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COMMENTARY

The parasitophorous vacuole membrane surrounding *Plasmodium* and *Toxoplasma*: an unusual compartment in infected cells

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SUMMARY

***Plasmodium* and *Toxoplasma* belong to a group of unicellular parasites which actively penetrate their respective mammalian host cells. During the process of invasion, they initiate the formation of a membrane, the so-called parasitophorous vacuolar membrane, which surrounds the intracellular parasite and which differs substantially from endosomal membranes or the membrane of phagolysosomes. The biogenesis and the maintenance of the vacuolar membrane are closely related to the peculiar cellular organization of these parasites and are unique phenomena in cell biology. Here we compare biological similarities and differences between the two parasites, with respect to: (i) the formation, (ii) the maintenance, and (iii) the biological role of the vacuolar membrane. We conclude that most differences between the organisms primarily reflect the different biosynthetic capacities of the host cells they invade.**

Key words: Host cell invasion, Membrane biogenesis, Parasitophorous vacuole, *Plasmodium*, *Toxoplasma*

INTRODUCTION

Apicomplexa are unicellular eukaryotes which are obligatory intracellular parasites with short-lived extracellular stages. Unlike many other microbial organisms which utilize phagocytic properties of their host cells for invasion, apicomplexa penetrate by a complex process which involves specialized organelles peculiar to these parasites. Inside the host cells most apicomplexan parasites reside and develop within a vacuole termed the 'parasitophorous vacuole' (Scholtysek and Piekarski, 1965), whereas some, such as *Theileria* and *Babesia*, escape from the vacuole shortly after invasion. The formation and the subsequent fate of the vacuole and its membrane (the parasitophorous vacuole membrane, PVM) clearly differs from that of phagolysosomes (reviewed by Sinai and Joiner, 1997), and it has no relation to digestive vacuoles. In fact, the PVM shelters the parasite inside the vacuole and prevents acidification of this compartment. These properties have attracted the interest of many investigators who have begun to study the molecular basis of PVM biogenesis and maintenance. As we will discuss below, these problems are closely related to the biological functions of specialized secretory organelles, called rhoptries and micronemes. These organelles are located at the apical end of the parasite cell and are a taxonomic criterion of the apicomplexa (Fig. 1).

Most of the research on the cell biology of apicomplexan parasites has been carried out using *Toxoplasma gondii* and several species of the genus *Plasmodium* as model systems. *T. gondii* and *P. falciparum* infect mammalian cells causing toxoplasmosis and human malaria, respectively. Both parasites have complex life cycles. Our discussion will centre primarily on the erythrocytic stages (merozoite→trophozoite→schizont) of *P. falciparum*, and on the actively replicating tachyzoite stage of *T. gondii*. They are the most extensively studied due to the experimental option to cultivate these stages in vitro and because these stages are most relevant to the pathology of the respective diseases. Since both parasites are related taxonomically, it is tempting to generalize experimental data and scientific interpretations. It is increasingly clear that this generalization is not always accurate, with regard to protein and membrane traffic within the infected cell. To illustrate this point, we will focus on: (i) the formation of the PVM, (ii) its maintenance during parasite growth and replication, and (iii) its biological role as interface between parasite and host cell. Other interesting aspects such as the molecular basis of the low affinity attachment of free parasites to host cells and the role of receptors involved in the invasion process are beyond the scope of this minireview. Our emphasis will be on biological similarities and differences between the two parasites, and we will consider the advantages and disadvantages of each as an experimental system.

From a conceptual and practical point of view, undoubtedly the most important difference between the parasites is the host cell (Fig. 2). After a temporary infection of liver cells, *P. falciparum* invades mature erythrocytes which are incapable of

