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Effect of repetitiveness on the immunogenicity and antigenicity of *Trypanosoma cruzi* FRA protein

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ABSTRACT

Repetitive proteins (RP) of *Trypanosoma cruzi* are highly present in the parasite and are strongly recognized by sera from Chagas' disease patients. Flagelar Repetitive Antigen (FRA), which is expressed in all steps of the parasite life cycle, is the RP that displays the greatest number of aminoacids per repeat and has been indicated as one of the most suitable candidate for diagnostic test because of its high performance in immunoassays. Here we analyzed the influence of the number of repeats on the immunogenic and antigenic properties of the antigen. Recombinant proteins containing one, two, and four tandem repeats of FRA (FRA1, FRA2, and FRA4, respectively) were obtained and the immune response induced by an equal amount of repeats was evaluated in a mouse model. The reactivity of specific antibodies present in sera from patients naturally infected with *T. cruzi* was also assessed against FRA1, FRA2, and FRA4 proteins, and the relative avidity was analyzed. We determined that the number of repeats did not increase the humoral response against the antigen and this result was reproduced when the repeated motifs were alone or fused to a non-repetitive protein. By contrast, the binding affinity of specific human antibodies increases with the number of repeated motifs in FRA antigen. We then concluded that the high ability of FRA to be recognized by specific antibodies from infected individuals is mainly due to a favorable polyvalent interaction between the antigen and the antibodies. In accordance with experimental results, a 3D model was proposed and B epitope in FRA1, FRA2, and FRA4 were predicted.

1. Introduction

Repeat proteins are extensively widespread among biological organisms and have been observed in entities as distant as virus and humans. These proteins present different biological and structural features, as it has been described for Zn finger motifs, WD40 repeats, armadillo repeats, HEATS repeats, leucine-rich repeats, and ANK repeats. Reviews of this topic are available in the literature (Andrade et al., 2001; Kloss et al., 2008). In *Trypanosoma cruzi,* as well as in other protozoan parasites infecting humans, these proteins have been systematically identified by immunological screening of cDNA libraries using antibodies from infected individuals (Hoft et al., 1989; Ibanez et al., 1988; Levin et al., 1989). Thus, 25% of the expressed sequences detected contained tandem repeat motifs composed of 5–68 aminoacids. As *T. cruzi* repetitive proteins correspond only to 2% of the genomic ORFs, they are clearly more strongly recognized by antibodies than non-repetitive proteins. Furthermore, by screening repetitive ORFs in *T. cruzi* genome and checking their antigenicity, at least 13 out of 20 main repetitive proteins from *T. cruzi* were found to be highly antigenic (Goto et al., 2008).

Since tandem repeat antigens were described in different pathogenic microorganisms, the most widely accepted hypothesis to explain their antigenicity was that cross linking of B lymphocyte mIgs triggers a T-independent response (Vos et al., 2000). However, some *T. cruzi* repetitive antigens have been shown to induce a T-dependent immune response (Abel et al., 2005; Alvarez et al., 2004; Pereira et al., 2005). Furthermore, evidences showing that they are highly recognized by IgG antibodies from chronic patients also suggest that in *T. cruzi* infection these antigens are T-dependent (da Silveira et al., 2001; Vercosa et al., 2007). Although the role of the high antigenicity and immunogenicity of the tandem repeat proteins observed in the *T. cruzi*-host interaction is unknown, it was suggested that the repetitive regions of these proteins would act as a decoy to divert immune response from functional regions (Frasch, 1994). In support of this hypothesis, it has been recently reported that ribosomal tandem repeat proteins would bring forth a non-effective immune response against intracellular components of the parasite (Pais et al., 2008). Furthermore, in *Leishmania* infection, which is caused by a phylogenetically close parasite, repetitive proteins have been found to be involved in the

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immunological escape by enhancing humoral response to the detriment of protective cellular response (Goto et al., 2010).

Flagellum Repetitive Antigen (FRA) is one of the proteins most widely used in the diagnosis of Chagas' disease due to its high level of recognition by antibodies from *T. cruzi*-infected patients (da Silveira et al., 2001). This antigen has repeats of 68aa, which is two to sixfold the size of other tandem repeat proteins from *T. cruzi*. The coding gene of this molecule has been described in different strains and at least two copies per haploid genome are present. These common arrays between different strains (Cotrim et al., 1995), indicate that the genomic duplication may have been an early event in the evolution of the parasite. This protein has also non-repetitive regions and is present in the flagellum root region (Cotrim et al., 1995). Once the complete genome of *T. cruzi* was sequenced, the protein was renamed as Calpain Cistein Proteinase because it shares a putative domain with this enzyme (El-Sayed et al., 2005). However, this enzymatic activity has still not been described in *T. cruzi*.

