Identification of a *Trypanosoma cruzi* antigen that is shed during the acute phase of Chagas’ disease

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A *Trypanosoma cruzi* antigen which is shed into the culture medium by the trypomastigote stage of the parasite and detected in blood of acutely infected mice was cloned and characterized. We designate this antigen shed acute phase antigen (SAPA). Five protein bands with apparent molecular masses ranging from 160 to 200 kDa were detected by immunoblotting of plasma from infected mice and in supernatants of cultured trypomastigotes upon reaction with antibodies against SAPA. A serum obtained from a patient acutely infected with Chagas’ disease revealed a similar set of polypeptides in supernatants of cultured trypomastigotes when tested by immunoblotting. SAPA seems thus to be a major shed protein during the acute period of the disease. Twenty-six of 28 sera from human acute cases of Chagas’ disease tested reacted with SAPA. Conversely, only 8–10% of sera from chronic cases of the disease contained detectable levels of antibody against SAPA. Sera from rabbits infected with six different parasite strains all contained antibodies against SAPA. Antibodies against SAPA are detectable 15 days after the manifestation of acute Chagas’ disease symptoms in humans and 15 days post-infection in sera from mice and rabbits. The nucleotide sequence of a genomic clone encoding the 3' end of the SAPA gene revealed the presence of 14 tandemly arranged 12-amino acid-long repeats. A 39-amino acid-long region that is very hydrophobic precedes the stop codon. Due to its early appearance it might be possible to design diagnostic assays which are based on SAPA for identification of recently infected cases of Chagas’ disease.

**Key words:** *Trypanosoma cruzi*; Shed antigen; Gene cloning; Amino acid repeats; Chagas’ disease

**Introduction**

*Trypanosoma cruzi* is the agent of Chagas’ disease, a severe endemic illness affecting several million people in Central and South America. The parasite has a complex life cycle involving an epimastigote stage in the insect vector and two main stages in the mammalian host, one present in the blood (trypomastigote) and a second which is intracellular (amastigote) [1].

The acute phase of the disease is in most cases asymptomatic, and the infection may remain quiescent for decades. Some patients may, however, develop a progressive chronic form of the disease with cardiac and/or digestive tract alterations [2]. After the acute phase with parasitemia, parasite growth is in most cases controlled by the host, and patients and animals enter into a chronic phase where few parasites are present in the blood [3]. Several factors may be involved in the control of parasite growth, and antibodies seem to play an essential role (for a review, see ref. 3). Antibodies against *T. cruzi* are able to lyse trypomastigotes [4–5] and partially to block par-
parasite penetration of host cells [6-7]. Given these results, analysis of individual parasite antigens seems relevant.

Unlike the blood stage of African trypanosomes [8] and the sporozoite stage of plasmodia [9], several different antigens are present on the T. cruzi surface, some of which are stage-specific [10-12]. Therefore, we decided to clone genes for T. cruzi antigens in order to analyze each one separately. In a recent study, we characterized 7 antigens isolated from a genomic expression library with the aid of a chronic Chagasic serum [13-14]. The results showed that T. cruzi, like plasmodia [15] possesses antigens made up of arrays of tandemly repeated amino acid sequences [16]. Some of the isolated antigens reacted with a large fraction of sera from chronic Chagasic patients [16]. In this paper we describe a cloned T. cruzi antigen that reacts preferentially with sera from acute cases of Chagas’ disease.

**Materials and Methods**

**Parasites.** Epimastigotes of the Tulahuen, RA and CAI strains [17] were grown in liquid media [13]. Trypomastigotes, RA strain [17] from infected Vero cell cultures were obtained as described by Zingales et al. [18].

**Patients and sera.** Sera from acute cases were collected before treatment, from 28 patients (14 females) who had acquired the disease in the States of Minas Gerais and Bahia (Brazil) [19]. Half of these were children, all 28 displayed acute symptoms of the disease, and parasitemia was easily detectable by direct methods. The symptoms started between 15 and 80 days before collection of blood. Thirty-seven sera from patients in the chronic phase of the disease were from cases who had acquired the disease in the states of Goias and Bahia (Brazil). All of these had been positive by xenodiagnosis [19]. Sera from rabbits immunized with the fusion proteins from clones 1, 7, and 30 produced in the λgt11 vector were obtained as described previously [16].

