Epitope mapping of a single repetitive unit of the B13 Trypanosoma cruzi antigen as fusions to Escherichia coli LamB protein

Cátia M. Pereira a,*, José Franco da Silveira a, Márcia Duranti b, Edécio Cunha-Neto c, Bianca Zingales b, Beatriz A. Castilho a

a Departamento de Microbiologia, Imunologia e Parasitologia, Escola Paulista de Medicina, UNIFESP, Rua Botucatu 862, CEP 04023-062 São Paulo, SP, Brazil
b Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, Brazil
c Laboratório de Imunologia de Transplantes, Instituto do Coração, Faculdade de Medicina, Universidade de São Paulo, São Paulo, Brazil

Received 23 September 2003; received in revised form 9 January 2004; accepted 22 April 2004

First published online 10 May 2004

Abstract

B13, one of the immunodominant antigens of Trypanosoma cruzi, is composed of repeats of a 12-amino-acid motif. Using synthetic peptides, the sequence FGQAAAGDK was previously shown to contain the B13 immunodominant epitope recognized by chagasic patients sera. To investigate the effects of neighboring sequences in the immunodominance, we tested serum recognition of two B13 sequences fused to LamB. GDKPSPFGQAAA–LamB and FGQAAAGDKPSP–LamB were recognized, respectively, by 15% and 80% of 80 sera reactive to B13 antigen. Recognition of FGQAAAGDKPSP–LamB was inhibited by AAAGDK-containing synthetic peptides. FGQAAAGDKPSP–LamB competed with a B13 recombinant protein containing 16.6 repeats for binding to chagasic antibodies. These results strengthen previous conclusions on the immunodominant epitope of B13 and provide a comparison of two methods for epitope mapping.

© 2004 Published by Elsevier B.V. on behalf of the Federation of European Microbiological Societies.

Keywords: Trypanosoma cruzi; Immunodominant repetitive antigen; B13 antigen; B cell epitope mapping; Escherichia coli LamB protein

1. Introduction

Several pathogenic protozoa, such as Trypanosoma cruzi, Trypanosoma brucei, Plasmodium and Leishmania, express many antigens carrying repeated amino-acid epitopes that are immunodominant in natural infections [1–3]. Thus, the expression of peptide repeats seems to be a generalized phenomenon among protozoan parasites that evade the host immune system [1,2]. Although a large number of parasite repeated antigens have been isolated, little is known on the molecular mechanisms underlying the immunodominance of the repetitive epitopes. Studies on the conformational structure of these repetitive epitopes are mandatory to better define the molecular aspects of immunodominance.

The T. cruzi B13 antigen, located on the surface of infective trypomastigotes, is composed of arrays of a tandemly repeated 12-amino-acid motif – P(L)P(S,A)-P(L)FGQAA(A)E(G)A(D)GK, where residues within parentheses can be found replacing the preceding residue in different repeat copies [4]. B13, purified as recombinant protein from Escherichia coli, has been shown to present high sensitivity and specificity in the serological diagnosis of Chagas disease [4,5]. The characterization of B13 immunodominant epitopes has been based on the use of synthetic 9-mer peptides, derived from the B13 sequence, in competitive ELISA with B13 recombinant protein and sera from chagasic patients [6]. This analysis indicated that the peptide FGQAAAGDK contained the immunodominant epitope of B13 and that the 7-mer sequence QAAAGDK should be the core of
that epitope [6,7]. Conformational preferences of synthetic peptides bearing the immunodominant epitope of the B13 antigen have been investigated by our group by circular dichroism and $^1$H nuclear magnetic resonance [8]. The data suggested a correlation between the propensity of the peptides to adopt a helical configuration centered in the AAAGDK sequence and their antigenicity. Even though such studies thoroughly analyzed the antigenicity of short synthetic peptides representing the amino-acid sequences of selected regions of the antigenic B13 protein, they did not provide insights into how epitopes are recognized in the context of a native protein antigen. It has been reported that antibody recognition of B cell epitopes as free synthetic peptides can differ from that of the same epitopes embedded into a folded protein [9].

Bacterial surface display techniques based on in-frame fusion of heterologous sequences to a carrier protein had initial interest for the development of live bacterial vaccines. Peptide antigens from a variety of pathogens have been expressed on the surface of Salmonella and E. coli fused to flagellin or outer membrane proteins, respectively [10–12]. A number of other applications, including selection of high-affinity antibodies, production of anti-peptide antibodies, development of recombinant whole-cell adsorbents and biocatalysts, cell-based solid-phase reagents for diagnosis and tools for the study of adhesion–receptor interactions, shows the versatility of systems to express protein antigens on the bacterial surface [13–15].

