PROTEIN SYNTHESIS
POST-TRANSLATION MODIFICATION
AND DEGRADATION:

Protein Farnesyltransferase and Protein Prenylation inPlasmodium falciparum

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Comparison of the malaria parasite and mammalian protein prenyltransferases and their cellular substrates is important for establishing this enzyme as a target for developing antimalarial agents. Nineteen heptapeptides differing only in their carboxyl-terminal amino acid were tested as alternative substrates of partially purified *Plasmodium falciparum* protein farnesyltransferase. Only NRSCAIM and NRSCAIQ serve as substrates, with NRSCAIM being the best. Peptidomimetics, FTI-276 and GGTI-287, inhibit the transferase with IC_{50} values of 1 and 32 nM, respectively. Incubation of *P. falciparum*-infected erythrocytes with [^{3}H]farnesol labels 50- and 22–28-kDa proteins, whereas [^{3}H]geranylgeraniol labels only 22–28-kDa proteins. The 50-kDa protein is shown to be farnesylated, whereas the 22–28-kDa proteins are geranylgeranylated, irrespective of the labeling prenol. Protein labeling is inhibited more than 50% by either 5 μM FTI-277 or GGTI-298. The same concentration of inhibitors also inhibits parasite growth from the ring stage by 50%, decreases expression of prenylated proteins as measured with prenyl-specific antibody, and inhibits parasite differentiation beyond the trophozoite stage. Furthermore, differentiation specific prenylation of *P. falciparum* proteins is demonstrated. Protein labeling is detected predominantly during the trophozoite to schizont and schizont to ring transitions. These results demonstrate unique properties of protein prenylation in *P. falciparum*: a limited specificity of the farnesyltransferase for peptide substrates compared with mammalian enzymes, the ability to use farnesol to label both farnesyl and geranylgeranyl moieties on proteins, differentiation specific protein prenylation, and the ability of peptidomimetic prenyltransferase inhibitors to block parasite differentiation.

Malaria continues to be a major disease in tropical areas of the world. Parasite resistance to current drugs used in the treatment of malaria has lead investigators to seek out new drug targets. Among those targets are proteins and enzymes, which are necessary for cellular division and differentiation. Such targets include the protein prenyltransferases, which are necessary for the post-translational modification of proteins involved in the signal transduction pathways and in regulation of DNA replication and cell cycling (1–3). These enzymes are currently a major focus of efforts to design drugs that inhibit unregulated cell growth in cancer (4, 5). Several candidate compounds show great promise as antitumor drugs and are currently being tested in clinical trials. The potential for the application of such drugs to inhibit malarial parasite division and differentiation motivates one to examine the properties of the protozoan enzyme with the goal of identifying unique features of this enzyme that would make it a target for the development of parasite-specific drugs.

A variety of proteins including small G-proteins, such as Ras, Rac, Rap, Rho, Rab (6), heterotrimeric G protein γ subunits (7), nuclear lamins (8), protein kinases (9), and protein-tyrosine phosphatases, PTP_{CAAX} (10), are post-translationally prenylated near the carboxyl terminus with farnesyl (C_{15}) or geranylgeranyl (C_{20}) groups. Some of these, notably Ras, are farnesylated and play an important role in the regulation of DNA replication and cell cycling (1–3). The C_{20}-modified proteins have a variety of cellular functions including intracellular vesicular trafficking. The attachment of the farnesyl or geranylgeranyl groups to these proteins generally promotes membrane association. Inhibition of farnesylation of oncogenic Ras, for example, decreases association of Ras with membrane and blocks cell transformation (11, 12).

The protein prenyltransferases catalyzing these lipid addition reactions show specificity for the carboxyl-terminal amino acid sequence to be modified. Mammalian protein farnesyltransferase (PFT)[1] farnesylates the cysteine residue in carboxyl-terminal cysteine-$aa_{aliphatic}$-$aa_{aliphatic}$-aa_x (*CAAX*) sequences, where aa_x is methionine, glutamine, serine, threonine, or cysteine (13–16). Protein geranylgeranyl transferase-I (PGGT-I) shows specificity for cysteine-$aa_{aliphatic}$-$aa_{aliphatic}$-leucine sequences (13, 17–20). Both PFT and PGGT-I can recognize synthetic short peptides as substrates and inhibitors (13, 18, 19). Protein geranylgeranyl transferase-II (Rab-PGGT), in contrast, modifies proteins with a cysteine-cysteine, cysteine-cysteine-aa_x-aa_x, or cysteine-aa_x-cysteine carboxyl-terminal sequences but cannot prenylate or be inhibited by synthetic short peptides (13, 21).

Although knowledge of the protein prenyltransferases in lower eukaryotes other than yeast is very limited, protein prenylation has been demonstrated in the parasites *Giardia lamblia* (22), *Schistosoma mansoni* (23), *Trypanosoma brucei* (24, 25), *Trypanosoma cruzi* (26), *Leishmania mexicanan* (26), and *Toxoplasma gondii* (27). Yokoyama *et al.* (25) reported PFT and PGGT-I activities in *T. brucei*, cloned the *T. brucei* PFT, and

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[1] The abbreviations used are: PFT, protein farnesyltransferase; Pf-PFT, *Plasmodium falciparum* protein farnesyltransferase; PGGT-I, protein geranylgeranyl transferase-I; FTI, farnesyltransferase inhibitor; GGTI, geranylgeranyl transferase inhibitor; SPA, scintillation proximation assay.

