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Heat Shock Protein 90 Function Is Essential for *Plasmodium falciparum* Growth in Human Erythrocytes*

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Hsp90 is important for normal growth and development in eukaryotes. Together with Hsp70 and other accessory proteins, Hsp90 not only helps newly synthesized proteins to fold but also regulates activities of transcription factors and protein kinases. Although the gene coding for heat shock protein 90 from *Plasmodium falciparum* (PfHsp90) has been characterized previously, there is very little known regarding its function in the parasite. We have analyzed PfHsp90 complexes and addressed its role in parasite life cycle using Geldanamycin (GA), a drug known to interfere with Hsp90 function. Sedimentation analysis and size exclusion chromatography showed PfHsp90 to be in 11 $s_{20,w}$ complexes of ~300 kDa in size. Similar to the hetero-oligomeric complexes of Hsp90 in mammals, PfHsp70 was found to be present in PfHsp90 complexes. Homology modeling revealed a putative GA-binding pocket at the amino terminus of PfHsp90. The addition of GA inhibited parasite growth with LD_{50} of 0.2 μM. GA inhibited parasite growth by arresting transition from Ring to trophozoite. Transition from trophozoite to schizonts and reinvasion of new erythrocytes were less significantly affected. While inducing the synthesis of PfHsp70 and PfHsp90, GA did not significantly alter the pattern of newly synthesized proteins. Pre-exposure to heat shock attenuated GA-mediated growth inhibition, suggesting the involvement of heat shock proteins. Specificity of GA action on PfHsp90 was evident from selective inhibition of PfHsp90 phosphorylation in GA-treated cultures. In addition to suggesting an essential role for PfHsp90 during parasite growth, our results highlight PfHsp90 as a potential drug target to control malaria.

Plasmodium falciparum is responsible for the most severe form of malaria in humans, causing approximately 2 million deaths every year. During its asexual life cycle in human erythrocytes, the parasite progresses through three growth phases (1). The early form following invasion called the Ring stage is the phase of establishment in the erythrocyte. Trophozoite stage is the metabolically most active biosynthetic phase, whereas the schizont stage represents the phase of nuclear division before release of merozoites from the erythrocyte. Heat shock proteins of the class Hsp60, Hsp70, and Hsp90 are known to be expressed by the parasite during the intraerythrocytic stages in the vertebrate host (2–4). Although these heat shock proteins share significant homologies with their mammalian counterparts, there is very limited information available regarding their functional roles in parasite development. We have focused our study on the role of parasite-heat shock protein 90 expressed during erythrocytic cycle in humans.

Previous reports have shown that the gene coding for Hsp90 from *P. falciparum* is present on chromosome 7, has a single intron of 800 bp, and encodes protein with 745 amino acids, giving a molecular mass of 86 kDa (5, 6). Sequence comparison shows 59% identity and 69% similarity to human Hsp90. There is significant similarity at the NH_2-terminal nucleotide-binding domain in the central acidic hinge region as well as at the COOH-terminal substrate-binding domain. The presence of EEVD motif at the COOH terminus suggests cytosolic localization of heat shock protein 90 from *P. falciparum* (PfHsp90)[1] (7).

In higher eukaryotes, cytosolic Hsp90 is a highly abundant protein organized in the form of a multi-chaperone complex. Two obvious roles have been ascribed to Hsp90. 1) Together with Hsp70 and Hsp60, Hsp90 helps newly synthesized proteins to fold and 2) it helps modulate the activities of transcription factors (steroid hormone receptors and nuclear receptors) and protein kinases (8–10). The latter activity of Hsp90 puts it at the center stage of signal transduction events, crucial for cell survival and growth. Indeed, experiments performed in yeast, fruit fly, plant, and animal systems support the idea that in addition to helping newly synthesized proteins to fold, Hsp90 also regulates cell cycle and development (11–13).

In addition to the common approaches of gene disruption in yeast and mutational analysis in Drosophila, a new approach of pharmacologically interfering with Hsp90 function has been described in the literature (14–16). The approach involves the use of a benzoquinone ansamycin drug called Geldanamycin (GA), which specifically binds to Hsp90 and interferes with its ATP-dependent chaperone function (14). The specificity of GA interaction with Hsp90 has been described at the molecular level through the crystal structure of human Hsp90 complexed to GA (17). The use of GA in Drosophila and Arabidopsis has provided important insights into the profound involvement of Hsp90 in various cellular functions.

We have examined the complexes of PfHsp90 and analyzed its role in parasite growth in human erythrocytes using GA. Using an antibody specific to PfHsp90 and by employing sucrose gradient sedimentation as well as gel filtration chromatography, we have characterized the complexes of PfHsp90 in the parasite cytoplasm. In addition to demonstrating its pres-

[1] The abbreviations used are: PfHsp90; heat shock protein 90 from *P. falciparum*; GA, geldanamycin; HA, herbimycin A; DSP dithiobissuccinimidyl propionate; IP, immunoprecipitation; STAMP, Structure Alignment of Multiple Protein; HPLC, high pressure liquid chromatography.

ence in a complex similar in size to mammalian Hsp90, we also show PfHsp70 to be a part of the PfHsp90 multi-chaperone complex. Most importantly, we show that GA inhibits parasite growth in human erythrocytes. Modeling the NH_2-terminal domain of PfHsp90 based on its high degree of sequence identity to human Hsp90 uncovered a putative GA-binding pocket at its amino terminus. The addition of GA to parasite cultures inhibited parasite growth with an LD_{50} of 0.2 μM. The involvement of heat shock proteins in GA-mediated growth inhibition was evident from the observation that parasites pre-exposed to heat shock resisted growth inhibitory effects of GA. The specificity of GA action on PfHsp90 was evident from a selective inhibition of PfHsp90 phosphorylation in GA-treated cultures without a significant change in overall protein phosphorylation. Our results show that GA inhibits parasite growth through its interaction with PfHsp90 and suggests an essential role for PfHsp90 in parasite growth in human erythrocytes.

MATERIALS AND METHODS

Reagents and Antibodies—Antisera to PfHsp70 and PfHsp90 were generated against the recombinant COOH-terminal fragments of proteins in rabbit (18). DSP was obtained from Pierce. GA, herbimycin A (HA), and apyrase were purchased from Sigma.

Sedimentation on Sucrose Gradient and Gel Filtration Analysis—[^{35}S]Cys- and [^{35}S]Met-labeled trophozoites were cross-linked *in vivo* using DSP (9). Cells were hypotonically lysed (18), and the lysates were layered onto 5–25% continuous sucrose gradient. The gradient was spun at 40,000 rpm for 15 h at 4 °C in SW41 rotor. 500-μl fractions were immunoprecipitated with PfHsp90 antiserum and analyzed by SDS-PAGE and fluorography. For gel filtration analysis, saponin-released parasites (18) were sonicated briefly in phosphate-buffered saline containing protease inhibitors on ice. The lysate was clarified by centrifugation at 20,000 × g for 20 min at 4 °C and passed through Superdex 200 column (Amersham Biosciences) at a flow rate of 0.5 ml/min. Manually collected fractions (500 μl each) were trichloroacetic acid-precipitated and immunoblotted for PfHsp90. Thyroglobulin (669 kDa), alcohol dehydrogenase (150 kDa), and bovine serum albumin (66 kDa) were used as molecular size standards.