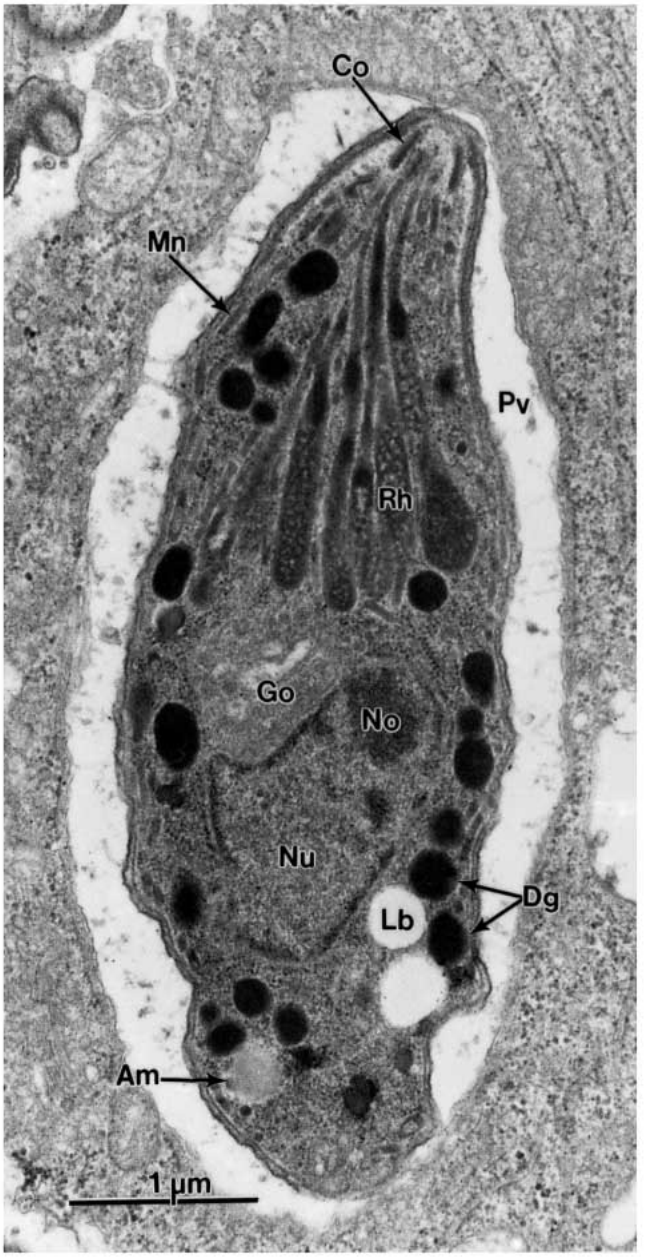

Fig. 1. Transmission electron micrograph of the tachyzoite stage of *Toxoplasma gondii* in a mouse peritoneal exudate cell. Am, amylopection granule; Co, conoid; Dg dense granule; Go, Golgi complex; Lb, lipid complex; Mn, microneme; No, nucleolus; Nu, nucleus; Pv, parasitophorous vacuole; Rh, rhoptry. ×25,000. Micrograph kindly provided by J. P. Dubey, D. S. Lindsay and C. A. Speer.

protein, nucleic acid or lipid synthesis and which survive in the absence of the exogenous components needed for these processes. In contrast, *T. gondii* replicates within nucleated cells replete with a full biosynthetic machinery. This presents an experimental advantage to *Plasmodium* in the study of vacuole formation and modification as well as nutrient acquisition, since it is easier to ascribe observed effects to modifications induced by the parasite.

FORMATION OF THE PVM

The PVM is formed during invasion, and its formation coincides with a release of the contents from specialized secretory organelles (reviewed by Schwartzman and Saffer, 1992). Therefore we will describe the secretory compartments of both parasites before we discuss the maintenance and the possible biological functions of the PVM.

Ubiquitous secretory compartments, namely the endoplasmic reticulum (ER) and the Golgi complex, differ in some aspects between *Toxoplasma* and *P. falciparum*. Likewise, protein trafficking between these organelles shows peculiarities not observed in other eukaryotic cells.

In *T. gondii*, an ER is apparent throughout the cell. A well developed stacked Golgi is observed anterior to the nucleus, although documented post-translational modifications of secreted proteins are minimal and only limited N-glycosylation of a subset of surface proteins is documented (reviewed by Schwarz and Tomavo, 1993). Vesicles bearing a fuzzy coat bud from the anterior nuclear envelope/ER, and vesicles bearing a clathrin-like coat are observed budding from the trans-face of the Golgi (L. Tilney, M. Shaw, and D. Roos, personal communication). ADP-ribosylation factor 1 (ARF-1) has been cloned, is 83% identical at the amino acid level to human ARF-1, and localizes by IFA to a structure consistent with the Golgi, following transient transfection in the parasite (T. Stedman and K. A. Joiner, unpublished). Brefeldin A (BFA) at high concentrations (20 µg ml^{-1}) blocks delivery of plasma membrane proteins to the parasite surface (Karsten et al., unpublished), and leads to extensive proliferation of the ER and nuclear envelope (L. Tilney, M. Shaw and D. Roos, personal communication), providing some biochemical evidence for the involvement of coats in protein transport. In fact, genes for components of both COPI (β-COP) and COPII (Sec23) coats are present (Ajioka et al., 1998), and an antibody to bovine β-COP stains a structure anterior to the nucleus, consistent with the Golgi, by immunofluorescence. Rab6 has been localized to this same region, using an antibody to *P. falciparum* Rab6 (see below), and also using an antibody to detect hemagglutinin-tagged endogenous Rab 6 expressed in the parasite (T. Stedman and K. A. Joiner, unpublished observations).

In *Plasmodium* an ER has been identified by electron microscopy and by localization of the parasite BIP homologue (Kumar et al., 1988). N-glycosylation occurs at low levels only if it occurs at all (Dieckmann-Schuppert et al., 1992; Kimura et al., 1996). Whether *P. falciparum* has a stacked Golgi complex as a stable, intermediary post ER compartment still is a matter of dispute. A single cisterna which may correspond to the parasite Golgi has been observed by electron microscopy (Bannister and Mitchell, 1995; Ward et al., 1997). Recently, the gene encoding parasite Rab6 has been identified (de Castro et al., 1996; van Wye et al., 1996). Imunofluorescence studies using PfRab6 as a marker argue against a stacked organization of the putative parasite Golgi (van Wye et al., 1996). Sphingomyelin synthase which localizes predominantly to the Golgi in other eukaryotes, is transported from the parasite cell into the expanding PVM (Haldar et al., 1991) suggesting that, at least in the case of this enzyme, retention within an intracellular compartment of the parasite does not occur. In contrast to *Toxoplasma*, the use of antibodies to mammalian COPs has not been successful in the identification of the

