Nutrient transporters play critical roles in parasite metabolism, but the membranes in which they reside have not been clearly defined. The transport of purine nutrients is crucial to the survival of the malaria parasite Plasmodium falciparum, and nucleoside transport activity has been associated with a number of different membrane components within the parasitized erythrocyte. To determine the location of the PfNT1 nucleoside transporter, the first component of the nucleoside permeation pathway to be studied at the molecular level in P. falciparum (Carter, N. S., Ben Mamoun, C., Liu, W., Silva, E. O., Landfear, S. M., Goldberg, D. E., and Ullman, B. (2000) J. Biol. Chem. 275, 10683–10691), polyclonal antisera against the N-termin- al 36 amino acids of PfNT1 were raised in rabbits. Western blot analysis of parasite lysates revealed that the antibodies were specific for PfNT1 and that the level of PfNT1 protein in the infected erythrocyte is regulated in a stage-specific fashion. The amount of PfNT1 polypeptide increases dramatically during the early trophozoite stage and reaches its maximal level in the late trophozoite and schizont stages. Deconvolution and immunoelectron microscopy using these monospecific antibodies revealed that PfNT1 localizes predominantly, if not exclusively, to the plasma membrane of the parasite and not to the parasitophorous vacuolar or erythrocyte membranes.

Plasmodium falciparum is the etiologic agent of the most pernicious form of human malaria and is responsible for 1.5–2.7 million deaths per annum, >1 million of which occur in children <5 years of age (1, 2). P. falciparum is an intracellular protozoan parasite that exhibits a complex life cycle, going through a sexual phase in the insect vector, the Anopheles mosquito, and multiplying asexually within the human host to precipitate its morbidity and lethal sequelae. Chemotherapy and prophylaxis for malaria are available but are severely stymied by drug toxicity and pervasive drug resistance (1, 2). Thus, the need for more efficacious and less toxic antimalarial agents, particularly drugs that are directed toward pathways or targets that are unique to the parasite, is acute.

One striking metabolic discrepancy between all parasites and their mammalian hosts is the purine biosynthetic pathway. Whereas mammalian cells are capable of synthesizing purine nucleotides de novo, all protozoan parasites studied to date (3), including Plasmodium spp. (4), are incapable of making the purine ring. As a consequence, each genus has evolved a unique complement of purine salvage enzymes to scavenge purines from their hosts. This purine salvage process is initiated by the translocation of purines across the parasite membrane. Thus, purine base and nucleoside transporters serve an indispensable nutritional function for the parasite.

The discovery by Gero et al. (5) in 1988 that erythrocytes exhibit altered nucleoside transport properties after P. falciparum infection has stimulated considerable interest into the underlying mechanisms by which this unusual phenomenon occurs. The predominant nucleoside carrier expressed in uninfected human red blood cells (RBCs) is the human equilibrative nucleoside transporter 1 (6). The human equilibrative nucleoside transporter 1 is a facilitated carrier that recognizes virtually all purine and pyrimidine nucleosides and is extraordinarily sensitive to inhibition by 4-nitrobenzyl-6-thioinosine (6). Upon infection with P. falciparum, the paramount nucleoside transporter activity in the red blood cell becomes insensitive to 4-nitrobenzyl-6-thioinosine (6). This unusual capability to translocate L-nucleosides is accounted for by the growth sensitivity of P. falciparum to L-nucleoside analogs (7) and implies that nucleoside uptake components could serve as potential drug targets.

The enclosure of P. falciparum in a parasitophorous vacuole necessitates that the uptake of purines, or for that matter any nutrient, from the human host into P. falciparum occurs across a multiplicity of membranes. Nutrients generally must be transported across the erythrocyte membrane (RBCM), the parasitophorous vacuolar membrane (PVM), and the parasite plasma membrane (PPM); these nutrient permeation pathways may involve a variety of complex and novel components including transporters, channels, ducts, and the tubovesicular mem-
branes (TVM) (9, 11–16). The altered transport capabilities of the infected RBC, referred to as the new permeation pathway (NPP), appear 10–20 h post-invasion, are attributed at least in part to a channel on the RBCM that transports nucleosides, polyols, amino acids, sugars, and cations (17–19), and can be distinguished by sensitivity to anion-selective inhibitors such as furosemide (9, 18). Selective inhibition of parasite protein synthesis prevents the formation of the NPP, suggesting that it originates from parasite proteins. However the specific protein components of the NPP have yet to be determined, and it is possible that more than one NPP exists (15). Recently Desai et al. (17) showed that the nonsaturable, voltage-dependent channel is abundant after invasion, supporting the theory that the channel is a major component of the NPP. Within the parasitized red blood cell, the TVM, an interconnected membrane network extending from the PVM to the periphery of the infected erythrocyte, has also been implicated in nucleoside transport (16).

