Active isoprenoid pathway in the intra-erythrocytic stages of *Plasmodium falciparum*: presence of dolichols of 11 and 12 isoprene units

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N-glycosylation of proteins is required for the intra-erythrocytic schizogony of *Plasmodium falciparum*. In eukaryotic cells, this process involves the transfer of oligosaccharides from a dolichyl pyrophosphate derivative to asparagine residues. We have identified dolichol, dolichyl phosphate and dolichyl pyrophosphate species of 11 and 12 isoprenoid residues by metabolic labelling with [^{3}H]farnesyl pyrophosphate, [^{3}H]geranylgeranyl pyrophosphate and [^{14}C]acetate in the different intra-erythrocytic stages of *P. falciparum*. This is the first demonstration of short-chain dolichols in the phylum Apicomplexa. The results demonstrate the presence of an active isoprenoid pathway in the intra-erythrocytic stages of *P. falciparum*. Parasites treated with mevastatin, a 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor, show depressed biosynthesis of dolichol, dolichyl phosphate and isoprenoid pyrophosphate. This effect is observed in all intra-erythrocytic stages of the parasite life cycle, but is most pronounced in the ring stage. N-linked glycosylation of proteins was inhibited in the ring and young-trophozoite stages after mevastatin treatment of parasite cultures. Therefore the isoprenoid pathway may represent a different approach to the development of new anti-malarial drugs.

Key words: [1(n)-^{3}H]farnesyl triammonium pyrophosphate, [1(n)-^{3}H]geranylgeranyl triammonium pyrophosphate, malaria, mevastatin.

INTRODUCTION

Malaria is a major cause of morbidity and mortality in developing countries. About 2300 million people inhabit tropical and subtropical areas where malaria transmission occurs. Of these, 300–500 million people are infected, and between 1.5 and 2.7 million, including about 1 million children, die each year [1]. The disease is caused by protozoan parasites of the genus *Plasmodium*, and is transmitted by *Anopheles* mosquitoes. Four *Plasmodium* species infect humans, the most common being *Plasmodium falciparum*. This parasite is associated with a potentially fatal disease and has become resistant to most of the currently available anti-malarial drugs. Because of this, it is essential that new anti-malarial drugs are developed. To do this efficiently, we require a thorough knowledge of the biochemistry of *P. falciparum*.

Recently, several reports on the glycobiology of *P. falciparum* have been published. We have demonstrated that the presence of N-linked glycoproteins is related to schizogony of the intra-erythrocytic stages of *P. falciparum* [2]. Gowda et al. [3] showed that glycosylphosphatidylinositol anchors represent the major carbohydrate protein modification of the intra-erythrocytic stages of *P. falciparum*.

Anchors and N-linked glycoproteins require dolichyl phosphate (dolichyl-*P*) and dolichyl pyrophosphate (dolichyl-*PP*) as carriers of the different monosaccharide constituents. The biosynthetic pathways for cholesterol, ubiquinones and dolichol share the same initial steps. Usually, cholesterol is the most abundant product of the isoprenoid pathway, but *P. falciparum* does not biosynthesize steroids [4,5]. Ubiquinone, another end-product of the isoprenoid pathway, is a component of the mitochondrial respiratory chain [6]. In the last few years, proteins covalently modified by isoprenoid groups have been described [7–11].

N-linked glycosylation is a protein modification that occurs co-translationally in the endoplasmic reticulum [12]. This process is dependent of *de novo* synthesized dolichyl-*P*, a long-chain non-sterol isoprene which acts as a membrane-bound carrier of oligosaccharides in the assembly of glycoproteins [13]. The family of dolichols is present in all membranes, but in highly variable amounts [14], and consists of lipids ranging from 15 to 23 isoprene residues. It has been reported that polyisoprenoids contain short-chain α-saturated polyisoprenols [15–17].

Published results on the isoprenoid pathway in *P. falciparum* are contradictory. An active isoprenoid pathway has only been demonstrated up to the synthesis of farnesyl-*PP* upon incubation of *P. falciparum*-infected erythrocytes with [^{14}C]acetate. This pathway was stage-dependent, as shown by the increase in radiolabelled farnesyl-*PP* at the beginning of the schizogonic phase (30–36 h of culture) [5]. On the other hand, no isoprenol-cycle intermediates were detected among glycolipids metabolically labelled with [^{3}H]glucosamine [18]. Similar results were obtained using a cell-free system, although dolichol or isoprenoid-*P*-mannose and glucose from radioactive exogenous GDP-mannose or UDP-glucose were detected [19]. However, Walter [20] demonstrated that dolichol kinase, a rate-limiting enzyme for the supply of dolichyl-*P*, is present in *P. falciparum*.

In both prokaryotic and eukaryotic cells, the isoprenoid pathway is highly regulated through feedback at the level of two sequential enzymes involved in the synthesis of mevalonate: 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase and HMG-CoA reductase [21]. Inhibitors of HMG-CoA reductase

Abbreviations used: dolichol 11 (etc.), dolichol of 11 isopene units (etc.); HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; -*P*, phosphate; -*PP*, pyrophosphate.

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(lovastatin, simvastatin) are available as hypocholesterolaemic agents for humans. These drugs have strong anti-malarial activity *in vitro* [22], but the concomitant biochemical modifications in the parasite are unknown. The recent finding that N-glycosylation of proteins is required for the intra-erythrocytic schizogony of *P. falciparum* [2] raises the possibility that HMG-CoA reductase regulates this process.

In this paper, we demonstrate the presence of dolichol, dolichyl-*P* and dolichyl-*PP* species of 11 and 12 isoprene residues in cultured asexual *P. falciparum* blood stages. Treatment of cultures of parasites with mevastatin blocks the synthesis of isoprenoids and inhibits the synthesis of glycoproteins in *P. falciparum*. Inhibition of the development of *P. falciparum* by mevastatin is clearly stage-dependent, occurring only when parasites are treated before differentiation into schizonts. These findings indicate that the isoprenoid pathway may be an important regulatory target for the mechanism of differentiation of *P. falciparum* asexual intra-erythrocytic stages.

