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Multiple *Trypanosoma cruzi* antigens containing tandemly repeated amino acid sequence motifs

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Chromosomal DNA from *Trypanosoma cruzi*, the agent of the American trypanosomiasis (Chagas' disease), was used for construction of a DNA library, employing the expression vector lambda gt11. Nine clones encoding different parasite antigens were isolated from this library by screening with an antiserum from a Chagasic patient. Nucleotide sequence analysis showed that seven out of the nine isolated clones code for antigens which contain tandemly repeated amino acid sequence motifs. Each of the seven antigens contains a unique repeat, ranging in length between 5 and 68 amino acids. The length of the repeats is highly conserved within each clone. Fusion proteins, expressed from two of the clones, reacted with a large proportion of sera collected from Chagasic patients in Argentina, Brazil and Chile. These clones appear thus to encode antigens which are shared between different strains of *T. cruzi*. Immunofluorescence experiments with live parasites showed that three of the antigens were detectable on the surface of trypanosomes.

Key words: *Trypanosoma cruzi*; Antigen; Tandem repeat; Lambda gt11

Introduction

Studies of cloned antigens from plasmodia have shown that many of them have an internal repeated structure which is the target of the immune response [1–5]. Tandem repeats in malaria proteins may moreover generate a 'smokescreen' that prevents the production of effective antibodies against critical epitopes. *Trypanosoma cruzi*, the agent of the American trypanosomiasis (Chagas' disease), exists in two main stages in the human host. The trypomastigotes are present in blood and are consequently exposed to the host immune system. Trypomastigotes are capable of invading cells, giving rise to amastigotes which replicate intracellularly. *T. cruzi* parasites must thus carry on their surface molecules that allow interactions with host cells [6,7]. These surface molecules seem to elicit an immune response, protecting against subsequent infection [8]. So far very few studies have been reported on the structure of *T. cruzi* antigens. Recently, the genes for two proteins with a similar overall structure were described in *Trypanosoma brucei* [9] and in *T. cruzi* [10]. However, the *T. brucei* protein does not seem to play a role in cellular invasion or in the interaction with the immune system as it is expressed only in the insect/vector stage of the parasite [9]. The *T. cruzi* protein has a molecular mass of 85 kDa and contains tandemly repeated amino acid sequence motifs [10]. An internal *T. cruzi* antigen which is homologous to a heat-shock protein has also been described [11].

In a previous paper we described the isolation of a collection of clones encoding *T. cruzi* anti-

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Abbreviation: H2, human Chagasic serum.

gens [12]. In the present study nine clones from this collection were selected for further analysis. Our results show that seven of them encode antigens containing tandemly repeated amino acid sequence motifs. Some of these are likely to represent important *T. cruzi* antigens since they are recognized by a large proportion of sera from Chagasic patients obtained in Chile, Brazil, and Argentina.

Materials and Methods

Parasites and sera. T. cruzi epimastigotes, CA1 strain [13] were grown in liquid media [14]. Trypomastigotes, RA strain [13] from an infected Vero cell-culture were obtained as described by Zingales et al. [6]. Human sera from chronic infections were collected in Argentina, Chile and Brazil. Sera from patients with visceral Leishmaniasis (Kala-azar) were obtained in Brazil. Rabbit sera against fusion proteins from clones #1, #2 and #30 were obtained after two subcutaneous inoculations. Fusion proteins were partially purified from sodium dodecyl sulfate polyacrylamide gels and homogenized with Freund's complete adjuvant.

DNA purification and hybridization. Nuclear DNA from trypanosomes was prepared [15], digested with restriction endonucleases, fractionated on 0.8% agarose gels, blotted onto nitrocellulose filters and hybridized with cloned DNA probes labelled by nick translation as previously described [12]. The *T. cruzi* clones were isolated from a lambda gt11 library [12].

Detection of antibodies against cloned proteins in sera from infected humans. Lambda gt11 clones were grown on a lawn of *Escherichia coli* Y1090. Filters containing 10 mM of IPTG (isopropyl-β-D-thiogalactopyranoside) were placed on the plaques. The filters were then processed and incubated with antiserum as previously described [12].

DNA sequencing. DNA sequences were determined by the chain termination method [16] after subcloning of the phage inserts into pUC19. Selected parts of the inserts were sequenced by the chemical degradation method [17]. Partial restriction endonuclease digests as well as Bal31 digestions were in some cases performed to order the repeat units within a given clone.