LamB maltoporin, a 421-amino-acid, trimeric, integral, outer membrane protein of E. coli, has been used successfully to display foreign peptides on the bacterial surface [10,15,16]. The X-ray structure of LamB revealed 18 antiparallel β strands connected to each other on the cell exterior surface by long unstructured loops [17]. One of these loops, named L4, spanning amino acids 149–166 has been used as a site for insertions of several foreign sequences without disruption of the proper protein structure, localization, or ability to form trimers [16,18]. LamB can be expressed in high amounts in E. coli and is easily purified.

In a previous study, we described the use of the LamB protein as carrier of heterologous sequences in order to map the immunodominant B cell epitope of the T. cruzi JL8 repetitive antigen [19]. Our results showed that fusions to LamB may provide a simple and useful method to define and map B cell epitopes, overcoming obstacles inherent to synthetic peptides, such as insolubility and conformational variations. The fact that the site for insertions in LamB is part of a nonstructured loop provides the possibility for the heterologous sequence to take on a conformation that mimics its own in the native protein.

In this work, to confirm the conclusions obtained with synthetic peptides and to extend the results to in vivo systems, we have expressed two sequences derived from B13 (GDKPSPFGQAAA and FGQAAAGD-KPSK) in the LamB system and the fusion proteins were tested for antigenicity. The results confirmed previous conclusions on the primary structure of the immunodominant epitope of B13 and provide a comparison of this peptide mapping method with data from synthetic peptide-based mapping.

2. Materials and methods

2.1. Bacterial strain and plasmid

The E. coli strain POP6510 (thr leu tonB thi lacYi recA dex-5 metA supE) and plasmid pAJC264, which carries the lamB gene under the control of the tac promoter were a kind gift of Dr. M. Hofnung (Institute Pasteur, Paris, France). The lamB gene in pAJC264 contains a BamHI site for insertions between amino acids 153 and 154. Luria broth and Luria agar supplemented with ampicillin (100 μg·ml$^{-1}$) were used for growth of bacteria. The expression of LamB was induced by addition of isopropyl-β-D-thiogalactoside (IPTG) (10$^{-3}$ M) to the cultures at 0.7$A_{600}$ followed by an incubation of 4 h.

2.2. Synthetic oligonucleotides, fusion proteins and synthetic peptides

Complementary oligonucleotide pairs corresponding to two different sequences of the B13 gene (GenBank U15616) (Table 1), B13.1 (coding strand 5’-gatccagttgacaaccatactctggacgcggcctgaaat) and B13.2 (coding strand 5’-gatccatttgacagcgcgctgagctgacaaa-ccatcaccataat), were inserted in the BamHI site of pAJC264. The 5’ end of the coding oligonucleotides contained a BamHI half site to provide a rapid restriction analysis screen for the plasmids carrying the insertions. Ampicillin-resistant transformants of strain POP6510 had their plasmids analyzed for the presence of the insert by digestion with the enzymes BamHI and NcoI. The insertions were sequenced using a 20-mer primer complementary to nucleotides located 69 bp upstream from the BamHI insertion site in the lamB gene. A glutathione S-transferase (GST) fusion protein carrying 16.6 repeats of the 12-amino-acid motif of B13 (B13–GST) was also produced in E. coli (GST fusion system, Pharmacia) [5]. The recombinant protein was purified from IPTG-induced E. coli lysates by affinity chromatography.

Peptides encompassing linear epitopes of B13 antigen (Table 1) were synthesized by solid-phase Merrifield “teabag” technology from $t$-Boc derivatives, HPLC purified, and checked by mass spectrometry [6,8].
2.3. Purification of the LamB proteins

Bacterial cultures were grown in 50 ml of LB-ampicillin, harvested, washed in 50 mM Tris–HCl, pH 8.0, and resuspended in 0.5 ml of 20% sucrose–100 mM Tris–HCl, pH 8.0. After the addition of 0.1 ml of 0.5 M EDTA and 0.2 ml of 10 mg ml\(^{-1}\) lysozyme, the cell suspensions were incubated at 37°C for 15 min. Extraction buffer (0.5 ml) (2% Triton X-100, 10 mM MgCl\(_2\), 50 mM Tris–HCl, pH 8.0) was added, followed by incubation at 30°C for 30 min. The cell membrane fraction was sedimented by centrifugation at 2500 g for 10 min and resuspended in 0.3 ml of Laemmli sample buffer. Samples were boiled for 5 min and the proteins were separated on preparative 10% SDS–PAGE. The LamB protein was visualized by staining a strip of the gel with Coomassie blue. The corresponding portion of the unstained gel was isolated and LamB was eluted by incubation of the gel slice in 10 ml ddH\(_2\)O at 4°C for 18 h. The eluted protein was concentrated on SpeedVac\(^\circ\) and quantitated by SDS–PAGE using bovine serum albumin as standard.

