The human malarial parasite *Plasmodium falciparum* (Pf) is exposed to wide temperature fluctuations during its life cycle, ranging from 25 °C in the mosquito vector and 37 °C in humans to 41 °C during febrile episodes in the patient. The repeated occurrence of fever at regular intervals is a characteristic of human malaria. We have examined the influence of repeated exposure to elevated temperatures encountered during fever on the intra-erythrocytic development of the parasite. Using flow cytometry, we show that repeated exposure to temperatures mimicking febrile episodes promotes parasite development in human erythrocytes. Heat shock-mediated cytoprotection and growth promotion is dependent on the heat shock protein 90 (PfHsp90) multi-chaperone complex. Inhibition of PfHsp90 function using geldanamycin attenuates temperature-dependent progression from the ring to the trophozoite stage. Geldanamycin inhibits parasite development by disrupting the PfHsp90 complex consisting of PfHsp70, PfPP5, and tubulin, among other proteins. While explaining the contribution of febrile episodes to the pathogenesis of malaria, our results implicate temperature as an important environmental cue used by the parasite to coordinate its development in humans.

The pathogenesis of human malaria is caused by intra-erythrocytic growth of the parasite. Entry of the parasite into an erythrocyte triggers its metamorphosis to a complex cell, rich in endomembrane system and capable of complex trafficking events (1). The transformation is evident during the progression of the parasite from the early stage of establishment, namely rings, to the biosynthetically active stage called the trophozoite. Trophozoites finally convert to schizonts and undergo nuclear division to form multiple merozoites. Molecular signals and components orchestrating the asexual cycle of the parasite remain poorly understood. Coordination of stage transitions is likely to depend on environmental cues available to the parasite.

The occurrence of febrile episodes, resulting in body temperatures as high as 41 °C, is a common manifestation of malaria (2). The release of the proinflammatory cytokine tumor necrosis factor (TNF) is believed to be responsible for the appearance of fever in malaria patients (3), and fever is known to augment the pathogenesis of malaria (4). There are a number of reports in the literature addressing the influence of elevated temperature encountered during malarial fever on the host (5) as well as the parasite (6, 7). Keeping in view the augmentative effects of fever on the severity of malaria, antipyretics have been suggested to have beneficial effects on the patient (5). Details about the influence of repeated occurrences of fever on the growth of the malarial parasite are only partly explored.

In addition to the periodic appearance of fever, which is a characteristic of tertian malaria caused by *Plasmodium vivax*, subtertian fever is commonly seen in patients infected with *P. falciparum* (2, 5). Body temperatures of these patients reach as high as 41 °C, extending irregularly over a period of >24 h (3). To mimic the repeated appearance of fever, we have examined the influence of two heat shocks separated by an interval of 10 h on the growth of the malarial parasite in culture. Using flow cytometry to examine parasite stage transitions, we find that the exposure of parasites to a prior heat shock accelerates their development from the ring stage to the trophozoite stage during the subsequent heat shock. In the absence of the prior heat shock, exposure to second heat shock alone results in significant death of the parasite. Acceleration of the critical stage switch resulting from the exposure to prior heat shock also translates into a robust increase in the productivity of the infection cycle. Using geldanamycin (GA), a specific inhibitor of Hsp90 function, we show that the growth-promoting effects of prior heat shock depend on the PfHsp90 multi-chaperone complex. Exposure to GA following exposure to prior heat shock (PHS) interfered with parasite stage conversion. The accelerative effect of PHS was only attenuated and not blocked by GA, indicating the dominant potentiating influence of PHS on parasite development. GA brings about the inhibition of PfHsp90 function by disrupting its complex consisting of PfHsp70, PfPP5, and tubulin, among other proteins. Our results provide an additional basis for the augmentative effects of fever on the pathogenesis of malaria.

**EXPERIMENTAL PROCEDURES**

Flow Cytometry—*P. falciparum* strain 3D7 was cultured using standard protocols (8). Flow cytometry was carried out essentially as described earlier but with a few modifications (9). Briefly, parasites were purified on 75% Percoll. Parasite cultures were suspended in phosphate-buffered saline (PBS) and layered on 75% Percoll prepared in PBS. Following centrifugation at 2000 rpm for 25 min, uninfected erythrocytes form pellets at the bottom, whereas the infected erythrocytes appear at the interface between Percoll and PBS. Percoll purification of infected erythrocytes was carried out thrice to remove uninfected red blood cells from the samples. We obtained a purity of infected cells of >98% using this protocol, and this result was confirmed by Giemsa staining and microscopic examination.

