LETTERS

Sodium-dependent uptake of inorganic phosphate by the intracellular malaria parasite

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As the malaria parasite, *Plasmodium falciparum*, grows within its host erythrocyte it induces an increase in the permeability of the erythrocyte membrane to a range of low-molecular-mass solutes, including Na^+ and K^+ (ref. 1). This results in a progressive increase in the concentration of Na^+ in the erythrocyte cytosol[2,3]. The parasite cytosol has a relatively low Na^+ concentration[2,4] and there is therefore a large inward Na^+ gradient across the parasite plasma membrane. Here we show that the parasite exploits the Na^+ electrochemical gradient to energize the uptake of inorganic phosphate (P_i), an essential nutrient. P_i was taken up into the intracellular parasite by a Na^+-dependent transporter, with a stoichiometry of $2Na^+$:$1P_i$ and with an apparent preference for the monovalent over the divalent form of P_i. A P_i transporter (PfPiT) belonging to the PiT family was cloned from the parasite and localized to the parasite surface. Expression of PfPiT in *Xenopus* oocytes resulted in Na^+-dependent P_i uptake with characteristics similar to those observed for P_i uptake in the parasite. This study provides new insight into the significance of the malaria-parasite-induced alteration of the ionic composition of its host cell.

P_i is an important nutrient in cell metabolism and is required for the synthesis of DNA, RNA and numerous phosphorylated metabolic intermediates. Removal of P_i from the growth medium resulted in a $92 \pm 3\%$ ($n = 5$; mean ± s.e.m.; $P < 0.0001$) decrease in parasite proliferation (measured over 96 h). The intraerythrocytic parasite is therefore dependent on an external supply of P_i to maintain normal growth.

The mechanism by which the parasite takes up P_i from the host erythrocyte was investigated by using parasites 'isolated' from their host cells with saponin[5]. Initial uptake experiments were performed in 'de-energized' (glucose-depleted and therefore ATP-depleted) cells to avoid complications from metabolism. The influx of $^{33}P_i$ into de-energized parasites was linear with time for more than 20 min, with the $^{33}P_i$ distribution ratio ($[^{33}P_i]_{in}/[^{33}P_i]_{out}$) increasing to more than 40. Replacement of Na^+ in the medium with either K^+ (Fig. 1a) or choline (not shown) decreased P_i influx by more than 95%, which is consistent with the involvement of a Na^+-dependent transporter. P_i influx showed a sigmoidal dependence on the Na^+ concentration (Fig. 1b) with a Hill coefficient of 2.1 ± 0.2 ($n = 9$; mean ± s.e.m.), consistent with two Na^+ ions binding to the transporter for each P_i molecule. The stoichiometry was confirmed by a 'static head' experiment[6] in which $^{33}P_i$ was allowed to equilibrate in the absence of a Na^+ gradient, then the suspension was diluted in $^{33}P_i$-free medium containing a range of Na^+ concentrations. The $[Na^+]_{out}$ required to keep the intracellular $[^{33}P_i]$ at the same level as in parasites maintained in the original loading solution was 69 ± 12 mM ($n = 7$, mean ± s.e.m.), which is consistent with a stoichiometry of about 2 (1.7 ± 0.3) Na^+ ions being transported per P_i molecule (Fig. 1c).

The apparent K_m for the uptake of P_i ($K_m(P_i)$) decreased with increasing extracellular $[Na^+]$, with values of 445 ± 31, 258 ± 16 and $106 \pm 8\,\mu M$ at Na^+ concentrations of 5, 15 and 100 mM, respectively ($P < 0.01$; Fig. 1d). Thus, as the concentration of Na^+ in the erythrocyte increases with parasite maturation[2,3], the efficiency of the parasite's P_i uptake mechanism increases.

