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Differentiation of *Leishmania donovani* in host-free system: analysis of signal perception and response

Efrat Barak[a,1], Sigal Amin-Spector[a], Elena Gerliak[a], Sophie Goyard[b], Neta Holland[a], Dan Zilberstein[a,*]

[a] *Department of Biology, Technion – Israel Institute of Technology, Haifa 32000, Israel*
[b] *Unit' Postulante Biologie et Pathog'nicit' fongiques, Institute Pasteur, 28 rue du docteur Roux 75724, Paris Cedex 15, France*

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Abstract

Leishmania donovani are the causative agents of kala-azar in humans. They undergo a developmental program following changes in the environment, resulting in the reversible transformation between the extracellular promastigote form in the sand fly vector and the obligatory intracellular amastigote form in phagolysosomes of macrophages. A host-free differentiation system for *L. donovani* was used to investigate the initial process of promastigote to amastigote differentiation. Within an hour after exposing promastigotes to differentiation signal (concomitant exposure to 37 °C and pH 5.5), they expressed the amastigote-specific A2 protein family. At 5 h they started to transform to amastigote-shaped cells, a process that was completed 7 h later. This morphological transformation occurred synchronously, while cells arrested at G1. By sequential exposure to elevated temperature (for 24 h) and then acidic pH, we found that heat was responsible for the growth arrest and acidic pH to its release and subsequent route to differentiation into amastigotes. Lastly, ethanol and Azetidine 2 carboxylic acid (a synthetic proline analog) that induced heat shock response in promastigotes were capable of replacing heat in the differentiation signal.

Keywords: *Leishmania donovani*; Differentiation; Cell cycle; Heat shock response; Signaling

1. Introduction

During their lifetime, parasitic protozoa cycle between vectors (that transmit the parasites) and hosts (to whom they are pathogenic), and as a result encounter extreme environmental changes. Parasitic protozoa respond to these changes by differentiating to highly adapted forms for each of these new environments, which enables them to invade and proliferate inside their hosts. Our knowledge of the molecular mechanisms that regulate these processes is limited. We aim to reveal these mechanisms using *Leishmania donovani* as a model parasite.

L. donovani, the causative agents of visceral leishmaniasis, are intracellular parasitic protozoa that cycle between phagolysosomes of mammalian macrophages and alimentary tract of sand flies [1,2]. In the vector, they grow as extracellular flagellated promastigotes, in the host they proliferate as intracellular aflagellated amastigotes. Normally, in the insect mid-gut, the actively dividing, immature, procyclic promastigotes differentiate into non-dividing metacyclic forms, which migrate to the thoracic mid-gut and proboscis. These latter forms have been shown to be the infective stage of the parasite [3]. They are introduced into the host during a vector's blood meal and are subsequently phagocytosed by macrophages, where they differentiate to amastigotes [2].

Studies of the last decade indicated that shifting promastigotes to an intralysosomal-like environment (e.g. 37 °C and pH 5.5 in a 5–7% CO_2 environment for visceral species) induced differentiation into amastigotes in host-free culture [4–8]. Such an experimental system has been developed in

* Corresponding author. Tel.: +972 4 8293647; fax: +972 4 8225153.
E-mail address: danz@tx.technion.ac.il (D. Zilberstein).
[1] Present address: Center for Biologics Evaluation and Research, FDA Division of Cellular and Gene Therapies, Bethesda, MD, USA.

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our laboratory and has already been used to investigate various stage-regulated functions in *L. donovani* [9–14]. These studies revealed that concomitant exposure to acidic pH and high temperature signal for differentiation. Regardless of the method used for differentiation, all axenic amastigotes express the known stage-specific proteins including A2, amastin, specific proteases, nucleotidases and phosphatases, and down-regulate the expression of surface coat lipophosphoglycan. Axenic amastigotes are virulent as they infect hamsters and macrophage cell lines. Moreover, differentiation in host-free culture resumes virulence of long-term attenuated promastigotes [4,6]. Hence, it is established that these cultures are excellent representatives of animal-derived amastigotes.

Whereas we know what trigger promastigotes to differentiate, there is no data available on the course of events that occur between the signal and completion of differentiation. The objective of this work was to determine these activities. We found that the differentiation signal induced cell cycle arrest at G1 and that morphological transformation into amastigotes initiated during this time. The role of each parameter of the differentiation signal was assessed. Heat induced growth arrest in promastigotes, acidic pH released the arrest and route the heat adapted promastigote to differentiate into amastigotes. Furthermore, modulators of heat shock can replace heat in the differentiation signal.

2. Materials and methods

2.1. Materials

[^{3}H]thymidine was obtained from New England Nuclear; propidium iodide and hydroxyurea from Sigma; medium 199 and fetal calf serum from Biological Industries, Inc.; flavopiridol was donated by Dr. Jeremy C. Mottram from Glasgow University. All other reagents were of analytical grade.

2.2. Cell culture

A cloned line of *L. donovani* 1SR was used in all experiments [6]. This cell line was maintained as a clone by inoculating single colonies of promastigotes from medium 199 agar plates. Promastigotes were grown in medium 199 and supplemented with 10% fetal calf serum at 26 °C.

Promastigote to amastigote differentiation in a host-free culture and the maintenance of axenic amastigotes were performed either as described by Saar et al. [6], or by inoculating late log phase promastigotes in medium 199 at pH 5.5 containing 25% fetal calf serum. The axenic amastigotes remained stable in culture for a long time. They were recycled routinely every 10 weeks by differentiating them back to promastigotes, in parallel initiating a new line of amastigotes.

2.3. Cloning the green fluorescence protein gene (GFP) into promastigotes chromosome

A 0.75 Kbp of *GFP* coding region was cut from pEGFP (NcoI/Not1, Clontech Inc) and subsequently cloned into pF4X1.HYG (Jena Bioscience, Germany; pF4X.HYG-GFP, S. Goyard, unpublished). In this construct 18s rRNA flanked the HYG-GFP genes. Following linearization with SwaI, HYG.GFP was integrated into 18s rRNA locus in *L. donovani* promastigotes chromosome (Ld1S-GFP).

