International Journal for Parasitology 46 (2016) 75–82

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Protein kinase A signaling during bidirectional axenic differentiation in *Leishmania*

Sabine Bachmaier[a,1], Ronit Witztum[b,1], Polina Tsigankov[b], Roni Koren[b], Michael Boshart[a,*], Dan Zilberstein[b]

[a] *Ludwig-Maximilian-University Munich (LMU), Faculty of Biology, Genetics, 82152 Martinsried, Germany*
[b] *Technion-Israel Institute of Technology, Faculty of Biology, Haifa 32000, Israel*

ARTICLE INFO

Article history:
Received 10 March 2015
Received in revised form 14 August 2015
Accepted 6 September 2015
Available online 13 October 2015

Keywords:
Leishmania donovani
Development
Stage differentiation
Axenic culture
Protein kinase A
Protein phosphorylation

ABSTRACT

Parasitic protozoa of the genus *Leishmania* are obligatory intracellular parasites that cycle between the phagolysosome of mammalian macrophages, where they proliferate as intracellular amastigotes, and the midgut of female sand flies, where they proliferate as extracellular promastigotes. Shifting between the two environments induces signaling pathway-mediated developmental processes that enable adaptation to both host and vector. Developmentally regulated expression and phosphorylation of protein kinase A subunits in *Leishmania* and in *Trypanosoma brucei* point to an involvement of protein kinase A in parasite development. To assess this hypothesis in *Leishmania donovani*, we determined proteome-wide changes in phosphorylation of the conserved protein kinase A phosphorylation motifs RXXS and RXXT, using a phospho-specific antibody. Rapid dephosphorylation of these motifs was observed upon initiation of promastigote to amastigote differentiation in culture. No phosphorylated sites were detected in axenic amastigotes. To analyse the kinetics of (re)phosphorylation during axenic reverse differentiation from *L. donovani* amastigotes to promastigotes, we first established a map of this process with morphological and molecular markers. Upon initiation, the parasites rested for 6–12 h before proliferation of an asynchronous population resumed. After early changes in cell shape, the major changes in molecular marker expression and flagella biogenesis occurred between 24 and 33 h after initiation. RXXS/T re-phosphorylation and expression of the regulatory subunit PKAR1 correlated with promastigote maturation, indicating a promastigote-specific function of protein kinase A signaling. This is supported by the localization of PKAR1 to the flagellum, an organelle reduced to a remnant in amastigote forms. We conclude that a significant increase in protein kinase A-mediated phosphorylation is part of the ordered changes that characterise the amastigote to promastigote differentiation.

© 2015 Australian Society for Parasitology Inc. Published by Elsevier Ltd. All rights reserved.

1. Introduction

Leishmania donovani are intracellular parasitic protozoa that cause kala azar, a fatal form of visceral leishmaniasis, in humans. During their life cycle, the parasites shuttle between the midgut of female sand flies, where they proliferate as extracellular flagellated promastigotes, and the phagolysosomes of human macrophages, where they multiply as amastigotes with a flagellum remnant (Herwaldt, 1999). During the blood meal of an infected sand fly, virulent metacyclic promastigotes are transmitted to the host and phagocytosed by resident macrophages near the bite location (Chang and Dwyer, 1976). Following infection, the parasites encounter a temperature increase of >10 °C (from 26 to 37 °C) followed by a significant pH shift, from pH 7 in the vector to approximately pH 5 in the phagolysosome (Zilberstein, 2008).

Development of host-free systems using axenic culture conditions has enabled a better understanding of the molecular mechanism of *Leishmania* intracellular differentiation. *Leishmania donovani* differentiation can be induced by exposing promastigotes to the high temperature and acidity (37 °C, pH 5.5, 5% CO_2) typically found in the phagolysosome (Saar et al., 1998; Burchmore and Barrett, 2001; Zilberstein, 2008). The axenic amastigotes of both old and new world species have been shown to resemble animal-derived amastigotes as assessed by monitoring biochemical and molecular markers (Bates, 1994; Saar et al., 1998; Goyard et al., 2003; Debrabant et al., 2004). Based on morphological criteria, axenic *L. donovani* differentiation consists of four phases (Barak