The apparent $K_m(P_i)$ also showed a strong dependence on the pH of the medium, decreasing as the extracellular pH decreased (Fig. 1e). P_i has a pK_a of 6.8 under physiological conditions[7], and as extracellular pH varies so, too, do the relative proportions of monovalent ($H_2PO_4^-$) and divalent (HPO_4^{2-}) P_i. If P_i influx is plotted as a function of the (calculated) concentration of $H_2PO_4^-$, similar apparent K_m values are obtained at each of the three extracellular pH values tested (Table 1). If the same analysis is performed for HPO_4^{2-}, the apparent K_m values diverge (Table 1). These data are consistent with the influx of P_i being by means of a transporter with a strong preference for $H_2PO_4^-$ over HPO_4^{2-}, and an apparent $K_m(P_i)$ that varies little with pH.

A stoichiometry of $2Na^+$:$1H_2PO_4^-$ predicts that net uptake of P_i should be electrogenic, involving an influx of positive charge. Under physiological conditions the parasite has a large, inwardly negative plasma membrane potential[8], which should therefore influence P_i uptake. To test this, $^{33}P_i$ influx was measured in energized (ATP-replete) parasites. Depolarization of the parasite plasma membrane with carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) or by the plasma membrane H^+-pump inhibitor bafilomycin A_1 (ref. 8) resulted in a significant decrease in $^{33}P_i$ influx (Fig. 1f), consistent with the predicted electrogenicity of transport.

Results of additional (efflux) experiments with isolated parasites were consistent with the transporter catalysing not only the net influx and efflux of P_i but also a non-productive exchange of intracellular P_i for extracellular P_i (Supplementary Fig. 2). These transport characteristics may be accounted for by the mechanistic model presented in Supplementary Fig. 3. $^{33}P_i$ efflux by the exchange reaction was much faster than net efflux, which is consistent with net efflux being rate limited by the reorientation of the empty transporter, whereas exchange involves the faster reorientation of the $[H_2PO_4^- + 2Na^+]$-loaded transporter. Depolarization of the parasite plasma membrane slowed net P_i efflux but not the exchange reaction, which is consistent with the presence of a negative charge on the transporter, rendering the $[H_2PO_4^- + 2Na^+]$-loaded transporter complex electroneutral. Exchange was slowed (but not abolished) by the removal of extracellular Na^+, whereas the same manoeuvre enhanced net efflux. The efflux of $^{33}P_i$ into Na^+-free medium might be explained by a partial reaction of the transporter (Supplementary

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Fig. 3), although the involvement of additional transport pathways cannot be excluded.

The annotated *P. falciparum* genome has a single putative plasma membrane P_i transporter (PlasmoDB accession no. MAL13P1.206)[9,10], designated here as PfPiT. Sequence comparisons show PfPiT to be a member of a family of H^+- or Na^+-coupled P_i transporters (the PiT family, or solute carrier family 20), other members of which are present in prokaryotes, plants, fungi and animals (Supplementary Fig. 4). Phylogenetic analysis reveals that PfPiT bears a closer resemblance to Na^+-dependent transporters from fungi and animals than to the H^+-dependent PiT transporters of bacteria and plant chloroplasts (Fig. 2a). An alignment of the hydropathy plots for PfPiT and mouse PiT-1 (Supplementary Fig. 5) shows the strong structural homology of the parasite protein to animal PiT family members and suggests the presence of 12 transmembrane domains (Fig. 2b), consistent with the proposed topology of the human homologue PiT-2 (ref. 11). PiT-2 has both the amino and carboxy termini located at the extracellular face of the membrane[11], and the same is likely to be true of PfPiT.

