Infection, Genetics and Evolution 9 (2009) 1083–1091

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

cDNA cloning and partial characterization of amastigote specific surface protein from *Trypanosoma cruzi*

Marybell Olivas-Rubio [a], Salvador Hernández-Martínez [b], Patricia Talamás-Rohana [a], Victor Tsutsumi [a], Pedro A. Reyes-López [c], José Luis Rosales-Encina [a,*]

[a] Departamento de Infectómica y Patogénesis Molecular, Centro de Investigación y de Estudios Avanzados del I.P.N., México D.F. 07360, Mexico
[b] Departamento de Genética, Centro de Investigaciones Sobre Enfermedades Infecciosas, Instituto Nacional de Salud Pública, Cuernavaca, Morelos, 62508, Mexico
[c] Dirección de Investigación, Instituto Nacional de Cardiología "Ignacio Chávez", México D.F. 14080, Mexico

ARTICLE INFO

Article history:
Received 18 December 2008
Received in revised form 20 May 2009
Accepted 20 May 2009
Available online 27 May 2009

Keywords:
Trypanosoma cruzi
Amastigotes
Surface protein
Gene expression
Molecular mimetism

ABSTRACT

Trypanosoma cruzi amastigote surface proteins are the target of both humoral and cell-mediated immune responses; however, few such molecules have been thoroughly studied. In order to study a *T. cruzi* amastigote-specific protein (SSP4), we used antibodies against the deglycosylated form of this molecule to clone cDNA. The selected cDNA clone (2070 bp) encodes for a 64 kDa protein product whose sequence analysis revealed no N-glycosylation signal. The DNA sequence showed high homology with a member of a previously reported dispersed repetitive gene family of *T. cruzi*. Antibodies against the recombinant protein reacted strongly with a 66 kDa protein and weakly with an 84 kDa protein in amastigote extracts. Immunoelectron microscopy studies showed that intracellular amastigotes express the native protein on their surfaces and flagellar pockets. The antibody label was also associated with an amorphous material present in the parasitic cavity and in direct contact with the parasite surface, which suggest that amastigotes are releasing this material. On cell-free amastigotes, the antibody showed strong decoration of the cell surface and labeling of intracellular vesicles. Immunofluorescence analysis showed that the superficial protein is expressed shortly after trypomastigotes begin to transform into amastigotes. Anti-recombinant protein antibodies recognized proteins of 100 kDa and 50–60 kDa in protein extracts of rat heart and skeletal muscle, respectively.

1. Introduction

Trypanosoma cruzi, the causative agent of Chagas' disease, undergoes morphological and physiological changes during the course of its life cycle. This cycle is initiated when metacyclic trypomastigotes are eliminated in the feces of the triatomine vector and enter mammalian cells. Inside the cell, trypomastigotes transform into amastigotes in a process that is characterized by changes in major surface glycoproteins (Andrews et al., 1987). Amastigotes are found both inside the host cells and in circulation and, as in trypomastigotes, they can infect cells both *in vivo* and *in vitro* (Ley et al., 1988; Noisin and Villalta, 1989; Mortara, 1991). To continue the cycle, amastigotes multiply, transform again into trypomastigotes, and lyse the cell. They are then released into circulation and spread the infection to other tissues.

It has been suggested that intracellular amastigotes play an important role in the persistence of *T. cruzi* infection (Verbisck et al., 1998). Using monoclonal antibodies, it has been shown that amastigotes from distinct strains and clones express different epitopes in a polymorphic manner, similar to patterns described for epitopes of other parasite stages (Verbisck et al., 1998). Previous work has shown that newly transformed amastigotes – both intracellular and extracellular – express a major surface glycoprotein (SSP4) that is bound to the plasma membrane by a GPI anchor (Andrews et al., 1987, 1988a,b). The cloning of a closely related gene family that is highly expressed in amastigotes (accomplished by differential screening) has also been reported. These genes encode for amastin (Teixeira et al., 1994), surface glycoproteins that show a half-life 7 times longer in amastigotes than in epimastigotes and a level of mRNA 68-fold higher in amastigotes than in epimastigotes (Coughlin et al., 2000). Other amastigote-specific genes (ASP-1 and ASP-2) were shown to be members of the sialidase/trans-sialidase gene superfamily 2 of *T. cruzi* (Low and Tarleton, 1997; Santos et al., 1997).

