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Intracellular Signaling Pathways Involved in Cell Differentiation in Trypanosomatids

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Abstract: Knowledge of cell signaling pathways in trypanosomatids is crucial for the future design of new drugs to treat diseases caused by these parasites. The publication of the complete genome sequences of three pathogenic trypanosomatids, *Trypanosoma brucei*, *T. cruzi* and *Leishmania major*, revealed numerous protein members of signaling pathways that modulate important processes, such as cell differentiation. Even so, little is known about the role that these proteins play in the physiology of trypanosomatids. This review aims to stimulate discussion on this subject to encourage further studies of the signaling pathways involved in the cell differentiation of trypanosomatids.

Keywords: Cellular differentiation, signaling pathways, protein kinases, *Trypanosoma cruzi*, *Trypanosoma brucei*, *Leishmania*.

INTRODUCTION

Cell differentiation during the life cycle of trypanosomatid parasites comprises integrated changes in cell morphology, metabolism, gene expression and signaling pathways. These processes are different from most other eukaryotic life forms, especially the multicellular organisms. While in higher eukaryotes cell differentiation is a "one way" phenomenon, in trypanosomatids the characteristic morphotypes of a given genus are interchangeable throughout its life cycle, adjusting to environmental factors present in each one of their hosts [1]. These structural and physiological changes are progressive and result in altered infectivity, a critical feature of a parasite [2]. The morphotypes that characterize a certain genus are cell shape, dimensions and the position of the complex kinetoplast-flagellar pocket relative to the nucleus [3,4] (Fig. **1**).

The complex morphological and biochemical changes during cell differentiation in trypanosomatids are directed by different ligands and/or stimulatory molecules present in their environment [5,6]. These changes are mediated by signaling pathways that coordinate processes such as cell growth, development and differentiation. In mammalian systems, this coordination involves several signaling scaffolds, such as protein kinases and phosphatases, G proteins and second messengers. Interestingly, some receptors for mammalian ligands have been described in a few trypanosomatids [5], although genes encoding for G proteins have not been found in their genomes [7-9]. The presence of receptors for several vertebrate extracellular signaling molecules has been well documented [5,6]. These signaling molecules display a wide range of activities, such as the induction or inhibition of target cell survival, proliferation and differentiation. When directly interacting with different parasites, these molecules exert similar activities as they do in mammals [6].

Protozoa in the genus *Herpetomonas* are typically taxonomically characterized by the presence of promastigote, paramastigote and opisthomastigote developmental stages [10] (Fig. **1**). This genus is characterized by a life cycle involving only an invertebrate host, although in some cases these protozoans can parasitize plants; some *Herpetomonas* species can be human pathogens, causing a diffuse cutaneous leishmaniasis-like infection in immunodeficient and immunocompetent patients [11,12]. Platelet-activating factor (PAF), a naturally-occurring phospholipid mediator [13], triggers the cell differentiation of *H. muscarum muscarum* and *T. cruzi*, which is completely abrogated by a specific PAF receptor antagonist (WEB 2086), indicating that these parasites may have receptors for PAF [14,15]. Additionally, PAF-stimulation of cell differentiation in *H. m. muscarum* involves a complex signal transduction pathway that includes Ser/Thr and Tyr protein phosphatases, PLC, PKC and MAP kinases as upstream elements as well as CK2 as a target. This study demonstrated for the first time that a lipid mediator could stimulate the activation of CK2, leading to parasite differentiation [16]. The isolation of a PAF-like synthesized by *T. cruzi*, which stimulates the differentiation of the parasite itself from epimastigotes to metacyclic trypomastigotes, highly infective to mammalian cells [17], suggests that PAF-triggered cell differentiation in trypanosomatids is a phenomenon that may actually occur in nature.

Genomic and proteomic studies of *T. brucei*, *T. cruzi* and *L. major* have allowed for a description of the entire kinome for these trypanosomatids [18]. This analysis demonstrated that although these protozoans lack members of the receptor-linked or cytosolic tyrosine kinase families, they have abun-

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dant quantities of the STE and CMGC families of protein kinases [18]. The STE and CMGC families of protein kinases are involved in regulating cell cycle control, responses to stress during their complex life cycles and cell differentiation [7-9,18].

