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Genetic Evidence for the Essential Role of PfNT1 in the Transport and Utilization of Xanthine, Guanine, Guanosine and Adenine by *Plasmodium falciparum*

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Abstract

The malaria parasite, *Plasmodium falciparum*, is unable to synthesize the purine ring *de novo* and is therefore wholly dependent upon purine salvage from the host for survival. Previous studies have indicated that a *P. falciparum* strain in which the purine transporter PfNT1 had been disrupted was unable to grow on physiological concentrations of adenosine, inosine and hypoxanthine. We have now used an episomally complemented *pfnt1Δ* knockout parasite strain to confirm genetically the functional role of PfNT1 in *P. falciparum* purine uptake and utilization. Episomal complementation by *PfNT1* restored the ability of *pfnt1Δ* parasites to transport and utilize adenosine, inosine and hypoxanthine as purine sources. The ability of wild-type and *pfnt1Δ* knockout parasites to transport and utilize the other physiologically relevant purines adenine, guanine, guanosine and xanthine was also examined. Unlike wild-type and complemented *P. falciparum* parasites, *pfnt1Δ* parasites could not proliferate on guanine, guanosine or xanthine as purine sources, and no significant transport of these substrates could be detected in isolated parasites. Interestingly, whereas isolated *pfnt1Δ* parasites were still capable of adenine transport, these parasites grew only when adenine was provided at high, non-physiological concentrations. Taken together these results demonstrate that, in addition to hypoxanthine, inosine and adenosine, PfNT1 is essential for the transport and utilization of xanthine, guanine and guanosine.

Keywords

Plasmodium falciparum; malaria; PfNT1; purine; transport

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1. INTRODUCTION

More than three billion people live at risk of contracting malaria, a disease caused by intra-erythrocytic protozoan parasites of the genus *Plasmodium* [1,2]. Four species of *Plasmodium* are responsible for the majority of human malaria infections (*P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*), and cases of *P. knowlesi* infection in humans have recently been reported [3]. *P. falciparum* is the most lethal species, causing more than one million deaths annually [4]. With no effective vaccine available and drug resistance on the rise, the need for new antimalarial therapies is critical.

Protozoan parasites, unlike the human host, are incapable of purine biosynthesis and so are reliant upon the uptake of purines from their environment. As a consequence of this nutritional deficiency, each genus of parasite has evolved a unique complement of purine permeases and salvage enzymes that enables it to scavenge host purines effectively [5]. The first step in purine acquisition by *P. falciparum* is the translocation of host purines into the parasite, a process that fulfills an obligatory nutritional function and, as a result, offers potential targets for pharmacologic intervention.

The *P. falciparum* genome sequencing project uncovered four genes (*PfNT1*, *PfNT2*, *PfNT3*, and *PfNT4*) that harbor the majority of the signature sequences and topological features of the equilibrative nucleoside transporter family [6–8]. Of these, only PfNT1 has been functionally characterized at the molecular level [9,10]. Immunofluorescence and immunoelectron microscopy analyses using antibodies directed toward this protein's NH₂-terminus revealed that PfNT1 is localized to the parasite plasma membrane [11]. Furthermore, western blot analysis demonstrated that PfNT1 is expressed throughout the intraerythrocytic life cycle but is maximally expressed prior to parasite schizogony, at the peak of nucleic acid synthesis and utilization [11]. Expression studies in *Xenopus laevis* oocytes established that PfNT1 exhibits broad ligand specificity for nucleosides and nucleobases [9,10]. Studies by Downie and colleagues have shown that the transport properties of nucleosides and nucleobases across the parasite plasma membrane match closely those of PfNT1 expressed in *Xenopus* oocytes [12, 13].

Initial evidence for the functional importance of PfNT1 in purine metabolism was achieved by creating a *pfnt1Δ* null mutant in which the *PfNT1* locus was disrupted by gene replacement [14]. Growth of this strain was only permissible on high, non-physiological concentrations of adenosine, inosine and hypoxanthine (presumably at high concentrations purines are able to cross the parasite plasma membrane by simple diffusion) [14], indicating that PfNT1 plays a key role in the utilization of host adenosine, inosine, and hypoxanthine as purine sources. The role PfNT1 plays in the transport and utilization of the other naturally occurring purines xanthine, guanine, guanosine and adenine has not yet been investigated.

We have now extended our analysis of purine transport to other physiologically relevant purines and demonstrated that PfNT1 is essential for parasite growth on guanine, guanosine, xanthine and adenine.