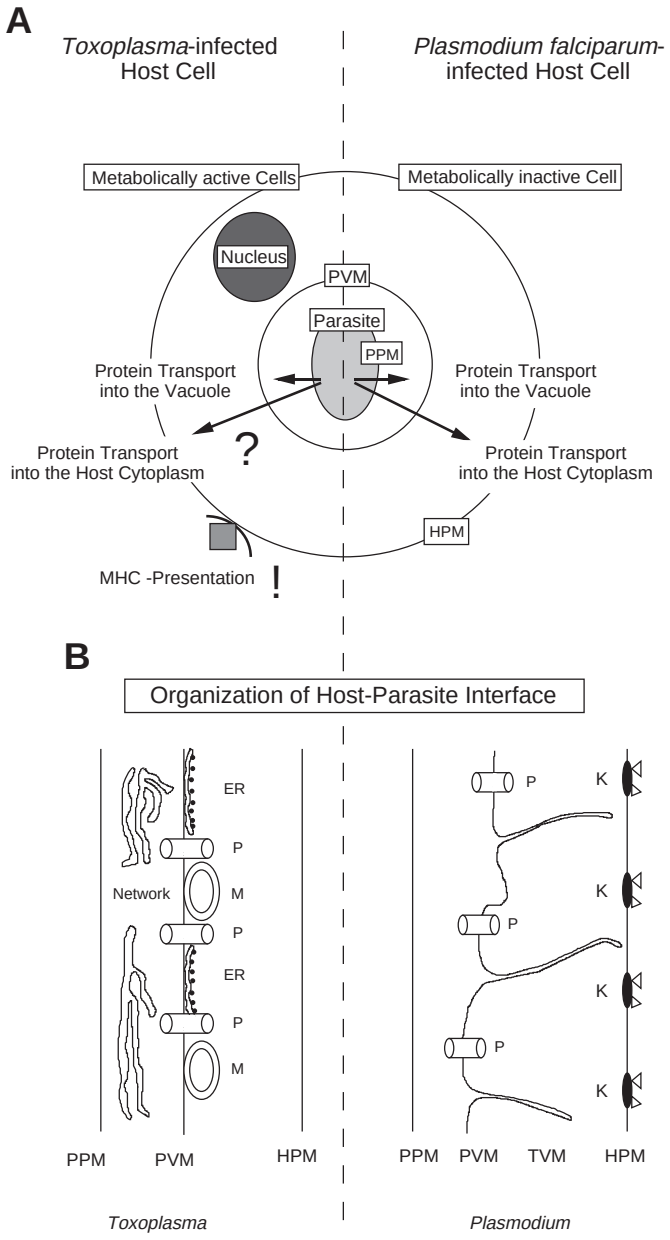

Fig. 2. Host-parasite relationship in *Toxoplasma*- vs *Plasmodium*-infected cells. (A) *Toxoplasma* invade a wide variety of nucleated cells which are metabolically active and which have the potential of presenting parasite proteins in an MHC context. Secretion of parasite proteins into the vacuole and into the vacuolar membrane (PVM) occurs. Translocation of parasite proteins across the PVM is still a matter of debate. In contrast, *Plasmodium falciparum* invades mature erythrocytes which lack intracellular organelles and which are devoid of protein and lipid biosynthesis. Parasite proteins are secreted into the vacuolar space and translocated across the PVM. HPM, host cell plasma membrane; PPM, parasite plasma membrane. (B) The vacuolar space of *Toxoplasma*-infected cells contains a membranous network which contains proteins of dense granule origin. Protein pores (P), presumably of parasite origin, are inserted into the PVM. Mitochondria (M) and endoplasmic reticulum (ER) from the host cell associate tightly with the PVM. The PVM of *Plasmodium* extends into the host cell cytoplasm and forms a network of tubovesicluar membranes (TVM). As in *Toxoplasma*, the PVM of *P. falciparum* contains pores. The HPM is modified extensively. Parasite proteins interact with the submembranous cytoskeleton which results in the formation of electron-dense, knob-like protrusions (K). In the knob region, parasite proteins, such as PfEMP1, are inserted into the erythrocyte membrane and are exposed on the outer face.

corresponding plasmodial proteins (K. Lingelbach, unpublished). As the anterograde transport of proteins can be inhibited by BFA, which inactivates ARF by interfering with the exchange of GDP by GTP, it is likely that ARFs contribute to protein transport from the ER and/or the formation of subsequent transport vesicles. In fact, genes encoding plasmodial ARFs and ARF-like proteins have recently been cloned and characterized (Truong et al., 1997) but not yet localized at the ultrastructural level. In conclusion, the organization of the ubiquitous classical secretory pathway, the dynamics of constitutive protein secretion and the contribution of regulatory proteins at individual steps are still poorly understood in *Plasmodium* compared to higher eukaryotes. A more detailed analysis clearly requires a considerable improvement in the genetic manipulation of the parasite and the development of cell-free assays to study protein and membrane trafficking.