Supernatants from infected Vero cell cultures (RA strain) were collected five days post-infection. The supernatants were centrifuged three times in order to remove parasites. In some experiments, the trypomastigotes were incubated for 5 and 15 h at 37°C in cell-free media and the supernatants collected by centrifugation.

**Immunoblotting.** Plasma from acutely infected mice (RA strain, 7 days post-infection) and supernatants from infected Vero cell cultures (RA strain) were concentrated 20 times by lyophilization. 50 μl from each sample was layered on 7.5% polyacrylamide gels. Plasma from uninfected mice and supernatants from uninfected Vero cells, prepared under identical conditions, were used as controls. Polyacrylamide gels were electrophoretically blotted onto nitrocellulose filters [20] and reacted with the indicated sera [13]. Blotted gels of supernatant samples were then reacted with 125I-protein A, whereas for plasma samples the blots were reacted with a rabbit secondary antibody using the biotin/avidin/peroxidase system (Vecta stain, Vector laboratories). Spots of parasites (3 × 10^5 cells/spot) and from supernatants (2 μl of 20 × concentrated samples) were layered onto nitrocellulose filters and immunologically detected using the indicated serum and rabbit secondary antibody as described above. Crithidia samples were used as negative controls in some of the experiments.

**Detection of antibodies in sera from cases of Chagas’ disease.** To test sera from human cases of the disease against nine recombinant T. cruzi antigens [13], nitrocellulose filters embedded in IPTG (isopropyl-β-d-thio-galactopyranoside) were layered on plaques of λgt11 recombinant phages grown on a lawn of Escherichia coli Y1090. Filters were then processed with the indicated sera and 125I-protein A, as previously described in detail [13]. Sera were depleted of non-specific antibodies [13] and used in 1:100 to 1:200 dilution. Recombinant phages reacting significantly more strongly than the background observed with λgt11 were recorded as positive (see Fig. 1). Each serum sample was tested at least twice.

**DNA sequence analysis.** The recombinant λgt11 clones have been described previously [13,16]. The 5’ end of clone 7 (SAPA) was mapped using a single PstI site present in the DNA insert. The nucleotide sequence was determined by the chain
termination method [21] after subcloning of the phage insert in pUC19. Partial exonuclease III digestions, starting at the 5' end of the insert, were performed in order to generate subclones for sequencing experiments.

**Results**

Antibodies to SAPA are present in sera from acute cases of Chagas' disease. Twenty-eight sera from patients with acute Chagas' disease were tested using as antigens the fusion proteins expressed from nine previously described *T. cruzi* clones [13,16]. Twenty-six of the sera reacted preferentially with the fusion protein of clone 7, and to a lesser extent with those of clones 13 (11 positive sera) and 36 (10 positive sera; Table I). The two sera that did not react with clone 7 recognized the fusion proteins from clones 1, 13, and 36 (data not shown). These results are in marked contrast with those obtained previously with sera from chronic cases [16]. In the latter case the fusion protein of clone 7 was only detected by 10% of the chronic sera, whereas fusion proteins from clones 1 and 2 were detected by 66% and 67% of sera, respectively (Table I). Due to its properties we designate the antigen encoded by clone 7 SAPA.

Since all the sera from the acute cases were collected in one region of Brazil, the above results might be due to local differences in parasite strains rather than to differences between sera collected during acute and chronic phases of the disease. To exclude this possibility, 37 new sera from chronic cases were collected in Goias and Bahia and tested. Again, most sera detected fusion proteins from clones 1 and 2, and few (8%) detected SAPA (Table I). This confirms our previous results (Table I). Examples of autoradiograms obtained with three acute and three chronic sera are shown in Fig. 1. In this figure, typical results obtained with sera from rabbits infected with three different *T. cruzi* strains are included for comparison. As in the case of sera from acute human infections, sera from infected rabbits reacted strongly with SAPA, and to a lesser degree with the other cloned *T. cruzi* antigens (see also ref. 13).