Immunofluorescence. Immunofluorescence studies were performed with living epimastigotes from culture media and with living trypomastigotes from Vero cell cultures. Antibodies were either selected from a human Chagasic serum (H2) by affinity-chromatography, using the fusion proteins [18] or were obtained from rabbits immunized with fusion proteins. The secondary antibody consisted of human or rabbit anti-immunoglobulins conjugated with fluorescein.

Results

Isolation of T. cruzi clones encoding antigens with tandemly repeated amino acid sequence motifs. In a previous paper we described the isolation of

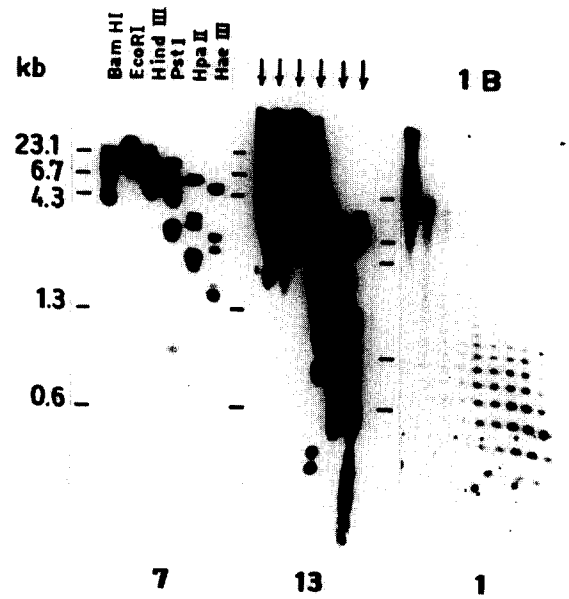

Fig. 1. Nuclear DNA from *T. cruzi* epimastigotes, digested with different restriction enzymes, was analysed by Southern blotting. The filters were hybridized with the DNA inserts from the clones, indicated under each panel. In the right-hand panel (1B), the *T. cruzi* DNA was partially digested with HindIII and hybridized with the insert from clone #1.

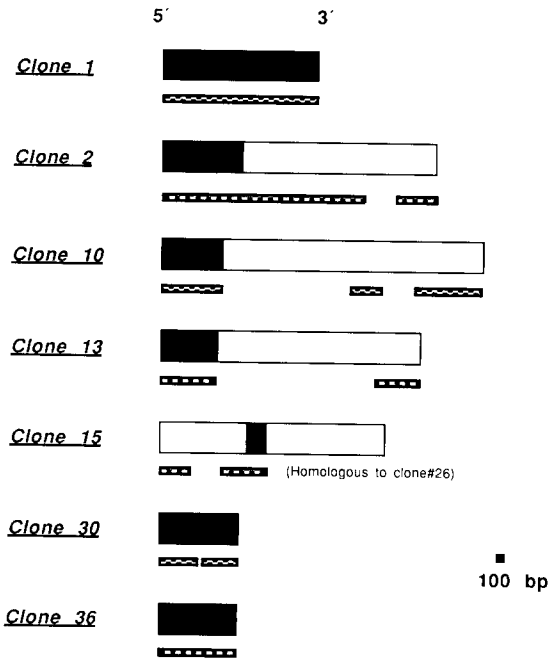

Fig. 2. Size and location of the repeated sequences, present in seven different clones. The regions sequenced (hatched) as well as regions containing repeats (filled) are indicated.