2. METHODS

2.1. Construction of the *pfnt1Δ*+PfNT1 complemented strain

To generate the *pfnt1Δ*+*PfNT1* complemented strain, the open reading frame of *PfNT1* was amplified by PCR from *P. falciparum* cDNA using the forward primer 5'-CCGCTCGAGATGAGTACCGGTAAAGAGTCATCT-3' (*Xho*I site in bold, start codon underlined) and the reverse primer 5'-CCGCTCGAGTTATTGTGTTACATCGATGGGTGG-3' (*Xho*I site in bold, stop codon

underlined), digested and cloned into the *Xho*I site of the pHC1 vector [15], which encodes the *P. falciparum* CAM1 promoter and HSP86 terminator. Clones were selected and used to transform the *pfnt1Δ* strain using standard protocols [16]. Transgenic parasites harboring the *pfnt1Δ*+*PfNT1* were selected by addition of 60 nM pyrimethamine to the culture medium and characterized by PCR to demonstrate both the loss of the *PfNT1* chromosomal locus and the presence of the episome.

2.2. Microscopy

The intraerythrocytic development of *P. falciparum* was monitored by light microscopy on Giemsa-stained smears. Localization of PfNT1 in wild-type (3D7), *pfnt1Δ* and *pfnt1Δ* +*PfNT1* parasites was determined by immunofluorescence as previously described [14]. Coverslips containing *P. falciparum*-infected erythrocytes were fixed and incubated for 1 h at room temperature with rabbit anti-PfNT1 polyclonal antibodies (diluted 1:500) and mouse anti-Band3 monoclonal antibody (1:500). Nuclei were stained by incubating the coverslips in phosphate buffered saline containing 3 μg/ml Hoechst 33258 (Molecular Probes) for 5 min at room temperature. The coverslips were then washed and mounted on slides with Prolong Antifade (Invitrogen), and images were analyzed by high-resolution fluorescence using deconvolution protocols. Microscopy was performed using an inverted microscope (Eclipse TE2000-E; Nikon) and filter 96320/HYQ (excitation 480 – 440 nm/emission 440 nm) for FITC, filter 96312/G2EC (excitation 540 – 525 nm/emission 620 – 660 nm) for rhodamine, and filter 96310/UV2EC (excitation 360–340 nm/emission 460–450 nm) for DAPI.

2.3. Parasite lactate dehydrogenase assay

The parasite-specific lactate dehydrogenase assay was performed in 96-well plates as described [17–19], 48 hours after synchronized, ring-stage infected red blood cells were added to RPMI containing increasing concentrations of purines. For each reaction, 20 μl of infected red blood cells were mixed with 10 μl of 1 mg/ml diaphorase, 10 μl of 1 mg/ml nitroblue tetrazolium and 100 μl of malstat reagent [17–19]. Parasite viability is indicated by the production of a purple color.

2.4. Transport assays in saponin-isolated parasites

Synchronized, mature trophozoite-stage (36 – 40 h post-invasion) parasites were ‘isolated’ from their host erythrocytes by treatment with saponin as described elsewhere [20]. Following saponin treatment, parasites were washed at least three times in a HEPES-buffered saline (125 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 20 mM glucose and 25 mM HEPES, pH 7.1). In order to distinguish between the transport and subsequent metabolism of radiolabeled purines, all experiments were carried out on parasites that had been depleted of their ATP content by incubation in a glucose free HEPES buffered saline (135 mM NaCl, 5 mM KCl, 1 mM MgCl₂ and 25 mM HEPES, pH 7.1) followed by incubation at 37 °C for 15 min [21]. Parasites are wholly reliant on glycolysis for ATP synthesis [22]. Transport measurements were carried out essentially as reported previously [12,13,20], except that all experiments were carried out at room temperature (in order to allow measurable choline uptake, which acted as a control in all transport experiments). Briefly, uptake of radiolabelled solute was initiated by addition of a 200 μL volume of isolated parasites to an equal volume of radiolabeled substrate solution (at twice the intended final concentration) layered over a 200 μL dibutyl phthalate/dioctyl phthalate (5:4, v/v) inert oil mix. Uptake was stopped at pre-determined times by sedimenting the parasites below the oil layer using centrifugation in a rapid-acceleration microcentrifuge. The cell pellets were processed for scintillation counting as described previously [20], and estimates of the volume of extracellular fluid trapped in the cell pellets were made as described previously [12]. Uptake levels are expressed as ‘distribution ratios’ (i.e. the calculated intracellular concentration relative to the extracellular concentration of a particular solute).

2.5 Curve fitting

All curves were fitted using SigmaPlot version 7.0. For all time-course experiments, the curves were fitted to the full five data point, three minute time-course. To aid clarity the 3 min time points have been omitted from the scatter plots and are represented as bar graphs. Full time courses are provided as supplemental figures (Fig S1, Fig S2 and Fig S3)

3. RESULTS

3.1. Expression of the PfNT1 gene in the *pfnt1Δ* background rescues adenosine, inosine and hypoxanthine transport and nutritional defects of the knockout