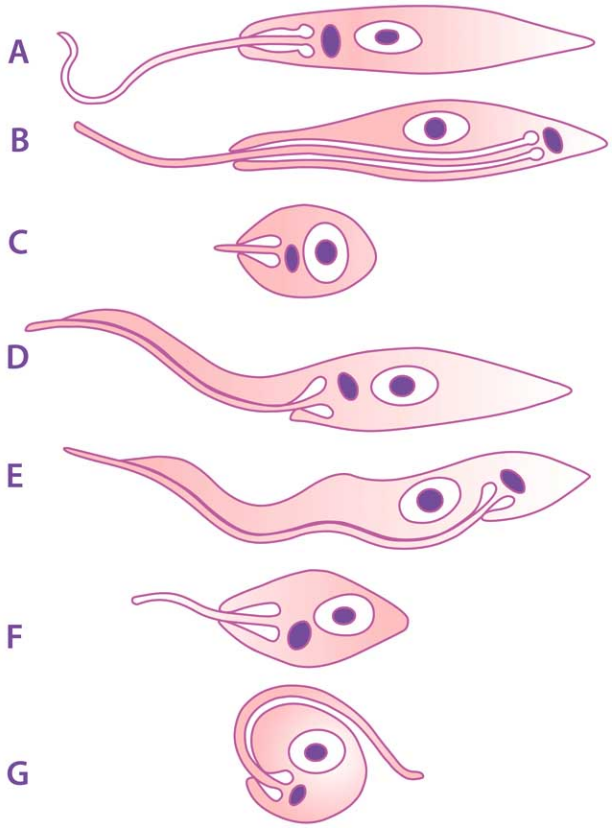

Fig. (1). The most common morphotypes of trypanosomatids. A: promastigote; B: opisthomastigote; C: amastigote; D: epimastigote; E: trypomastigote; F: choanomastigote; G: spheromastigote. Diagram based on Hoare and Wallace [3].

Trypanosoma cruzi

Trypanosoma cruzi, the etiological agent of Chagas disease, has a complex life cycle, alternating between a triatomine insect vector and a mammalian host. Epimastigote forms, after replicating in the insect midgut, differentiate into infective metacyclic trypomastigotes, which are released with feces when the insect feeds on the mammalian host, thus enabling infection of the latter and completion of the parasite's life cycle [19]. The differentiation enables *T. cruzi* to adapt to environmental changes through morphological (Fig. **2**) and functional transformations that involve the activation of protein kinase cascades and a variety of complex physiological signals [6]. Little is known about the potential of the mammalian or vector-derived molecules to regulate the growth and differentiation of the parasites. Even so, a number of *in vitro* systems have been proposed to induce metacyclogenesis, including the use of artificial triatomine urine media [20].

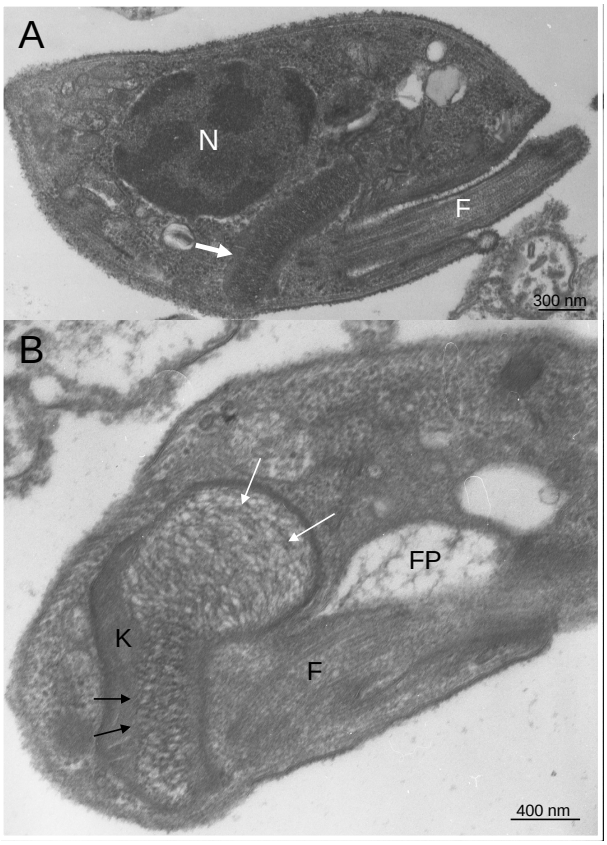

Fig. (2). Cell differentiation of *Trypanosoma cruzi*. A. Transitional form between amastigote and trypomastigote obtained from the supernatant of LLC-MK2 cell culture previously infected with trypomastigotes. The kinetoplast (white arrow) although showing a morphology (bar- or rod-shaped) typical of the amastigote form, is located close to the nucleus. N = nucleus; F = flagellum. **B.** Transitional form between epimastigote and trypomastigote obtained from a stationary growth phase culture. The kinetoplast (K) shows a morphology in which part of the DNA fibers are arranged as in epimastigotes (black arrows) and part as in trypomastigotes (dispersed filaments – white arrows). FP = flagellar pocket; F = flagellum. *T. cruzi* epimastigotes of the CL-Brener clone were maintained at 28°C by weekly transfers in liver infusion tryptose (LIT) medium supplemented with 10% fetal calf serum (FCS). Four-day-old culture forms at the mid-log phase of growth were used in all experiments. Tissue culture trypomastigotes and amastigotes were obtained from the supernatants of 5 to 6-day-old infected LLC-MK2 cells maintained in RPMI-1640 medium supplemented with 2% FCS at 37°C in a 5% humidified CO_2 atmosphere [21]. The parasites in **A** and **B** were prepared for transmission electron microscopy as described [21]. Ultra-thin sections obtained with a Leica (Nussloch, Germany) ultramicrotome were stained with uranyl acetate and lead citrate, and observed using a FEI Morgagni F 268 (Eindhoven, The Netherlands) transmission electron microscope operating at 80 kV [21].