Metabolic Labeling, Radioiodination, and Immunoprecipitation—The peak fraction of PfHsp90 (corresponding to elution volume 10.5 ml) was collected, and protein complexes were cross-linked using DSP (see above). Iodination of cross-linked proteins was carried out using [^{125}I] by chloramine T method (19). The iodinated fraction was precleared using protein A-agarose beads and immunoprecipitated using anti-PfHsp90, anti-PfHsp70, or preimmune serum. The immune pellets were solubilized in Laemmli buffer and analyzed as above.

Parasites were metabolically labeled with [^{35}S]Cys and Met and lysed in 20 volumes of NETT buffer (150 mM sodium chloride, 1 mM EDTA, 10 mM Tris, and 1% Triton X-100) (18) containing 20 units/ml apyrase, protease inhibitors, and phosphatase inhibitors. This lysate was used for non-denaturing immunoprecipitation (IP). For denaturing IP, cells were directly lysed in Laemmli buffer, heated at 95 °C for 5 min, and diluted to 0.1% SDS concentration with NETT buffer. PfHsp70 or PfHsp90 immunoprecipitated from these lysates were solubilized in two-dimensional lysis buffer or Laemmli buffer for analysis. To examine induction of heat shock proteins following GA treatment, cells were treated with Me_2SO or 10 μM GA for 14h and metabolically labeled in the last 2 h. Cells were lysed in 2× Laemmli buffer, boiled at 95 °C for 5 min, and diluted with NETT buffer to 0.1% SDS concentration. Clarified lysate was used for immunoprecipitation of PfHsp70 and PfHsp90.

Early Ring-infected erythrocytes at 2% hematocrit and with 1–2% parasitemia were treated with Me_2SO (as a control) or various concentrations of GA (0.05, 0.5, 1, 2, and 5 μM) for 24 h (21). At the end of 24 h, [^{3}H]hypoxanthine at 10 $\mu Ci/ml$ was added and incubated for an additional 3 h. At the end of labeling, cells were lysed and DNA was collected in glass fiber filters using a cell harvester and the radioactivity incorporated was measured by liquid scintillation counting.

For phosphate labeling of proteins, parasites were cultured in phosphate-free medium supplemented with 0.5% human serum, [^{32}P]-orthophosphoric acid, and 1 mCi/ml medium at 37 °C for 6 h in the absence or presence of GA. Cells were lysed at the end of treatment in Laemmli buffer in presence of 2 mM NaF and 1 mM sodium orthovanadate and heated at 95 °C for 5 min. The lysate was treated with DNase and RNase in the presence of protease inhibitors. The lysate was diluted to 0.1% SDS concentration with NETT buffer, which was used for IP or measurement of cpm in a liquid scintillation counter.

Modeling the Amino Terminus of PfHsp90—Amino-terminal sequence of PfHsp90 (1–177 amino acids) was submitted to SWISS MODEL program (www.expasy.ch) to obtain the three-dimensional structure using the crystal structure of amino-terminal human Hsp90 (Protein Data Bank code 1YET) as a template. This model was superimposed over the structure of human Hsp90·GA complex using STAMP (Structure Alignment of Multiple Protein) program (20).

Effect of GA on Parasite Growth—Sorbitol-synchronized Ring-infected cells were suspended in RPMI 1640 medium to 5% hematocrit. 200-μl cell suspension was treated with Me_2SO or with GA in Me_2SO at various concentrations for 24 h. Smears were taken at the end of treatment, Giemsa-stained, and viewed under microscope. The same experiment was performed with early trophozoites and also schizonts. To examine the effect of GA and HA on parasitemia, Ring-infected erythrocytes were either treated with Me_2SO or with 5 μM drug for 48 h (culture was replenished with medium and GA every 24 h) and parasitemia was calculated. A change in percentage of parasitemia with respect to control was plotted against the duration of drug treatment. To analyze stage-specific expression of PfHsp90, synchronous cultures of infected erythrocytes were harvested at 10 h (Ring), 20 h (early trophozoite), 30 h (late trophozoite), and 40 h (schizont) and lysed in 10 volumes of NETT buffer. The protein content of the lysates was estimated by the Bradford method. Equal protein from the different stages was analyzed for the presence of PfHsp90 by Western blotting.

Prior Heat Shock and GA Treatment—An asynchronous culture of infected erythrocytes at ~20% parasitemia was given heat shock at 41 °C for 1 h in complete RPMI 1640 medium and then recovered at 37 °C for 2 h. As a control, an equal number of cells were kept at 37 °C for 3 h. Parasitemia was calculated at the end of recovery period in both control and heat-shocked cells following which the cells were treated with either Me_2SO alone or 1 μM GA for 24 h. Parasitemia was calculated again at the end of treatment period.

In Vitro Kinase Assay—Ring stage parasites were treated with Me_2SO or 0.5 μM GA for 6 h. Parasites were released using 0.01% saponin in phosphate-buffered saline, and hypotonic lysates were prepared from these parasites. 6 μg of lysate from GA-treated or untreated parasites was used in a kinase assay using 2 μCi of [γ-^{32}P]ATP with or without 5 μg of dephosphorylated bovine milk casein as substrate in 1× kinase buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 5 μM $ZnSO_4$) in the presence of 1 mM sodium orthovanadate and 1 mM NaF. The kinase assay was performed at 37 °C for 30 min and terminated by the addition of Laemmli buffer and boiling. Phosphorylation of casein was analyzed by 10% SDS-PAGE and fluorography.

Two-dimensional Gel Electrophoresis—Synchronous Ring-infected erythrocytes were treated with 10 μM GA for 16 h at 37 °C followed by the addition of [^{35}S]Cys and Met at 100 $\mu Ci/ml$ for 3 h. As a control, Ring stage parasites (normal Rings) were labeled with [^{35}S]Met at 100 $\mu Ci/ml$ for 3 h. The cells were washed twice in phosphate-buffered saline, and parasites were released by saponin lysis. Parasites were sonicated in 50 volumes of 20 mM Tris buffer containing 1% Triton X-100 supplemented with protease inhibitors. Lysates containing equal cpm were acetone-precipitated, solubilized in two-dimensional lysis buffer, and analyzed on a pH 3.5–9.5 tube gel in the first dimension and 7.5% SDS-PAGE in the second dimension followed by fluorography.

