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Short communication

A Protein Phosphatase 1 involved in correct nucleus positioning in trypanosomes

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ABSTRACT

Reversible protein phosphorylation is a key regulator in intracellular functions. In the African trypanosome, *Trypanosoma brucei*, the serine–threonine phosphatase PP1-3, is localised in the cytoplasm. RNAi mediated knockdown of PP1-3 leads to a coordinated rearrangement of cellular organelles and compartments in the procyclic trypanosome. These parasites display their nucleus at the very posterior end of the cell. The kinetoplast is very close to the nucleus, and often located in a more anterior position. The lysosomal compartment, which in a normal procyclic cell is situated between nucleus and kinetoplast, is now positioned towards the anterior end of the cell. The Flagellum Attachment Zone, essential for cytokinesis, is still constructed, allowing initiation of the cleavage furrow and cell division. These adaptations allow dividing cells to distribute their organelles among the daughter cells and to proliferate normally. PP1-3 is therefore essential in conserving the intracellular organisation of the procyclic trypanosome cell.

Protein phosphorylation is one of the major mechanisms for the control and regulation of cellular functions in eukaryotic cells. Phosphorylation occurs mainly on serine (Ser) or threonine (Thr) residues, making Ser/Thr protein phosphatases (PP) exceedingly important players in the eukaryotic cell. The Ser/Thr protein phosphatases of type 1 (PP1) are the most frequent and among the best characterised PP [1]. The PP1 catalytic subunit can interact with a large number of regulatory proteins, resulting in a conformational change of the phosphatase and a specific dephosphorylation of the target. This association of various regulatory domains with the catalytic domain explains the involvement of PP1s in cellular regulations as diverse as glycogen metabolism, glycolysis, microtubular functions, cell cycle progression, mitosis, apoptosis, cell signalling or motility [2].

In protozoan parasites, the physiological activity of PP1 was mainly studied using potent inhibitors, such calyculin A or okadaic acid (OA). These inhibitors act on both PP1 and PP2A with different specificities and revealed an involvement of these enzymes in the attachment process of the parasites *Trichomonas vaginalis* [3], *Toxoplasma gondii*, [4] and *Plasmodium falciparum* [5] to their host cells and in the differentiation of the extracellular stage into the intracellular stage of *Trypanosoma cruzi* [6]. In contrast to these parasites, *Trypanosoma brucei*, the agent of human sleeping sickness, does not invade host cells. Treatment of procyclic *T. brucei* with OA resulted in multinucleated cells with a kinetoplast segregation defect, indicating a role for PP1 and/or PP2a in cytokinesis and organelle positioning [7]. Calyculin A also inhibits *T. brucei* growth (C. Gallet, P. Grellier, unpublished). Using these inhibitors to determine whether the responsible PP was a type 1 or a type 2A is not possible. Taking advantage of the inducible RNA interference system in *T. brucei*, Li and co-workers simultaneously knocked down the expression of 7 of the 8 PP1, as well as the expression of the PP2A catalytic subunit in procyclic trypanosomes [8]. The authors observed a slight reduction in growth of the cells, but no defect in cytokinesis or organelle positioning [8]. As the knockdown had been performed on several PP1 at the same time [8], it was not possible to quantify the efficiency of the knockdown for a particular PP1 enzyme. Inefficient knockdown, different roles of PP1 enzymes at different life cycle stages or the presence of another OA-sensitive PP in *T. brucei* could explain the differences in phenotype observed between the inhibitor and the RNAi studies.

In order to determine whether a specific PP1 was involved in organelle positioning and cytokinesis in procyclic *T. brucei*, we decided to specifically knock down the expression of the first PP1 identified in this organism (TriTrypDB: Tb927.4.5030 [9]). Since this protein was also studied in the simultaneous RNAi knockdown study [8], it will be referred to as 'PP1-3', according to the PP1 nomenclature introduced by Li and coworkers.

We first determined the localisation of PP1-3 in procyclic *T. brucei* (Fig. 1A and B) using a specific anti-PP1-3 antiserum raised in mice against the complete recombinant *T. cruzi* PP1-3, which

Abbreviations: PP, protein phophatase; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; RNAi, RNA interference; K, kinetoplast; N, nucleus; OA, okadaic acid; FAZ, Flagellum Attachment Zone; Tet, tetracycline.