Rhoptries (derived from the Greek word for club) are unique club-shaped organelles (Fig. 1). They are connected by thin necks (the 'handle' of the club) to the extreme anterior (apical) end of the organism. Although no analogous structures are described in other cells, rhoptries can be compared to multicellular glands since the enclosing wall structure of the rhoptry is maintained after the electron dense contents are discharged, leading to 'empty rhoptries'. The view that rhoptries are part of the classical secretory pathway is based on several observations: (i) in *Plasmodium* small vesicles, presumably transport vesicles, have been seen by electron microscopy to fuse with the rhoptry compartment (Bannister and Mitchell, 1995), (ii) rhoptry proteins are synthesized with N-terminal signal sequences which mediate translocation of the respective proteins across the ER membrane (reviewed by Lingelbach, 1993), and (iii) post-translational proteolytic maturation of rhoptry proteins is inhibited at 15°C and blocked by BFA, both of which disrupt transport through the classical secretory pathway (Ogun and Holder, 1994). Invasive stages of *P. falciparum* contain 2 rhoptries per parasite while these organelles are morphologically undetectable in the intracellular stages preceeding the schizont. In contrast, rhoptries (6-12 per parasite) are present throughout the intracellular replicative phase of *T. gondii* tachyzoites, although they disappear transiently and are regenerated during each parasite division.

Micronemes are apical organelles common to *T. gondii* and *Plasmodium* (Fig. 1). Micronemal marker proteins contain N-terminal signal sequences responsible for targeting of the proteins to the ER (Sim et al., 1990; Adams et al., 1990; Galinski et al., 1992; Formaux et al., 1996; Wan et al., 1997). Some microneme proteins contain linear hydrophobic stretches which potentially serve as trans-membrane domains. Micronemes, like rhoptries, are absent from intracellular stages of *Plasmodium* preceeding the schizont stage. It is not clear whether cryptic precursor forms of these organelles exist in developing *Plasmodium* and which cellular events trigger their ultimate formation.

Dense granules are present in both parasites. These

secretory organelles which are similar in appearance to dense matrix granules in mammalian exocrine or neuroendocrine cells are distributed throughout the cell (Fig. 1). All *T. gondii* dense granule proteins identified to date contain N-terminal signal sequences (reviewed by Cesbron-Delauw, 1994), whereas some dense granule proteins in *Plasmodium* lack typical signal sequences. Dense granules are not known to contribute to initial formation of the PVM.

Sorting in the secretory pathway

In both *T. gondii* and *P. falciparum* it remains an open question at which branch point proteins are sorted from the ubiquitous secretory pathway to these organelles. Such studies are complicated by the limited option to use structural analyses of N-linked carbohydrates or other post-translational modifications as a means to follow individual steps along the secretory pathway. The analysis of sorting signals has progressed in *T. gondii* because genetic manipulation of this parasite is far more advanced than in *Plasmodium*. In *T. gondii*, all known soluble secreted proteins are packaged either into rhoptries, dense granules or micronemes. In *P. falciparum* secretion via the apical organelles is not obligatory, particularly since these organelles are absent throughout most of the intracellular development. In *T. gondii*, soluble proteins lacking targeting signals are delivered by default to dense granules (Karsten et al., unpublished; Striepen et al., 1998). This includes both endogenous proteins (soluble SAG1-GFP) and foreign molecules (*Escherichia coli* β-lactamase and alkaline phosphatase). Targeting signals for delivery to micronemes and rhoptries are not yet defined.

Organelle secretion and host cell invasion

Drugs which disintegrate the parasite cytoskeleton interfere with host cell invasion of *Toxoplasma* and *Plasmodium* (Bannister and Mitchell, 1995; Bejon et al., 1997; Dobrowolski and Sibley, 1996). Rhoptries and micronemes could contribute to the invasion process in several ways. While in *P. falciparum* both rhoptries are discharged, only a few of the rhoptries in *T. gondii* discharge. In *T. gondii* (Carruthers and Sibley, 1997) and presumably also in *P. falciparum*, microneme discharge preceeds rhoptry discharge, which may occur in part after the parasites have entered the cell. Overall, the stage of invasion appears very similar in *T. gondii* and *Plasmodium*, where the morphology of the process, and the biogenesis of the PVM appear highly congruent.

The precise triggers for invasion and for rhoptry and microneme discharge are not known, the invasion of host cells is completed within a few seconds, and invasion mutants cannot currently be rescued for further analysis. All of these factors contribute to the difficulty in studying the process. It is assumed that micronemal and rhoptry proteins participate in the invasion process. In principle, these proteins could be involved in two distinct functions: (i) in *Plasmodium* it has been demonstrated that rhoptry and micronemal proteins which are released prior to invasion have an affinity to noninfected erythrocytes (Sim et al., 1990; Orlandi et al., 1990; Ogun and Holder, 1996). It has been suggested that these proteins mediate an initial contact of the free parasite with the host cell. (ii) At a later stage enzymes from apical organelles could be involved in the dissociation of the erythrocyte membrane. Thus, a parasite protease has been implicated in the degradation of band 3 (Roggwiller et al., 1996), and rhoptry proteins have been found associated with the plasma membrane of newly infected erythrocytes (Sam-Yellowe, 1992; Ndengele et al., 1995). As these events preceed the formation of the PVM, we will not discuss them in more detail.