2.4. Flow cytometry

Flow cytometry of promastigotes, amastigotes and differentiating promastigotes was performed as follows: For each assay, 5 ml of cell culture (1×10^6–1×10^7 cells/ml) was aliquoted, washed twice with a phosphate buffered saline and then suspended in 90% ice-cold methanol for fixation. These cells were kept at −20 °C for further use. Prior to analysis, the cells were treated with 20 mg/ml RNase for one hour at 37 °C. Subsequently, DNA was stained using propidium iodide and analyzed for DNA content using FACSCalibur (Becto Dickinson, San Jose, CA.). In each assay, 20,000 cells were counted. The distribution of G1, S and G2/M phases in each experiment was calculated from each histogram using the Phoenix algorithm (Phoenix Flow Systems Inc., San Diego, CA).

2.5. Thymidine incorporation

Thymidine incorporation into cell nuclei was determined according to Noll et al. method [15] as follows:

To 1 ml of cells (0.5–1×10^7 cell/ml), 1–2 μCi/ml of [^{3}H] thymidine was added in 24-well ELISA plates. After 0.5 h of incubation, the cells were washed in 10 ml cold phosphate buffered saline (PBS), then suspended in 2 ml lysis buffer (140 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl pH 8.6 and 0.5% NP-40) and supplemented with a 4 mM Vanadyl ribonucleoside complex. The suspension was vortexed for 10 s and then centrifuged for three minutes ($6000 \times g$ at 4 °C). The nuclei-containing pellet was suspended in 10 ml ice-cold 10% trichloroacetic acid (TCA), kept at 4 °C for at least 20 min and subsequently filtered through GF/C filters (Whatman International Ltd.). The filters were washed with 10 ml 10% TCA and then with 10 ml 95% ethanol. The filters were then subjected to scintillation counting.

2.6. Western blot analyses

Cellular proteins from the indicated time points were separated on 12% SDS-PAGE and subsequently transferred to a nitrocellulose membrane [16] reacted with anti-A2 monoclonal antibody [17], and developed using Super-Signal® kit for peroxidase-conjugated antibody detection (Pierce Inc., Rockford).

2.7. Northern blot analyses

An 2.4 Kb EcoRI-XhoI fragment from A2XE31 pBLSC (Kindly provided by Prof. G. Matlashewski) [18] was used as a probe for Northern hybridization. The probe was random prime radiolabeled with ^{32}P-dCTP using the Prime-It II kit (Stratagene). Hybridization was performed in 7% SDS, 0.125 M phosphate buffer at 65 °C. Washes were carried out 65 °C in 5% SDS in 40 mM phosphate solution.

3. Results

3.1. Time course of L. donovani differentiation

As previously described, *L. donovani* promastigote to amastigote differentiation in the host-free culture initiates only when parasites are exposed to both acidic pH and elevated temperature [6]. In the following experiments differentiation was initiated by concomitant exposure of promastigotes to 37 °C and pH 5.5 in a 5% CO_2 environement (differentiation signal), and changes in morphology and expression of specific genes as a function of time was determined. In order to follow cell viability during differentiation we used parasites that constitutively express the green fluorescence protein gene *(Ld1S-GFP)*. As shown in Fig. 1A1, promastigotes of Ld1S-GFP are completely green as *GFP* is expressed in the cell body, the flagella and the nucleus. These cells grow in culture equally well as wild type promastigotes. As shown in Fig. 1B, promastigotes of Ld1S-GFP efficiently infect and proliferate inside the THP-1 macrophage cell line indicating that they retain wild type virulence [19]. Control experiments indicated that *GFP* expression in *L. donovani* did not affect the physiological behavior of promastigotes and amastigotes (e.g. transport of sugar and amino acids). Hence, *Ld1S-GFP* is suitable for the study of differentiation.

Fig. 1A illustrates change in morphology of the *Ld1S-GFP* promastigotes as a function of time after the differentiation signal. As shown, no change in morphology was observed during the first 4 h (Fig. 1A1, 2). Between five and 7 h cells aggregate and start to round (Fig. 1A3, 4). At 9 h > 70% of the parasites rounded and started to lose their flagella (Fig. 1A5), a process that completed at 12 h (Fig. 1A6). This population of axenic amastigotes developed large aggregates of green cells, hardly any single cell was found in these cultures (Fig. 1C). Transferring these axenic amastigotes to pH 7 and 26 °C induced differentiation back to promastigotes, a process that completed within 24–48 h (data not shown).

Fig. 2 illustrates the expression of the amastigote-specific A2 gene family [17] during differentiation. As early as one hour after the signal promastigotes synthesized the low molecular weight A2 proteins (lane 2) and from 5 h on, the time they started to change to amastigote-shaped cells, parasites expressed all A2 proteins at a level similar to that of amastigotes (lanes 3–6). The cellular level of A2 mRNA abundance in these cells correlated with A2 protein expression (Fig. 2B). The results further indicate that the A2 gene family can be used as a molecular marker for *L. donovani* differentiation.

Note, that our Ld1S clone expressed two inducible A2 transcripts of 3.5 and 2.8 Kbp, unlike a single 3.5 Kbp band observed in previous studies [18]. Expression of two transcripts has recently been observed by others as well (Matlashewski, G., personal communication). This could be the reason for the relatively higher abundance of the low molecular weight proteins (Fig. 2A), which was not observed with a single transcript expression [17].

3.2. Promastigote to amastigote differentiation initiates at G1

The results in Fig. 1 suggest that promastigotes transform to amastigotes almost synchronously. We assessed this phenomenon by subjecting differentiating promastigotes to time course flow cytometry. Fig. 3A illustrates histograms of propidium iodide-stained log phase axenic *L. donovani* promastigotes and amastigotes (AI and AII, respectively). As shown, 53 ± 6% of the growing amastigote population is in G1, 17 ± 4% in S, and 30 ± 2% in the G2/M phase (Fig. 3AII). A similar distribution was observed in the axenic promastigote population, i.e. 57 ± 1%, 18 ± 5% and 25 ± 4% in G1, S and G2/M, respectively (Fig. 3AI). The resemblance of the DNA distribution pattern indicates that both axenic amastigotes and promastigotes are stably growing populations.