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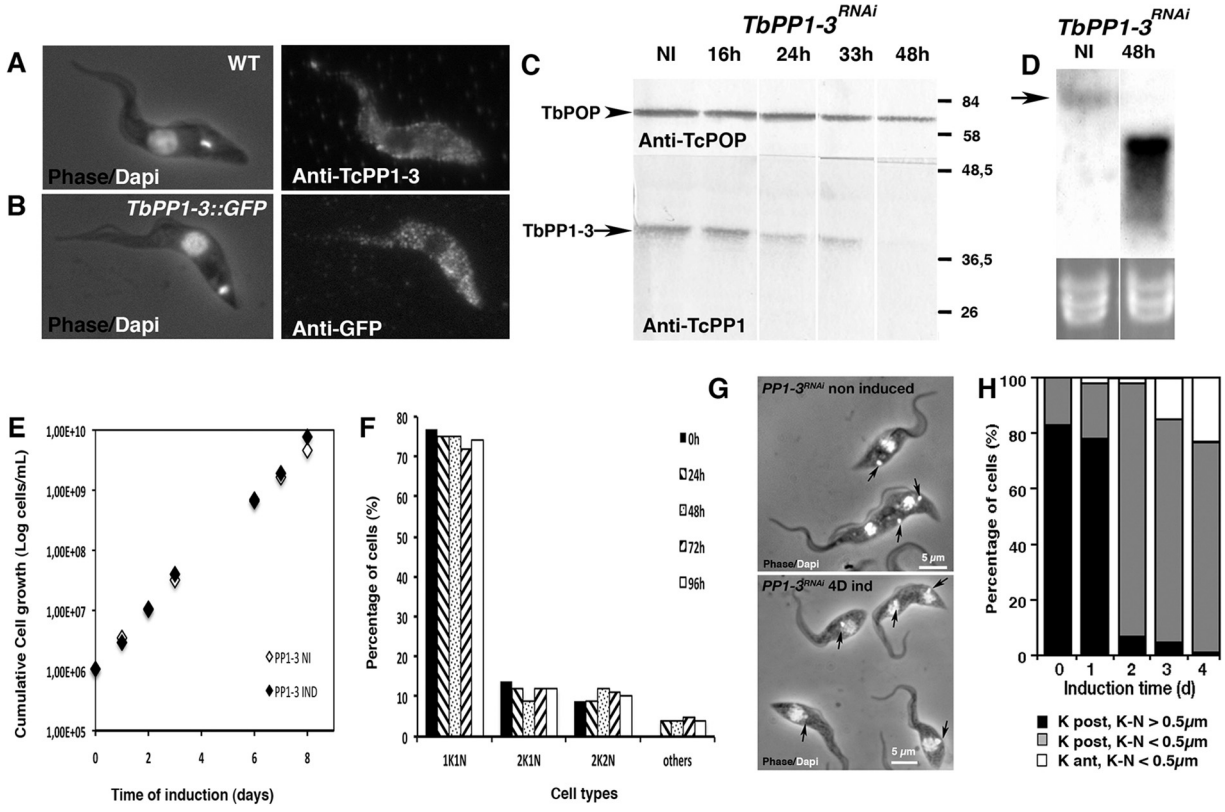