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**Recurrent Fever Promotes *Plasmodium falciparum* Development in Human Erythrocytes**

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The abbreviations used are: GA, geldanamycin; PHS, prior heat shock; non-HS, non-heat shock; Pf, *Plasmodium falciparum*; PP5, protein phosphatase 5; PBS, phosphate-buffered saline.
RESULTS

Exposure to Prior Heat Shock Confers Protection against Subsequent Heat Shock—To examine the influence of recurrent febrile episodes on the growth of the parasite, we exposed parasite cultures to 40 °C and analyzed their development during the subsequent exposure to 40 °C using flow cytometry. The increase in DNA content seen during ring to trophozoite transition allowed us to differentiate between these two stages through flow cytometry by measuring green fluorescence due to the acridine orange staining of parasites. To define scattergrams corresponding to the ring (0–24 h post-synchronization) and trophozoite (24–36 h post-synchronization) stages, we purified highly synchronous populations of these stages on 75% Percoll and analyzed their scattergrams by flow cytometry. Fig. 1A shows scattergrams corresponding to rings (left) and trophozoites (middle). The insets show the corresponding Giemsa-stained stage. The superimposition of these scattergrams as shown at the right in Fig. 1A clearly indicated that it was possible to differentiate between rings and trophozoites by this approach.

Ring-infected erythrocytes were exposed to 40 °C for 2 h (PHS, phase A) and, after an interval of 10 h at 37 °C (phase B), were exposed to 40 °C (phase C) again. The effect of heat shock on overall parasitemia and the conversion to trophozoite stage was monitored by examining scattergrams at 0-, 6-, and 12-h time intervals during the second heat shock (phase C) by flow cytometry as described under “Experimental Procedures.” As control, a sample was maintained at 37 °C without prior exposure to heat shock (control, phase A), and stages were monitored during the second heat shock (phase C) at equivalent time points. Cultures kept at 37 °C during all three phases (phases A, B, and C) were also used as control (non-HS control). The scheme of temperature shifts is depicted in Fig. 1B (right). As shown in Fig. 1B (left), control cultures incubated at 37 °C without a prior heat shock showed a significant drop in parasite count during the second heat shock. Parasites exposed to a prior heat shock, on the other hand, did not show any growth inhibition during the second heat shock. As expected, the non-HS control cultures did not show any drop in parasite number. Estimation of parasitemia by Giemsa staining and microscopic examination produced the same results (not shown). Furthermore, comparison of scattergrams (Fig. 1C, left) showed that whereas control cultures exposed to the second heat shock alone persisted in the ring stage at the 6-h time interval, parasites exposed to a prior heat shock had already progressed to the trophozoite stage at 6 h. The pattern of stage progression in cultures exposed to the second heat shock alone was similar to that of the non-HS control cultures, indicating that the second heat shock alone did not cause any retardation of parasite stage transition. These results were also corroborated by microscopic examination of Giemsa-stained smears at each time interval as shown in the inset (Fig. 1C) of each scattergram. The results showed that exposure to the first febrile episode may exert a protective effect against the febrile temperatures encountered subsequently. Growth-promoting effects of heat shock were also observed upon the exposure of parasites to 40 °C phases separated by an interval of 48 h to mimic conditions of tertian malaria (not shown).

Exposure to Prior Heat Shock Results in a Greater Productivity of the Infection Cycle—We examined whether the enhanced progression from ring to trophozoite seen in response to elevated temperature results in greater efficiency of the infection cycle. Ring stage parasites were subjected to a prior heat shock at 40 °C for 2 h (phase A) followed by incubation at 37 °C for 10 h (phase B). Parasites were then given a second heat shock at 40 °C for 24 h (phase C) and incubated at 37 °C for an
additional 12 h (phase D) to monitor new rings formed following an invasion of fresh erythrocytes (see bottom of Fig. 2A). Cultures kept at 37 °C throughout the intra-erythrocytic cycle were used as control. We found an almost 2-fold increase in the number of rings formed in cultures exposed to prior heat shock (Fig. 2A, top). The result indicated that exposure to heat shock brought about an overall increase in the productivity of the infection cycle. To rule out the possibility that this increase was due to a delay in stage progression of the control cultures, we examined the scattergrams of the control and PHS-treated cultures at the end of phase C. As shown in Fig. 2B, top, the control cultures had also progressed to trophozoites at the end of phase C.