* Corresponding author at: Biozentum, Genetik, Ludwig-Maximilians-Universität München, 82152 Planegg-Martinsried, Germany. Tel.: +49 89 2180 74600.
E-mail address: boshart@lmu.de (M. Boshart).
[1] These authors contributed equally.

http://dx.doi.org/10.1016/j.ijpara.2015.09.003
0020-7519/© 2015 Australian Society for Parasitology Inc. Published by Elsevier Ltd. All rights reserved.

et al., 2005): signal perception with no morphological change (phase I, 0–5 h); movement cessation and aggregation (phase II, 5–10 h); morphological transformation to amastigote-shaped cells (phase III, 10–25 h); and amastigote maturation (phase IV, 25–144 h). Analyses using high-coverage transcriptomic and proteomic approaches showed that *L. donovani* differentiation is well-regulated with ordered and coordinated changes in mRNA and protein abundance (Saxena et al., 2007; Rosenzweig et al., 2008b) that result in the parasites retooling their metabolic pathways for life in the new host environment. We recently described stage-specific protein phosphorylation patterns (Tsigankov et al., 2013) and have now completed an extensive examination of phosphorylation changes during promastigote-to-amastigote differentiation (Tsigankov et al., 2014).

When amastigote parasites residing in host macrophages are ingested by the vector during a blood meal, sensing of a colder and more alkaline environment in the fly gut induces development into the promastigote stage. Temperature and pH are also well-known developmental triggers in a wide range of other organisms such as *Trypanosoma brucei* (Czichos et al., 1986; Overath et al., 1986; Rolin et al., 1998; Engstler and Boshart, 2004; Szöör et al., 2013) and *Plasmodium falciparum* (Billker et al., 1997). *Trypanosoma brucei* is a kinetoplastid parasite closely related to *Leishmania* and the causative agent of human sleeping sickness. The regulatory pathway that controls its differentiation from the mammalian to the fly stage in culture involves a cascade of the protein phosphatases PTP1B and PIP39 (Szöör et al., 2006, 2010) and protein kinases RDK1 and RDK2 (Jones et al., 2014), indicating the dominant role of protein phosphorylation in the process. The high phosphorylation dynamics during promastigote to amastigote differentiation (Tsigankov et al., 2014) also suggests that protein phosphorylation cascades are involved in the developmental regulation of *Leishmania* parasites.

Several observations suggest an involvement of protein kinase A (PKA) signaling in regulation of the differentiation process in *Leishmania*: (i) a catalytic subunit of PKA termed c-Lpk2 (the orthologue of *T. brucei* PKAC3 (Bachmaier and Boshart, 2013)) is downregulated in the amastigote stage relative to promastigotes at the mRNA level and undergoes temperature-dependent regulation at the level of mRNA stability (Siman-Tov et al., 1996); (ii) the regulatory subunit LdPKAR1 has been suggested to be important for metacyclogenesis based on reverse genetic analysis (Bhattacharya et al., 2012); (iii) the abundance of phosphopeptides with consensus PKA phosphorylation motifs (RRXS, RXXS) shows stage-specific regulation (Tsigankov et al., 2013). These observations prompted us to study the phosphorylation kinetics of consensus PKA target motifs during the complete life cycle of *L. donovani*, i.e. during bidirectional axenic differentiation from promastigote to amastigote as well as from amastigote to promastigote forms. Using antibodies recognising the phosphorylation motif RXXS*/T* (*phosphorylation), we observed that phosphorylation faded completely and rapidly at the end of phase I of promastigote differentiation to amastigotes. During the reverse differentiation process from the amastigote to the promastigote stage, RXXS/T phosphorylations reappeared more gradually and peaked during terminal promastigote maturation. This study provides evidence that PKA-mediated phosphorylation is correlated with stage differentiation and is particularly important for promastigote-specific functions.