MATERIALS AND METHODS

Materials

Reagents were obtained from the following sources. RPMI 1640 medium, RPMI 1640 medium without glucose and methionine, Hepes, hypoxanthine, glucose, gentamycin, Tris, EDTA, SDS, 2-mercaptoethanol, Nonidet P40, Triton X-100, DMSO, PMSF, iodoacetamide, *N*-α-p-tosyl-lysylchloromethane ('TLCK'), leupeptin, mevastatin, farnesyl-*PP*, geranylgeranyl-*PP*, dolichols of 11 isoprene residues (dolichol 11), dodecaprenol, dolichols of 18–19 isoprene units (dolichol 18–19), dodecaprenyl-*P*, dolychyl-*P*s of 18–19 isoprene units (dolychyl-*P* 18–19) and acid phosphatase from potato were purchased from Sigma (St. Louis, MO, U.S.A.). Geraniol and farnesol were kindly donated by Dr. N. Franca Roque (Dept. Fundamental Chemistry, Chemistry Institute, U. S. P., Brazil). Percoll® was purchased from Pharmacia Chemicals (Uppsala, Sweden). Plasmagel® was purchased from Laboratoire Roger Bellon (Nevilly-sur-Seine, France). L-[^{35}S]Methionine, D-[U-^{14}C]glucose, [1(n)-^{3}H]geranyl-geranyl-*PP* triammonium salt, [1(n)-^{3}H]farnesyl-*PP* triammonium salt, sodium [1-^{14}C] acetate and Amplify® were obtained from Amersham International. N-Glycanase® and O-Glycanase® were purchased from Genzyme (Cambridge, MA, U.S.A.). DEAE-cellulose was acquired from Bio-Rad (Hercules, CA, U.S.A.). AlbuMax I® was obtained from Gibco-BRL Life Technologies (Grand Island, NY, U.S.A.). All solvents were of analytical or HPLC grade.

Parasite cultures

The experiments were performed using an isolate (S20) of *P. falciparum* obtained from a patient living in Porto Velho (Rondônia, Brazil) [23]. Cultures were grown as described by Trager and Jensen [24], modified as described in Kimura et al. [2]. Briefly, the parasites were cultivated in 100 mm-diam. Falcon Petri dishes and maintained in RPMI 1640 medium supplemented with 25 mM Hepes, 21 mM sodium bicarbonate, 370 μM hypoxanthine, 11 mM glucose, 40 μg/ml gentamycin and 0.5 % (v/v) AlbuMax I® [25,26]. Washed human O^{+} erythrocytes were added to the culture medium to a haematocrit of 5 % (w/v). The Petri dishes were incubated at 37 °C in a candle jar with daily medium changes.

Development and multiplication of the culture were monitored by microscopic evaluation of Giemsa-stained thin smears. Synchronization was obtained by two treatments with Plasmagel® (6 %, w/v) as described in [27]. Starting with asynchronous cultures, schizonts were concentrated by flotation in Plasmagel and subcultured with fresh erythrocytes at 48-h intervals.

All experiments were carried out using extracts from ring-infected erythrocytes (0–20 h cultures), young-trophozoite-infected erythrocytes (20–30 h cultures), old-trophozoite-infected erythrocytes (30–40 h cultures) and schizont-infected erythrocytes (40–48 h cultures), purified on a 40/70/80 % discontinuous Percoll® gradient (centrifuged at 15000 ***g***, 30 min, 25 °C). This yielded an upper band from old-trophozoite- or schizont-stage cultures (40 %), another band from the young-trophozoite-stage cultures (70/80 % interface), and a pellet of ring stages and uninfected cells [28].

Inhibition tests

Mevastatin was diluted as described; 25 mM lactone solutions in DMSO were mixed with 2 equiv. of NaOH in an equal volume of water, heated at 40 °C for 45 min, and diluted to 5.0 mM in water with adjustment to pH 7.80 with HCl [29,30]. Various concentrations of mevastatin (240, 120, 60 and 30 μM) were tested. A control with DMSO was performed in parallel.

Inhibition tests were carried out in flat-bottomed microtitration plates (Falcon). Freshly synchronized cultures of 5 % (w/v) haematocrit and 1 % parasitaemia (ring-stage parasites) were exposed to serial dilutions of the compound to be tested in normal culture medium. After 48 h and 96 h (if not otherwise stated), the multiplication rate was assessed from the number of newly formed ring-stage parasites.

Metabolic labelling

The experiments with labelled parasites were performed using four different protocols.

Protocol 1

Synchronous *P. falciparum* ring-stage cultures, untreated or treated with 120 μM mevastatin for 48 h were labelled with 25 μCi/ml L-[^{35}S]methionine (> 1000 Ci/mmol) in methionine-deficient RPMI medium containing 10 μM methionine, from time zero or after 10 h of treatment. Another synchronous culture at the ring stage was treated with 120 μM mevastatin, and from time zero or after 10 h of treatment was labelled with 6.25 μCi/ml D-[U-^{14}C]glucose (291 mCi/mmol) in RPMI 1640 medium without the addition of glucose (see Figure 1, arrows 1 and 2). Aliquots were collected at different times (0–50 h) and precipitated with 12.5 % (w/v) trichloroacetic acid, and radioactivity was measured with a Beckman 5000 β-radiation counter.

Protocol 2

Cultures of *P. falciparum* with parasitaemia of around 15 % (85 % ring forms, 10 % trophozoites, 5 % schizont forms), pretreated with 120 μM mevastatin for 20 h were labelled for 15 h with D-[U-^{14}C]glucose in the presence of 120 μM mevastatin in RPMI 1640 medium without the addition of glucose, as described above (see Figure 1, arrow 3 and point C). All cultures were incubated in glucose-deficient medium for 1 h before the addition of the radioactive precursor, in order to deplete endogenous stores. The cultures were then washed three times in 10 vol. of RPMI 1640 medium without AlbuMax I. Ring, young-trophozoite and old-trophozoite forms were purified on a discontinuous Percoll gradient as described above, followed by lysis of the cells in 2 vol. of ice-cold buffer comprising 10 mM Tris/HCl, pH 7.2, 150 mM NaCl, 2 % (v/v) Triton X-100, 1 mM

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PMSF, 5 mM iodoacetamide, 1 mM *N*-α-p-tosyl-lysylchloromethane and 1 μg/ml leupeptin. After incubation for 15 min at 4 °C, lysates were centrifuged at 10000 ***g*** for 30 min, and supernatants were stored in liquid N_2 for subsequent SDS/PAGE analysis.

Protocol 3

P. falciparum was cultured for 10 h with an initial parasitaemia of around 25 % (70 % ring forms, 20 % trophozoites and 10 % schizonts), and was labelled for 15 h with [1(n)-^{3}H]geranylgeranyl-*PP* triammonium salt (16.5 Ci/mmol; 6.25 μCi/ml) or with [1(n)-^{3}H]farnesyl-*PP* triammonium salt (16.5 Ci/mmol; 6.25 μCi/ml) (see Figure 1, arrow 2 and point B). Each parasite stage (ring, trophozoite and schizonts forms) was purified as described, and was stored in liquid N_2 for subsequent TLC and HPLC analysis.

Protocol 4

Cultures of *P. falciparum* with parasitaemia of around 15 % (85 % ring forms, 10 % trophozoites and 5 % schizonts), pretreated with 120 μM mevastatin for 20 h were labelled for 15 h in the presence of 120 μM mevastatin with 7.14 μCi/ml sodium [1-^{14}C]acetate (56 mCi/mmol) (Figure 1, arrow 3 and point C). Each stage (ring, young-trophozoite and old-trophozoite forms) was purified on a Percoll gradient as described. Parasite pellets were stored in liquid N_2 for subsequent TLC and HPLC analysis.

