A Perspective in Parasitology: Transmembrane solute transport in the apicomplexan parasite *Plasmodium*

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Abstract

Apicomplexa are a large group of eukaryotic, single-celled parasites, with complex life cycles that occur within a wide range of different microenvironments. They include important human pathogens such as *Plasmodium*, the causal agent of malaria, and *Toxoplasma*, which causes toxoplasmosis most often in immunocompromised individuals. Despite environmental differences in their life cycles, these parasites retain the ability to obtain nutrients, remove waste products and control ion balances. They achieve this flexibility by relying on proteins that can deliver and remove solutes. This reliance on transport proteins for essential functions makes these pathways excellent potential targets for drug development programmes. Transport proteins are frequently key mediators of drug resistance by their ability to remove drugs from their sites of action. Study of transport processes mediated by integral membrane proteins and in particular identification of their physiological functions and localization, and differentiation from host orthologues has already established new validated drug targets. Our understanding of how apicomplexan parasites have adapted to changing environmental challenges has also increased through study of their transporters. This brief introduction to membrane transporters of apicomplexans highlights recent discoveries focusing on *Plasmodium*, and emphasizing future directions.

Introduction

Apicomplexans

Apicomplexa are a large and diverse group of eukaryotic, unicellular organisms, consisting almost entirely of obligate endoparasites (*i.e.* those that live within hosts). The phylum includes protozoan parasites of the genera *Plasmodium, Toxoplasma, Babesia,* and *Cryptosporidium*. These phyla contain species that cause serious illness in humans and livestock with consequent global impacts. Their defining feature is an apical complex that is involved in cellular invasion [1,2]. During invasion apicomplexan parasites also form a parasitophorous vacuole membrane (PVM) that surrounds the intracellular parasite [3]. Many also contain a novel organelle called the apicoplast, which is homologous to the chloroplast of plants, and harbours critical metabolic pathways that are typical of plastid function such as type II fatty acid biosynthesis, isoprenoid biosynthesis, and haem biosynthesis [4,5]. Apicomplexan parasites undergo highly specialised life cycles, which consist of both asexual and sexual reproductive stages. Often there is transmission between an invertebrate vector (*e.g.* mosquitoes or ticks) and a vertebrate host, invasion of more than one host cell type can occur (*e.g.* hepatocytes and erythrocytes in the case of *Plasmodium*) and spore formation (*e.g.* in the case of *Crytosporidium* and *Toxoplasma*).

Membrane transport

For apicomplexan parasites to prosper within a range of different intracellular and extracellular microenvironments, they need systems to provide i) a constant supply of nutrients, ii) waste removal of potentially toxic metabolites (or drugs in the case of resistance) and iii) control of their ion balances . These systems are formed by a network of solute transport proteins (*e.g.* for *Plasmodium* [6,7]). Transport proteins (or transporters) are integral membrane proteins that facilitate that movement of polar solutes across the lipid bilayers that form biological membranes. Transporters are classified depending on whether they are pore-like (channels) or if they require solute binding and subsequent conformational change (carriers) to enable transport (Fig.1). Carriers are further classified depending on their energy requirements into primary active secondary active and facilitative carriers (Fig. 1).

Parasite transporters can be characterised *in situ*, although this can be difficult because of the complex multi-membrane nature of intracellular parasites and the variety of transporters that function in a single membrane. Therefore, heterologous expression systems are often used (Box 1). Transport can be measured using several different techniques including radio tracers, biosensors and electrophysiological approaches [6]. Transporters can be characterised in the same way as enzymes (albeit measuring transport rates rather than rates of chemical reactions) and can conform to Michaelis-Menten kinetics. However, it is important that interpretation of transport data is not confounded by metabolism of the solutes being studied, as this can lead to rate limiting steps in metabolism being measured instead of kinetics of transport [8].

