structures in this initial stage of merozoite development. Here the IMC forms cramp-like structures embedding the two centrosomes. With ongoing schizogony the crescent IMCs grow into rings that enlarge before the IMC ultimately surrounds the fully formed merozoites. The group B contains proteins of the Alveolin family as well as MAL13P1.228. They all appear approximately 3-4h later in schizogony compared to their group A counterparts (17). Group B proteins appear as rings with a wider diameter surrounding the already established compartment (see Figure 3). By the time the merozoite and also IMC development is completed, the alveolins are located at the periphery of the merozoite where the IMC encircles the entire cell.

The final step of daughter cell development includes the formation of the plasma membrane around the fully matured bud. In *Toxoplasma* the two zoites develop inside the cytoplasm and inherit the maternal plasma membrane as they bud from the mother cell. However, *Plasmodium* parasites undergo various replication rounds and form a multinucleated syncytium with up to 32 nuclei. The nascent buds are located in the periphery of the cell and in close proximity to the plasmalemma with the apical pole pointing outwards. After the completion of organelle division or development respectively, the plasma membrane folds inwards at the apical regions and with this defines the elliptical shape of the merozoite (19). How exactly the process of invagination of the plasma membrane takes place and if this includes the formation of proteinacous connections between the outer IMC membrane and the plasmalemma remains elusive and is currently under investigation (Kono and Gilberger, unpublished).

7. PROTEIN RECRUITMENT AND MODIFICATION OF IMC PROTEINS

IMC proteins can also be classified according to their structural characteristics as well as their level of co- and post-translational modifications, which might represent important modulators for trafficking and function or determine their mode of membrane association (Figure 4). The latter includes N-terminal acylation and unknown mechanisms that possibly involve protein-protein interactions or hydrophobic and electrostatic interaction with the lipid bilayer. Many IMC proteins appear to be subjected to multiple palmitoylations (64,65) and some of display additional N-terminal lipid modification motifs. For instance, N-terminal myristoylated and palmitoylated proteins like PF14_0578 and PF10_0107 (a putative homologue of the *T. gondii* ISP1/2, 32) could get trapped into the IMC membrane by an IMC-specific palmitoyl acyl transferase (PAT) as previously suggested for the ISPs and GAP45 in *T. gondii* (33,34,41) and recently for *P. falciparum* (64). For members of the ISP family there seems to be a coordinated acylation governing the localization even within specific areas of the IMC network of the *T. gondii* tachyzoite (33,34). In contrast the transmembrane spanning proteins likely use the vesicle mediated transport mechanism of secretory proteins for delivery to the IMC membranes (17,35). Furthermore in the case of some *Toxoplasma* alveolins, the alveolin domains are implicated in membrane association (45). Phosphorylation is another important posttranslational modification that might be crucial for the proper orchestration of the initiation and operation of the invasion process. In different studies it has been shown that GAP45 for instance is heavily phosphorylated, albeit the exact function of this phosphorylation step as well as the phosphorylation of other IMC proteins is still unknown (44,66-68).

8. SUMMARY

The Inner Membrane Complex of the Apicomplexa appears to have evolved as a highly versatile system fulfilling various conserved and lineage-specific functions throughout the apicomplexan lifecycle. This is reflected in the divergent evolutionary background of the protein members of this compartment. The conservational level and overall quantity of pellicular proteins seems to be highly variable, depending on the basic or specific requirement of pellicle complexity of different alveolate organisms. Especially in *Plasmodium spp.* the identification and characterization of novel IMC proteins could provide important insight into the coordinated hierarchy of pellicle assembly as well as the late and unknown processes coordinating IMC and plasma membrane biogenesis during the final steps of cytokinesis.

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Send correspondence to: Tim-Wolf Gilberger, McMaster University, MDCL 2301, M.G. DeGroote Institute for Infectious Disease Research, 1280 Main Street West, Hamilton, ON L8S 4K1, Canada, Tel.: 001-905-525-9140, ext. 21674, Fax: 905-522-9033 ext. 5330, E-mail: tgilber@mcmaster.ca