Trypanosome TOR as a major regulator of cell growth and autophagy

Antonio Barquilla and Miguel Navarro*

Instituto de Parasitología y Biomedicina "López-Neyra"; Consejo Superior de Investigaciones Científicas, CSIC; [Spanish National Research Council], Granada, Spain

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Trypanosomatid protozoa parasites are responsible for tropical diseases, and undergo complex life cycles involving developmental forms adapted to insect vectors and vertebrate hosts. During their life cycle these parasites proceed through different forms in response to dramatic environmental changes and/or developmentally regulated programs. Successful progression of the parasite through its life cycle is highly dependent on the capacity of adaptation to distinct stresses involving processes such as autophagy. In eukaryotes, target of rapamycin (TOR) protein kinases act as a sensor, which integrates the nutritional and energetic status, adjusting cell metabolism and growth. Compromising cell viability in yeast and mammals leads to a reduction of TOR function, triggering processes aimed to overcome unfavorable conditions. This is partly achieved by TOR-mediated regulation of protein synthesis and recycling of cellular components by autophagy. In the last few years, autophagy has been described during developmental differentiation processes in Trypanosomatidae. However, no link between TOR signalling, autophagy, and differentiation has been described so far. This addendum is a commentary to the work published by our group, in which we discuss the possible role of TOR kinases, as a controller of cell growth and autophagy, in the regulation of differentiation processes during Trypanosomatidae life cycles.

Fitness of eukaryotic cells to variations in environmental or metabolic cues is a crucial parameter for survival. The modulation of cellular growth in response to nutritional or energetic status is exerted through the regulation and coordination of a wide variety of processes including metabolism, transcription and translation. There is currently substantial work pointing to the target of rapamycin kinase as being responsible for coupling nutrient availability to activation of anabolic processes leading to cell growth or, in contrast, those processes leading to cell adaptation to stress situations. TOR kinases function in two functionally and structurally multiprotein complexes or TORCs. This dual function of TOR kinases confers the capability of governing spatial and temporal cell growth separately. TOR complex 1 (TORC1) controls temporal aspects of cell growth through processes such as ribosome biogenesis, transcription, translation and repression of autophagy, while TORC2 controls spatial aspects of cell growth by actin cytoskeleton remodelling (reviewed in refs. 4 and 5).

Recently, we described the growth of the highly proliferative Trypanosoma brucei bloodstream form as being controlled by two functional independent TOR kinases, TbTOR1 and TbTOR2. TbTOR1 and TbTOR2 bind to and control signaling through TbTOR1 and TbTOR2, respectively. Whereas TbTOR2 regulates autophagy, TbTOR1 promotes cell growth and proliferation by positively regulating protein synthesis, cell size, cell cycle progression and RNA polymerase I localization to the nucleus. All data suggested that overall TOR functions were conserved in this early-branched eukaryote, including the two distinct TOR complexes described in metazoans and yeast. However, several new findings have emerged that differ from those described for other eukaryotes. We found two additional TOR-like proteins, TbTOR-like 1 and 2, with a similar domain structure present in TOR proteins, although our results suggest that they are not components of the classical TORC1 or TORC2. Furthermore, rapamycin treatment of bloodstream trypanosomes resulted in a pronounced reduction of cell proliferation but, interestingly, by inhibiting TORC2 signalling, rather than affecting TORC1 signalling as occurs in other eukaryotes. Thus, it is important to bear in mind that rapamycin cannot be used for triggering the autophagic process in T. brucei because TbTOR1 is rapamycin-insensitive. Treatment of the T. brucei bloodstream form in the nanomolar range inhibits TORC2, producing actin depolarization which interferes with endocytosis and cytokinesis.

**Autophagy is Triggered upon TbTOR1 Loss-of-Function**

We asked whether TbTOR1 was involved in the regulation of autophagy in this ancient eukaryote. To address this question, cells depleted of TbTOR1 were examined by electron microscopy. TbTOR1-depleted cells showed an increased number of double-membrane structures containing cytoplasmic material, dilatation or abnormal appearance of endoplasmic reticulum and membranous cellular components that rearrange in membranous whorls called myelin figures or multi-lamellar membrane (Fig. 1). This suggests that an autophagic process takes place in the T. brucei bloodstream.
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The bloodstream and procyclic forms of Trypanosoma brucei are found in the vertebrate, and the procyclic and epimastigote form in the tsetse fly. The preadapted nonproliferative stages are the stumpy bloodstream form in the vertebrate and metacyclic in the tsetse fly. It is tempting to speculate that TbTOR1 signaling may play an important role in these developmental transitions, as TOR inactivation confers resistance to overcome unfavorable conditions.

During the last few years, autophagy has been characterized in life stages of Leishmania and T. cruzi characteristic of the invertebrate host. In the insect vector, noninfective proliferating epimastigotes differentiate to the infective metacyclic trypomastigotes by a process called metacyclogenesis, a process triggered by nutritional stress. It has been demonstrated that autophagy is triggered during metacyclogenesis, transition from the log phase to stationary phase of growth, and starvation periods. Moreover, a defect in the autophagic process compromises cell viability upon these situations and interferes with the differentiation to the infective metacyclic form, a crucial process for survival and infectivity in the mammalian host.