described its substrate specificity (28, 29). Recently Buckner *et al.* (30) reported the cloning and substrate specificity of *T. cruzi* and *Leishmania major* PFT. We described PFT and PGGT-I activity in *P. falciparum*, the protozoan malaria parasite, and hence presented the first indication that protein prenylation is functional in this parasite (31). Our description of the strong inhibition of *P. falciparum* PFT (PfPFT) by peptidomimetics illustrated the potential of targeting these enzymes in developing drug therapy for malaria. We describe here the prenylation of *P. falciparum* proteins in culture from exogenously supplied prenols and inhibition of this process with peptidomimetics. Ohkanda *et al.* (32) have recently demonstrated the potency of a variety of other peptidomimetics as inhibitors of *P. falciparum* growth and PfPFT activity. Moura *et al.* (33) have also shown that the monoterpene, limonene, inhibits parasite development and prenylation of *P. falciparum* proteins. These findings clearly present PFT as a target for the development of antimalarial drugs. The results presented here provide the first description of PfPFT substrate specificity, further focus the target for inhibitors on competitors of peptide binding, and identify specific *P. falciparum* proteins for further characterization as natural substrates for prenylation.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]Farnesol and [^{3}H]geranylgeraniol were purchased from American Radiolabeled Chemicals. The SPA PFT kit was purchased from Amersham Biosciences. Inhibitors of protein prenyltransferases were purchased from Calbiochem, except FTase IV, D-tryptophan-D-methionine-D-p-chlorophenylalanine-L-γ-carboxyglutamic acid (Bachem). All other substrates were available from other commercial suppliers. Nineteen heptapeptides were synthesized by solid phase methodology using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry, purified by high performance liquid chromatography, and characterized by mass spectroscopy at the Protein Chemistry Core Facility, University of Florida Biotechnology Program. Twelve of the peptides were synthesized in two groups with a mixture of COOH-terminal amino acids, NRSCAI{Ile,Glu,Arg,Gly,Asn,His} and NRSCAI{Tyr,Trp,Val, Asp,Lys,Cys}. Individual peptides from each of these groups were separated by high performance liquid chromatography. Because NRSCAIN and NRSCAIH were not separated by high performance liquid chromatography they were tested as a mixture. All stock solutions of peptides were stored in 50 mM HEPES, pH 7.7, 5 mM dithiothreitol, 5 mM $MgCl_2$, and 20% glycerol.

Growth and Isolation of P. falciparum—*P. falciparum* 3D7 was grown *in vitro* in a modified erythrocyte culture as previously described (31, 34). The parasite pellet was isolated from infected erythrocytes by treatment with 0.1% (w/v) saponin for 5 min followed by two washes with phosphate-buffered saline, pH 7.2, at 1,800 × *g* for 7 min. When needed, cultures were synchronized with 5% sorbitol (35). Typically parasites were harvested, post-synchronization, at 6 h for rings, 20 h for trophozoites and 32 h for schizonts. The yield was about 4–5 × 10^7 parasites ml^{-1} of culture. The growth of parasites was monitored by light microscopy of Leukostat (Fisher)-stained thin smears. Pellets of parasites were stored at −80 °C. They were stable in this form for at least 6 months.

Partial Purification of Protein Farnesyltransferase—Conceptual translation of two open reading frames, chr12_1.glm_484 and chr11_1.glm_536, obtained from the malaria genome data base (www.plasmodb.org) yields protein sequences that undoubtedly describe the α and β subunits, respectively, of PfPFT. However, multiple attempts to express PfPFT in *Escherichia coli* as a translationally coupled heterodimer have not been successful to date. In the absence of recombinant enzyme, native PfPFT, which was partially purified by ammonium sulfate fractionation or Mono Q chromatography, has been used for *in vitro* assays. Parasite pellets from 1 liter of cultures (100 plates at 10–20% parasitemia, predominantly trophozoites) were homogenized at 4 °C and the cell suspension was sonicated to disrupt the cells as previously described (31). The lysate was centrifuged at 100,000 × *g* with a 50.1 Ti rotor for 1 h at 4 °C and the protein precipitated between 0 and 50% saturated ammonium sulfate was collected. The ammonium sulfate precipitate was suspended in and dialyzed against 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 20 μM $ZnCl_2$, 20 mM NaCl at 4 °C. The ammonium sulfate fraction was partially purified by Mono Q chromatography by modifications of previously described methods at 4 °C (14, 31). Peak fractions were pooled, concentrated by ultrafiltration, and stored at −80 °C.

Enzyme Assays and Inhibitor Studies—Assays for PfPFT were carried out with the scintillation proximation assay (SPA) according to the manufacturers (Amersham Biosciences) recommendations using 0.12 μM [^{3}H]farnesyl diphosphate and 0.1 μM biotinylated lamin B peptide (biotinyl-YRASNRSCAIM) as substrates, unless otherwise stated. For studies with peptidomimetics with limited aqueous solubility (all except FTase II), each was dissolved in dimethyl sulfoxide. Aliquots (2–4 μl) of these solutions were added to 100-μl SPA assay mixtures. Controls for inhibition of PFT activity in the presence of dimethyl sulfoxide were included in each experiment. Control values for reaction mixtures containing no biotinylated peptide were subtracted from values obtained from complete incubations before calculations of percent inhibition. The effectiveness of each inhibitor is reported as an IC_{50} value, the concentration of inhibitor that reduces the activity of PfPFT to 50% of its uninhibited activity under the conditions tested.

Enzyme assays using nonbiotinylated peptides as substrates were incubated under the same conditions as described for the SPA, except that different nonbiotinylated heptapeptides (2 μM) were substituted for the biotinylated lamin B peptide. The amount of prenylated product was quantitated after purification on 1-ml SPE anion exchange chromatography columns (J. T. Baker Inc.) (28). The first two eluted fractions were pooled and subjected to TLC on silica G sheets in 1-propanol, NH_4OH, water (6:3:1, v/v/v). The positions of migration of the prenylated peptides were determined by cutting each lane into 1-cm sections and analyzing for radioactivity. Prenylated peptides (1000–2000 cpm) migrated with R_F values of 0.69, which is consistent with previously reported R_F values (0.67–0.74) for this system (37). [^{3}H]Farnesol, the product of phosphatase action on [^{3}H]farnesyl diphosphate, is a common by-product of the reaction mixtures and chromatographs, as is unlabeled farnesol with an R_F of 0.87.

Nineteen nonbiotinylated heptapeptides at 10 μM concentration were also tested as alternative substrates or inhibitors by an indirect method using the SPA and 2.5 μg of Mono Q-purified PfPFT. Enzyme activity without added heptapeptide (control) was assigned the value of 100%. The activity of PfPFT in the presence of each heptapeptide was assessed relative to the 100% control. A decrease (inhibition) in percentage of control activity indicates the peptide functions as an alternative substrate.

