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SHORT COMMUNICATION

Trypanosomatid Pin1-Type Peptidyl-Prolyl Isomerase Is Cytosolic and Not Essential for Cell Proliferation

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Keywords

MORN domain; parvulin; peptidyl-prolyl isomerase; Pin1; PPIase; *Trypanosoma cruzi*.

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ABSTRACT

Pin1-type peptidyl-prolyl *cis/trans* isomerases (PPIases) isomerise the peptide bond of specific phosphorylated (Ser/Thr)-Pro residues, regulating various cellular events. Previously, we reported a Pin1-type PPIase in *Trypanosoma cruzi*, but little is known about its function and subcellular localization. Immunofluorescence analysis revealed that in contrast with Pin1-like proteins from diverse organisms, *Tc*Pin1 mainly localized in the cytoplasm and was excluded from the nuclei. In addition, RNAi-mediated downregulation of *Tb*Pin1 in *Trypanosoma brucei* did not abolish cell proliferation. Using yeast two-hybrid assay, we identified a MORN domain-containing protein as putative Pin1-binding partners. These data suggest that Pin1-mediated signaling mechanism plays a different role in protozoan parasites.

PIN1 belongs to the parvulin family of peptidyl-prolyl *cis/trans* isomerases (PPIases) (EC 5.2.1.8). Enzymes of this subfamily display a striking preference for substrates containing phosphorylated side chains of serine or threonine residues preceding the proline position (Ranganathan et al. 1997). Pin1 function is dependent on its nuclear localization, consistent with its substrates being involved in transcription and cell cycle progression (Reviewed in Liou et al. 2011). Pin1-type PPIases contain two domains that are important for the in vivo function of these proteins: an N-terminal WW domain and a C-terminal PPIase domain. The WW domain acts as a phosphoprotein-binding module regulating substrate interaction (Lu et al. 2002). In several organisms, Pin1 localizes almost exclusively in the cell nucleus and concentrates at discrete structures (Huang et al. 2001; Lu et al. 1996). Phosphorylation within the catalytic active site of hPin1 impairs nuclear localization. This has been attributed to the predicted interactions between the phosphorylated site and a conserved Arg residue, which lies within Pin1's nuclear localization (Lee et al. 2011; Lufei and Cao 2009). We have previously described the biochemical and molecular characterization of the parvulin-type PPIase family from *Trypanosoma cruzi*. Three distinct parvulins were identified in this parasite and annotated as *Tc*Pin1 (Erben et al. 2007), *Tc*Par14, and *Tc*Par45 (Erben et al. 2010). Only *Tc*Pin1 possesses the characteristics of Pin1-type parvulins and shows a high degree of similarity with the previously characterized plant Pin1-type parvulins. *Tc*Pin1 is formed by a single-domain polypeptide and contains only the main catalytic core of PPIases, as do previously reported homologs in different plants species (Kouri et al. 2009; Landrieu et al. 2000; Metzner et al. 2001; Yao et al. 2001). However, all the Pin1 homologs examined from

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plants have been localized in both nuclear and cytosolic fractions (Kouri et al. 2009; Metzner et al. 2001; Wang et al. 2010). Despite the well-studied roles of parvulin-type PPIases in higher eukaryotic, very little is known about the physiological functions of these enzymes in the TriTryp parasites (causative agents of Chagas' disease, African sleeping sickness, and leishmaniasis), where Pin1-like PPIases display conserved features (Erben et al. 2007, 2010; Goh et al. 2010). To further characterize the Pin1 cellular function in trypanosomatids, we looked at its subcellular localization and studied the effect of downregulation of *Tb*Pin1 in procyclic (PCF) *T. brucei* using RNA interference (RNAi). Finally, using a yeast two-hybrid screen, we identified a Membrane Occupation and Recognition Nexus (MORN)-containing protein as putative substrate.

MATERIALS AND METHODS

Immunofluorescence microscopy

Immunofluorescence was essentially performed as described previously (Erben et al. 2010). Pin1 was detected by incubating cells for 1 h at 25 °C with the Pin1 rabbit affinity-purified antibody (1:400 dilution), and subsequently incubated for 1 h at 25 °C with the Alexa Fluor 546-conjugated anti-rabbit (Molecular Probes, 1:500) as the secondary antibody and DAPI to label nuclear and kinetoplast DNA (blue). The rabbit polyclonal anti-green fluorescent protein (anti-GFP) antibody was from Anaspec (San Jose, CA).

