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Original article

Characterization of a 21 kDa protein from *Trypanosoma cruzi* associated with mammalian cell invasion

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Abstract

Trypanosoma cruzi genomic database was screened for hypothetical proteins that showed high probability of being secreted or membrane anchored and thus, likely involved in host-cell invasion. A sequence that codes for a 21 kDa protein that showed high probability of being secreted was selected. After cloning this protein sequence, the results showed that it was a ubiquitous protein and secreted by extracellular amastigotes. The recombinant form (P21-His_6) adhered to HeLa cells in a dose-dependent manner. Pretreatment of host cells with P21-His_6 inhibited cell invasion by extracellular amastigotes from G and CL strains. On the other hand, when the protein was added to host cells at the same time as amastigotes, an increase in cell invasion was observed. Host-cell pretreatment with P21-His_6 augmented invasion by metacyclic trypomastigotes. Moreover, polyclonal antibody anti-P21 inhibited invasion only by extracellular amastigotes and metacyclic trypomastigotes from G strain. These results suggested that P21 might be involved in *T. cruzi* cell invasion. We hypothesize that P21 could be secreted in the juxtaposition parasite-host cell and triggers signaling events yet unknown that lead to parasite internalization.

Keywords: *Trypanosoma cruzi*; Cell invasion; Amastigote; Metacyclic trypomastigote

1. Introduction

In the past 20 years, many laboratories have attempted to identify surface components of *Trypanosoma cruzi* implicated in host-cell invasion by metacyclic trypomastigotes and tissue culture derived trypomastigotes (TCT). The picture emerging from these studies is that *T. cruzi* penetration into host cells is a multi-step process involving various parasite and host-cell molecules that, in a concerted series of events, leads to intracellular Ca^{2+} mobilization in both cells [1,2]. To invade mammalian cells metacyclic trypomastigotes engage surface glycoproteins such as gp82, gp35/50, or gp30, a gp82 variant expressed in gp82-deficient isolates [2]. These parasites may also take advantage of secreted components such as protein from the SAP (serine-, alanine-, and proline-rich protein) family [3]. TCTs engage a series of components to traverse the extracellular matrix and invade host cell such as Tc-85, gp83, Tc-1, cruzipain, oligopeptidase B, and POP Tc80 [1,2].

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On the other hand, little is known about the molecules involved in the extracellular amastigote (EA) invasion process into mammalian cells. Prior findings that amastigotes are present in the circulation of mice during the acute phase of the disease [4] raised the intriguing possibility that amastigotes may initiate an alternative subcycle of this parasite in the mammalian host [5]. This alternative mode of propagation may be significant for survival of the parasite in the presence of a host cytotoxic response, in which lysis of infected cells would lead to the release of viable amastigotes [5]. Unlike trypomastigotes, which show a marked preference for invading at the host-cell margins, amastigotes attach seemingly at random to surface microvilli and become internalized at the dorsal surface of cells [6]. Previous studies from our laboratory also showed that EA from G and Tulahuen strains were more infective than those from the traditionally more infectious strains (CL and Y) [6]. Furthermore, we observed that carbohydrate epitopes from Ssp-4 [4], an amastigote specific surface component, are necessary for the parasite attachment into host cells prior to invasion [7]. Also, we have shown that EA invasion involves the formation of small cup-like membrane expansions in HeLa cells and larger crater-like structures in Vero cells [6]. Amastigote invasion of either HeLa or Vero cells required functional actin microfilaments in order to achieve internalization. Clustering of actin filaments is detected at the site of amastigote attachment, and the process is inhibited by the protein kinase inhibitors staurosporine and genistein [6]. In addition, the expression and activation of Rho GTPase Rac1 are involved in the invasion of EA from G strain [6] and microdomains present in mammalian cell membranes, that are enriched in cholesterol and GM1 are implicated as well [8].