Radiolabeling of P. falciparum Prenylated Proteins—Asynchronous cultures (1 ml) of *P. falciparum* were labeled with 10 μCi of [^{3}H]farnesol or [^{3}H]geranylgeraniol for 24 h in the presence or absence of different peptidomimetics. Inhibitors (5 μl in dimethyl sulfoxide) were added at the same time as the labeling agent. Labeled parasites were released from infected erythrocytes by saponin treatment as previously described (31). The parasite sample was washed several times with phosphate-buffered saline and lysed by addition of 24 μl of M-Per lysis reagent (Pierce). Lysed parasite extracts were resolved by SDS-PAGE on 12% gels according to the method of Laemmli (38). The gel was subjected to fluorography following treatment with Amplify (Amersham Biosciences). Typically gels were exposed to x-ray films for 7–28 days at −80 °C. The effect of FTI-277 and GGTI-298 on protein synthesis was tested by labeling cells with ^{35}S-Pro Mix (Amersham Biosciences) in RPMI 1640 medium deficient in methionine and cysteine for 24 h in the presence of different inhibitors. Cell extracts were prepared, resolved on SDS-PAGE, and subjected to autoradiography. No inhibition of protein synthesis was observed at concentrations of inhibitors that significantly inhibited prenylation. Synchronized parasite cultures (1 ml at 6% parasitemia) were labeled as described above with either 5 μCi of [^{3}H]farnesol or [^{3}H]geranylgeraniol for 16 h starting at ring, trophozoite, and schizont stages.

Determination of Mode of Protein Prenylation—The nature of the radiolabeled prenyl moiety attached to *P. falciparum* proteins was determined following metabolic labeling with [^{3}H]farnesol and [^{3}H]geranylgeraniol. Gel segments corresponding to labeled proteins were cut out, and treated with methyl iodide and base according the the method of Casey *et al.* (39) as modified by Dugan and Allen (40). The free prenols released by this treatment were separated by reverse phase TLC on KC_8 plates (Whatman) in acetonitrile, H_2O (9:1, v/v) and identified by comparison to the mobilities of standards of farnesol and geranylgeraniol.

Two-dimensional Analysis of Stage-specific Labeled Proteins—Proteins isolated from cultures (1 ml) labeled with 10 μCi of [^{3}H]farnesol or [^{3}H]geranylgeraniol were analyzed by two-dimensional electrophoresis according to the method of O'Farrell (41) by Kendricks Labs, Inc. (Madison, WI). Isoelectric focusing was carried out in glass tubes of 2.0 mm inner diameter, using 2.0% pH 4–8 ampholines (BDH, Gallard

Schlesinger, Long Island, NY) for 9600 volt h. Following equilibration for 10 min in 10% glycerol, 50 mM dithiothreitol, 2.3% SDS, and 62.5 mM Tris, pH 6.8, the tube gel was sealed to the top of a stacking gel of a 10% acrylamide slab gel (0.75 mm thick). SDS-gel electrophoresis was carried out for 4 h at 12.5 mA/gel. The gels were treated with EN^{3}HANCE (PerkinElmer Life Sciences) for 1 h, rehydrated in water for 30 min, and dried onto filter paper. Fluorography was carried out using Kodak X-Omat AR film with exposures at −70 °C for 28 days.

Effect of Prenyltransferase Inhibitors on P. falciparum Maturation—Various concentrations of inhibitors (in dimethyl sulfoxide) were added to synchronized *P. falciparum* 3D7 culture at the ring stage (6 h post-synchronization). The inhibition of growth was measured in terms of inhibition of incorporation of [^{3}H]hypoxanthine (0.2 μCi/well) into nucleic acids. Cells were harvested in a cell harvester (TomTec MachIIM Harvester 96) and counted for radioactivity. Images of stained thin smears of inhibitor-treated cells were captured with a Zeiss Axioplan2 microscope equipped with a Hamamatsu color chilled 3CCD camera.

Indirect Immunofluorescence Microscopy—Synchronized parasite-infected erythrocytes were washed twice with RPMI 1640 and at a cell density of 1×10^7 cells/ml were allowed to adhere to poly-L-lysine-coated coverslips at 37 °C for 30 min. The slides were washed four times with phosphate-buffered saline and fixed in 4% paraformaldehyde (10 min at room temperature). Following four washes with phosphate-buffered saline, the cells were permeabilized and blocked with a solution containing 0.05% saponin, 5% bovine serum albumin for 20 min at room temperature. The cells were probed with an affinity purified rabbit anti-farnesyl polyclonal antibody (Calbiochem) at a dilution of 1:50 and affinity purified Cy2-labeled goat anti-rabbit IgG (Jackson Immunoresearch Laboratories) at a dilution of 1:200 in phosphate-buffered saline containing 5% bovine serum albumin, respectively, for 90 min followed by three washes with phosphate-buffered saline. Nuclear staining was done with 1 μM 4′,6-diamidino-2-phenylindole for 5 min. The coverslips were mounted with gel/mount (Biomeda) on slides. The slides were viewed on a DeltaVision restoration microscope system (Applied Precision) equipped with a Nikon TE200 microscope and a Photometrix cooled CCD camera. DeltaVision software (SoftWoRx) was used for image deconvolusion.

RESULTS

In Vitro Inhibition of PfPFT with Peptidomimetics—Our earlier studies showed that PfPFT was poorly inhibited by the farnesyl diphosphate analogues FPT inhibitor-I and -II, whereas the peptidomimetic PFT inhibitor, L-745631, was an effective inhibitor with an IC_{50} of 3–4 nM (31). Several additional peptidomimetics (Table I) were tested here with Mono Q-purified PfPFT. PFT inhibitor, FTI-276, and geranylgeranyl transferase inhibitors, GGTI-287 and GGTI-297, each inhibit at concentrations comparable with those reported for the mammalian PFTs (Table II). FTI-276 shows the best inhibition and is slightly more inhibitory than L-745631. Other commercially available peptidomimetic PFT inhibitors, FTase-I, FTase-II, and FTase-IV are relatively ineffective inhibitors of PfPFT (IC_{50} of 1.7–6.3 μM).

Peptide Substrate Specificity of PfPFT—Seven heptapeptides, NRSCAIX, with carboxyl-terminal residues of methionine, alanine, serine, threonine, phenylalanine, glutamine, or leucine were tested directly as substrates of PfPFT. Each unbiotinylated peptide was incubated with [^{3}H]farnesyl diphosphate and ammonium sulfate-fractionated enzymes as described under "Experimental Procedures." Prenylated peptides were then purified by SPE anion exchange chromatography and TLC. Among the peptides tested, NRSCAIM and NRSCAIQ were the best substrates. Quantitation of the prenylated peptides showed that the reactivities of the different NRSCAIX peptides, relative to NRSCAIM taken as 100%, were 40, 7.5, 3.0, 3.0, 1.5, and 0%, respectively, when the carboxyl termini were of glutamine, leucine, serine, threonine, alanine, and phenylalanine.