RESULTS

Complexes of PfHsp90—Mammalian Hsp90 is known to be organized in a 11 $s_{20,w}$ hetero-oligomeric complex consisting of Hsp70 and other accessory co-chaperones. To examine whether PfHsp90 is organized in a complex similar to that of mammalian Hsp90, we employed techniques of sucrose density gradient ultracentrifugation and size exclusion chromatography. Synchronous cultures of *P. falciparum* were metabolically labeled with radiolabeled amino acids for 24 h, and the lysates were subjected to centrifugation on sucrose gradients as described under "Materials and Methods." Fractions collected after run were immunoprecipitated with antibodies to PfHsp90 and analyzed by SDS-PAGE and fluorography. As shown in Fig. 1*A* (*top panel*), PfHsp90 was found in complexes ranging in size from 4.5 to 9 $s_{20,w}$, indicative of heterogeneous populations ranging from monomeric forms to complexes as high as 150 kDa in size. To examine whether our lysis protocol was dis-
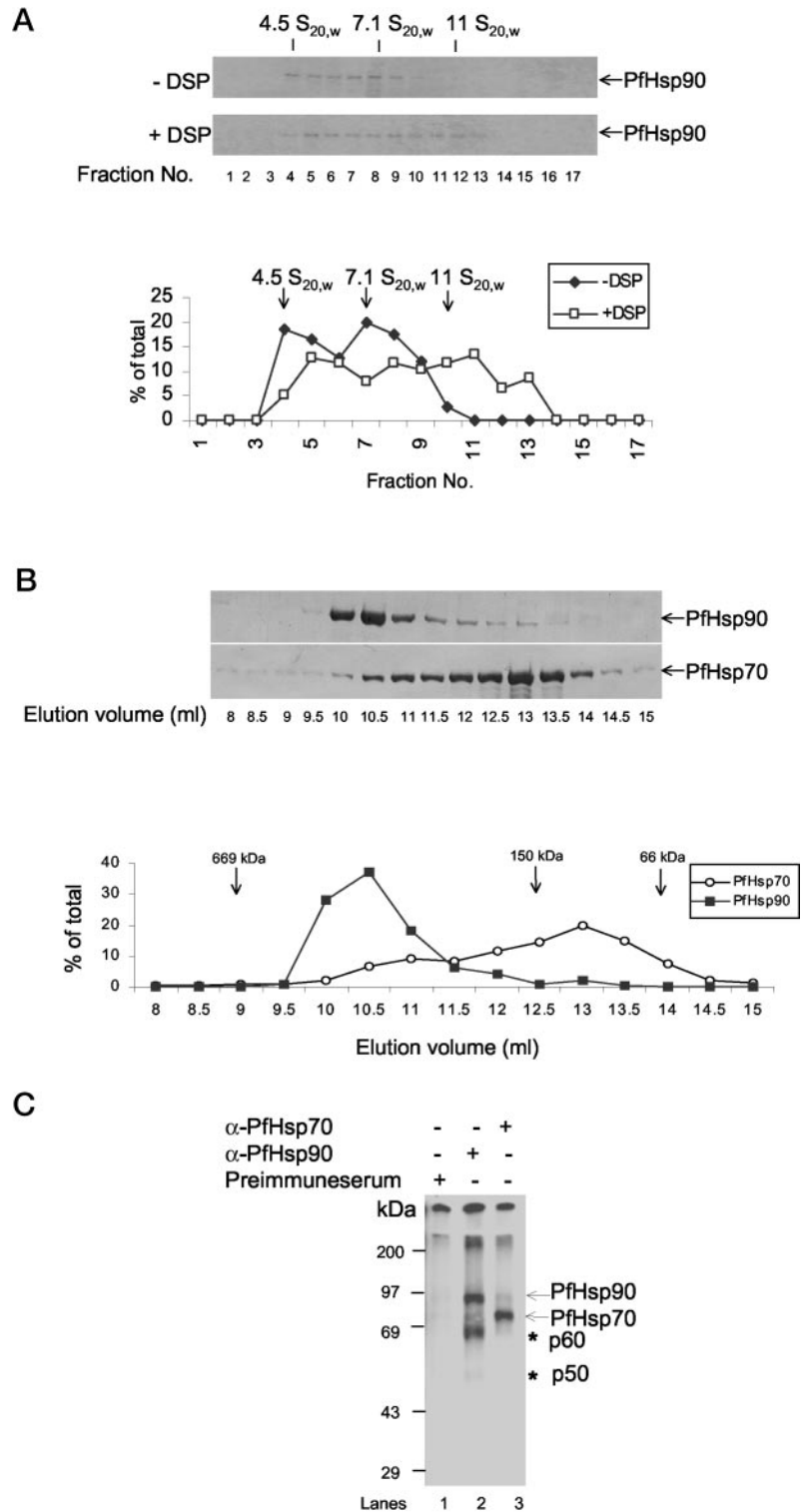
Geldanamycin Blocks P. falciparum Growth

A

B

C

FIG. 1. **Analysis of PfHsp90 complexes.** *Panel A*, sedimentation analysis. Two aliquots of *P. falciparum* infected erythrocytes were labeled with [^{35}S]Cys and [^{35}S]Met. One aliquot was lysed as such, and the other aliquot was lysed after cross-linking. Lysates were layered on sucrose gradients and centrifuged as described under "Materials and Methods." 500-μl fractions were collected from the top and immunoprecipitated using anti-PfHsp90 antibody. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. Bovine serum albumin (4.5 $s_{20,w}$), IgG (7.1 $s_{20,w}$), and catalase (11 $s_{20,w}$) were used as sedimentation standards. The *bottom panel* shows quantitation of these results. *Panel B*, gel filtration analysis. Lysates from saponin pellets were clarified and analyzed using Superdex 200 gel filtration column. 500-μl fractions were collected and immunoblotted for PfHsp90 and PfHsp70. Thyroglobulin (669 kDa), alcohol dehydrogenase (150 kDa), and bovine serum albumin (*BSA*) (66 kDa) were used as molecular size standards. *Bottom panel* shows quantitation of the profile. *Panel C*, radioiodination and immunoprecipitation analysis. HPLC fractions corresponding to 10–10.5-ml elution volume were pooled, cross-linked, and iodinated. Iodinated fractions were immunoprecipitated using preimmune serum (*lane 1*), anti-PfHsp90 (*lane 2*), or anti-PfHsp70 antisera (*lane 3*). Immunoprecipitates were analyzed by SDS-PAGE and fluorography.

rupting complexes of PfHsp90 in the cell, we used an approach of *in vivo* cross-linking to freeze complexes of PfHsp90 before preparing parasite lysates. We used DSP, which is a membrane-permeable, reversible cross-linker to cross-link PfHsp90 complexes. As seen in Fig. 1*A*, upon cross-linking (+DSP), PfHsp90 was found in complexes ranging from 5 $s_{20,w}$ to as high as 11 $s_{20,w}$, corresponding to approximately 300 kDa in size. Fig. 1*A*, *bottom panel*, shows quantitation of these results.

We also employed size exclusion chromatography using HPLC to analyze PfHsp90 complexes. Lysates were prepared from synchronous cultures of the parasite as described under

"Materials and Methods" and analyzed on a gel filtration column Superdex 200 interfaced to HPLC. Fractions were collected up to a one-bed volume of the column and analyzed for the presence of PfHsp90 and PfHsp70 by trichloroacetic acid precipitation of fractions and Western blotting using specific antibodies. As shown in Fig. 1*B* (*top panel*), PfHsp90 eluted as a single peak at a volume of 10–11 ml. A comparison with the elution volumes of standards indicated that the complex corresponded to ~300 kDa in size. This was in agreement with the 11 $s_{20,w}$ complexes observed on sucrose gradient analysis of cross-linked samples. On the other hand, PfHsp70 was found to