clones belonging to ten groups according to cross-reactivity, which all encode *T. cruzi* antigens [12]. They were isolated from a genomic lambda gt11 library by screening with a serum from a Chagasic patient. In the present study we have selected nine clones for further analysis (clones #1, #2, #7, #10, #13, #26, #30, #36 and #54). DNA from each of these nine clones was hybridized to nuclear DNA from *T. cruzi*, digested with six restriction endonucleases. All clones with the exception of #7 and #13 gave rise to hybridization patterns which are compatible with the presence of a single gene copy, i.e. single fragments of similar intensity were observed after cleavage with several enzymes (data not shown). Clone #7 hybridized with several fragments. Interestingly, similar sets of fragments (groups of four fragments) were obtained after cleavage of *T. cruzi* DNA with PstI, HpaII and HaeIII (Fig. 1). Hybridization with clone #13 revealed a large number of bands, thus suggesting the presence of a repetitive sequence element in this clone (Fig. 1). Certain clones gave rise to small bands after cleavage with some enzymes, for instance, in the case of HpaII and HindIII digestion of clone #1. This was apparently due to the presence of tandemly repeated sequences as shown by partial HindIII digestion of nuclear DNA in the case of clone #1 (Fig. 1B). An explanation was also provided by the sequence data for these clones (see below). Complete or partial nucleotide sequences were obtained from the nine clones described above. Seven of them were found to contain an internal repeat structure which in most cases was located immediately downstream of the β-galactosidase coding region of the lambda gt11 vector (Fig. 2). However, in the case of clone #15, which belongs to the same subgroup as clone #26 (Table I; see also ref. 12), the repeated sequence follows after a stretch of unique DNA. The length of the repeat units varied among the different clones, although it is constant for each individual clone (Fig. 3). Thus, some clones contain short repeat units of 15 bp (clone #13) or 24 bp (clone #10) while others contain very long repeats (114

TABLE I

Reactivity between fusion proteins from nine recombinant clones and sera from chronic Chagasic patients, collected in Brazil, Argentina and Chile[a]

Country of origin	# sera tested	% sera positive with 1 or more clones	% positive sera reacting with individual clones								
			1	2	7	10	13	26	30	36	54
Brazil	49	84	59	61	2	5	41	7	29	39	5
Argentina	23	87	80	75	30	25	45	45	90	35	15
Chile	12	75	67	78	0	0	11	0	44	11	0
Total	84	83	66	67	10	10	39	17	49	34	7

[a] The percentages of sera that reacted with one or more of the nine cloned proteins are shown. The right side of the table shows the fraction of the positive sera that reacted with each individual fusion protein.