GA Blocks Protection Due to Prior Heat Shock—To examine
the involvement of heat shock proteins in growth protection, we made use of GA, a specific inhibitor of Hsp90 function (10, 12). We included 10 µM GA in cultures during the shift from 37 °C (phase B) to 40 °C (phase C). Fig. 3A, right, shows a schematic representation of the experiment. There was significant conversion from ring to trophozoite in PHS cultures treated with the Me$_2$SO carrier at 6 h (Fig. 3A, left, top row), but cultures exposed to GA remained blocked in the ring stage until 6 h (Fig. 3A, left, second row from top). At 12 h, however, a significant population of parasites exposed to PHS had progressed to the trophozoite stage despite the presence of GA. This was in contrast to cells that did not experience a heat shock, remaining blocked as rings even at 12 h of development (Fig. 3A, left, bottom two rows) as reported previously (11). The result showed that exposure to PHS was able to overcome GA-mediated growth inhibition. This was also evident from an examination of the Giemsa-stained smears for these samples as shown in the inset of each scattergram (Fig. 3A, left). This result suggested that PfHsp90 function was important to trigger parasite transformation from ring to trophozoite in response to heat shock. The quantitation of parasite number by flow cytometry at 0 and 12 h showed that there was a drop in
parasite number at 12 h for GA-treated cultures but not for the control cultures (Fig. 3B). This finding was also confirmed by estimating parasitemia from the Giemsa-stained smears of these samples (not shown). Similar results were observed upon GA exposure of parasites to two heat shock episodes separated by an interval of 48 h to mimic conditions of tertian malaria (not shown). The stimulatory effect of heat shock and the inhibitory effect of PfHsp90-interference by GA together sug-
gested a central role for PfHsp90 in triggering stage development in the malarial parasite.

The binding of GA to mammalian Hsp90 has been studied extensively (10, 13). To confirm directly the interaction of GA with PfHsp90, parasite lysate was incubated with GA-coupled beads, and the bound proteins were analyzed for the presence of PfHsp90 by Western blotting. Control beads did not show any signal, whereas GA-coupled beads showed a clear band for PfHsp90 (Fig. 3C, top). This result supported our previous modeling studies showing the presence of a GA-binding pocket in PfHsp90 (11) and confirmed that GA was capable of binding to PfHsp90.

GA Disrupts PfHsp90 Complex—We have previously shown PfHsp90 to be associated with PfHsp70 in a ~300 kDa complex (11). To examine if, similar to mammalian Hsp90, PfHsp90 is also organized in a multi-chaperone complex, we analyzed its size under conditions that would preserve protein-protein interactions (see “Experimental Procedures”). Using mild lysis conditions followed by gel filtration chromatography, we found the PfHsp90 complex to be ~450 kDa in size (Fig. 4A). The size of the complex suggested that, in addition to dimeric PfHsp90 (~180 kDa) and PfHsp70 (~70 kDa), it contained other proteins. To examine the identities of the proteins associated with PfHsp90, we used an approach of co-immunoprecipitation. Trophozoites were metabolically labeled for 24 h, and the lysate was immunoprecipitated with antibodies to PfHsp90. Protein A beads alone were used as control. The immunoprecipitates were analyzed by two-dimensional gel electrophoresis and fluorography. As shown in Fig. 4B, in addition to PfHsp90, three other proteins could be co-precipitated using antibodies to PfHsp90. Among these proteins, spot number 2 (Fig. 4B) corresponded to PfHsp70 (11). To examine the identities of the proteins, we excised spot numbers 3 and 4 (Fig. 4B) from a two-dimensional gel electrophoresis that was run in parallel and subjected the protein spots to in-gel trypsin digestion and MALDI-TOF analysis using the Ettan Pro system (Amersham Biosciences). Peptide mass fingerprinting by MALDI-TOF showed that spot numbers 3 and 4 (Fig. 4B) correspond to Ser/Thr protein phosphatase 5 (PfPP5) (gene identifier 23619476) and α-tubulin (gene identifier 23510202), respectively (not

![Figure 4](http://www.jbc.org/)

**Fig. 4. GA disrupts PfHsp90 complex.** A, trophozoite lysate was fractionated on a Superdex 200 column, and the fractions were analyzed for the presence of PfHsp90 by Western blotting. B, a parasite lysate from metabolically labeled cells was immunoprecipitated using an α-PfHsp90 antibody and subjected to two-dimensional gel electrophoresis and fluorography. On the left is the total proteome and on the right is the immunoprecipitate. IEF, isoelectric focusing. C, the trophozoite lysate was passed through a Superdex 200 column at a flow rate of 0.5 ml/min. Fractions 20 and 21 were collected. Fractions from five such runs were pooled and concentrated. Following treatment with Me2SO (DMSO) or GA, the concentrate was rerun through the Superdex-200 column, and fractions 17–28 were analyzed for the presence of PfHsp90 by SDS-PAGE and immunoblotting.
shown). Previously, co-immunoprecipitation of PfHsp90 using antibodies to PfPP5 has been demonstrated (14). Both PfPP5 and tubulin have been shown to interact with Hsp90 in mammalian cells (15, 16). Whereas PfPP5 is known to be a bona fide co-chaperone component of the mammalian Hsp90 multi-chaperone complex, tubulin is its known client protein (17, 18). Overall, the results showed PfHsp90 to be organized in a multi-chaperone complex consisting of PfHsp70, PfPP5, tubulin, and an additional protein that needs to be identified. The complex was akin to that found in higher eukaryotes both in terms of its size as well as composition.