Although the high reactivity of FRA with specific antibodies from infected humans or animals has been repeatedly described by several authors, there is limited information about the causes of its antigenic behavior. In this work we analysed if the high reactivity of this antigen with antibodies developed in *T. cruzi*-infected individuals is due to an enhancement of immune response induced by tandem repeated motifs or is a consequence of the antigenic nature of the molecule evidenced in the immunochemical reactions. The immunogenicity of one FRA motif, and of two and four tandem repeats was assessed in mice. The same variants of repeat arrangements were also evaluated for their antigenic properties by immunochemical assays. Finally, in an attempt to interpret the results observed, molecular modeling together with B-epitope predictions along the repeat sequence were carried out.

2. Materials and methods

2.1. FRA coding sequences and construction of expression plasmids with different repeat arrangements

The coding sequence of *T. cruzi* FRA protein was obtained by PCR of genomic DNA isolated from CL Brener strain, as described by Camussone et al. (2009). DNA fragments containing one and two tandem FRA repeats were obtained and inserted into the expression vector pET32a (Novagen, Madison, WI, USA); these plasmid constructions were named FRA1 and FRA2, respectively. The DNA sequence encoding four tandem repeats of FRA was obtained by PCR, using FRA2 as template and the following primers: A1 Forward: CTC GAG AAG GCC AAG CTT CGC GAC and A2 Reverse: GTC GAC TCA CAG TGC GCG CTG CTC TGC. A1 contains the restriction site *Sac* I and A2 incorporates the *Sal* I restriction sequence (underlined), allowing the subsequent cloning steps. The amplified DNA sequence was cloned into the FRA2 plasmid, thus generating four tandem repeats of FRA inserted in pET32a. This construction was named FRA4. As pET32a expression vector encodes the sequence of TRX upstream of the cloning site, all sequences cloned in the vector are expressed with TRX attached at the N-terminus. Cells of *Escherichia coli* BL 21 (DE 3) were transformed with each plasmid described and the clones selected were sequenced to verify the identity of the cloned sequences.

2.2. Expression and purification of recombinant proteins

E. coli cells transformed with the different plasmids were grown in LB medium (10 g/l peptone, 5 g/l yeast extract, 10 g/l NaCl, pH 7.5) supplemented with 0.1 mg/ml of ampicillin up to values of A_{550nm} = 0.50–0.60. The expression of recombinant protein was induced with 0.5 mM isopropyl-thio-β-D-galactoside. After an incubation of 3 h at 37 °C, cells were recovered by centrifugation, resuspended in buffer (50 mM Na_2HPO_4 pH 8, 300 mM NaCl, 10 mM Imidazol, 1 mM phenyl methyl sulfonyl fluoride) and sonicated with 50% pulses for 2 min at 600 W; after centrifugation at 13000 rpm for 10 min, the supernatant was recovered. Proteins were purified from supernatant with a nickel pseudo-affinity IDA-Sepharose column (Novagen, Madison, WI, USA). To remove the fusion protein TRX, the recombinant protein was incubated with 2 ng of enterokinase per mg of protein (New England Biolabs, Ipswich, MA, USA) followed by purification by pseudo-affinity to nickel. The purity and concentration of the proteins were evaluated with 15% polyacrylamide gel electrophoresis under denaturing conditions (SDS–PAGE) and subsequent Coomassie Blue staining (Fig. S1, supplementary information). Quantification was performed by densitometry using Gel Doc XR System (Bio-Rad-life science) and the software Quantity One (Bio-Rad Inc., Hercules, CA) and bovine serum albumin as calibration standard.

2.3. Mouse immunization and human sera

Rockland female mice 6- to 8-weeks-old were used for the inoculation protocols. All experimental procedures were performed according to the recommendations of the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources (US), 1996). Mice were inoculated with the purified recombinant proteins by intraperitoneal route four times at three-week intervals. The animals were separated into seven groups, each receiving a different inoculum, as follows: 1.11 nmol of FRA1, 0.55 nmol of FRA2, 0.27 nmol of FRA4, 1.11 nmol of TRX-FRA1, 0.55 nmol of TRX-FRA2, and 0.27 nmol of TRX-FRA4 or PBS (control of buffer solution). Inocula were administered in the presence of Freund's Complete Adjuvant for the first dose and incomplete adjuvant for the following doses. Details about the number of animals used in each experiment are included in the Results section. One week before each inoculation and one week after the last dose administration, blood samples were taken by tail puncture into capillary tubes. Serum was isolated by centrifugation and stored at −20 °C.

Human sera were obtained from chronic chagasic patients and uninfected individuals, all from the same endemic region, who provided written consent for the study. In all cases, human sera showing significant reactivity in ELISA and indirect hemagglutination test (Wiener Lab, Rosario, Santa Fe, Argentina) were classified as positive. All protocols were approved by the Ethics Committee of the institution.