Origin of lipids for the PVM

The contribution of parasite lipids to the formation of the PVM has long been a matter of interest. Since the PVM is a large membrane, estimated to have a surface area of 30-33 μm^2 (Suss-Toby et al., 1996), yet forms de novo in 10-20 seconds, an unusual biological process must be involved. The possibility that rhoptries contain internal membranes or at least lipid stores, which participate in the de novo formation of the PVM, is a controversial but fascinating cell biological issue with little precedent in biology.

The lipid composition of the *Plasmodium* PVM is unknown, as is the lipid composition of the *Plasmodium* rhoptries. Treatment of parasites with cytochalasin, which blocks parasite entry but not attachment or rhoptry discharge, led to the appearance in the erythrocyte of empty vesicular structures, suggesting a rhoptry origin for these structures (Aikawa et al., 1981). While these structures were generally smaller than the PVM surrounding intracellular parasites, they are consistent with the notion that rhoptries participate in part in formation of the PVM. In *Plasmodium*, lipid probes have been used to examine the origin of lipids in the PVM. Initial reports suggested that PVM lipids were derived from the parasite. More recent studies in which erythrocyte membranes were labeled with non-exchangable fluorescent lipid probes prior to parasite invasion indicated that PVM lipids were largely derived from the erythrocyte plasma membrane (Ward et al., 1993; Pouvelle et al., 1994). On the other hand, measurements on the loss of surface area from newly infected erythrocytes appear to contradict a major internalization of lipids from the host cell plasma membrane (Dluzewski et al., 1995).

Fewer investigations are available on the origin of lipids in the *T. gondii* PVM. No studies are published using lipid probes. Empty vesicular structures containing the rhoptry protein ROP1 are present in host cells in which parasite invasion is blocked by cytochalsin (Carruthers and Sibley, 1997), analogous to the results for *Plasmodium*. However, a recent elegant study using patch clamping to measure host cell capacitance during cell invasion by *T. gondii* concluded that the majority of lipids comprising the PVM were host cell derived (Suss-Toby et al., 1996). This suggests that host cell lipids can rapidly 'flow' past the moving junction (described below) to generate the bulk of the PVM. Nonetheless, up to a 19% contribution of lipids from the parasite could occur, and be beyond the limits of sensitivity of the assay.

A unifying hypothesis for both *Plasmodium* and *Toxoplasma* is that rhoptry lipids do indeed participate in the formation of the PVM, but that the bulk of the lipids in the newly formed PVM come from the host cell.

Host cell and parasite proteins in the PVM

Unlike lipids, erythrocyte membrane proteins are excluded from the PVM upon invasion of *P. falciparum* (Atkinson et al., 1987; Dluzewski et al., 1989; Ward et al., 1993). Rhoptry proteins on the other hand are associated with newly formed PVM (Sam-Yellowe et al., 1988; Bushell et al., 1988). It is not

clear, whether they span the PVM in a transmembrane topology and whether, still inside the rhoptry, they are membrane associated. Since one of these proteins has also been detected at the erythrocyte membrane shortly after invasion (Sam-Yellowe et al., 1988) it is more likely that they are synthesized as soluble proteins which associate with membranes after the discharge of the rhoptry. A post-delivery association of parasite proteins with biological membranes, though unusual in other cells, appears to be very common in apicomplexan parasites. This issue will be discussed in more detail below.

Host cell proteins are also completely excluded from the *T. gondii* PVM. The site of exclusion of proteins with both *T. gondii* and *Plasmodium* is thought to be the moving junction. This is a transient and uncharacterized structure, which bridges the parasite and host cell and appears as a crystalline array by electron microscopy. Selected rhoptry proteins are associated with the *T. gondii* PVM, but not with the host cell plasma membrane. A total of nine *T. gondii* rhoptry proteins are identified by molecular cloning (ROP1,2,4,7,8) or monoclonal antibody reactivity (Sadak et al., 1988); additional family members are present in the *T. gondii* EST data base. *T. gondii* ROP1 was the first of these genes to be cloned (Ossorio et al., 1992), the ROP1 gene product is recognized by a monoclonal antibody which inhibits a functional activity termed penetration enhancing factor (Schwartzman, 1986), reported to increase *T. gondii* invasion of host cells (Lycke and Norrby, 1966). Nonetheless, disruption of the ROP1 gene was not associated with any change in parasite invasion (Kim et al., 1993). ROP1 is a proline rich protein without a putative transmembrane domain; the protein is associated with the lumenal face of the PVM shortly after invasion (Beckers et al., 1994; Saffer et al., 1992), but is no longer detectable within 12-24 hours after invasion (Dubremetz et al., 1993). Genes for ROP2,4,7, and 8 are related to one another (approximately 55-60% identity at the nucleotide level). All have putative transmembrane domains and are highly basic (pI >9.0). ROP2,3,4, and 7 are detected in the PVM shortly after invasion, are exposed on the cytoplasmic face of the PVM, are present in a punctate localization in mature vacuoles, and behave as integral membrane proteins (Beckers et al., 1994). Taken altogether, these results further support the notion that rhoptries contribute to PVM formation, by contributing membrane containing imbedded rhoptry proteins. Since the lipid composition of *T. gondii* rhoptries is unusual (Foussard et al., 1991), this may influence the final lipid composition of the newly formed PVM (Joiner, 1991).