Fraidenraich and co-workers [22] showed that intestinal extracts of *Triatoma infestans*, containing peptides derived from hemoglobin breakdown, stimulated adenylate cyclase and induced metacyclogenesis. In addition, peptides released from the proteolysis of fibronectin increased cAMP levels in

trypomastigotes [23]. cAMP analogs and phosphodiesterase inhibitors have also been reported to induce the differentiation of this parasite [24,25] because proliferation decreased due to decreased DNA or RNA synthesis [26,27]. In trypanosomatids, like in most eukaryotes, cAMP is synthesized by adenylyl cyclases (ACs) and degraded by phosphodiesterases (PDEs) [28]. In most mammalian systems, cAMP regulates numerous physiological processes through cAMP-dependent protein kinase (PKA). By binding the regulatory subunit of the enzyme and releasing it from the catalytic subunit, cAMP stimulates PKA activity; however, little is known about PKA in trypanosomatids. A putative regulatory subunit of PKA was identified in *T. brucei* with an associated kinase activity [29], but cGMP and not cAMP stimulated this kinase activity [29]. The presence of PKA activity was also suggested in *T. cruzi* [30] and the expression of this putative PKA catalytic subunit appears to be developmentally regulated [31]. Putative PKA catalytic subunits have also been identified in *L. major* and *L. donovani* [28,32,33]. *T. cruzi* genome contains all of the members of the cAMP cascade, such as adenylate cyclase, phosphodiesterases and catalytic and regulatory subunits of PKA [7-9,34]. Additionally, trypanosomatid adenylyl cyclases have a completely different structure in comparison to their mammalian counterparts. *T. cruzi* adenylyl cyclase is similar in structure to receptor guanylate cyclases and contains a putative extracellular ligand-binding domain and a cytoplasmic domain. The extracellular domain of the receptor may directly activate the intracellular components of the adenylyl cyclase without requiring activation by heterotrimeric G-proteins or other regulatory factors [35,36]. Even though G proteins cannot be found in the now complete trypanosome genomes [7-9], Coso and co-workers [37] characterized a Gi-protein from *T. cruzi* epimastigote membranes. Blocking the PKA function using a genetic approach allowed them to identify some putative proteins that interact with the PKA catalytic subunit of *T. cruzi* (TcPKA), such as PI3 kinase, a mitogen-activated extracellular signal-regulated kinase (MAPK) and a cAMP-specific phosphodiesterase (PDEC2) [28,37]. Intriguingly, an adenylyl cyclase stimulated by calcium, which interacts with paraflagellar rod protein [38] was suggested to be one of these proteins.

"Crosstalk" between adenylyl cyclase and phospholipase C during events of growth and differentiation in *T. cruzi* has also been described. *T. cruzi* exposure to mitogenic factors in fetal calf serum (FCS) promoted the inhibition of cAMP production and stimulated phospholipase C (PLC), leading to the accumulation of inositol phosphates (InsP) and diacylglycerol (DAG) [27]. In a similar way, chicken α^D-globin was shown to produce dose-dependent inositol triphosphate (InsP3) accumulation as a consequence of phosphatidylinositol phosphate-PLC (PtdIns-PLC) activation [39] and transient calcium mobilization [40]. Lipids and lipid kinases related to the inositol cycle were shown to display important roles in signal transduction processes, such as cell differentiation [40]. Wainszelbaum and co-workers [41] showed that free fatty acids (FFA), primarily oleic acid (OA), found in the intestinal tract of *T. infestans* induced *T. cruzi* metacyclogenesis through a signaling pathway involving *de novo* diacylglycerol biosynthesis, protein kinase C (PKC) activation and Ca^{2+} mobilization [41]. The PKA pathway does not appear to be activated in these cells, as previously described by Fraidenraich and co-workers [22]. It is possible that activation of the PKA pathway is blocked in the presence of the PKC-activating stimuli. Thus, activation of one signaling pathway may block an alternative pathway, leading to the same differentiating effects [41]. In addition, these authors found that OA directly stimulated the *in vitro* activity of *T. cruzi* PKC in a dose-dependent manner and induced the selective translocation of PKC isoenzymes. Interestingly, PKC isoforms were differentially expressed in the infective and non-infective *T. cruzi* stages, suggesting that each isoenzyme possesses specific functions that could be related to the diverse environments that the parasite encounters during its complex life cycle [42]. Indeed, activation of stage-specific genes during metacyclogenesis was documented [43]. Nevertheless, very little is known about the differentiation process and the changes that occur during the stage-regulated gene expression program.

