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Identification of a functional prostanoid-like receptor in the protozoan parasite, *Trypanosoma cruzi*

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Abstract

Trypanosoma cruzi infection in humans and experimental animals causes Chagas disease which is often accompanied by myocarditis, cardiomyopathy and vasculopathy. *T. cruzi*-derived thromboxane A₂ (TXA₂) modulates vasculopathy and other pathophysiological features of Chagasic cardiomyopathy. Here, we provide evidence that epimastigotes, trypomastigotes and amastigotes of *T. cruzi* (Brazil and Tulahuen strains) express a biologically active thromboxane prostanoid (TP) receptor that is responsive to TXA₂ mimetics, e.g. IBOP. This putative receptor, *TcTP*, is mainly localized in the flagellar membrane of the parasites and shows a similar glycosylation pattern to that of TPs obtained from human platelets. Furthermore, TXA₂-TP signal transduction activates *T. cruzi* specific MAPK pathways. While mammalian TP is a G-protein coupled receptor (GPCR); *T. cruzi* genome sequencing has not demonstrated any GPCRs in these parasites. Based on this genome sequencing it is likely that *TcTP* is unique in these protists with no counterpart in mammals. TXA₂ is a potent vasoconstrictor which contributes to the pathogenesis of Chagasic cardiovascular disease. It may, however, also control parasite differentiation and proliferation in the infected host allowing the infection to progress to a chronic state.

Keywords

Thromboxane; thromboxane receptor; MAPK; *Trypanosoma cruzi*; Chagas disease; Prostanoid receptor

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Introduction

American trypanosomiasis or Chagas disease is caused by infection with the protozoan parasite, *Trypanosoma cruzi*. Major consequences of this infection are acute myocarditis, vasculitis and chronic cardiomyopathy (Machado et al. 2012). Acute infection is accompanied by an increase in inflammatory mediators including cytokines, chemokines and eicosanoids. There is also enhanced platelet aggregation, focal ischemia and myonecrosis (Tanowitz et al. 1990; Tanowitz et al. 1992). Endothelin-1 (ET-1), a 21 amino acid peptide, and the eicosanoid thromboxane A₂ (TXA₂) are up regulated in experimental *T. cruzi* infection. Both ET-1 and TXA₂ share similar biological properties including enhancement of vasoconstriction, inflammation and platelet aggregation (Tanowitz et al. 1990; Petkova et al. 2000; Petkova et al. 2001; Tanowitz et al. 1999).

T. cruzi has a complex life cycle involving mammalian hosts and insect vectors (Hidron et al. 2010). Two-life stages of the parasite are found within the insect vector; epimastigotes and metacyclic trypomastigotes. Epimastigotes multiply extracellularly within the insect midgut and transform into infective non-dividing metacyclic trypomastigotes that are introduced into the mammalian host through insect feces deposited at the site of the vector bite. Metacyclic trypomastigotes gain entry into mammalian host through the skin or conjunctivae. Within the mammalian host, metacyclic trypomastigotes transform into non-dividing, blood stage trypomastigotes that can infect any nucleated mammalian cell type where they transform to intracellular amastigotes (Ferreira et al. 2006). Amastigotes transform into blood-stage trypomastigotes and spread to adjacent uninfected host cells or are spread via the lymphatic and blood stream to distant parts of the body. The transmission cycle is completed when trypomastigotes are taken up by the insect vector during an ensuing blood meal from the infected host. Blood transfusion, oral, organ transplantation, and congenital are other recognized modes of transmission.

