Mapping of B cell epitopes in an immunodominant antigen of *Trypanosoma cruzi* using fusions to the *Escherichia coli* LamB protein

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Abstract

The JL8 protein antigen from *Trypanosoma cruzi*, a dominant immunogen in man, has been characterized as containing tandem amino acid repeats. Here, we describe the use of the LamB protein of *Escherichia coli* as a carrier of JL8 derived sequences in order to map the immunodominant B cell epitopes in this antigen. Five different sequences of JL8 were inserted in the LamB protein and the JL8-LamB fusion proteins were tested by ELISA with human chronic chagasic sera. The fusion carrying the sequence AEKQKAAEATKVAE was recognized by most sera. This protein was also capable of inhibiting the binding of human chagasic antibodies to GST-JL8 in competitive ELISA suggesting that it contains an immunodominant B cell epitope of JL8. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

1. Introduction

Chagas' disease is a zoonosis caused by the protozoan parasite *Trypanosoma cruzi*, which affects a large number of individuals in Central and South America. Because in many cases the infection becomes chronic, with hardly any parasite detectable in the blood of patients, serological tests are invaluable for the diagnosis of this disease [1]. Routinely, crude preparations of the parasite's antigens are used in these assays. Many antigenic proteins have had their coding sequences cloned and characterized by serological screening of expression libraries in attempts to obtain better reagents for more sensitive and specific diagnostic tests [2]. Most of the cloned antigens contain tandem amino acid repeats that are immunodominant in natural infections. One of these is JL8, a cytoplasmic repetitive antigen, homologous to antigens CRA, Ag30 and TCR27, composed of arrays of tandemly repeated 14 amino

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acid sequences [3–6]. JL8 recombinant proteins, purified from *E. coli*, as fusions to either glutathione *S*-transferase (GST) or β-galactosidase, have been tested as alternatives to the routine serological tests based on the fact that anti-JL8 antibodies were found in a great majority of chronically infected individuals [7,8].

Synthetic peptides derived from the repeat sequences of JL8 have been tested as substitutes for the complex antigen in ELISA. This approach, although showing high specificity, has proven less sensitive than the classical methods for detection of antibodies against *T. cruzi*, requiring the use of combinations of different peptides of large sizes for obtaining a sensitivity comparable to the complex mixture of antigens [9,10]. There is an increasing need for the development of diagnostic tools that are as sensitive as the complex antigen for the detection of antibodies in sera of patients. Ideally, this would comprise a mixture of defined molecules. Alternatively, recombinant proteins expressing several epitopes from different *T. cruzi* antigens could be employed to improve the sensitivity of the immunological assays. This improvement requires the determination of immunodominant epitopes in known major antigens. Thus a detailed mapping of epitopes present in JL8 is relevant to this approach.

LamB is a 421 amino acid, trimeric, integral outer membrane protein of *Escherichia coli*, involved in the permeation of maltose and maltotriose, and is required for the transport of higher dextrins [11]. Heterologous sequences can be inserted within 'permissive sites' of the LamB protein known to be exposed at the surface of the cell and at the surface of the protein [12,13]. LamB can be expressed in high amounts by *E. coli* and can be easily purified.

Here we describe the use of the LamB protein as a carrier of JL8 sequences in order to map immunodominant B cell epitopes in this protein, as an alternative for 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 [12], were a kind gift of Dr. M. Hofnung (Institute Pasteur, Paris, France). The *lamB* gene in pAJC264 contains a *Bam*HI site for insertions between amino acids 153 and 154. Luria broth and Luria agar supplemented with ampicillin (100 μg/ml) were used for the growth of bacteria. The expression of LamB was induced by the addition of isopropyl-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 and DNA constructions

Complementary oligonucleotide pairs corresponding to five different sequences of JL8 (GeneBank