Enzymic hydrolysis

Lysates from the three intra-erythrocytic parasite stages (not treated with mevastatin), obtained as described above (protocol 2), were submitted to cleavage by O-Glycanase (BSA-free) and recombinant N-Glycanase (glycerol-free). Samples were treated as described in [2,31,32]. After enzymic hydrolysis, samples were analysed by SDS/PAGE.

Gel electrophoresis

SDS/PAGE was carried out on 7 % (w/v) polyacrylamide gels as previously described [33]. The same number of parasites at each stage, lysed in sample buffer, was applied to each well for analysis of mevastatin-treated or untreated parasites. All gels were treated with Amplify®, dried, and exposed to Kodak X-Omat film with intensifying screens at −70 °C.

Cell extracts

Each purified parasite stage was freeze-dried and successively extracted with hexane (3 × 0.5 ml) (extract I), chloroform/methanol (1:1, v/v; 3 × 0.5 ml) (extract II) and chloroform/methanol/water (10:10:3, by vol.; 3 × 0.5 ml) (extract III). Aliquots of each extract were monitored for radioactivity.

Reverse-phase HPLC

Samples were analysed on an Ultrasphere ODS Beckman column (4.6 mm × 25 cm) with a Pharmacia LKB HPLC 2150 pump and a Pharmacia LKB LCC 2252 gradient module connected to a Micromeritics 787 variable UV/visible detector and a Hewlett Packard HP 3395 registrator. The eluent was monitored at 210 nm. Fractions of 0.5 min were collected, and aliquots were subjected to liquid scintillation counting. Two different gradient elution systems were used, one for the neutral lipid fraction and one for the charged fraction. In the first case (condition 1), solvent A was methanol/water (9:1, v/v) and solvent B was hexane/propan-2-ol/methanol (1:1:2, by vol.). A linear gradient was run from 5 % to 100 % B over a period of 25 min; 100 % B was then pumped through for an additional 5 min. The flow rate was 1.5 ml/min [17]. Geraniol, farnesol, dolichol 11, dodecaprenol and a mixture of dolichols 18–19 were used as standards. It is well known that dolichols and polyprenols containing the same number of isoprene units are not separated by reverse-phase HPLC [34].

In the second case (condition 2; charged lipid analysis), solvent A was methanol/water (9:1, v/v) and solvent B was hexane/propan-2-ol/methanol (1:1:1, by vol.), both containing 20 mM phosphoric acid. Here, a gradient from 25 % to 80 % B was run for 15 min, followed by 80 % B for an additional 5 min [17]. Dodecaprenyl-*P* and dolichyl-*P* 18–19 were used as standards.

TLC analysis

TLC of lipid extracts was performed using plates precoated with silica gel 60 (Merck) with the following solvent systems: (A) hexane/propan-2-ol (49:1, v/v); (B) hexane/propan-2-ol (24:1, v/v); (C) propanol/NH_4OH/water (6:2:1, by vol.).

Reverse-phase TLC was performed with plates precoated with RP-18 F254 (Merck) using hexane/propan-2-ol/methanol (1:1:1, by vol.) containing 20 mM phosphoric acid. The same number of parasites (untreated or treated with 120 μM mevastatin) of each stage, extracted in organic solvents, was applied to each spot for analysis.

Plates were dried and exposed to iodine vapour for detection of lipid standards. Labelled samples were detected after spraying with EN^{3}HANCE spray (Dupont NEN) and exposure to X-Omat AR films with intensifying screens at −70 °C. Spots were quantified by scanning the films with a GS-700 Imaging Densitometer with Molecular Analyst software (Bio-Rad). Dodecaprenyl-*P*, dolichyl-*P* 18–19, farnesyl-*PP* and geranylgeranyl-*PP* were used as standards in reverse-phase TLC systems.

Purification of polar lipids

Parasites labelled with sodium [^{14}C]acetate, [^{3}H]farnesyl ammonium-*PP* or [^{3}H]geranylgeranyl ammonium-*PP* were extracted as above. When necessary, extracts were fractionated further on a DEAE-cellulose column (acetate form) (10 cm × 0.8 cm) [35]. Neutral lipids were eluted from the column with 10 vol. of chloroform/methanol/water (10:10:3, by vol.). Charged lipids were then eluted with chloroform/methanol/water (10:10:3, by vol.) containing 0.2 M ammonium acetate.

Analysis of the lipid moiety of sugar-*P*-polyisoprenols

Sugar-*P*-polyisoprenol lipids obtained from labelled parasites and retained in the DEAE-cellulose column were dissolved in 90 % (v/v) methanol containing 0.1 M HCl and hydrolysed for 1 h at 50 °C [17]. Acid was removed by several rounds of the addition of methanol followed by evaporation, and the hydrolysate was analysed by HPLC.

Sugar-*PP*-polyisoprenols present in chloroform/methanol/water extracts (extract III) were treated as above and further hydrolysed with potato acid phosphatase [36].

Alkaline treatment of the pyrophosphate derivatives

Samples were treated with 20 % (w/v) KOH in methanol/water (2:1, v/v) in a screw-capped test tube for 1 h at 100 °C. After incubation, the sample was extracted with chloroform/methanol (2:1, v/v; 2 × 0.5 ml) [34].

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RESULTS

P. falciparum growth inhibition *in vitro* by mevastatin is stage-dependent

Treatment of *P. falciparum* (synchronous in ring stage) with 120 μM mevastatin for 30 h caused 95% inhibition in the development of the young trophozoites to the schizont stage without pigment formation. Parasites at the old-trophozoite stage (30–40 h culture time) in the first cycle were stopped in their development and died (Figure 1, panels 3′ and 4′). When 30 μM mevastatin was used for 96 h 50% inhibition in the development of the young trophozoites to the old-trophozoite stage occurred after 78–80 h of treatment in the second cycle. The percentage inhibition increased to 95% after 78–80 h of treatment in the second cycle when parasites were treated with 60 μM mevastatin, although to obtain 45% inhibition with this drug concentration, 40 h of treatment were necessary (results not shown). When treatment with 120 μM mevastatin started at the old-trophozoite stage (after 30–40 h in culture), inhibition was detected only after 40–45 h of treatment. Cytotoxic effects giving a complete inhibition of growth only occurred for all parasite stages with drug concentrations above 240 μM. Parasites developed through three cycles in erythrocytes pretreated for 48 h with 120 μM mevastatin; this suggests that inhibition does not occur as a result of drug accumulation in the erythrocytes, and agrees with the results obtained with other drugs such as tunicamycin [2,37].

The effects of mevastatin showed variability, with between 60 and 95% inhibition, when AlbuMax I was substituted for human sera in the culture medium (results not shown), probably due to the variable composition of the sera.

Protein biosynthesis was estimated by addition of L-[^{35}S]methionine and measurement of the radioactivity incorporated into the trichloroacetic acid precipitate. No differences in protein synthesis were observed when parasites treated with 120 μM mevastatin were compared with untreated parasites (Figure 1, bottom panel). On the other hand, when the precursor used was D-[U-^{14}C]glucose, 70–80% inhibition of sugar incorporation was detected. This effect was more pronounced during differentiation of young trophozoites into old trophozoites at 25–48 h of culture (Figure 1).

