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The role of protein phosphorylation in differentiation of *Leishmania* parasites

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B) Table of content

C) Executive summary..... 3

D) Research objectives..... 4

E) Methods and Results..... 5

E.1. In vitro differentiation of *L. donovani*..... 5

E.2. Developmentally-regulated synthesis of lipophosphoglycan..... 6

E.3. A2 expression in axenic amastigotes..... 7

E.4. Proline transport in axenic amastigotes..... 8

E.5. Metabolic activities of axenic amastigotes..... 8

E.6. Promastigote to amastigote differentiation initiates at G1..... 10

E.7. Differentiation in G1 and G2 synchronized promastigotes..... 11

E.8. Acidic pH induces differentiation-specific protein phosphorylation..... 12

E.9. Cloning of LDCDC from L. donovani genomic DNA library..... 13

E.10. Characterization of LDCDC gene..... 13

E.11. Differentiation signal induce accumulation of misfolded proteins..... 16

E.12. Modulators of heat shock can replace heat in the differentiation signal.... 17

F) Impact, Relevance, and Technology Transfer..... 19

G) Project Activities/Outputs..... 19

H) Project Productivity..... 21

I) Future Work..... 21

J) Reference list..... 22

C) Executive summary

Leishmania donovani are the causative agents of kala azar in humans. These organisms 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 human macrophages.

The aim of this research was to identify signal transduction pathway that trigger *Leishmania* differentiation and virulence inside its host. By mimicking intralysosomal environment in the growth conditions promastigotes differentiate into amastigotes in host-free systems. Using specific markers we showed that these axenic amastigotes resemble real amastigotes.. The concomitant exposure of promastigotes to pH 5.5 and 37^{0}C (differentiation signal) induced phosphorylation and dephosphorylation of cellular proteins within ten minutes. Subsequently, these cells started to express of the amastigote-specific A2 protein family within one hour, arrest the cell cycle at G1 within 3 hours and initiated morphological change to amastigote-shaped cells at five hours. Synchronization at G1 and G2 using hydroxyurea and flavopiridol, respectively, indicated that G1 promastigotes differentiate earlier than G2 cells. G1 cells expressed A2 within one hour after exposure to the differentiation signal, whereas G2 cells expressed these proteins six hours later. G1 promastigotes changed to amastigote-shaped cells after five hours, whereas G2 promastigotes started to do so only 12 hours after exposure to the differentiation signal. Hence, G1 cells start to differentiate in less than a generation time (nine hours), whereas it takes more than a generation to initiate differentiation for G2 parasites. Therefore, we propose that *L. donovani* promastigote to amastigote differentiation initiate at G1. Using anti ubiquitin antibodies and molecular markers of *L. donovani* differentiation we found that exposing promastigotes to DS induces accumulation of high level of ubiquitinated proteins. The same level of ubiquitylation and differentiation was observed using modulators of protein misfolding (e.g. ethanol) at pH 5.5 and 26^{0}C. Exposure to heat or acidic pH only induced low level of ubiquitylation and the promastigotes did not differentiate. We propose that the DS-induced ubiquitylation represent cellular accumulation of misfolded proteins, which subsequently play a role in triggering initiation of *L. donovani* promastigotes to amastigotes differentiation.

D) Research Objectives

D.1. Research objectives of the Israeli PI

The ultimate goal of this research was to identify signal transduction pathway that trigger *Leishmania* differentiation and virulence inside its host. The motivation to this project came from previous observations that acidic pH and elevated temperature play a role in parasites recognition of final destination in the host, e.g. macrophage phagolysosomes, and in triggering differentiation form promastigotes, the extracellular form of the parasite into amastigotes, the intracellular form of the parasite. Specific objectives were:

1. To develop methods for *L. donovani* differentiation in host-free culture.
2. To characterize initial process of differentiation.
3. Posttranslational characterization of *L. donovan*. Differentiation.
4. Identification signal transduction pathway that mediate *L. donovani* differentiation signal

Our original objectives were to focus on protein phosphorylation as a major mechanism that controls differentiation. As project developed we found other processes such as protein misfolding and ubiquitination play major role in these processes and therefore changed the emphasis of this research accordingly.

The results of the PI project were very well accepted by the scientific community. Parts of the results have already been published (Mengeling *et al.*, 1997;Saar *et al.*, 1998) and the rest have been completed using other sources and are in process for submission.

The result of this project that was funded by CDR was highly appreciated by other foundations. This subject was supported by the Tropical Disease Program Foundation of the World Health Organization, The Israel Science Foundation and The Israel Ministry of Health.

E) Methods and Results

Objective 1: To develop methods for *L. donovani* differentiation in host-free culture

E.1. In vitro differentiation of L. donovani

The methods devised in this work for in vitro transformation of *L. donovani* promastigotes to amastigotes mimics the in situ process. First, *L. donovani* promastigotes (Fig. 1A) were shifted to grow at 37°C in a 5% CO_2 environment. Subsequently, they were transferred to a medium that had been titrated to pH 5.5 and kept under the same incubation conditions. Differentiation was then completed within 4–5 days (Fig. 1B).

As shown in Fig. 1B, cells exposed to both pH 5.5 and 37°C differentiated to amastigote-shaped cells. Differentiation of promastigotes to amastigotes did not complete at extracellular pHs exceeding 6 or at temperatures below 37°C. Changing the pH alone without changing the temperature also failed to induce promastigotes to fully differentiate to amastigotes (not shown, see(Zilberstein *et al.*, 1991).

Amastigotes were induced to differentiate back to promastigotes by shifting them to grow at pH 7 at 26°C. Under these conditions, parasites differentiated back to promastigotes within 48 h. The results indicated that under the conditions outlined above both dividing stages of the *L. donovani* cycle were successfully maintained.

In control experiments, we observed that about 8 h after shifting promastigotes from 26 to 37°C, they started to form large aggregates. The aggregates remain even after shifting them to pH 5.5. The addition of 10 μM cycloheximide before the heat shock inhibited the aggregation, thus indicating that this process required a synthesis of new proteins.

The *L. donovani* 1S clone cells lost their infectivity due to long-term passage in culture. Therefore, it was interesting to determine whether in vitro cycling of these cells could induce parasite virulence. Hamsters were inoculated with axenic amastigotes (1×10^{7} cells per hamster), and the level of infectivity in their spleen was determined after 6 weeks. The results indicated that 68±7.5% of the spleen cells were infected with 10±2.5 parasites per cell. In addition, promastigotes of *L. donovani* 1SR successfully infected peritoneal macrophages in culture. Macrophages were infected with late stationary promastigotes for 1.45 h at a promastigotes to macrophages ratio of 2:1. Within 24 h, 90±3.7% of these macrophages were infected with an initial number of 5.1±0.2 parasites $cell^{-1}$ (Carter, K.C., unpublished results).

Fig. 1. Confocal microscopy of axenic *L. donobani* culture. Promastigotes (A) and amastigotes (B) of *L. donobani* were fixed with 2% *p*-formaldehyde and then reacted with rabbit anti-plasma membrane antibodies (kindly provided by Dr Dennis M. Dwyer). The antibodies were conjugated to FITC via secondary antibodies (goat anti-rabbits). Subsequently, the cells were treated with 20 mg:ml RNase at 37°C for 1 h. The cells were then stained with 10 mg:ml propidium iodide. The green color represents anti-membrane antibodies and the red color represents the DNA stained with propidium iodide. Labels are: cell nucleus (N) and kinetoplast DNA (K). Scale bars indicate 1.7 mm..