2.4. ELISA

Polystyrene plates of 96 wells (Greiner Bio-One, Frickenhausen, Germany) were sensitized with the purified protein in concentrations ranging from 1.75 to 112 fmol/well, depending on the assay. Proteins were diluted in carbonate/bicarbonate buffer at pH 9.6. After blocking nonspecific binding sites with PBS-5% skimmed milk, mouse, and human sera were tested in 1:200 and 1:60 dilution, respectively, in PBS-1% skimmed milk. Antibody binding was evaluated by incubation with anti-mouse IgG conjugated to peroxidase, 1:30,000 (Jackson, West Grove, PA, USA) or anti-human IgG peroxidase conjugate (1:10,000) (Dako, Corp., Carpinteria, CA), as appropriate, and further incubation with hydrogen peroxide and tetramethylbenzidine. Optical density (OD) was measured at 450 nm using a microplate reader (Emax Microplate Reader, Molecular Devices, Sunnyvale, CA).

2.5. Relative avidity assessment

An ELISA was performed as described above with 10 different sera, and using decreasing amounts of antigen (FRA1: 112-7

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fmol/well; FRA2: 56–3.5 fmol/well; FRA4: 28–1.75 fmol/well). Results were expressed as the amount of protein leading to a 50% reduction of the maximum OD reached for each serum.

2.6. Statistical analysis

Data were analyzed by ANOVA or Kruskal–Wallis test, depending on the assay, followed by multiple comparison tests when significant differences between means or medians were found. Differences were considered significant at $p < 0.05$. Normal data distribution was performed when necessary using Shapiro Wilk test. Statistical analysis was performed using the software GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego, CA, USA).

2.7. Circular dichroism (CD)

All CD spectra were recorded at 15 °C on a JASCO J-800 spectropolarimeter using quartz cells of 1 mm path length. Measurements were performed at 1-nm intervals between 190 and 260 nm, and the averaged spectrum of six scans was obtained. The data were processed with the software Spectra Manager 1.53 (Jasco Corporation).

2.8. Molecular modeling and antigenicity prediction

2.8.1. Three-dimensional model

Three-dimensional models of FRA1, FRA2, and FRA4 were first generated using comparative modeling. Because all sequence alignment methods reported less than 30% sequence identity between our sequences and three-dimensional structures available on the Protein Data Bank (PDB), in this work we used methods of Fold-Recognition (Tramontano, 2006) to obtain the 3D models. After an exhaustive analysis of several Fold-Recognition servers, the I-TASSER server (http://zhang.bioinformatics.ku.edu/I-TASSER/, Zhang, 2008) was selected. This server implements various comparative modeling techniques and has shown to be the automatic Internet server with the best performance in the latest edition of the biennial competition "Critical Assessment of Techniques for Protein Structure Prediction" (CASP8, 2008, http://predictioncenter.org/casp8). Using techniques of sequence–sequence, sequence–profile and profile–profile alignment, the server identifies potential protein patterns that will guide the construction of the models. Moreover, since their algorithm allows the consideration of possible patterns obtained by the user, we have included consideration of previous models obtained by our group (Peralta et al., 2007) for similar peptides. At the end of the calculations, the server provided five possible models for validation. For the validation step, we performed a minimization of energy and a systematic analysis of the resulting Ramachandran maps, and analyzed the amino acid score in the verifier called Verify3D (http://nihserver.mbi.ucla.edu/Verify_3D/, Luthy et al., 1992), which is considered one of the most reliable verifiers at present. The structural model selected for each of the studied peptides in the present work is the one that provides the best combination of all these validation techniques.

2.9. Antigenicity prediction

The antigenic regions in our models were predicted using the Internet server DiscoTope (http://www.cbs.dtu.dk/services/DiscoTope, Haste Andersen et al., 2006), which can predict linear and conformational epitopes from the models obtained.

3. Results

3.1. Influence of tandem repeats on immunogenicity

An immunization protocol was conducted to assess the possible influence of the number of FRA tandem motifs on the magnitude of humoral response in a mouse model. Three groups of mice ($n = 6$ per group) were inoculated with equal amounts of total repetitive units of FRA1, FRA2, and FRA4 per dose. Thus, 1.110, 0.555, and 0.277 nmol of FRA1, FRA2, and FRA4 were administered, respectively. Another immunization protocol was conducted with the same amounts of protein fused with TRX. In the first protocol, the control group was inoculated with vehicle (PBS + adjuvant), whereas in the second, the control group received 0.555 nmol of TRX per dose. Antibody detection was performed using FRA4 as antigen for sera from all groups, since this protein contains all the antigenic determinants. All mice from the immunized groups showed a specific IgG response after the fourth inoculation, compared with control groups ($p < 0.05$, Kruskal–Wallis test) (Fig. 1A and B). To rule out the possibility of an immune reaction to *E. coli* contaminating proteins in immunogen preparation, sera from FRAs immunized mice were tested against TRX, which was obtained using the same expression system and purification protocol and we observed no reaction. No significant differences between groups were observed in either protocol. Although proteins fused with TXR induced higher antibody levels than those digested with enterokinase, this difference was statistically significant only for FRA2 vs TRX-FRA2 ($p < 0.05$, Mann–Whitney test). The comparison of the immune response specific to TRX showed a significant reduction in the levels of anti-TRX antibodies in