As shown in Fig. 3B, the log phase promastigotes population ($t = 0$) reacted to the differentiation signal by synchronizing at G1 within 3 h. At 6 h after the signal, they synchronously returned to the cell cycle, as indicated by the reappearance of S phase cells ($t = 4$–9). Cell counting (Fig. 3C) indicated that during the first 5 h after the signal this population ceased their growth. They doubled in number between 6 and 8 h after the signal and then returned to normal growth. This indicates that following the differentiation signal parasites remained viable and their growth rate was in agreement with the results of the flow cytometry assays.

In order to ascertain the role of G1 as a differentiation initiation point, we used synchronized populations. *L. donovani* promastigotes were synchronized at G1 and G2 at 26 °C using hydroxyurea and flavopiridol, respectively (Fig. 4A). Hydroxyurea blocks the cell cycle at the G1/S boundary [20,21]. Incubating log phase promastigotes with 4 mM hydroxyurea for 12 h at 26 °C and pH 7 synchronized 76% of the cells at G1 (Fig. 4AII). These cells remained single, promastigote-shaped and were fully viable. Flavopiridol is an ATP analog that inhibits CDK28 and CDC2 in mammalian and yeast cells, respectively [22–24]. It synchronizes *L. mexicana* promastigotes at G2 [25]. As shown in Fig. 4AIII, treating log phase *L. donovani* promastigotes with 2.5 μM flavopiridol at 26 °C for 12 h synchronized 98% of them at G2.

Within an hour after shifting the G1-synchronized promastigotes to differentiation conditions in a hydroxyurea-free medium, they started to express the A2 protein family (Fig. 4BII, G1). Differentiation to amastigote-shaped cells in this population occurred synchronously at 5 h after the signal (Fig. 4BI, G1), the same time it took untreated promastigotes to initiate differentiation (Fig. 1A). Control flow cytometry assays indicated that when the G1-synchronized promastigotes that were treated with hydroxyurea at 26 °C were shifted to differentiation conditions in a hydroxyurea-free medium, immediately and synchronously resumed cell cycle progression (Fig. 5AI). G1-synchronized promastigotes started to grow as soon as they reached G2/M phase, e.g. 4 h after the signal (Fig. 5A and B). Hydroxyurea-treated promastigotes that were transferred to either pH 7 at 37 °C or pH 5.5 at 26 °C showed a similar cell cycle time course but did not differentiate to amastigotes.

When G2-synchronized promastigotes were exposed to the differentiation signal (pH 5.5, 37 °C) in a flavopiridol-free medium, they immediately and synchronously resumed cell cycle progression (Fig. 5AII) and growth (Fig. 5B). It took G2 promastigotes 6 h to reach S phase, the stage at which G1 cells started to express A2. In correlation with this, G2-synchronized promastigotes started to express A2 proteins