2. Materials and methods

2.1. Cell culture and differentiation of L. donovani

A cloned line of *L. donovani* strain 1SR was used in all experiments (Saar et al., 1998). Promastigotes were grown at 26 °C in medium 199 (M199, Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% foetal calf serum (FCS). Differentiation of promastigotes to amastigotes in axenic culture was carried out as in Barak et al. (2005). To initiate differentiation of axenic amastigotes to promastigotes, log phase axenic amastigotes ($0.5–1 \times 10^7$ cells/ml) were centrifuged using a table swing out centrifuge at 1244*g* for 10 min at room temperature (22–25 °C). Subsequently, the cell pellet was resuspended in promastigote medium (M199 at pH 7 + 10% FCS) to a density of 0.5×10^6 cells/ml and incubated at 26 °C. Aliquots were taken after 3, 6, 10, 16, 20, 24, 30, 33, 48 and 96 h. At each time point, parasites were collected, washed in PBS and used for microscopy and lysate sampling.

2.2. Cultivation of trypanosomes and generation of transgenic lines

Bloodstream forms of the monomorphic *T. brucei brucei* strain Lister 427, variant MiTat1.2 (Cross and Manning, 1973) were cultivated at 37 °C in a 5% CO_2 atmosphere in modified HMI-9 medium (Vassella et al., 1997) supplemented with 10% (v/v) heat-inactivated FBS. A homozygous gene deletion of the regulatory subunit of PKA (*PKAR*, Tb427tmp.02.2210) was generated. The targeting constructs pBSK.PKARko.puro and pBSK.PKARko.hygro contain a puromycin or hygromycin resistance cassette, respectively, flanked by actin untranslated regions (UTRs) and the *PKAR* UTRs. They were digested with *Kpn*I and *Xmn*I restriction enzymes prior to transfection. *PKAR* knock out cells were grown in the presence of 0.05 μg/ml of puromycin and 1 μg/ml of hygromycin B. The *L. donovani PKAR* open reading frame (ORF) (LdBPK_130160.1) was amplified from genomic DNA of *L. donovani* strain Lo8 and cloned into the pBSK.phleo backbone containing the phleomycin resistance cassette flanked by actin UTRs and *T. brucei PKAR* UTRs. The construct was digested with *Kpn*I and *Xmn*I for transfection, and antibiotic selection was performed by the addition of 2.5 μg/ml of phleomycin to the culture medium.

2.3. Antibodies

The gene encoding the *L. donovani* 2,3-trans-enoyl CoA isomerase precursor (LdteCi1, LdBPK_312400.1) was cloned into plasmid pGEX4T-1 and expressed in *Escherichia coli*-E151. Bacteria forming inclusion bodies of expressed LdteCi1 were extracted according to Sambrook and Russell (2001), and 0.5 mg of recombinant LdteCi1 protein was injected into rabbits as a contracted service by Sigma–Aldrich with five consecutive boosts. Antiserum was used at a dilution of 1:1000. For production of a polyclonal antibody against *T. brucei* PKAR, the full-length protein was expressed as a C-terminal hexahistidine fusion protein in *E. coli* M15 and purified under denaturing conditions using Ni–NTA columns (Qiagen, Hilden, Germany). After separation of the concentrated protein fractions on a 10% SDS gel, rabbits were immunised with Coomassie-stained PKAR containing gel slices and Freund's adjuvant followed by two consecutive boosts as a contracted service by BioScience (Göttingen, Germany). Affinity purification of the antibody was performed according to the method of Olmsted (1981). A dilution of 1:250–1:500 was used for western blot analysis. Other antibodies used were: anti-heat shock protein (Hsp)90 and anti-Hsp100 (Hubel et al., 1997), kindly provided by Dr. Joachim Clos, Bernhart Nocht Institute, Hamburg, Germany); L13D6 monoclonal antibodies against *Leishmania* paraflagellar rod (PFR) protein PFR2C (LinJ.16.1510 and LinJ.16.1520) and *T. brucei* PFR-A/C (Kohl et al., 1999), kindly provided by Dr. Philippe Bastin, Institute Pasteur, Paris, France), and Phospho-(Ser/Thr) PKA substrate antibody (1:1000; Cell Signaling Technology/New England Biolabs, Frankfurt, Germany; catalogue number 9621).

Download English Version:

https://daneshyari.com/en/article/2435961

Download Persian Version:

https://daneshyari.com/article/2435961

Daneshyari.com