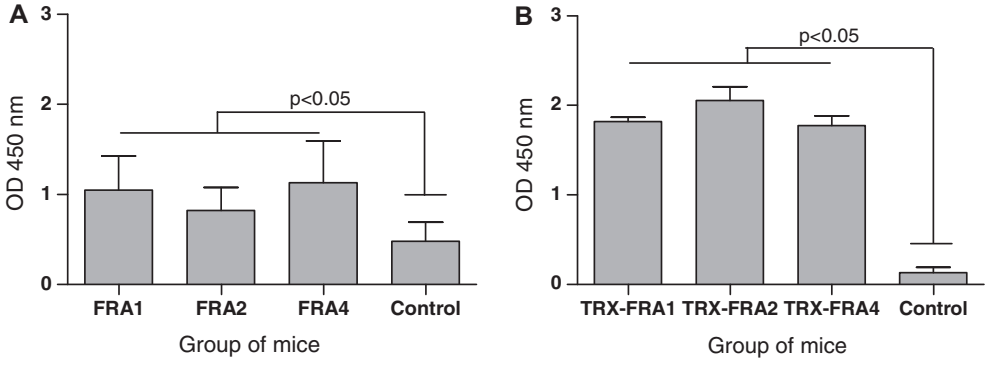

Fig. 1. Immune response of mice to FRA1, FRA2, and FRA4 (A), or the same proteins fused to TRX (B). Mouse sera were diluted 1:200, specific IgG were detected by indirect ELISA using FRA4 as antigen, and a peroxidase conjugated anti-mouse IgG antibody. No significant differences were observed between groups immunized with FRA containing proteins. Results are expressed in OD units, as mean and SEM. Statistical analysis was performed by Kruskal–Wallis test.

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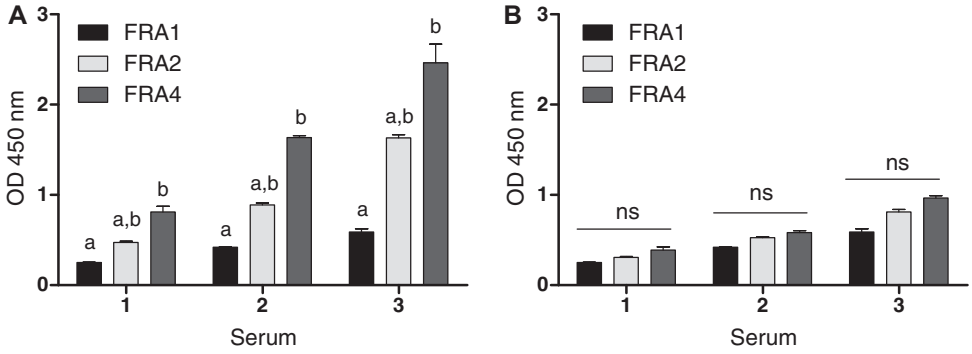

Fig. 2. Analysis of antigenicity of proteins with human sera from patients infected with *T. cruzi* by indirect ELISA. FRA1, FRA2, and FRA4 were immobilized, in equal number of moles per well (A) or equal number of repetitive units per well (B). Sera were diluted 1:60 and human antibodies were detected using a peroxidase conjugated anti-human IgG antibody. Significant differences were observed between FRA1 and FRA 4 reactivity, for the three tested sera, when using the same number of moles (A). When equal number of repetitive units was used, no significant differences were detected between different antigens (B). Results are expressed in OD units, as mean and SEM. Different superscripts denote significant differences ($p < 0.05$, ns: non significant, Kruskal–Wallis test).

TRX-FRA4 vs. TRX-inoculated mice ($p < 0.05$) (Fig. S2, supplementary information).

3.2. Influence of tandem repeats on antigenicity

To analyze if the presence of tandem repeats in the molecule can enhance the strength of the recognition by antibodies generated during natural infection, human sera from infected patients were assessed by an indirect ELISA using the recombinant proteins. The specificity of the sera was previously verified. The results showed that negative sera of individuals from the same geographic region did not bind to recombinant proteins. The assay was performed using 28 fmol of antigen per well, since it was previously determined that this is the minimum amount of protein allowing the detection of antibodies in all sera tested. Under this condition, the proteins were distributed in the well, leaving a great number of free binding sites; this was corroborated with the peroxidase complementation assay, which showed only a small difference between sensitized and empty wells when 28 fmol of protein was loaded (Fig. S3, supplementary information). Due to the small molecular size of repeats, it was important to first determine if antibodies could bind to more than one FRA repeat when these units were displayed in tandem. A primary assay was then performed using an identical molar concentration of each protein. The three sera evaluated showed a significant increase in their recognition ability with increasing number of tandem repeat units in the molecule ($p < 0.05$, Kruskal–Wallis test) (Fig. 2A). It was then confirmed that divalent or tetravalent diplays of FRA repeats had not conformational impediments to allow antibodies recognition against different repeats of the molecules. Then, we aimed to assess if the binding between these molecules and antibodies recognition increased ELISA OD signals when FRA tandem repeats are present. Then a new assay was conducted using the antigens in a proportion of 4:2:1 moles of FRA1:FRA2:FRA4. This condition gives the same number of repetitive units per well, regardless of the recombinant protein used. Evaluation of sera under this condition showed no differences in their ability to bind to the different proteins ($p > 0.05$, Kruskal–Wallis test, Fig. 2B). As it is schematically represented in Fig. 4, the different phenomena that might be involved in our results are that the addition of motifs to the molecule is associated with; (i) a higher number of antibodies bound per molecule or; (ii) with higher avidity interactions (Fig. 4C and E). Because the concentration of antigen used to coat the well was extremely low, we assume that the results are representative of isolated interactions between the antigen molecules and their specific antibodies, without interference of neighboring complexes. Indeed, taking into account this low molar concentration and the available surface in the well, if molecules are distributed uniformly in the well during the sensitizing step, a minimum separation of 600 nm between them can therefore be assumed.