TXA₂ is an eicosanoid generated via the metabolism of arachidonic acid (AA). Eicosanoids have a diverse range of biological properties including modulation of vascular tone, inflammation, ischemia and tissue homeostasis (Haeggstrom et al. 2010; Rossi et al. 2010; Factor et al. 1985; Tanowitz et al. 1996). AA is cleaved from membrane phospholipids by the action of phospholipase A1 (PLA1). The free AA is further hydrolyzed through three pathways, the lipoxygenase (LOX) pathway (producing leukotrienes, lipoxins, hydroxyeicosatetraenoic and hydroperoxyeicosatetraenoic acids), the cyclooxygenase (COX) pathway (producing prostaglandins, prostacyclins and thromboxane) and the Cyt P450 pathway (producing epoxides and hydroxyeicosatetraenoic acids). Cyclooxygenase converts AA to prostaglandin H₂ (PGH₂), the central metabolite, from which different terminal prostaglandins (PGE₂, PGD₂, PGF_{2α}), prostacyclin (PGI₂) and TXA₂ are generated by species-specific synthases (Santovito et al. 2009).

Prostaglandins and leukotrienes act via seven membrane spanning G-protein coupled receptors (GPCRs) located on the plasma membrane of multiple cells types in mammals. In addition, lipoxin A₄ and PGJ₂ bind to the nuclear ligand-activated transcription factor, the Aryl hydrocarbon receptor (AhR) and to PPAR-γ respectively (Schaldach et al. 1999; Miwa et al. 2004). The signal transduction through surface receptors occur via heterotrimeric G-Proteins to manifest their biological effects (Beller et al. 2004; Hui et al. 2004; Tager et al. 2003). Although AA metabolism in mammalian cells is well described, it is unclear if these pathways are present in unicellular parasites such as *T. cruzi*. Since *T. cruzi* is an intracellular parasite, it could potentially scavenge substrates from the host, thus bypassing important metabolic steps that are otherwise required in mammals. Although there is evidence of existence of phospholipase A2 (PLA 2) in *T. cruzi* and *T. brucei* (Opperdoes et al. 1982; Sage et al. 1981; Belaunzaran et al. 2007; Shuaibu et al. 2001), PGF_{2α} Synthase

(PGF_{2α}S) and TXA₂ synthase (TXA₂S) (Machado et al. 2011; Ashton et al. 2007; Mukherjee et al. 2011) in *T. cruzi*, the relevance of these enzymes and their products are not well understood in the context of host-parasite interactions. There are no reports of cyclooxygenase (COX), the critical enzyme for the biosynthesis of eicosanoids in *T. cruzi*. The COX enzyme family has different pathophysiological roles; COX-1 is constitutively produced and maintains vascular tone, platelet activation and gastric mucus production, while COX-2 is inducible and implicated in inflammation and cancer (Mukherjee et al. 2011).

Kinetoplastids have adaptable lipid biosynthetic pathways that appear to be dictated, in part, by environmental constraints where they complete their life cycle (Mukherjee et al. 2011; Rouzer et al. 2008; Kabututu et al. 2003; Kubata et al. 2002). There is evidence of presence of PGF_{2α} Synthase (PGF_{2α}S) in *Leishmania*, and *T. brucei* (Kabututu et al. 2003), Old Yellow Enzyme (similar to PGF_{2α} S) in *T. cruzi* (Kubata et al. 2002) and TXA₂S in *T. cruzi* (Ashton et al. 2007). *Leishmania* and *T. brucei* also produce smaller quantities of PGE₂ and PGD₂. (Kabututu et al. 2003; Kubata et al. 2002; Sterin-Borda et al. 1996). The life cycles of *Leishmania*, *T. brucei* and *T. cruzi* are different and this difference may be reflected in their eicosanoid productions. For example, *Leishmania* and *T. brucei* predominantly produce PGF_{2α} and *T. cruzi* preferentially synthesizes TXA₂ (Ashton et al. 2007). Recently, Frire-de-Lima et al. (2000) reported that *T. cruzi*-derived eicosanoids might contribute to parasite differentiation, phagocytosis and host survival (Kabututu et al. 2003) by acting as immunomodulators to aid transition and maintenance of the chronic phase of the disease.