Searches of The Institute for Genomic Research P. falciparum data base uncovered a gene, PfNT1, that encoded a polypeptide with significant homology to the equilibrative nucleoside transporter family (20, 21). PfNT1 is the first component of the nucleoside permeation pathway to be characterized at the molecular level from P. falciparum. Expression of PfNT1 cRNA in Xenopus laevis oocytes revealed PfNT1 to be a nucleoside transporter of broad specificity that also recognizes purine nucleosides and 1-nucleosides as ligands and exhibits insensitivity to inhibitors of human equilibrative nucleoside transporter 1 (20, 21). Given the complexity of the intraerythrocytic environment in which the parasite resides, the location of PfNT1 becomes particularly important because a restricted milieu can be a determinant of a specialized function and its potential as a therapeutic target.

To localize PfNT1, we raised polyclonal antisera against PfNT1 in rabbits and determined by Western blot analysis that the antibodies were specific. These monospecific antibodies were subsequently used to determine by deconvolution and immunoelectron microscopy that the predominant location of PfNT1 is the PPM and that the absolute level of PfNT1 protein is stage-dependent.

**Experimental Procedures**

**Parasite Cell Culture**—The chloroquine-sensitive HB3 clone of P. falciparum was propagated in human RBCs at 2% hematocrit and 10% parasitemia by the method of Trager and Jensen (22) except that the serum component in the culture medium was replaced with 0.5% Albumax (Life Technologies, Inc.) (23).

**PfNT1 Antibodies**—A 108-base pair fragment of the P/NTI open reading frame encoding the 36 NH2-terminal amino acids of PfNT1 was amplified using the polymerase chain reaction (PCR) and a sense primer, 5'-TCTGGATCCATGAGTACCGGTAAAGAGTCATCTAAAGC-3', and antisense primer, 5'-TTGGATCTCGAATATTGTTTACTGCTTAATGGTAGAT-3', with engineered BamHI and EcoRI restriction sites (underlined), respectively. The PCR product was subcloned first into the PCR 2.1-TOPO vector (Invitrogen) and then into the BamHI and EcoRI sites of the pGEX-KT expression plasmid (24) to create pGEX-KT-PfNT1-1–108. The pGEX-KT vector directs the synthesis of the PfNT1 fragment fused to the NH2 terminus of the glutathione S-transferase from Schistosoma japonicum (25). The segment of pGEX-KT-PfNT1-1–108 adjoining and encompassing the PfNT1 fragment was sequenced in both directions to verify that the PCR product was subcloned in frame and faithfully. Expression from pGEX-KT-PfNT1-1–108 was induced in 0.4 mM isopropyl β-D-thiogalactoside, and the fusion protein was purified over a glutathione-agarose matrix (Sigma) according to the manufacturer’s recommendations. Purity was verified by SDS-polyacrylamide gel electrophoresis (26), and a purified fusion protein was sent to CoCalico Biologicals for polyclonal antisera production in rabbits.

To generate an antigen for screening and purifying PfNT1-specific antibodies, the 1,075-base pair BamHI/PstI fragment encompassing the 108-base pair P/NT1 insert was excised from pGEX-KT-PfNT1-1–108 and subcloned into the pmal-C2 vector (New England Biolabs) to generate pmal-C2-PfNT1-1–108. The pmal-C2-PfNT1-1–108 construct synthesizes the PfNT1 NH2-terminal fragment as a fusion protein to the NH2 terminus of the E. coli malto-β-binding protein. pmal-C2-PfNT1-1–108 was expressed in 0.4 mM isopropyl β-D-thiogalactoside, and the fusion protein, PfNT1-1–108-malto-binding protein, was purified according to the New England Biolabs brochure.

The PfNT1 antisera obtained from CoCalico Biologicals were screened initially against PfNT1-1–108-malto-binding protein by enzyme-linked immunosorbent assay (27) and Western blotting (28). After we established the presence of PfNT1 antibodies, the crude serum was affinity-purified over an Affi-15 gel (Bio-Rad) matrix to which PfNT1-1–108-malto-binding protein was bound covalently. Antibody specificity was then confirmed by enzyme-linked immunosorbent assay and immunoblotting with PfNT1-1–108-malto-binding protein antigen and glutathione S-transferase and malto-binding protein proteins as negative controls.

