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Stage and strain specific expression of the tandemly repeated 90 kDa surface antigen gene family in *Trypanosoma cruzi*

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A recombinant cDNA library constructed in the expression vector λ gt11 using mRNA from the trypomastigote stage of *Trypanosoma cruzi* was screened with two monoclonal antibodies that have been shown to react with a 105 kDa and a 90 kDa surface antigen in trypomastigotes of the Peru and Y strains of *T. cruzi*. One recombinant λ phage, designated Tcc-20, was reactive to both monoclonals. The β -galactosidase/*T. cruzi* hybrid protein encoded in Tcc-20 is recognized by the monoclonal antibodies and by serum antibodies from mice infected with strains of *T. cruzi* which contain the 90 kDa antigen. Antibodies immunoselected from serum of mice infected with the Peru strain by adsorption to Tcc-20 fusion protein react specifically with a 90 kDa polypeptide in trypomastigote but not epimastigote lysates of *T. cruzi*. The mRNA complementary to the DNA insert in Tcc-20 is present only in those stages and strains of *T. cruzi* which express the 90 kDa surface antigen. These characteristics are strong evidence that the *T. cruzi* DNA fragment cloned into Tcc-20 encodes a portion of the 90 kDa surface antigen. The gene(s) which encodes this polypeptide is shown to be present in approximately 20 copies per haploid genome and most, and possibly all, of the copies are found in a tandemly linked multigene family.

Key words: *Trypanosoma cruzi*; Surface antigen; Gene family

Introduction

We have recently reported the production of monoclonal antibodies directed against the major 90 kDa antigen present on the surface of the bloodstream trypomastigote form of *Trypanosoma cruzi* [1]. This antigen has been shown to be specific to the trypomastigote stage, and to be present on the surface of some but not all strains of *T. cruzi* [2]. In our studies, monoclonal antibody 4.2 recognized a polypeptide doublet of 90 and 105 kDa in Western blots of trypomastigote lysates of the Peru and Y strains of *T. cruzi* and a single polypeptide of 130 kDa in the Esmeraldo strain. No bands were seen in blots of the CL or Silvio X10 clone 1 strains of *T. cruzi*.

To better understand the heterogeneity and distribution of this surface antigen among different strains of *T. cruzi*, we chose to isolate the gene sequence which encodes this antigen and study its structure and expression. Here we report the results of these studies.

Materials and Methods

Parasite strains and culture. *T. cruzi* Peru, Y, and CL strains were obtained from Stuart M. Krasner, University of California, Irvine. The cloned *T. cruzi* strains Esmeraldo clone 3 and Silvio X10 clone 1 were obtained from James Dvorak, National Institutes of Health, Bethesda, MD. Growth and maintenance of epimastigotes and tissue-culture derived trypomastigotes of these five strains are as described elsewhere [1].

Nucleic acid isolation, radiolabeling, Southern and Northern transfer, and restriction enzymes. Parasites were harvested and DNA, RNA, and

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Abbreviations: bp, base pairs; kb, kilobase (pairs).

poly(A)⁺ mRNA were isolated as described previously [3]. Plasmid DNA was amplified, harvested by lysis in sodium dodecyl sulfate, and purified by banding in cesium chloride as described [4]. Lambda phage were amplified, harvested by precipitation with polyethylene glycol, banded in cesium chloride and the DNA extracted as described [4]. DNA restriction fragments were radiolabeled with [α -³²P]dNTP by fill-in reaction with Klenow enzyme [4]. RNA was partially hydrolyzed at alkaline pH and 5' end-labeled as described [5]. Agarose gel electrophoresis of DNA, Southern transfer, prehybridization, hybridization, and filter washing were performed as described [6] except the gels were 1% agarose and the wash temperature was 68°C. RNA was electrophoresed in a formaldehyde gel, blotted to nitrocellulose, baked, prehybridized, hybridized, and washed as described [7] except the transfer apparatus was according to Thomas [8]. All restriction enzymes were purchased from International Biotechnologies, Inc. (New Haven, CT) and used as recommended.

Partial restriction endonuclease mapping of genomic DNA cloned into λ EMBL4 was performed according to Rackwitz et al. [9] by partial digestion with BamHI [4].