It has been reported that essential fatty acid synthesis is important in regulating *T. cruzi* parasitemia and survival rates during acute infection (Santos, 1992). In addition, mice resistant to *T. cruzi* infection have higher rates of eicosanoid synthesis than susceptible mice (Cardoni et al. 2004). Zuniga et al. (2005) reported that CD11b+ myeloid cells from *T. cruzi*-infected mice secrete an unidentified prostaglandin that mediates the loss of immature B-cells by apoptosis, thus compromising host defense leading to the chronic stage of infection.

Herein, we report the existence of a TXA₂ prostanoid (*Tc*TP) receptor-like moiety in *T. cruzi* and an active TXA₂ signaling pathway. This TP receptor, *Tc*TP, is expressed in all life-stages of the parasite and mainly localized in the flagellar membrane. *Tc*TP also has glycosylation patterns similar to that of TPs obtained from human platelets. Furthermore, we provide evidence that this moiety is functionally active when stimulated with TXA₂ mimetics. The presence of an active parasite TXA₂ signaling pathway further extends the role of TXA₂ in the pathogenesis of Chagas disease. Although we do not know the extent of host-parasite participation in eicosanoid production, we have demonstrated that parasite-derived TXA₂ may be important in controlling parasitemia, tissue parasitism and mortality in the infected host (Ashton et al. 2007; Mukherjee et al. 2011). In addition, the parasite may employ TXA₂ as a quorum-sensing lipid by controlling parasite differentiation and proliferation in the infected host, thus contributing to cardiovascular remodeling.

Materials and methods

Reagents

Tissue culture reagents were purchased from Invitrogen (Carlsbad, CA). Plastic ware was purchased from Costar (Cambridge, MA). The TXA₂-mimetic compounds, IBOP and U46619 were obtained from Cayman Chemicals (Ann Arbor, MI). Rabbit polyclonal antibodies directed against human thromboxane A₂ synthase (TXA₂S) and TP were purchased from Cayman Chemicals (Ann Arbor, MI) and Imgenex (San Diego, CA). Rabbit polyclonal antibodies directed against total *T. cruzi* MAPK and phospho MAPK 2 was

developed in our laboratory (Huang et al. 2011). Alexa-Fluor-488 conjugated Goat Anti-Rabbit IgG and DAPI were purchased from Molecular probes (Carlsbad, CA), Goat serum was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were of the highest grade available.

Parasite Maintenance

The Tulahuen and Brazil strains of *T. cruzi* were used in our experiments. The Tulahuen strain was maintained by syringe passage in A/J mice (Jackson Laboratories, Bar Harbor, ME) while the Brazil strain was maintained in C3H/HeJ mice (Jackson Laboratories) (Tanowitz et al. 2005). Trypomastigotes were maintained in cultured L₆E₉ myoblasts at a multiplicity of infection of ~2:1 (Rowin et al. 1983). Parasitism was determined by fixing the infected cells with methanol and staining with Giemsa.

Immunoblot Analysis

Parasite lysates were prepared in lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100) containing protease inhibitors (Roche Applied Science, Indianapolis, IN) for 30 mins in ice and homogenized. Homogenates were further sonicated for 20sec in ice and centrifuged at 16,000g at 4°C for 10 min to pellet insoluble material. Protein concentrations were estimated with the bicinchoninic acid protein assay (Bio-Rad, Hercules, CA) in Nanodrop (Thermo Fisher Scientific, Waltham, MA). Equal amounts of parasite lysates (50 µg) protein were separated by SDS-PAGE under reducing conditions. For *T. cruzi* phosphorylated MAPK 2 immunoblotting, parasite lysates were boiled to destroy endogenous phosphatases prior to gel separation. Proteins were transferred onto nitrocellulose membrane (Protran BA 85 Nitrocellulose from Whatman, Dassel, Germany) and analyzed using antibodies against human TP, TXA₂S and *T. cruzi* specific phospho MAPK 2. Primary antibodies were used at a dilution of 1:500 and anti-rabbit AP-conjugated secondary antibodies at a dilution of 1:5,000 and were visualized with BCIP/NBT color detection system (Promega, Madison, WI).