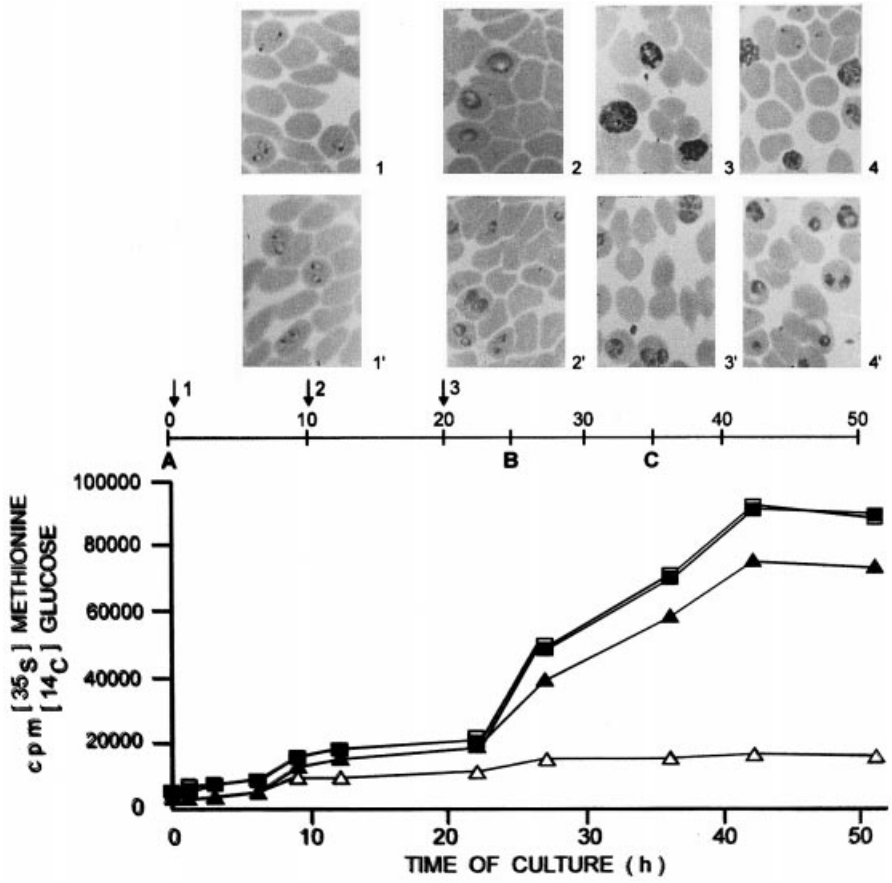

Figure 1 ***P. falciparum*** **parasites treated or not with 120 μM mevastatin**

Upper panels: the micrographs of Giemsa-stained thin smears (magnification × 840) show the sequence of events when parasites of *P. falciparum*, starting at the ring stage (1% parasitaemia), were left untreated (panels 1–4) or were treated with mevastatin (panels 1′–4′). No differences could be detected between treated and untreated parasites at the ring stage, as shown in panels 1 and 1′. At the young-trophozoite stage (20–30 h of culture time), a delay in maturation could be detected in treated parasites, which were also deformed (panels 2 and 2′). At the old-trophozoite stage (30–40 h of culture time) and schizont stage (40–48 h of culture time), untreated parasites continued to develop (panels 3 and 4), whereas treated parasites continued to display deformities (panels 3′ and 4′). The bottom panel shows the incorporation of L-[^{35}S]methionine into untreated parasites (■) and parasites treated with mevastatin (□) for 48 h of culture time, measured as incorporation into trichloroacetic acid-precipitable pellets. The incorporation of D-[U-^{14}C]glucose into untreated parasites (▲) and parasites treated with mevastatin (△) over 48 h of treatment was measured in the same way. Arrows 1, 2 and 3 indicate the times at which pulses of radioactive precursor were applied to cultures of untreated or mevastatin-treated parasites. Point A, start of culture of *P. falciparum*; points B and C, times when parasites were collected after the pulses of radioactive precursors (arrows 2 and 3 respectively) for analysis by SDS/PAGE, HPLC, TLC and reverse-phase TLC.

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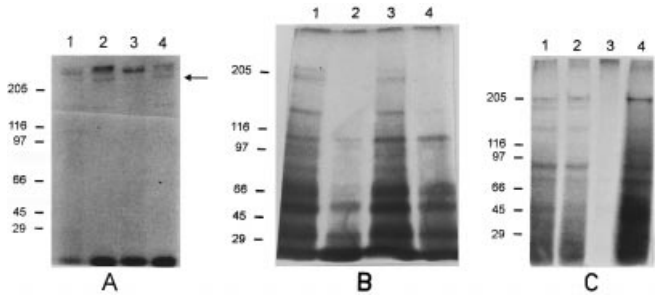

Figure 2 Analysis by SDS/PAGE of the profiles of D-[U-^{14}C]glucose-labelled glycoproteins in *P. falciparum* treated or not with mevastatin

Labelled glycoproteins obtained from untreated parasites were incubated with N-Glycanase or O-Glycanase. (**A**) Ring stage. Lane 1, treated with 120 μM mevastatin; lane 2, treated with O-Glycanase; lane 3, treated with N-Glycanase; lane 4, untreated parasites. The arrow on the right indicates the band of $>$ 200 kDa. (**B**) Young-trophozoite stage. Lane 1, untreated parasites; lane 2, treated with 120 μM mevastatin; lane 3, treated with O-Glycanase; lane 4, treated with N-Glycanase. (**C**) Old-trophozoite stage. Lane 1, untreated parasites; lane 2, treated with 120 μM mevastatin; lane 3, treated with O-Glycanase; lane 4, treated with N-Glycanase. Lanes showing the effects of N-Glycanase or O-Glycanase treatment were loaded with 50% more parasites than those containing untreated or mevastatin-treated parasites. Molecular mass markers (kDa) are indicated at the left of each figure.

Biosynthesis of N-linked glycoproteins is inhibited by mevastatin

Both treated and untreated parasites displayed asynchronous forms after 30–42 h of culture (see the Materials and methods section, Protocol 2 under 'Metabolic labelling'). Nevertheless, while untreated parasites displayed 20% young trophozoites, 70% old trophozoites and 10% ring forms, mevastatin-treated parasites displayed 85% young trophozoites, 10% old trophozoites and 5% ring forms. Developmental stages were purified on a discontinuous Percoll gradient, processed as described in the Materials and methods section, and the presence of glycoproteins was analysed for each stage.