New discoveries

The essential *Plasmodium* permeome

In the case of *Plasmodium falciparum* parasites, just over 140 known and putative transporter sequences have been identified and are collectively termed the *Plasmodium* “permeome” [9,10]. This is less than 3% of the ~5300 gene sequences in plasmodia. This turns out to be a relatively small percentage compared with other organisms, although it is worth noting that ~50% of the plasmodial genome still awaits annotation. Even so, the apparent lack of transporters in *Plasmodium* parasites suggests there is little functional redundancy and reinforces their potential therapeutic possibilities [11]. Though not studied

in as much detail in other apicomplexan parasites (see for example [12]), *Toxoplasma* has a greater number of transporters than *Plasmodium* (including within transporter classes), suggesting far more redundancy and thus fewer targeting opportunities. Interestingly, *Cryptosporidium* and *Babesia* parasites may have reduced numbers of transporters and/or transporter classes compared with *Plasmodium* (in this case targeting opportunities could be increased due to less functional redundancy and/or decreased due to druggable transport classes not being present).

An important validation step to determine the therapeutic potential of a protein is to determine whether it is essential by gene disruption. While there has been a steady flow of studies that target single transport proteins (*e.g.* [13]), recent genome-wide essentiality studies in the mouse model of malaria, *P. berghei* (and *T. gondii* [14,15]) and a large targeted gene knockout study in *P. berghei* [16] has increased greatly our understanding of the importance of transporters individually and as a family. Out of the identified transporters in *Plasmodium* parasites, gene disruption has been attempted in just over 100 (including the few previous studies in *P. falciparum*), with evidence that ~33% are likely to be essential during the asexual erythrocyte stage. A further 21% of transporter gene knockouts produce slow-growth phenotypes, while the remaining 46% are dispensable. In some cases, complete life-cycle studies have shown that many of those transporters that are not essential during the asexual erythrocyte stage are important at other life cycle stages [16]. Therefore, it is clear that transporters play critical roles during the plasmodial life cycle and offer opportunities for therapeutic intervention.

Plasmodium falciparum P-type ATPase 4, PfATP4

While there has long been interest in transporters that are involved in resistance (*e.g.* the *P. falciparum* chloroquine resistance transporter, PfCRT, see below), the discovery that a novel antimalarial drug class, the spiroindolones [17], most likely acts by inhibition of PfATP4 has heightened interest in targeting transport proteins in *Plasmodium* and other apicomplexan parasites. The P-type ATPase family of cation and lipid pumps, to which PfATP4 belongs, has long been postulated to contain antimalarial drug targets [18]. Currently in phase II trials [19], spiroindolones were discovered from a library produced following large phenotypic drug screens [20-22]. *In vitro* spiroindolone drug pressure experiments generated resistant parasites with mutations in PfATP4 [17]. This finding and subsequent functional experiments that demonstrate spiroindolones alter Na^+ (and H^+) homeostasis by inhibition of Na^+/H^+ pump-like activity in *P. falciparum* suggest that PfATP4 is directly targeted by spiroindolones [23]. However, the current evidence is unable to exclude the possibility that spiroindolones target regulators of PfATP4 and/or other Na^+/H^+ homeostasis processes. Furthermore, a range of additional chemotypes have been found to work via a similar mechanism and, where tested, selected for mutations in PfATP4 [24-27]. This has led to the possibility that PfATP4 is not the direct target but acts as a drug efflux resistance mechanism. A recent study was undertaken to address this issue [28]. Using directed evolution of a yeast line (the "ABC_{16} Monster") that is susceptible to spiroindolones at low micromolar concentrations, it was shown that spiroindolones select for mutations in a P-type ATPase (ScPMA1, a H^+ pump). Furthermore, spiroindolones were shown directly to inhibit ScPMA1 in a cell-free model system [28], adding weight to the suggestion that PfATP4 is targeted directly by spiroindolones. These studies also highlight the problem of linking functional data to a specific gene and alternative hypotheses will remain until PfATP4 can be studied in isolation.