E.2. Developmentally-regulated synthesis of lipophosphoglycan

McConville and Blackwell (McConville and Blackwell, 1991) previously showed that LPG synthesis in *L. donovani* is stage-specific; promastigotes synthesize approximately 10^6 copies per cell, whereas amastigotes express less than 10^3 copies per cell (see also (Turco and Sacks, 1991;Sacks *et al.*, 1995). Therefore, LPG expression can be used as a marker to evaluate successful in vitro differentiation. LPG synthesis was determined in *L. donovani* by metabolic labeling of amastigotes and promastigotes with [^{3}H]mannose. LPG was subsequently purified and the level of [^{3}H]mannose incorporation was determined (Table 1). As expected, late logarithmic phase *L. donovani* promastigotes synthesized large amounts of LPG as indicated by the high level of [^{3}H]mannose incorporation. In contrast, axenic amastigotes hardly synthesized any LPG (between 2.5 and 8.9% of the promastigotes level). The level of [^{3}H]-mannose incorporation decreased as a function of time following parasite differentiation (2100 and 800 cpm $(10^9 \text{ cells})^{-1}$ in amastigotes cultured for 1 and 5 weeks, respectively). LPG synthesis resumed after shifting amastigotes to differentiate back to promastigotes (recycled promastigotes), indicating the stage-specific LPG synthesis.

The LPG level on the axenic amastigotes surface was assessed using indirect immunofluorescence with the monoclonal antibody CA7AE that is specific against the disaccharide-phosphate repeating units of *L. donovani* LPG (McNeely *et al.*, 1990). As shown in Fig. 2A, promastigotes were strongly stained on their surface by the monoclonal antibodies. Furthermore, binding of the anti-LPG antibodies to the promastigotes surface cross-linked the cells to each other, resulting in the formation of large stained parasite aggregates. Conversely, axenic amastigotes did not stain on their surface, and no cell aggregates were formed by the antibodies (Fig. 2B). Most of the cells did not stain at all. However, a weak intracellular fluorescence staining, which could be attributed either to internalization of LPG glycan or to the accumulation of LPG precursors, was observed in some. In control experiments, antibodies against *L. donovani* plasma membranes were used to show that both promastigotes and axenic amastigotes were intact in these experiments (Fig. 2C and D). Fluorescence-activated cell-sorter analyses using CA7AE indicated that the level of fluorescence staining was three to five times higher in promastigotes than in amastigotes (not shown).

Table 1
LPG synthesis in *L. donovani* promastigotes and amastigotes

Parasites	[^{3}H]mannose incorporation into LPG (cpm)
Stationary phase promastigotes	73 900
1-Week amastigotes	2100
5-Week amastigotes	800
Recycled promastigotes	129 650

Promastigotes and amastigotes were metabolically labeled with [^{3}H]mannose at their corresponding pH. LPG was purified from either 10^9 promastigotes or 10^9 axenic amastigotes as described in Section 2. This experiment is representative of five independent experiments

Development of a method for in vitro culture of amastigotes enabled an examination of time-dependent changes in LPG expression during parasite differentiation. Axenic amastigotes were induced to differentiate to promastigotes, and aliquots were taken every 2 h to measure LPG expression. As shown in Fig. 3, the amount of LPG observed at zero time (lane 1) with axenic amastigotes is similar to the amount obtained using splenic-derived amastigotes. At 2 h after inducing differentiation, the LPG level is still rather low (lane 2), it increases dramatically at 4 h (lane 3), and reaches a steady-state level of promastigotes at about 6 h (lane 4). In these experiments, differentiation of amastigotes to promastigotes was complete within 48 h.

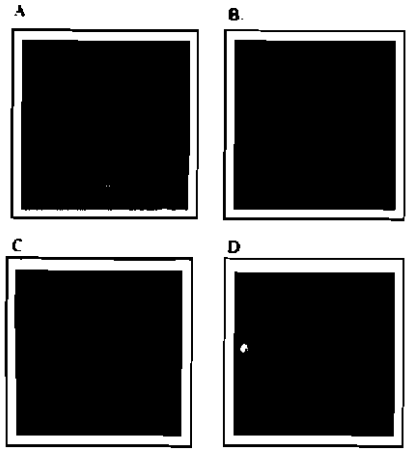

Fig. 2. Stage-specific expression of LPG on the *L. donovani* urface. Axenic promastigotes (A, C) and amastigotes (B, D) of *L. donovani* reacted with C7AE anti-LPG monoclonal antibody (A and B) or rabbit anti-plasma membranes antibodies (C and D) as described in Section 2. The antibodies were conjugated to fluorescein isothiocyanate via secondary antibodies (rabbit anti mouse, and goat anti-rabbit, respectively, for the monoclonal and polyclonal antibodies). Scale bars indicate 8 mm.

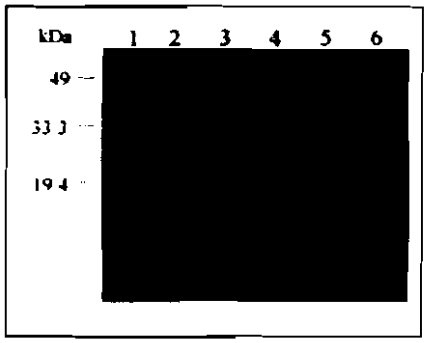

Fig. 3. Up-regulation of LPG synthesis during differentiation of amastigotes to promastigotes. Axenic amastigotes were induced to differentiate by shifting them to promastigotes' growth conditions, as described in Section 2. Aliquots with a constant number of cells were taken every 2 h. Subsequently, LPG was purified from the cells, separated on 9% SDS-PAGE, transferred to nitrocellulose paper, and reacted with C7AE anti-LPG antibodies. Lane 1, amastigotes; lanes 2–5 cells, 2, 4, 6 and 8 h in promastigotes' growth conditions; lane 6, 2 mg of promastigotes LPG.

E.3. A2 expression in axenic amastigotes

A2 represents a multi-gene family in *L. donovani* that is only expressed in amastigotes (Charest and Matlashewski, 1994). Using both polyclonal and monoclonal antibodies against this protein, Zhang et al. (Zhang *et al.*, 1996) showed that A2 encodes for seven proteins that are expressed in both animal-derived and axenic amastigotes of *L. donovani*.

This anti-A2 monoclonal antibody was used to further evaluate axenic amastigotes (Fig. 4). As shown, promastigotes did not synthesize A2 proteins at all (lane 1). The axenic amastigotes, on the other hand, expressed seven proteins that reacted with anti-A2 antibodies (lane 2), forming two very close bands at about 100 kDa and single bands at 81, 59, 53, 49 and 45 kDa. In a control experiment, anti-*L. donovani* hsp83 antiserum was used to show that

similar amounts of promastigote and amastigote proteins were loaded on the gel (Fig. 4, lanes 3 and 4).

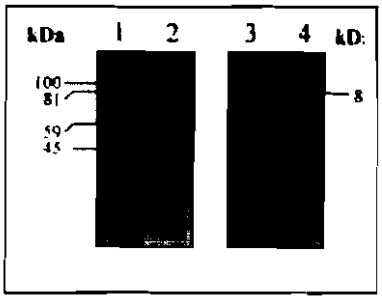

Fig. 4. Stage-specific expression of A2 proteins in *L. donovani*. A total of 20 mg of cellular *L. donovani* promastigote (lanes 1 and 3) and amastigote (lanes 2 and 4) proteins were separated on 9% SDS-PAGE, transferred to nitrocellulose paper and reacted with anti-A2 monoclonal (lanes 1 and 2) or anti-hsp83 antiserum (lanes 3 and 4).