Table 1
Oligonucleotides corresponding to the JL8 sequences

5′ GATCCAGTTGCCGAAGCGGAGAAGCAGAAGGCAGCTGAAGCCACGAAGAAT 3′	JL8.1
5′ GATCATTCTTCGTGGCTTCAGCTGCCTTCTGCTTCTCCGCTTCGGCAACTG 3′	
5′ GATCCAGCGGAGAAGCAGAAGGCAGCTGAAGCCACGAAGGTTGCCGAAAAT 3′	JL8.2
5′ GATCATTTTCGGCAACCTTCGTGGCTTCAGCTGCCTTCTGCTTCTCCGCTG 3′	
5′ GATCCACAGAAGGCAGCTGAAGCCACGAAGGTTGCCGAAGCGGAGAAGAAT 3′	JL8.3
5′ GATCATTCTTCTCCGCTTCGGCAACCTTCGTGGCTTCAGCTGCCTTCTGTG 3′	
5′ GATCCAGCTGAAGCCACGAAGGTTGCCGAAGCGGAGAAGCAGAAGGCAAAT 3′	JL8.4
5′ GATCATTTGCCTTCTGCTTCTCCGCTTCGGCAACCTTCGTGGCTTCAGCTG 3′	
5′ GATCCAACGAAGGTTGCCGAAGCGGAGAAGCAGAAGGCAGCTGAAGCCAAT 3′	JL8.5
5′ GATCATTGGCTTCAGCTGCCTTCTGCTTCTCCGCTTCGGCAACCTTCGTTG 3′	

accession number J04016), representing permutations that would encompass one repetition unit (Table 1), were inserted in the *Bam*HI site of pAJC264. The 5′ end of the coding oligonucleotides contained a *Bam*HI half site to provide a rapid restriction analysis screen for the plasmids carrying the insertions. Ampicillin resistant transformants of strain POP6510 had their plasmids analysed for the presence of the insert by digestion with the enzymes *Bam*HI and *Nco*I. The insertions were sequenced using a 20-mer primer complementary to nucleotides located 69 bp upstream from the *Bam*HI insertion site in the *lamB* gene. A GST recombinant protein carrying multiple repeats of JL8 was also produced. The bacterial expression system (GST fusion system, Pharmacia) [14] was used to subclone and express a 351-bp fragment [5] encoding 6.7 repeats of 14 amino acids of JL8. The recombinant protein was purified from IPTG-induced *E. coli* lysates by affinity chromatography [14].

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 EDTA 0.5 M and 0.2 ml lysozyme 10 mg/ml, the cell suspensions were incubated at 37°C for 15 min. 0.5 ml of extraction buffer (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 $2500 \times g$ for 10 min and resuspended in 0.3 ml of Laemmli sample buffer [15]. 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_2O at 4°C for 18 h. The eluted protein was concentrated on Speed Vac® and its concentration was estimated by SDS-PAGE using bovine serum albumin as standard.

2.4. Sera

Human chagasic 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 complement fixation tests. All the serum samples used in this work were positive in ELISA tests with GST-JL8. For the ELISA assays against the 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-JL8 fusion protein was raised on 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 incomplete Freund's adjuvant. The rabbits were bled 30 days after the third injection.

3. Results and discussion

3.1. Expression of the JL8 sequences as fusions with LamB protein

Five different 14 amino acid sequences, derived from JL8, representing permutations that would encompass one repetition unit, 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 (Fig. 1) [13]. The expression of the recombinant LamB proteins was determined by immunoblots of whole cell extracts of bacteria induced for the expression of the fusion proteins (Fig. 2A). All five JL8-LamB proteins were recognized by antiserum raised against a GST-JL8 recombinant fusion protein, as indicated by the presence of bands with the expected molecular mass of about 48 kDa. The recombinant LamB proteins were expressed at the same levels as the wild-type LamB

JL8.1	V A E A E K Q K A A E A T K
JL8.2	A E K Q K A A E A T K V A E
JL8.3	Q K A A E A T K V A E A E K
JL8.4	A E A T K V A E A E K Q K A
JL8.5	T K V A E A E K Q K A A E A