Here, we report the purification of an 84-kDa protein reactive to the monoclonal antibody 2C2, which defines the SSP4 amastigote-specific surface glycoprotein, and the partial cDNA cloning of an

* Corresponding author at: Departamento de Infectómica y Patogénesis Molecular, Centro de Investigación y de Estudios Avanzados del I.P.N., Avenida Instituto Politécnico Nacional No. 2508, Col. San Pedro Zacatenco, Delegación Gustavo A. Madero, CP 07360, México D.F., Mexico. Tel.: +52 55 5747 3349; fax: +52 55 5747 3377.
E-mail address: rosales@cinvestav.mx (J.L. Rosales-Encina).

1567-1348/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.meegid.2009.05.016

amastigote-specific surface protein (SSP4). Surface expression of SSP4 was also detected in intermediate forms between trypomastigotes and amastigotes, indicating that the gene starts expressing when trypomastigotes begin the transformation into amastigotes. Anti-fusion protein *MBP::SSP4* antibodies recognized proteins of 100 kDa and 50–60 kDa in protein extracts of rat heart and skeletal muscle, respectively.

2. Materials and methods

2.1. Parasites

Strain Y of *T. cruzi* was maintained in monolayers of LLC-MK2 cells in Dulbecco's minimum essential medium (DMEM) containing 5% fetal calf serum (FCS) at 37 °C, epimastigote forms were grown in LIT medium at 28 °C with gentle shaking (Andrews et al., 1987). One to two weeks after infection, the supernatant containing trypomastigote, intermediate, and amastigote forms was collected. To obtain trypomastigotes, the heterogeneous parasite suspension was centrifugated at 2000 × *g* for 5 min and then incubated at 37 °C. After 2 h, the motile, slender, and highly infective trypomastigotes were collected from the supernatant. Trypomastigotes released from infected cells were incubated in LIT medium at 37 °C for 24–48 h for extracellular transformation into amastigotes (Andrews et al., 1987, 1988a).

2.2. TcSSP4 purification and deglycosylation

*Tc*SSP4 purification was carried out as described (Iida and Ley, 1991). Briefly, 8×10^9 parasites were suspended in 10 mM Tris–HCl, pH 7.7, containing 10 mM NaCl and a cocktail of protease inhibitors (10 μg/ml leupeptin, 0.2 mM phenylmethane sulfonate fluoride, and 0.1 mM EDTA), quickly frozen in liquid nitrogen and thawed in a 37 °C water bath. After three cycles of freezing and thawing procedure, disrupted parasites were centrifugated at 10,000 × *g* for 30 min. The supernatant was passed through a DEAE-5PW column (Pharmacia LKB) and washed with 10 mM NaCl, 10 mM Tris–HCl, pH 7.7 containing 0.02% Tween-20 (Buffer A), bound proteins were eluted with a linear gradient of NaCl (0.1–0.5 M) in Buffer A. In order to check for the presence of SSP4 glycoproteins, fractions were assayed by silver staining of 10% SDS–PAGE and Western blot with the monoclonal antibody (mAb) 2C2 (Andrews et al., 1987). Positive fractions were desalted in a 3.0 ml Sephadex G-25 column equilibrated with 10 mM NaCl, 10 mM acetate buffer pH 4.1 containing 0.02% Tween-20 (Buffer B), then passed through a Mono-S HR 5/5 column (Pharmacia LKB) previously equilibrated with Buffer B. Proteins were eluted with a linear gradient of NaCl (0–0.5 M) in the same buffer. SSP4 purity was assayed by silver staining and Western blot with the mAb 2C2. SSP4 protein (25–100 μg) was deglycosilated under denaturating conditions using N-glycanase (Genzyme). Samples were heated at 100 °C during 3 min in the presence of 0.25 M sodium phosphate buffer, pH 8.6 and 0.5% SDS. o-Phenantroline, NP-40, and N-glycanase were added to a final concentration of 10 mM, 0.6%, and 100 U/ml respectively. Samples were incubated for 16 h at room temperature.