E.4. Proline transport in axenic amastigotes

Previous studies indicated that proline transport and its metabolism in *L. donovani* are developmentally regulated (Mukkada *et al.*, 1985;Zilberstein, 1993;Mazareb *et al.*, 1999). Proline transport in promastigotes is optimal at around pH 7.0 (Zilberstein and Gepstein, 1993), whereas in amastigotes it is optimal at pH 5.5, and the uptake level is 10-fold less that in promastigotes (Glaser and Mukkada, 1992). Hence, proline transport can be useful to evaluate parasite differentiation. As shown in Fig. 5A, proline transport activity in log phase *L. donovani* promastigotes was optimal at around pH 7.0, with maximal activity of 6 nmol min^{-1} $(10^8$ cells$)^{-1}$. Axenic amastigotes take up proline at a much lower level (1.26 nmol min^{-1} $(10^8$ cells$)^{-1}$), and at a different pH optimum (pH 5.0, Fig. 5B). As indicated in the figure, the uptake in axenic amastigotes was much more sensitive to changes in extracellular pH than in promastigotes, as indicated by the pH optimum curve, which was sharper in the amastigotes. A small peak of proline transport activity at pH 7.0 in axenic amastigotes was also observed.

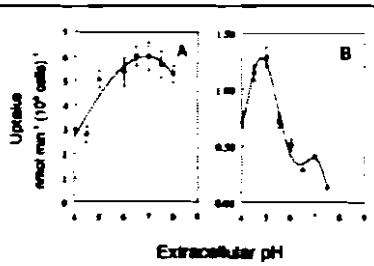

Fig. 5. pH response of proline transport in axenic *L. donovani* amastigotes and promastigotes. Logarithmic phase promastigotes (A) and amastigotes (B) were harvested, washed and resuspended in Earl's salt solution at either pH 7 or pH 5.5 ($5X10^8$ cells/ml), then 10 ml of each cell suspension was transferred to Earl's solution at indicated pH and incubated for 10 min at either 30°C (promastigotes) or 37°C (amastigotes) for equilibration. Assays were carried out for 5 min.

E.5. Metabolic activities of axenic amastigotes

Fig. 6 illustrates thymidine incorporation into the various life stages of *L. donovani*. As shown, promastigotes incorporate thymidine at high levels, with optimum activity at around pH 7.0. Changing the growth medium pH to 5.5 caused a shift in the pH optimum to about pH 6.0, and the level of incorporation decreased by 50% (Fig. 6A). In axenic amastigotes, the optimum pH of thymidine incorporation further shifted to pH 5.0, and the incorporation level decreased to one eighth that of promastigotes (6.4 and 53 pmol h^{-1} $(10^8$ cells$)^{-1}$ in amastigotes and promastigotes, respectively, Fig. 6A and 6B).

Protein synthesis was evaluated by measuring the incorporation of the radiolabeled proline (Fig. 7). Proline is a zwiterionic amino acid that stays neutral throughout a wide range of pHs, and is therefore useful for pH response analyses. As shown in Fig. 7, proline incorporation into *L. donovani* promastigotes was more sensitive to extracellular pH than thymidine incorporation. Optimum incorporation activity in promastigotes was at pH 7.0. Either increasing or decreasing the extracellular pH resulted in a marked decrease in the level of proline incorporation. Proline incorporation was not observed below pH 5.0. Similar to thymidine incorporation, shifting the growth medium pH of promastigotes to 5.5 decreased both the proline incorporation level and its pH optimum. In addition, proline incorporation activity in the pH 5.0-grown promastigotes was much less sensitive to changes in extracellular pH than thymidine incorporation. The proline incorporation level in axenic amastigotes was one ninth that of promastigotes. Similarly, the pH optimum for the incorporation activity shifted to about 5.0 (Fig. 7B), resembling thymidine incorporation activity (compare with Fig. 6B).

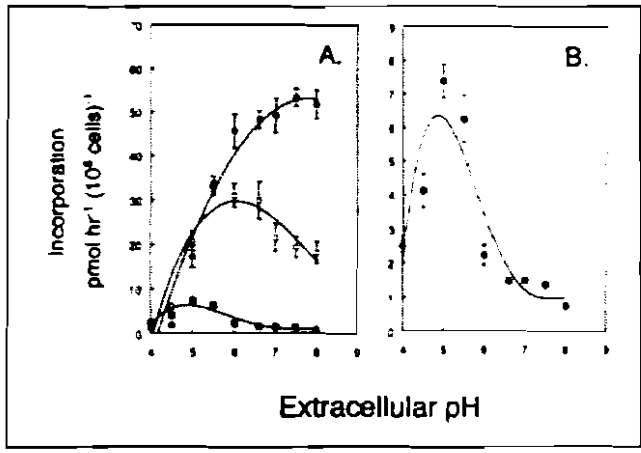

Fig. 6. pH response of thymidine incorporation by axenic *L. donovani* promastigotes and amastigotes. Cells were labeled with [3H]thymidine as described in Section 2. (A), (●) promastigotes, (O) promastigotes grown at pH 5.5 at 26°C, (■) axenic amastigotes. (B), axenic amastigotes (enlarged scale). The results are expressed as means±SD (n=3).

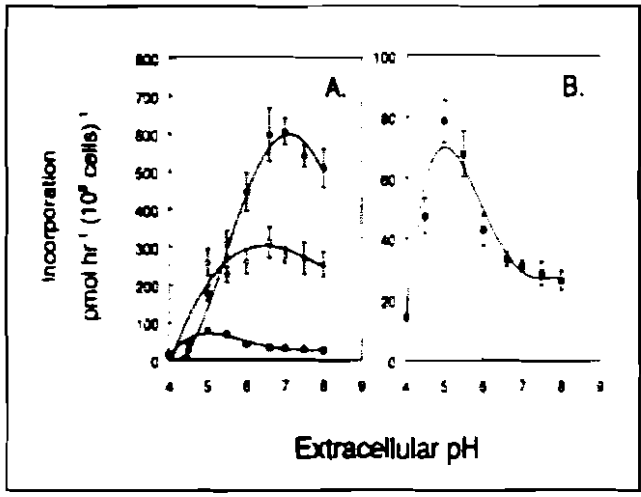

Fig. 7. pH response of protein synthesis in *L. donovani* promastigotes and axenic amastigotes. Protein synthesis was determined by measuring [3H]proline incorporation as described in Section 2. (A), (●) promastigotes, (O) promastigotes grown at pH 5.5 at 26°C, (■) axenic amastigotes (B), axenic amastigotes (enlarged scale). The results are expressed as means±SD (n=3).

Objective 2: To characterize initial process of differentiation

E.6. Promastigote to amastigote differentiation initiates at G1

As previously described, *Leishmania donovani* promastigote to amastigote differentiation in the host-free culture is accomplished in two steps (Saar *et al.*, 1998): the heat-adaptation period (37°C at pH 7 in a 5% CO_2 environment for 12-14 hours), followed by a pH shift to 5.5. Differentiation initiates only when parasites are exposed to both acidic pH and elevated temperature (differentiation signal). Figure 8A presents flow cytometry histograms of propidium iodide-stained log phase axenic *L. donovani* amastigotes and promastigotes. As shown, 53±6% of the growing amastigote population is in G1, 17±4% in S, and 30±2% in the G2/M phase. 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. The resemblance of the DNA distribution pattern indicates that both axenic amastigotes and promastigotes are stably growing populations.