For immunoblot analysis, proteins were transferred to nitrocellulose membranes (Hybond-C Extra, Amersham) at 1 A for 2 h using the buffer conditions as previously described [20]. The membranes were blocked with 5% milk in phosphate-buffered saline (PBS) for 1 h at room temperature, and incubated for 1 h with rabbit sera raised against a B13–GST recombinant fusion protein, diluted 1:100. After three washings with 0.05% Tween 20 in PBS, bound antibodies were detected with goat anti-rabbit IgG conjugated with peroxidase (Sigma), diluted 1:1000. After incubation for 1 h with the conjugate and washings in PBS, the bound antibodies were detected using the 4-chloro naphtol chromogen.

2.4. Sera

Human sera were obtained from patients with chronic Chagas disease diagnosed by serological and clinical symptoms. Serological analyses were performed by indirect immunofluorescence, indirect hemagglutination and ELISA. All the serum samples used in this work were positive in ELISA with B13-fused to GST. For the ELISA against recombinant LamB fusion proteins, the sera were absorbed with purified wild-type LamB protein (1 µg of protein per µl of serum). Serum against the GST–B13 fusion protein was raised in New Zealand female rabbits immunized with the recombinant protein purified as described. The protein (50 µg) was injected subcutaneously with complete Freund’s adjuvant. After 15 and 30 days from the first injection, 50 µg of protein were injected in the presence of incomplete Freund’s adjuvant. The rabbits were bled 30 days after the third injection.

2.5. Enzyme-linked immunosorbent assay

Microplates (Nunc) were coated with the purified recombinant LamB or GST proteins (50 ng/well) diluted in carbonate buffer (15 mM Na\(_2\)CO\(_3\), 35 mM NaHCO\(_3\), pH 9.6) by overnight incubation at 4°C. The wells were washed three times with PBS-Tween 0.05% and blocked with 5% milk for 2 h at 37°C. Sera from chagasic patients (diluted 1:200) were added and the plates were incubated for 1 h at 37°C. After three washes with PBS-Tween 0.05%, rabbit anti-human IgG peroxidase-conjugate diluted 1:2000 (DAKO) was added and the plates were incubated for 1 h at 37°C, before development with O-phenylenediamine (OPD). For competitive ELISA, the microplates were coated with B13–GST recombinant fusion protein (50 ng/well) diluted in carbonate buffer (15 mM Na\(_2\)CO\(_3\), 35 mM NaHCO\(_3\), pH 9.6) at 4°C overnight. The wells were washed three times with PBS-Tween 0.05% and blocked with 5% milk for 2 h at 37°C. Sera from chagasic patients (diluted 1:400), incubated previously with different concentrations of the B13–LamB fusion proteins, were added and the plates were processed as above. Competitive inhibition of B13 ELISA was performed as described [6]. Briefly, appropriate dilutions of serum samples (yielding \(A_{492}\) in the 0.3–0.8 range) were preincubated with the synthetic peptides (0.2 M) overnight at 4°C. Each serum/peptide mixture was then incubated 1 h at 37°C in

<table>
<thead>
<tr>
<th>LamB fusion proteins</th>
<th>B13.1</th>
<th>G</th>
<th>D</th>
<th>K</th>
<th>P</th>
<th>S</th>
<th>P</th>
<th>F</th>
<th>G</th>
<th>Q</th>
<th>A</th>
<th>A</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>B13.2</td>
<td></td>
<td>F</td>
<td>G</td>
<td>Q</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>G</td>
<td>D</td>
<td>K</td>
<td>P</td>
<td>S</td>
<td>P</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Peptides</th>
<th>pB13</th>
<th>G</th>
<th>D</th>
<th>K</th>
<th>P</th>
<th>S</th>
<th>L</th>
<th>F</th>
<th>G</th>
<th>Q</th>
<th>A</th>
<th>A</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>S4</td>
<td></td>
<td>F</td>
<td>G</td>
<td>Q</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>G</td>
<td>D</td>
<td>K</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S5</td>
<td></td>
<td>Q</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>G</td>
<td>D</td>
<td>K</td>
<td>P</td>
<td>S</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S3</td>
<td></td>
<td>S</td>
<td>L</td>
<td>F</td>
<td>G</td>
<td>Q</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S2</td>
<td></td>
<td>K</td>
<td>P</td>
<td>S</td>
<td>L</td>
<td>F</td>
<td>G</td>
<td>Q</td>
<td>A</td>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1</td>
<td></td>
<td>G</td>
<td>D</td>
<td>K</td>
<td>P</td>
<td>S</td>
<td>P</td>
<td>L</td>
<td>F</td>
<td>G</td>
<td>Q</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pNR</td>
<td>N</td>
<td>K</td>
<td>S</td>
<td>A</td>
<td>K</td>
<td>Q</td>
<td>F</td>
<td>S</td>
<td>L</td>
<td>H</td>
<td>I</td>
<td>M</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>Q</td>
<td>P</td>
<td>D</td>
<td>G</td>
<td>S</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1