The molecular events involved in PVM formation, although still unresolved, are a fascinating issue and, undoubtedly, a unique process in cell biology. The mechanism is broadly similar for *Plasmodium* and *Toxoplasma*, and only with further study will biologically meaningful differences be defined.

MAINTENANCE AND ENLARGEMENT OF THE PVM

The vacuole in which the parasite resides must expand as the parasites grow and replicate. In erythrocytes infected with *P. falciparum*, the PVM extends into the host cell cytosol as a network of so-called tubovesicular membranes (TVM). The source of lipids for this expansion and the insertion of proteins into the *Plasmodium* and *Toxoplasma* PVM has been of considerable interest. Studies of this issue have been complicated by technical obstacles to purifying the PVM from infected cells.

Plasmodium is a substantially easier system than *Toxoplasma* to analyze with regard to maintenance and enlargement of the PVM. The mature erythrocyte cannot contribute to the expansion of the *Plasmodium* PVM because: (i) the red cell lacks the machinery for de novo lipid biosynthesis, and (ii) because erythrocytes do not endocytose. In fact, when erythrocyte membranes were labeled with non-exchangeable lipid probes after parasite invasion, no labeling of the TVM or other intracellular membranes was observed (Ward et al., 1993; Haldar and Uyetake, 1992; Pouvelle et al., 1994). Some data suggest that lipids in the erythrocyte membrane inner leaflet are taken up by the parasite, whereas analogues confined to the outer leaflet may not be. While synthesis of phospholipids is detected in infected erythrocytes, the de novo synthesis of fatty acids remains below detectable levels. Thus, the parasite relies on an exogenous supply of components for lipid biosynthesis. The site where lipids for the PVM and the TVM are synthesized, and the lipid composition of this endomembrane system still remains obscure. The parasite synthesizes and exports a sphingomyelin synthase activity, which is detectable within the PVM and the TVM (Elmendorf and Haldar, 1994). More recent data suggest the existence of two distinct sphingomyelin synthase activities. One of these activities, characterized by its unusually high sensitivity to a specific inhibitor of sphingomyelin synthase appears to be specifically required for TVM enlargement (Lauer et al., 1995). Since it is unlikely that sphingomyelin is the only lipid component of the PVM it is conceivable that additional enzymes involved in lipid biosynthesis are also exported from the parasite and that lipid biosynthesis occurs in the PVM/TVM. Experimental evidence for this hypothesis, however, is still lacking. An alternative model proposes a junction between the parasite plasma membrane and the vacuolar membrane at which lipids could be transfered from the parasite into the PVM (Haldar, 1996). Again, there is still no experimental evidence for the transfer of lipids from a junction.

A number of membrane proteins of the PVM/TVM have been identified which are synthesized in the parasite cytoplasm and transported to their final destinations. One of these proteins, EXP-1, is synthesized as a type I integral membrane protein (Günther et al., 1991). Its orientation within the PVM has been determined experimentally. The N-terminal end of EXP-1 is located at the vacuolar site of the PVM, and the C-terminal end is directed to the erythrocyte cytosol (Ansorge et al., 1997). This topology is consistent with the view that such proteins are delivered to the PVM via a vesicular transport which involves transport vesicles that bud from the parasite plasma membrane and fuse with the PVM. Futhermore, according to this model, the vacuolar face of the PVM would have to correspond to the extracytoplasmic face of the parasite plasma membrane. Although this vesicular flow could account for the enlargement of the PVM and the insertion of integral membrane proteins, transport vesicles within the vacuolar space have not been identified so far.

In *T. gondii*-infected cells, the maintenance of the PVM is far more difficult to assess than in infected erythrocytes. There