Figure 8B illustrates the expression of the amastigote-specific A2 protein family (Zhang *et al.*, 1996) during promastigote to amastigote differentiation. Promastigotes do not express A2 (lane 1), whereas amastigotes express all seven proteins of this family (lane 4). Heat-adapted promastigotes express a low level of two proteins of this family (lane 2). At twenty-four hours, parasites express all seven A2 proteins (lane 3). Expression of these proteins correlates with the morphological changes: amastigote-shaped cells start to appear 5 hours after the pH shift, and at 24 hours most of the parasites have the shape of amastigotes (not shown). The results further indicate that the A2 family can be used as a molecular marker for *L. donovani* differentiation.

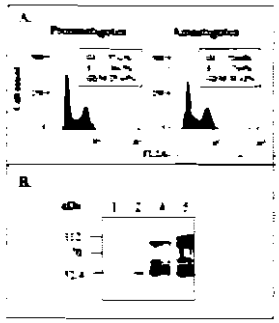

Fig. 8: Cell cycle distribution of *L. donovani* promastigotes and amastigotes. A) Log phase *L. donovani* promastigotes and amastigotes were stained with propidium iodide and analyzed by Flow cytometry. B) A2 protein expression during promastigote to amastigote differentiation. Each lane was loaded with 10 µg of cellular proteins. Lane 1, promastigotes; lane 2, 24 hours at 37°C and pH 7; lane 3, 24 hours after the shift to pH 5.5; lane 4, amastigotes.

Since only concomitant exposure to elevated temperature and acidic pH induce differentiation of *Leishmania donovani*, we decided to find out whether this process is regulated by the cell cycle. In order to do so propidium iodide-stained parasites were subjected to time course flow cytometry (Fig. 9A). As shown, the log phase promastigotes population (t=0) reacted to the differentiation signal by synchronizing at G1 within three to four hours. At 6 hours after the differentiation signal, they synchronously returned to the cell cycle, as indicated by the reappearance of S phase cells (Fig. 9A 4-9). In parallel, thymidine incorporation ceased at 2 hours and resumed at 5-6 hours after the differentiation signal, suggesting that the parasites had undergone growth arrest (data not shown). Cell counting (Fig. 9B) indicated that during the first 5 hours after the signal this population ceased their growth. They doubled in number between 6 and 8 hours after the signal and then returned to normal growth. This indicates that following the differentiation signal, the parasites remained viable and their growth rate was in agreement with the results of the flow cytometry assays. As shown in Fig. 9C, one hour after differentiation initiated, the promastigotes expressed a high level of the low molecular weight proteins of the amastigote-specific A2 family. After

five hours, these cells expressed all of the A2 proteins at a level similar to that of amastigotes. Morphological analyses indicated that these promastigotes started to change to amastigote-shaped cells at five hours after the differentiation signal (Fig. 9D). At approximately 24 hours, most of the parasite population obtained the shape of amastigotes (not shown).

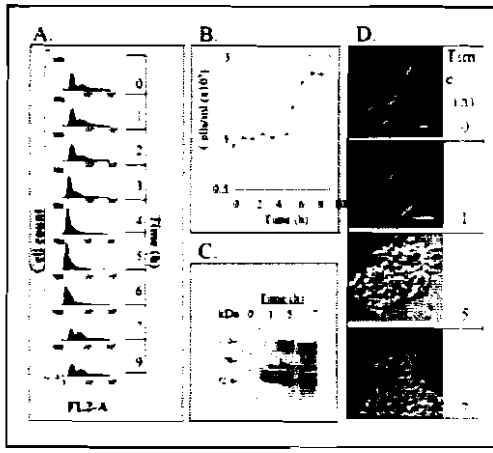

Fig. 9: Cell cycle arrest of *L. donovani* promastigotes upon exposure to the differentiation signal. Late-log phase *L. donovani* promastigotes were shifted to grow at 37°C and pH 5.5. (A) At the indicated times, aliquots of cell suspension were stained with propidium iodide and analyzed for DNA content using flow cytometry; (B) Growth curve of differentiating promastigotes. The number of cells were determined for each sample at the indicated times; (C) Time course of A2 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; (D) Nomarski images of the cells at 0, 1, 5, and 7 hours after the differentiation signal. Scales indicate 10 µm.

E.7. Differentiation in G1 and G2 synchronized promastigotes

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. 10). Hydroxyurea blocks the cell cycle at the G1/S boundary (Simpson and Braly, 1970;Mutomba and Wang, 1996). Incubating log phase promastigotes with 4 mM hydroxyurea for 10 hours at 26°C and pH 7 synchronized 76% of the cells at G1 (Fig. 10B). These cells remained single, promastigote-shaped and were fully viable (Fig. 3E, in comparison with Fig. 10D). Flavopiridol is an ATP analog that inhibits CDK28 and CDC2 in mammalian and yeast cells, respectively (Patel *et al.*, 1998;Chien *et al.*, 1999;Lee *et al.*, 1999). It synchronizes *L. mexicana* promastigotes at G2 (Hassan *et al.*, 2001). As shown in Fig. 3C, treating log phase *L. donovani* promastigotes with 2.5 µM flavopiridol at 26°C for 24 hours synchronized 98% of them at G2. These cells remained promastigote-shaped and were fully viable (Fig. 10F).

When the G1-synchronized promastigotes that were treated with hydroxyurea at 26°C were shifted to differentiation conditions in a hydroxyurea-free medium, they immediately and synchronously resumed cell cycle progression. These cells expressed the A2 protein family within one hour after exposure to the differentiation signal (Fig. 11B, G1). Differentiation to amastigote-shaped cells in this population occurred synchronously five hours after the differentiation signal (Fig. 11A, G1). In a control experiment, the 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 amastigote-shaped cells.

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. Unlike the G1 cells, G2-synchronized promastigotes started to express A2 proteins only seven hours after exposure to the differentiation signal (Fig. 11B, G2). These cells formed aggregates but remained promastigote-shaped cells for at least 12 hours (Fig. 11A, G2). The results in Figures 2–4 indicate that upon exposure to the differentiation signal, promastigote to amastigote differentiation initiates at G1.

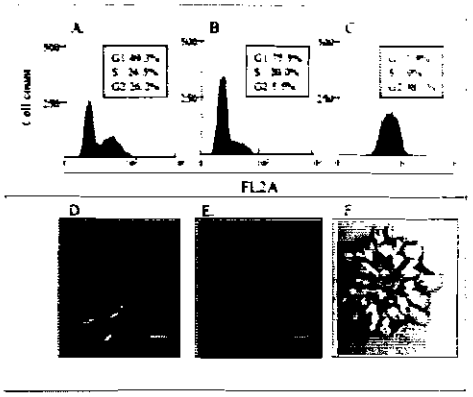

Fig. 10: Synchronization of *L. donovani* promastigotes at G1 and G2. Promastigotes (A and D) were incubated with 4 mM hydroxyurea for 10 hours at 26°C (B and E), or 2.5 μM flavopiridol for 24 hours (C and F). Each of these cells were stained with propidium iodide and analyzed for DNA content using flow cytometry. (A-C) are flow cytometry histograms and (D-F) are Nomarski phase contrast images. Scales indicate 10 μm.