Immunofluorescence

For immunofluorescence analysis, parasites were washed in TBS (20 mM Tris pH 7.4, 500 mM NaCl), fixed in 1% paraformaldehyde (EMS, Hatfield, PA) for 10 min and blocked in 10% goat serum in TBS containing 1% Triton X-100 (TBS-T) for 30 min. The parasite and or cultured myoblasts were immunostained with 2 µg/ml anti-human TP or TXA₂S antibodies for one hour, washed three times in TBS-T and stained with Alexa 488 conjugated goat anti rabbit secondary antibody and DAPI for one hour in the dark. The parasite or cultured myoblasts were washed in TBS-T three times and observed under immunofluorescence microscopy. Infected L₆E₉ myoblasts were grown on cover slips or on eight-well Labtek chambers (Thermo Fisher Scientific, Rochester, NY) overnight and then processed for immunofluorescence analysis.

Image acquisition and processing

Immunofluorescence images were acquired with a 60× (1.4) Olympus objective on an Olympus 1×71 inverted microscope containing automated excitation and emission filter wheels. Data were collected through a CoolSNAP HQ cooled charge-coupled device (Photometrics, Roper Scientific, Tucson, AZ) camera regulated by MetaMorph (Molecular Devices, Sunnyvale, CA) software. Exposure times (100 ms) and brightness adjustments (image normalization) were kept constant for images from different cell types and only secondary antibody negative control. Fluorescence images were analyzed using ImageJ 1.39u (National Institutes of Health public domain; rsb.info.nih.gov/ij/) and Adobe Photoshop CS2 version 9.0.2 (Adobe Systems, San Jose, CA).

Statistical Analysis

Immunoblot, immunofluorescence and live cell imaging studies were performed at least three times and representative data are presented. Data were pooled and statistical analysis was performed with Student's t-test as appropriate and significance of difference was determined as $p < 0.05$.

Results

Identification of a *T. cruzi* thromboxane prostanoid receptor (TP)-like protein by immunoblotting

A specific, 37 kDa reactive protein was identified in all the life-forms of *T. cruzi* (from both Tulahuen and Brazil strains) by immunoblotting. We used two different anti-human TP antibodies obtained from Cayman Chemicals and Imgenex, recognizing two specific cytoplasmic domains of human TP receptor. The Cayman antibody recognizes a peptide corresponding to amino acids 323–343 of human TP receptor, while the antibody from Imgenex recognizes a peptide sequence corresponding to the second cytoplasmic domain of human TP receptor. Both the antibodies detected a 37 kDa major protein and several high molecular weight proteins ranging from 37–100 kDa in the parasite lysates (Fig. 1A). These may represent dimeric forms of the receptor (Laroche et al. 2005). The detection of *T. cruzi* TP receptor (*TcTP*) is specific as the anti-TP antibody does not react with either putative *TcTXA₂S* or *TcOYE* both of which we have cloned from *T. cruzi* and expressed in bacteria (Fig. 1A, data from Brazil strain with Cayman Chemicals antibody shown). The parasite specific TP receptor was detected in the heart of CD-1-infected (Brazil strain) cardiac tissue (Fig. 1B). We did not detect similar reactive proteins from other protozoan parasites such as the Apicomplexan *Toxoplasma gondii* and the Microsporidian *Encephalitozoon cuniculi* suggesting that other protists do not share similar thromboxane signaling pathways (Fig. 1A). Both *T. gondii* and *E. cuniculi* are also negative for *TXA₂* synthesis, as we did not detect *TXA₂* in the lysates from these species with *TXA₂* ELISA (data not shown).