3.3. Influence of the number of repeats on Ag–Ab avidity

An immunoassay was conducted to assess possible differential avidity in the interaction between human antibodies and FRA motifs with different number of repeats in a molecule. Sera from *T. cruzi*-infected individuals were incubated with decreasing amounts of each recombinant protein, and the amount of protein leading to a 50% decrease of the maximum OD obtained in each case was determined. Our results showed that a higher decrease in antigen amount of FRA4 was needed to reach the same decrease in the ELISA signal obtained with FRA1 ($p < 0.05$, ANOVA), indicating that antibodies from these sera were able to bind to FRA4 with higher avidity than to FRA1 (Fig. 3).

3.4. Peptide modeling and B-epitope predictions

Three-dimensional models for FRA1, FRA2, and FRA4 antigens were obtained (Fig. 5) and validated using the procedures described in Section 2. The 3D model of FRA1 shows a pattern of three alpha-helices connected by two loops, A and B: N-terminal coil – helix1 – loop A – helix2 – loop B – helix3 – C-terminal coil.

The corresponding 3D model obtained for FRA2 basically shows that the same structural arrangement is repeated twice, except

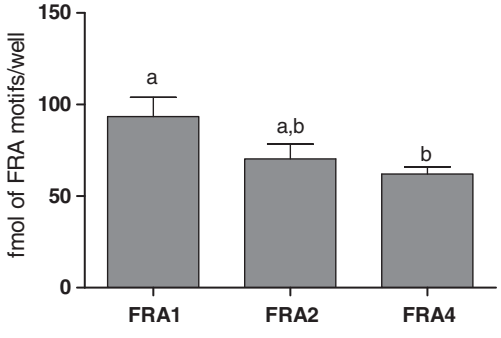

Fig. 3. Comparison of avidity of antibodies from human sera to antigens carrying different number of repetitive units. Human sera from patients infected with *T. cruzi* ($n = 10$) were incubated with decreasing amounts of each antigen, in an indirect ELISA. Results are expressed as the amount of protein leading to a 50% decrease in the maximum reached OD. Significant differences were observed between FRA1 and FRA4. Different superscripts denote significant differences ($p < 0.05$, ANOVA).

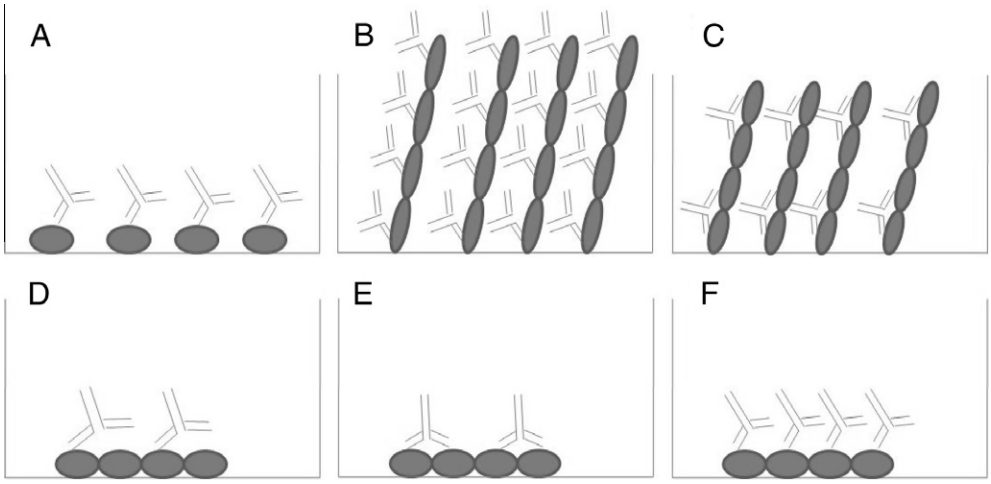

Fig. 4. Possible interpretations of ELISA results: A, B, and C are schematic representations of possible interactions between antibodies and FRA antigens when the same number of molecules of FRA1 (A) or FRA4 (B and C) are adsorbed in the well. Figure B represents a model where multiple antibodies may bind a single molecule of FRA, and C represents the case of bivalent binding of antibodies to FRA. A, D, E, and F are schematic representations of possible interactions between antibodies and FRA repeats when the same amount of FRA motifs are absorbed in the well as single FRA motif (A) or as four tandem repeat motifs (D, E, and F).