To examine the effect of GA on the PfHsp90 complex, parasite lysate was fractionated using a Superdex 200 gel filtration column. Fractions corresponding to PfHsp90 were pooled, concentrated, and divided into two aliquots (see “Experimental Procedures”). Following treatment with Me2SO or GA (100 μM), the concentrate was rerun through the Superdex 200 column, and the fractions were analyzed for the presence of PfHsp90 by immunoblotting. Although the Me2SO-treated sample showed PfHsp90 to be in a complex of ~450 kDa (Fig. 4C, top), the aliquots exposed to GA showed that ~50% of PfHsp90 appears in fractions corresponding to ~160 kDa in size (Fig. 4C, bottom). The result indicated that the binding of GA to PfHsp90 resulted in dissociation of the PfHsp90 complex. We also examined the effect of GA on the PfHsp90 complex in vivo using much lesser concentrations of GA (10 μM). By metabolic labeling and co-immunoprecipitation, we found that co-precipitation of PfHsp90 with antibodies to PfHsp70 was reduced in GA-treated cells (not shown). The results clearly indicated that GA treatment in cells resulted in destabilization of the PfHsp90-PfHsp70 complex.

DISCUSSION

The occurrence of chills followed by a febrile episode at 48–h intervals is a common manifestation of P. falciparum malaria. Body temperatures ranging from 38 to 41 °C are commonly encountered during febrile episodes in malaria patients (2). The influence of such repeated exposures to high temperatures on parasite growth has not been studied systematically. We have examined the influence of two repeated febrile episodes on parasite growth by exposing them to elevated temperatures twice with a recovery phase in between. Our rationale was to expose parasites to elevated temperatures under conditions mimicking subtertian as well as tertian malaria. Parasites were exposed to 40 °C twice with either an interval of 10 or 48 h, and parasite numbers as well as stages were monitored at 0, 6, and 12 h after the second heat shock.

Using a conventional method of counting parasites from Giemsa-stained smears as well as a flow cytometry-based approach, we found that exposure to a prior heat shock conferred significant protection against the second heat shock. Parasites not exposed to prior heat shock did not withstand elevated temperatures, as seen by a drop in overall parasite count. Our observation is in agreement with the inhibitory effect of heat shock on parasite growth reported previously (6). Importantly, we found that parasites exposed to a prior heat shock were not only cytoprotected but also developed efficiently to the trophozoite stage in response to the second heat shock. The promotion of ring to trophozoite transition in response to a prior heat shock resulted in an overall increase in the efficiency of the infection cycle. Parasites subjected to prior heat shock gave rise to a 4-fold greater number of newly invaded rings in the next infection cycle. The results clearly showed that a prior heat shock had a stimulatory effect on parasite development during the subsequent exposure to heat shock. These observations imply that the appearance of repeated febrile episodes in malaria patients can hasten intra-erythrocytic development of the parasite.

Heat shock is also known to regulate stage transformation in protozoan parasites like Leishmania and Trypanosoma (19, 20). In Leishmania, the temperature-dependent developmental transition from promastigote to amastigote forms is triggered through Hsp90 (19). Although there is awareness about the regulatory role played by temperature in Plasmodium (7), this is the first demonstration that increased temperature can promote intra-erythrocytic stage maturation in the malarial parasite.

We implicated PfHsp90 in heat shock-dependent cytoprotection using GA, a specific inhibitor of Hsp90 function (10, 12, 13). In Leishmania, the temperature-dependent developmental transition from promasitgote to amastigote forms is triggered through Hsp90 (19). Although there is awareness about the regulatory role played by temperature in Plasmodium (7), this is the first demonstration that increased temperature can promote intra-erythrocytic stage maturation in the malarial parasite.

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In eukaryotic cells, the Hsp90 multichaperone complex has evolved to respond to extracellular signals by effecting the ligand-mediated expression of specific genes (21–23) or modulating the activities of protein kinases involved in signal transduction pathways (24–26). The system has been optimized to respond to hormonal regulation (27) and cell-cell communication (28, 29) in multicellular organisms. It appears that in unicellular intracellular parasites like Plasmodium, the PfHsp90 complex is fine-tuned to sense and respond to the milieu within the host.

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