is no evidence for an extensive array of parasite-induced membranes in the cytosol of the host cell, although, unlike erythrocytes lacking internal membrane structures, their detection in nucleated cells infected by *Toxoplasma* is completely dependent upon the presence of parasite protein markers. Instead, the most extensive collection of membrane-like structures is a tubuloreticular network which accumulates within the vacuolar space. The network appears fused at points with the PVM, providing a potential physical connection for lipid traffic from the PVM to the vacuolar space, and potentially to the parasite, or in reverse. Thread-like extensions from the PVM into the host cell cytosol are observed for nearly all vacuoles, detected by immunofluorescent staining for the dense granule protein GRA3, and potentially reflecting the analogue of the *Plasmodium* TVM. Despite these structural connections, the contribution of the host cell to lipid incorporation into the expanding PVM is not clear. The host cell lipid biosynthetic apparatus (i.e. ER) is tightly opposed to the *Toxoplasma* PVM. Hence, the mechanisms and/or necessity for acquiring lipids from the host cell are very different when *Toxoplasma* and *Plasmodium* are compared. Export of parasite proteins involved in lipid biosynthesis, such as sphingomyelin synthase, is unexplored and would be technically nearly impossible to study in the context of the host cell. Instead, secreted proteins, which are of dense granule origin, localize to structures within the vacuolar space, either to the membranous intravacuolar network, to the PVM, or both. The GRA3 protein, for example, is soluble within the dense granule but, upon release into the vacuolar space, spontaneously inserts into the PVM (Ossorio et al., 1994). In contrast, the dense granule protein GRA5 contains a putative transmembrane domain and localizes to the PVM (Lecordier et al., 1993), suggesting the possibility that vesicular transport delivers the protein from the parasite to its final destination. Although discrete transport vesicles are not observed in the vacuolar space, the extensive array of tubules provides a potential conduit for delivering protein and lipid from the parasite to the PVM, or vice versa.

Overall, the mechanisms for maintenance and enlargement of the PVM appear to be substantially different for *Plasmodium* and *Toxoplasma*, reflecting differences in the lipid biosynthetic capabilities of the host cells.

BIOLOGICAL FUNCTIONS OF THE PVM

The biological functions of the PVM are substantially different when *T. gondii* and *P. falciparum* are compared. The reasons for these differences are largely based on the necessities imposed by the differences in the host cell. The *Toxoplasma* vacuole does not fuse with any organelle of the host cell endocytic cascade from the nucleated host cell. This attribute is most likely due to the absence from the PVM of the protein signals from the host cell needed for docking and fusion (reviewed by Sinai and Joiner, 1997). Since fusion results in killing of the parasite, e.g. when it is taken up via the host cell's phagocytic pathway, this is an essential feature of the PVM. Understanding which SNAREs and additional proteins of the docking and fusion machinery are missing, and/or how the lipid composition of the PVM influences fusion, represents an interesting cell biological issue for which there are few paradigms. This question, while relevant for the liver stages of *Plasmodium*, has little applicability to the erythrocytic stages. An open but important question is whether the formation and maintenance of the PVM in erythrocytic and in liver stages of *P. falciparum* is controlled by the same or by different genes.

Despite the absence of fusion with endocytic organelles, the *T. gondii* PVM forms tight associations with host cell mitochondria and ER, commencing immediately after cell invasion (Porchet-Hennere and Torpier, 1983; Sinai et al., 1997). Recent evidence from our laboratory implicates rhoptry proteins in the association (A. P. Sinai and K. A. Joiner, unpublished). While the function of the tight interaction is not known, it is likely that lipid acquisition from the host cell is the favored process. The intimate finger-glove type association of the PVM with mitochondria and ER, which obliterates the normal organelle exclusion zone seen between organelles in nucleated cells, has few precedents in biology. Tightly opposed mitochondria can be seen around fat droplets in adipocytes and cardiac myocytes. Mitochondrial-associated membranes (MAM), the sight for decarboxylation of phosphatidylserine to phosphatidylethanolamine, may reflect functionally the same process (Shiao et al., 1995). Nonetheless, understanding this process in *T. gondii* will undoubtedly reveal novel mechanisms for organelle association.

In both parasites, the PVM forms a barrier between the host cell cytosol and the parasite surface and prevents access of host cell proteins to the vacuolar space. Whereas the question of whether soluble secreted parasite proteins are transported beyond the PVM has long been a matter of debate, there is now plentiful evidence that this process occurs with *P. falciparum*. The transported proteins either remain within the erythrocyte cytosol or, alternatively, associate with the submembranous erythrocyte cytoskeleton. For example, a family of proteins, collectively called PfEMP1, is exposed on the erythrocyte surface (Baruch et al., 1995). These proteins are believed to mediate adherence of infected erythrocytes to endothelial cells, a phenomenon largely responsible for the pathogenesis of malaria.

The transport of plasmodial proteins across the PVM is selective, as some secreted proteins remain within the vacuolar space (Ansorge et al., 1996). From a cell biological standpoint, this observation raises a number of apparent questions: (i) what are the transport pathways of these proteins, (ii) what are the signals that direct these proteins to a specific destination within the infected cell, and (iii) what are the sorting mechanisms which operate in a cell that normally does not distribute newly synthesized proteins? Most of these questions are still unanswered. The transport of one protein, called the glycophorin-binding protein, has been followed in some detail. The protein is first secreted into the vacuolar space via a BFA sensitive pathway and, in a second step, is translocated across the PVM into the erythrocyte cytosol, where the protein remains soluble (Ansorge et al., 1996). Likewise, the secretion from the parasite of some proteins which associate with the erythrocyte cytoskeleton is sensitive to BFA (Hinterberg et al., 1994) but it has not been determined whether the vacuolar space is also a transit compartment for these proteins. Proteins which are released into the vacuolar space are likely to have a folded conformation. A recent hypothesis therefore proposes the presence of chaperones within the vacuole which assist in unfolding of these proteins prior to their translocation across

the PVM. Another prediction would have to be the existence of a membrane bound translocator (Lingelbach, 1997).