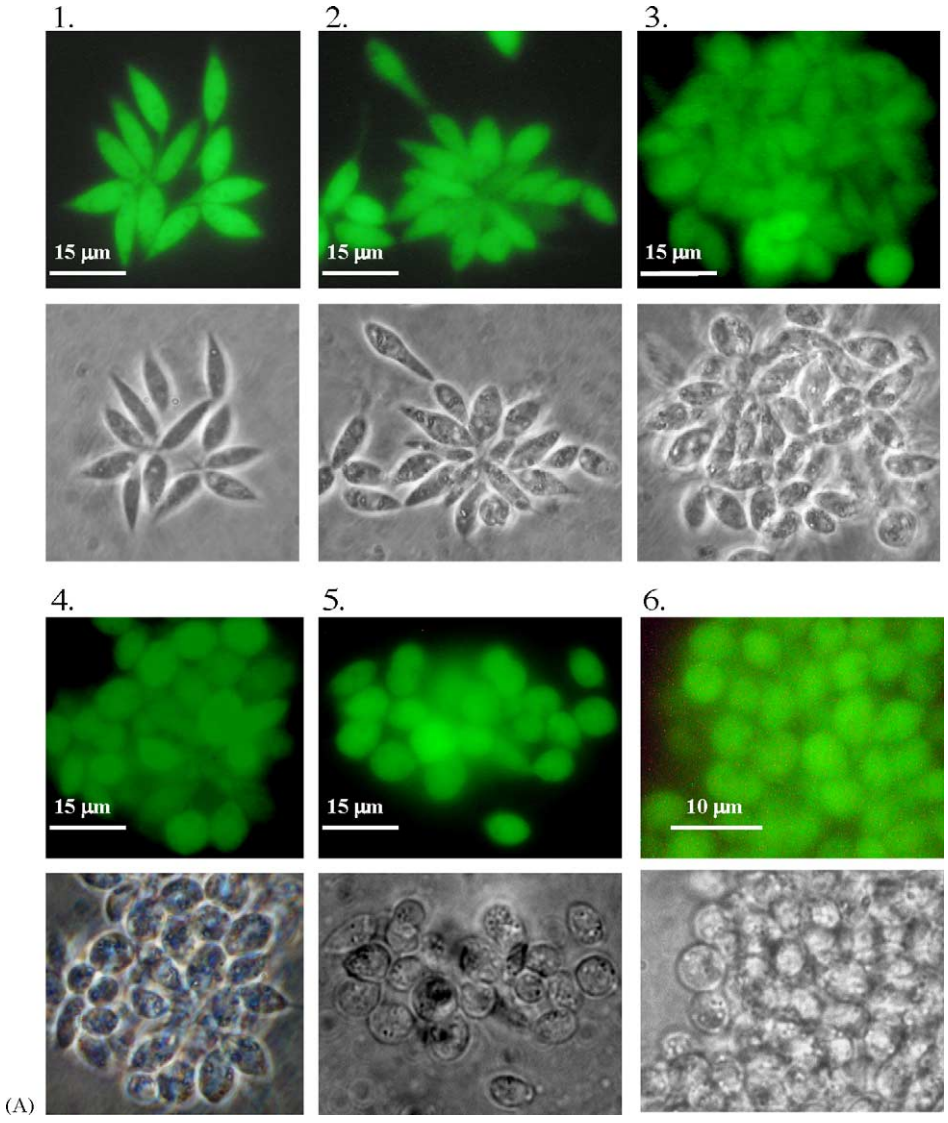

Fig. 1. Differentiation of *L. donovani* promastigote into amastigote. (A) Time course of differentiation in axenic culture. Log-phase GFP-expressing promastigotes (Ld1S-GFP; 5×10^6 cells/ml) were shifted to amastigotes medium at the same density (Materials and methods section) and differentiation initiated by incubating these cells at 37 °C, pH 5.5 in a 5% CO_2 environment. At different time points, a few microliter aliquots were taken for microscopic analysis, both fluorescence (upper panels) and phase contrast (lower panels). (1) promastigotes, (2) 4 h after differentiation initiated, (3) 5 h, (4) 7 h, (5) 9 h and (6) 12 h. (B) Growth of Ld1S-GFP amastigotes inside THP-1 macrophages cell line. Infection was carried out as described in Materials and methods section. (1) Fluorescence image of amastigotes inside THP1 macrophage and (2) phase image of the same infected macrophage. (C) A cluster of amastigotes. Arrows indicate dead cells that are seen in the phase contrast (lower panel) but do not fluoresce (upper panel).

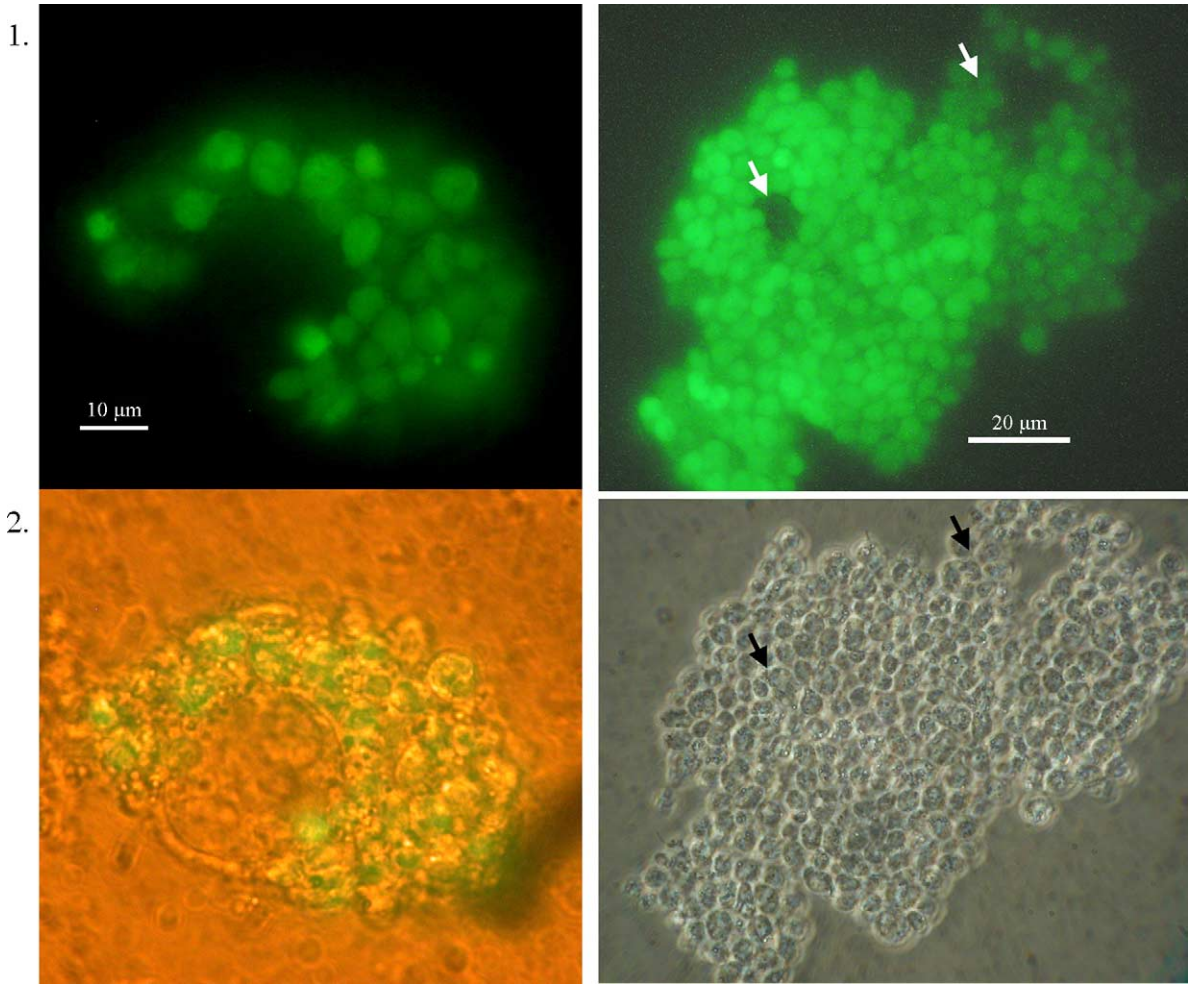

Fig. 1. (*Continued*).

between 5 and 7 h after exposure to the differentiation signal (Fig. 4BII, G2). These cells formed aggregates but remained promastigote-shaped cells for at least 7 h (Fig. 4BI, G2). The results in Figs. 4 and 5 indicate that upon exposure to the differentiation signal, promastigote to amastigote differentiation initiates at G1.