FIG. 2. **Co-immunoprecipitation of PfHsp70 with PfHsp90.** *Panel A, P. falciparum* infected erythrocytes in early trophozoite stage were metabolically labeled with [^{35}S]Cys and [^{35}S]Met for 2 h. At the end of labeling period, cells were lysed in 20 volumes of NETT buffer in the presence of apyrase (*apy*) or under denaturing condition using SDS (*den.*). Clarified lysates were immunoprecipitated using antibodies to PfHsp70 (*lanes 2* and *5*) or PfHsp90 (*lanes 3* and *6*) and analyzed by SDS-PAGE and fluorography. *Lanes 1* and *4*, protein A controls. *Panels B–D*, analysis of the immunoprecipitates by two-dimensional gel electrophoresis (*2D GE*). *Panel B*, PfHsp70 IP. *Panel C*, PfHsp90 IP. *Panel C*, PfHsp90 IP mixed with denaturing PfHsp70 IP. *Arrow*, PfHsp90 spot; *arrowhead*, PfHsp70 spot.
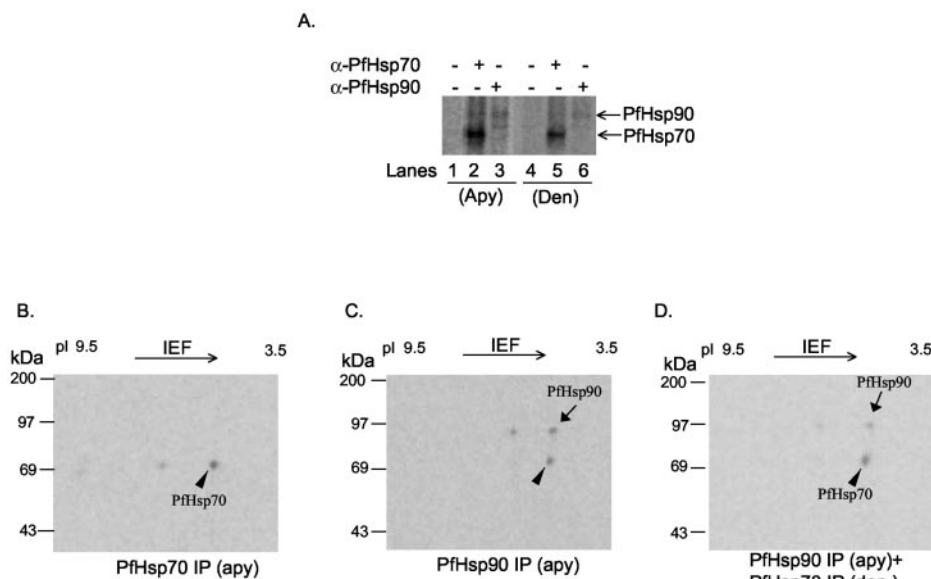
elute at a volume of 10.5–13.5 ml, ranging in size from monomeric forms to complexes as high as 300 kDa (*bottom panel*). Quantitation of this profile is presented in the *bottom panel* of Fig. 1*B*.

To analyze the composition of the 300-kDa complex seen on HPLC, we iodinated the peak fractions corresponding to elution volumes 10–10.5 ml after cross-linking and immunoprecipitated the labeled complex using antibodies to PfHsp90 and PfHsp70. An equal volume of the labeled fraction was also incubated with protein A beads and pre-immune serum to ascertain the specificity of immunoprecipitation. Fig. 1*C* shows an analysis of the immunoprecipitate by SDS-PAGE and fluorography. As expected, a clear band corresponding to PfHsp90 (*lane 2*) was visible on SDS-PAGE, which was absent in the control (*lane 1*). In addition to PfHsp90, three other bands corresponding in size to ~75-, 60-, and 50-kDa proteins were also seen in PfHsp90 immunoprecipitate. To examine whether the 75-kDa protein was PfHsp70, an equal volume of iodinated fraction was immunoprecipitated using antibodies to PfHsp70. As shown in Fig. 1*C* (*lane 3*) in addition to a signal corresponding to PfHsp70, we also found a signal for PfHsp90. The result indicated that PfHsp70 was included in the 300-kDa complex of PfHsp90 in the parasite cytoplasm.

Interaction of PfHsp90 and PfHsp70—To further examine possible interactions between PfHsp70 and PfHsp90, we used a more direct approach of co-immunoprecipitation from labeled parasite lysates. Synchronous cultures of *P. falciparum* were metabolically labeled for 2 h with radiolabeled amino acids and lysed with detergent containing buffer in the presence of apyrase as described under "Materials and Methods." Apyrase was included in the lysis buffer to stabilize possible association of PfHsp70 and PfHsp90 (9). The lysates were divided into three equal aliquots. One aliquot was immunoprecipitated with antibodies to PfHsp90. The second aliquot was immunoprecipitated with antibodies to PfHsp70; whereas the third aliquot was incubated with preimmune serum alone to serve as a control. The immunoprecipitates were analyzed by SDS-PAGE and fluorography as described under "Materials and Methods." As seen in Fig. 2*A*, in samples immunoprecipitated with antibodies to PfHsp70 (*lane 2*), we could detect a band corresponding to itself but, in addition, a band corresponding in size to PfHsp90 was also evident. Similarly, in sample immunoprecipitated with antibodies to PfHsp90, we could find a band corresponding to PfHsp70 in addition to itself (*lane 3*). Preim-

mune serum control (*lanes 1* and *4*) did not show any specific signal. Immunoprecipitation done under denaturing conditions for Pfhsp70 and PfHsp90 pulled down only the respective proteins (*lanes 5* and *6*).

To ascertain that the co-precipitating bands indeed correspond to PfHsp70 and PfHsp90, we also analyzed the immunoprecipitates by two-dimensional gel electrophoresis. We have previously defined the positions of PfHsp70 and PfHsp90 spots on two-dimensional gels (18). As shown in Fig. 2*B*, in PfHsp70 immunoprecipitates, we found a spot of PfHsp70 (*arrowhead*). In PfHsp90 immunoprecipitate (Fig. 2*C*) in addition to a spot corresponding to PfHsp90 (*arrow*) at a position expected from its size (90 kDa) and pI (4.8), we also found a distinct 75-kDa spot (*arrowhead*) corresponding in size (74.3 kDa) and pI (5.4) to PfHsp70. The preimmune serum control on the other hand did not show the presence of either of these spots on two-dimensional gels (data not shown). When a small aliquot of PfHsp90 IP (apyrase) was mixed with denaturing PfHsp70 IP (which pulls down only PfHsp70 as shown in Fig. 2*A*, *lane 5*), there was an increase in the intensity of the 75-kDa spot (compare PfHsp90:PfHsp70 ratios between *panels C* and *D*) showing that PfHsp70 is co-precipitated with PfHsp90 under non-denaturing conditions. The results confirmed that PfHsp90 and PfHsp70 were present in a common complex.

A Putative GA-binding Domain at the Amino Terminus of PfHsp90—GA, a benzoquinone ansamycin drug with antitumor properties, is known to specifically bind the amino-terminal domain of human Hsp90. The structure of the complex of human Hsp90 with GA has shown that the drug interacts with the amino-terminal ATP-binding domain of Hsp90 (17). To examine the presence of putative GA binding site in PfHsp90, we aligned sequences corresponding to the amino-terminal GA-binding domain of human Hsp90 with PfHsp90 (see Fig. 3*A*). The amino-terminal domain of PfHsp90 shows 69% identity with human Hsp90 and contains a GXXGXG motif essential for ATP binding. The contact-making residues, critical in the binding to GA, were conserved in PfHsp90 (indicated by *asterisk*). Based on the high degree of sequence similarity between human Hsp90 and PfHsp90 in this region, we modeled the structure of the PfHsp90. We used the structure of human Hsp90 in complex with GA as a template for this purpose (Protein Data Bank code 1YET). Modeling the amino-terminal domain of PfHsp90 (residues 3–177) indicated that the overall fold in this region was highly similar to that in human Hsp90 (Fig. 3*B*).
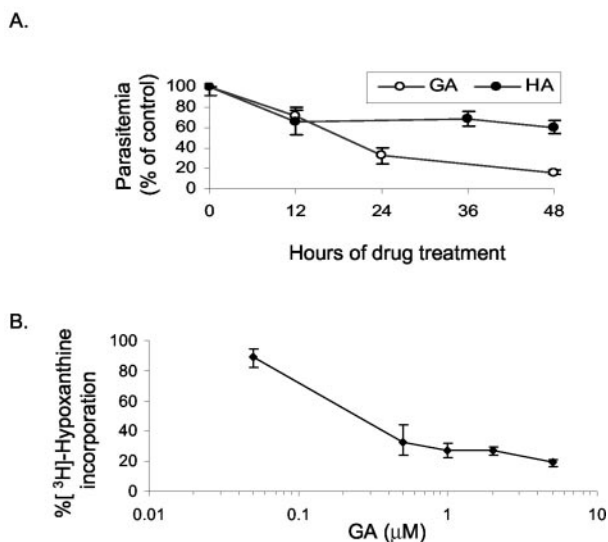
A.