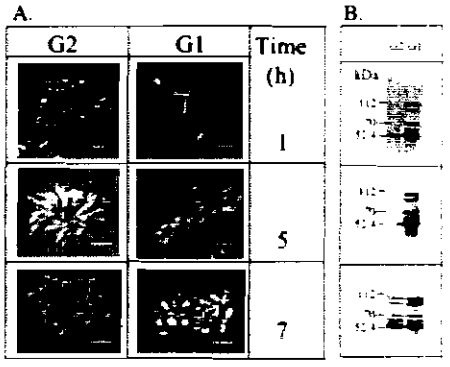

Fig. 11: A) Cell shape changes during promastigote to amastigote differentiation. Nomarsky images were taken at the indicated time point of G2 synchronized cells (G2) and G1 synchronized cells (G1). B) A2 protein expression during differentiation of G2 synchronized cells (G2) and G1 synchronized cells (G1). Each lane was loaded with 10 μg of cellular proteins. The protein-containing membranes were reacted with anti A2 monoclonal antibodies.

E.8. Acidic pH induces differentiation-specific protein phosphorylation

As shown in our previous work (Saar *et al.*, 1998) and in the results above, only acidic pH shift that either follow a heat adaptation period or comes concomitantly with temperature elevation initiate differentiation into amastigote-shaped cells. It is likely that acidic pH activate a signaling pathway that together with heat shock response induces differentiation. This activation might involve post-translational mechanisms such as protein phosphorylation. In order to assess this hypothesis, we investigated the effect of acidic pH on protein phosphorylation ten minutes after the acidic pH shift.

Heat-adapted promastigotes (e.g. promastigotes incubated at 37°C and pH 7 for 24 hours) were metabolically labeled with inorganic ^{32}P (Fig. 12A) and subsequently shifted to pH 5.5 for 10 minutes (Fig. 12B), conditions which initiate differentiation. Two-dimensional PAGE of the lysates of these cells indicated that eight proteins were specifically phosphorylated (Fig. 12B 1-8) only after the shift to pH 5.5 at 37°C. Three proteins numbered 9-11 (Fig. 12A 9-11), that are constitutively phosphorylated in heat-adapted promastigotes and promastigotes at 26°C either at pH 7 or pH 5.5, were dephosphorylated following the shift to differentiation conditions (Fig. 12B). It is interesting to note that fewer changes in protein phophorylation occurred following the shift of promastigotes to pH 5.5 at 26°C (Fig. 5C and D) than of heat-adapted promastigotes to pH 5.5 at 37°C (Compare Fig. 12B with D). This further supports the notion that only concomitant exposure to 37°C and pH 5.5 activates intracellular processes related to differentiation.

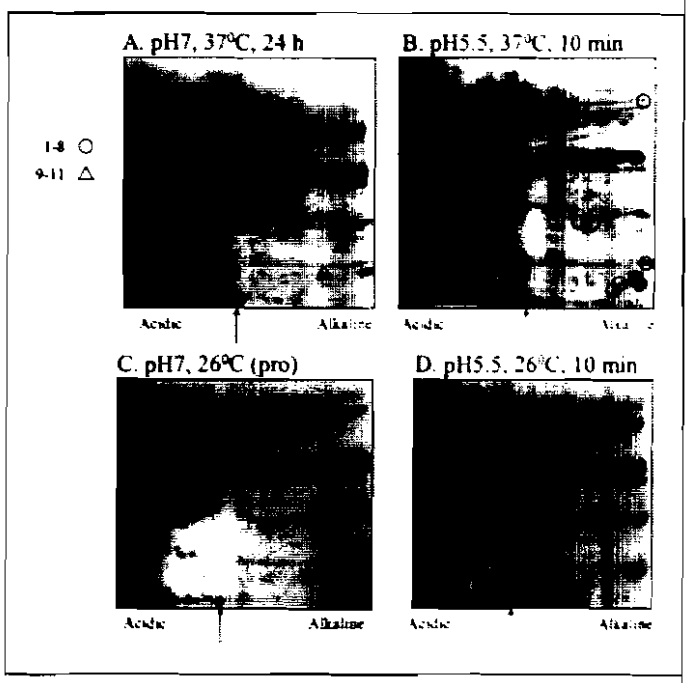

Fig. 12: Differentiation signal-induced protein phosphorylation in heat-adapted *L. donovani* promastigotes. Late-log phase promastigotes were incubated for 24 hours at pH 7 and at either 37°C (A) or 26°C (C) and subsequently were metabolically labeled with [^{32}P]H_3PO_4 (50 μCi/ml) for 3 hours at the respective temperatures. These cells were then washed to remove excess ^{32}P and divided into 2 groups: one group remained at pH 7 and the other was shifted to pH 5.5 at either 37°C (B) or 26°C (D) respectively. After 10 minutes, 1 ml of aliquot of each cell suspension was mixed with 10 ml ice-cold 10% trichloroacetic acid and incubated on ice for 30 min. Proteins were then washed and subjected to 2-dimensional gel electrophoresis (isoelectric focusing at a pH range of 3-10 followed by 9% SDS-PAGE). Phosphorylation (o) and dephosphorylation (Δ) specific to the differentiation signal (heat + acidic pH) are indicated.

Objective 3: Posttranslational characterization of *L. donovan*. Differentiation

E.9. Cloning of LDCDC from L. donovani genomic DNA library.

A genomic DNA library of *L. donovani* promastigotes have been constructed in λ zap expression vector(Stratagene Inc.). *L. donovani* promastigotes genomic DNA was partially digested with SauIII restriction enzyme. A mean size of 4000 bp DNA was packed into BamH1 site of the λ zap.

A1, the PCR product that have been described in detail in our previous report (section B.2.), was used as a probe to screen the genomic DNA library. A few positive clones have been identified, enriched and amplified. One clone with an insert of 2.2 Kbp, which was named LDCDC, was sequenced using automatic sequencing service of the Weizman Institute (Rehovot Israel). The insert contained 2249 nucleotides. Nucleotides 1 through 2004 encode for a single frame that ends with a TGA termination codon. No initiation codon was found near the 5' end of the insert, not even a stop codon that might indicate a non-translating region was identified. Therefore, we conclude that this insert do not contain the 5' end of LDCDC. Currently we are doing experiment to reveal this region.

E.10. Characterization of LDCDC gene.

The putative primary structure of LDCDC protein was deduced from the nucleotide sequence. The ORF encodes for a 668 amino acids that form a protein with a molecular mass of 71055 kDa. Its amino acid composition indicate a relatively alkaline protein with a pI of 7.54. It contains high level of alanine (bold letters in Fig. 13) and serine. The alanines are distributed in doubles, triples and tetrads throughout the molecules. Three double serine repeats exist between amino acids 82 and 244. Longer stretches of serine appear between

amino acid 315 and 336. Inside this serine repeats we have identified interesting sequences that appears in 3 repeats: "SCSP", "SESP", and "SESP" (shaded and undermarked with stars). Four serines appear between each of these sequences.

The following known motifs have been identified in LDCDC:

1. A single N glycosylation site between amino acids 427- 430.
2. A leucine zipper consensus sequence between amino acids 540-561. There are 3 segments of amino acids in which every seventh amino acid is leucine and the amino acid are hydrophobic.
3. A few phosphorylation sites have been identified: one for cAMP dependent kinase, six for protein kinase C and 10 for casein kinase 2.
4. Ten myristylation sites have been identified.

The following motifs in LDCDC indicate that it encodes for a protein kinase:

1. Amino acids 512-567 (underline are the primer sequences that were used for PCR) the highly conserved protein kinase sequences.
2. Amino acids 208-213, QGVYGV is an ATP binding site motif of proteins of the cdc2 family.
3. Amino acids 337-344, PSTEQHAE is homologous to PSTAIRE, a motif common to most cdc2 kinases.