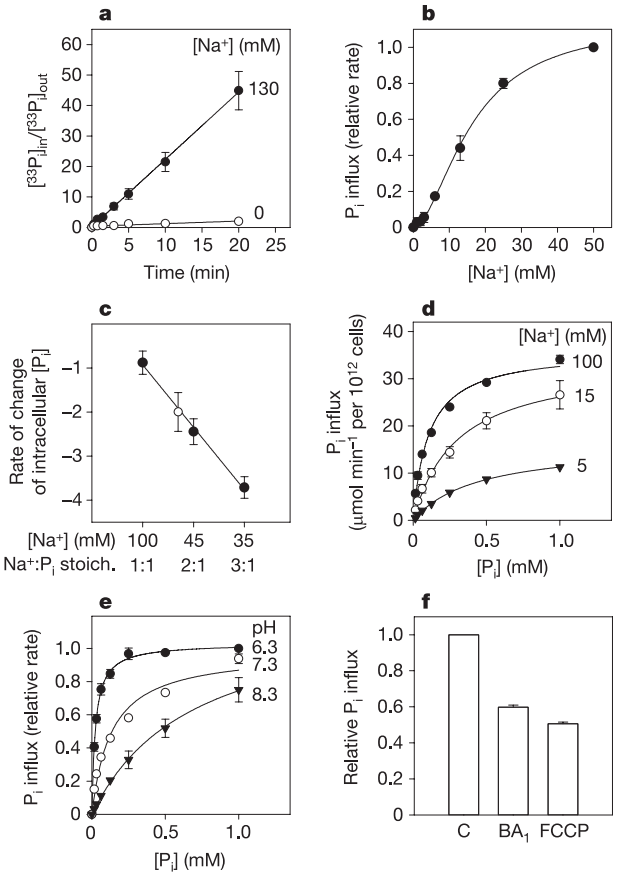

Figure 1 | P_i transport in malaria parasites 'isolated' from their host erythrocytes by permeabilization with saponin. **a**, Time courses for the uptake of $^{33}P_i$ into isolated parasites, de-energized by preincubation in the absence of glucose and suspended in 130 mM Na^+-containing medium (filled circles) or in medium in which Na^+ was replaced iso-osmotically with K^+ (open circles). **b**, $[Na^+]$ dependence of the influx of $^{33}P_i$ into de-energized isolated parasites. **c**, Estimate of Na^+:P_i stoichiometry in a 'static head' experiment in which de-energized parasites were pre-equilibrated with $^{33}P_i$ in the absence of a Na^+ gradient ($[Na^+]_{in} \approx [Na^+]_{out} = 20$ mM), then either maintained in the loading solution (under which conditions $[^{33}P_i]_{in}$ slowly decreased) or diluted fivefold in medium of three differing Na^+ concentrations, corresponding to the $[Na^+]$ values required by a $1Na^+$:$1P_i$, $2Na^+$:$1P_i$, and $3Na^+$:$1P_i$ transporter to maintain the rate of decrease of $[^{33}P_i]_{in}$ the same as that in cells maintained in the original loading solution. The filled symbols show the rate of decrease of $[^{33}P_i]_{in}$ in the three solutions with differing $[Na^+]$ and the open symbol shows the rate of decrease observed in cells maintained in the original loading solution. **d**, $[P_i]$ dependence of the influx of $^{33}P_i$ into de-energized isolated parasites suspended in medium containing 100 mM (filled circles), 15 mM (open circles) or 5 mM (filled triangles) Na^+ (pH 7.4). **e**, $[P_i]$ dependence of the influx of $^{33}P_i$ into de-energized isolated parasites suspended in medium at pH 6.3 (filled circles), 7.3 (open circles) or 8.3 (filled triangles) ($[Na^+]_{out} = 130$ mM). The corresponding kinetic constants are given in Table 1. **f**, Effect of depolarization of the parasite membrane potential by either bafilomycin A_1 (BA_1; 100 nM) or FCCP (10 μM) on the influx of P_i into isolated parasites. The intracellular and extracellular pH values were both 7.35. Influx rates are normalized relative to that measured in energized parasites under control (C) conditions. Results are mean ± s.e.m. from multiple ($n \geq 3$) experiments.

Messenger RNA encoding the *P. falciparum* protein is expressed throughout the entire blood stage of the parasite's life cycle[9,12,13]. The level of transcript inside the cell increases markedly between 16 and 24 h after invasion[9], during which time the parasite-induced increase in erythrocyte $[Na^+]$ is predicted to occur[3]. Transfection of the parasite with PfPiT complementary DNA, tagged at the C terminus with a haemagglutinin (HA) tag, enabled the localization of the protein by immunofluorescence (Fig. 2c). Fluorescence was restricted to the parasite (Fig. 2c, top three panels), and colocalized with that obtained with an antibody against the (unrelated) plasma membrane protein PfENT1 (ref. 14) (Fig. 2c, bottom three panels).