3.3. Acidic pH and high temperature play different roles in the differentiation signal

It was interesting to find out the role of each parameter of the differentiation signal, elevated temperature (37 °C) and acidic pH (pH 5.5), in the transient growth arrest at G1 and

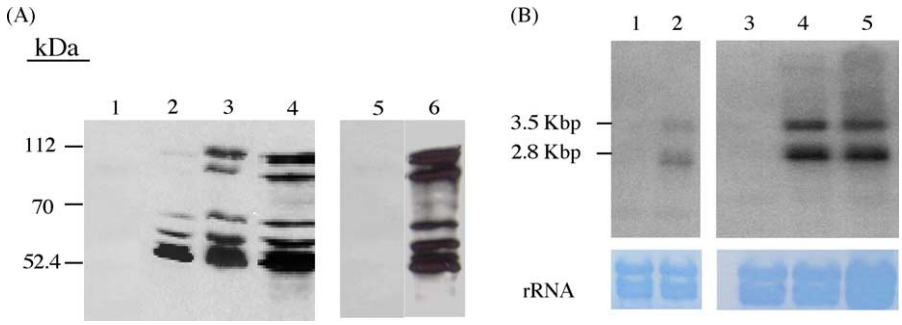

Fig. 2. A2 expression during *L. donovani* differentiation. (A) Time course of A2 protein expression during promastigote to amastigote differentiation. A total of 10 μg of cellular proteins were separated by SDS-PAGE, transferred to nitrocellulose paper, and reacted with anti-A2 monoclonal antibody. Lanes 1 and 5, promastigotes; lane 2, 1 h; lane 3, 5 h and lane 4, 7 h after exposure to differentiation signal; lane 6, amastigotes. (B) A2 mRNA abundance during *L. donovani* differentiation. Total RNA was isolated from promastigotes (lanes 1 and 3), parasites one hour (lane 2) and 24 h (lane 4) after the differentiation signal and from amastigotes (lane 5). Northern analysis using A2 gene as a probe was done as in Experimental Procedures.

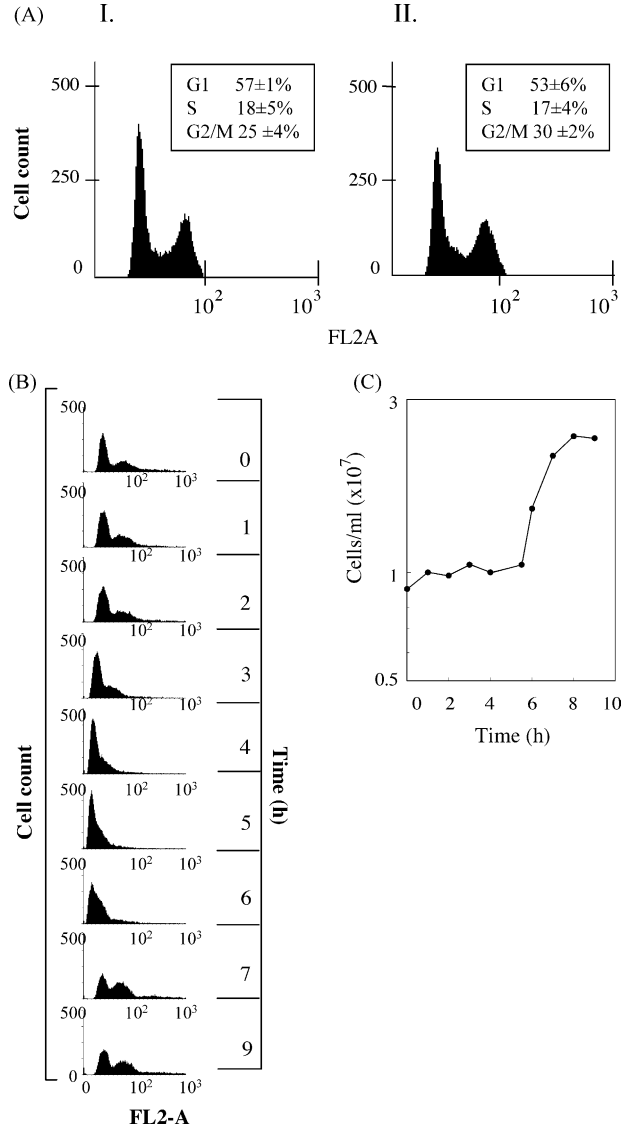

Fig. 3. Flow cytometry time course of differentiating *L. donovani*. (A) Log phase *L. donovani* promastigotes (AI) and amastigotes (A2) were stained with propidium iodide and analyzed by flow cytometry. (B) Late-log phase *L. donovani* promastigotes (5×10^6 cells/ml) were exposed to differentiation signal (pH 5.5 at 37 °C). At the indicated times, aliquots of cell suspension were stained with propidium iodide and analyzed for DNA content using flow cytometry; (C) Growth curve of differentiating promastigotes.

initiation of shape transformation. For this we exposed logarithmic phase promastigotes to growth at 37 °C and pH 7 for 24 h (heat adapted promastigotes) and the time course of proliferation was determined by measuring thymidine incorporation into the cells' nuclear DNA (Fig. 6A). As shown, shifting the *L. donovani* promastigotes growth temperature from 26 to 37 °C caused a rapid increase in the rate of thymidine incorporation, which reached a maximum after 4 h and then decreased to almost zero within 14 h. Similar results were obtained using flow cytometric analysis of propidium iodide stained cells, indicating parallel change in the relative number of parasite cells in S phase (Fig. 6B). Shifting these to pH 5.5 released the growth arrest as indicated by resumed thymidine incorporation at either 10 or 26 h after the shift to 37 °C (arrows in Fig. 6A and B). After 3 h of the acidic pH shift these cells started to transform into amastigotes. On the other hand, parasites that remained at 37 °C and pH 7 remained in growth arrest for at least 24 h and when resumed growth did not differentiate.

A major concern in the experiments described in Fig. 6 is that the rapid transient increase in DNA synthesis reflects a process that might lead to DNA damage, which usually results in cell death. However, the immediate resumption of cell cycle progression and thymidine incorporation that we observed in these experiments rule out the possibility that parasite cells are damaged. Hence, we propose that elevated temperature induce growth arrest and acidic pH releases it and route the heat adapted promastigote to differentiate into amastigotes.