Library construction and screening. 10 μ g of Peru strain trypanomastigote poly(A)⁺ mRNA was transcribed using AMV reverse transcriptase (Molecular Genetics Resources, Tampa, FL) [4]. Second strand synthesis was performed using RNase H and DNA polymerase I [10]. The double-stranded cDNA was blunt ended with T4 DNA polymerase, ligated to phosphorylated EcoRI linkers (Collaborative Research, Inc., Bedford, MA) using T4 DNA ligase, and digested with EcoRI as described [4]. The cDNA product was separated from free linker fragments by extraction with phenol, precipitation with ethanol out of 2 M ammonium acetate, and ligated into the EcoRI site of λ gt11 [11]. Prior to ligation the λ gt11 DNA had been digested with EcoRI, and the 5' terminal phosphates removed by treatment with calf alkaline phosphatase [4]. The recombinant molecules were in vitro packaged [4] and plated on *Escherichia coli* strain Y1090 [11]. The recombinant λ phage library was determined to contain

10⁶ independent λ phage of which 97% were determined to be recombinant phage by plating on the indicator dye 5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside. Also, 80% of the λ phage showed positive hybridization with ³²P-labeled trypanomastigote poly(A)⁺ mRNA.

A Peru strain trypanomastigote genomic library was constructed by ligating a partial MboI digest of *T. cruzi* nuclear DNA into the BamHI sites of λ EMBL4 according to the manufacturer's suggested protocol (Promega Biotec, Inc., Madison, WI). The library was screened by the Benton-Davis plaque hybridization method [4] using the radiolabeled 154 base pair (bp) insert from Tcc-20.

Fusion protein analysis. Tcc-20 fusion protein and β -galactosidase were partially purified [12], electrophoresed on a 10% polyacrylamide gel (15 μ g per lane [13]) and Western blotted to nitrocellulose [1]. The Western blots were reacted with either monoclonal antibody 4.2 (20 μ g ml⁻¹), immune mouse sera (1/1000 dilution) or sera from human Chagasic patients (1/1000 dilution). Immune mouse sera were obtained from mice bled at week 6 of a *T. cruzi* infection. Sera from human Chagasic patients were obtained from the Bambui region of Brazil [14].

Antibodies directed against Tcc-20 fusion protein were obtained by immunoaffinity absorption from immune mouse sera by a modification [15] of the procedure of Hall et al. [12].

Results

Isolation of the 90 kDa surface antigen gene. To isolate a DNA fragment which encodes a portion of the 90 kDa surface antigen, a recombinant cDNA library of trypanomastigote poly(A)⁺ RNA sequences was constructed in the expression vector λ gt11 [11]. Approximately 30 000 recombinant phage were screened with monoclonal antibody 4.2 and 4.13 [16] and four positive plaques were identified, of which one, Tcc-20, rescreened positive. The cDNA insert in phage Tcc-20 was excised by digestion with EcoRI and subcloned into the plasmid vector pUC9. The insert size in the subclone, pTcc-20, was determined to be 154 bp by electrophoresis in an acrylamide gel and by di-

rect DNA sequence analysis (data not shown).

That Tcc-20 contains sequences that encode a portion of the 90 kDa surface antigen was confirmed by two methods. First, Tcc-20 was shown to encode a hybrid protein of M_r 121 000 which in Western blots was recognized by monoclonal antibody 4.2 (Fig. 1, Lane E), thus indicating that an antigenic determinant found in the 90 kDa surface protein is present in the polypeptide encoded in the *T. cruzi* DNA sequences present in Tcc-20. In the second method, antibody selection [12] was used. Tcc-20 fusion protein bound to nitrocellulose was used to affinity purify antibodies

Fig. 1. Identification of *T. cruzi* native proteins that share antigenic determinants with Tcc-20 fusion protein. Western blots of trypomastigote lysates of the Peru strain reacted with: (A) sera from mice infected with Peru strain; (B) anti-Tcc-20 fusion protein antibodies affinity purified from sera of mice infected with Peru strain; (D) monoclonal antibody 4.2. (C) Western blot of Peru epimastigote lysate reacted as in (B). (E) Western blot of Tcc-20 fusion protein reacted with monoclonal antibody 4.2. Markers for calibration of the gel are 14 C-methylated myosin (M_r 200 000), phosphorylase-b (M_r 92 500), bovine serum albumin (M_r 69 000), ovalbumin (M_r 46 000), and carbonic anhydrase (M_r 30 000).