A BLAST search for the epitope for the Cayman anti-TP antibody (LSTRPRSLSLQPQLTQRSGLQ) in EuPathDB.org identified a protein of unknown function from *T. brucei gambiense* (peptide PRS+SLQP+ QR of 13 kDa) and a protein of unknown function from *T. cruzi* CL Brener strain (peptide motif R+L LQ QLT SG, 33 kDa). Although the predicted protein from the blast search is of slightly smaller than that observed in the immunoblot, the variation could be because of difference in strains or in the resolution of gel electrophoresis. Thus, there is a good possibility that one of these proteins could be the potential orthologue identified in our immunoblotting results. The reaction is authentic as antibodies raised against two different conserved TP peptide sequences identify a specific reactive protein in *T. cruzi* lysate and specific as the anti-TP antibodies does not react with either *TcTXA₂S* or *TcOYE* (*TcPGF_{2α}*) or with *T. gondii* or *E. cuniculi*.

T. cruzi TP receptor-like protein is predominately localized at the flagellar membrane

We performed an immunofluorescence assay to localize *TcTP*, using antibodies from both the Cayman Chemicals and Imgenex. The flagella and the flagellar membrane in trypomastigotes (Fig. 2A, B) and epimastigotes (Fig. 2C, D) from both Tulahuen and Brazil strain were brightly stained along with the parasite plasma membrane, which is a hallmark location for receptors in the parasite (data from Tulahuen strain with Cayman Chemicals antibody shown). We also detected *TcTP* over the plasma membrane of amastigotes within heavily infected cultured L₆E₉ myoblasts (Fig. 2C, arrow pointing to circular amastigotes with deep green staining on the plasma membrane). Similar plasma membrane staining was also seen in L₆E₉ myoblasts. In contrast when we stained the parasites with anti-human

TXA₂S antibody, only the cytoplasm of the parasite was stained indicating cytoplasmic localization of this enzyme (Fig. 2D).

***T. cruzi* TP receptor is glycosylated**

In humans, the amino terminal extracellular region of TP α and TP β contains two highly conserved N-linked glycosylation sites at Asn4 and Asn16. TP glycosylation is essential for ligand binding, G protein coupling and intracellular signaling (Chiang et al. 1998; Walsh et al. 1998). Therefore, we examined the possibility that *Tc*TP is similarly glycosylated. Immunoblotting employing anti-human TP antibodies indicates that *Tc*TP reveals similar glycosylation patterns to that of human platelets (Fig. 1A & 3). The molecular weight of TP ranges from 37 kDa to 100 kDa depending upon the degree of glycosylation and strain of *T. cruzi*. Fig. 3 shows the TP glycosylation pattern obtained from the Tulahuen strain of *T. cruzi*. When we treated the epimastigote and trypomastigote lysates with PNGase, an enzyme that cleaves N-linked glycosylated residues from aspartate, we were able to remove 90% of glycosylation from TP and the nascent protein could be detected as a 37 kDa with anti TP antibodies by Western blotting (Fig. 3).

Analysis of signal transduction through a *T. cruzi*-like receptor

Signal transduction mechanisms in *T. cruzi* are not fully defined (Huang 2011). In mammals, TP stimulation results in activation of the ERK pathway (Gao et al. 2001; Miggin et al. 2002; Gallet et al. 2003). Recently, we identified and cloned *T. cruzi* mitogen-activated protein kinase (*Tc*MAPK) genes. Some of these genes are unique compared to the mammalian counterparts. Phospho-specific antibodies against *Tc*MAPK 2 were generated (Huang et al. 2011). To investigate responsiveness to TP ligands we challenged the parasite with the mimetic IBOP (200 nM for 30 min) and determined *Tc*MAPK activation by immunoblotting. All life stages of *T. cruzi* from both Brazil and Tulahuen strains showed *Tc*MAPK activation (Fig. 4). The anti *Tc*MAPK antibody specifically detects a 55 kDa MAPK protein from *T. cruzi* but also reacts with a unknown protein of 72 kDa. However, anti *Tc*MAPK antibody does not react with mammalian ERK1/2 (endothelial cells) nor did antibodies against mammalian phospho ERK show reaction in the parasite lysates (data not shown).