Divalent cation transport

Calcium (Ca^{2+}) is an important signalling cation and its concentration, or more specifically the free intracellular Ca^{2+}concentration $[Ca^{2+}]_i$, is tightly regulated by Ca^{2+} buffers and Ca^{2+} transporters. In *Plasmodium* and other apicomplexan parasites, $[Ca^{2+}]_i$ regulates key processes, including motility, cellular invasion and egress, and intracellular development, during different life cycle stages [29-33]. Unlike *Toxoplasma* that encodes a range of putative Ca^{2+} transporters, those annotated in the databases of *Plasmodium* parasites are scanty [34,35]. Only two Ca^{2+} transporters have been characterised in *P. falciparum*. The first is a sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) orthologue, PfATP6 [36]. PfATP6 is refractory to knockout attempts despite being amenable to homologous recombination, suggesting it is essential for the asexual erythrocytic stage of development [37]. It has also been identified as a target for artemisinins [36], see below also, and linked to a resistance mechanism against a novel antimalarial compound that is being put forward for clinical development [38]. The second Ca^{2+} transporter is the *P. falciparum* Ca^{2+}/H^{+} exchanger, PfCAX [13,39,40], which is localised intracellularly (though the exact location is debated). The *P. berghei* homologue is predominantly expressed during parasite transmission and acts as a critical Ca^{2+} tolerance mechanism for the free living parasite developing within the mosquito gut [13]. It may also play a role in signalling, given new evidence that CAXs are directly involved in this process [41]. The lack of genomic evidence for more transporters involved in Ca^{2+} homeostatic control in *Plasmodium* parasites, even in light of evidence for multiple storage sites (*e.g.* acidocalcisomes [42]) and functional data for known Ca^{2+} homeostatic processes such as IP_3R-like release mechanisms (reviewed in [43]), suggests novel Ca^{2+} transporters remain to be characterised.

Iron is another important cation due to its ability to act as an electron donor and acceptor, existing in the ferric (Fe^{3+}) and ferrous (Fe^{2+}) forms physiologically. It has a central role in a range of cellular processes such as DNA, pyrimidine and haem synthesis, glycolysis and electron transport. While essential, iron can also be toxic by mediating the production of oxygen free radicals and, thus, its regulation is tightly controlled. However, little is known about the molecular basis of iron acquisition and its homeostatic control in malarial parasites. Several plasmodial genes encode putative iron transporters [9] and three have been characterised in recent years. The first is an orthologue of the zinc/iron permease, ZIP, family (of which two exist in *Plasmodium* genomes), which is termed the ZIP domain-containing protein, ZIPCO. While not essential, it was found to be important to parasite development during the liver stage, and while transport function was not characterised directly, increasing extracellular iron could, in part, rescue *P. berghei* parasites in which ZIPCO was genetically disrupted [44]. The latter result, coupled with plasma membrane localisation, suggests that ZIPCO acts to import iron into the parasite [44]. The second is an orthologue of the vacuolar iron transporter, VIT, family, members of which are proposed to transport Fe^{2+} into acidic vacuoles. Using the yeast heterologous expression system, *P. falciparum* VIT was shown to transport Fe^{2+} with low micromolar affinity, in the first functional characterisation of a member of the VIT family [45]. It was later demonstrated to exchange Fe^{2+} for protons [46]. As with ZIPCO, *P. berghei* VIT was found not to be essential. However, it is important for both blood and liver stages of parasite growth, providing a tolerance mechanism against excess iron, and may localise to the parasite's endoplasmic reticulum [45]. The third and most recently characterised iron transporter is PfCRT. PfCRT has a primary role in the development of resistance in *P. falciparum* to the antimalarial drug chloroquine. Localised to the parasite's digestive vacuole, it has long been known that PfCRT mutants are able to transport chloroquine. However, the essential physiological role of PfCRT has received far less attention but is hotly debated [47-52]. Expressed in *Xenopus laevis* oocytes (frogs eggs), both wild-type

and mutant PfCRT transport Fe^{2+} and Fe^{3+}, albeit with slight different kinetics [53]. How this relates to the physiological role of PfCRT and iron homeostasis in the parasite remains to be determined.