3.4. Heat shock modulators can replace heat in the differentiation signal

If heat shock response plays an important role in mediating the differentiation signal, then we should be able to induce differentiation by using stress-inducing compounds that mimic heat. Azetidine 2 carboxylic acid (AZC) and ethanol are well-established modulators of heat shock response in cells [17,26–28]. We assessed whether these compounds activate a heat shock response in *L. donovani*. As shown in Fig. 7A, treating log phase promastigotes with 5% ethanol at 26 °C caused a decrease in thymidine incorporation into nuclear DNA to almost zero within 6 h. These cells remained viable for at least 48 h but they arrest growth as their density remained unchanged. These cells also expressed a higher level of HSP100 than untreated promastigotes (Fig. 7B), indicating that ethanol induced heat shock response in *L. donovani*.

The similar effects of ethanol and heat in concomitant with acidic pH prompted us to assess whether ethanol as well as other modulators of heat shock response are able to take the place of temperature elevation in the differentiation signal. Late-log phase promastigotes were incubated at pH 5.5 and 26 °C in the presence of 5% ethanol, and aliquots were taken every 2 h to determine the cellular level of the amastigotes-specific A2 protein family [17]. As shown in Fig. 8A, these cells started to express proteins of the A2 family 7 h after the shift and at 12 h they expressed all seven proteins of this family. Within 24 h the majority of these promastigotes differentiated into amastigote-shaped cells (Fig. 8C1). A similar effect on A2 expression and differentiation was observed when promastigotes were incubated for 24 h at pH 5.5 and 26 °C in the presence of 200 mM azetidine 2-carboxylic acid (AZC; Fig. 8B3 and C2). In control experiments, neither A2 expression nor morphological differentiation was observed in cells grown at pH 7 with either ethanol or AZC or at pH 5.5 without ethanol or AZC (not shown).

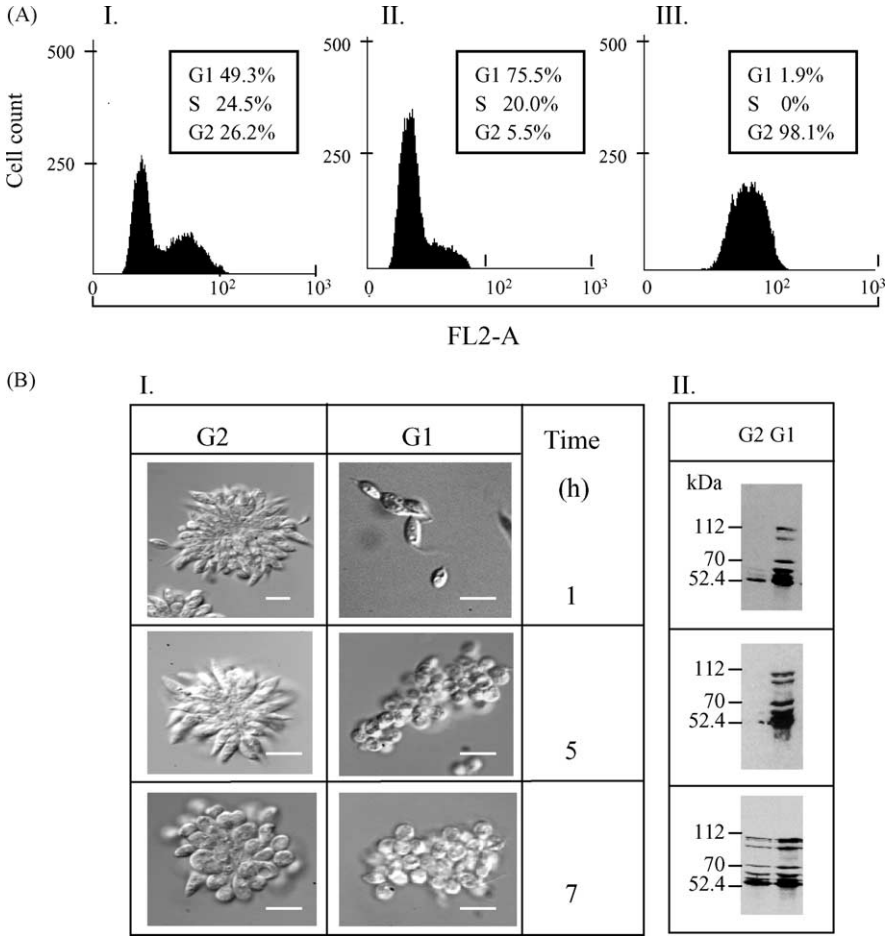

Fig. 4. Differentiation of G1 and G2 synchronized *L. donovani* promastigotes. (A) Parasites synchronization. Promastigotes (AI) were incubated with 4 mM hydroxyurea for 12 h at 26 °C (AII), or 2.5 μM flavopiridol for 12 h (AIII). These cells were stained with propidium iodide and analyzed for DNA content using flow cytometry. (B) Cell shape changes (BI) and A2 expression (BII) as a function of time of the synchronized promastigotes during differentiation were carried out as described in the previous figures. Nomarsky images of G2 synchronized cells (BI, G2) or G1 (BI, G1) synchronized cells were taken at indicated time points (scales = 10 μm). A2 protein expression during differentiation of G2 synchronized cells (BII, G2) and G1 (BII, G1) synchronized cells. Each lane was loaded with 10 μg of cellular proteins.

4. Discussion

The recent development of host-free differentiation systems for *Leishmania* has enabled better insight into the early differentiation processes in these parasites. Previous studies indicated that concomitant exposure to elevated temperatures and acidic pH signals promastigotes to differentiate into amastigotes [4,6,29,30]. In this work, we determined time course of differentiation for *L. donovani* and assessed the role of each parameter of the signal. The major observations were: (1) morphological transformation to amastigote-shaped cells initiates 5 h after the signal, (2) differentiation occurs synchronously, while cells arrest at G1, (3) heat induces the cell cycle arrest in promastigotes, (4) subsequently, acidic pH resume growth and route promastigotes to differentiate into amastigotes and (5) modulators of heat shock can replace heat in the differentiation signal.