<table>
<thead>
<tr>
<th>Peptides</th>
<th>pB13</th>
<th>G</th>
<th>D</th>
<th>K</th>
<th>P</th>
<th>S</th>
<th>L</th>
<th>F</th>
<th>G</th>
<th>Q</th>
<th>A</th>
<th>A</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>pB13</td>
<td></td>
<td>F</td>
<td>G</td>
<td>Q</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>G</td>
<td>D</td>
<td>K</td>
<td>P</td>
<td>S</td>
<td>P</td>
</tr>
<tr>
<td>S4</td>
<td></td>
<td>F</td>
<td>G</td>
<td>Q</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>G</td>
<td>D</td>
<td>K</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S5</td>
<td></td>
<td>Q</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>G</td>
<td>D</td>
<td>K</td>
<td>P</td>
<td>S</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S3</td>
<td></td>
<td>S</td>
<td>L</td>
<td>F</td>
<td>G</td>
<td>Q</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S2</td>
<td></td>
<td>K</td>
<td>P</td>
<td>S</td>
<td>L</td>
<td>F</td>
<td>G</td>
<td>Q</td>
<td>A</td>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1</td>
<td></td>
<td>G</td>
<td>D</td>
<td>K</td>
<td>P</td>
<td>S</td>
<td>P</td>
<td>L</td>
<td>F</td>
<td>G</td>
<td>Q</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pNR</td>
<td>N</td>
<td>K</td>
<td>S</td>
<td>A</td>
<td>K</td>
<td>Q</td>
<td>F</td>
<td>S</td>
<td>L</td>
<td>H</td>
<td>I</td>
<td>M</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>Q</td>
<td>P</td>
<td>D</td>
<td>G</td>
<td>S</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

microplates wells previously sensitized with the B13.2–LamB protein (50 ng/well). The reaction was developed using an anti-human IgG peroxidase-conjugate with OPD as chromogenic substrate. The antigenic activity for each serum/peptide combination was scored as the percentage inhibition of binding in B13 ELISA, measured according to the following equation, where the nonrelated peptide sequence (pNR), derived from the human T-cell receptor V \( \alpha \) chain (TCR V \( \alpha \) 13), was used as a blank [6]:

\[
\frac{A_{492}(\text{serum} + \text{pNR}) - [A_{492}(\text{serum} + \text{test peptide})]}{[A_{492}(\text{serum} + \text{pNR})]} \times 100.
\]

The values of the percentage inhibition were then plotted for each peptide/serum combination.

3. Results and discussion

3.1. Expression of the B13 sequences as fusions to LamB

Previous mapping of the antigenic regions of B13 using synthetic peptides suggested that the sequence FGQAAAGDK contains the immunodominant epitope [6]. A 12-amino-acid sequence of B13 containing the potential immunodominant epitope (B13.2 – FGQAAAGDKPSP) and another sequence in which the epitope was truncated (B13.1 – GDKPSPFGQAAA) were inserted between residues 153 and 154 of LamB, in an unstructured loop known to be exposed on the surface of the protein, facing the cell exterior. For these constructions, complementary oligonucleotides corresponding to the B13 sequences were ligated into the BamHI site of the plasmid pAJC264 and the resulting plasmids were introduced in strain POP6510. The expression of the two recombinant LamB proteins was determined by immunoblots of whole cell extracts of bacteria induced for the expression of the fusion proteins with IPTG (Fig. 1(a)). The two B13–LamB proteins were recognized by antisera raised against GST–B13, as indicated by the presence of bands with the expected molecular mass of about 48 kDa (Fig. 1(a)). The recombinant LamB proteins were expressed at the same levels as the wild-type LamB and were fractionated as outer membrane components. B13–LamB fusions and wild-type LamB to be used in ELISA were further purified from this preparation by eluting from preparative gels. Molecular mass standards are indicated on the left, in kDa.