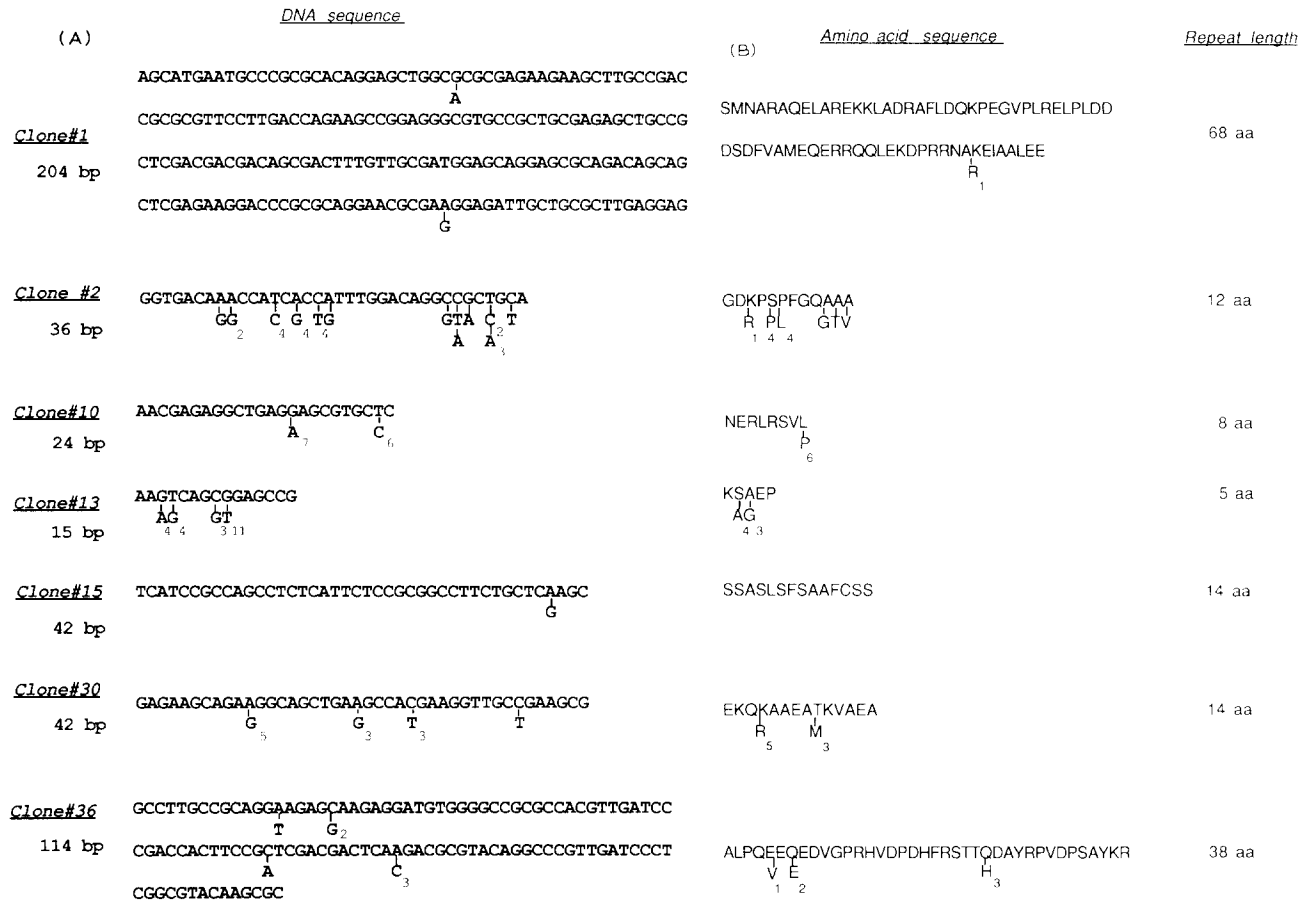

Fig. 3. Nucleotide (A) and amino acid (B) sequences of the repeat units present in seven clones. The consensus sequences are shown and variable positions are indicated. The numbers connected with the mutated nucleotides indicate the number of repeats in which the mutation was present.

bp in clone #36 and 204 bp in clone #1). Some sequence divergency was observed among the repeats within a given clone (Fig. 3). Those containing longer repeat units (clones #1 and #36) seem to be more conserved than those containing smaller ones (clones #2, #10 and #13). Variable bases are usually restricted to certain positions (Fig. 3). No sequence homology was evident when repeat units of different clones were compared, neither at the nucleotide nor at the amino acid sequence levels. None of the sequences appears to be related to previously described *T. cruzi* antigens [10,11]. The number of repeat units present in most of the antigens appears to be large. In clone #1, as many as 17 repeat units, each consisting of 204 bp, were detectable in partial HindIII digests of nuclear DNA (Fig. 1B). Clones #2, #10 and #13 were estimated to contain at least 17, 19, and 30 units, respectively (Fig. 3). Clone #46, belonging to the same subgroup as clone #30, consisted entirely of repeats, and we estimate that there are 57 units of a 42 bp long repeat present in this clone (data not shown).

Proteins encoded by some of the clones react with a wide collection of sera from Chagasic patients. In order to study whether the isolated clones encode important antigens, fusion proteins expressed from each one of the sequenced clones were tested with 84 sera from chronic Chagasic patients, obtained in Chile, Brazil, and Argentina. The results showed that 83% of these sera

reacted with one or more of the fusion proteins (Table I). Further analysis of the reactivity of individual fusion proteins showed that two of them (#1, #2) detected antibodies in 66–67% of the Chagasic sera tested and sera obtained from the above mentioned countries showed similar reactivities with these two fusion proteins. We thus conclude that antigens represented by these clones are present in parasites from widely different geographical regions. The fusion proteins from clones #13, #30 and #36 also reacted with a large proportion of the sera, although greater regional preferences were noticed (Table I). Some of the fusion proteins displayed a very strong preference for sera from Argentina; for instance, the fusion proteins of clones #10, #26 and #30. Also clones #7 and #54, which encode proteins that lack repeats, reacted almost exclusively with sera from Argentina. None of the nine fusion proteins reacted with 21 sera from cases of Leishmaniasis (Kala-azar) nor with 37 sera, collected in Argentina and Brazil from healthy individuals.

Three of the inserts consisted exclusively of repeats (clones #1, #30 and #36). Since fusion proteins encoded by them reacted with several sera from Chagasic patients (Table I) we conclude that the amino acid repeat itself in these cases must elicit an immune response during a chronic *T. cruzi* infection.