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HumanHsp90 :   1   MPEETQTQDQPMEEEEVETFAFQAEIAQLMSLIINTFYSNKEIFLREL   48

PfHsp90    :   1   -------------------------MSTETFAFNADIRQLMSLIINTFYSNKEIFLREL   34

HumanHsp90 :  49   ISNSSDALDKIRYESLTDPSKLDSGKELHINLIPNKQDRTLTIVDTGIGM   98
                            *                                              *      *
PfHsp90    :  35   ISNASDALDKIRYESITDTQKLSAEPEFFIRIIPDKTNNTLTIEDSGIGM   84

HumanHsp90 :  99   TKADLINNLGTIAKSGTKAFMEALQAGADISMIGQFGVGFYSAYLVAQ   146
                           *                                    *
PfHsp90    :  85   TKNDLINNLGTIARSGTKAFMEAIQASGDISMIGQFGVGFYSAYLVAD   132

HumanHsp90 : 147   KVTVITKHNDDEQYAWESSAGGSFTVRTDT-GEPMGRGTKVILHL   190
                                                                *
PfHsp90    : 133   HVVVISKNNDDEQYVWESAAGGSFTVTKDETNEKLGRGTKIILHL   177
```

B.

C.

FIG. 3. **A putative GA-binding domain in PfHsp90.** *Panel A*, amino terminus of human Hsp90 and PfHsp90 are aligned. Identical residues are indicated in *black color*, whereas *blue-colored residues* correspond to similar amino acids and dissimilar residues are denoted by *red color*. Residues marked with an *asterisk* are involved in hydrogen bonding with GA. G*XX*G*X*G motif is indicated in *boldface*. *Panel B*, amino terminus domain structure of PfHsp90 (modeled using SWISS MODEL) is superimposed on that of human Hsp90 with GA (Protein Data Bank code 1YET) using STAMP program. *Blue line* corresponds to PfHsp90, and *yellow line* corresponds to human Hsp90. GA is shown in the center in *yellow color*. *Panel C*, a magnified view of the superposed model proximal to GA is shown. *Thick* and *thin lines* correspond to human Hsp90 and PfHsp90, respectively. Amino acid residues crucial for hydrogen bonding with GA are indicated.

The contact-making residues, namely Lys-58, Asp-93, Gly-97, Lys-112, Phe-138, and Gly-183, were similarly positioned in PfHsp90 (Fig. 3*C*). The structures could be superimposed with a root mean square deviation of 0.07. The analysis indicated that GA is likely to interact with PfHsp90 in a manner similar to its association with human Hsp90.

A.

B.

FIG. 4. **Effect of GA on parasite growth.** *Panel A*, synchronous culture in Ring stage was treated with 5 μM GA or 5 μM HA or Me$_2$SO (as a control) for 48 h, and percent parasitemia was calculated every 12 h. *Solid circles* represent percent parasitemia in HA-treated culture, whereas *open circles* represent that in the GA-treated culture. *Panel B*, *P. falciparum* Ring-infected erythrocytes were treated with various concentrations of GA (0.05, 0.5, 1, 2, and 5 μM) for 24 h and incubated with [^{3}H]hypoxanthine for 3 h. Samples were prepared as described under "Materials and Methods," and cpm incorporation was estimated. The experiment was done in triplicates, and the mean values of cpm incorporation against concentrations of GA were plotted to calculate the LD$_{50}$.

GA Inhibits Parasite Growth—To examine the effect of GA on parasitemia, synchronous Ring stage parasites were treated with 5 μM GA as described under "Materials and Methods" and percentage parasitemia was determined every 12 h until 48 h. We also examined the effect of a closely related benzoquinone ansamycin, HA, on parasite growth. The results were plotted as percentage parasitemia with respect to control *versus* hours of drug treatment. As shown in Fig. 4*A*, there was a progressive decline in the percentage parasitemia in cultures exposed to 5 μM GA. Although 5 μM HA treatment also resulted in reduction in parasitemia, the effect was less drastic. [^{3}H]Hypoxanthine incorporation in GA-treated cultures (Fig. 4*B*) confirmed growth inhibitory effects of GA, and the LD$_{50}$ value was determined to be 0.2 μM.
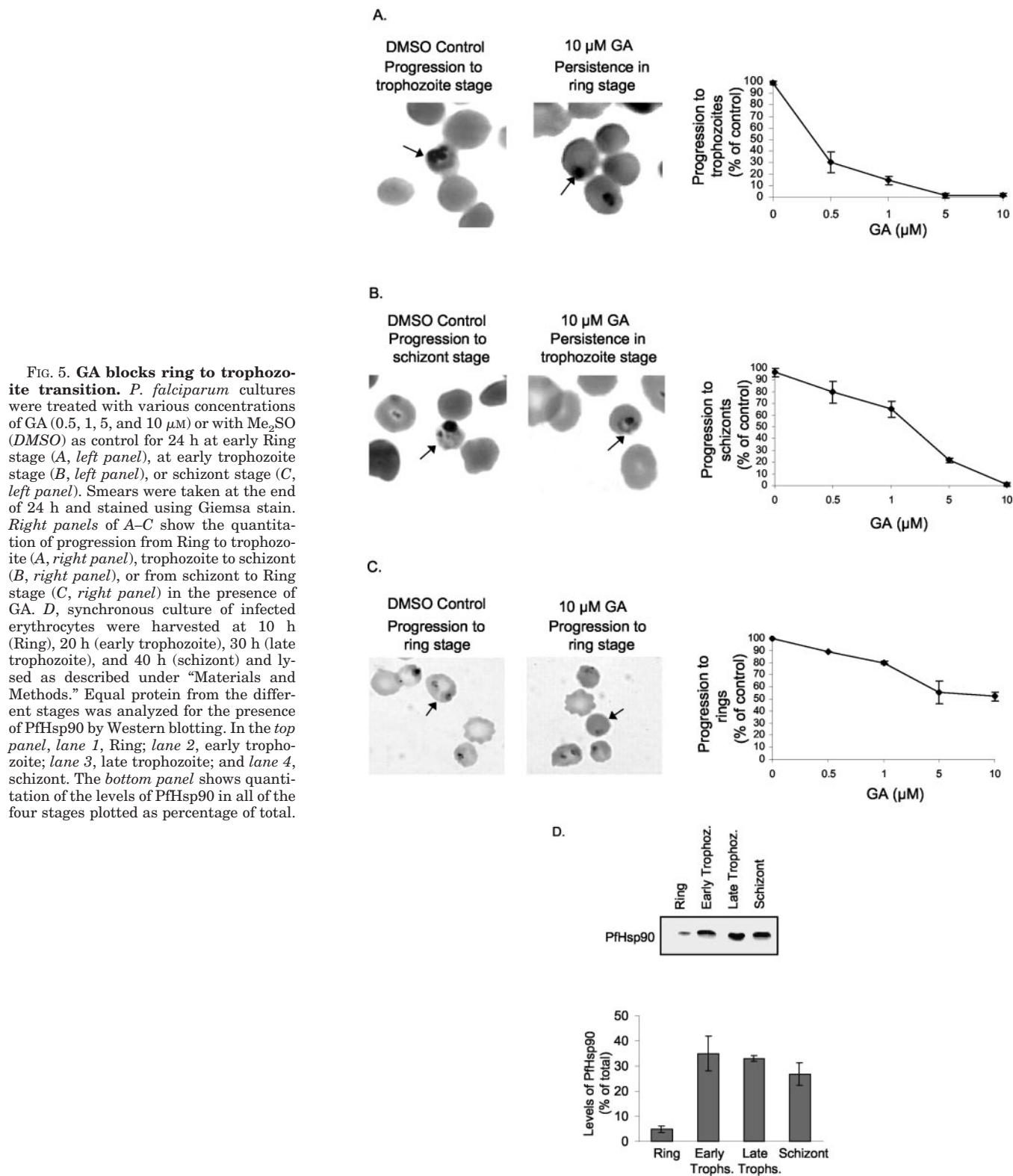
GA Blocks Ring to Trophozoite Stage Progression—To examine the effect of GA on growth of the parasite, we used highly synchronous cultures of *P. falciparum* in Ring stage and treated them with different concentrations of GA. At the end of 24 h of GA treatment, smears were taken to determine the stage of the parasite and percent parasitemia. The results were compared, in parallel, with mock-treated Ring stage culture. As expected, in the absence of GA treatment, Ring stage cultures progressed to the trophozoite stage (see Fig. 5*A*, *left panel*, *arrow*). Fig. 5*A*, *middle panel*, also shows a smear from culture treated with 10 μM GA. Most of the parasites persisted in the Ring stage (see *arrow*) even at the end of 24 h in the presence of the drug. In cultures treated with GA at a concentration of 0.5, 1, 5, or 10 μM for 24 h, we found a progressive decline in the number of parasites progressing to the trophozoite stage. *Right panel* shows the extent of progression from Ring to trophozoite stage at increasing concentrations of GA. A similar experiment was carried out with synchronous cultures in the trophozoite stage progressing to schizont stage in the presence of different concentrations of GA. As shown in Fig. 5*B*, in the absence of GA treatment (*left panel*), trophozoite stage culture effectively progressed to schizonts (*arrow*), but in the presence of GA (*middle panel*), there was persistence of trophozoites (*arrow*) even after 24 h. The degree of inhibition in stage progression increased with an increase in GA concentration. Fig. 5*B*, *right panel*, shows quantitation