**Stage Regulation of PfNT1 Expression**—24 plates, each containing 12 ml of P. falciparum-infected erythrocytes at ∼10% parasitemia were synchronized with 0.15% sorbitol (29), and 3 plates were harvested every 6 h. Erythrocytes were washed once in phosphate-buffered saline (PBS), and parasites were released from the RBCs by incubation in 0.15% saponin for 15 min on ice. The released parasites were washed...
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Fig. 2. Deconvolution immunofluorescence microscopy of PfNT1 in *P. falciparum*-infected red blood cells. Parasitized RBCs were fixed and prepared as described under “Experimental Procedures.” Parasites were examined by deconvolution microscopy using illumination at 546 nm to visualize PfNT1 (panels A–D), PfHT1 (panel E), and *P. falciparum* protein phosphatase 2C (panel F) conjugated to the FITC-conjugated anti-rabbit secondary antibody or at 488 nm to visualize Band 3 complexed with the Texas Red-conjugated anti-mouse secondary antibody (all panels). Panel A depicts a ring stage parasite, panel B shows two trophozoites, and schizonts are exhibited in panels C–F. Each panel is accompanied by an inset displaying nuclear staining with Hoechst dye.

three times in PBS and lysed by sonication. Hemoxin was separated from the parasite lysate by centrifugation at 10,000 × g for 10 min, and protein concentration was determined by the method of Bradford (30). Parasite lysate was mixed with an equal volume of 2% suspension buffer containing 8 M urea and incubated at 70°C for 1 h at room temperature. The cover slips were rinsed three times in wash buffer and then incubated in Hoechst nuclear stain (Molecular Probes) for 5 min. Cover slips were mounted on slides with Molecular Probes Antifade and images acquired with the Applied Precision DeltaVision® image restoration system. Deconvolution was accomplished using the iterative constrained algorithm of Agard et al. (33), and additional image processing was performed on an SGI Octane workstation located in the Oregon Health and Science University-Molecular Microbiology and Immunology Imaging Core Facility.

**Immunoelectron Microscopy**—Parasitized RBCs were enriched for schizonts (15), pelleted, and fixed on ice for 1 h in a solution of 4% paraformaldehyde, 200 mM PIPES and 0.5 mM MgCl₂, pH 7.0. After rinsing in PIPES buffer, the cell pellet was embedded in 10% gelatin and fixed again for 30 min at room temperature. Fixed samples were infiltrated with 20% polyvinylpyrrolidone and 2.3 µm sucrose in PIPES buffer overnight at 4°C. Samples were rapidly frozen in liquid nitrogen, cryosectioned and collected on carbon-formvar-coated grids at the Washington University Molecular Microbiology Imaging Facility. Grids were blocked in 5% fetal bovine serum, 5% goat serum in PIPES buffer and incubated in purified PfNT1 antibody diluted 1:50 in blocking buffer for 1 h at room temperature. Grids were rinsed, incubated with anti-rabbit antibodies conjugated to 15 nm gold particles (Jackson Immunoresearch) diluted in blocking buffer overnight at 4°C. The grids were rinsed in PIPES buffer, the cell pellet was embedded in 10% gelatin and fixed again for 30 min at room temperature. Fixed samples were infiltrated with 20% polyvinylpyrrolidone and 2.3 µm sucrose in PIPES buffer overnight at 4°C. Samples were rapidly frozen in liquid nitrogen, cryosectioned and collected on carbon-formvar-coated grids at the Washington University Molecular Microbiology Imaging Facility. Grids were blocked in 5% fetal bovine serum, 5% goat serum in PIPES buffer and incubated in purified PfNT1 antibody diluted 1:50 in blocking buffer for 1 h at room temperature. Grids were rinsed, incubated with anti-rabbit antibodies conjugated to 15 nm gold particles (Jackson Immunoresearch) diluted in blocking buffer, washed, and counterstained with 0.3% uranyl acetate and 2% polyvinyl alcohol. Images were viewed on an a Zeiss 902 transmission electron microscope.