To investigate the function of PfPiT we expressed the protein in *Xenopus laevis* oocytes. Oocytes injected with PfPiT cRNA showed a fivefold to tenfold increase in $^{33}P_i$ uptake relative to non-injected controls (Fig. 3a) or to oocytes expressing PfENT1 (data not shown). The increase was comparable in magnitude to that seen in oocytes expressing the mouse homologue mPiT-1 (data not shown). Replacement of extracellular Na^+ by *N*-methyl-D-glucamine$^+$ abolished PfPiT-induced P_i uptake (Fig. 3a; $P < 0.0001$), which is consistent with PfPiT being a Na^+-dependent transporter. An analysis of uptake as a function of phosphate concentration at pH 7.4 yielded an apparent $K_m(P_i)$ of 20 ± 2 μM and a V_{max} of 40 ± 1 pmol h^{-1} per oocyte (Fig. 3b). The former figure is close to the K_m values obtained for the human PiT-1, rat PiT-2 and mouse PiT-2 phosphate transporters when expressed in oocytes[15,16], although it is lower than the apparent $K_m(P_i)$ determined in the experiments with isolated parasites under similar conditions (Table 1). Discrepancies between the apparent K_m determined for a transport protein in its native cell and that measured for the same protein expressed in a heterologous system have been observed previously[17,18].

As was observed in isolated parasites (Fig. 1e), the rate of P_i influx into oocytes expressing PfPiT increased with decreasing pH (Fig. 3c), which is consistent with PfPiT having a strong preference for $H_2PO_4^-$. Depolarization of the oocyte by the replacement of 50 mM NaCl with 50 mM KCl in the medium[19] halved P_i uptake (Fig. 3d; $P < 0.0001$), a similar decrease to that seen for the influx of P_i into isolated parasites after membrane depolarization (Fig. 1f).

Thus, PfPiT, the only putative plasma membrane P_i transporter

Table 1 | pH dependence of the kinetic parameters for P_i influx into isolated and de-energized *P. falciparum* parasites

pH	V_{max} (μmol min^{-1} per 10^{12} parasites)	Apparent K_m (μM)		
		$[P_i]$	$[H_2PO_4^-]$	$[HPO_4^{2-}]$
6.3	27 ± 6	30 ± 6*	23 ± 4	7 ± 1**
7.3	22 ± 5	118 ± 15*	28 ± 4	90 ± 11**
8.3	28 ± 6	621 ± 103*	19 ± 3	601 ± 100**

The three different apparent K_m values at each pH are those estimated when the analysis was performed using the total P_i concentration ($[P_i]$, as in Fig. 1e), the calculated concentration of monovalent $H_2PO_4^-$, and the calculated concentration of divalent HPO_4^{2-}, in the extracellular medium. All values are mean ± s.e.m. for four different experiments. Asterisk, values obtained at the three different pH values differ significantly from one another (ANOVA; $P < 0.0002$). Two asterisks, values obtained at the three different pH values differ significantly from one another (ANOVA; $P < 0.0001$). No asterisk, values obtained at the three different pH values do not differ significantly from one another (ANOVA; $P = 0.268$).