To confirm that the defects in the utilization of adenosine, inosine and hypoxanthine in the *pfnt1Δ* strain [14] were not caused by secondary genetic alterations due to the loss of the *PfNT1* chromosomal locus, a complemented strain (*pfnt1Δ+PfNT1*) was constructed in which the *PfNT1* gene was expressed episomally within the *pfnt1Δ* genetic background. To generate the *pfnt1Δ+PfNT1* strain, the *PfNT1* ORF was cloned into the pHC1 vector, which encompasses the *Toxoplasma gondii* DHFR-TS marker conferring resistance to pyrimethamine [23]. In the resulting pHC1-PfNT1 vector, *PfNT1* expression is under the regulatory control of the *P. falciparum* *CAM1* promoter and *HSP86* terminator (Fig 1A). This vector was then transfected into the *pfnt1Δ* knockout and parasites resistant to both pyrimethamine and blasticidin (by virtue of the replacement of the *PfNT1* chromosomal locus with the BSD cassette) were selected. *PfNT1* expression in the *pfnt1Δ+PfNT1* strain was confirmed by reverse transcriptase PCR (Fig 1B) and immunofluorescence using affinity-purified anti-PfNT1 antibodies [11] (Fig 1C).

To determine whether the episomal expression of *PfNT1* rescues the growth defects of the *pfnt1Δ* strain, the nutritional requirements of wild-type, *pfnt1Δ* and *pfnt1Δ+PfNT1* parasites were assessed by examining parasite growth when adenosine, inosine, and hypoxanthine were supplied as sole purine sources at concentrations ranging from 0 to 500 μ M using the parasite-specific lactate dehydrogenase growth assay (Fig 2A) [17–19]. Whereas the *pfnt1Δ* strain was capable of growth only when hypoxanthine was present as a sole purine source at concentrations above 50 μ M, wild-type and *pfnt1Δ+PfNT1* strains exhibited robust growth when hypoxanthine was present as a sole purine source at concentrations as low as 2 μ M (Fig 2A). Similar results were obtained with adenosine and inosine (data not shown). Giemsa-stained smears of parasite cultures were used to monitor development and life-cycle progression of each strain at different hypoxanthine concentrations (Fig 2B). Whereas wild-type and *pfnt1Δ+PfNT1* parasites proceeded through the intraerythrocytic development cycle at hypoxanthine concentrations as low as 2 μ M, the *pfnt1Δ* chromosomal knockout required 50–100 μ M hypoxanthine in order to complete the intraerythrocytic life cycle (Fig 2B). Similar results were observed for growth on adenosine and inosine as sole purine sources (data not shown).

In order to determine if the episomal expression of *PfNT1* can complement the transport defects seen in *pfnt1Δ* parasites, time-courses for the uptake of radiolabeled purines were carried out with ATP-depleted, saponin-isolated wild-type, *pfnt1Δ* and *pfnt1Δ+PfNT1* parasites. To test the viability of the ‘isolated’ ATP-depleted parasites, the ATP-independent, equilibrative transport of [14 C]-choline, a compound that is not a PfNT1-ligand, was investigated. Previous work has indicated that the accumulation of [14 C]-choline in energized parasites reflects a combination of transport and metabolic trapping (i.e. phosphorylation of choline by choline kinase) [24]. Depleting cells of their ATP content inhibits this metabolic trapping (as the phosphorylation of choline is energy dependent) and results in choline reaching a distribution ratio of 1 (indicating equal concentrations of choline inside and outside the cell) [24]. Consistent with previously published results [24,25], ATP-replete wild-type and *pfnt1Δ* cells

accumulated [^{14}C]-choline in a linear fashion, reaching a distribution ratio (i.e. the concentration of a particular substrate inside the cell relative to that outside the cell) of ~ 20 after 10 min. In contrast, when cells from both strains were depleted of their ATP content, [^{14}C]-choline levels reached a distribution ratio of ~ 1 (indicating equal concentrations inside and outside the cell) over a 30 s time period, a ratio that was maintained over the 10 min time course of the experiment (Fig. 3A). The transport of [^{14}C]-choline was therefore used as a control for cell viability and ATP depletion in all subsequent purine transport experiments.

Time courses for the uptake of adenosine, inosine and hypoxanthine by isolated wild-type, *pfnt1Δ* and *pfnt1Δ+PfNT1* parasites depleted of their ATP content are shown in Fig. 3 (panels B – E; full timecourses are shown in supplemental data, Fig S1). As observed previously [12], [^3H]-adenosine (Fig 3B) and [^3H]-inosine (Fig 3C) were taken up rapidly by wild-type 3D7 parasites with each [^3H]-nucleoside reaching a distribution ratio of 2 – 3 in the first 10 s when the relevant unlabelled nucleoside was present at an extracellular concentration of 10 μM . This level of accumulation continued to rise, such that after three min the level of [^3H]-adenosine within wild-type cells had reached a distribution ratio of $7.1 (\pm 0.7, n=2)$, and that of [^3H]-inosine had reached a distribution ratio of $5.7 (\pm 0.9, n=2)$ (Fig 3E). In contrast, however, knockout parasites exhibited no initial uptake of [^3H]-adenosine (Fig 3B) or [^3H]-inosine (Fig 3C), and uptake remained negligible even after 3 min (Fig 3E). Similarly, knockout parasites showed negligible uptake of [^3H]-adenosine and [^3H]-inosine when the relevant unlabelled nucleoside was present at an extracellular concentration of 0.1 μM (data not shown). Nucleoside transport capability was restored in *pfnt1Δ+PfNT1* parasites (closed triangles). In each case, the [^3H]-nucleoside reached a distribution ratio approximating 1 within the first 30 s after addition of the radiolabel to the cell suspension (Fig 3B & 3C), and a distribution ratio of ~ 2 after three min (Fig 3E).