Parasite transfection and RNAi experiments

The entire coding region of *Tc*MORN2 was amplified from *T. cruzi* CL Brener genomic DNA by PCR using *Pfu* polymerase. PCR reaction conditions were as follows: initial denaturation cycle at 94 °C for 1 min, followed by 25 cycles of 94 °C for 1 min, 60 °C for 20 s, 72 °C for 3 min, and a final extension of 10 min at 72 °C. The primers for PCR cloning of the *TcMORN2* gene were MORN2F 5′-AGGATATCATGCCAGGCGCACG-3′ and MORN2R 5′-GAGATATCATCCGCAGAATGACGC-3′ as sense and antisense primers, respectively. The inserted restriction sites are underlined. The PCR product was cloned into the *Eco*RV digested pTEX vector in fusion with the GFP. *Trypanosoma cruzi* epimastigote cells of the CL Brener strain were transfected with the plasmid as previously described (Erben et al. 2007). For RNAi, *T. brucei* 29-13 cells were transfected with the pZJM vector as previously described (Erben et al. 2010). To test the effects of RNAi, we added tetracycline (1 μg/ml) and cultivated the parasites for up to 9 days, diluting as required and assessed mRNA levels by Northern blotting.

Other procedures

The yeast two-hybrid screening and the synthesis and screening of the cellulose-bound overlapping oligopeptides of *Tc*MORN2 can be found in the Data S1 associated with this article.

Results and Discussion

To ascertain that the endogenous *Tc*Pin1 was accurately detected, previously obtained polyclonal antibodies (Erben et al. 2007) were affinity purified as described (Erben et al. 2010). Indirect immunofluorescence assays in epimastigote cells of *T. cruzi* indicated that *Tc*Pin1 is mainly localized in the cytoplasm but also revealed some degree of flagellar localization (Fig. 1A). On immunoblots of cell lysates from *T. cruzi* epimastigotes, the purified antibodies recognize a single polypeptide that matched the predicted molecular mass of 13 kDa (Fig. 1B). In contrast with Pin1-like proteins from diverse organisms (Huang et al. 2001; Kouri et al. 2009; Lu et al. 1996, 2002; Metzner et al. 2001), *Tc*Pin1 localizes outside the nucleus.

To gain further insight into the function of Pin1 in trypanosomatids, we performed a large-scale yeast two-hybrid screen using *Tc*Pin1 as bait using a *T. cruzi* cDNA library. After verify expression of trypanosome *Tc*Pin1 in yeast cells by Western blotting (data not shown), we obtained two uncharacterized "hypothetical" proteins as positive clones. One of these clones comprised a fragment corresponding to the TcCLB.505999.140 open reading frame (ORF), and the other clone carried a fragment from the TcCLB.509617.40 ORF. Whereas the bioinformatics analysis of the *Tc*CLB.505999.140 sequence did not reveal any domains with a highly significant score, the *Tc*CLB.509617.40 analysis revealed a MORN-containing protein. We named to this protein as MORN2. *Tc*MORN2 encodes a 916-amino-acid-predicted protein containing 17 MORN domains along its sequence. The MORN repeat is a $\sim$22 amino acid repeat originally identified in studies of junctophilins, mammalian proteins involved in transmembrane junctional complexes between the ER and the plasma membrane (Takeshima et al. 2000). To determine the subcellular localization of *T. cruzi* MORN2, epimastigotes expressing GFP-tagged MORN2 were generated. Production of ectopic *Tc*MORN2-GFP (126 kDa) proteins of the correct molecular weight was verified by Western blotting (Fig. 1C). In addition to the flagellum, GFP-MORN2 proteins are also abundant at the base of the flagellum where they colocalize partially with *Tc*Pin1 (Fig. 1D). However, we have been unable to validate, despite numerous coimmunoprecipitation assays, a direct interaction between the GFP-MORN2 and *Tc*Pin1 in epimastigote cells. This might be a reflection of the *Tc*Pin1 structure as it lacks the WW domain. Furthermore, weak and transient interactions are more readily detected in two-hybrid screens as the genetic reporter gene strategy results in a significant amplification. However, the possibility that the binding affinities are affected by the GFP tagged to the Pin1 protein cannot be ruled out. In addition, it could be possible that such interaction is parasite stage specific. Therefore, to ascertain whether the *Tc*MORN2-*Tc*Pin1 interaction occurs, a different approach was taken. We used a cellulose-bound array to