SAPA is shed by trypomastigotes. Supernatants of infected (RA strain) and uninfected Vero cells as well as from trypomastigotes incubated in cell-free media for 5 and 15 h at 37°C were tested for the presence of parasite proteins. Epimastigotes and trypomastigotes (RA strain) were included as controls (Fig. 2). Serum from an infected rabbit (Tulahuen strain) gave a positive reaction with all three trypomastigote supernatants, but proved negative with supernatants from uninfected Vero cells, thus revealing the presence of shed antigens. Antibodies to SAPA also reacted strongly with trypomastigote supernatants but not with supernatants from uninfected Vero cells. The reactivity increased when the trypomastigotes were incubated for longer periods in cell-free medium (Fig. 2). To exclude the possibility that reactivity was due to parasite contamination in the supernatants, similar filters were incubated with antisera against fusion proteins from clones 1 and 30 [16]. None of these antibodies reacted with any of the supernatants (Fig. 2), thus showing that they are free from parasites and parasite debris. The results depicted in Fig. 2 also show that SAPA is preferentially expressed during the trypomastigote stage, although a small amount may also be present in epimastigotes.

SAPA could also be detected by immunoblot-

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**TABLE I**

Reactivity of nine cloned proteins sera from acute and chronic cases of Chagas' disease

<table>
<thead>
<tr>
<th>Disease phase</th>
<th>Number of sera tested</th>
<th>% Sera Reacting with Individual Clones</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Acute</td>
<td>28</td>
<td>18</td>
</tr>
<tr>
<td>Chronic</td>
<td>37</td>
<td>73</td>
</tr>
<tr>
<td>Chronic*</td>
<td>70</td>
<td>66</td>
</tr>
</tbody>
</table>

*Taken from ref. 16.
Fig. 1. Reaction of human and rabbit sera with fusion proteins from different *T. cruzi* clones. Nitrocellulose filters containing recombinant products, distributed as indicated at the bottom of the figure, were reacted with sera from: (A), three human chronic cases; (B), three human acute cases; and (C), three rabbits infected with *T. cruzi* strains AWP (30 days post-infection), RA (30 days post-infection) and Y (60 days post-infection) [13]. \( \lambda \), Agt11 phage.
Thus confirming that SAPA is preferentially synthesized during the trypomastigote stage (Fig. 3 and ref. 13).

Nucleotide sequence analysis of the SAPA gene. The 3' end of the SAPA gene contained in the genomic clone 7 [13] was sequenced. Starting from the 5' end of the insert, there are 139 bp of non-repeated DNA, followed by 504 bp containing 14 tandemly arranged 36-bp-long repeats and finally 129 bp of non-repeated DNA (Fig. 4A). The latter region starts with an incomplete repeat of 12 bp. Few differences were detected among the repeats and the nucleotide substitution were in all cases restricted to positions 8, 16, 27 and 35 of each repeat. These substitutions give rise to four kinds of amino acid repeats (Fig. 4B). The deduced amino acid sequence of the repeats is weakly hydrophilic, and each repeat has two conserved prolines which preclude the formation of α-helix or β-sheet structures. The repeat region is unusually rich in serine, as each repeat contains 3-4 serine residues. The amino acid sequence of the non-repeated region at the 3' end is very hydrophobic (Fig. 4). A putative glycosylation site is present around position 655 of the nucleotide sequence, which is located after the end of the amino acid repeats.