A convenient and more rapid method of assessing the peptide specificity of the enzyme is to test unbiotinylated peptides as alternative substrates or inhibitors of prenyltransferase activity as measured by the SPA assay. Binding of unbiotinylated peptide to the enzyme active site will decrease binding of the biotinylated SPA peptide substrate and hence decrease the amount of biotinylated [^{3}H]farnesyl peptide formed and trapped with SPA streptavidin beads. Nineteen C*AAX* containing heptapeptides (NRSCAI*X*), with sequences corresponding to lamin B, but having different carboxyl termini, were tested as alternative substrates of the Mono Q-purified PfPFT using the SPA assay. Among the peptides tested only NRSCAIM and NRSCAIQ functioned as alternative substrates. The effect of varying concentrations of NRSCAIM and NRSCAIQ on enzyme activity is illustrated in Fig. 1. Respective IC_{50} values of 1 and 21 μM were obtained.

Stage-dependent Prenylation of Parasite Proteins—Development stage-specific prenylation of proteins during intraeryth-

TABLE I
Peptidomimetic prenyltransferase inhibitors

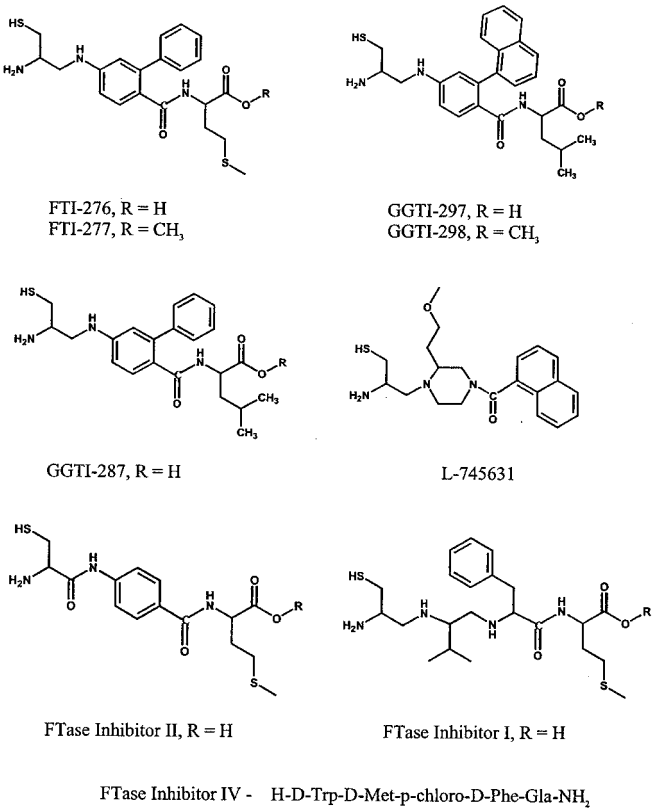

TABLE II
IC_{50} values for peptidomimetic inhibitors of PfPFT

Peptidomimetics FTI and GGTI were tested as inhibitors of Mono Q-purified PfPFT. FTase inhibitors were tested with ammonium sulfate-purified enzyme. The scintillation proximation assay was used in each case. The values reported for the human PFT are literature values.

Inhibitor	IC_{50}	
	P. falciparum	Human
	nM	
FTI-276	0.9	0.5[a]
GGTI-287	32	25[b]
GGTI-297	190	200[a]
FTase inhibitor II	1,700	50[c]
FTase inhibitor IV	5,000	2[d]
FTase inhibitor I	6,300	21[e]

[a] Ref. 42.
[b] Ref. 43.
[c] Ref. 44.
[d] Ref. 45.
[e] Ref. 46.

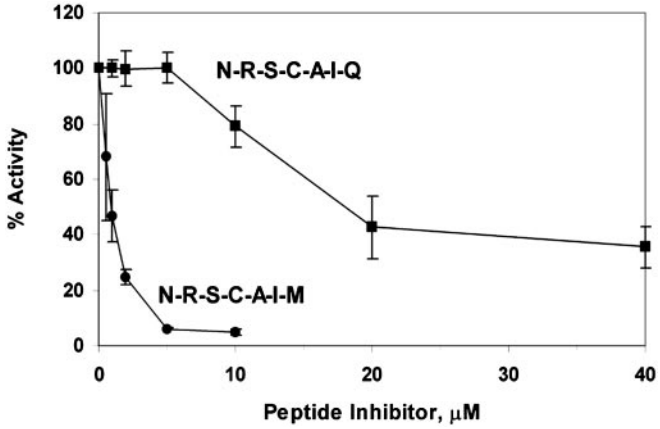

FIG. 1. **Concentration dependence of NRSCAIM and NRSCAIQ as alternative substrates of PfPFT.** The ability of different concentrations of peptides NRSCAIM (●) and NRSCAIQ (■) to decrease the apparent activity of ammonium sulfate-purified PfPFT was measured using the SPA. PfPFT activity without added heptapeptide was assigned the value of 100% activity. The relative PfPFT activities and standard errors at various concentrations of heptapeptide are plotted.

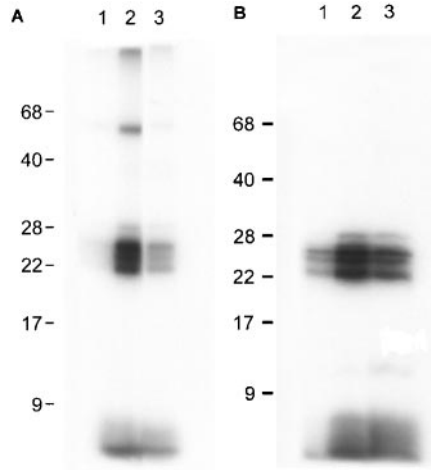

FIG. 2. **Labeling of synchronized parasite cultures.** Synchronized parasite cultures were labeled with [^{3}H]farnesol (*A*) or [^{3}H]geranylgeraniol (*B*) for 16 h starting at ring (*1*), trophozoite (*2*), and schizont (*3*) stages as described under "Experimental Procedures." Radiolabeled parasite proteins were isolated, resolved by SDS-PAGE, and detected by fluorography.