Detection of the cloned antigens on the surface of live parasites. Immunofluorescence studies were performed, using live epimastigotes and trypomastigotes. The experiments were performed in such a way that the parasites remained mobile during the whole treatment and after the completion of the study. For these experiments antibodies from a Chagasic patient (H2; see ref. 12) were used. The antibodies were affinity-purified by adsorption to immobilized fusion proteins prior to use. In the case of clones #1, #2 and #30, rabbit sera against the corresponding fusion proteins were also tested. Previous results [12], obtained by immunoblotting, have shown that antibodies against fusion proteins from clones #1, #10, #30 and #36 react with epimastigotes. The immunofluorescence study showed that of these only clone #10 encodes an antigen that is detectable on the surface of the living epimastigote (Table II). Using live trypomastigotes it was shown that antibodies against fusion proteins from clones #2, #10 and #13 were reactive (Table II). Thus, three of the seven proteins, having an internal repeat structure, were detectable on the surface of trypanosomes.

TABLE II

Immunofluorescence studies of intact *T. cruzi* parasites[a]

Clone	Affinity-purified human antibodies		Rabbit antibodies	
	Epimastigote	Trypomastigote	Epimastigote	Trypomastigote
#1	–	–	–	–
#2	NT	+	NT	+
#7	NT	–	NT	NT
#10	+	+	NT	NT
#13	NT	+	NT	NT
#26	NT	–	NT	NT
#30	–	–	NT	–
#36	–	–	NT	NT
#54	NT	–	NT	NT

[a] Live epimastigotes or trypomastigotes of *T. cruzi* were incubated with human antibodies, affinity-purified from a Chagasic serum, or with rabbit sera, prepared against the indicated fusion proteins. Sera obtained from normal rabbits and healthy humans were used as negative controls. Positive control sera were obtained from a rabbit, infected with the AWP strain of *T. cruzi* and from an infected patient (H2; see ref. 12). Only some antibodies were tested against epimastigotes as it was known from our previous studies [12] that only antigens encoded by clones #1, #10, #30, and #36 are expressed in epimastogotes.

NT = not tested.

Discussion

Nine clones encoding *T. cruzi* antigens have been characterized in the present study. Some of the fusion proteins, expressed from these clones, reacted with a large proportion of Chagasic sera, collected in distant geographical regions, thus showing that they represent parasite antigens which are conserved in different *T. cruzi* isolates. We have recently tested three sera obtained in Honduras, a country far away from Argentina. All these three sera recognized one or more of the cloned proteins (data not shown). Proteins encoded by clones #1, #2, #13 and #30 are together able to detect antibodies in 70 of the 84 sera tested (Table I). It is noteworthy that the other 14 sera lacked antibodies against any of the cloned proteins. Two of the latter sera were used to screen our genomic DNA library with negative results. Given the diversity of *T. cruzi* parasites [19], it is possible that the trypanosomes infecting the persons from which these 14 negative sera were obtained are very different from the rest. The cloned antigens might provide means to improve current serological methods for diagnosis since they did not react with control sera nor with sera positive for Leishmaniasis (Kala-azar) which usually give rise to cross-reactions when conventional tests are performed [20]. Our finding that a large proportion of the isolated clones encode *T. cruzi* antigens with repeated sequence motifs, was surprising. Out of nine clones which were selected by screening with serum from a Chagasic patient, seven turned out to contain tandem repeats. A remote possibility is that the remaining clones (#7 and #54) also contain repeats which have escaped detection due to their length. The reason why our screening procedure appears to select for antigens with a repeat structure is unknown. Several possibilities can be considered. The antigens might be prominent surface components which due to their location and abundance elicit a strong antibody response. Another possibility is that proteins which contain repeat structures in general are very antigenic. An interesting finding was that three of the antigens with repeats are located on or associated with the surface of the parasite. The others may be secreted antigens or, alternatively, become reactive only after disruption of the parasite. For some of the clones, it was demonstrated that the repeat unit itself was antigenic since it was recognized by several Chagasic sera and because they gave rise to an antibody response in rabbits. The cloned *T. cruzi* antigens resemble to some extent surface proteins of plasmodium, another intracellular parasite. In the latter, antigens with a repeated structure are presumed to play a role for the interaction between the parasite and the host immune system [1–5]. *T. cruzi* is an intracellular parasite, able to invade a wide range of eukaryotic host cells. Consequently, one possible explanation for the presence of several antigenic proteins in *T. cruzi* with a repeat structure might be related to the fact that the parasite can interact with many different cell types. If so, these antigens may be essential for parasite survival and antibodies against them might interfere with parasite invasion. Some of the cloned proteins may thus be candidate antigens to be included in a putative vaccine against *T. cruzi*.

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