of the inhibitory effect of GA on progression from trophozoite to schizont stages. The inhibitory effect of GA was more pronounced for progression from Ring to trophozoite stage than from trophozoite to schizont stage. Importantly, GA did not significantly affect the release of merozoites from schizonts, and their reinvasion to gave rise to new Rings (5*C*). To examine the levels of PfHsp90 present in different stages of parasite growth, we prepared lysates from equal number of Rings, early trophozoites, late trophozoites, and schizonts and examined equal amounts of protein from different stages by SDS-PAGE and Western blotting. Fig. 5*D*, *top panel*, shows Western blot of PfHsp90 in the Ring (*lane 1*), early trophozoite (*lane 2*), late trophozoite (*lane 3*), and schizont (*lane 4*) stages, and the quantitation is presented in the *bottom panel*. The result indicated that PfHsp90 was present in maximal amounts during trophozoite stage.

GA Induces Heat Shock Protein Synthesis—To examine the effect of GA treatment on overall protein synthesis and heat shock protein induction, we used two aliquots of synchronous Ring stage culture. One was cultured for 14 h in the presence of GA and labeled for 2 h (see "Materials and Methods"). Giemsa staining of smears from the cultures confirmed that GA-treated aliquot was blocked in the Ring stage (as also described above). The second Ring stage culture was labeled for 2 h without any treatment and lysed as described under "Materials and Methods." Aliquots of lysates from both the samples were precipitated with acetone, and radioactivity incorporated was counted. As shown in Fig. 6*A*, the amount of radioactivity incorporated in GA-treated culture was not drastically different from that seen in untreated Ring stage culture (normal Rings). When lysates of normal Ring and GA-blocked Ring containing equal cpm were analyzed by two-dimensional gel electrophoresis, overall protein profile looked similar in both (Fig. 6*B*). The result suggested that the overall profile of proteins made in the presence of GA was qualitatively and quantitatively similar to that in controls.

To examine the effect of GA on the levels of PfHsp70 and PfHsp90, we immunoprecipitated these proteins from lysates containing equal cpm from GA-treated and untreated cultures using specific antibodies. As shown in Fig. 6*C*, *top panel*, higher amounts of PfHsp70 and PfHsp90 were seen in GA-treated parasites (*lanes 2* and *4*) compared with untreated cultures (*lanes 1* and *3*). *Bottom panel* shows quantitation of these data, indicating ~3-fold induction of PfHsp70 and 5-fold induction of PfHsp90 upon GA treatment.

Prior Heat Shock Counteracts GA-mediated Growth Inhibition—To analyze the involvement of heat shock proteins in GA-mediated parasite growth inhibition, we examined whether prior heat shock is able to attenuate GA-mediated growth arrest. An asynchronous culture of *P. falciparum* was divided into two aliquots. One aliquot was exposed to heat shock for 1 h at 41 °C, whereas the other aliquot was used as a control at 37 °C. The culture was allowed to recover for 2 h following heat shock, and percent parasitemia was determined in both of the aliquots. Following recovery, both of the aliquots were incubated with 1 μM GA or Me$_2$SO for 24 h. At the end of 24 h of GA treatment, parasitemia was again determined in both the cultures. Fig. 7 shows quantitation of the drop in parasitemia upon GA treatment in control culture and culture exposed to prior heat shock. Although there was >50% drop in parasitemia upon GA treatment in control cells, the decrease was significantly attenuated in culture pre-exposed to heat shock. The result suggests that pre-induction of heat shock proteins as

Fig. 5. **GA blocks ring to trophozoite transition.** *P. falciparum* cultures were treated with various concentrations of GA (0.5, 1, 5, and 10 μM) or with Me$_2$SO (*DMSO*) as control for 24 h at early Ring stage (*A*, *left panel*), at early trophozoite stage (*B*, *left panel*), or schizont stage (*C*, *left panel*). Smears were taken at the end of 24 h and stained using Giemsa stain. *Right panels* of *A–C* show the quantitation of progression from Ring to trophozoite (*A*, *right panel*), trophozoite to schizont (*B*, *right panel*), or from schizont to Ring stage (*C*, *right panel*) in the presence of GA. *D*, synchronous culture of infected erythrocytes were harvested at 10 h (Ring), 20 h (early trophozoite), 30 h (late trophozoite), and 40 h (schizont) and lysed as described under "Materials and Methods." Equal protein from the different stages was analyzed for the presence of PfHsp90 by Western blotting. In the *top panel*, *lane 1*, Ring; *lane 2*, early trophozoite; *lane 3*, late trophozoite; and *lane 4*, schizont. The *bottom panel* shows quantitation of the levels of PfHsp90 in all of the four stages plotted as percentage of total.

a result of prior heat shock may confer protection against GA-mediated growth inhibition.

GA Blocks PfHsp90 in a Dephosphorylated State—Chaperoning function of mammalian Hsp90 has been linked to its phosphorylation state in the cell, and GA has been shown to abrogate Hsp90 phosphorylation in mammalian cells (22–24). To examine whether phosphorylation state of PfHsp90 was affected in GA-treated parasites, we labeled Ring stage culture with [^{32}P]phosphoric acid with or without GA treatment (0.5