that loop A is longer than predicted in the FRA1 model. The FRA2 model presents the same type of loop (A or B), oriented in the same spatial direction, generating a tandem structure similar to that described for other repetitive peptides (Kloss et al., 2008). The 3D model obtained for FRA4 preserves the pattern of alpha-helices linked through long (A-type) and short (B-type) loops, with perturbation of the extended tandem pattern as the number of repetitions increases. In this case, the first two B loops are oriented toward the same region of the space, while the last two B loops are facing the same area of space, nearly perpendicular to the direction faced by the first two ones (Fig. 5).

Although the models illustrated in Fig. 5 should be considered only approximate, the predominance of an alpha-helix secondary structure that they predict has been confirmed by circular dichroism (CD) measurements (Fig. 6). In agreement with the models obtained by Fold-Recognition techniques, the pattern of the three spectra observed in this figure, with minimums at 208 and 222 nm, clearly indicates that proteins containing either one, two, or four tandem repeat FRA acquire an alpha-helix conformation in solution.

Finally, we have searched for theoretical (linear or conformational) B epitopes within the different constructs using the internet server DiscoTope. The sequence Lysine-Aspartic Acid-Proline-Arginine-Arginine-Asparagine was the best ranked sequence to be considered a B epitope for the peptides investigated (FRA1, FRA2, and FRA4). Each peptide is identified by both the single letter code and the number in the sequence, as follows: FRA1: *K52-D53-P54-R55-R56-N57*; FRA2: *K52-D53-P54-R55-R56-N57* and *K120-D121-P122-R123-R124-N125*; FRA4: *K52-D53-P54-R55-R56-N57*, *K120-D121-P122-R123-R124-N125*, *K189-D190-P191-R192-R193-N194* and *K257-D258-P259-R260-R261-N262*. All these sequences are located at the corresponding type-B loops in the models found for each peptide (Fig. 5).There are no predicted B epitopes in the A-type loops, or in residues involving the joining region of two repetitive sequences.

4. Discussion

We obtained the FRA coding sequences from CL Brener strain genomic DNA for 1 and 2 repeats. Our sequence for 1 and 2 repetitions has an identity of 100% with the one submitted by El-Sayed (El-Sayed et al., 2005) for this protein in the same strain. In the second repeat of the codificant sequence there is a substitution of an arginine by a lysine at position 50, the polymorphism being a feature of these repetitive antigens (Andrade et al., 2001; Cotrim et al., 1995; Krieger et al., 1991). Then we fused the two repeats to obtain four repeated coding sequences and we expressed and purified proteins carrying 1, 2, or 4 repeats fused to TRX, as well as non-TRX fusion proteins.

In this work, the relationship between the repetitive characteristic of FRA protein of *T. cruzi* and its immunogenicity was directly analyzed for the first time in a mouse model, using equimolar amounts of FRA motifs. Groups of six mice were inoculated with equimolar amounts of FRA motifs of each antigen to assess if tandem repeat arrangement enhances the immunological response. As early as 15 days post-inoculation we found high levels of IgG in all groups. Although we obtained a significant immune response for all proteins, we found no difference between groups inoculated with 1, 2, or 4 tandem repeats of FRA motif. This indicates that the immunogenicity of the domain do not depend on its repetition and would lie in the individual motif, at least up to the four tandem repeats studied here. We also evaluated the immunogenicity of FRA repeats with a non-repetitive molecule (TXR protein) fused to the N-terminal extreme of the different FRA constructs. Antigen immunogenicity was previously found to be increased with the presence of a TRX peptide fused to it (Bertini et al., 1999; Blum et al., 1996; Rubio et al., 2009). The presence of the fusion peptide in the protein did not induce significant differences between the constructs with increasing number of repeats, although the IgG specific responses against FRA sequences were greater and more homogeneous for each group when this fusion peptide was present in the immunogen. These results confirm that the number of repetitions does not affect FRA immunogenicity. Our results indicate that the presence of FRA motif decreases the immune response toward TRX protein, supporting the immune distractive function hypothesis of repetitive domains. However, further research is needed to assess this role of repeats.