1 QNWYGPPSRS TVSISLISNS CSPGDPLCVP FTCDMVVPVL NALLSNQQHG

51 STSTVAAAAS GGLNGAVVPM MTEAVNSIDA TGGDTRDVSA LSSASAEIPG

101 AGRDSIGKES QSIVVAKAVV RMREVLAAQG PPQMPQKAAV ESVPSITATG

151 TDTAAGAPVC SLRPSSASLF ADAAYNTACA VARVDVSGAV RPICRDVNYY

201 TRVGRISQGV YGVVFRAVTT ADYERQHRQQ YRHRVTRPGS AASSAAAPPG

•••• ••••

251 HVRTYALKHI KKMWLEDSQV GLPPYLMREI DLLLRLQHPN IMGALELVLL

301 DPTPVPRRLA SPPKSCSPSS SSSESPSSSS SLSSESPSTE QHAEEAAECD

**** **** ****oooo ooooo

351 RALRRLRSTA EDTAGQNLAK KAKIDNAEEP LPQREEARPA TQAQTKGEKG

401 AAQDAAATRP LAAVGAASKA KEVFPCNGTK SLPCNGYCPY
DLGSYMRRYA

451 TAAELYGDGD DCKSTHASAQ VPYFHITPRN AHPQAAASYV ARAKSIVYQI

501 LRAVAFLHDS RILHRDLKTS NVLLGEDGYV KVCDFGLGR[redacted]

****

551 [redacted]HFGVVDYSH KMDVWSVGCI FAELFLRRPL FHASTDSHHL

****

601 LAVCEVLGIP TEESFPGLVS PSPDEDNDAV AAALEPHVAA GEPLPTWRCP

651 PHRSTRRCSG GSGGRAIA

Fig. 13: Deduced amino acid sequence of A1. See detail in text.

Blast analyses indicated that LDCDC is homologous to a wide range of protein kinases, all of which belong to the cdc2 family. Most of the homology is located around the conserved active site and cdc2-specific regions. Overall, the level of identity between at the region of the active site is around 50%. However, most of LDCDC protein contains amino acid sequences that are unique to *L. donovani*.

Southern analyses of *L. donovani* promastigotes with LDCDC as a probe revealed that this gene appear as single copy (Fig. 14). This is an important information because many leishmanial genes appear as gene family, usually in clusters. Northern analyses of both promastigotes and amastigotes failed as we could not detect any hybridization in both life stages. Many kinases appear at very low level of expression, which might be the reason for the negative results. In order to overcome this problem we have conducted reverse transcriptase PCR (RT PCR) assay. We have synthesized 2 oligonucleotide primers that are shaded in Fig. 13. Messenger RNA was isolated from *L. donovani* promastigotes and axenic amastigotes. The first cDNA cDNA strand was synthesized using reverse transcriptase and oligo dT as primer.

As shown in Fig. 15, both promastigotes and amastigotes express LDCDC at similar level. Time course analysis was carried out where samples were taken from the PCR reaction mixture at cycles 15 through 35. In all of them the amplified DNA appeared in the same fractions, indicating that LDCDC is constitutively expressed in both promastigotes and amastigotes.

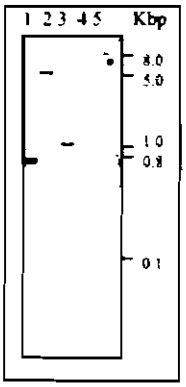

Fig. 14: Southern analysis of LDCDC. Genomic DNA of *L. donovani* was digested with restriction enzymes, separated on agarose and transferred to nitrocellulose paper. Subsequently, A1 was labeled with ^{32}P and used to probe the DNA. 1) PstI digest; 2) PvuII digest; 3) HindIII; 4) MspI digest, and 5) XhoI digest.

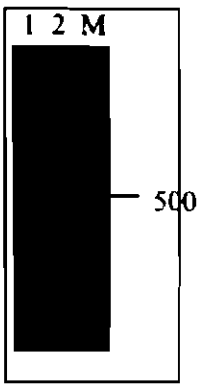

Fig. 15: Expression of A1 in *L. donovani* promastigotes and amastigotes One µg of total RNA of *L. donovani* promastigotes (lane 1) and amastigotes (lane 2) were used to synthesize cDNA strands, using oligo dT as a primer. Subsequently, oligonucloetide primers that contained sequences around the active site of LDCDC were used for PCR reaction. M indicate size markers.

Objective 4: Identification signal transduction pathway that mediate *L. donovani* differentiation signal

E.11. Differentiation signal induce accumulation of misfolded proteins.

The signal that induces promastigotes differentiation into amastigotes is composed of two physical parameters: high temperature (37^{0}C) and acidic pH (pH 5.5) (Saar *et al.*, 1998). We investigated the role of each parameter on differentiation initiation. Logarithmic phase promastigotes were exposed to growth at 37^{0}C and pH 7 for 24 hours (heat adapted promastigotes) and the time course of proliferation was determined by measuring thymidine incorporation into the cells' nuclear DNA (Fig. 16A). As shown, shifting the *L. donovani* promastigotes growth temperature from 26^{0}C to 37^{0}C caused a rapid increase in the rate of thymidine incorporation, which reached a maximum after four hours and then decreased to almost zero within 14 hours. 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. 16B).

Recent studies in yeast indicate that temperature up-shift induced cell cycle arrest via cellular protein misfolding (Trotter *et al.*, 2001;Trotter *et al.*, 2002). It is likely that a similar mechanism is responsible for the heat-induced growth arrest in *L. donovani* promastigotes. Generally, cells respond to the accumulation of misfolded proteins by either activating chaperone-aided protein refolding and or by labeling proteins with poly-ubiquitin (ubiquitylation) for degradation by the proteasome pathway (Glickman and Ciechanover, 2002). In order to assess our hypothesis, the level of ubiquitylation was determined using anti ubiquitin antibodies. In such assays, the accumulation of ubiquitinated proteins is observed as a smear of wide range molecular weight poly ubiquitin conjugated proteins. Fig. 16C illustrates Western blot analysis of heat- and acidic pH-adapted promastigotes. The results indicate a significant increase in protein ubiquitylation over time in the heat adapted promastigotes, but not in promastigotes at pH 5.5 and 26^{0}C.

Recently, we showed that concomitant exposure to pH 5.5 and 37^{0}C (the differentiation signal) induced promastigotes differentiation into amastigotes in a process that initiates at G1 (Barak, E. *et al.*, submitted). Promastigotes undergo cell cycle arrest at G1 three hours after the differentiation signal and during this process they start to change to amastigotes-shaped cells (at five hours after the signal). Fig. 16D shows that following exposure to the differentiation signal promastigotes accumulate ubiquitylated proteins at a level that is three times higher than that determined in the heat-adapted parasites. Furthermore, whereas the level of ubiquitylation in the heat adapted promastigotes remains constant over a long period of time, it is transiently expressed in cells that are exposed to both acid pH and high temperature. As shown in Fig. 16D, the level of ubiquitylation reaches a maximum at about 10 hours and then starts to decrease.