Differences were observed in the electrophoretic profiles of parasites treated with 120 μM mevastatin for 20 h and pulsed for 15 h with D-[U-^{14}C]glucose in the presence of the drug, when compared with those of untreated parasites (cf. Figure 1, arrow 3 and point C). In the ring-stage form treated with mevastatin, one $>$ 200 kDa band had disappeared when compared with untreated parasites (Figure 2A, lanes 1 and 4). When ring forms that had not been exposed to mevastatin were treated with N-Glycanase, the absence of the same band was shown (Figure 2A, lane 3), although this band remained after treatment with O-Glycanase (Figure 2A, lane 2). Mevastatin-treated young trophozoites did not show bands of between 205 kDa and 110 kDa, while other bands (110, 93, 80, 70 and 50 kDa) were less intense than in the corresponding untreated controls (Figure 2B, lanes 1 and 2). A similar profile was detected when young trophozoites not treated with mevastatin were incubated with N-Glycanase (Figure 2B, lane 4), although no differences were detected when they were treated with O-Glycanase (Figure 2B, lane 3) [2]. In contrast, mevastatin-treated old trophozoites showed a similar pattern to that in untreated parasites (Figure 2C, lanes 1 and 2). Differences were detected when old trophozoites were treated with O-Glycanase (Figure 2C, lane 3), as shown previously [2]. Each stage of parasites not treated with mevastatin was incubated in digestion buffer without enzyme as a control, and the patterns obtained were the same as those of non-incubated parasites (results not shown) [2]. Under identical experimental conditions, uninfected erythrocytes did not incorporate labelled sugars into glycoproteins [2,37,38].

Table 1 Radioactivity recovered in the different extracts from each stage of *P. falciparum* after metabolic labelling with [^{3}H]farnesyl-*PP* or [^{3}H]geranylgeranyl-*PP*

		$10^{-4} \times {}^3$H (c.p.m.)		
Precursor	Extract	Ring form	Trophozoites	Schizonts
[^{3}H]Farnesyl-*PP*	I	164.0	7.4	70.8
	II	86.9	36.0	13.8
	III	43.4	3.0	10.0
[^{3}H]Geranylgeranyl-*PP*	I	143.0	13.7	88.0
	II	147.0	70.5	19.4
	III	46.3	3.0	7.6

Presence of dolichol and dolichyl derivatives of 11 and 12 isoprene units in all stages of *P. falciparum*

The previous findings on the presence of N-glycosidic chains in the glycoproteins of *P. falciparum* [2], together with our present results, led us to search for the presence of lipid metabolites from the isoprenoid pathway. The precursors generally used for the biosynthesis of dolichol, i.e. acetate and mevalonate, were chosen first. However, the very low incorporation of radioactive acetate or mevalonate (results not shown) was in accordance with published results [5,39]. Next we used the ammonium salts of [^{3}H]farnesyl-*PP* and [^{3}H]geranylgeranyl-*PP* for 15 h to search for other precursors. Each labelled stage was purified as described in the Materials and methods section and freeze-dried. The pellets were extracted with hexane (extract I), chloroform/methanol (1:1, v/v) (extract II) and chloroform/methanol/water (10:10:1, by vol.) (extract III). Table 1 shows the total radioactivity incorporated into each extract. There was no incorporation of [^{3}H]farnesyl-*PP* or [^{3}H]geranylgeranyl-*PP* ammonium salts into uninfected erythrocytes submitted to identical experimental conditions.

Samples of the less polar extracts (extract I) were analysed by C_{18} reverse-phase HPLC. Figure 3 shows the radioactive profiles obtained with each precursor for each stage. [^{3}H]Geranylgeranyl-*PP* incorporation into samples resulted in three major peaks: one eluting at 8 min (fraction nos. 15–16), coincident with an authentic sample of geraniol (probably representing a degradation product), and two others, eluting at 21 min (fraction nos. 42–43) and 23 min (fraction nos. 46–47), coincident with authentic samples of dolichol 11 and dodecaprenol respectively used as standards. The latter peaks were the major ones in trophozoites and schizonts (Figures 3B and 3C), while they were minor ones in ring forms (Figure 3A).

Samples incorporating [^{3}H]farnesyl-*PP* showed a number of other labelled products. Although these products were not all identified, they probably correspond to intermediate metabolites of the isoprenoid pathway (Figures 3A′–3C′). The presence of important peaks at 21 min (fraction nos. 42–43) and 23 min (fraction nos. 46–47) was again evident, corresponding to polyisoprenols of 11 and 12 units. Interestingly, while dolichol 11 was predominant in the [^{3}H]farnesyl-labelled extracts, [^{3}H]geranylgeranyl was mainly incorporated into dolichol 12.

A sample of extract I obtained from schizonts metabolically labelled with [^{3}H]geranylgeranyl-*PP* was analysed by TLC on

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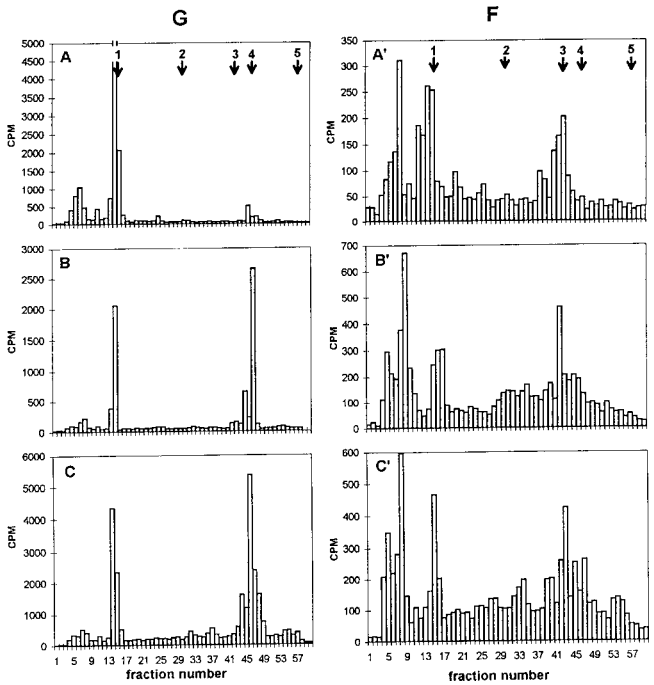

Figure 3 Reverse-phase HPLC analysis of less polar extracts

Reverse-phase HPLC analysis (condition 1) of the hexane extracts (extract I) from the different stages of *P. falciparum* metabolically labelled with ammonium [^{3}H]geranylgeranyl-*PP* (G; left panels) or ammonium [^{3}H]farnesyl-*PP* (F; right panels), as described in the Materials and methods section. Radioactivity in fractions was monitored every 0.5 min. Panels: (**A**) and (**A′**), ring forms; (**B**) and (**B′**), trophozoites; (**C**) and (**C′**), schizont forms. Arrows indicate the elution positions of authentic isoprenoid standards co-chromatographed in each case: 1, geraniol; 2, farnesol; 3, dolichol 11; 4, dodecaprenol; 5, dolichol 18–19.