μM) as described under "Materials and Methods" and measured total cpm incorporated in the cell lysates. Lysates corresponding to equal cpm were immunoprecipitated with antibodies to PfHsp90 or PfHsp70, and the immunoprecipitates were analyzed by SDS-PAGE and fluorography. As shown in Fig. 8*A* (*top panel*), both PfHsp70 (*lane 3*) and PfHsp90 (*lane 5*) immunoprecipitates showed labeled bands in GA-untreated cultures, confirming phosphorylation of these proteins in the parasite. In parasite cultures labeled in the presence of 0.5 μM GA, while
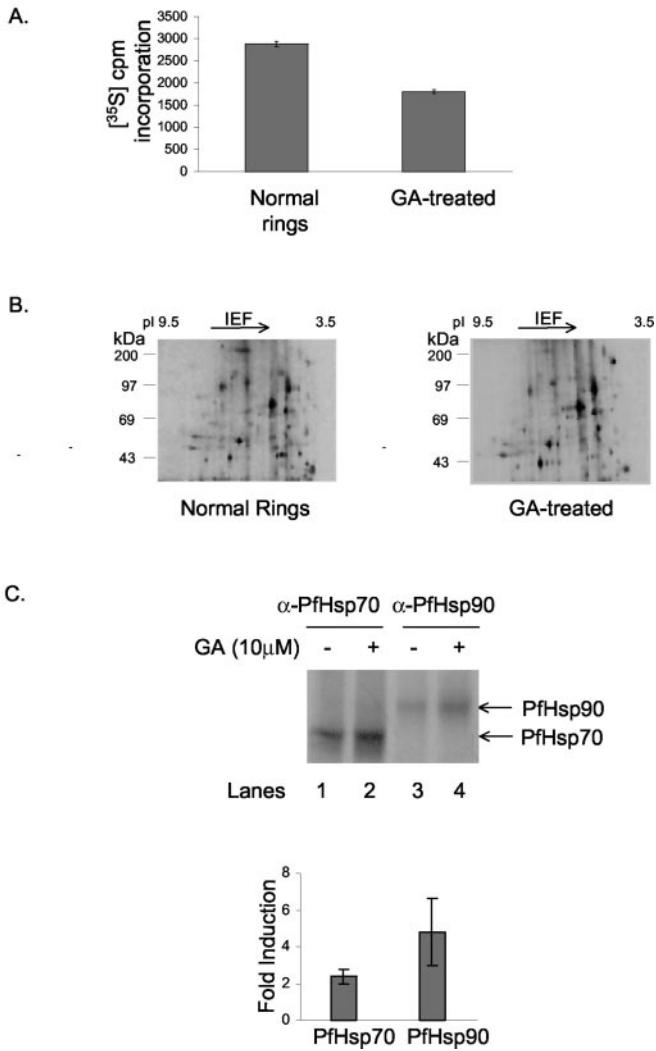
FIG. 7. **Prior heat shock counteracts GA-mediated growth arrest.** An asynchronous culture of infected erythrocytes was given heat shock at 41 °C for 1 h and recovered for 2 h. Control or heat shocked cells (*prior HS*) were treated with Me₂SO (*DMSO*) or 1 μM GA for 24 h. Parasitemia was calculated in both sets before and after GA treatment. Effect of GA on parasitemia is shown in the form of a *bar diagram*.

FIG. 6. **PfHsp70 and PfHsp90 are induced by GA treatment.** Ring stage parasites were labeled metabolically (*Normal rings*) or treated with 10 μM GA and labeled as described under "Materials and Methods." Normal and GA-treated parasites were lysed, and an aliquot of each was used to measure cpm incorporation. *A*, total cpm incorporated in normal Rings and GA-treated parasites. *B*, two-dimensional profiles of ³⁵S-labeled normal Rings and GA-treated parasites. *IEF*, isoelectric focusing. *C*, equal cpm from GA-treated (*lanes 2* and *4*) and untreated (*lanes 1* and *3*) parasite lysates were immunoprecipitated with antibodies to PfHsp70 and PfHsp90 and analyzed by SDS-PAGE and fluorography. In *top panel*, *lanes 1* and *2*, PfHsp70 immunoprecipitates; *lanes 3* and *4*, PfHsp90 immunoprecipitates. Quantitation of PfHsp70 and PfHsp90 signals is shown in *bottom panel*.

FIG. 8. **GA blocks PfHsp90 in a dephosphorylated state.** Trophozoites were labeled with [³²P]phosphoric acid in the presence of 0.5 μM GA or Me₂SO for 6 h. Cells were lysed at the end of treatment, and the lysates were used for immunoprecipitation. *Panel A*, immunoprecipitates of PfHsp70 and PfHsp90 from GA-treated and untreated lysate were analyzed by 7.5% SDS-PAGE and autofluorography. *Top panel*, *lanes 1* and *2*, immunoprecipitates using preimmune serum; *lanes 3* and *4*, PfHsp70 immunoprecipitates; and *lanes 5* and *6*, PfHsp90 immunoprecipitates. *Lanes 1*, *3*, and *5*, untreated lysates; *lanes 2*, *4*, and *6*, GA-treated lysates. Quantitation for PfHsp90 phosphorylation levels is shown in *bottom panel*. *Panel B*, lysates from GA-treated or untreated parasites were incubated with [γ-³²P]ATP and milk casein as described under "Materials and Methods." The lysates were analyzed by SDS-PAGE and fluorography to visualize the band corresponding to labeled casein.

the signal corresponding to PfHsp70 (*lane 4*) was similar to control that for PfHsp90 were significantly reduced (*lane 6*). Quantitation of bands indicated a 60% drop in signal for phosphorylated PfHsp90 in GA-treated cultures (Fig. 8*A*, *bottom panel*).

To rule out the possibility that GA was inhibiting overall kinase activity in the parasite, we also examined the ability of lysates from GA-treated parasites to phosphorylate exogenously added bovine milk casein. Ring stage parasites were treated with 0.5 μM GA or Me₂SO for 6 h. Hypotonic lysates prepared from saponin-released parasites were incubated with casein in the presence of [γ-³²P]ATP for 30 min (see "Materials and Methods"). Casein in the reaction mixture was then analyzed by SDS-PAGE and fluorography. As shown in Fig. 8*B*, casein was phosphorylated to a similar extent by lysates from both GA-treated and untreated parasites. These results indicated that GA did not affect total kinase activity in the infected
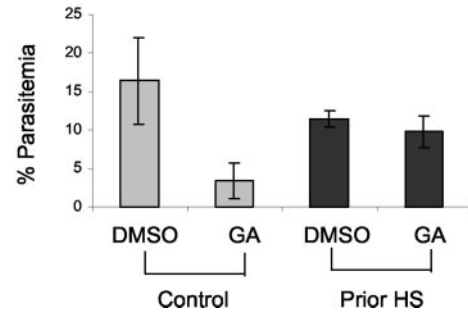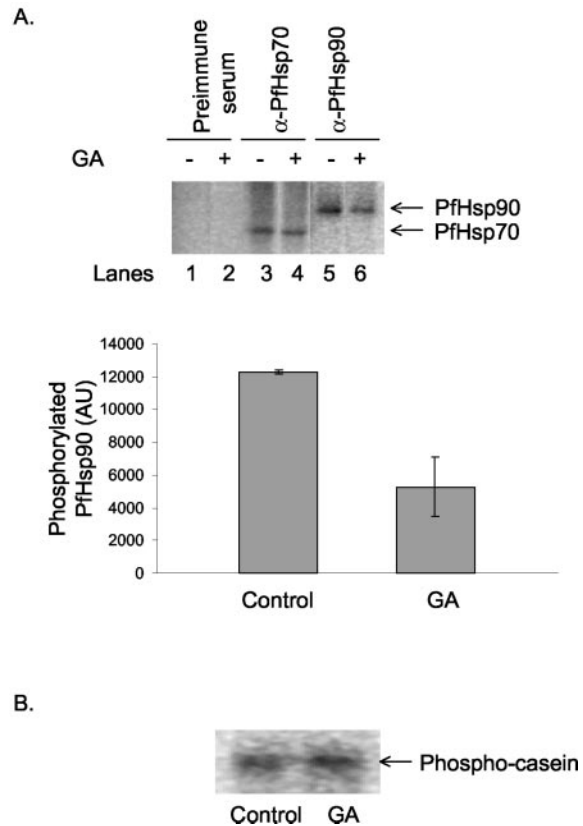
erythrocytes and that decrease in phosphorylation of PfHsp90 seen in *in vivo* phosphate-labeling experiment was a result of a specific effect of GA on PfHsp90.

DISCUSSION

Among the different heat shock proteins described in biological systems, heat shock protein 90 plays a particularly

important role in cell growth and development. In addition to participating in the folding and assembly events of newly synthesized proteins, Hsp90 also regulates signal transduction events by interacting with transcription factors and protein kinases. In mammalian cells, Hsp90 is one of the most abundant proteins in the cytoplasm, existing as a multichaperone complex associated with Hsp70, Hsp60, p23, and cyclophilin (25). The lethal phenotype of Hsp90 disruption in yeast underlines its pivotal role in cell function (26). Mutations in Hsp90 from *Drosophila melanogaster* are known to result in a variety of developmental abnormalities (12). Similarly, defects in Hsp90 function in *Arabidopsis thaliana* result in phenotypic changes (13). The results have been interpreted to suggest that normal functioning of Hsp90 is essential to buffer phenotypic variations.