Consistent with published results [13], [^3H]-hypoxanthine was transported rapidly by isolated wild-type parasites. When unlabelled hypoxanthine was present at an extracellular concentration of 10 μM , [^3H]-hypoxanthine reached a distribution ratio of 1 within the first 10 s (Fig 3D), and this distribution ratio was maintained over 3 min (Fig 3E). Under the same conditions, *pfnt1Δ* parasites exhibited no uptake of [^3H]-hypoxanthine over the first 60 s (Fig 3D), and only slight uptake after 3 min, reaching a distribution ratio of $0.2 (\pm 0.1; n = 3)$. Similar results were obtained when unlabelled hypoxanthine was present at 1 μM and 0.1 μM (data not shown). The transport of [^3H]-hypoxanthine by *pfnt1Δ+PfNT1* parasites (Fig 3D) was similar to that obtained for the transport of adenosine and inosine by this strain, with [^3H]-hypoxanthine reaching a distribution level of $1.3 (\pm 0.2, n = 2)$ over 30 s, and $1.9 (\pm 0.4, n = 2)$ after 3 min (Fig 3E). Interestingly, however, this level of hypoxanthine uptake was actually higher than that detected in the wild-type strain (Fig 3D). These results clearly indicate that the transport deficits in *pfnt1Δ* parasites can be complemented by episomal expression of *PfNT1*. As such, subsequent transport experiments compared purine uptake in wild-type and knockout strains only.

3.2. PfNT1 is essential for the utilization but not the transport of adenine

The abilities of wild-type, *pfnt1Δ* and *pfnt1Δ+PfNT1* strains to undergo asexual reproduction in the presence of varying concentrations of adenine was assessed using parasite-specific lactate dehydrogenase growth assays. Adenine supported growth of wild-type and *pfnt1Δ+PfNT1* parasites at concentrations as low as 2 μM when present as a sole purine source (Fig 4A), similar to the concentrations of hypoxanthine, adenosine and inosine necessary to support growth of these strains (Fig 2). The *pfnt1Δ* knockout, however, was unable to grow when adenine was present as a sole purine source at concentrations below 50 μM , although higher concentrations of adenine permitted *pfnt1Δ* growth and progression through the intraerythrocytic development cycle (Fig. 4A). These results suggest that either PfNT1 is

essential for the transport of adenine into the parasite or that adenine is converted in the red blood cell cytoplasm into a substrate that requires PfNT1 for its transport across the parasite plasma membrane. In order to distinguish between these two possibilities, transport experiments to measure the uptake of [³H]-adenine by *pfnt1Δ* parasites were performed. The conditions used were similar to those described above for adenosine, inosine and hypoxanthine (Fig 3), and to those used previously for the characterization of adenine transport in isolated wild-type parasites [13]. As shown in Fig 4, [³H]-adenine was taken up by both wild-type and *pfnt1Δ* parasites when adenine was present at 1.0 μM (Fig 4B & 4C) or 0.1 μM (Fig 4D & 4E). The data are also represented as 3 min time courses in Fig S2 (supplemental data). At each concentration, the total amount of adenine taken up by *pfnt1Δ* parasites was similar to that seen for wild-type parasites. When unlabelled adenine was present at an extracellular concentration of 1 μM (Fig 4B), [³H]-adenine was taken up rapidly by both wild-type and *pfnt1Δ* parasites, reaching a distribution ratio close to 2 within 30 seconds of the addition of radiolabel. After 3 min (Fig 4C), [³H]-adenine had reached a distribution ratio of 2.1 (± 0.1; n=3) in wild-type cells and 1.9 (± 0.1; n=4) in *pfnt1Δ* cells. A difference in the rate of uptake of [³H]-adenine between wild-type and *pfnt1Δ* cells was observed when the concentration of extracellular unlabelled adenine was 0.1 μM (Fig 4D). Whereas wild-type parasites showed rapid uptake of [³H]-adenine, reaching a distribution ratio of ~ 3 within 30 s, the amount of [³H]-adenine within *pfnt1Δ* parasites did not reach this level until 60 s after the addition of radiolabel. Intracellular adenine concentrations were comparable between the two strains after 3 min (wild-type: 3.2 ± 0.7, n=3; *pfnt1Δ*: 3.4 ± 0.9, n=4; Fig 4E). These transport results imply that while PfNT1 does account for some adenine transport a second uptake process exists at the parasite plasma membrane, which in addition to PfNT1 can enable entry of adenine into the parasite.