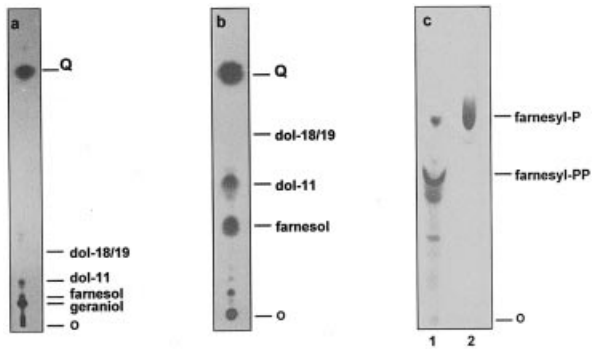

Figure 4 TLC of the lipid extracts obtained from schizont forms of *P. falciparum*

(**a**) Extract I (hexane) obtained from parasites metabolically labelled with [1(n)-^{3}H]geranylgeranyl-*PP* triammonium salt (solvent system A). (**b**) Extract I (hexane) obtained from parasites metabolically labelled with sodium [^{14}C]acetate (solvent system B). (**c**) Lane 1, extract III (chloroform/methanol/water) from parasites metabolically labelled with sodium [^{14}C]acetate; lane 2, same as lane 1, but subject to alkaline treatment (solvent system C). Q, ubiquinone; dol-18/19, dolichol 18–19; dol-11, dolichol 11; O, origin.

silica plates using solvent A (Figure 4a). Spots coincident with authentic samples of geraniol (R_F 0.07), farnesol (R_F 0.08) and dolichol 11 (R_F 0.14) were revealed. The presence of a fast-moving component with an R_F (0.83) coincident with that of ubiquinone was also evident. This product has previously been

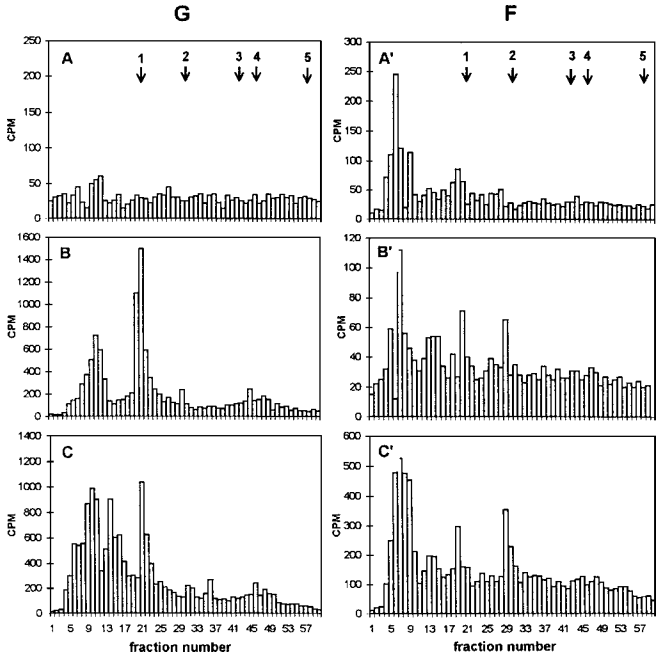

Figure 5 Reverse-phase HPLC analysis of the dolichol obtained from dolichyl-*PP* species present in extract III

Extracts III (chloroform/methanol/water) from parasites labelled with [^{3}H]geranylgeranyl-*PP* (G; left panels) or [^{3}H]farnesyl-*PP* (F; right panels) were subjected to DEAE-cellulose column chromatography, acid hydrolysis and acid phosphatase digestion. Hexane extracts were analysed using condition 1, as described in the Materials and methods section. Radioactivity was monitored every 0.5 min. Panels: (**A**) and (**A′**), ring forms; (**B**) and (**B′**), trophozoites; (**C**) and (**C′**), schizont forms. Arrows indicate the elution positions of authentic isoprenoid standards co-chromatographed in each case: 1, geraniol; 2, farnesol; 3, dolichol 11; 4, dodecaprenol; 5, dolichol 18–19.

described as a component of different *Plasmodium* species [6], and would not be resolved in the HPLC analysis under the conditions used.

Extracts II (chloroform/methanol) of each stage were fractionated [34] and the charged lipid fractions obtained were hydrolysed and analysed by HPLC. Two peaks were obtained; one was eluted with a retention time similar to that reported by Low et al. [17], and may correspond to isoprenyl-*P* of 11 isoprenoid units, and the retention time of the other peak was similar to that of an authentic standard of dodecaprenyl-*P* (results not shown)

Extracts III were fractionated as above, hydrolysed with mild acid and digested further with potato acid phosphatase [36]. The hydrolysate was extracted with hexane and analysed by HPLC (Figure 5). Although the [^{3}H]geranylgeranyl-labelled extract III contained a large amount of radioactivity (Table 1), after fractionation only a very small amount was retained in the charged lipid fraction. Analysis of the different stages with each precursor also suggested the presence of dolichol 11 (fraction nos. 42–43) and dolichol 12 (fraction nos. 45–46) in these extracts, particularly when using [^{3}H]geranylgeranyl-*PP* as precursor (Figures 5A–5C).

At this point, the presence of an active isoprenoid pathway in the intra-erythrocytic stages of *P. falciparum* became evident.

Mevastatin inhibits the isoprenoid pathway in *P. falciparum*

Sodium [^{14}C]acetate was not incorporated efficiently into the different *P. falciparum* stages. However, it was used to evaluate

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Table 2 Radioactivity recovered in the different extracts after metabolic labelling with sodium [^{14}C]acetate of the different stages of *P. falciparum* treated or not with mevastatin

The same quantities of parasites, estimated as final volumes of each stage recovered after the Percoll gradient, were used to give total radioactivity.

	^{3}H (c.p.m.)					
	Ring form		Young trophozoites		Old trophozoites	
Extract	Control	Treated	Control	Treated	Control	Treated
I	5218	1800	1800	1600	62000	46000
II	80000	42600	22600	14285	34000	23000
III	14300	8900	8800	3700	12000	8000

the effects of mevastatin, a HMG-CoA reductase inhibitor, on the synthesis of isoprenoid intermediates. Metabolic incorporation of sodium [^{14}C]acetate into parasites treated or not with mevastatin was performed. The different stages were purified, freeze-dried and extracted, and the total radioactivity in each extract is shown in Table 2. Further analyses of some of these extracts were carried out by TLC and HPLC. Under identical experimental conditions, uninfected erythrocytes did not incorporate sodium [^{14}C]acetate.

Lipids present in extract I obtained from schizont forms metabolically labelled with sodium [^{14}C]acetate were analysed by TLC in solvent system B (Figure 4b). In accordance with the results obtained by metabolic labelling with [^{3}H]isoprenyl-*PP* precursors, a spot (R_F 0.43) migrating similarly to the dolichol 11 standard was obtained. As expected, farnesol (R_F 0.28), present in high amounts, and a faint spot (R_F 0.38) probably corresponding to other polyisoprenol intermediates of the biosynthetic pathway, were also observed. Ubiquinone (R_F 0.75) could also be detected using this precursor.