**RESULTS AND DISCUSSION**

**Antibody Characterization and PfNT1 Levels in Intact Parasites**—To determine the location of PfNT1 in the parasitized erythrocyte, antibodies directed against the NH₂-terminal 36 amino acids of PfNT1 were generated in rabbits. These antibodies, which had been purified over a PfNT1-1–108-maltose-binding protein affinity matrix, recognized only a single polypeptide on a Western blot of fractionated parasite lysate that migrated with a molecular mass of 45 kilodaltons (Fig. 1A). The size of PfNT1 on the immunoblot is consistent with that predicted from the PfNT1 translation product (20, 21). The PfNT1 antibodies are specific for the PfNT1 epitope because they recognized PfNT1-1–108-maltose-binding protein but not glutathione S-transferase or maltose-binding protein alone (data not shown). Binding to PfNT1 was abolished when antibodies were preincubated with excess PfNT1-1–108-maltose-binding protein, and no recognition of the 45-kilodalton polypeptide was observed with preimmune serum (data not shown).

Although PfNT1 polypeptide is observed through all stages of the intraerythrocytic life cycle, the level of PfNT1 protein increases dramatically from the ring (4 h after release) to the early trophozoite stage (20 h after release) and then remains relatively constant through the late schizont stage (40–46 h after release). The pattern of expression is mimicked by that of *P. falciparum* elongation factor-1α (Fig. 1B), a *P. falciparum* protein known to be up-regulated at the trophozoite stage (34). The stage-dependent variation in PfNT1 protein levels observed among RBC *P. falciparum* life cycle stages is consistent with the need for purine precursors prior to and during DNA replication of intraerythrocytic parasites (35). The amount of PfNT1 protein detected at each time point does not coincide with the levels of PfNT1 transcript, which is maximal in early trophozoites and subsequently down-regulated in late stage trophozoites and schizonts (20). Since the pattern of the extent of gene expression are observed at the transcript and protein levels with *P. falciparum* elongation factor-1α (20, 34, 35).

**Immunolocalization**—The location of PfNT1 within the parasitized erythrocyte was examined initially by deconvolution microscopy. Immunofluorescence analysis of PfNT1 stained...
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Both the deconvolution immunofluorescence and immunoelectron microscopy established that PfNT1 is localized primarily, if not exclusively, to the PPM, although the possibility cannot be ruled out that PfNT1 is a minor constituent of other structures and uptake components of the complex nucleoside permeation pathway of *P. falciparum*. The lack of PfNT1 antibody recognition of the TVM and RBCM in which the NPP is found, both of which accommodate nucleoside uptake capacities (15, 16), is consistent with the biochemical evidence that PfNT1 is a saturable, nucleoside-specific carrier (20, 21).

The localization of PfNT1 implies that this saturable transporter is not directly responsible for the dramatically augmented 4-nitrobenzyl-6-thioinosine-insensitive nucleoside transport observed upon *P. falciparum* infection of human erythrocytes (5). Rather, these biochemical alterations, also observed with other nutrients (17, 18), are more likely ascribed to a RBCM component such as the anion channel, known to be part of the NPP, which exhibits more generalized transport capabilities. These other components of the nucleoside permeation pathway remain to be dissected at the molecular level. Recently, our laboratory has uncovered a second nucleoside transporter gene from the malaria genome sequencing data base that exhibits most of the structural features of a member of the equilibrative nucleoside transporter family. The biochemical properties and cellular milieu of this protein remain to be established.

Acknowledgments—We thank Dr. Sanjeev Krishna of the St. George’s Hospital Medical School, Department of Infectious Diseases, for generously providing antibodies to PHT1. We acknowledge the help of Aurelie Snyder of the Oregon Health & Science University-Molecular Microbiology and Immunology Imaging Core Facility for the deconvolution images and thank Jaime Dante and Wandy Beatty of Washington University School of Medicine, Molecular Microbiology Imaging Facility for help with the immunoelectron microscopy.

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**FIG. 3.** Transmission electron micrographs of ultrathin cytosections of *P. falciparum* intraerythrocytic schizont stages using PfNT1 antibodies. Immunogold labeling (15 nm gold particles) of PfNT1 antibodies bound to intraerythrocytic *P. falciparum* is depicted. Magnification is ×12,000 in panel A and ×50,000 in panel B. MC, Maurer’s clefts; N, nucleus; R, rhoptry; TVM, tubovesicular membrane; RBCM, red blood cell membrane; PPM, parasite plasma membrane; PVM, parasitophorous vacuole; FITC, fluorescein isothiocyanate; Texas Red, tetramethylrhodamine isothiocyanate.
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Localization of the *Plasmodium falciparum* PfNT1 Nucleoside Transporter to the Parasite Plasma Membrane

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*J. Biol. Chem. 2001, 276:41095-41099.*

doi: 10.1074/jbc.M107037200 originally published online September 10, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M107037200

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