Repetitive antigens have been frequently described as highly immunogenic; based on this evidence, some authors have tandemly repeated certain epitopes to enhance the immunological performance of vaccine candidates (Kim et al., 2005; Liu et al., 2004; Rubio et al., 2009; Yankai et al., 2006). Using this approach, immunogenicity was improved for many of the antigens studied and effective immunological therapies were successfully achieved in animal models. However, in some cases the positive effects of

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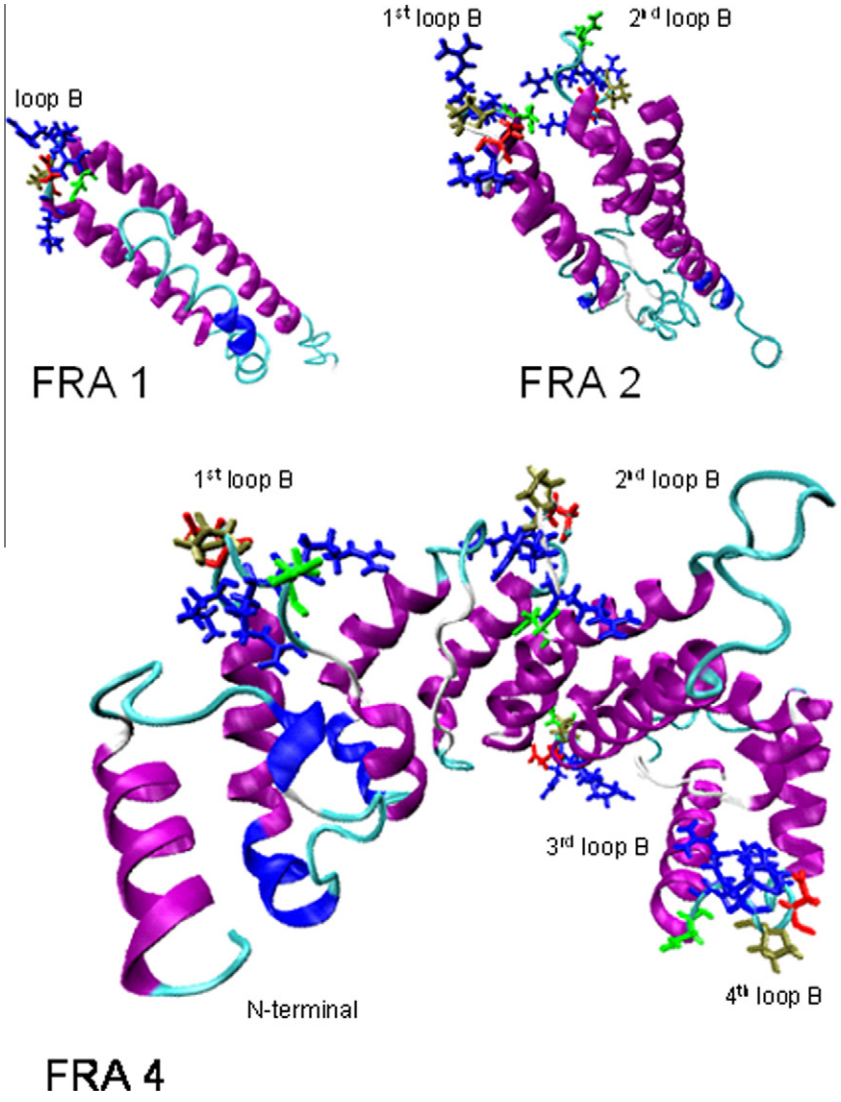

Fig. 5. Molecular modeling of peptides: FRA1, FRA2, and FRA4. 3D models obtained with the Fold-Recognition server I-TASSER. The B-cell epitopes predicted by DiscoTope server, are represented with residue details. The same sequence (Lys-Asp-Pro-Arg-Arg-Asn) was predicted to be the antigenic determinant in all B loops. The Lys and Arg residues (positive charged) are shown in blue; the Asp residue (negative charged) is shown in red; the Pro residue (neutral and non polar) is presented in tan; and the Asn residue (neutral but polar) is depicted in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

antigenic repetition on the immunogenicity of these constructs can be influenced by the specific aminoacid composition and the length of the tandemly repeated sequences, as it was described by Rubio et al. (2009). Since we found no differences after adding repetitive units to the molecule, the length of the FRA single motif could determine a behavior similar to that reported in the work performed by Rubio for a 108 aminoacids sequence.

We also analyzed the consequences of increasing the number of FRA repeats on FRA antigenicity. We used sera from *T. cruzi*-infected humans since antibodies from infected patients are developed against the native protein in Chagas' disease (da Silveira et al., 2001; Umezawa et al., 1999). Furthermore, we performed this analysis by ELISA, the assay most widely used in immunochemistry and used by the cited authors. We found that at identical molar concentration of FRA1, FRA2, or FRA4 in the assay, optical densities increased with increasing number of repeats. This result confirmed that antibodies could bind to more than one FRA repeat when these units were displayed in tandem. However, when ELISA wells were sensitized with the same number of FRA repeats, optical density values did not show any significant differences. This result could indicate that four single repeats displayed in four single molecules react with the same amount of antibodies than four FRA repeat displayed in a tetravalent protein (Fig. 4A, B, and F). However, taking into account that the large size of antibody molecules in relation to the size of repeats could prevent antibody binding in adjacent repeats (Fig. 4D), another hypothesis should be considered; less amounts of antibodies react with multimeric proteins but with an increased affinity caused by a bivalent interaction of the antibodies with the repetitive proteins (Fig. 4C and E). To assess this hypothesis we compared the avidity of the different proteins with a panel of 10 human sera. We found that avidity was higher with increasing multimerization. These results are in agreement with a recent study performed with Leishmania repetitive antigens (Goto et al., 2010).