Platelet-activating factor (PAF) was shown to promote the enhancement of the secreted phosphatase activity through the activation of PKC in *T. cruzi* [44] and a PAF-like phospholipid produced by these parasites triggered its own cell metacyclogenesis [17]. The signaling pathways are modulated through the antagonistic activities of highly specific protein kinases and protein phosphatases that control a number of processes, including metabolic pathways, cell-cell communication, cell growth and proliferation and gene transcription. Analyses of *T. cruzi* phosphatome identified 86 phosphatase catalytic domains in these parasites [45].

Recently, Nakayasu *et al.* [46] sequenced 221 phosphorylation sites in 119 proteins from *T. cruzi*. Of those 221 newly identified phosphorylation sites, 148 (65.5%) were on serines, 57 (25.2%) on threonines and 8 (3.5%) on tyrosines. The identified phosphoproteins were categorized based on their role in cell structure, motility, transportation, metabolism, pathogenesis, DNA/RNA/protein turnover and signaling [46]. In addition, approximately 2% of the *T. cruzi* genome encodes protein kinases, suggesting a major regulatory role in controlling parasite development and function [47].

Trypanosoma brucei

Trypanosoma brucei is a protozoan flagellate that causes sleeping sickness in tropical Africa and is responsible for ~30,000 deaths per year [19]. The life cycle of *T. brucei* alternates between the midgut of the tsetse fly vector and the bloodstream of mammalian hosts. These trypanosomes are pleomorphic in the mammalian bloodstream, transforming from a replicating long slender form into a non-dividing short stumpy form pre-adapted for transmission into the tsetse fly. The differentiation is signaled through a density-sensing mechanism as parasite numbers increase in response to an unidentified parasite derived signaling factor, stumpy induction factor (SIF) [48,49]. In the midgut of the vector, the short stumpy form differentiates into a replicating procyclic form, migrating to the salivary glands and differentiating into infective forms [50]. The most remarkable marker for differentiation of these parasites is the exchange of the main surface antigens: variant surface glycoprotein (VSG) in the bloodstream stage [51] and procyclin in the procyclic stage [52]. Both proteins are attached to the cell surface by a glycosylphosphatidylinositol (GPI)-anchor [53]. VSG is a