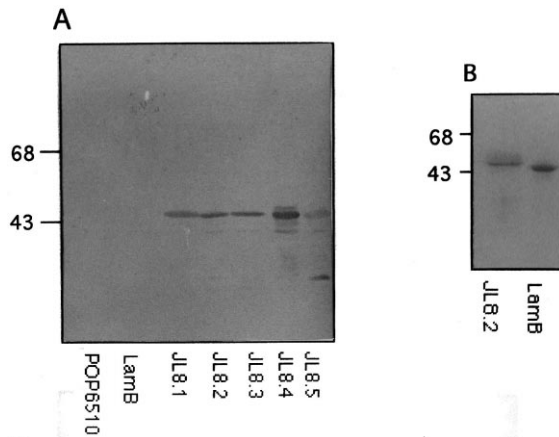Fig. 1. Amino acid sequences derived from the JL8 repeats inserted in the LamB protein.

Fig. 2. Expression and purification of the JL8-LamB fusion proteins. A: Immunoblot of whole cell extracts of *E. coli* expressing the JL8-LamB fusion proteins reacted with an antiserum raised against GST-JL8. The strains POP6510 and POP6510 carrying the *lamB* gene without insert (LamB) were used as negative controls. *E. coli* cells were lysed by boiling for 5 min in Laemmli sample buffer [15]. Proteins were transferred to nitrocellulose membranes (Hybond-C Extra, Amersham) at 1A for 2 h using the buffer conditions described [16]. The membranes were blocked with 5% low-fat milk in phosphate-buffered saline (PBS) for 1 h at room temperature, and incubated for 1 h with rabbit serum raised against purified GST-JL8 recombinant fusion protein, diluted 1:100. After three washings with 0.05% Tween 20 in PBS, bound antibodies were reacted with goat anti-rabbit IgG conjugated with peroxidase (Sigma), diluted 1:1000. After incubation for 1 h with the conjugate and washing in PBS, the bound antibodies were detected using the 4-chloro naphtol chromogen. B: Coomassie blue staining of 10% SDS-PAGE of wild-type LamB and JL8.2-LamB fusion proteins after elution from preparative gels. Molecular mass standards are indicated on the left, in kDa.

and fractionated as outer membrane components. The JL8-LamB fusions to be used in ELISA assays were further purified from this preparation by elution from the gel (Fig. 2B). The yield of this procedure was about 5 mg of purified protein per liter of culture.

3.2. Mapping of antigenic regions of JL8

The five JL8-LamB fusion proteins were tested by ELISA with 12 serum samples from chronic chagasic patients (Table 2). We have selected sera with different reactivity values against the GST-JL8 fusion protein. All the sera reacted with JL8.2 and the reactiv-

ity values obtained with this construction were significantly higher than those obtained with any of the other LamB fusion proteins. These data strongly suggest that the sequence AEKQKAAEATKVAE, present in JL8.2, contains the immunodominant epitope of JL8.

The presence of other antigenic determinants along the JL8 protein was assessed by testing all five JL8-LamB fusion proteins against 80 serum samples from human chronic chagasic patients, which had been previously shown to react with the GST-JL8 fusion protein. A panel of 52 normal human sera was used as a negative control. About 74% of the chagasic sera reacted with any one of the five JL8-LamB fusion proteins, assuming a cut off absorbance value equal to three times the average absorbance value of the normal sera. The JL8.2-LamB fusion was recognized by 64% of the chagasic sera

Table 2
Recognition of LamB fusion proteins by chagasic sera

Sera	Fusion proteins				
	JL8.1	JL8.2	JL8.3	JL8.4	JL8.5
Normal.1	0.009	0	0	0	0.025
Normal.2	0.052	0	0	0	0.079
P.1	0	0.558	0	0	0
P.2	0	0.980	0	0	0
P.5	0	0.210	0	0	0
P.7	0.008	0.058	0	0	0
P.8	0.067	0.326	0	0	0
P.9	0.038	0.175	0	0	0
P.10	0	0.484	0	0.039	0
P.11	0.130	0.614	0	0	0
CHB	0	1.061	0	0	0
DPG	0.050	0.139	0.016	0	0.014
DPJ	0.297	0.480	0	0	0
DPL	0.019	0.102	0.016	0.013	0
DPM	0.062	0.693	0.185	0	0