Samples of extract I obtained from treated and untreated parasites were analysed by HPLC (Figure 6). Peaks corresponding to farnesol, dolichol 11 and other isoprenoid intermediates were detected with this precursor. However, even though no significant differences were found between treated and untreated parasites for young trophozoites and old trophozoites (Figures 6B and 6C), the ring stage showed a dramatic inhibition of isoprenoid biosynthesis (Figure 6A).

When extracts III obtained from sodium [^{14}C]acetate-labelled schizonts were analysed by TLC in solvent C, a major component corresponding to farnesyl-*PP* (R_F 0.43), together with minor spots appearing in the region where other isoprenoids-*PP* migrate (R_F 0.40–0.50), were observed (Figure 4c, lane 1). The susceptibility of these compounds to basic treatment, with concomitant transformation into isoprenyl-*P* species (R_F 0.68), confirmed the presence of the pyrophosphate group (Figure 4c, lane 2). Mbaya et al. [5] have previously reported the presence of farnesyl-*PP* as a major labelled compound when sodium [^{14}C]mevalonate was incorporated into *P. falciparum*.

As the amounts of phosphorylated species did not allow HPLC analysis, samples of extract II for each stage, treated or not with mevastatin, were compared by reverse-phase TLC. Spots co-migrating with standards of dolichyl-*P*, dodecaprenyl-*P*, farnesyl-*PP* and geranylgeranyl-*PP* were detected (results not shown). Under the conditions used, isoprenoid-*PP* species of different chain lengths are not resolved. The densitometry of spots showed that pyrophosphate species were reduced by 90% in the ring stage, 20% in young trophozoites and 48% in old

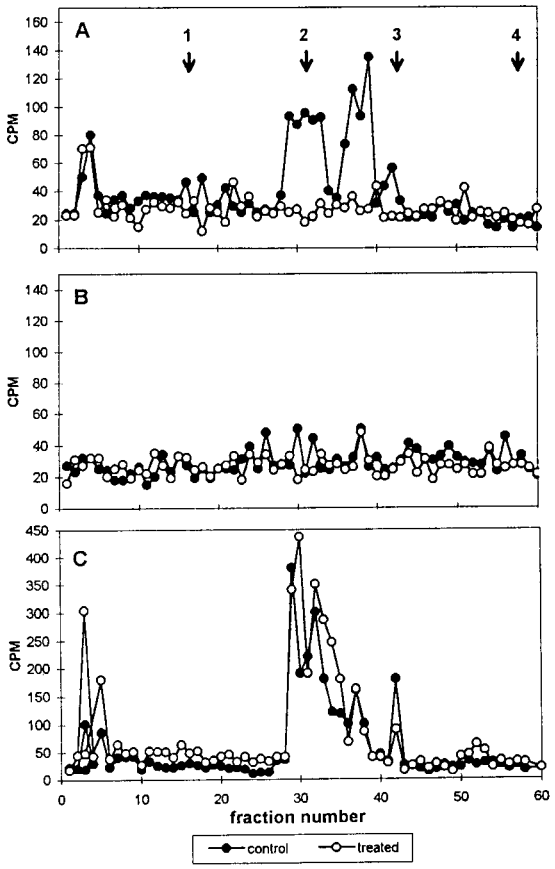

Figure 6 Reverse-phase HPLC analysis of extract I obtained from the different stages of *P. falciparum* treated or not with mevastatin and metabolically labelled with sodium [^{14}C]acetate

Condition 1 was used for HPLC analysis (see the Materials and methods section). (**A**) Ring forms; (**B**) young trophozoites; (**C**) old trophozoites. Arrows indicate the elution positions of authentic isoprenoid standards co-chromatographed in each case: 1, geraniol; 2, farnesol; 3, dolichol 11; 4, dolichol 18–19. ● Control parasites; ○, parasites treated with mevastatin.

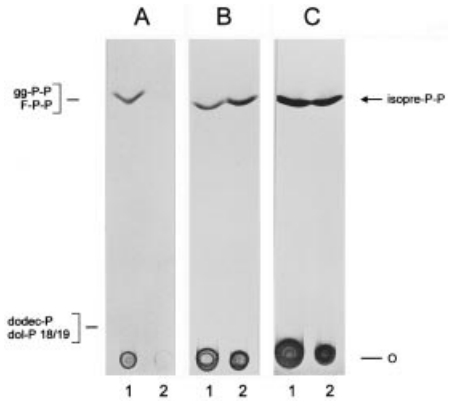

Figure 7 Analysis by reverse-phase TLC of extract III from the different stages of *P. falciparum* treated or not with mevastatin and metabolically labelled with sodium [^{14}C]acetate

(**A**) Ring forms; (**B**) young trophozoites; (**C**) old trophozoites. Lane 1, untreated parasites; lane 2: parasites treated with 120 μM mevastatin. Positions of standards of dolichyl-*P* 18–19 (dol-P 18/19), dodecaprenyl-*P* (dodec-P), farnesyl-*PP* (F-P-P) and geranylgeranyl-*PP* (gg-P-P) are indicated at the left of figure. The arrow at right of figure indicates a spot of isoprenyl-*PP* (isopre-P-P). O, origin.

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trophozoites, while phosphate species were reduced by 71% in the ring stage, 28% in young trophozoites and 43% in old trophozoites.

When analogous samples of extract III were analysed in the same way, spots corresponding to pyrophosphate derivatives were observed (Figures 7A–7C, lanes 1). Mevastatin treatment resulted in a 90% decrease in these derivatives in ring forms and a 33% decrease in old trophozoites, while no significant differences ($< 5\%$) were detected in the young-trophozoite stage (Figures 7A–7C, lanes 2).

DISCUSSION

The results presented here clearly demonstrate the presence of an active isoprenoid pathway in *P. falciparum* intra-erythrocytic stages. We have identified dolichol, dolichyl-*P* and dolichyl-*PP* of 11 and 12 isoprene residues by metabolic labelling of parasites with [^{3}H]farnesyl-*PP*, [^{3}H]geranylgeranyl-*PP* and [^{14}C]acetate. Parasite treatment with mevastatin depressed the biosynthesis of dolichol, dolichyl-*P* and isoprenyl-*PP* species in all intra-erythrocytic stages, but the inhibition was most pronounced in the ring stage. This fact might be correlated with inhibition of protein N-glycosylation in the ring and young-trophozoite stages, and with a concomitant arrest of parasite development.

The presence of N-linked glycoproteins in *P. falciparum*, mainly in the ring and young-trophozoite stages of the intra-erythrocytic cycle, has been unequivocally demonstrated [2], in spite of reports claiming that glycosylation in the malaria parasite is mostly associated with the synthesis of glycosylphosphatidylinositol-anchor structures [3,40]. Nevertheless, our results on the presence of N-glycosylation in *P. falciparum* glycoproteins prompted us to study the effects of inhibitors of this biosynthetic pathway on parasite growth.