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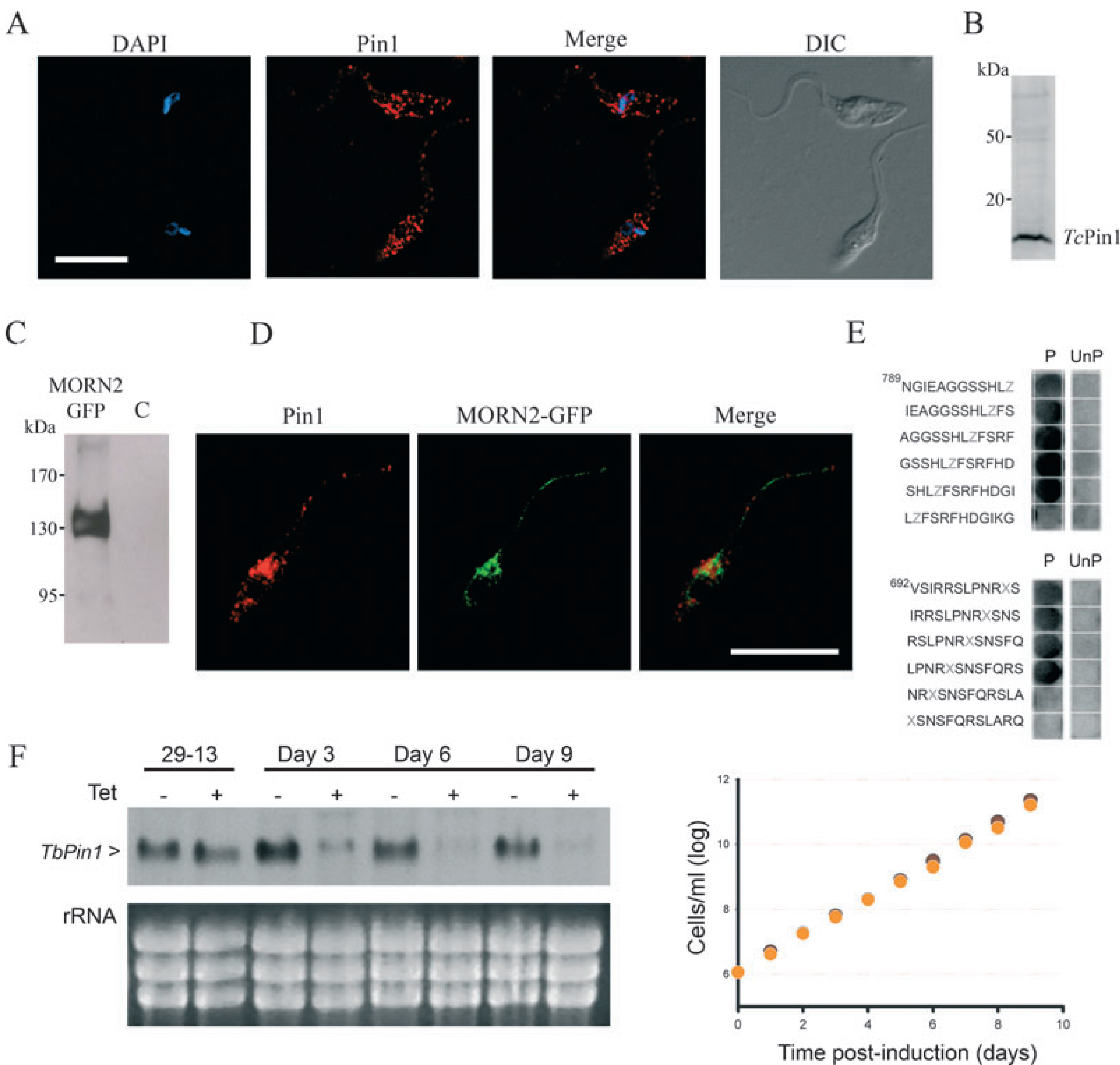