homodimer that surrounds the cell and forms a surface coat that is impenetrable to host serum macromolecules. Procyclin genes encode a more restricted family composed of two basic isoforms, EP and GPEET, defined by amino acid sequences of C-terminal repeat domains, glu-pro for EP and gly-pro-glu-glu-thr for GPEET [52,54,55]. These domains confer resistance to hydrolysis while the parasite is located in the vector. *In vitro* experiments showed that 12 hours after the initial stimulus for differentiation, the surface coat is complete and differentiating trypanosomes enter into their first cell cycle as fully transformed procyclics [56]. During the differentiation process, the VSG coat is actively removed through GPI hydrolysis and endoproteolysis [57-59]. GPI hydrolysis is mediated by an endogenous GPI-specific phospholipase C (GPI-PLC) that is found exclusively in the bloodstream stage of the parasite [60,61]. The VSG GPI-anchor is present early in the differentiation process in the starting stumpy population. The other mode of release, endoproteolysis, is mediated by a zinc metalloprotease that is up regulated during differentiation [59]. A zinc metalloprotease gene (MSP), coding for three different proteins (MSP-A, -B, -C) that are differentially expressed into the differentiation forms, was found in the *T. brucei* genome [62,63]. In addition, to prevent loss of the newly synthesized procyclin coat, the structure of the GPI anchor precursor that is attached to surface proteins must change. The structure changes from that found in the bloodstream cells, which is susceptible to GPI-PLC activity [64,65], to that found in procyclic parasites, which is resistant [66]. There are differences in GPI anchors in the different forms and the bloodstream form has a diacylglycerol structure, while the procyclic form presents a lysoacylglycerol acylinositol structure. Thus, the GPI biosynthetic shift prevents the newly synthesized procyclin from being shed [56]. Many factors have been shown to induce the differentiation process *in vitro*; for example, the addition of the Krebs cycle intermediate citrate/*cis*-aconitate (CCA) to the culture medium speeds up the rate of differentiation [67-69]. A variation in the temperature range from 37ºC to 20ºC induces the immediate and reversible expression of the insect stage-specific EP procyclin surface protein in slender and stumpy-stage bloodstream trypanosomes *via* a post-transcriptional mechanism. In stumpy cells, the cold shock elicits up to a 1000-fold increased sensitivity to CCA [70]. To differentiate into the next developmental stage, parasites must have evolved mechanisms to sense and respond to the microenvironment imposed by their different hosts to adapt their biology to extracellular or intracellular survival [71]. These mechanisms are post-translational events like signaling pathways that are mediated by proteins kinases and phosphatases. A recent study of the phosphoproteome of *T. brucei* showed that most *T. brucei* phosphorylation sites were detected on serine residues (75%) and threonine residues (21.5%) and, to a lesser extent, on tyrosine residues (3.5%). Of the 491 *T. brucei* phosphoproteins viewed in the phosphoproteome study, 44 were kinases [72]. The recent kinome study showed the existence of 170 ePKs (conventional protein kinases) and 12 aPKs (atypical protein kinases) [72]. The trypanosomatid kinome lacks members of the receptor-linked or cytosolic tyrosine kinase families, but there is evidence that several proteins are phosphorylated on tyrosine residues in kinetoplastids [73, 74], presumably though the activity of dual-specificity protein kinases [such as Wee and dual specificity tyrosine phosphorylation-regulated kinase (DYRK) family kinases] [47,72]. Tyrosine phosphatase activity also shows marked differences among different life cycles stages in *T. brucei* [75]. The trypanosomatid-specific protein tyrosine phosphatase *Tb*PTP1 has a prominent role in the differentiation process. When *Tb*PTP1 activity is inhibited by RNAi or biochemically inhibited with the specific inhibitor BZ3, the process of differentiating into procyclic forms occurs spontaneously in the absence of any exogenous trigger. Thus, the form shows all of the markers from the procyclic stage, such as EP and GPEET procyclins and the stage-specific cytoskeletal antigen CAP5.5 [76]. The stumpy cells were uniformly arrested in G1/G0 and initiated differentiation synchronously and efficiently [77]. The enzyme *Tb*PTP2 seems not to contribute to the observed differentiation phenotype because RNAi against this enzyme did not elicit differentiation of the bloodstream forms [76], which suggests that *Tb*PTP1 normally inhibits this differentiation step [76,78]. Although only a small proportion of cultured monomorphic bloodstream forms were differentiated, uniformly arrested stumpy forms differentiated with an efficiency of nearly 100% when exposed to the inhibitor. This result suggested a model in which cell-cycle arrest was required for differentiation and in which this arrest was prevented by the activity of *Tb*PTP1 in the bloodstream. Once in the fly, inactivation of this enzyme would allow those cells that were able to differentiate to do so [2]. The trypanosomatid kinome has an abundance of STE and CMGC family protein kinases, which include the members of the canonical MAP kinase cascade (MAPKs, MAPKKs and MAPKKKs) involved in environmentally regulated cell cycle control, differentiation and the cellular response to various stress signals [18]. These two families alone make up more than 40% of the conserved trypanosomatid kinome [76]. Various members of the MAPK family and MAPK-like kinases were found in *T. brucei*. The first MAPK to be identified and characterized, KFR1, is essential for procyclic forms. The bloodstream form of KFR1 shows an increased abundance of mRNA and proteins compared with the procyclic form [79]. Gene-targeting experiments revealed that MAPKs were directly implicated in the growth control and differentiation of *T. brucei*. Null mutants of *Tb*MAPK2, the ortholog of MPK 4 in *Leishmania*, proliferated normally in cultures in the bloodstream stage but differentiated less efficiently in the procyclic insect stage, resulting in non-synchronous cell cycle arrest [80]. Genetic deletion of *Tb*MAPK5, the ortholog of MPK 5 of *Leishmania*, did not interfere with the proliferation of *T. brucei* at the procyclic stage, both in cultures and during the development of the tsetse fly [81]. However, the absence of *Tb*MAPK5 resulted in low-rate infections in mice, accompanied by premature differentiation in the non-proliferative stumpy form. These results suggest that *Tb*MAPK2 and *Tb*MAPK5 may have complementary roles in the control of proliferation in procyclic and bloodstream *T. brucei* developmental stages, respectively. The cyclin-dependent kinase (CDK) family is comparatively large in trypanosomatids, with 11 members in *T. brucei*. CDK requires an activating cyclin partner for activity and analysis of the genome revealed 10 orthologous cyclins in the parasite (CYC2-CYC11) [43]. Some CDKs require phosphorylation in the conserved threonine residue by a cdc2-activating kinase (CAK). Down-regulation of