These data suggest TXA₂ signaling is intact in *T. cruzi* and can potentially influence parasite behavior. Indeed, depriving the host of responsiveness to parasite-derived TXA₂ promotes dysregulation of parasite growth and increased parasitemia (Ashton et al. 2007). Direct stimulation of ERK 1/2 signaling in mammals induces proliferative changes in cells (Zhang et al. 2002). Using immunoblotting and immunofluorescence assays we found no increase in parasite division, cyclin D1/2 or G expression in parasites due to IBOP stimulation (although the antibodies used were raised against mammalian proteins, data not shown). Thus, if *Tc*TP signaling is a necessary component of the parasite metabolic system it must be responsible for controlling cellular function other than growth.

Discussion

The role of TXA₂ in the pathogenesis of *T. cruzi* infection has only recently been appreciated. We demonstrated that *T. cruzi* life stages were capable of synthesizing TXA₂ and thus potentially modulating the pathophysiological features of Chagasic cardiomyopathy including inflammation, vasoconstriction and platelet activation (Tanowitz et al. 1990; Ashton et al. 2007; Mukherjee et al. 2011). We have shown that TXA₂ from the parasite interacts with host TP expressed on endothelial cells modulating peripheral parasitemia, tissue parasitism and mortality in murine *T. cruzi* infection (Ashton et al. 2007). Moreover, parasite-derived TXA₂ appears to balance the rate of parasite proliferation with the

continued survival of the host so that the disease can progress to a chronic infection. However, the signaling cascade that ensues due to TP activation that controls *T. cruzi* differentiation and proliferation is not known. Here we provide the first evidence that *T. cruzi* expresses a functional TXA₂ receptor, *TcTP*.

Our knowledge of the characteristics of the TP receptor (TP) is based on the human TP. The human TP mediates in part, platelet aggregation, vasoconstriction and inflammation when stimulated with TXA₂. In humans, two TP isoforms have been identified, with different C-terminal tails resulting from alternative splicing. Human TP is a seven transmembrane domain G protein coupled receptor (GPCR), which predominantly couples to the Gα_q and activates phospholipase (PL) Cβ (Camps et al. 1992; Jhon et al. 1993; Katz et al. 1992), leading to an increase in inositol triphosphate (Berridge et al. 1984; Brass et al. 1987), diacylglycerol (Rink et al. 1988), and intracellular free calcium concentrations (Rink et al. 1988). To date, there are no reports as to the presence of prostaglandin or other eicosanoid receptors in parasites.

GPCRs are ubiquitously present with more than 1000 members in invertebrate and vertebrate genomes (Drews et al. 2000; Wise et al. 2004). The importance of GPCRs in human medicine is supported by the fact that 30–50% of drugs target this class of receptors (de Castro et al. 1987; Oliveira et al. 1984). To date there is no report of GPCR in trypanosome genome, nor are the downstream effector components of GPCR signaling known. Although no GPCR-like receptors have been cloned from *T. cruzi*, there are reports of radioligand binding characterization of β-adrenergic receptors in *T. cruzi* (Oliveira et al. 1984) and of the effect of cAMP on the growth of *T. cruzi* epimastigotes through adrenergic receptors (Sanchez et al. 1995). This suggests that the *TcTP* receptor is unique in structure when compared to that of higher vertebrates. Study of *TcTP* would could uncover a unique receptor phenomenon found nowhere in other representative eukaryotes with a further possibility of the presence of a unique cAMP-dependent signaling system in these organisms.