In *T. gondii*-infected cells, parasite proteins which are transported beyond the PVM have not been identified so far. The identification of such proteins would be technically very difficult due to de novo protein synthesis within the host cell. In addition, a conceptual bias against an extensive transport of parasite proteins across the PVM is the host cell's potential to present such proteins to the effector cells of the immune system, whereas infected erythrocytes cannot present molecules on the surface in the context of MHC class I or II. An alternative point of view is equally tenable, that the parasite induces a selective immune response as part of the beneficial (to the parasite) process of conversion to the dormant bradyzoite stage within the cells. In either case, these are considerations which are not applicable to *Plasmodium*, and define an important difference between the two host-parasite systems.

Nutrient acquisition

It is a logical assumption that the intracellular parasites acquire nutrients from either the host cell cytosol, the extracellular medium or from both. For example, both parasites are purine auxotrophs and are incapable of synthesizing fatty acids de novo. Hence, the PVM has a dual function since it has to protect the parasite from potentially harmful substances on the one hand and has to allow if not to facilitate nutrient access on the other hand. The latter aspect applies particularly to nutrients derived from the host cell cytosol. As discussed above, there is no evidence that host cell proteins gain access to the vacuolar space. In fact, eukaryotic cells that are part of multicellular organism (and endoparasites can be considered as such) do not necessarily require uptake of proteins for growth and development, although selected amino acid auxotrophies are documented. Nevertheless, *Plasmodium* ingests approximately 80% of the host cell hemoglobin. A prominent organelle, called the cytostome, constantly phagocytoses the PVM including hemoglobin. The PVM is degraded within the primary food vacuole, and the hemoglobin is digested. It is not clear to what extent this pathway is physiologically relevant in terms of nutrient acquisition. It has been suggested that degradation of hemoglobin may be a prerequisite to provide space for the growing parasite inside the erythrocyte. Although in *T. gondii* an organelle called micropore is believed to be involved in phagocytosis, a major depletion of host cell cytosol has not been observed.

The PVMs of both parasites contain proteinaceous pores, presumably of parasite origin, which allow the passive bidirectional diffusion of small molecules <1,300 Daltons (Schwab et al., 1994; Desai and Rosenberg, 1997). In the case of *T. gondii*-infected cells, this pore should allow parasite access to the entire range of small molecules present within the nutrient rich host cell cytoplasm, precluding any necessity to acquire such nutrients from the extracellular environment. For example, it has been suggested that host cell ATP, capable of diffusing across the PVM pore, may serve as a source of purines for salvage by the intracellular parasite. The development of *P. falciparum*, in contrast, depends on the presence of essential amino acids in the culture medium, indicating that an uptake of these molecules is a physiological requirement. During parasite development, the infected erythrocyte becomes permeable to a variety of solutes (for reviews see Ginsburg, 1994; Gero and Kirk, 1994, Cabantchik, 1990; Gero and Upston, 1992). Two fundamentally different pathways have been proposed whereby the intracellular parasite could gain access to nutrients from the extracellular milieu. According to a conventional view, the parasite alters the permeability of the erythrocyte membrane, either by a biochemical modification of pre-existing transporters or by the insertion of novel, parasite derived transporters into the host cell membrane. Solutes transported into the erythrocyte cytosol could gain access to the vacuolar space via the pores in the PVM. Alternative models involve biological functions of the TVM. According to the 'parasitophorous duct hypothesis' the TVM extends to the erythrocyte membrane and forms an aqueous channel which allows direct access of macromolecules to the vacuolar space and thus to the parasite surface (Pouvelle et al., 1991). This model has been challenged by many investigators (e.g. Haldar, 1994; Hibbs et al., 1997). It is difficult to envisage the co-existence of an open duct and non-selective pores within the PVM. This would result in an equilibrium of the Ca^{2+} concentration in the medium and in the host cell cytosol which, however, is not the case (Ginsburg, 1994). A more differentiated view of the TVM as an intracellular transport system has recently been presented by Lauer et al. (1997). According to their data, the TVM acts as a molecular sieve which forms a junction with the erythrocyte membrane and which allows import of specific nutrients such as adenosine, glutamate and orotic acid to the parasite. The view that the PVM/TVM forms a network within the infected cell, responsible for the acquisition of nutrients and, possibly, for the delivery of parasite proteins to the surface of the infected cell is intriguing and will stimulate future research.

SUMMARY AND CONCLUSIONS

The PVM surrounding *Plasmodium* and *Toxoplasma* is a unique membrane, unlike nearly any other in biology. The biogenesis of this membrane, which is dependent on the unusual and shared entry mechanism of the parasites into cells, is similar for the two parasites. Beyond this initial step, the maintenance, expansion, and function of the PVM is substantially different when the two organisms, are compared, reflecting the biosynthetic capacities of the host cells.

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