rocytic development of the parasite was analyzed next. Synchronized parasites were labeled with [^{3}H]farnesol and [^{3}H]geranylgeraniol for 16 h starting at ring, trophozoite, and schizont stages. As seen from Fig. 2*A*, labeling with [^{3}H]farnesol results in detection of 50 kDa as well as 22–28-kDa prenylated proteins, predominantly during the trophozoite to schizont transition (*lane 2*). Although significantly less, prenylated proteins of these sizes are also detected during schizont to ring transition (*lane 3*). Very little prenylation of proteins is detected during ring to early trophozoite transition (*lane 1*). Incubation with [^{3}H]geranylgeraniol resulted in labeled proteins in the 22–28-kDa range in all stages of intraerythrocytic growth (Fig. 2*B*, *lane 1*-3) reaching a peak during maturation of trophozoites into schizonts. At times, trace labeling of a 50-kDa protein is observed. It is to be noted that we have not detected protein prenylation in uninfected erythrocytes (data not shown). Furthermore, the appearance of little or no detectable protein prenylation in the ring stage (Fig. 2, *lanes A1* and *B1*) also suggests no contribution from prenylation of erythrocytic proteins.

Two-dimensional Separation of Proteins Labeled with Prenyl Groups during Malaria Parasite Intraerythrocytic Differentiation—Proteins radiolabeled with [^{3}H]farnesol or with [^{3}H]geranylgeraniol during *P. falciparum* differentiation in erythrocytes were also separated by two-dimensional electrophoresis. Multiple labeled proteins are observed in both the 50- and 22–28-kDa range when using [^{3}H]farnesol (Fig. 3). Proteins in the 50,000 range are found predominately in the trophozoite stage (Fig. 3*B,* also see Fig. 2*A*, *lane 2*) and they appear to be of the same molecular weight but of different charge. Multiple proteins are found in the 22–28-kDa range of cells in both the trophozoite (Fig. 3*B*) and schizont (Fig. 3*C*) stages. Several proteins appear to be common to the two stages judging from their position of migration, however, there is differential labeling with some and at least one protein is labeled specifically at the schizont stage. With [^{3}H]geranylgeraniol labeling, the 22–28-kDa protein population appeared with charge heterogeneity (data not shown).

Mode of Prenylation of P. falciparum Proteins—The nature of the prenyl moiety attached to *P. falciparum* proteins was determined following metabolic labeling with [^{3}H]prenols. Proteins were separated by SDS-PAGE and gel segments corresponding to labeled prenylated proteins were subjected to a methyl iodide treatment, which releases the prenyl moieties as free prenols. Reverse phase TLC analysis of prenols released from the 50-kDa protein, which was radiolabeled in cultures containing [^{3}H]farnesol, shows only farnesol (Fig. 4). In contrast, the prenol released from each of the three bands in the 22–28-kDa range is geranylgeraniol. Similar analysis of the 22–28-kDa proteins from cultures incubated with [^{3}H]geranylgeraniol showed that these proteins were geranylgeranylated (data not shown). Analysis of the prenols associated with proteins labeled with [^{3}H]prenols in the stage dependent study (Fig. 2*A*) shows the same prenylation pattern. The 50-kDa protein is farnesylated with [^{3}H]farnesol labeling, whereas the 22–28-kDa proteins are geranylgeranylated with either [^{3}H]farnesol or [^{3}H]geranylgeraniol labeling.

Inhibition of P. falciparum Intraerythrocytic Maturation—The effects of peptidomimetic prenyltransferase inhibitors were tested on the growth of *P. falciparum* in the erythrocytic culture. Both FTI-277 and GGTI-298, the methyl esters of FTI-276 and GGTI-297, respectively, exhibit an IC_{50} of 5 μM (data not shown). The FTI-277-treated culture forms a vacuole-like structure (Fig. 5, *panel 3*). Similar vacuoles are not observed in control cells (Fig. 5, *panel 2*). Furthermore, the inhibitor-treated cells do not mature beyond the trophozoite stage and the few parasites that escape inhibition at the IC_{50} inhibitor concentration mature normally but are arrested at the trophozoite stage in the next developmental cycle (data not shown).

Changes in the Intracellular Localization of Prenylated Proteins following Treatment with Peptidomimetics—To analyze the localization of prenylated proteins in *P. falciparum*, a commercially available rabbit farnesyl polyclonal antibody was used in an indirect immunofluorescence experiment. This antibody was generated using farnesyl cysteine conjugated to keyhole limpet hemocyanin and cross-reacts also with geranylgeranylated proteins. Prenylated proteins exhibit distinct foci of localization and are detected in all stages of intraerythrocytic maturation (Fig. 6). Inhibitor treatment was initiated in the ring stage (0 h) and in 24 h the cells matured to late trophozoites. At 36 h the cells are in the schizont/segmentor stage as evident from multinucleated cells (Fig. 6, *panel I*). Upon treatment with 5 μM FTI-277 or GGTI-298 (Fig. 6, *panels II* and *IV*) there is a significant reduction of intracellular prenylated protein. The fluorescence-labeled prenylated proteins are almost undetectable in the presence of 10 μM peptidomi-

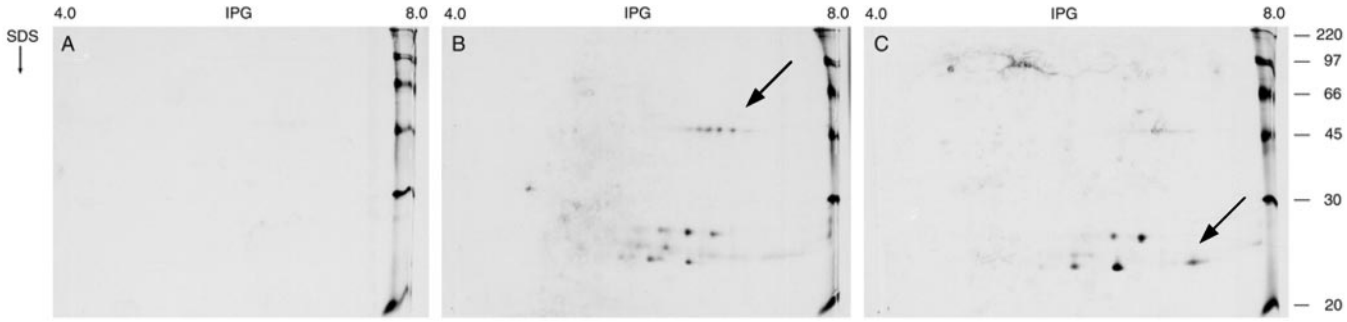

FIG. 3. **Two-dimensional separation of proteins labeled with [^{3}H]farnesol during erythrocytic stages of differentiation.** Parasites were labeled with [^{3}H]farnesol during the ring (*A*), trophozoite (*B*), and schizont (*C*) stages of *P. falciparum* growth. Labeled proteins were isolated and separated as described under "Experimental Procedures," then were detected by fluorography. ^{14}C-Labeled molecular weight markers (myosin, 200,000; phosphorylase *b,* 97,400; bovine serum albumin, 68,000; ovalbumin, 46,000; carbonic anhydrase, 30,000; and lysozyme, 14,300) appear at the basic edge of the autoradiographs. The *arrows* represent the proteins, which are observed uniquely at that stage of differentiation.