Characterization of autophagy requires, in addition to ultrastructural analysis by electron microscopy, further confirmation by a marker such as the Atg8/LC3 protein. This marker has been used successfully in the characterization of autophagy in the related organisms T. cruzi and Leishmania. Unfortunately, on the basis of our initial observations, the Atg8/LC3 T. brucei orthologues did not behave this way. A yellow fluorescent protein fused with either TbAtg8A or TbAtg8B (TcAtg8.1 orthologues) did not show punctate distribution or cleavage upon nutrient starvation or TbTOR1 depletion in monomorphic bloodstream trypanosomes (Barquilla A and Navarro M, unpublished data), consistent with a previous report. Similarly, an additional Atg8 orthologue, TcAtg8.2, did not function as an autophagosome marker in T. cruzi. Future work will be required to identify an autophagosomal marker in T. brucei.

In other eukaryotes, TORC1 inhibition by distinct stresses leads to an enhanced resistance to unfavorable conditions. Accordingly, it is reasonable that reduced protein synthesis and activation of autophagy mediated by TORC1 inactivation would confer partial resistance to unfavorable conditions in trypanosomatids. To test whether TbTOR1 and TbRaptor (TORC1)-depleted cells showed enhanced viability during stress situations, we cultured these cell lines and the untransfected control without changing the medium, and followed the cell number daily (Fig. 2). Interestingly, TbTOR1- and TbRaptor-depleted cell lines, but not untransfected or uninduced controls, exhibited enhanced resistance and survived for a longer period of time. At day 4, no living cells were detected in controls, whereas induced RNAi cell lines remained viable. This result suggests that reduction of TbTORC1 signaling confers partial resistance to stress situations, essential for overcoming temporal unfavorable conditions.

**Role of Autophagy during Developmental Differentiation in Trypanosomatids**

Trypanosomatids exhibit extraordinarily complex life cycles involving an insect vector and a vertebrate host. Accomplishment of the life cycle requires accurate adaptation to distinct hosts and distinct compartments within the vector. Little is known about parasite adaptation to the drastic environmental changes encountered as the parasite traverses through the insect, which is accomplished by modulating several processes, including metabolic and morphological readjustments. In addition, trypanosomatids undergo complex developmentally-regulated differentiation processes as a mechanism for preadaptation to the successive host. The T. brucei life cycle includes proliferation stages, the long-slender bloodstream form in the vertebrate, and the procyclic and epimastigote form in the tsetse fly. The preadapted nonproliferative stages are the stumpy bloodstream form in the vertebrate and metacyclic in the tsetse fly. It is tempting to speculate that TbTOR1 signaling may play an important role in these developmental transitions, as TOR inactivation confers resistance to overcome unfavorable conditions.

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**Figure 1.** Ultrastructural (TEM) analysis of cellular structures (black arrows) reminiscent of autophagy in the bloodstream form trypanosomes upon TbTOR1 RNA interference. (A) TbTOR1-depleted cells contain abundant vesicles with a range of distinct morphologies similar to those of autophagic vacuoles (AV) in mammals and yeast. AV morphologies include double-membraned vesicles exhibiting heterogeneous intralumenal contents. A rare or dilated endoplasmic reticulum profile can also be seen. (B) Vacuole containing membrane whorls. The punctate pattern of membranes resembles those of rough endoplasmic reticulum. (C) Detail of a double-membraned vacuole. (D) Multilamellar bodies, another variant of AV, are also common upon TbTOR1 RNAi. Bars, 0.25 μm.
Figure 2. Cells with reduced TORC1 signalling showed enhanced resistance to unfavorable conditions. Bloodstream form cell lines for inducible TbTOR1 RNAi, TbRaptor RNAi and untransfected control were seeded at 2 x 10^5 cells per ml in order to achieve the maximum density (8–9 x 10^6 cells per ml) before cell cycle blockade occurs in induced RNAi cells. Cells reached 2.5 x 10^6 cells per ml the first day, and 8–9 x 10^6 cells per ml in the second, but cell density quickly drops afterwards. RNA interference was induced by addition of doxycycline (1 mg/ml) to the cultures at day 0. (A) Uninduced (DOX-) RNAi cell lines and the untransfected control quickly died after reaching maximum density at day 3. No live cells were detected on day 5. (B) Induced (DOX+) TbTOR1 and TbRaptor RNAi cell lines are more resistant than the untransfected control. At day 4, cell density in RNAi cell lines was approximately ten-fold higher than the untransfected control. At day 5, RNAi cell lines were still viable, whereas no live cells were detected in the untransfected control.

During T. brucei infection, cells multiply in the bloodstream where a constant and nutrient-rich milieu allows rapid proliferation of the long-slender bloodstream form. Once parasitemia reaches certain levels, trypanosoma differentiate to a nondividing short stumpy bloodstream form by a quorum-sensing mechanism.10 Stumpy forms are metabolically preadapted for the drastic environmental change that occurs upon transmission to the fly’s midgut, being more resistant to distinct stresses.13 When the insect ingests the blood meal infected with trypanosomes, quiescent stumpy forms are capable of differentiating rapidly and synchronously to procyclic forms, activating cell cycle progression and establishing an effective infection. Interestingly, Herman and coworkers verified in an elegant study that glycosomes, peroxisome-like organelles required for compartmentalization of enzymes involved in carbohydrate metabolism, are targeted for degradation by autophagy upon differentiation programs in T. brucei.8 This autophagic process is triggered during the differentiation from long-slender to short stumpy, but mainly from short stumpy to procyclic forms. TOR function may have a major role in this differentiation process as occurs in fungi.14 Transition from the short stumpy bloodstream to the procyclic form involves drastic changes in the source of energy from a glucose- and nitrogen-rich environment to a glucose-depleted and proline-rich one. In this situation, it is conceivable that TbTOR1 activity may be reduced as occurs in other eukaryotes, thus triggering an autophagic process as occurs in mammals and yeast.15

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References