In this study we used EA of two *T. cruzi* strains that showed distinct infectivities [9] to further investigate the surface or secreted molecules that promote parasite entry into mammalian cells. First, we screened *T. cruzi* genomic database in order to find hypothetical proteins that showed high probability of being secreted or membrane anchored and thus, likely involved in host-cell invasion. We have cloned and characterized a novel *T. cruzi* protein that is ubiquitous, secreted and its recombinant form (P21-His_6) adhered to mammalian cells in dose-dependent manner and upregulated a phagocytosis-like activity by triggering a yet unknown host-cell signaling response.

2. Material and methods

2.1. Gene predictions

Motif scanning in the hypothetical proteins from *T. cruzi* genomic database was performed in the ExPASy proteomics server at http://www.expasy.org/, which screens for signal peptide cleavage sites (SignalP); glycosylphosphatidylinositol (GPI) anchor and cleavage sites (DGPI); *O*-GalNAc (mucin-type) glycosylation sites (NetOGlyc); *N*-glycosylation sites (NetNGlyc); Ser, Thr, and Tyr phosphorylation sites (NetPhos) and protein destination (SecretomeP, TargetP).

2.2. Cloning and purification of a recombinant P21 (P21-His_6)

A fragment of P21 allele gene (GenBank XM_812182) encoding the molecule without the predicted signal peptide was cloned in fusion with His tag in plasmid pET-28a(+) (Novagen). In order to amplify the fragment from G strain genomic DNA, the following primers were used: forward NdeI-5′-GCG CGC TCA TAT GGA GGA GGT GGT GAA TCG GGG-3′ and reverse EcoRI-5′-GGA ATT CTT ACT GGC GTC TGT GGA ATC C-3′. The amplification was performed using *Taq* DNA polymerase in a final volume of 50 µl. PCR conditions were 35 cycles of 1 min at 94 °C, 30 s at 58 °C and 1 min at 72 °C, and a final extension at 72 °C for 10 min. The insert was cloned into the plasmid pET-28a(+) and was used to transform *Escherichia coli* BL-21 to produce recombinant P21. The recombinant protein was purified in pre-packed Ni-Sepharose columns (Quiagen) then dialyzed for 48 h at 4 °C against PBS. The amount of purified protein was determined in microtiter plates by using the Coomassie Plus assay reagent (Pierce) and measuring the optical density at 620 nm. To ascertain that the correct protein was obtained, the purified preparations were analyzed by Coomassie blue staining SDS-polyacrylamide gels.

2.3. Similarity searches

Total RNA was extracted from extracellular amastigotes from G and CL strains with TRIZOL® Reagent (Invitrogen). First-strand cDNA was prepared using SuperScript® II Reverse Transcriptase (Invitrogen) and oligo (dT) primers according to the manufacture's instructions (Life Technologies). Primer forward: Splice leader 5′-CTA TTA TTG ATA CAG TTT CTG TAC TAT ATT-3′ and reverse: 5′-GGA ATT CTT ACT GGC GTC TGT GGA ATC C-3′ were used to amplify P21 sequence by PCR. The PCR product was used to sequence P21 by the dideoxynucleotide chain termination method using *Taq* dye terminator cycle sequencing chemistry in an ABI 3100 DNA sequencer. BLASTN and BLASTP [10] algorithms were used to search for homologous nucleic acid or protein sequence in the *T. cruzi* GeneDB and GenBank databases, respectively, at http://www.genedb.org/and http://www.ncbi.nlm.nih.gov/.

2.4. Parasites, mammalian cells and invasion assays

G and CL *T. cruzi* strains were used in this study. EA from these strains were obtained after differentiation of TCT in LIT medium as previously described in Ref. [7]. Epimastigotes and metacyclic trypomastigotes were obtained essentially as previously described in Ref. [11].

Vero and HeLa cells (obtained from Instituto Adolfo Lutz, São Paulo, SP, Brazil) were cultured in Dulbecco's minimal essential medium (DMEM) (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Cultilab, Campinas, SP, Brazil), 10 µg/ml streptomycin, 100 U/ml penicillin and 40 µg/ml gentamycin at 37 °C in a 5% CO_2 humid atmosphere. For experiments with intracellular