from hyperimmune mouse serum. As shown in Fig. 1, Lane A, antibodies present in the hyperimmune serum recognized at least 20 polypeptides of different molecular weight when reacted with a Western blot of a trypomastigote lysate. In contrast, the affinity purified antibodies reacted only with a 90 kDa polypeptide in Western blots of a trypomastigote lysate (Fig. 1, Lane B). No reaction with a 90 kDa polypeptide was observed in Western blots of epimastigotes (Fig. 1, Lane C), consistent with previous observations that show the 90 kDa polypeptide is specific to the trypomastigote stage. In a control experiment using native β -galactosidase under the same conditions as for antibody selection with Tcc-20, no strong reaction was observed with polypeptides in either of the *T. cruzi* lysates (data not shown). Based upon these results we conclude that Tcc-20 contains DNA sequences that encode an epitope present in the 90 kDa surface antigen, and that the *T. cruzi* DNA insert in Tcc-20 likely represents a portion of the 90 kDa surface antigen gene.

Additional support that Tcc-20 encodes a portion of the 90 kDa surface antigen was obtained by reaction of Tcc-20 fusion protein with serum antibodies from mice infected with five different strains of *T. cruzi*. As shown in Fig. 2, only serum from mice infected with the Peru, Y and Esmeraldo strains contains antibodies that react with Tcc-20 fusion protein in Western blots. This result is consistent with Tcc-20 encoding a portion of the 90 kDa surface antigen since our previous studies [2] have shown that monoclonal 4.2 reacts with a surface protein(s) in Western blots of trypomastigote lysates of the Peru, Y and Esmeraldo strains but not the CL or Silvio X10 strains.

Structural organization and copy number of the 90 kDa surface antigen gene. To isolate a genomic copy of the 90 kDa gene, the 154 bp insert in pTcc-20 was excised, labeled with [α - 32 P]dATP and used as a probe to screen a *T. cruzi* genomic recombinant DNA library cloned into λ EMBL4 [17]. Approximately 40 000 plaques were screened and two plaques, designated Tcg-20 and Tcg-21, showed positive hybridization signals upon subsequent rescreening. Partial restriction endonuclease mapping of cleavage sites in Tcg-20 DNA

Fig. 2. Western blot analysis of Tcc-20 fusion protein recognized by sera from mice infected with different strains of *T. cruzi*. Lanes A, C, E, G, I, and K contain 15 μ g each of Tcc-20 fusion protein. Lanes B, D, F, H, J, and L contain 15 μ g each of partially purified β -galactosidase isolated from λ gt11. Pairs of lanes were reacted with sera from mice infected with the following strains of *T. cruzi*: (A,B) Peru; (C,D) Y; (E,F) CL; (G,H) Silvio X10; (I,J) Esmeraldo; (K,L) normal mouse serum. Molecular weight markers are as in Fig. 1.

indicated a tandemly repeating unit of 2.3 kilobase pairs (kb) in the DNA insert (Fig. 3A). Southern blots of Tcg-20 and Tcg-21 DNA digested with BamHI, EcoRI, or MboI probed with the radiolabeled 154 bp pTcc-20 insert (data not shown), in conjunction with the partial digest data, indicate that Tcg-20 contains six tandem repeats of a 2.3 kb BamHI fragment and a seventh partial repeat which has been truncated at the MboI site and inserted into the vector (Fig. 3B). Tcg-21 contains six tandemly repeating BamHI units which are flanked by two partial repeats both of which are truncated and inserted into the vector at the MboI site (Fig. 3B). One of the 2.3 kb repeat units from Tcg-20 was subcloned into the BamHI site of pUC9 and designated pTcg-20. Partial restriction mapping of trypanomastigote nuclear DNA confirmed that the tandem repeat arrangement is present in genomic DNA (data not shown).

The average copy number and organization of the 90 kDa gene sequence in the genome of *T. cruzi* was determined by hybridization of the 154 bp DNA insert of pTcc-20 to a Southern blot containing trypanomastigote nuclear DNA digested with BamHI (Fig. 4). Included on the Southern blot was DNA from subclone pTcg-20 containing the 2.3 kb BamHI repeat fragment equivalent to 1, 5, 10, 15, and 20 copies per haploid genome. The calculations for copy number determination

Fig. 3. (A) Tandem arrangement of the 2.3 kb repeat. Tcg-20 was partially digested with BamHI, hybridized to 32 P-labeled oligonucleotide complementary to the λ cos-R site and electrophoresed on a 0.5% agarose gel. Markers are high molecular weight standards from Bethesda Research Laboratories (Gaithersburg, MD). (B) Restriction map of λ Tcg-20 and λ Tcg-21. B, BamHI; E, EcoRI; M, MboI.