Importantly, the trypanosome genome project reveals the presence of a number of genes that encode for membrane bound adenylate cyclase (AC) (*T. brucei*, *T. cruzi*, *T. congolense*, and *T. equiperdum*) (Sanchez et al. 1995) and in *Leishmania donovani* (Naula et al. 2001), which are structurally and functionally similar to guanylyl cyclase (GC)-like receptors of mammals (Paveto et al. 1995). Trypanosome extracellular AC domains are structurally similar to the serpentine receptors and the tyrosine kinase receptors of higher vertebrates, with the ability to interact with specific ligands (Paveto et al. 1995), indicating the numerous *TcACs* function both as a receptor and effector, all in the same protein. Therefore, it is highly possible that *TcTP* could belong to one of the parasite AC family of receptors.

The predicted trypanosomal ACs are devoid of any structures that can interact with known heterotrimeric G proteins, indicating that *TcAC* functions as both receptor and effector with an inbuilt transducer component for effective signal amplification. The possible second messengers generated could be adenylyl cyclase, guanylyl cyclase, phospholipase C, phosphatidylinositol 3-kinase, and other ion channels. Specific guanylyl cyclase activity is reported in epimastigotes, generated by NMDA-receptor agonists (Shpakov et al. 2008; Pereira et al. 1997), indicating a NO-synthase pathway in the parasites similar to higher mammals (Shpakov et al. 2008; Pereira et al. 1997). However, it has not been proven that the GC-cGMP system in these parasites is involved in regulation of these receptors. Furthermore, there are reports that single pass membrane bound AC/GC receptor in *T. cruzi* can effectively bind to natriuretic factor. These findings support the hypothesis that such a signaling moiety could function as the putative *TcTP* identified here.

In higher eukaryotes, mitogen-activated protein kinases (MAPK) are known to regulate important cellular processes, such as cell proliferation, differentiation, stress response, apoptosis and also been linked to the aging process (Haeggstrom et al. 2010; Rossi et al. 2010; Factor et al. 1985; Tanowitz et al. 1996). MAPKs are evolutionary conserved from unicellular protozoans, including *T. cruzi* (Lorache et al. 2005) to vertebrates where they are activated by phosphorylation at a specific threonine and tyrosine residue in response to extracellular stimuli (Camps et al. 2000; Avruch 2007; Hsieh et al. 2010). In mammals, several MAPKs are known, including extracellular signal-regulated kinase (ERK); c-Jun NH2-terminal kinase (JNK), stress-activated protein kinase (SAPK), p38 and big MAPK-1/ERK5 (BMK-1/ERK5) (Camps et al. 2000; Avruch 2007; Balasubramanian et al. 2010). ERK is also important in the post-translational regulation of MAPK phosphatase-2 (Ramos et al. 2008; Peng et al. 2010). Recently, our laboratory group has identified thirteen MAPK genes from *T. cruzi* and cloned five of them (Huang et al. 2011). The characterization of *T. cruzi* MAPK 2 revealed that they are unique to the parasites with no mammalian homology. Furthermore, our anti *T. cruzi* phospho MAPK 2 antibody was found to be *T. cruzi* specific with no reaction to mammalian ERK (p42/44) (Huang et al. 2011). Interestingly, like mammals, we have observed activation of *T. cruzi* MAPK 2 when stimulated with the TXA₂ mimetic IBOP. This observation indicates the presence of unknown adaptor molecules in *T. cruzi* that couple to *TcTP* to activate MAPKs. Currently we do not know the downstream pathways that are activated in the parasites by IBOP in order to activate MAPK signaling. Although in mammals increase in ERK results in proliferative changes in cells, we found no increase in parasite division or in cyclin D1/2, and G in parasites due to IBOP stimulation either by immunoblotting or by immunofluorescence using antibodies directed to mammalian cyclin D1/2 and G (data not shown).

Additional study of *TcTP* is needed to elucidate this unique receptor phenomenon that has not been previously described in other representative eukaryotes. In addition, these organisms may have a unique cAMP-dependent signaling system. Insight into the structure of *TcTP* would provide us with functional information of how the coupling between *TcTP* and adaptor molecules occurs. If the *TcTP* glycosylation sites were different from the mammals, then these may be useful as a potential drug target.