Microplates (Nunc) were coated with the purified wild-type or recombinant LamB proteins (50 ng/well) diluted in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). The wells were washed three times with PBS-Tween 0.05% and blocked with 5% low-fat milk for 2 h at 37°C. Chagasic sera (1:200) that were previously absorbed with the wild-type LamB proteins overnight at 4°C 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 dihydrochloride. Numbers correspond to the average A_{492} value of triplicate ELISA's, discounted of the A_{492} value obtained with the purified wild-type LamB protein.
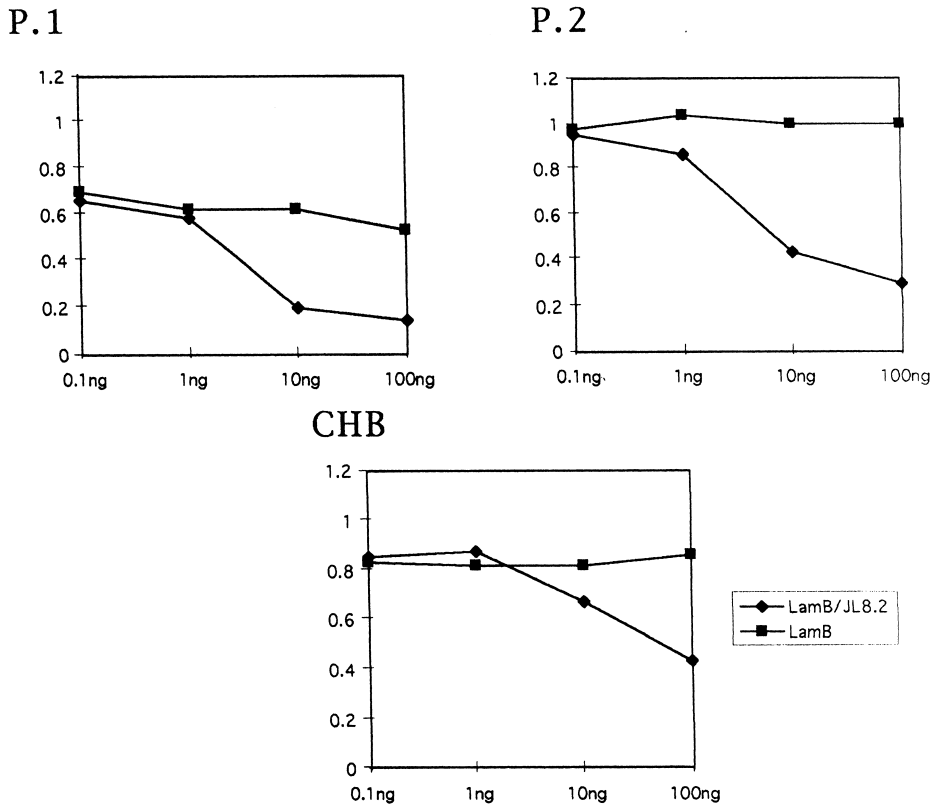
Fig. 3. Inhibition ELISA. Human chagasic sera (P.1, P.2 and CHB) diluted to 1:400 were pre-incubated with the JL8.2-LamB protein, in the indicated concentrations (ng), and added to microtiter plate wells previously coated with GST-JL8 recombinant fusion protein (50 ng/well), diluted in carbonate, at 4°C overnight, washed three times with PBS-Tween 0.05% and blocked with 5% milk for 2 h at 37°C. The wild-type LamB protein was used as a negative control. The average A_{492nm} values from duplicate experiments are indicated on the vertical axis.