3.3. PfNT1 is required for xanthine, guanine and guanosine transport

Human serum contains measurable quantities of xanthine, guanine and guanosine [26], which could serve as potential purine sources for *P. falciparum*. To assess the role of PfNT1 in the transport of these physiologically relevant purines across the *P. falciparum* parasite plasma membrane, the transport and nutritional requirements for these 6-oxypurines was compared in wild-type, *pfnt1Δ* and *pfnt1Δ*+PfNT1 parasites (Fig 5). Wild-type and *pfnt1Δ*+PfNT1 strains were able to grow when guanine, guanosine, and xanthine were present as sole purine sources at concentrations as low as 2 μM, while *pfnt1Δ* parasites were unable to complete the intraerythrocytic life cycle at concentrations of these ligands up to 50 μM (Fig. 5A and B). Interestingly, xanthine, guanine and guanosine were found to be toxic to wild-type parasites at concentrations higher than 50 μM (data not shown). Thus, whether higher concentrations of xanthine, guanine, or guanosine could meet the purine requirement of *pfnt1Δ* parasites could not be determined.

To examine the involvement of PfNT1 in the transport of xanthine, guanine and guanosine across the parasite plasma membrane, time courses for the uptake of [³H]-xanthine, [³H]-guanine and [³H]-guanosine by isolated, ATP-depleted mature trophozoite-stage parasites were carried out (in the presence of 0.1 μM concentrations of the unlabelled substrate of interest). As shown in Fig 6A, wild-type cells transported xanthine rapidly, reaching a distribution ratio of 0.9 (± 0.2, n=2) within the first 10 sec, a level that was maintained for the 3 min duration of the time-course (Fig 6B). *pfnt1Δ* parasites, however, showed negligible uptake of xanthine over the first 60 s (Fig 6A), although measurable uptake of [³H]-xanthine was detected after 3 min, reaching a distribution ratio of 0.4 (± 0.1, n=2) (Fig 6B). This rate of uptake was much slower than that seen in wild-type cells and may represent simple diffusion of the compound over time. The data are shown as 3 min time-courses in the supplemental data, Fig S3A.

[³H]-Guanine and [³H]-guanosine were also rapidly taken up by isolated wild-type cells, attaining distribution ratios of ~2 within the first 10 sec (Fig 6C & 6E; see also Fig S3B & S3C). Uptake continued over time with each radiolabeled purine compound attaining a distribution ratio of ~4 after 3 min (Fig 6D & 6F). In contrast, under the same conditions, *pfnt1Δ* parasites displayed slight uptake of guanine (0.3 ± 0.1 ; Fig 6C & 6D; similar to that seen for xanthine) and only negligible uptake of radiolabeled guanosine (Fig 6E, F). These results are consistent with PfNT1 being essential for the transport of the 6-oxypurines xanthine, guanine and guanosine across the *P. falciparum* plasma membrane.

4. DISCUSSION

It has recently been demonstrated that PfNT1 plays an essential function in the uptake of host hypoxanthine across the parasite plasma membrane [14]. Under physiological conditions of purine supply, transgenic *pfnt1Δ* parasites lacking PfNT1 activity display a conditionally lethal growth phenotype that could be circumvented by the provision of excess concentrations of hypoxanthine, inosine, or adenosine to the culture medium. We have now created and exploited a *PfNT1* complemented strain, *pfnt1Δ+PfNT1*, to prove definitively that the transport and nutritional deficits observed in the *pfnt1Δ* strain were due to the deletion of *PfNT1* rather than to any other chromosomal rearrangements resulting from the knockout of the *PfNT1* locus. The *pfnt1Δ+PfNT1* strain, like wild-type parasites, was able to grow on physiological concentrations of adenosine, inosine, hypoxanthine, adenine, xanthine, guanine and guanosine, thus complementing the *PfNT1* genetic lesion in the *pfnt1Δ* knockout.

In order to ensure that the saponin-isolated, ATP depleted parasites used in uptake experiments were viable, time-courses for the uptake of [¹⁴C]-choline acted as a control in all experiments. The uptake of choline by isolated parasites is highly temperature dependent, with little uptake occurring at 0°C [25] or 4°C (this study, data not shown). As such, all transport experiments presented here were carried out at room temperature. This may account for the differences observed between the equilibration of adenine and adenosine seen previously for isolated, ATP-depleted cells [12,13] and the accumulation seen here (all other conditions were identical). Performing the uptakes at this temperature also meant that we were unable to acquire data within the linear phase of uptake for these substrates, meaning that kinetic characterization of transport (where observed) was not feasible, as determination of initial rates of transport was not possible. The kinetics of such transport, however, have previously been studied in some detail for wild-type parasites [12,13].