Fig. 4. Determination of the 90 kDa gene copy number in Peru trypomastigote DNA. Nuclear DNA (2.5 μ g) was digested with BamHI and electrophoresed on a 1% agarose gel (A). Included in the gel was BamHI digested DNA from subclone pTeg-20 containing the equivalent of 1, 5, 10, 15, and 20 copies per haploid genome (B–F, respectively). A Southern blot of the gel was hybridized with [32 P]pTcc-20 insert DNA. Numbers in kb on the margin refer to the migration of 32 P-labeled HindIII fragments of λ phage DNA.

were based on a haploid genome size in *T. cruzi* of 1.25×10^8 bp [19]. Strong hybridization of the 154 bp probe was observed to a genomic fragment of 2.3 kb and less intense hybridization was seen with a genomic fragment of 3.7 kb, presumably representing a truncated repeat unit at the end of the tandem array. The positioning of the 3.7 kb fragment at the end of a tandem array is based on nucleotide sequence data (not shown) which places the 154 bp insert of Tcc-20 within the 1567 bp MboI/BamHI fragment of the repeat unit. When the amount of hybridization in the genomic DNA is compared to that of the various equivalents in the cloned DNA, the hybridization band at 3.7 kb is present once per haploid genome while the fragment at 2.3 kb has approximately 20 copies per haploid genome.

Stage and strain specific expression of the 90 kDa surface antigen gene. Our previous studies have shown that the surface antigen(s) recognized by

monoclonal antibody 4.2 is both stage and strain specific. To determine whether the mRNA(s) which codes for this surface antigen is also stage and strain specific, Northern blots containing total RNA from trypomastigotes and epimastigotes of the Peru, Y, Esmeraldo, CL and Silvio X10 strains were hybridized with the 154 bp insert of pTcc-20. Hybridization was observed to a single mRNA band with an apparent molecular length of 1250 bases in trypomastigote RNA (Fig. 5). No hybridization was observed in epimastigote RNA (data not shown). Also, hybridization was observed only to RNA from the Peru, Y and Esmeraldo strains, thus confirming that the stage and strain specificity of the mRNA encoding the 90 kDa antigen correlates with those strains that were identified as expressing the protein by

Fig. 5. Identification of mRNA complementary to the 154 bp insert of Tcc-20 in total cellular RNA isolated from trypomastigotes of five strains of *T. cruzi*. A Northern blot containing 25 μ g of trypomastigote total cellular RNA per lane isolated from 5 different strains of *T. cruzi* was hybridized with [32 P]pTcc-20 insert DNA. (A) Peru RNA; (B) Y RNA; (C) CL RNA; (D) Silvio X10 RNA; (E) Esmeraldo RNA. Numbers in bp on the margin refer to the migration of a mixture of 32 P-labeled fragments of pBR322 plasmid DNA digested with the following sets of restriction endonucleases: EcoRI; EcoRI/PstI; EcoRI/AvaI; EcoRI/BamHI/PvuII/AvaI.

Western blot and immunofluorescence analysis [2]. Identical results have been observed with poly(A)⁺ mRNA rather than total cellular RNA (data not shown).

To determine the relative genomic organization of the 90 kDa gene in the five different strains of *T. cruzi*, total trypomastigote genomic DNA was digested with BamHI, separated by gel electrophoresis, blotted to nitrocellulose and hybridized with the 154 bp insert of pTcc-20 (Fig. 6). Intense hybridization was observed to a 2.3 kb fragment in the Peru, Y and Esmeraldo strains. Less intense hybridization was also observed to bands of size 3.7 kb and 4.3 kb in the Peru and

Fig. 6. A Southern blot containing 25 µg per lane of trypomastigote nuclear DNA digested with BamHI from the following strains of *T. cruzi* was hybridized with [³²P]pTcc-20 insert DNA. (A) Peru DNA; (B) Y DNA; (C) CL DNA; (D) Silvio X10 DNA; (E) Esmeraldo DNA. Size markers on left margin as in Fig. 4.