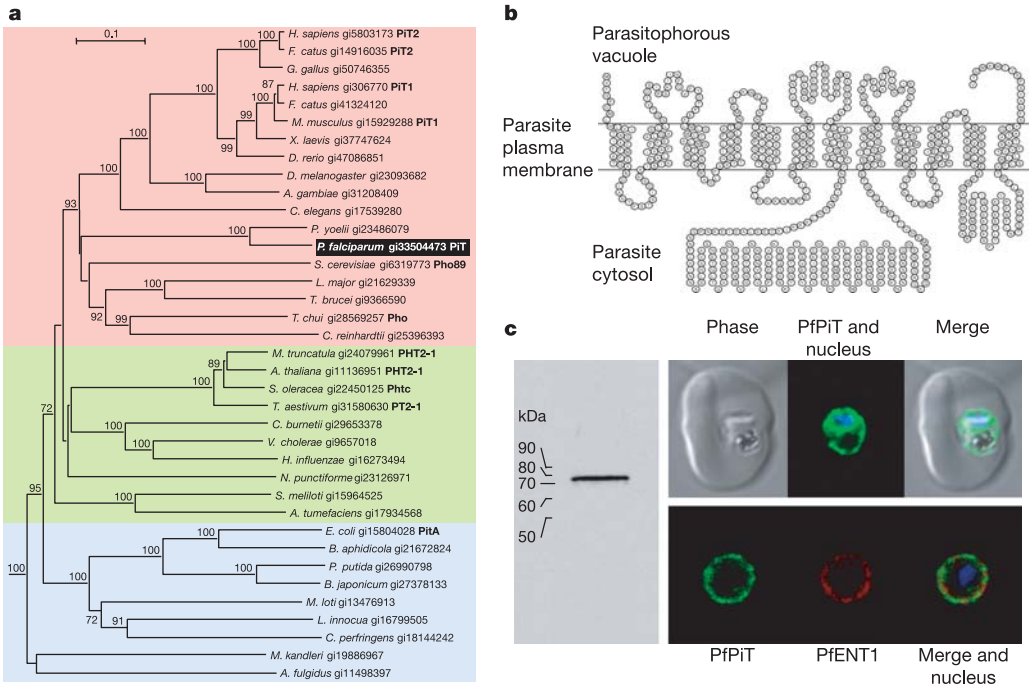

Figure 2 | The *P. falciparum* P_i transporter, PfPiT. a, Phylogenetic tree showing relationships between PfPiT and homologues in other organisms. The main branches of the tree are indicated by backgrounds as follows: red, proteins from animals, fungi and protists; green, proteins from plants and bacteria; blue, a second cluster of bacterial proteins and two Archaean proteins. Bootstrap scores of more than 70% are shown on the branches. The scale bar represents the number of substitutions per site for a unit branch length. **b**, Topographical model of PfPiT derived from hydropathy plots (Supplementary Fig. 5), from a protein sequence alignment of the PiT family (Supplementary Fig. 4) and from topological data for the related human PiT-2 transporter[11]. **c**, Western blot and immunolocalization of PfPiT in the *P. falciparum*-infected erythrocyte. Left: western blot analysis of cells expressing PfPiT-HA. A single band corresponding to the predicted size of PfPiT–HA (about 78 kDa) was detected. Protein from erythrocytes infected with wild-type 3D7 *P. falciparum* did not bind the HA antibody (data not shown). Top row of three panels: a *P. falciparum*-infected erythrocyte expressing PfPiT-HA (green) and nucleus stained with Hoechst 33258 (blue). Within the infected cell, PfPiT-HA localizes to the parasite. Lower row of three panels: PfPiT localizes to the parasite surface, colocalizing with PfENT1 (red), a transport protein shown previously to reside on the plasma membrane of the parasite[14].