Other transporters

The wealth of genomic information, our growing understanding of apicomplexan transporters and a touch of serendipity have led to the characterisation of a number of new transporters in recent years, some of potential therapeutic interest. Asexual blood stage *Plasmodium* parasites and other stages are wholly dependent on glycolysis for their energy requirements. The *P. falciparum* hexose transporter (PfHT [54]) is the entry point for glucose into this process and its critical role has been demonstrated with both genetic and chemical approaches [55-57]. Yet the nature of the transporter responsible for the removal of the major byproduct of glycolysis, lactate, had remained elusive until recently. Two groups demonstrated that the surface (and digestive vacuole) expressed *P. falciparum* member of the microbial formate–nitrite transporter family, PfFNT, transports lactate and a range of other monocarboxylates, in a H^+-coupled manner [58,59]. Furthermore and like PfHT [60,61], PfFNT is amenable to inhibition by a range of antiplasmodial compounds [62,63], highlighting its therapeutic potential.

The Major Facilitator Superfamily includes numerous transporters found in the plasmodial parasites, including PfHT [54] and the more recently characterised vitamin B_5 pantothenate transporter PfPAT [64], yet an intriguing group of transporters within this large family shared no obvious homology with other characterised members. This led to them being named the novel putative transporters (NPT), of which there are 5 in *Plasmodium* [10]. While the essential role of one in *P. berghei* (PbNPT1) in the transmission of parasites was highlighted several years ago [65], its role was unknown. It was not until researchers studying a homologue in *T. gondii* (TgNPT1) undertook gene disruption experiments that the role was revealed. They demonstrated that conditional knock-down of the TgNPT1 gene killed the parasites when grown in Dulbecco's Modified Eagle's medium but surprisingly not when grown in RPMI 1640 medium [66]. By comparison of the composition of the two mediums, they were able to determine that TgNPT1 transports arginine in a selective manner and this was confirmed after expression of the transporter in *Xenopus* oocytes. Further experiments with PbNTP1 demonstrated that it also transported arginine along with other cationic amino acids [66]. These findings and the fact that there are 5 NPT sequences in *Plasmodium* and 16 in *T. gondii* suggests that the NPT may be a large novel family of amino acid transporters, and it will be interesting to see if this holds true.

Future directions

While our understanding of transport processes in apicomplexan parasites is increasing there is still much to learn. In the case of *Plasmodium*, our knowledge of the function of half of the ~5300 genes that form the plasmodial genomes is lacking and there will almost certainly be novel transport proteins awaiting discovery. As with many of the current putative transporters, identifying physiological substrates is often challenging, even if comparative analysis provides obvious candidates. In addition, identifying transporter location is also critical to interpretation of function and can be hindered by low copy number. Developments in super resolution microscopy may help with the latter, while the former could be circumvented using functional profiling of *Plasmodium* genomes (*e.g.* [14]), coupled to appropriate solute transport assays (with a similar approach used to identify novel glucose transporters in plants [67]).

In addition to the identification and characterisation of novel transporters, there are a number of important future directions. The majority of essentiality (and localisation) studies have been undertaken in the genetically amenable *P. berghei* mouse model. Studies in human infections, especially *P. falciparum*, are limited presently (*e.g.* PfHT [57]). As genetic studies in *P. falciparum* increase and become more efficient, it will be interesting to see if current discrepancies remain or are resolved. For example, two related putative K^+ channels have been refractory to attempts at genetic disruption in *P. falciparum in vitro*, while both can be knocked out in *P. berghei in vivo* [68-70].

Even where transporters have been identified as essential, and potential drug targets, there remains an almost complete lack of structural studies. It has been nearly a decade since the crystal structure (to 2.05 A) of the likely non-essential *P. falciparum* aquaglyceroporin, PfAQP, was published [71] and this remains the only plasmodial transporter with a reported crystal structure. Structures for eukaryotic transporters in the literature are increasing (*e.g.* [72-74]), along with efforts to express plasmodial transport proteins of sufficient quality for structural determination (*e.g.* [46,75-77]). This suggests structural information will be forthcoming.

