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The identification and characterization of epitopes in the 30–34 kDa *Trypanosoma cruzi* proteins recognized by antibodies in the serum samples of chagasic patients

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ABSTRACT

Trypanosoma cruzi proteins with molecular weight between 30 and 34 kDa have shown high reactivity in western blot assays with serum samples from chagasic individuals. However, in-depth analysis of the constituents of these protein fractions has not been performed. This is the first report of an immunoaffinity proteomic approach to identify the immunodominant 30–34 kDa proteins of *T. cruzi* that could eventually be used for the diagnosis of Chagas disease. We used two different sample preparation protocols for protein digestion coupled to mass spectrometry to identify proteins in the protein fraction. The immunodominant proteins and their respective epitopes were then identified by co-immunoprecipitation and excision-epitope mapping/mass spectrometry, using human sera followed by the prediction and three-dimensional structural modeling of reactive epitopes. The use of different sample preparation methods allowed the identification of a relatively high number of proteins, some of which were only identified after one or multiple sample preparation and digestion protocols. Seven immunodominant proteins were identified by co-immunoprecipitation with purified IgGs from chagasic serum samples. Moreover, six reactive peptide epitopes were detected in four of these proteins by excision-epitope mapping/mass spectrometry. Three-dimensional structural models were obtained for the immunoreactive peptides, which correlated well with the linear B-cell epitope prediction tools.

1. Introduction

Chagas disease (CD) is an important public health problem that affects almost 8 million people in South and Central America [1,2]. Over the years, the disease has spread to other areas, including North America and Europe, where blood transfusion has become the main route of transmission of the parasite [3]. These facts forced the implementation of a

Abbreviations: CD, Chagas disease; 2-DE, two-dimensional gel electrophoresis; Co-IP, co-immunoprecipitation; MS, mass spectrometry

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rigorous and systematic screening program of blood donors in the affected countries [4].

The diagnosis of *Trypanosma cruzi* infection is based on clinical data, and supported by laboratory analyses that can be accomplished by parasitological methods for the direct or indirect detection of the parasite or by the immunological detection of antigens of the parasite or host-specific antibodies in the serum of infected individuals. In the latter case, the use of total parasite extracts as antigens usually generates specificity problems in the immunoassays. For this reason, several purified and recombinant proteins have been identified as candidates to be used in serological commercial tests [5–8]. *T. cruzi* proteins in the molecular range of 30–34 kDa have shown strong reactivity with the antibodies in serum samples from chagasic patients and have shown higher sensitivity and specificity than total parasite extracts in western blot analyses [9–11]. Interestingly, proteins in this molecular weight range have also been specifically detected in chagasic individuals during evaluation and standardization of a western blot test for the diagnosis of leishmaniasis, which confirms the potential utility of these proteins as immunogenic biomarkers for Chagas disease [12]. High-throughput proteomic approaches have been developed to identify thousands of proteins of *T. cruzi* [13,14], including when combining microarrays or co-immunoprecipitations (Co-IP) techniques and mass spectrometry (MS) to discover new diagnostic biomarkers for Chagas disease [15,16]. However, in-depth analysis of the constituents of the 30–34 kDa immunoreactive proteins of *T. cruzi* epimastigotes has not been defined. Here, we use two protocols for the preparation and digestion of samples to identify proteins in the 30–34 kDa protein fraction and to combine Co-IP and MS techniques to determine which immunodominant proteins and epitopes are present in this important fraction. The results were further reinforced by the prediction and tridimensional structure modeling of immunologically relevant proteins and reactive epitopes.

2. Materials and methods

2.1. Partial purification of the 30–34 kDa protein fraction

T. cruzi epimastigotes (Y strain) were cultivated in LIT medium at 28 °C [17]. The parasites were lysed and the extracted proteins were obtained by following the protocol of Teixeira et al. [9]. The protein concentration was determined by using the commercial reagent kit BCA Protein Assay Reagent (Pierce, Rockford, Illinois, USA), and the solution containing the total extracted proteins was stored at −80 °C until use [18].

To obtain the protein fractions, the total extracted proteins from the parasite lysate (10 mg/mL) were diluted 1:2 in sample buffer (50 M Tris–HCl, 1% SDS, 20% glycerol and 10% 2-mercaptoethanol), boiled for 5 min, centrifuged at 14,000 ×*g* for 5 min and run on a preparative sodium dodecyl sulphate poliacrylamide gel electrophoresis (SDS-PAGE) system Prep Cell Model 490 (BioRad Laboratories, Inc., Hercules, CA, USA) using 12% acrylamide column according to the manufacturer's instructions. The eluted material was recovered in small fractions using a fraction collector. SDS detergent was removed from each fraction by precipitation with 10% tricloroacetic acid (TCA) and washed five times with 50% ethanol–ether (v/v) by centrifugation at 13,000 ×*g* at 4 °C for 5 min. The dried pellet, which contained proteins in the range of 30–34 kDa, was resuspended in MilliQ water and analyzed by SDS-PAGE [19], and the protein bands were visualized by staining with colloidal Coomassie blue G-250 reagent.

2.2. Processing protocols for mass spectrometry analysis of the 30–34 kDa protein fraction

To identify the maximum number of proteins of this fraction, two different and complementary protocols were carried out to prepare trypsin digests of proteins in the 30–34 kDa fraction. The protocols are as follows.

2.2.1. Protocol 1 (SDS-PAGE and in-gel digestion)

Ten micrograms of proteins from the 30–34 kDa fraction was mixed (1:2, v/v) with the SDS-PAGE sample buffer and subjected to electrophoresis on a 12% SDS-PAGE vertical gel (Mini Protean II Dual Slab Cell, Bio Rad)). Protein bands on the gel were visualized by staining with the colloidal Coomassie blue G-250, sliced horizontally in six sections of gel pieces and reduced and alkylated with 50 mM DDT and 100 mM iodocetamide, respectively. The proteins were trypsin-digested with 25 μL of the enzyme (80 ng/mL in 25 mM NH_4HCO_3, pH 7.8) (MS grade, Promega, Maddison, WI, USA) per piece of gel at 37 °C for 14 h. After the gel extraction step, the resultant peptide mixture was loaded onto a ZipTip C-18 microcolumn (Millipore, Billerica, MA, USA) and eluted in 50% ACN. Finally, the solution was dried in a speed vac and resuspended with a solution containing 0.1% formic acid in 3% ACN for MS analysis.

2.2.2. Protocol 2 (in-solution digestion)

After precipitation with 10% TCA (item 1), a 1 μL aliquot of the 30–34 kDa fraction containing 10 μg of proteins was reduced and alkylated according to the protocol described above. The digestion of proteins in solution was carried out with 100 ng of MS-grade Trypsin (Promega) in 25 mM NH_4HCO_3 at 37 °C for 14 h. The cleaning, elution and preparation of the peptide solution for MS analysis were performed according to protocol 1.

2.3. Mass spectrometry, data analysis and criteria for protein identification

The protein samples prepared using protocol 1 were analyzed using an ESI-Q-TOF micro in DDA acquisition mode (Waters MS Technologies, Manchester, UK) and an ESI-Q-TOF Premier in MSE acquisition mode (Waters MS Technologies), and the proteins that were trypsin-digested using protocol 2 were analyzed using the ESI-Q-TOF micro (Waters MS Technologies,) and the ESI-Q-TOF ultima in DDA acquisition mode (Waters MS Technologies).

The raw data obtained by DDA acquisition were collected and processed using the ProteinLynx 2.4 software (Waters MS Technologies), and the data were used to search and identify proteins using the MASCOT Server2.3. The data obtained