(51 out of 80 sera tested). Twenty-one of these were also capable of recognizing JL8.1 and/or JL8.3, although with lower reactivities. Only eight sera displayed reactions against epitopes other than JL8.2. These data strongly support the suggestion that the sequence AEKQKAAEATKVAE, present in JL8.2, contains the immunodominant JL8 epitope. Twenty-one sera (26%) did not react with any of the fusion proteins, indicating the existence of other conformational and/or discontinuous antibody binding site(s) in the JL8 antigen, which is not present in the LamB constructs. Discontinuous and/or conformational dependent epitopes formed by two or more repeats may have been excluded from this analysis, due to the constraint in size imposed by our mapping strategy.

3.3. The anti-JL8.2 reactivity of the chagasic sera is specifically directed against the JL8 antigen

In order to determine whether the antibodies that recognize JL8 in chagasic chronic patients sera were directed mainly to the epitope in JL8.2, this LamB fusion was tested for its ability to inhibit the binding of human chagasic sera to GST-JL8 in a competitive ELISA. Fig. 3 shows the results obtained with 3 chagasic sera, that showed high levels of anti-JL8 reactivity. JL8.2-LamB was capable of inhibiting the recognition of the JL8 antigen by the human chagasic sera, in a concentration dependent manner, giving further support for the suggestion that the sequence AEKQKAAEATKVAE contains the immunodominant JL8 epitope.

The constructions used in this work start at different points of the 14 amino acid consensus repeat of JL8 antigen, with their sequences shifted by 3 amino acids relative to each other. JL8.2 differs from the other constructions by the presence of three pairs of alanine and glutamic acid residues (AE pair), separated by a stretch of 4 amino acids. In JL8.1, JL8.3, JL8.4 and JL8.5, the AE pair is repeated three times, but two of them are contiguous. The strong reactivity of JL8.2 with chagasic sera could be explained by the existence of a discontinuous epitope made up of three non-contiguous AE pairs which could be brought together by folding of the polypeptide chain. This discontinuous epitope is contained within a sequence that has been identified as a linear epitope using the algorithm developed by Pellequer et al. [17]. In fact, this may be a general trend of epitopes from proteins that are antigenic in the *T. cruzi* infection. Recently, fine epitope mapping of the C-terminal epitope of the *T. cruzi* ribosomal P protein revealed that the epitope was not linear but discontinuous and defined by two acidic residues separated by 9 amino acids [18]. The low reactivities of other JL8 constructions with the chagasic sera could be due to a continuous linear epitope (VAEAEK) present in these constructions.

The presence of an immunodominant discontinuous epitope in JL8 antigen is reinforced by the results obtained with a synthetic peptide composed of two 14 amino acid repeats of JL8, which reacted with 58% of the human chronic chagasic sera tested [10]. On the other hand, JL8 recombinant proteins that carry many 14 amino acid repeats, such as GST-JL8 and β-galactosidase-JL8 fusions, are recognized by a large proportion of human chagasic sera (from 85 to 100%) [7,8]. This is a clear indication that synthetic peptides are not able to mimic the immunodominant epitopes present in JL8 recombinant proteins.

Our results show 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. In this regard, the fact that the site for insertions in LamB is part of a non-structured loop provides the possibility for the heterologous sequence to take on a conformation that mimics the native protein. Although LamB proteins

can be easily purified from the outer membrane of *E. coli* in large amounts, the presence in human sera of antibodies directed to LamB domains can impose some restrictions in their use for diagnostic purposes. Previous absorption of sera with wild-type LamB can overcome this problem as shown here. These natural antibodies are mainly directed towards a recently identified immunodominant sequence of LamB, and the deletion of this region drastically reduces reaction with human sera (Pereira, C.M., Newton, S. and Castilho, B.A., unpublished results). Therefore, the LamB fusion proteins devoid of this sequence could be used in diagnostic tests for Chagas disease. Moreover, it should be mentioned that the epitopes described here were detected by immunofluorescence on the surface of the bacteria (data not shown); thus, the intact cells adsorbed directly to the microtiter plates could potentially be used in diagnostic tests. However, since some human sera may be highly reactive to *E. coli*, they should be previously absorbed with an extract of *E. coli* that does not express JL8.

Acknowledgments

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