Overall, the present results indicate that even when multimerization of FRA do not have a positive impact on the immunogenicity of the protein, it increases the antigenicity observed in ELISA assays. To interpret the antigenicity results obtained with FRA1, FRA2, and FRA4 molecules, we obtained structural models for all the constructs since the crystallographic structure of this protein has still not been obtained and no bioinformatics models have been published up to now. The final models presented show an

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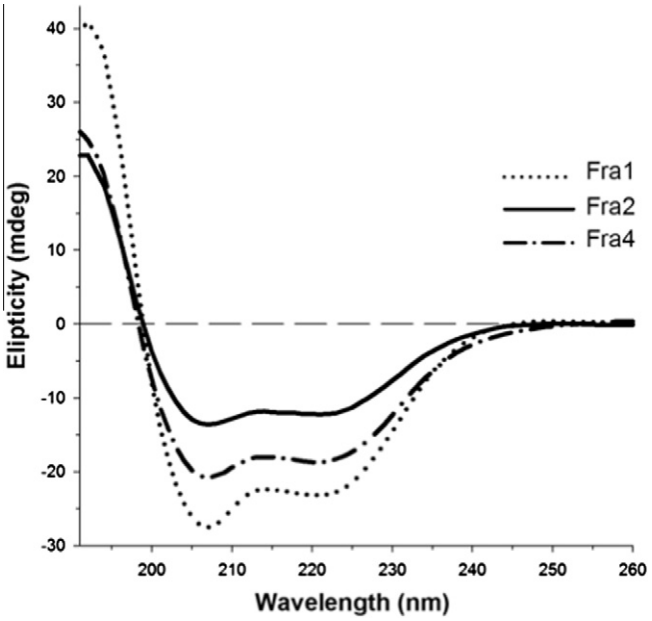

Fig. 6. Circular dichroism spectroscopy of constructs containing one, two, and four tandem repeats of FRA. All three spectra show minimums at 208 and 222 nm which are typical from alpha helical conformation.

extended macromolecular arrangement where the repetition of the FRA unit sequence translates into a repetition of a defined structural domain. Repetitive tandem domains do not always have an extended arrangement; for example, they can arrange in a closed circular structure. The structural models here proposed are in agreement with those reported by Kloss et al. (2008) for many tandem repeat proteins. These authors described that there is an apparent degree of repetitions at all levels of structures, where the relative orientation between repetitions is conserved and the structural similarity between repeats allows a highly uniform tertiary structure in different regions of the protein. Based on these characteristics, they proposed that the folding of the different repeats composing these proteins can be studied individually. In our models, the secondary structure for each FRA motif corresponds to alpha-helix domains disrupted by two loops. The secondary structure of these models was consistent with the CD spectra of the proteins.

In our study, B-cell epitope predictors indicated a major lineal and conformational epitope in the second loop, corresponding to the KDPRRN sequence. This antigenicity predictor uses the information of the protein structure or its model (and not just its sequence, as did previous predictors), and therefore is able to predict the formation of epitopes either linear or conformational. The structural repetition of the antigenic motif (loop B) in an extended conformation along the FRA repetitive region establishes a model where the B-epitopes are exposed at the surface of the protein structure and implies that the number of epitopes available increases when increasing the number of repetitions. According to Rubinstein (Rubinstein et al., 2008), who studied the features of crystallized epitopes by statistical methods, B loop regions are highly coincident with epitope regions, because they present Lys, Arg, and Asp, which are charged residues with long lateral chains. In addition, B loop region displays asparagine aminoacid, which holds a polar lateral chain. Furthermore, this portion of the molecule protrudes from the surface and corresponds to a non-defined structure from the molecule, which is also a feature of antigenic determinants. A major epitope in this region would be compatible with experimental data, as in FRA4 the distances between these epitopes in the model (48 Å between the first and the fourth B loop) are compatible with inter-epitope distances reported for divalent antibodies binding (Hewat and Blaas, 1996). Furthermore, as optical densities do not increase when FRA motifs are displayed in tandem for an equivalent number of FRA motifs (Fig. 2), we concluded that there are no inmunodominant epitopes in the inter-repeat regions. This experimental result as well as the theoretical model confirm the absence of epitopes close to the inter-repeat region and localize the immunodominant epitope inside the repetitive unit.

It can therefore be concluded that the high reactivity of sera from individuals with Chagas disease against FRA antigen is mainly due to the polyvalent nature of B-cell epitopes within FRA repetitive unit. Using immunological techniques combined with theoretical structural and antigenic studies we propose a structural model of FRA domains supporting their antigenic properties. In future works, the integration of information from experimental and bioinformatic analysis involving repetitive proteins could contribute to rationally design molecules using tandem repeats of appropriate non-immunogenic framework regions linked to antigenic loops, to confer highly antigenic properties.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.exppara.2010.11.011.

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