The revelation that TXA₂-responsive moieties exist in non-vertebrates, and more specifically in parasitic organisms, has several compelling implications. TXA₂ signaling from the host has previously been identified as a potential quorum sensor that could control parasite proliferation, transformation into invasive trypomastigotes and ultimately survival for the host during acute infection (Ashton et al., 2007). The ability of the parasite to respond directly to its own TXA₂ generation may add another dimension to the quorum sensing effects of the TXA₂ signaling axis in this infection. TXA₂ responsiveness for the intracellular amastigote suggests that TXA₂ biosynthesis by the host cell may be an important pro-survival mechanism regulating intracellular parasite proliferation and support our observation that in infected TXA₂ synthase null mice have increased parasitemia during acute infection (Mukherjee et al., 2011). Additionally, these findings may explain why aspirin administration during acute infection increases both parasitemia and mortality. Theoretically, our findings suggest that non-steroidal anti-inflammatory drugs not be utilized in patients during acute Chagas disease. Furthermore, the ability to sense TXA₂ by trypomastigotes in their surroundings could be both chemotactic (i.e., drawing parasites to sites of damage where compromised cell membranes enhance parasite invasion of host cells) or chemorepulsive (i.e., driving parasites away from areas of inflammation or ischemia which are incompatible with their survival or replication). As such, the ability of the parasite to respond to TXA₂ in its immediate extracellular milieu could contribute to the progression of the disease.

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Abbreviations

Tc	<i>T. cruzi</i>
TXA₂	Thromboxane A ₂
TXA₂S	TXA ₂ synthase
TP	thromboxane prostanoid receptor
IBOP/U46619	TXA ₂ mimetics
GPCR	G-protein coupled receptor
ERK	extracellular signal-regulated kinase
MAPK	mitogen-activated protein kinase
AA	arachidonic acid
LOX	lipoxygenase
COX	cyclooxygenase
Cyt P450	cytochrome P450
PG	prostaglandin
AhR	aryl hydrocarbon receptor
PLA₂	phospholipase A ₂

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Fig. 1.

(A) Detection of a specific 37 kDa reactive protein with anti TP antibody in all the life-forms of *T. cruzi* by immunoblotting. Human platelets and endothelial cells are shown as control. The multiple bands represents various glycosylated forms of TP. Anti TP antibodies could not detect similar reactive proteins from apicomplexan, *T. gondii* and the microsporidian, *E. cuniculi*. (B) Representative Western blot demonstrating glycosylated TP detected in the heart lysate obtained from infected CD-1 mice but not from uninfected heart.

Fig. 2.

Immunofluorescence analysis of *T. cruzi* for the presence of TP like protein. Both trypomastigotes (A) and epimastigotes (B) of the Brazil and Tulahuen strain of *T. cruzi* were positive for TP (Tulahuen strain shown). No staining was observed in secondary antibody control in both parasites and L₆E₉ myoblasts (data not shown). TP was mainly detected in the flagella and plasma membrane of the parasite. Amastigotes in the infected myoblasts were also positive for TP. (C), the arrow pointing to the deeply stained amastigote membrane. On the other hand, TcTXA₂S was predominantly localized in the cytoplasm of the parasites (D). Bar= 10μM

Fig. 3.

T. cruzi TP like protein is N-linked glycosylated like that of human platelet TP. The multiple bands ranging from 37–100 kDa indicates the various glycosylated forms of TP. Treatment of parasite lysate with PNGase removed N- linked glycosylation and the nascent protein appeared as a 37 kDa reactive protein by immunoblotting (lysates obtained from the Tulahuen strain shown).

Fig. 4.

Increased expression of *T. cruzi* phospho MAPK 2 was observed when epimastigotes and trypomastigotes were treated with IBOP, a TXA₂ mimetic. *T. cruzi* phospho MAPK 2 expression (55 kDa, arrow) was detected by immunoblotting using a specific antibody that does not react with mammalian ERK1/2.