Grellier et al. [22] have reported that both lovastatin and simvastatin possess anti-parasitic activity *in vitro* against *P. falciparum*. Our results, using mevastatin as an inhibitor, agree with theirs in terms of inhibitory concentrations, duration of treatment and stage-dependence. As they also found, our attempts to reverse the parasite growth inhibition by competition with an excess of exogenous mevalonate were unsuccessful (results not shown).

Mevastatin-treated parasites showed 70–80% inhibition of sugar incorporation between 25 h and 48 h of treatment, coincident with the arrest of differentiation of young trophozoites into old trophozoites and schizonts (Figure 1). Protein biosynthesis was not affected, as previously described for tunicamycin [2]. Moreover, mevastatin-sensitive glycoproteins from ring and young-trophozoite stages were similar to those affected on incubation with N-Glycanase (Figures 2A and 2B) and tunicamycin [2]. These results provide evidence for the importance of N-linked glycoproteins in the schizogony of the intra-erythrocytic stages.

In order to characterize the presence of an isoprenoid pathway, mevalonic acid is usually incorporated [8,41,42]. Unfortunately, all attempts to label the *P. falciparum* isoprenoid constituents with this precursor were unsuccessful (cf. [5,39]). It is well known that yeast prenylated proteins cannot be labelled directly with [^{3}H]mevalonate, presumably because this compound cannot be transported into the cell [7]. Despite the reported inability of other cellular systems, such as yeast and some mammalian cells, to take up advanced precursors of the isoprenoid pathway, such as [^{3}H]farnesyl-*PP* and [^{3}H]geranylgeranyl-*PP*, all the intra-erythrocytic stages of *P. falciparum* did incorporate both of them (Table 1). The process of erythrocyte invasion by *P. falciparum* is associated with a sudden and dramatic increase in the total membrane area and a considerable rise in the total lipid content [43]. It is well known that *Plasmodium* spp. do not biosynthesize cholesterol or fatty acids [4,44]. Thus the only possible source of these lipids is their acquisition from the plasma of their host. This suggests the existence of an intense lipid transport system in infected erythrocytes that may explain the efficient uptake of the isoprenoid precursors found in the present study. As far as we know, this is the first report on the incorporation of these precursors into dolichol.

By labelling with [^{3}H]farnesyl-*PP*, it was possible to characterize a free dolichol of 11 isoprene units. Minor amounts of a compound eluting at the same time as a standard of dodecaprenol were also detected, mainly in schizont forms. Using [^{3}H]geranylgeranyl-*PP*, a precursor carrying one more isoprene unit, the presence of a dolichol of 12 isoprene units was predominant (Figures 3A–3C). The lengths of dolichol-*P* derivatives in both cases were consistent with these findings. Differences between the levels of dolichol 11 and dolichol 12 labelled with each precursor obtained from the pyrophosphate derivatives were not so evident, because they are only detectable in very small amounts (Figure 5). Low levels of dolichyl-*PP* derivatives are usually found in eukaryotic cells [13,14].

Thus geranylgeranyl-*PP* and farnesyl-*PP* were found to be effective substrates of *cis*-prenyltransferase, the enzyme responsible for the elongation steps of isoprenoid biosynthesis. Adair et al. [45] reported that the same number of isoprene units is transferred regardless of the length of the precursor used.

When [^{14}C]acetate was incorporated, TLC analysis of the free-dolichol-containing extract (extract I) of schizont forms showed a main spot with an R_F coincident with that of an authentic standard of farnesol. Mbaya et al. [5] had previously reported an accumulation of farnesol using the same conditions. A spot coincident with dolichol 11 was also evident (Figure 4b). TLC analysis of extract III from schizont forms showed the presence of spots migrating in the region of pyrophosphate derivatives. The fact that alkaline treatment turned these compounds into monophosphate forms confirmed the presence of pyrophosphate compounds in this extract (Figure 4c).

Despite the fact that few reports exist on the characterization of dolichols in protozoa, the presence of short-chain species seems to be a common feature. Dolichols of 13 isoprene units in *Trypanosoma cruzi* [15], of 11 isoprene units in *Crithidia fasciculata* [16], and of 11 and 12 isoprene units in *Trypanosoma brucei* [17] have been reported. The present findings are the first in Apicomplexa.

Inhibitors of HMG-CoA reductase are used to decrease blood levels of cholesterol. There is much debate on the extent of the effect of these inhibitors on dolichol levels, although a decrease in dolichol content would be expected after drug treatment. In neuroblastoma cells, treatment with mevinolin suppressed by 90% the incorporation of [^{3}H]acetate into cholesterol, dolichol and ubiquinone [46]. In a melanoma cell line, a depression of the biosynthesis of dolichyl-*P* was observed [47]. On the other hand, the effects of HMG-CoA reductase inhibitors in rats were tissue-dependent, with an increase in dolichol levels in brain and liver [48,49].

Treatment of *P. falciparum* cultures with mevastatin caused a pronounced delay in parasite development; ring forms and young trophozoites seem to be the stages most affected. During the time that control ring forms were transformed into old trophozoites, only 85% of the mevastatin-treated ring forms became young trophozoites, while others failed to develop.

We have shown that mevastatin decreases dolichol, dolichyl-*P* and isoprenyl-*PP* levels in ring forms (Figures 6A and 7A). However, only minor inhibition of these compounds in the old-

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trophozoite stage was detected, while young trophozoites showed no significant differences (Figures 6B, 6C, 7B and 7C). This stage-specific inhibition may suggest that dolichol biosynthesis takes place predominantly in the ring stage. This is in accordance with a recent report by Chakrabarti et al. [39] showing that protein prenyltransferase activity is highest in the ring stage, coincident with the greatest inhibitory effects of specifics drugs. It has also been suggested that, during stages where inhibitors seem to have no effect, alternative mechanisms of prenylation may be operative. In fact, a new alternative pathway for isoprenoid biosynthesis has been described for Eubacteria and plants [50]. This pathway involves glyceraldehyde-3-phosphate/pyruvate, and is not inhibited by mevastatin [50].

In conclusion, we have shown that intra-erythrocytic stages of *P. falciparum* synthesize dolichol, dolichyl-*P* and dolichyl-*PP* species of 11 and 12 isoprenoid units. Mevastatin treatment of parasites decreased levels of isoprenoids, especially in the ring stage, and also affected the N-glycosylation of glycoproteins in the young-trophozoite stage, as shown by SDS/PAGE analysis (Figure 2). These results may be related to the fact that mevastatin treatment affects *P. falciparum* development. Obviously, the possibility that other end-products of this pathway, such as isoprenylated proteins and/or ubiquinones, may also be inhibited by this treatment cannot be ruled out. Therefore the isoprenoid pathway may represent a different approach for the development of new anti-malarial drugs. Studies to address these questions are currently in progress in our laboratory.

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