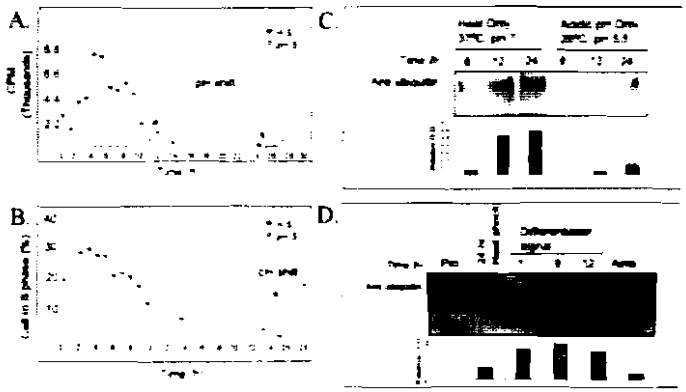

Fig. 16: Heat-shock-response-derived cell cycle arrest and its releases of acidic pH (A) Time course of DNA synthesis of differentiating promastigotes as indicated by thymidine incorporation into the nucleus. (B) Change in S phase population as a function of time in differentiating promastigotes. Promastigotes were shifted first to 37^{0}C at pH 7 and subsequently to pH 5.5 and then subjected to flow cytometric analysis. (•) Promastigotes at 37^{0}C and pH 7 (heat adapted parasites); (o) heat adapted promastigotes are shifted to pH 5.5 at 37^{0}C; (C) protein ubiquitylation in heat- and acidic pH- treated promastigotes. At each time point aliquots were treated with 1 mM N-Ethylmaleimide and subsequently subjected to western blot analysis using anti ubiquitin polyclonal antibodies; (D) protein ubiquitylation in differentiating promastigotes. Density of each lane was calculated using TINA software and normalized against the level of glyceraldehyde phosphate dehydrogenase.

E.12. Modulators of heat shock can replace heat in the differentiation signal.

Azetidine 2 carboxylic acid (AZC) and ethanol are well-established modulators of heat shock response by increasing cellular protein misfolding. AZC competes with proline on the incorporation into proteins (Fowden *et al.*, 1967), and biophysical methods showed that it disrupts protein folding (Zagari *et al.*, 1990a;Zagari *et al.*, 1990b;Zagari *et al.*, 1990c;Zagari *et al.*, 1994). Treating yeast cells with ethanol induces the HOG signaling pathway via protein misfolding (Schuller *et al.*, 1994). Recent studies indicated that by inducing cellular protein misfolding, both ethanol and AZC modulate heat shock-like response in *Saccharomyces cerevisiae* (Trotter *et al.*, 2001;Trotter *et al.*, 2002). Other amino acid analogs such as canavanin (an alanine analogue) failed to induce heat shock response (Trotter *et al.*, 2002). We assessed whether these compounds activate a heat shock response in *L. donovani*. As shown in Fig. 17A, treating log phase promastigotes with 5% ethanol at 26^{0}C caused a decrease in thymidine incorporation into nuclear DNA to almost zero within six hours. These cells remained viable for at least 48 hours but they arrest growth as their density remained unchanged. These cells also expressed a higher level of HSP100 than untreated promastigotes do (Fig. 17B), indicating that ethanol induces heat shock response in *L. donovani*. Hence, we expect that ethanol can replace temperature in the differentiation signal. In Fig. 2C we show that concomitant exposure of promastigotes to ethanol and pH 5.5 at 26^{0}C induce an accumulation of ubiquitinated proteins to a level similar to that induced by the differentiation signal.

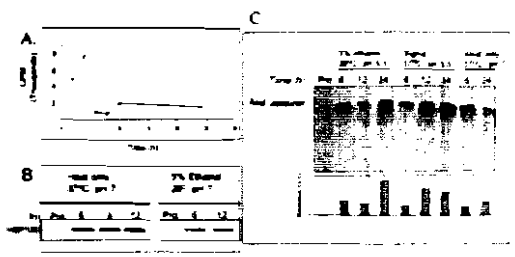

Fig. 17: Modulators of heat shock response can induce a high level of protein ubiquitylation. (A) Thymidine incorporation as a function of time in promastigotes treated with 5% ethanol at pH 7; (B) expression of HSP100 in heat – and ethanol- treated promastigotes; (C) polyubiquitylation in heat-, ethanol- and differentiation signal- treated promastigotes. Densities were determined as in Fig. 16.

The similar effects of ethanol and heat in concomitant with acidic pH on protein ubiquitylation 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^{0}C in the presence of 5% ethanol, and aliquots were taken every two hours to determine the cellular level of the

amastigotes-specific A2 protein family (Zhang *et al.*, 1996). As shown in Fig. 18A, these cells started to express proteins of the A2 family 7 hours after the shift and at 12 hours they expressed all seven proteins of this family. Within 24 hours the majority of these promastigotes differentiated into amastigote-shaped cells (Fig. 18C1). A similar effect on A2 expression and differentiation was observed when promastigotes were incubated for 24 hours at pH 5.5 and 26^{0}C in the presence of 200 mM azetidine 2- carboxylic acid (AZC; Fig. 18B3 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). Unlike AZC, incubating promastigotes at pH 5.5 and 26^{0}C with canavanin, a synthetic analog of arginine, at concentrations up to 20 mM did not induce A2 expression or morphological change, indicating the specific effect of the proline analog (not shown). The IC_{100} for canavanin and ethanol were 20 mM and 15%, respectively.

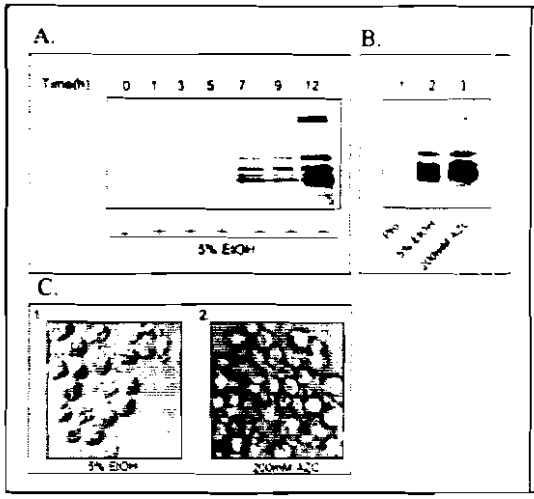

Fig. 18: Modulators of protein misfolding can induce differentiation at 26^{0}C. (A) Western blot analysis of A2 expression in promastigotes that were incubated with 5% ethanol at 26^{0}C and pH 5.5; (B) promastigotes were incubated with 5% ethanol or 200mM AZC at 26^{0}C and pH 5.5 for 24 hours and assayed for A2 proteins. (C) Cell shape of the cells in (B) was determined using Nomarski phase contrast microscope. The bars indicate 10 μm.

Unfolded protein response (UPR) is a signaling pathway that senses accumulation of unfolded proteins in the endoplasmic reticulum (Kaufman *et al.*, 2002). This pathway can be activated specifically by tunicamycin (an N glycosylation inhibitor) or thapsigargin (a calcium pump inhibitor). We assessed the possible role of the UPR pathway in initiating *L. donovani* differentiation by incubating promastigotes in the presence of sub lethal concentrations of these compounds and determined their ability to induce differentiation at pH 5.5 and 26^{0}C (Fig. 19). As shown, these compounds induced the expression of A2 in a fashion similar to that of 5% ethanol, reaching maximum expression in cells exposed to 1.5 μM thapsigargin and 20 μg/ml tunicamycin (Fig. 19A). However, neither of these drugs was able to induce morphological differentiation of these cells as did ethanol (Fig. 19B & 3C1). None of these treatments induced activation of HSP100 expression (not shown).

A2 expression without subsequent morphological differentiation could be due to either inhibition of protein modifications in the ER that are essential for differentiation progression or that UPR only partially activates the differentiation cascade. This was assessed by subjecting promastigotes to the differentiation signal in the presence of these inhibitors. The results in Fig. 19C support the latter, e.g. promastigotes were able to complete differentiation in the presence of tunicamycin and thapsigargin. The results of the experiments in this work indicate that accumulation of misfolded proteins in the cytosol involve in the induction of promastigote to amastigote differentiation.