Fig. 1. Localisation of TbPP1-3 and *TbPP1-3*^{RNAi} characterisation. (A and B) TbPP1-3 is a cytoplasmic protein. (A) Immunofluorescence assay on paraformaldehyde fixed cells with the anti-TcPP1-3 antiserum (dilution 1/200 in PBS-0.1% BSA). The recombinant protein was expressed in BL21 (DE3) bacteria (Novagen). After purification under denaturing conditions (6 M urea) using the His-Bind purifying kit (Novagen) according to the manufacturers' instructions, the protein was solubilised using 2 M urea and used to immunise *balb-c* mice (3 injections, separated by 3 weeks). (B) Localisation by a GFP::PP1-3 fusion protein. The 1–900 bp of the *PP1-3* gene were cloned in frame behind the *GFP* gene in the vector pPCPFReGFP [28]. The vector was linearised by *Sph*I and integrated in the endogenous locus of wild-type trypanosomes, resulting in the reconstruction of a complete GFP::PP1-3 fusion protein. Resistant clones were selected by addition of 1 μg/mL puromycin. (B) Immunofluorescence assay on methanol fixed cells with an anti-GFP antiserum raised in rabbit (Invitrogen, dilution 1/400 in PBS-0.1% BSA). (C) Knock down of TbPP1-3 expression by RNAi reveals the specificity of TcPP1-3 antiserum. 5×10^6 cells were loaded per well on a 12% SDS-PAGE gel. Proteins were transferred on C-Hybond membranes (Amersham) in the presence of 20% methanol at 4 °C. PP1-3 is not detectable after 48 h induction by Tet. Blots were probed with anti-TcPP1-3 (dilution 1/200), respectively anti-TcPOP (loading control, dilution 1/300) [12]) mouse polyclonal antibodies and revealed using by alkaline phosphatase revelation. The arrows indicate TbPOP (top), TbPP1-3 (bottom). (D) PP1-3 RNA is no longer detected on a Northern blot after 48 h of induction. (E) Induced *PP1-3*^{RNAi} cells do not show a reduction in growth, over the course of 8 days of induction, when compared to non-induced (NI) cultures. (F) Induced *PP1-3*^{RNAi} cultures contain few abnormal cells. Cells were fixed methanol and DAPI stained. The cell types were determined for at least 500 cells in total, at different time points in the induction. After 96 h of induction 95% of the cells belonged to the classical cell types (1K1N, 2K1N, 2K2N) and less than 5% of other cells were identified. (G) 48 h induced *PP1-3*^{RNAi} cells (bottom) display a reduced N/K distance when compared to non-induced cells (top). Phase contrast images combined with DAPI staining. The arrows indicate the kinetoplasts. (H) Apparition of the phenotype during the course of the induction. 100 uniflagellated cells (1K1N cells) were analysed and divided in three groups, depending on the relative distance between K and N and K position in the cell (posterior or anterior).

displays 71% AA identity with its *T. brucei* counterpart (Fig. 1A). PP1-3 is a cytoplasmic protein and the localisation was confirmed using a GFP::PP1-3 fusion (Fig. 1B). The nucleus seemed excluded from the labelling (Fig. 1A and B).

To determine the physiological role of PP1-3, we constructed the vector pZJM-PP1-3, expressing double-stranded RNA corresponding to *PP1-3* under the control of two tetracycline (Tet)-inducible T7 promoters facing each other in the pZJM vector [10]. After linearisation by *Cla*I, the vector was transformed in 29–13 procyclic cells that express the T7 RNA polymerase and the Tet repressor [11]. Resistant transfectants were selected and cloned by addition of 1 μg/mL phleomycin and production of dsRNA was induced by addition of 1 μg/mL Tet to the medium. Knockdown of TbPP1-3 expression was verified by Western blot (Fig. 1C) and Northern blot (Fig. 1D) and efficient knockdown was seen after 48 h of *TbPP1-3* dsRNA expression. We used an antibody against TcPOP as loading control. TcPOP is a 80 kDa prolyl oligopeptidase required for parasite entry into mammalian cells [12].

The cells grew normally up to 8 days in presence of Tet (Fig. 1E). Trypanosomes possess two DNA containing organelles: the nucleus (N) and the kinetoplast (K), a disc-shaped structure containing mitochondrial DNA and situated at the basis of the flagellum. These are two single-copy organelles and their faithful replication during the cell cycle is essential for cell survival. The N and K positions in the procyclic cell are well characterised [13]: at the beginning of the cell cycle, when the cell possesses 1K and 1N (1K1N cell), K is situated between N and the posterior end of the cell and the two organelles are distant by approximately 2–2.5 μm. K is first replicated, giving raise to a 2K1N cell. After mitosis, the cell displays 2K and 2N (2K2N), one couple K/N is towards the anterior end (KaNa), the other has migrated towards the posterior end of the cell (KpNp). DAPI analysis revealed the usual proportions of cell cycle intermediates and detected only few abnormal cell types in induced conditions (less than 5%, Fig. 1F). We noticed that N and K were very close to each other and that K could sometimes be seen in an anterior position with regard to N (Fig. 1G). We quantified this phenotype in non induced and induced cells at various time points. At each time point 100 uniflagellated cells were analysed. Both Three situations were observed and cells were grouped accordingly (Fig. 1H): (1) K in posterior position (K post), K and N separated by at least 0.5 μm, (2) K post, K and N separated by less than 0.5 μm, (3) K in anterior position (Kant), K and N separated by less than 0.5 μm (Fig. 1H). When K and N were separated by less than 0.5 μm, K was in close proximity and seemed almost on