In a previous study we have shown that affinity-purified antibodies against the clone 7 fusion protein (SAPA) cross-reacted in some cases with clone 36 protein, and that affinity-purified antibodies against the fusion protein from clone 36 also cross-reacted with the clone 7 protein [13]. The deduced amino acid sequence of SAPA revealed a tripeptide (Pro-Val-Asp) within the repeated region which also is present in the deduced amino acid sequence of the repeat of the clone 36 protein [16]. Thus, the Pro-Val-Asp sequence may constitute the cross-reacting epitope of both antigens.
GAA TTC GGG CCA ACC ATA AGC CAC GTG AGT AAT AAT GTT CTT TAC AAC GGT CAG CTG AAT GCC GAG GAG
   Glu Phe Gly Pro Thr Ile Ser His Val Thr Val Asn Asn Val Leu Leu Tyr Asn Arg Gin Leu Asn Ala Glu Glu
   25
ATC AAG ACC TGG TTC TGG AGC GAC CTG ATT GGC AGA GCA CAC ATG GAC AGC AGC AGC GAG GAG GCC AGT GGT
   Ile Lys Thr Leu Phe Leu Ser Gin Asp Leu Ile Gly Thr Glu Ala His Met Asp Ser Ser Asp Ser Ser Ala
   75
CAC GGT AGC CCC TCA ACT CCC GTT GAC AGC ACT GCC CAG GAC CTG ATT GGC ACG GAA GCA CAC ATG GAC AGC AGC
   His Gly Thr Pro Ser Thr Pro Val Asp Ser Thr Ala His Gly Thr Pro Ser Thr Pro Ala Asp Ser Ser Ala
   125
AGT AGC CCC TCG ACT CCT CCC GTT GAC AGC ACT GCC CAG GAC CTG ATT GGC ACG GAA GCA CAC ATG GAC AGC AGC
   Ser Thr Pro Ser Thr Pro Ala Asp Ser Ser Ala His Ser Pro Ser Thr Pro Ala Asp Ser Ser Ala His Ser
   175
AGC CCC TCG ACT CCT CCC GTT GAC AGC ACT GCC CAG GAC CTG ATT GGC ACG GAA GCA CAC ATG GAC AGC AGC
   Thr Pro Ser Thr Pro Ala Asp Ser Ser Ala His Ser Pro Ser Thr Pro Ala Asp Ser Ser Ala His Ser
   225
CCC TCA ACT CCT GGT GAC AGC ACT GCC CAG GAC CTG ACT CCT CCC GTT GAC AGC ACT GCC CAG GAC CTG ACT
   Pro Ser Ser Thr Pro Val Asp Ser Thr Ala His Gly Thr Pro Ser Thr Pro Ala Asp Ser Ser Ala His Ser
   275
TCA ACT CCT GGT GAC AGC ACT GCC CAG GAC CTG ACT CCT CCC GTT GAC AGC ACT GCC CAG GAC CTG ACT
   Ser Thr Pro Ser Thr Pro Ala Asp Ser Ser Ala His Ser Pro Ser Thr Pro Ala Asp Ser Ser Ala His Ser
   325
ACT CCT GGT GAC AGC ACT GCC CAG GAC CTG ACT CCT CCC GTT GAC AGC ACT GCC CAG GAC CTG ACT
   Thr Pro Ser Thr Pro Ala Asp Ser Ser Ala His Ser Pro Ser Thr Pro Ala Asp Ser Ser Ala His Ser
   375
CCC GAT GCC GCT GCA CTT TCC TGG GCC GGA GGT TTT CTT CTG TGT GGG GTG TGG CTG CGT CTC GAC
   Pro Gly Ala Ala Leu Thr Phe Ser Gly Gly Gly Leu Leu Leu Leu Cys Ala Cys Ala Leu Leu Leu His Val
   525
TTT TTT AGC GGT TTT TTC TGAT GTAG TGGAGAGTCTCC TAAACAAAGATAGATATAAATGATATGAGATGAGTCTCC TAAACAAAGATAGATAT
   Phe Phe Thr Ala Val Phe Phe * * *
   675
Fig. 4. Panel (A): Partial nucleotide sequence of the gene for SAPA. The nucleotide sequence and deduced amino acid sequence of the 3' end of the SAPA gene are shown. The 36-bp-long repeated motifs are boxed. A partial repeat near the 3' end is indicated with dashed lines. A putative glycosylation site is underlined and three in-frame stop codons are also marked (*). Panel (B): sequence of the repeated motifs. The positions of variant amino acids are shown and the number of copies of each repeat that is present in the determined sequence of SAPA are shown in parentheses.

Discussion
In this paper we have characterized a *T. cruzi* antigen (SAPA) which is shed into the medium by trypomastigotes and is present in plasma from acute Chagas' disease cases. Sequence analysis of the cloned fragment of SAPA showed internal amino acid repeats as observed in *T. cruzi* proteins detected in sera from chronic cases of Chagas' disease [16]. Interestingly, a 117-bp-long region located immediately before the stop codon encodes a highly hydrophobic amino acid region.