Synchronization at G1 and G2 indicated that G1 promastigotes differentiate earlier than G2 cells. This was demonstrated by the delayed expression of the amastigote-specific markers in the G2-synchronized promastigotes that were exposed to the differentiation signal. G1 cells expressed A2 within one hour after exposure to the signal, whereas G2 cells expressed these proteins 6 h later. G1 and G2 cells started to express A2 at the same phase of the cell cycles namely, at the entry to S. G1 synchronized cells reached this stage within an hour and G2 cells at around 6 h (Fig. 5). In both cases, morphological transformation to amastigote-shaped cells started 4 h after A2 expression when both synchronized populations reach G2/M again.

An interesting observation was that ethanol and AZC, at sub lethal concentrations induced promastigote to amastigote differentiation at acidic pH without raising the medium temperature. AZC and ethanol are well-established modulators of heat shock response by increasing cellular protein

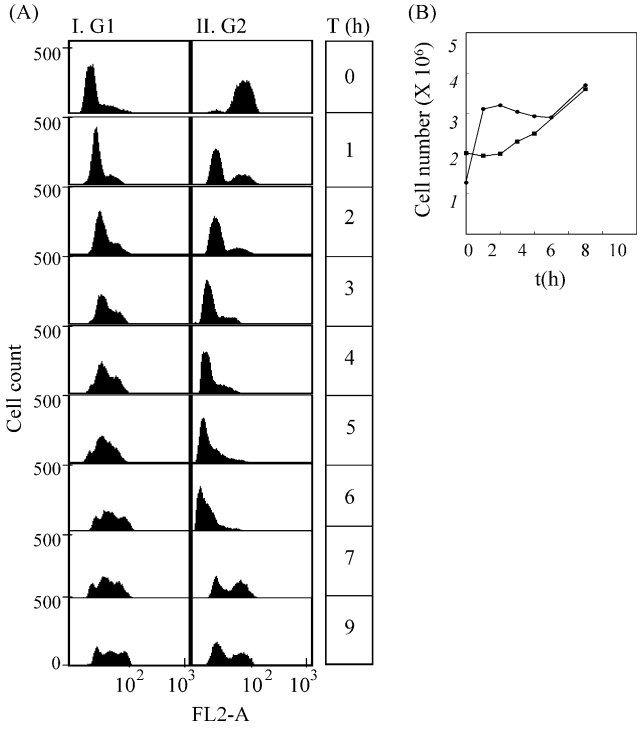

Fig. 5. Time course flow cytometry during differentiation of G1 and G2-synchronized promastigotes. (A) Promastigotes were synchronized as in Fig. 4 were washed twice in inhibitor-free PBS and then suspended in amastigotes medium and subsequently incubated at 37 °C in 5% CO2 environment. Aliquots were taken every hour, stained with propidium iodide and analyzed for DNA content using flow cytometry as in Fig. 4; (B) Growth curve of differentiating G1 (■) and G2 (●) cells.

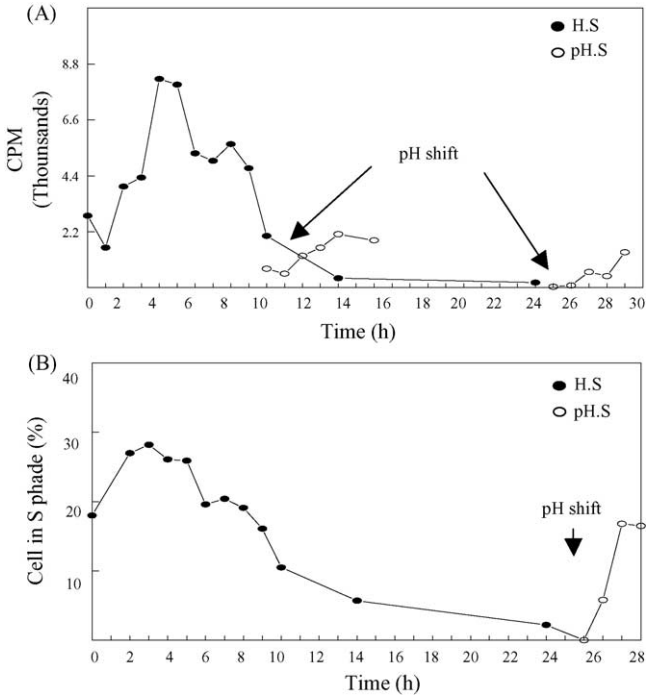

Fig. 6. Heat-shock-derived growth arrest and its release by acidic pH: (A) time course of DNA synthesis of differentiating promastigotes as indicated by thymidine incorporation into the nucleus; (B) change in S phase population as a function of time in differentiating promastigotes. Promastigotes were shifted first to 37 °C at pH 7 and subsequently to pH 5.5 and then subjected to flow cytometric analysis. (●) Promastigotes at 37 °C and pH 7; (◯) cells shifted to pH 5.5 at 37 °C.

misfolding. AZC competes with proline on the incorporation into proteins [31], and biophysical methods showed that it disrupts protein folding [32–35]. Treating *Saccharomyces cerevisiae* with ethanol induces the HOG signaling pathway that controls osmotic regulation of transcription via the stress response element of the CTT1 gene [36]. Induction of this pathway was mediated by protein misfolding. Recent studies indicated that by inducing cellular protein misfolding, both ethanol and AZC modulate heat shock-like response in *S. cerevisiae* [26,37]. Hence, the effects of ethanol and AZC on *L. donovani* differentiation indicate that heat shock response plays a role in mediating the differentiation signal. Furthermore, it is likely that heat-induced growth arrest in promastigotes is caused by elevation in cellular protein misfolding. Recent preliminary studies in our laboratory indicated a two-fold increase in protein ubiquitination in promastigotes that were exposed to the differentiation signal over heat treated cells (Barak, E. et al., unpublished results). This effect could be mimicked with ethanol, an agent that modulates differentiation at low temperature (Figs. 7 and 8). These results suggest that protein misfolding might play a role in mediating the differentiation signal. Currently, we are studying this phenomenon using molecular tools.