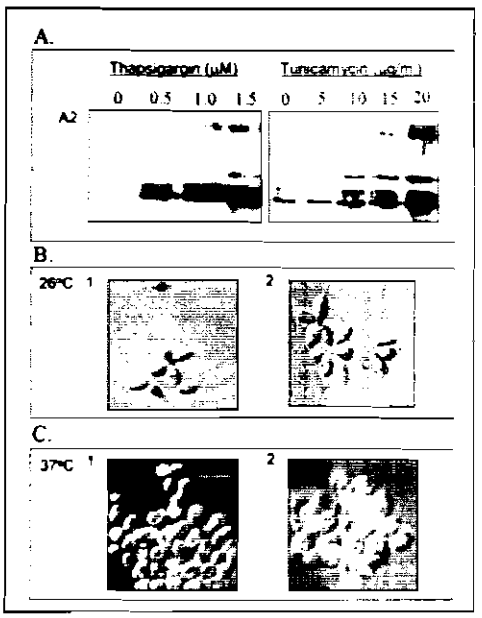

Fig. 19: Modulators of the ER unfolded protein response can not replace heat in the differentiation signal. (A) Promastigotes were exposed for 24 hours at pH 5.5 and 26°C to thapsigargin and tunicamycin and subsequently were assayed for A2 expression as was described in Fig. 2A; (B) Cell shape of promastigotes treated with 2 μM thapsigargin (panel 1) or 20 μg ml tunicamycin (panel 2) at pH 5.5 and 26°C for 24 hours; (C) Cell shape of promastigotes treated with 2μM thapsigargin (panel 1) or 20 μg ml tunicamycin (panel 2) at pH 5.5 and 37°C (differentiation signal) for 24 hours as was determined using Nomarski phase contrast. The bars indicate 10 μm.

F) Impact, Relevance, and Technology Transfer

F.1. Of the Israeli PI

The development of axenic culture of amastigotes, the intracellular form of *L. donovani* is useful not only for basic research but also for development of new drugs and studies on mechanism of drug resistance of existing drugs. An excellent example is the studies we have done on the mechanism of resistance to antimony, the anti leishmaniasis drug of choice. Our work lead to a breakthrough in our understanding drug action and mechanism of resistance (Ephros *et al.*, 1999;Shaked-Mishan *et al.*, 2001). In this present collaboration we helped Dr. lopez in his protein analyses using the help of our National Proteome Center. Dr. Lopez's laboratory learned and used our method of host-free axenization of *Leishmania* parasites.

G) Project Activities/Outputs

G.1. List the meetings held over the course of the entire project; include time, location, attendance

G.1.1. Of the Israeli PI

1994	Invited speaker at the Annual meeting of the Israel Society for Microbiology, Weizmann Institute, Israel, February, 1994. Subject: pH-regulated proline transport in *Leishmania donovani*.
1994	Invited speaker at the Laboratory of Parasitic Diseases, NIAID, NIH, USA, May 1994.
1995	Invited speaker at the Annual meeting of the Israel Society for Microbiology, Technion, Israel, February, 1995.

Subject: Protein phosphorylation in axenic amastigotes of *Leishmania donovani*.

1995 Invited speaker at the First Lower Saxony-Israel Synposium on the Life Sciences, Gottingen, Germany, October, 1995
Subject: The Ubiquitin-mediated proteolytic system and possible role in the life cycles of the parasitic protozoa *Leishmania donovani* and *Entamoeba invadens*.

1995 Invited speaker at the Federation of the Israeli Societies of Experimental Biology. Eilat, Israel, October, 1995
Subject: The role of pH and temperature in the host-parasite relations in leishmaniasis.

1996 Invited speaker at the Annual Swiss Tripanosomatid Meeting, Charmey, Switzerlan, February, 1996.
Subject: The role of pH and temperature in the development of *Leishmania* parasites.

1996 Invited speaker at the 1st International Conference on Emerging Zoonoses. Jerusalem, Israel November, 1996.
Subject: The use of axenic amastigotes in the study of drug resistance in *Leishmania donovani*.

1997 Invited speaker at the at the 1st World Congress on Leishmaniosis. Istanbul, Turkey, May, 1997.
Subjects: 1. pH and temperature regulation of *Leishmania* development. 2. Developmental regulation of amino acids transport in *Leishmania donovani*.

1998 Session chair and invited speaker at the 1st colloquium on *Trypanosoma* and *Leishmania* research in Germany. Baveria, Germany April, 1998. Subject: pH and temperature regulation of *Leishmania* differentiation.

1998 Session chair and invited speaker at the 2nd meeting of FISEB. Eilat, Israel. Subject: Cell cycle regulation of *Leishmania* differentiation

G.2. List the publications and patents for the entire project.

G.2.1. Of the Israeli PI

1. Wilkes, J.M., Peregrine, A.S. and **Zilberstein, D.** The accumulation and compartmentalization of isometamidium chloride in *Trypanosoma congolense*, monitored by its intrinsic fluorescence. Biochem. J. 312 (1995), 319-329.

2. Ephros, M., Waldman, E. and **Zilberstein, D.** Pentostam induces resistance to antimony and the preservative chlorocresol in *Leishmania donovani* promastigotes

and axenically grown amastigotes. Antimicrob. Agents Chemot. 41 (1997), 1064-1068.

3. Mengeling, B.J., **Zilberstein, D.** and Turco, S.J. Biosynthesis of *Leishmania* lipophosphglycan: solubilization and partial characterization of the initiating mannosylphosphate transferase. Glycobiology 7 (1997), 847-853.

4. Saar, Y., Asamoa, R., Waldman, E., Lumblee, J., Turco, S.J. and **Zilberstein, D.** Characterization of developmentally-regulated activities in axenic amastigotes of *Leishmania donovani*. Mol. Biochem. Parasitol. 95 (1998), 9-20.

5. Ephros, M., Bitnun, A., Shaked, P., Waldman, E. and **Zilberstein, D.** Stage-specific activity of pentavalent antimony on *Leishmania donovani*. Antimicrob. Agents Chemot. 43 (1999), 278-282.

6. Mazareb, S., Zhung, Y.F. and **Zilberstein, D.** Developmental Regulation of Proline Transport in *Leishmania donovani*. Exp. Parasitol. 31 (1999), 341-348.

7. Ulrich, N., Shaked-Mishan, P. and **Zilberstein, D.** Speciation of antimony(III) and antimony(V) in cell extracts by anion - chromatography – inductively – coupled – plasma – mass – spectrometry. Fresenius J. Anal. Chem. 368 (2000), 62-66.

8. Shaked-Mishan, P., Ulrich, N., Ephros, M. and **Zilberstein, D.** Novel intracellular Sb^{V} reducing activity correlates with antimony susceptibility in *Leishmania donovani*. J. Biol. Chem. 276 (2001), 3971-3976.

H) Project Productivity

Our original plan was to focus on protein phosphorylation and its role in *Leishmania* development. The studies on parasites development were very successful. The methods are well accepted by the scientific community and enabled for the first time in vitro analysis of the human for of *Leishmania*. This is now done in various aspects. It took us longer to start working on proteomics including protein phosphorylation. So, we are doing what we planned but it took us longer than we expected.

Overall, this was a successful and fruitful project.

I) Future Work

The group of the Israeli PI continue this project with Israel Science Foundation funds. Our goal is to identify key genes that regulate the post translational processes involved in *Leishmania* differentiation.

J)Reference List

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