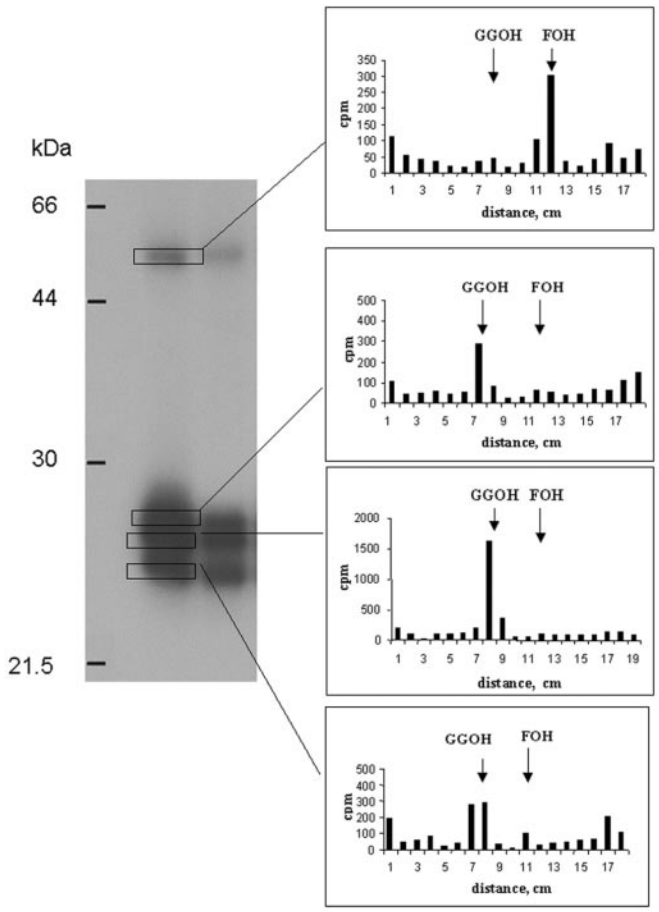

FIG. 4. **Mode of prenylation of *P. falciparum* proteins.** Proteins in asynchronous cultures of *P. falciparum* were radiolabeled with [^{3}H]farnesol. Gel segments corresponding to the indicated labeled prenylated proteins were cut out. The prenyl moieties were released as free prenols, separated by reverse phase TLC, and identified as described under "Experimental Procedures." The mobilities of farnesol (*FOH*) and geranylgeraniol (*GGOH*) standards are shown.

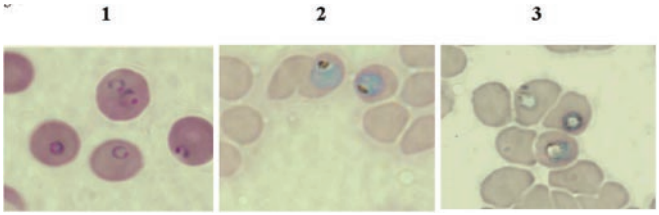

FIG. 5. **Effect of FTI-277 on the intraerythrocytic maturation of *P. falciparum*.** A synchronized parasite culture was treated with 5 μM FTI-277 at the late ring stage. *Panel 1*, control culture containing 2.5% dimethyl sulfoxide at 0 h; *panel 2*, control culture after 24 h; *panel 3*, culture containing 5 μM FTI-277 after 24 h.

metics and the maturation of cells is severely affected (Fig. 6, *panels III* and *V*). The effect of peptidomimetics on the distribution of prenylated proteins also provides evidence for the specificity of the antibody. There was no fluorescence-labeled prenylated proteins in uninfected erythrocytes.

Inhibition of in Vivo Radiolabeling of P. falciparum Prenylated Proteins with Peptidomimetics—To further test the effect of prenyltransferase inhibitors on protein prenylation in the malaria parasite, synchronous cultures of *P. falciparum*-infected erythrocytes were labeled with [^{3}H]farnesol or [^{3}H]geranylgeraniol in the presence or absence of different concentrations of the FTI-277 or GGTI-298. Labeling of 22–28- and 50-kDa proteins, independent of the source of labeled prenol, is inhibited by more than 50% by 5 μM peptidomimetic, FTI-277 or GGTI-298 (Fig. 7, *A* and *B*). At these inhibitor concentrations, no inhibition of protein synthesis was observed as evident from [^{35}S]methionine incorporation into proteins indicating specific inhibition of protein prenylation without affecting protein synthesis (data not shown).

DISCUSSION

Despite the fact that *P. falciparum* do not biosynthesize cholesterol (47, 48), isoprenoid metabolism is critical for normal parasite division and differentiation as evident from earlier studies, which showed that PFT inhibitors (31) and mevastatin (49), an inhibitor of β-hydroxy-β-methylglutaryl-CoA reductase, affect the transition of parasites from the ring state to the late trophozoite stage. The latter finding is intriguing as mevalonic acid-dependent synthesis of isopentenyl diphosphate is yet to be established in the malaria parasite.