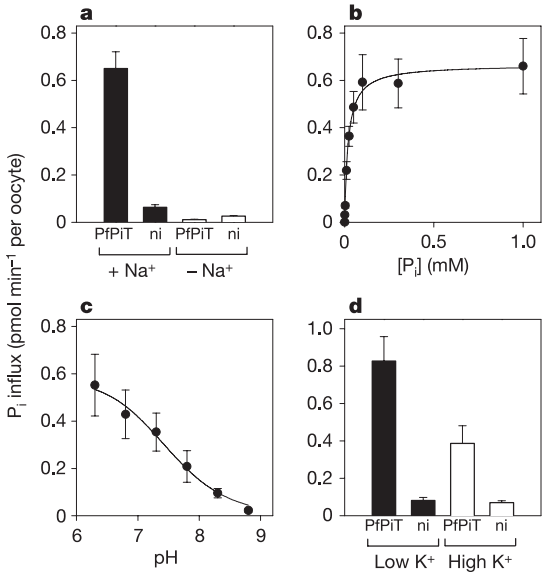

Figure 3 | Transport characteristics of PfPiT in *Xenopus* oocytes. **a**, Oocytes expressing PfPiT show a marked increase in $^{33}P_i$ uptake relative to non-injected (ni) oocytes. Replacement of NaCl by *N*-methyl-D-glucamine chloride in the incubation medium abolished PfPiT-induced $^{33}P_i$ uptake ($[P_i] = 10\,\mu M$). **b**, $[P_i]$ dependence of PfPiT-induced P_i uptake. PfPiT-induced uptake was calculated by subtracting the uptake measured in non-injected oocytes from that in oocytes expressing PfPiT. **c**, pH dependence of PfPiT-induced P_i uptake ($[P_i] = 25\,\mu M$). **d**, Depolarization of oocytes by the replacement of 50 mM NaCl with 50 mM KCl in the incubation medium[19] halved P_i uptake. P_i uptake is shown as mean ± s.d. measured in seven to ten oocytes.

identified so far in the *P. falciparum* genome[9,10], is localized to the parasite surface, and when expressed in *X. laevis* oocytes has P_i transport characteristics similar to those of isolated parasites. The data are consistent with PfPiT having an important role in the uptake of P_i by the intracellular parasite, although the involvement of additional (Na^+-dependent) transporters cannot be ruled out.

As an obligate intracellular parasite, *P. falciparum* inhabits an initially low-Na^+ environment, and in the original annotation of the *P. falciparum* genome it was assumed that the parasite would use H^+-coupled rather than Na^+-coupled transporters for the uptake of nutrients, including P_i (ref. 10). The results of the present study show that this is not necessarily the case, revealing the unexpected scenario of a Na^+-coupled nutrient transport mechanism in an intracellular parasite. Na^+-coupled transporters such as PfPiT provide the means for the malaria parasite to exploit the increased $[Na^+]$ in the infected erythrocyte cytosol (together with the parasite's plasma membrane potential[8]), to energize the uptake of solutes. The results of the present study (summarized in Supplementary Fig. 1) therefore provide new insight into the physiological role for the long-recognized perturbation by the parasite of the ionic composition of its host cell.

METHODS

P_i transport in isolated malaria parasites. *P. falciparum* parasites (strain FAF-6) were cultured and synchronized as described elsewhere[5]. The transport of P_i across the plasma membrane of mature 'trophozoite-stage' parasites (about 36 h after invasion) was measured in parasites 'isolated' from their host erythrocytes by permeabilization of the erythrocyte and parasitophorous vacuole membranes with saponin[5] (Supplementary Methods). Parasite growth was monitored by measuring the incorporation of [^{3}H]hypoxanthine over 96 h by parasites

suspended in a conventional medium (containing 5.6 mM P_i) or in an equivalent medium from which P_i was omitted.

Expression of PfPiT, mPiT-1 and PfENT1 in *X. laevis* oocytes. Genes encoding the parasite proteins PfPiT and PfENT1 and the mouse PiT-1 (mPiT-1) protein were cloned into oocyte expression vector pGem-He-Juel[20] (Supplementary Methods). *In vitro* transcription, oocyte preparation and cRNA injection (30 ng per oocyte) were performed as described elsewhere[21]. $^{33}P_i$ uptake was measured (4–6 days after injection) in medium that, unless specified otherwise, contained 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.4. Uptake of $^{33}P_i$ into oocytes expressing PfPiT was linear with time for at least 60 min (data not shown).

Protein sequence analysis. The phylogenetic tree, hydropathy plot alignment and topographical model were generated as described in Supplementary Methods.

Transfection and immunofluorescence analysis of parasites. Parasites were transfected with the gene encoding a HA-epitope-tagged PfPiT protein (Supplementary Methods). Western blots and immunofluorescence analysis were performed as described previously[22].

Statistics. Statistical comparisons were made with either a Student's *t*-test for paired or unpaired samples, or an analysis of variance.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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