3.2. Recognition of LamB fusion proteins by human sera

The B13–LamB fusion proteins were tested by ELISA with a panel of 80 human chronic chagasic sera, which had been previously shown to react with a B13–GST fusion protein [5]. A panel of 52 normal human sera was used as negative control. Fig. 2 shows the ELISA absorbance values of all serum samples analyzed. The B13.2–LamB fusion containing the sequence FGQAAAGDKPSP was recognized by 80% of the sera from chagasic patients, assuming a cut off absorbance value equal to the mean OD492 of normal sera plus three standard deviations (SD). On the other hand, the B13.1–LamB protein was recognized by only 15% of the sera from chagasic patients.

These results confirm that the major B cell epitope of B13 antigen is confined to the FGQAAAGDK sequence. In fact, truncation of the epitope in the B13.1–LamB protein drastically reduced recognition by human chagasic sera.

To determine whether the antibodies that recognize B13 in chronic chagasic patients’ sera were directed...
mainly to the epitope represented in B13.2, we tested the ability of B13.2–LamB to inhibit the binding of human chagasic sera to B13–GST in a competitive ELISA. A human chagasic serum that showed high chagasic sera to B13–GST in a competitive ELISA. A ability of B13.2–LamB to inhibit the binding of human mainly to the epitope represented in B13.2, we tested the
ability of B13.2–LamB to inhibit the binding of human chagasic sera to B13–GST in a competitive ELISA. A human chagasic serum that showed high A492 readings against B13–GST was incubated with different concentrations of B13.1–LamB or B13.2–LamB proteins before reacting to B13–GST (Fig. 3(a)). B13.2 was capable of inhibiting the recognition of the B13 antigen by the human chagasic serum, in a concentration-dependent manner, giving further support to the hypothesis that the sequence FGQAAAGDKPSP contains the immunodominant B13 epitope. As expected, B13.1–LamB did not interfere in the antibody binding to B13–GST.

Synthetic peptides derived from B13 (see Table 1) were incubated with five sera from chagasic patients in competitive ELISA experiments with B13.2–LamB as the solid-phase antigen. The median percentage inhibition for peptides pB13, S4 and S5 (all containing the core of the immunodominant epitope – AAAGDK) were 87%, 76% and 51%, respectively (Fig. 3(b)). Peptides S3, S2, S1, which do not contain the immunodominant epitope, and pNR, a negative control peptide derived from the human T-cell receptor Vα chain, did not inhibit the binding of chagasic sera to B13.2–LamB protein (Fig. 3(b)).

It is noteworthy that the B13.2–LamB construct, which contains a single repeat of the 12-amino-acid motif, is able to mimic the immunoreactivity of the whole B13 antigen in which the sequence is repeated several-fold. In fact, the data here presented indicate that B13.2–LamB protein is recognized by 80% of the sera from chagasic patients.

Previous work indicated that the sequence FGQAAAGDK (peptide S4) carries the immunodominant epitope of B13 antigen and that the sequence AAAGDK should be the core of the epitope [6,8]. B13.1–LamB and B13.2–LamB constructs display different antigenic activities, confirming that the residues flanking the core AAAGDK modulate the interaction of the human antibodies with B13 antigen. This was also confirmed by competitive ELISA between the B13.2–LamB construct and peptides S1, S2 and S3 which contain truncated forms of the immunodominant B13 epitope. The data also show that peptides pB13, S4 and S5 display different antigenic activities in agreement with a previous report of our group [8]. In fact, we have suggested that the distinct antigenic activities of these peptides correlate with their propensity to adopt a helical configuration centered in the AAAGDK sequence. It would be of interest to determine the configuration of the peptides expressed in the loop L4 of LamB.

Altogether, the results shown here clearly provide further experimental support for the immunodominance of the peptide FGQAAAGDK of B13. In addition, the finding that a single repeat unit of B13 protein, embedded into a foreign protein, can still be significantly recognized by sera from Chagas’ disease patients and can efficiently compete with a protein containing multiple repeats, indicates that the major epitope of native B13 protein is indeed contained in a single repetitive unit. These results are thus relevant for both the study of immunodominance in infections by T. cruzi as well as for further developments in diagnostic tools of Chagas disease.

References