Transport studies in isolated parasites showed that *pfnt1Δ+PfNT1* parasites, unlike *pfnt1Δ* parasites, were capable of efficient transport of adenosine, inosine and hypoxanthine. Adenosine and inosine reached lower concentrations in the complemented strain than they did in the wild-type strain, while complemented parasites took up slightly more hypoxanthine than wild-type parasites. These differences were minor and could be ascribed to the reintroduction of episomal *PfNT1* and its expression from the *CAM1* promoter in the complemented strain. The complemented strain showed similar levels of adenosine, inosine and hypoxanthine (where as hypoxanthine uptake was lower than adenosine or inosine in wild-type parasites). It is possible that the expression of purine salvage proteins is altered in *pfnt1Δ* parasites, and that this difference is maintained in the *pfnt1Δ+PfNT1* strain. Overall, the data obtained here using the *pfnt1Δ+PfNT1* parasites demonstrate that the purine transport and utilization defects seen in *pfnt1Δ* parasites are solely due to the loss of the *PfNT1* gene.

In the current study, the characterization of the *pfnt1Δ* parasites has been extended to examine transport and utilization of other physiologically relevant purines, specifically adenine, xanthine, guanine and guanosine. While wild-type and *pfnt1Δ+PfNT1* parasites were able to grow on adenine as a sole purine source at concentrations as low as 2 μM, *pfnt1Δ* parasites

were able to grow on adenine only when this compound was provided at concentrations above 50 μM (Fig 4A). Intriguingly however, these parasites were still able to transport [^3H]-adenine. At 0.1 μM adenine, the initial rate of transport of [^3H]-adenine was somewhat slower in *pfnt1Δ* parasites than in wild type cells, consistent with a PfNT1-dependent component of adenine transport (Fig 4D). However, the levels of adenine uptake after 3 min were comparable (Fig 4). The observation of a PfNT1-dependent adenine transport component is consistent with work by Downie et al [13], while the presence of adenine transport process(es) in *pfnt1Δ* parasites suggests that adenine can also cross the parasite plasma membrane by an additional transport mechanism. The lack of growth of the *pfnt1Δ* strain at physiological concentrations of adenine (the plasma concentration of adenine is $\sim 0.4 \mu\text{M}$) could be explained if all adenine entering the erythrocyte is rapidly converted to those purines dependent upon PfNT1 for transport into the parasite, either in the erythrocyte cytosol or the parasitophorous vacuole, such that adenine never reaches the parasite plasma membrane (see model in Fig. 7). Alternatively, although some adenine may reach the parasite plasma membrane of the *P. falciparum*-infected erythrocyte and be transported into the parasite by a PfNT1-independent mechanism, but the parasite may not be able to utilize this compound. Available biochemical data and BLAST searches of the *P. falciparum* genome [7], suggest that the parasite lacks the enzymes capable of metabolizing adenine [27]. The erythrocyte, however, has the metabolic machinery to convert exogenously provided adenine to PfNT1 substrates. Adenine can be phosphoribosylated by adenine phosphoribosyltransferase to AMP, AMP can be deaminated by AMP deaminase to IMP, and IMP can be desphosphorylated to inosine, a PfNT1 substrate, by mammalian cells. Thus, it is plausible that the *pfnt1Δ* growth defect could be attributed to an inability of the mutant parasite to take up adenine metabolites such as inosine formed in the erythrocyte cytosol.

Wild-type and complemented parasites were capable of growth on guanine, xanthine or guanosine as a sole purine source, even at concentrations as low as 2 μM . Conversely, *pfnt1Δ* parasites were unable to utilize these 6-oxypurines as a purine source at concentrations below 50 μM (Fig. 6), similar to results seen previously for adenosine, inosine and hypoxanthine [14]. Although we tested whether concentrations of guanine, xanthine, and guanosine between 50 and 1000 μM could rescue the growth defect of *pfnt1Δ* parasites, these high, non-physiological concentrations of these three purines inhibited growth of wild-type parasites (data not shown). Thus lack of growth of *pfnt1Δ* parasites at high concentrations of xanthine, guanine and guanosine cannot be imputed to a lack of functional rescue. Coupled with the impaired capacity of *pfnt1Δ* parasites to take up guanine, xanthine, and guanosine efficiently, the growth assays provide powerful genetic evidence that PfNT1 is the major route of entry of 6-oxypurines into the parasite.