Although the gene coding for Hsp90 in *P. falciparum* has been cloned (27), there is very limited information available regarding its function in the parasite. At the level of primary structure (as predicted from the nucleotide sequence), Hsp90 from *P. falciparum* is highly homologous to Hsp90 in mammalian cells. Previous studies indicate that PfHsp90 is predominantly cytoplasmic and is expressed in all three intraerythrocytic stages of human host (4). To begin to understand the biochemical role of PfHsp90, we have initiated studies on its size and composition of its complexes in the parasite cytoplasm. Furthermore, by using GA, a drug known to inhibit Hsp90 function (14, 15), we show that PfHsp90 is essential for parasite stage progression during intraerythrocytic growth.

Sedimentation analysis on sucrose gradients from *in vivo* cross-linked samples indicated that PfHsp90 was present in complexes ranging in size from 5 to 11 $s_{20,w}$ in the cell. The sedimentation coefficient of 5 $s_{20,w}$ corresponded to monomeric 90-kDa form of PfHsp90, whereas 11 $s_{20,w}$ corresponded to complexes of size 300 kDa. This was in agreement with the size of multi-chaperone complex reported for mammalian Hsp90 (28). Gel filtration analysis confirmed a size range up to ~300 kDa for PfHsp90. Similar to the Hsp90 complex reported in mammalian cells, we found PfHsp70 to be a part of the PfHsp90 complex. In addition, at least two other proteins of size 60 and 50 kDa could be seen in the PfHsp90 complexes. The identities of these proteins remain to be established. It is probable that these are parasite counterparts of Hop60 and cyclophilin known to be present in the mammalian Hsp90 multi-chaperone complex. Recently published genome sequences of the parasite indeed show the presence of Hop60 and cyclophilin homologs (29).

GA has been reported to interact with the nucleotide-binding domain of human Hsp90 (30). The crystal structure of the complex between amino-terminal domain of Hsp90 and GA has been reported previously (17). The high degree of sequence similarity between human Hsp90 and PfHsp90 allowed us to model the amino-terminal domain of PfHsp90 and compare its structure with that of human Hsp90. Superimposition of PfHsp90 amino-terminal domain with Hsp90 in complex with GA using STAMP program revealed that all of the points of contact could be juxtaposed. The model of PfHsp90 suggested that it possessed a putative GA-binding domain at the amino terminus.

The addition of GA to *P. falciparum* culture resulted in inhibition of its intraerythrocytic stage progression. Inhibition of parasite growth occurred in a narrow time window, affecting progression from Ring to trophozoite stage maximally. Progression from trophozoites to schizont stage release of merozoites from schizonts and reinvasion by newly released merozoites were affected less drastically by GA. Trophozoite being biosynthetically most active phase, the progression from Ring to

trophozoite, may depend heavily on PfHsp90 function, thereby showing an obvious sensitivity to GA. Indeed, Hsp90 synthesis was found to be maximal during Ring to trophozoite transition. Examination of cultures beyond one generation (>48h) revealed a significant decrease in the number of parasites in GA-treated cultures. This was probably a result of lysis of Ring stage-arrested parasites upon GA treatment.

Attenuation of GA-mediated growth arrest in parasites pre-exposed to heat shock provided direct evidence for the involvement of heat shock proteins in GA-mediated growth arrest. Such "cross-tolerance" conferred by pre-exposure to heat stress has been well documented in the literature (31). That GA-mediated arrest in parasite growth was a result of its effect on PfHsp90 function was evident from a specific drop in the levels of phosphorylated PfHsp90 without any decrease in overall protein phosphorylation in GA-treated parasites. A similar GA-mediated drop in Hsp90 phosphorylation has been reported in mammalian cells. Such a decrease in mammalian Hsp90 phosphorylation has been shown to be linked to inhibition of its chaperoning function (22).

GA has been shown to affect the growth of another protozoan parasite belonging to the genus *Leishmania* (32). GA arrests the growth of the promastigote stage (insect stage) of *Leishmania donovani* in culture. GA is also thought to mimick heat stress experienced by the parasite during transmission from the insect to the vertebrate host and induce its progression to amastigote stage, which is normally found only in the vertebrate host. Overexpression of cytosolic Hsp90 has been shown to overcome such growth inhibitory effect of GA in *Leishmania*. GA has also been reported to inhibit tumor growth in animal cells by interfering with tumor-inducing factors like mutated p53, Raf kinase *src* kinase, and steroid receptors, which are known substrates of cytosolic Hsp90 (14, 33, 34). GA is thought to bring about tumor inhibitory effects by abrogating interactions of the above tumor-promoting factors with Hsp90. The anti-tumor potential of GA is currently being evaluated under clinical trials in humans (33).

In all, our study shows PfHsp90 to be present in hetero-oligomeric complexes essential for parasite growth. Specific mechanisms of parasite growth inhibition by GA need to be addressed in future. Identification of PfHsp90 client proteins, *i.e.* nuclear receptors and protein kinase orthologs in the parasite, becomes an important priority for malaria biologists. The study of GA effects on gene expression profiles during stage progression will help uncover detailed mechanisms of parasite stage progression and may lead to identification of novel drug targets against malaria.

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