CRK3 (cdc2-related kinase protein) in both the procyclic and the bloodstream form resulted in a mitotic block and growth arrest [82]. It is currently postulated that cAMP plays an important role in the differentiation from slender to stumpy forms, although much of the data to support this hypothesis is circumstantial. Some genes within the *T. brucei* kinome have been found to be related to two genes in the AGC family (Tb09.211.2410 and Tb11.02.2210) that may code for proteins related with the catalytic and regulatory PKA subunits, respectively [72]. In *T. brucei*, two distinct peaks of adenylyl cyclase activity were observed during differentiation from the bloodstream form to the procyclic form. It has been suggested that the use of an analog of cAMP, 8-pCPT-cAMP, induced a slender-to-stumpy differentiation in the pleomorphic bloodstream form [49]. However, a requirement for cAMP or cyclase activity in this or in the subsequent release of variable surface glycoprotein was not apparent [83,84]. The use of another cell-permeable analog of cAMP, 8-pCPT-2'-*O*-Me-cAMP, inhibited the proliferation of the bloodstream form, but its PDE hydrolysis-resistant equivalent, Sp-8-pCPT-2'-*O*-Me-cAMP, does not show this capacity for inhibition. However, the most common downstream product of the hydrolysis of 8-pCPT-2'-*O*-Me-cAMP (8-pCPT-2'-*O*-Me-5'-AMP/-adenosine) is a more potent inhibitor [85]. High concentrations of extracellular cAMP, 5'-AMP or adenosine did not significantly affect the proliferation of *T. brucei*, suggesting that the antiproliferative effect caused by the nucleotide analogs was mediated by an intracellular "receptor" [85]. Membrane permeable 5'-AMP/adenosine analogs caused cell-cycle arrest and differentiation of slender forms into stumpy-like forms [85]. These data suggest that the observed effect of cAMP was due to the downstream products of PDE hydrolysis. The *T. brucei* line that was defective to differentiation (DiD1) showed an increased expression of PAD genes. The codes of the DiD1 were closely related to members of a family of 14 transmembrane-spanning proteins in the major facilitator superfamily [86]. RNAi against these genes was used in a pleomorphic lineage of T. *brucei* (AnTat 1.1 90:13). When this gene was ablated, the parasite presented a reduced differentiation rate of cells exposed to CCA and decreased the response of stumpy cells to citrate at 20ºC [86]. Another *T. brucei* that had mutated and lacked the *Tb*ICP locus encoded for an endogenous cysteine peptidase inhibitor (ICP) and showed marked differences during the differentiation process. The null mutant show an accelerated coat exchange when stimulated with CCA in comparison to the wild type. A synthetic inhibitor of cysteine peptidases (K11777) could act in the same way [87], suggesting a role for the CPs in the differentiation process of the parasite. The proteins *Tb*ZFP1 and *Tb*ZFP2 that have a RNA-binding zinc finger CCCH motif could be involved in the differentiation process as well. An RNAi against *Tb*ZFP2 inhibited the initiation of the process and overexpression of the RNAi generated a procyclic-specific morphological type [88]. *T. brucei* showed an alteration in kinetoplast positioning during differentiation; the bloodstream form presented this kinetoplast in the very posterior end, while the procyclic forms presented this kinetoplast half way between the nucleus and the posterior end. Ablation of *Tb*ZFP1 caused an alteration in kinetoplast migration in parasites and the null mutant changed the surface markers but maintained the kinetoplast in the same position as the bloodstream form and presented many cell cycle defects [88]. During the differentiating process, an alteration in the intracellular calcium concentration was shown to occur [89]. The functions of the proteins that require the binding of calcium for activity could be regulated in parallel with progression through the cell cycle as a consequence of the alteration in cytosolic calcium levels.

Leishmania

Leishmania species are the causative agents of leishmaniasis, which currently affects millions of people and threatens 350 million people in 88 countries [90]. Infection in humans may present as different forms of leishmaniasis depending on the *Leishmania* species causing the infection [91]. The clinical symptoms of this disease range from cutaneous to mucocutaneous to visceral. Some species, such as *L. major*, *L. tropica* and *L. mexicana*, may cause cutaneous forms of the disease that are typically displayed as self-healing skin ulcers on exposed parts of the body [90]. Infection by species such as *L. braziliensis*, *L. panamensis* and *L. guyanensis* may cause mucocutaneous forms of leishmaniasis that initially present as cutaneous lesions but may then spread and lead to partial or total destruction of mucous membranes, which are often resistant to treatment or cure [90]. *L. donovani* and *L. chagasi* infections may result in a chronic visceral disease that affects the liver and spleen and are usually fatal if left untreated [90,91].