In addition to PfNT1, the *P. falciparum* genome contains three putative transporters (PfNT2, PfNT3, and PfNT4) predicted to be equilibrative nucleoside transporters, suggesting that additional purine transport mechanisms exist in the parasite. While it is possible that these proteins may play a role in the transport of purines across the red blood cell membrane or across organelles within the parasite, the results accumulated thus far indicate that PfNT1 is the only purine transporter required for the uptake of physiologically relevant purine nucleosides and nucleobases across the parasite plasma membrane of intra-erythrocytic forms of *P. falciparum*. The roles of these transporters in the PfNT1-independent transport of adenine and in the biochemistry and physiology of other mammalian or insect stages of the *P. falciparum* lifecycle remain to be explored.

The data presented here for the uptake of adenosine, inosine, adenine, guanine and guanosine by wild-type parasites show that unlike choline (and xanthine), these substrates reached an intracellular concentration higher than the extracellular concentration. The conditions used here (glucose- and ATP-depletion) were chosen in order to prevent phosphorylation (as shown

for choline) and phosphoribosylation of the transported substrates. While it is possible that the accumulation is due to slow metabolism as a result of residual ATP content in the glucose-deprived cells, the fact that choline consistently reached distribution ratios of ~1 over a 3 min time course, rather than the distribution ratio of ~5 in ATP-replete cells would argue against this. Additionally, the observation that guanine is accumulated whereas xanthine is not suggests that this accumulation is not HGXPRT dependent, as both of these compounds are substrates of this enzyme. It is unlikely that the accumulation is due to active transport by PfNT1, as ATP is required to maintain H⁺ and Na⁺ gradients, and not all PfNT1 substrates are accumulated under these conditions. Additionally, multiple studies have shown that PfNT1 transport is neither H⁺ or Na⁺ dependent [9,10]. This suggests that upon their entry into the parasite, some of the transported substrates may be rapidly sequestered in one or more organelles of the parasite. Future studies aimed at the localization and characterization of PfNT2, PfNT3, and PfNT4 could help unravel this mechanism of accumulation, and help explain the need for additional transporters during the parasite's intraerythrocytic life cycle.

The transport results described here for adenosine and inosine differ slightly from our previously reported data, which showed residual uptake of these substrates in energized, isolated *pfnt1Δ* parasites [14]. The different methods used for parasite isolation and radiolabel uptake experiments as well as the energy status of the isolated parasites could account for some of the discrepancies seen. Nevertheless, the combined results of purine transport and utilization in *pfnt1Δ* parasites support the essential role of PfNT1 in the transport and utilization of all purines.

Recently, Quashie and colleagues [28] have suggested that there are four separate purine transport activities, and report the PfNT1-independent uptake of several purines. The data we have reported here does agree on one point (the existence of a PfNT1-independent adenine transport mechanism), however we did not observe any other PfNT1-independent purine transport mechanisms. We believe that the discrepancies could be due to differences in experimental methods as well as a failure on their part to distinguish between transport and metabolism.

In summary, the current study provides genetic evidence for the essential role of PfNT1 in the transport and utilization of xanthine, guanine and guanosine in *P. falciparum*. Future studies will focus on therapeutic strategies to target this transporter, either to block purine uptake into the parasite or as a route for the specific delivery of new antimalarial drugs to the parasite cytoplasm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Construction of a *pfnt1Δ*+*PfNT1* complemented strain. (A) Map of the pHC1-PfNT1 vector used to transfect the *pfnt1Δ* knockout to create the complemented *pfnt1Δ*+*PfNT1* strain. (B) Reverse transcriptase PCR analysis on wild-type, *pfnt1Δ* and *pfnt1Δ*+*PfNT1* RNA using primers specific to *PfNT1* or the *P. falciparum* phosphoethanolamine methyltransferase gene (*PfPMT*; used as a control). (C) Immunofluorescence microscopy of red blood cells infected with wild-type, *pfnt1Δ* or *pfnt1Δ*+*PfNT1* schizont stage parasites. PfNT1 is visualized in green, Band-3 in red and nuclei in blue.

Figure 2.

Growth of wild-type, *pfnt1Δ* and *pfnt1Δ+PfNT1* parasites in the presence of increasing concentrations of hypoxanthine. (A) The viability of parasites at different hypoxanthine concentrations was determined using the parasite-specific lactate dehydrogenase assay. The presence of parasite lactate dehydrogenase, and hence viable cells, is indicated by the production of a purple color. The observed color differences between *pfnt1Δ* and wild-type/complemented wells when purine concentration was below 2 μM are likely due to the presence of residual amounts of purine compounds in the erythrocytes, allowing limited parasite viability in the wild-type and complemented strains, whereas this minimal purine concentration is not sufficient to enable the presence of viable *pfnt1Δ* cells. (B) Synchronized wild-type, *pfnt1Δ* and *pfnt1Δ+PfNT1* parasites grown at different concentrations of hypoxanthine were collected at 8 h intervals, Giemsa-stained and evaluated by light microscopy. Similar results (for both assays) were obtained with inosine and adenosine (data not shown).

Figure 3.