Normally, in the insect mid-gut, the actively dividing procyclic promastigotes differentiate into non-dividing

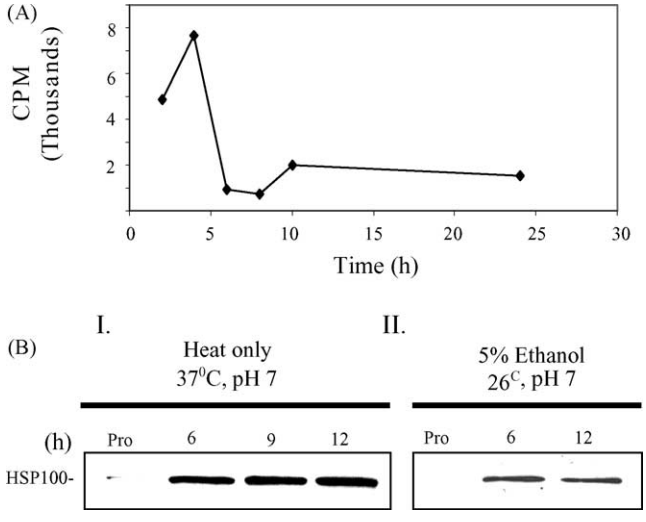

Fig. 7. Ethanol induces heat shock response in *L. donovani* promastigotes. (A) Thymidine incorporation as a function of time in promastigotes treated with 5% ethanol at pH 7 and 26 °C. Thymidine incorporation was carried out as described in Section 2; (B) expression of HSP100 in heat (BI) and ethanol (BII) treated promastigotes. Western blot of cellular proteins (10 μm, in each lane) and incubation with anti HSP100 polyclonal antibodies were carried out as described in Section 2.

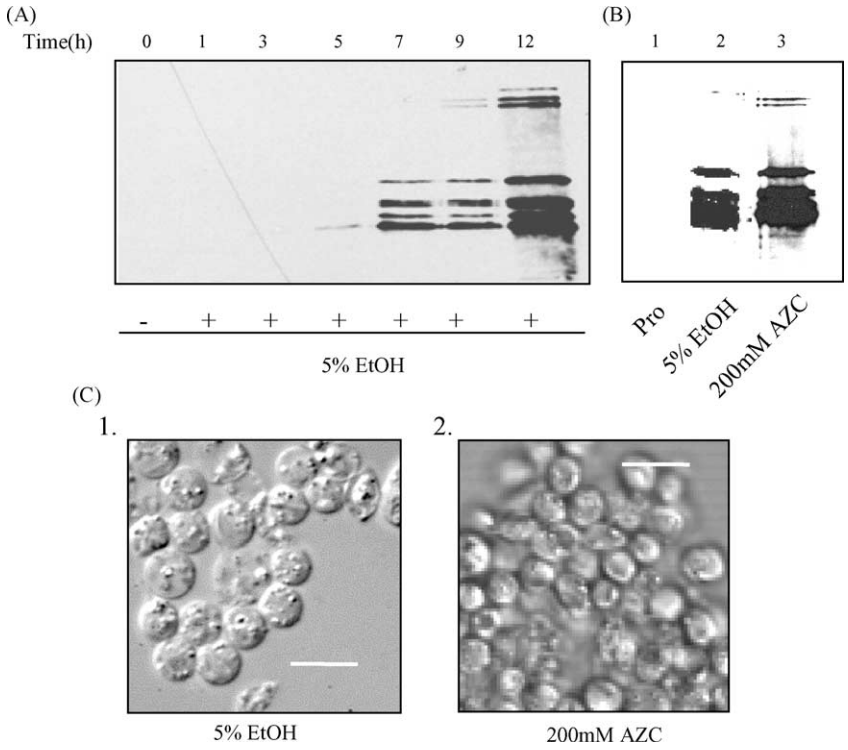

Fig. 8. Modulators of heat shock replace heat in the differentiation signal. Mid log promastigotes were incubated in amastigotes medium (pH 5.5) plus either 5% ethanol (A and C1) or 200 mM Azetidine 2 carboxylic acid (AZC, B and C2) at 26 °C for 24 h. (A) Western blot of A2 expression of 5% ethanol-treated promastigotes as function of time at pH 5.5; (B) Western blot of A2 expression of untreated (lane B1), 5% ethanol-treated (lane B2), or 200 mM AZC-treated (lane B3) promastigotes at 26 °C and pH 5.5 for 24 h; (lane C1) cell shape of the cells in B2 or (lane C2) in B3 was determined using Nomarsky phase contrast microscope. The bars indicate 10 μm.

metacyclic forms, the infective stage of the parasite, which is essential for successful invasion of the host [3]. Interestingly, it is not clear whether metacyclogenesis is an essential step in the differentiation process in vitro, since procyclic promastigotes appear to transform with equal efficiency as metacyclics [4,6,8]. The results of this work indicate common features of axenic promastigotes and de-novo metacyclics; they both start to differentiate to amastigotes while in growth arrest (G1 and G0, respectively) and differentiation initiates only when they encounter acidic pH, either in culture or in lysosomes. Furthermore, recent microarray analyses done in our laboratory in collaboration with Dr. Peter Myler's laboratory in Seattle indicated that *SHERP*, a metacyclic-specific gene [38], is expressed during differentiation that initiated with procyclic promastigotes (Saxena, A. et al., in preparation). Hence, *L. donovani* differentiation in host-free culture acquires cell cycle regulation and expression of selected genes that are characteristic of metacyclics. However, axenic promastigotes are able to perform this following exposure to differentiation signal, regardless of their developmental history.

In conclusion, the results of this paper illustrate time course of *L. donovani* promastigote to amastigote differentiation. It is a developmental process involving an initiation point at G1 at 5 h after exposure to differentiation signal. Studies are currently trying to identify and characterize genes that play key roles in this process.

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