Our earlier attempts to radiolabel prenylated proteins in *P. falciparum* with [^{3}H]mevalonic acid were not successful. This could be either attributed to the difficulty of transporting enough mevalonic acid through both the cellular membranes of the erythrocyte and the parasite to sufficiently label the isoprenoid pool, or to an alternative possibility that mevalonic acid is not an intermediate in the synthetic pathway of prenyl diphosphates used in protein prenylation, but the methyl erythritol phosphate biosynthetic pathway is used (50, 51). Recently, two key enzymes of this pathway, 1-deoxy-D-xylulose-5-phosphate synthase and 1-deoxy-D-xylulose-5-phosphate reductoisomerase, have been identified in *Plasmodium* (52). Consequently, metabolic labeling of prenylated proteins with radiolabeled prenols was used. Studies with mammalian cells have shown that both [^{3}H]farnesol and [^{3}H]geranylgeraniol can be used to label proteins (53, 54). The ability of these prenols to serve as prenylation agents, based on our current state of

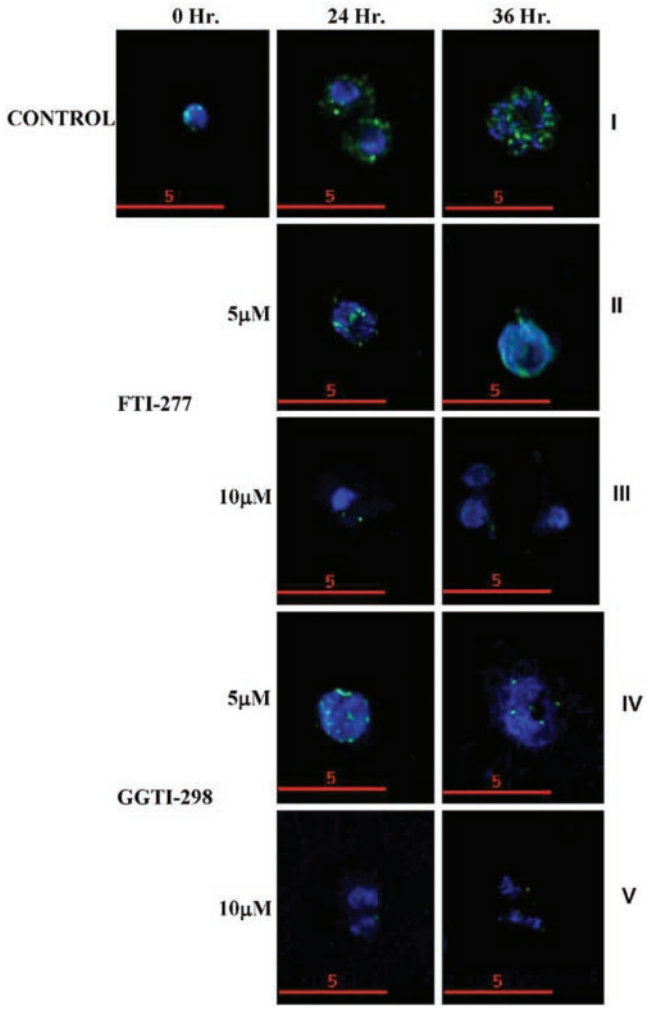

FIG. 6. **Localization of prenylated proteins.** *Panel I*, the synchronized control culture matured from the ring stage (0 h) to late trophozoites (24 h) to segmenters (36 h). The panel for 24 h shows two trophozoites in an erythrocyte. *Panel II*, 5 μM FTI-277 exposed cells at 24 and 36 h treatment. *Panel III*, 10 μM FTI-277 exposed cells at 24 and 36 h treatment; *panel IV*, 5 μM GGTI-298 exposed cells at 24 and 36 h treatment. The panel shows two overlapping trophozoites in an erythrocyte. *Panel V*, 10 μM GGTI-298 exposed cells at 24 and 36 h.

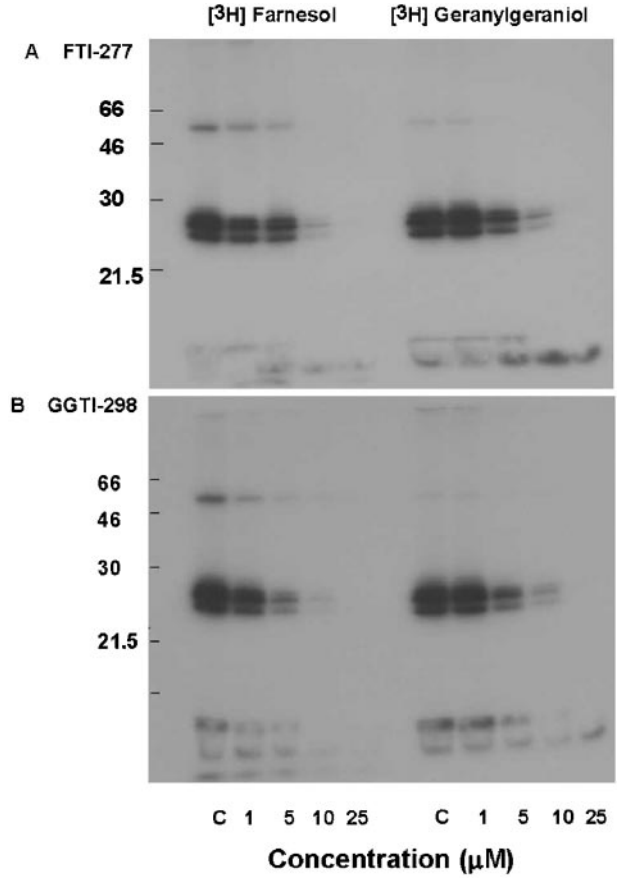

FIG. 7. **Inhibition of *in vivo* radiolabeling of *P. falciparum* prenylated proteins with peptidomimetics.** Asynchronous cultures (1 ml) of *P. falciparum* were labeled with 10 μCi of [^{3}H]farnesol or [^{3}H]geranylgeraniol for 24 h in the presence or absence of different concentrations of the FTI-277 (*A*) or GGTI-298 (*B*). Labeled parasite proteins were isolated as described under "Experimental Procedures" and resolved by SDS-PAGE. Radiolabeled prenylated proteins were detected by fluorography.

knowledge, requires the conversion of the prenols to their diphosphate derivatives. Enzymes involved in these phosphorylations have been described and are reviewed (55).

Radiolabeled proteins were observed in asynchronous cultures of *P. falciparum*-infected erythrocytes incubated with either [^{3}H]farnesol or [^{3}H]geranylgeraniol. A 50-kDa protein was shown to be metabolically labeled with [^{3}H]farnesol but not with [^{3}H]geranylgeraniol. Analysis of the mode of prenylation showed farnesylation. Although proteins of 22–28 kDa were labeled with either [^{3}H]farnesol or [^{3}H] geranylgeraniol, analysis of their mode of prenylation showed almost exclusively geranylgeranylation. It is apparent then that both [^{3}H]farnesol and [^{3}H]geranylgeraniol are phosphorylated to their diphosphates and used for prenylation. Furthermore, some of the [^{3}H]farnesyl diphosphate must be elongated to [^{3}H]geranylgeranyl diphosphate to account for the appearance of [^{3}H]geranylgeranyl groups in 22–28-kDa proteins isolated from cells labeled with [^{3}H]farnesol.