Leishmania spp. are heteroxenic parasites that present a complex life cycle that includes mammalian and insect hosts; the parasite forms are adapted to the environmental conditions within their hosts [92,93]. The promastigote form that resides in the sandfly insect vector occurs in the form of a multiplicative extracellular flagellated cell. The amastigote form resides in macrophages of the mammalian host, to which they are pathogenic. Upon infection of the mammalian host, metacyclic promastigotes are rapidly phagocytized by macrophages and differentiate into the proliferative intracellular amastigote form that survives and multiplies in a parasitophorous vacuole. Within the mammalian host, the amastigote life stage can survive for several months or years [92,93]. After transmission into the sandfly vector, the amastigotes rapidly undergo differentiation into the procyclic promastigote form, which colonizes the gut of the sandfly and then migrates to the sandfly foregut. In the foregut, the procyclic promastigote form differentiates into the metacyclic promastigote form and, upon a subsequent blood meal, is transmitted to a mammalian host [19,91,92,94].

Surviving the environmental variations during the life cycle of *Leishmania* spp. is a challenge that these parasites have overcome, evolving into more adapted and specialized forms. Studies of the differentiation events in *Leishmania* have mostly examined metacyclogenesis and the differentiation of metacyclic promastigotes into amastigotes in the macrophages of the mammalian hosts. Metacyclogenesis is the differentiation process that turns non-infective procyclic promastigotes into metacyclic infective promastigotes before they are injected by the sandfly vector into the mammalian hosts. Various biochemical changes occur that pre-adapt the parasite to living in the mammalian host. Although the steps for *Leishmania* differentiation *in vitro* have been recognized, the signaling pathways and molecular events that mediate

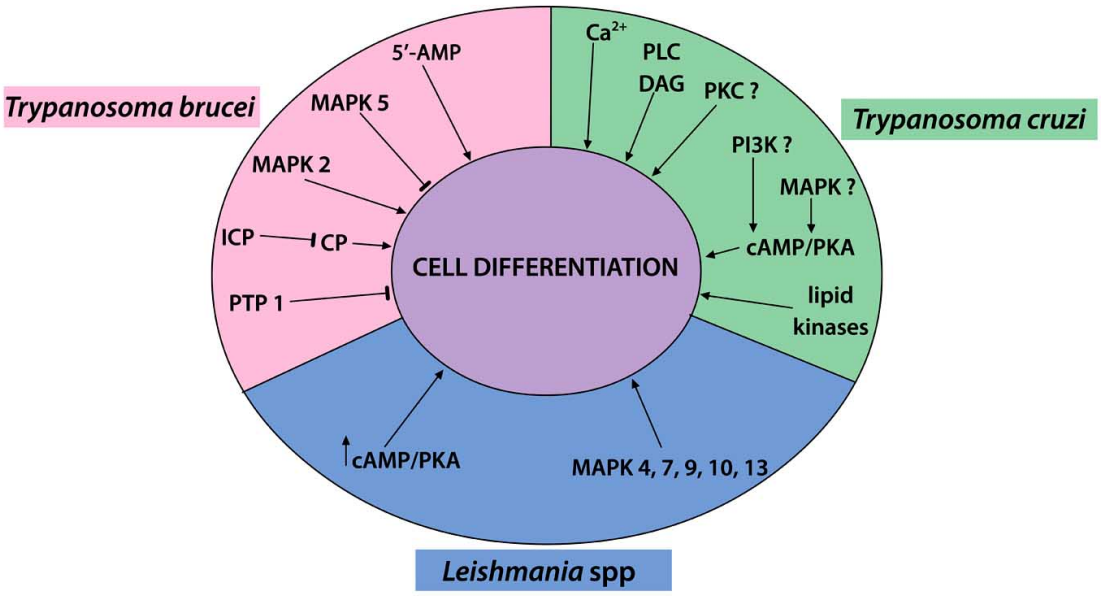

Fig. (3). Intracellular signaling pathways involved with cell differentiation in *Leishmania* spp., *Trypanosoma cruzi* and *Trypanosoma brucei*. The intracellular signaling pathways listed here are summarized in this diagram. ICP – endogenous cysteine peptidase inhibitor; CP – cysteine peptidase; PTP – protein tyrosine phosphatase; MAPK- mitogen-activated protein kinase; PLC- phospholipase C; DAG – diacyl glycerol; PKC- protein kinase C; PI3K- phosphoinositide 3 kinase; cAMP- cyclic AMP; PKA- protein kinase cAMP-dependent; ↑ - stimulation; ⊤ - inhibition.

cellular remodeling have yet to be established [92]. Some factors that can trigger this event *in vitro* have been characterized, such as low pH, lack of oxygen and nutritional depletion. Interestingly, endosome sorting and autophagy are required for metacyclogenesis [93,95-97].