2.3. DNA and cDNA expression library construction and screening

Genomic DNA was prepared from epimastigotes using a standard protocol (Maniatis et al., 1982). DNA was partially digested with HaeIII restriction enzyme, and fragments of 1000–6000 bp were isolated by a sucrose gradient. EcoRI adapters were ligated to the DNA HealII fragments and then ligated into λgt11 expression vector (Stratagene, La Jolla, CA). Also an amastigote cDNA expression library was constructed in the same vector (Gonzalez et al., 1990). A rabbit polyclonal antibody (see below) developed against purified and deglycosilated SSP4 molecule was used to screen 120,000 phages from the genomic expression library. After three rounds of screening one clone (C2-1) out of 10 highly positives was selected for further characterization. DNA inserts of λgt11 clones (genomic and cDNA) were subcloned in the EcoRI site of the plasmid pBluescript SK. A 5′-end 450 bp PstI fragment from clone C2-1 was used to screen the cDNA expression library (1.5×10^4 pfu) by standard procedures. A cDNA clone (*TcSSP4*) was isolated after three rounds of screening.

2.4. DNA sequencing

DNA inserts of selected clones were subcloned into the pBluescript SK-vector and subjected to automated double-stranded sequencing using Taq Fluorescence-Based Dye Terminator Cycle Sequencing on a PerkinElmer/Applied Biosystems 377-18Eln DNA sequencer, and also by the dideoxynucleotide chain termination method using Sequenase (United States Biochemicals, Cleveland, OH).

2.5. Fusion protein expression

The cDNA insert (GeneBank™, EMBL, and DDJ databases accession number AF480943) was cloned in the EcoRI site of the expression vector pMAL-C2 (New England Biolabs), and expressed in *Escherichia coli* DH-5α as fusion protein with Maltose Binding Protein (MBP). The fusion protein *MBP::SSP4* and MBP were affinity purified by using amylose resin according to the manufacturer. Purity of proteins was assayed by SDS–PAGE and Western blot. The *Tc*Hsp70 cDNA insert (GeneBank™ EMBL and DDJ databases accession number AY576621) was cloned in the same vector and the *MBP::Hsp70* was affinity purified.

2.6. Antibodies

Amastigote-specific 2C2 mAb has been previously described (Andrews et al., 1987). Polyclonal antibodies (R188) against the deglycosilated protein were obtained by immunization of rabbits with 50 μg of antigen in the presence of complete Freund's adjuvant. After 4 weeks rabbits were bled and the antibody titer was measured by Western blot against pure protein. Antibodies directed against *MBP::SSP4* and MBP were elicited by immunization of mice 4 times at 2-week intervals with 500 ng of protein per dose, and bled 2 weeks after the last booster injection. Complete Freund's adjuvant was used in the first immunization and incomplete Freund's adjuvant in the following.

2.7. Immunoblotting

Parasites (amastigotes, epimastigotes, and trypomastigotes) were pelleted by centrifugation and washed twice with cold phosphate-buffered saline (0.02 M sodium phosphate pH 7.4). Pelleted parasites were boiled in sample buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 5% 2-mercaptoethanol, 2% sodium dodecylsulfate, and 0.001% bromophenol blue), run in a 10% SDS–PAGE, and transferred to nitrocellulose paper. Blots were incubated with primary antibodies (anti-*MBP::SSP4* at a 1:1000), followed by incubation with a 1:3000 of the alkaline phosphatase conjugated secondary antibody.

2.8. Indirect immunofluorescence

Infected LLC-MK2 cell monolayers were washed twice with PBS and fixed in absolute methanol for 15 min at 4 °C. Collected free