Crick *et al.* (54) were able to demonstrate protein farnesylation in C_6 glioma cells using [^{3}H]farnesol, but no protein geranylgeranylation was seen. They raised the possibility of differential compartmentalization of the enzymes of isoprenoid metabolism. Our ability to demonstrate protein geranylgeranylation using [^{3}H]farnesol as a precursor, although different from the results of Crick *et al.* (54), also suggests that compartmentalization of prenol metabolism and protein prenylation may be a possibility. This is particularly evident when noting that radiolabeling of the proteins in the 22–28-kDa range was inhibited by the prenyltransferase inhibitors that are specific for PFT and PGGT-I and not the Rab PGGT (42). Therefore, although Rab proteins have been described in *P. falciparum* (56), the labeled 22–28-kDa proteins are apparently not those Rabs. The ability of PFT inhibitors to inhibit geranylgeranylation at low micromolar concentrations is also not readily explainable. We have reported the presence of PGGT-I in *P. falciparum* (31), but our inability to isolate a stable PGGT-I activity from *P. falciparum* extracts has prevented a determination of the sensitivity of PGGT-I to PFT and PGGT-I inhibitors.

Prenylated proteins labeled with [^{3}H]farnesol are observed in both the 50- and 22–28-kDa ranges during the trophozoite to schizont and schizont to ring stages of differentiation, whereas few prenylated proteins are observed in the ring to trophozoite stage. Our previous studies (31) demonstrated PFT and PGGT-I activity in each of these stages, therefore this suggests that the marked decrease in protein prenylation during the ring to trophozoite transition is because of a decrease in the production of suitable protein substrates for prenylation. It is noteworthy that peptidomimetic L-745,631, a PFT inhibitor, had no effect on the transition of schizont to early trophozoite

(31). We have also shown here that another PFT inhibitor, FTI-277, blocks maturation of the parasite during the trophozoite stage.

The indirect immunofluorescence experiment provides evidence for the presence of prenylated proteins in all stages of *P. falciparum* intraerythrocytic differentiation at distinct subcellular foci. In the segmenter stage, the prenylated proteins show punctated distribution around chromosomes that are being organized into individual nuclei. Interestingly, although we detected insignificant prenylation activity in the ring stage while labeling with prenol precursor, indirect immunofluorescence indicated the presence of prenylated proteins in rings. This suggests the possibility of a low turnover rate of preexisting prenylated proteins. Treatment of ring stage parasites with both PFT and PGGT inhibitors inhibited prenylation in trophozoites and schizonts. It is to be noted that at the IC_{50} concentration of 5 μM FTI-277 or GGTI-298 (*panels II* and *IV*, Fig. 6), separation of chromosomes into individual nuclei is affected and the whole chromosomal structure appears to be in the form of an unsegregated mass. Therefore, both metabolic labeling and immunofluorescence studies suggest that proteins made during the early to late trophozoite stages may be critical for the trophozoite to schizont transition and the viability of the parasite.

Earlier studies reported cell-cycle or differentiation dependent protein prenylation in synchronized HepG2 cells (57) and in the seminiferous epithelium of rats at different stages of spermatogenesis (40). The studies in HepG2 cells led the authors to suggest that protein prenylation could constitute an obligatory step leading to the duplication of the cellular genome. Several other studies have now shown that one of the main effects of prenyltransferase inhibitors is to alter the progression of cell cycle. PFT inhibitors such as FTI-277 have variable effects on cell cycle progression, causing either a G_0/G_1 or G_2/M block, depending on the cell line. On the other hand, PGGT-I inhibitors such as GGTI-298 invariably induce inhibition of G_0/G_1 (5, 58). Recently, FTI-2153 has been shown to inhibit formation of bipolar spindle formation and chromosome alignment (59). Another PFT inhibitor, SCH66336, inhibits microtubule association of farnesylated kinetochore proteins CENP-E (60). Our results with FTI-277 may represent a situation analogous to the mammalian cells where bipolar spindle formation is inhibited following PFT inhibitors.

The appearance of distinctive prenylated proteins during specific stages of *P. falciparum* differentiation is a novel observation for parasites. Therefore, it is of considerable interest to understand the role of prenylated proteins in cell cycle progression in malaria. Proteins in the 50-kDa range are found predominately in the trophozoite stage and appear to be of the same molecular weight but different charge as assessed by two-dimensional electrophoretic analysis. The migratory differences could be attributed to differences in phosphorylation or glycosylation. Phosphorylation seems more likely because there is little difference in the molecular weights of these proteins. Earlier studies on protein prenylation in murine lymphoma cells (61) and in rat seminiferous epithelium (40) also described multiple prenylated proteins of the same molecular weight that appeared to be phosphorylated. Prenylated proteins of different charge and molecular weight were also observed in the 22–28-kDa range from parasites at both the trophozoite and schizont stages. Although several proteins appear to be common to the two stages judging from their position of migration, there is differential labeling with some and at least one protein is labeled specifically at the schizont stage.

The specificity of PfPFT for the amino acid at the carboxyl terminus is similar to the mammalian PFT (13, 14, 36) in that peptides ending in methionine and glutamine are substrates but is different in that peptides ending in serine, threonine, and cysteine are not substrates. The specificity of PfPFT is more similar to that reported for the PFTs of the parasites *T. brucei* (28) and *Leishmania amazonesis* (30), which farnesylate peptides terminating in methionine and glutamine most effectively and have poor specificity for peptides terminating with other amino acids. It is difficult, however, to draw firm conclusions about PFT specificity among different species when different short peptides are used in the studies. As noted by Buckner *et al.* (28) the specificity of the *T. brucei* enzyme changed when a C*AAX* containing peptide with different amino acid sequence was used.

It is interesting that PFT and PGGT-I inhibitors are both effective at inhibiting PfPFT *in vitro* activity (31) and inhibiting labeling of *P. falciparum* proteins in culture with farnesol and geranylgeraniol. This unusual characteristic of PfPFT implies the potential for development of PFT inhibitors with specificity for the malarial parasite. An in depth analysis of protein prenylation in *P. falciparum* will provide us with a better understanding of the role of these proteins in malarial cell cycle progression.

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