Another area of research that has received little attention is the role of host transporters in the development of *Plasmodium* parasites. The relatively small permeome of *Plasmodium* suggests that the parasites have efficiently hijacked their host's functions to reduce their own genome and, thus, increase their fitness. A few studies have reported altered endogenous host transporter activity of varying importance in both erythrocyte (*e.g.* [78,79]) and liver stages of *Plasmodium* development (*e.g.* [80-82]) and further studies are warranted. In addition, there remains the open question of the involvement of host transporters in the altered permeability of host erythrocytes, following *Plasmodium* infection. Termed the new permeability pathways, NPP, and similar to volume-activated chloride channels [83,84] in function, their formation in the erythrocyte plasma membrane involves multiple parasite proteins [85-87] but may also involve host transporters [88,89].

A final and intriguing role for plasmodial transporters is in the action of artemisinins. Recent proteomic studies, using click chemistry, have identified a large pool of proteins that artemisinins interact with, suggesting a pleotropic mechanism of action [90,91]. The artemisinin interactome contains a variety of transporters, including PfATP4/6 and PfCRT. It will be interesting to determine the exact nature of each interaction and its importance, given our current reliance on artemisinins for successful malaria treatment.

Summary

- Transporters are a large group of proteins that facilitate the movement of solutes between membrane bound compartments.
- Recent genome-wide profiling studies have demonstrated the importance of transporters to apicomplexan parasites, including *Plasmodium* and *Toxoplasma*.
- High quality functional, structural and localisation data are required if the therapeutic potential of apicomplexan transporters is to be realised.

Abbreviations

PVM, parasitophorous vacuole membrane
PfATP4, *Plasmodium falciparum* P-type ATPase 4
PfCRT, *Plasmodium falciparum* chloroquine resistance transporter
ScPMA1, *Saccharomyces cerevisiae* plasma membrane H^+ pump 1
SERCA, sarco(endo)plasmic reticulum Ca^{2+} ATPase
PfATP6, *Plasmodium falciparum* P-type ATPase 6
PfCAX, *Plasmodium falciparum* Ca^{2+}/H^+ exchanger
IP_3R, inositol trisphosphate receptor
ZIPCO, zinc/iron permease domain-containing protein
VIT, vacuolar iron transporter
PfHT, *Plasmodium falciparum* hexose transporter
PfFNT, *Plasmodium falciparum* formate–nitrite transporter
PfPAT, *Plasmodium falciparum* pantothenate transporter
NPT, novel putative transporter
PfAQP, *Plasmodium falciparum* aquaglyceroporin
NPP, new permeability pathways

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Competing Interests

The authors have no conflicts of interest.