Transport of radiolabeled substrates by *P. falciparum* parasites isolated from their host cells by saponin permeabilization of the erythrocyte and parasitophorous vacuole membranes. (A) Uptake of [14 C]-choline by ATP-replete (squares, solid lines) or ATP-depleted (circles, dashed lines) wild-type (closed symbols) or *pfnt1Δ* (open symbols) parasites. Choline was present at an extracellular concentration of 1 μ M. Uptake of the purines [3 H]-adenosine (B); [3 H]-inosine (C) and [3 H]-hypoxanthine (D) by wild-type (closed circles), *pfnt1Δ* (open circles) and *pfnt1Δ* + *PfNT1* (closed triangles) parasites that had been depleted of their ATP content. The uptake of each purine substrate at 180 s by wild-type (black bars), *pfnt1Δ* (grey bars) and *pfnt1Δ* + *PfNT1* (white bars) parasites is shown in (E). In each case, the relevant unlabelled substrate

was present at an extracellular concentration of 10 μM . Similar results were observed for *pfnt1Δ* parasites when unlabelled substrate was present at 0.1 μM (data not shown). The data for adenosine uptake into wild-type parasites and all *pfnt1Δ* data are averaged from three or more independent experiments (shown $\pm$ SEM) each carried out in duplicate. Inosine and hypoxanthine data from wild-type parasites and all data from *pfnt1Δ+PfNT1* parasites are averaged from two independent experiments (shown $\pm$ SD), each carried out in duplicate. Where not shown, error bars fall within the symbols. All uptake data were obtained from 180 s time courses. For clarity, we have shown the 180 s time point on a separate graph (E). The curves in panels B, C and D were fitted to the complete 180 s time course, which has been provided as supplemental data, Fig S1.

Figure 4.

Utilization and transport of adenine. Parasite growth in the presence of increasing concentrations of adenine was measured using the parasite-specific lactate dehydrogenase assay (A). Transport of [3 H]-adenine by ATP-depleted parasites was measured when unlabelled adenine was present at an extracellular concentration of 1 μM (B, C) and 0.1 μM (D, E). Time courses for the uptake of adenine by wild-type (closed circles) and *pfnt1Δ* (open circles) parasites are shown in panels B and D. The curves are fitted to the data from the full 180 s time course (shown as supplemental data, Fig S2), but for clarity the 180 s data point was omitted from the time course and are shown in panels C & E. Black bars represent uptake by wild-type parasites, and grey bars that of *pfnt1Δ* parasites. *pfnt1Δ* data are averaged from four independent experiments (shown $\pm$ SEM), each carried out in duplicate. Wild-type data are averaged from three independent experiments (shown $\pm$ SEM), each carried out in duplicate.

Figure 5. Growth of wild-type, *pfnt1Δ* and *pfnt1Δ+PfNT1* parasites in the presence of increasing concentrations of guanine, guanosine and xanthine. (A) Parasite viability was assessed using the parasite-specific lactate dehydrogenase assay. (B) Synchronized wild-type, *pfnt1Δ* and *pfnt1Δ+PfNT1* parasites were cultured in increasing concentrations of guanine, collected at eight hour intervals, Giemsa stained and evaluated by light microscopy. Similar results were obtained with guanosine and xanthine (data not shown).

Figure 6.

Uptake of xanthine, guanine and guanosine by ATP-depleted, saponin-isolated wild-type (closed circles) and *pfnt1Δ* (open circles) *P. falciparum* parasites. Uptake of [3 H]-xanthine (A, B); [3 H]-guanine (C, D) and [3 H]-guanosine (E, F) measured in the presence of a 0.1 μ M concentration of the relevant unlabelled purine. Curves are fitted to the full 180 s time course, and in each case the 180 s data point has been shown on a separate graph to aid clarity and allow comparison to the choline control. The data are represented as a full time course in the supplemental data (Fig S3). Wild-type data are averaged from two independent experiments (shown $\pm$ SD), each carried out in duplicate. *pfnt1Δ* data are averaged from three independent experiments (shown $\pm$ SEM), each carried out in duplicate. Where not shown, error bars fall within the symbols.

Figure 7.

Model of the pathways for purine uptake in *P. falciparum*. Hypoxanthine (HXT); adenosine (ADO); inosine (INO); adenine (ADE); xanthine (XAN); guanine (GUA), and guanosine (GUO) are transported by means of endogenous purine transporters and the NPP. Adenine could be phosphoribosylated to AMP in the RBC cytoplasm by APRT, converted to IMP via AMP deaminase, dephosphorylated by an intracellular 5'-nucleotidase to inosine, and converted to hypoxanthine by PNP. The additional parasite plasma membrane permease(s) for the transport of adenine, represented by dotted lines, are unlikely to play an important role under physiological conditions. For clarity, the parasitophorous vacuole membrane has been omitted from this model. PNP, purine nucleoside phosphorylase; ADA, adenosine deaminase; PV, parasitophorous vacuole; RBCM, RBC membrane.