If this sequence constitutes a membrane anchor region, a cleavage step would be required in order to release SAPA into parasite supernatants and plasma from infected mice (Fig 2). Sera from rabbits infected with six different parasite strains all contained antibodies against SAPA, suggesting that SAPA from different *T. cruzi* strains contain common epitopes.

Ninety-three percent (26/28) of the sera from acute cases reacted strongly with SAPA (Fig. 1 and Table I), while only few of them reacted with fusion proteins from clones 1 and 2. On the other
hand, 66–73% of sera from chronic cases reacted with fusion proteins from clones 1 and 2 (Table I and ref. 16) and only a few of them reacted with SAPA (8–10%). Since all sera from acute human cases analyzed were derived from symptomatic patients, we cannot for the moment exclude the possibility that there are some *T. cruzi* subspecies that cause acute manifestations of the disease and which express SAPA, while those that do not give rise to acute symptoms fail to express this antigen. After the initial parasitemia, parasite growth is in most cases controlled by the host. Those antigens that are shed into the blood may preferentially give rise to an antibody response during the early period of an infection. This would be the case for SAPA. Indeed, this protein seems to be a major shed *T. cruzi* antigen during the acute period, since a serum from an acute case of Chagas’ disease reacted strongly with antigens in the size range of SAPA (Fig. 3). After a long-lasting chronic infection, exposure to the immune system of other parasite antigens will occur, and this might explain why antibodies in sera from chronic infections are directed towards other proteins [16].

Even though individual *T. cruzi* soluble antigens have not been characterized, they are known to be present in blood from infected humans and animals, as well as in supernatants of cells infected with *T. cruzi* (see references in refs. 22 and 23). Moreover, it has been suggested that circulating antigens, as well as circulating immune complexes, may mediate tissue lesions and thus be involved in the pathogenesis of Chagas’ disease [22]. Furthermore, soluble *T. cruzi* antigens may bind to the surface membrane of normal cells, possible being involved in the recognition and destruction of host cells by T cells and/or antibodies [23]. Recently, it has been shown that a protein with decay-accelerating activity is shed by *T. cruzi* trypomastigotes but not by epimastigotes, and it was suggested that this activity might make trypomastigotes resistant to complement lysis [24]. In this study a limited number of metabolically labelled radioactive components ranging in molecular mass from 86 to 155 kDa were shed by trypomastigotes [24]. However, the one which had decay-accelerating activity was not identified. SAPA is also shed by trypomastigotes and anti-SAPA antibodies recognize several components, ranging in molecular mass from 160 to 200 kDa [13]. Soluble antigens are also produced by other parasites and this may give some clues as to the function of SAPA. In *Plasmodium*, so-called S antigens are produced and released into the medium upon schizont rupture. However, unlike SAPA, this antigen is serologically diverse [25]. The circumsporozoite (CS) protein, like SAPA, contains a hydrophobic sequence at the C-terminus, and CS is shed upon reaction with antibodies [9]. Other *Plasmodium* antigens which have internal amino acid repeats may prevent the production of effective antibodies against critical epitopes through the generation of non-neutralizing antibodies [15]. Given the structure of SAPA, its release into the blood may prevent an effective immune response against critical epitopes during the acute period. Thus, several possible functions for SAPA need to be investigated, many of which might be essential for parasite survival.

Besides its possible biological function, SAPA may be useful for diagnosis of Chagas’ disease, since acute symptoms of the disease, if present, are not pathognomonic. SAPA does not react with control sera nor with sera positive for Leishmaniasis (Kala-azar) [16] which usually give rise to cross-reactions when conventional tests are performed [26]. Interestingly, it was possible to detect antibodies against SAPA as early as 15 days after appearance of clinical manifestations in humans and 15 days post-infection in rabbits [13] and mice (M.A. Basombrio et al., unpublished). Assays based on SAPA may thus be helpful in early diagnosis of Chagas’ disease, which is of clinical importance since the available chemotherapeutic drugs are mainly effective during the acute period of the disease [27].

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