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Figures and Boxes

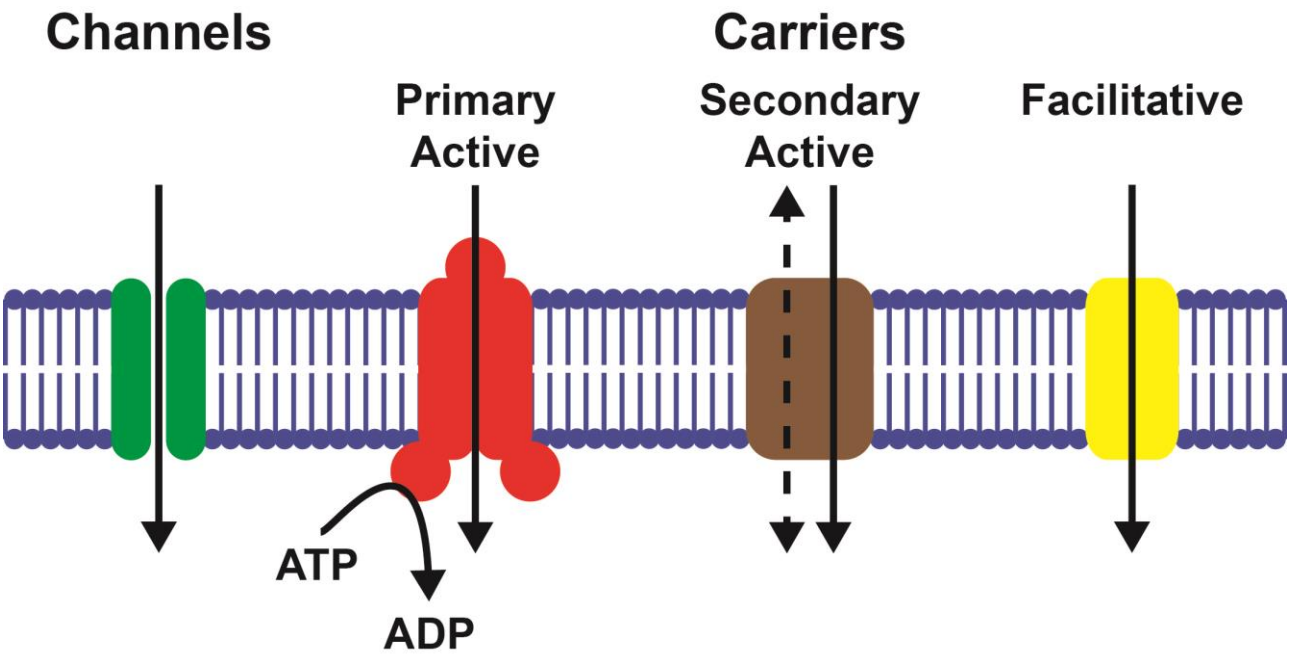

Figure 1. Graphical representation of different transporter classes.
Shown are channels - proteins that are essentially gated, water-filled pores and carriers - proteins that bind solutes and then undergo conformational change to move them across a membrane. Carrier proteins are further classified into three subclasses: primary, active carriers – these use energy derived directly from ATP, predominantly, to drive transport, secondary, active carriers – these use the energy derived from the electrochemical gradients of solutes such as H^+ and Na^+ to drive the transport of other solutes against their own electrochemical gradients, and facilitative carriers - these facilitate the transport of substrates down their electrochemical gradients.

Box 1. Heterologous expression systems

Heterologous expression systems provide less complicated environments in which to characterise proteins. They are a powerful and often necessary approach for the study of transport proteins, particularly those from organisms that are challenging to work with, such as intracellular parasites. Once expression of a transporter of interest in a heterologous system has been achieved (by either transfection or injection of RNA), the system (as a whole, as single cells or as membrane/vesicular preparations) can be used to characterise function, with various methodologies. Cell-free systems have also been developed [92]. However, it is important to note that information derived from expressions systems may not always relate to how transporters may function in their native environments. For example, they may not localise to the same region or there may be differential post-translational effects.

Xenopus oocytes (frog's eggs) are an attractive expression system for quantifying transport activity, particularly (although not exclusively) if the transporter of interest localises to the plasma membrane (e.g. PfHT [54]). They provide a relatively straightforward means for electrophysiological approaches and tracer transport experiments following transient expression by RNA injection [93]. Furthermore, a general low background level of endogenous transport activity is often a major advantage.

Another attractive whole cell heterologous expression system is the highly characterised and genetically amenable yeast, *Saccharomyces cerevisiae*. In particular, the availability of yeast mutants lacking a particular transport pathway provide systems for phenotype rescue following expression of a foreign transporter. For example, *S. cerevisiae* has three main Ca^{2+} transport pathways that accumulate Ca^{2+} into internal stores and can provide tolerance to excess Ca^{2+}: a Ca^{2+}-ATPase (PMC1) and a Ca^{2+}/H^{+} exchanger (VCX1) present at the vacuolar membrane, and a Ca^{2+}-ATPase (PMR1) present at the endoplasmic reticulum. When one or more of these Ca^{2+} transporters are deleted, the yeast cannot grow on high concentrations of Ca^{2+} in the growth medium [94,95]. Use of mutant yeast lines with these transporters deleted has been used for successful functional validation of Ca^{2+}-ATPases (*e.g.* PfATP6 [37]).