Fig. 1. *Tc*Pin1 location is cytosolic and downregulation is nonessential for proliferation of PCF *Trypanosoma brucei*. (A) *Tc*Pin1 (*red*) localizes to the trypanosome cytosol in epimastigote cells. DAPI (*blue*) was used to visualize DNA. Scale bar = 5 μm. (B) The purified anti-*Tc*Pin1 antiserum recognizes a 13-kDa protein in total cell extracts. (C) Production of an in-frame-tagged polypeptide of the correct molecular weight was verified by Western blot on epimastigote expressing *Tc*MORN2-GFP. Untransfected parasites (*C*) were used as negative control. (D) Fluorescence microscopy of epimastigotes transfected with GFP-*Tc*MORN2 (green) and stained with anti-*Tc*Pin1 antibodies (*red*). Scale bar = 5 μm. (E) Recombinant His-*Tc*Pin1 binding to multiple *Tc*MORN2-derived peptides is dependent on interactions with phosphorylated Ser (X = Ser(PO3H2)-Pro) and Thr (Z = Thr(PO3H2)-Pro) residues preceding proline bonds (P). Unphosphorylated sequences were used as a negative control (UnP). Complete sequences of peptides identified as interacting with *Tc*Pin1 are shown (*left*). More detailed methods are in the Data S1. (F) *Tb*Pin1 is not essential in 29-13 PCF cells. Representative cumulative growth curves of *Tb*Pin1 RNAi cells in the presence (orange circles) or absence (brown circles) of tetracycline. A time-course representative Northern blot is shown.

discover all the peptides in the *Tc*MORN2 protein that have the potential to bind to *Tc*Pin1. Peptide arrays prepared by spot synthesis on solid carriers are powerful tools for the study of protein–protein interactions (Frank 2002). As demonstrated by this approach, we observed that recombinant *Tc*Pin1 binds peptide motifs containing phosphorylated (Ser/Thr)-Pro residues (Fig. 1E). Although peptides containing the phosphorylated sequences bound the *Tc*Pin1 as anticipated, nonphosphorylated counterparts abolished detectable peptide binding to the PPIase

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enzyme. The small parvulin therefore appears to bind their targets through multiple interactions with motifs comprised phosphorylated targets. This assay strongly supports our previous results demonstrating that this parvulin is a phosphorylation-dependent PPIase and suggests that a *Tc*Pin1/*Tc*MORN2 interaction would occur in vivo.

Next, to investigate whether Pin1 depletion affects cell proliferation in trypanosome cells, as occur in different organisms (Devasahayam et al. 2002; Lu et al. 1996; Rippmann et al. 2000; Winkler et al. 2000), an inducible RNAi cell line was generated using the PCF Lister 427 29-13 strain. *Tb*Pin1 (Tb927.8.690; 66% identity with *T. cruzi* Pin1; Erben et al. 2007) was found to be nonessential in this stage (Fig. 1F). Knockdown efficiency was validated by Northern blot revealing that mRNA levels were reduced by > 90% after 3 days of induction. However, no proliferation defect was noted in PCF cells. In the same way, growth kinetics was not affected by the overexpression of the parvulin *Tc*Pin1 in epimastigote cells of *T. cruzi* (Erben et al. 2007).

The data reported here suggest that in *T. cruzi*, the orchestration of phosphorylation-dependent prolyl isomerization is carried out in a fundamentally different manner than that of other eukaryotes. First, only *Tc*Pin1 has localization other than nuclei. In hPin1, residues K63, R68, and R69 may actually represent a nuclear localization signal (NLS) (Lufei and Cao 2009). Although further studies are required, it is tempting to speculate that exclusion of trypanosomatid Pin1-like PPIases from nuclei could be due to its lack of the conserved NLS (Fig. S1). Nevertheless, probably other trypanosomatid-specific signatures may play a role. Second, in contrast to the mitotic arrest observed when Pin1 is silenced (Crenshaw et al. 1998; Lu et al. 1996; Shen et al. 1998), depletion of the *T. brucei* homolog Pin1 did not display detectible mitotic phenotype or flagellar defect. This is unlikely due to inefficient suppression of mRNA levels, as the PCF RNAi construct effectively declined to negligible levels after day 3 of induction. Finally, although Pin1 is able to bind specifically to phosphorylated oligopeptides derived from MORN2, the possible functional implication of such interaction remains unclear. Interestingly, analysis of the *T. brucei* phosphoproteome indicated that *Tb*MORN2 (Tb927.3.4270, 62% identity with MORN2 from *T. cruzi*) is indeed phosphorylated (Nett et al. 2009). These data may suggest that Pin1-mediated signaling mechanism is not conserved between higher eukaryotic and trypanosomes.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig S1. Phylogenetic tree and subcellular localization for the Pin1-like PPIases.

Data S1. Yeast two-hybrid and SPOTs membrane analysis.

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