The recent sequencing of the genome of three species of trypanosomatids has revealed the presence of several proteins involved in specific signaling pathways, such as intermediate messengers and kinases [82,98]. *Leishmania major* appears to have 179 protein kinases that are similar to eukaryotic counterparts, representing 2% of the *Leishmania* genome [9,99]. Nevertheless, the trypanosomatids lack receptor linked and cytosolic tyrosine kinase families and some groups of protein kinases, such as STE and GMGC (CDK cyclin-dependent kinases, MAP kinases, glycogen synthase kinase 3 kinase-dependent cell division cycle (CDC) as and casein kinase II family) are involved in important processes, including cell cycle regulation, stress responses, cellular signals and cell differentiation [9,82]. Moreover, some genes were found for certain families of protein kinases, such as CAMK (Ca^{+2} regulated by kinases) and AGC (which includes the protein kinase C, protein kinase A, protein kinase G etc) [9].

The MAP kinase family of proteins is involved in the regulation of cellular proliferation and differentiation in eukaryotic cells [9,100]. Fifteen MAPK genes were identified in *L. major* and *L. mexicana*, which are also present in *L. infantum* and *L. braziliensis* [99,47]. Studies using *L. major* and *L. donovani* showed that the increase in the recombinant MPK 4, MPK 7 and MPK 10 activity was similar to that observed during differentiation to the amastigote form. Additionally, MPK 3, MPK 13 and MPK 9 seem to be involved in the elongation and reduction of the flagellar length in *L. mexicana* and may be implicated in the regulation of cell differentiation [82,99,101-104]. Earlier reports suggested that MPK 4 in *L. mexicana* may have an important role in the progression of promastigotes to amastigotes because the *T. brucei* homologue of the enzyme TbMAPK2 null mutant showed a delay in differentiation kinetics [80]. Although several studies have highlighted the importance of MAP kinase proteins in cell differentiation, the signaling pathways involved in its activation are still unknown. Unusual surface receptors, the kinase tyrosine receptor, conventional substrates and transcription factors [99,47] are present in the *Leishmania* genome.

cAMP is an important second messenger that modulates several key cell processes. The *Leishmania* genome project showed that the parasite contains members of the cAMP cascade [35,105]. In *Leishmania*, studies have demonstrated that stress-induced elevations of the intracellular cAMP response are associated with cellular differentiation. Thus, cAMP may play a significant role in stress-induced differentiation because both intracellular cAMP and the activity of protein kinase A are elevated under stress [105].

Despite the fact that few members of the AGC kinase family have been identified in the trypanosomatid genome, three isoforms of PKA-C (catalytic subunit) and possibly an isoform of PKA-R (regulatory subunit) have been characterized in *Leishmania*. PKA is a tetrameric cAMP-dependent protein kinase that can modulate signaling pathways involved in cellular proliferation and differentiation processes [47,106,107]. However, unlike higher eukaryotes, very little is known about the role of PKA in *Leishmania*. In 2004, PKA activity was found to be higher in metacyclic forms compared to non-infective forms, which may suggest the

involvement of the enzyme in the process of parasite metacyclogenesis [108].

The presentation of the *Leishmania* genome showed the occurrence of gene members of environmental signaling pathways that may have an important role in the cell differentiation of these parasites. However, the actual contribution of these pathways to this process has yet to be determined.

PERSPECTIVES

The study of signaling pathways in trypanosomes is crucial for understanding the relationship of these parasites with their hosts (both vertebrates and invertebrates). Even so, our knowledge in this field today is still limited. To date, most studies of intracellular signaling pathways in trypanosomatids have used the simple algorithm of a single input or ligand to address the role of a single pathway or a small number of signaling pathways (Fig. **3**). This strategy was largely hampered by the unavailability of genomic and proteomic data. However, the first wide and more complex maps of cell signaling in such organisms could be constructed in the coming years. This undertaking will certainly be supported by both the availability of the data on parasite biology and bioinformatics and by the surge in novel high-throughput technologies to evaluate cell signaling and protein phosphorylation-dephosphorylation circuits. The first products of this new phase are becoming available in the literature, as are data generated on kinome, phosphatome and phosphoproteome projects in some groups of pathogenic trypanosomatids [45-47]. However, while such studies present an outstanding account of enzymes encoded in protozoan genomes and could eventually lead to the full collection of their target phosphoproteins, they are an initial platform for future investigation. It is of the utmost importance to put conventional signaling modulators of trypanosomatid biology on the map and to establish a detailed picture of the connection between molecules traditionally under study and their modes of action. We need to identify very specific signaling molecules or motifs within conventionally-studied molecules that could be a starting point for the development of novel targets to block diseases caused by trypanosomatids. These studies should take the place of those describing canonical signaling scaffolds in pathogenic protozoa. In conclusion, further investigation of the modulation of cellular differentiation in trypanosomatids with the aid of these novel technologies is of the utmost importance. Determining how the signaling pathways may contain potential targets for the development of immunotherapeutic and chemotherapeutic drugs in future years should be another research priority.

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