Esmeraldo strains, respectively. Hybridization was also observed to a diffuse band approximately 5.7 kb in size in both the Y and Peru strains and a very faint band of 2.3 kb in the CL strain. In general, these results are consistent with those previously observed for both the expression and transcription of the 90 kDa gene. In those strains which do not express this surface protein, the gene sequence is either absent (i.e. Silvio X10 strain), or found in many fewer copies (i.e. CL strain) than present in the expressing strains.

Recognition of the Tcc-20 fusion protein by Chagasic serum. The observation that the portion of the 90 kDa surface antigen encoded in Tcc-20 is recognized by antibodies from *T. cruzi* infected mice suggests that the epitope(s) encoded in the *T. cruzi* DNA insert in Tcc-20 may also be recognized by the human immune system. Also, since the 90 kDa surface antigen is not present in all strains of *T. cruzi* some Chagasic patients may be infected with strains of *T. cruzi* which carry this epitope(s) while others may be infected with strains which lack the epitope(s). To test these conjectures, Tcc-20 fusion protein was reacted in Western blots with sera from 16 Chagasic patients from the region of Bambui, Brazil (Fig. 7).

Fig. 7. Western blot analysis of Tcc-20 fusion protein recognized by sera from human Chagasic patients. Each lane contains 15 µg of Tcc-20 fusion protein reacted with sera from human Chagasic patients or normal human serum (NHS). Molecular weight markers on the right margin are as in Fig. 1.

The observation that 10 of the 16 sera tested contained antibodies that reacted with the fusion protein confirms the suggestion that the 90 kDa surface antigen contains determinants recognized by some, but not all, Chagasic patients. In addition, the lack of recognition of the fusion protein by six of the sera tested is consistent with the above observations that not all strains of *T. cruzi* infective to humans carry this epitope(s). A similar experiment in which β -galactosidase isolated from λ gt11 was reacted in Western blots with the sera from human Chagasic patients showed no reaction (data not shown).

Discussion

We have been able to select from a *T. cruzi* cDNA λ library a recombinant phage, Tcc-20, containing a portion of the 90 kDa surface antigen gene. Several experimental results support the presence of 90 kDa antigen coding sequences in Tcc-20. First, monoclonal antibodies directed against the 90 kDa surface protein also recognize the fusion protein encoded by Tcc-20. Second, antibodies selected by immunoabsorption to Tcc-20 fusion protein from a complex population of serum antibodies directed against numerous *T. cruzi* proteins react only with the 90 kDa antigen in a Western blot of a trypomastigote lysate. These same antibodies also fail to recognize any protein in a Western blot of an epimastigote lysate; a stage which has previously been shown to lack the 90 kDa antigen [2]. Finally, the stage and strain specificity of mRNA complementary to the putative 90 kDa gene is precisely that observed for the 90 kDa surface antigen [2]. Collectively, these results indicate that Tcc-20 contains sequences that encode the 90 kDa antigen.

We have also shown that there are approximately 20 copies of the 90 kDa gene per haploid genome equivalent in *T. cruzi*, and that most, and possibly all, of these copies are present as a tandemly linked gene family. To date, the structural organization of three other protein coding genes in *T. cruzi* has been determined. Gonzalez et al. [18] have shown that a gene whose predicted sequence resembles that of calcium-binding proteins is organized as a multicopy tandemly repeated gene family. A similar structural orga-

nization also has been observed for two separate *T. cruzi* genes whose predicted amino acid sequence shows strong homology to the 85 kDa and 70 kDa heat shock proteins of *Drosophila melanogaster* [3] (Dragon, personal communication). These four separate gene families can be placed within a lowly repetitive kinetic class of DNA sequences which previously has been shown to occupy approximately 51% of the *T. cruzi* genome and to have a repetition frequency of 10–50 copies per haploid genome [19]. Since this particular kinetic class of DNA does represent half of the total DNA sequences in the *T. cruzi* genome, and all of the genes which have been studied are members of this class of DNA, it seems likely that many of the protein coding genes in the *T. cruzi* genome may be organized in tandem repetitions.

The size of the mRNA complementary to the 90 kDa gene is 1250 nucleotides in those strains (i.e. Peru, Y and Esmeraldo) which express the gene. It is interesting to relate the apparent size of the mRNA in these strains to the apparent molecular weights of the polypeptides recognized by monoclonal antibody 4.2. In the Peru and Y strains monoclonal 4.2 reacted with a polypeptide doublet of 90 and 105 kDa in Western blots of trypomastigote lysates, while in the Esmeraldo strain a single polypeptide of 130 kDa was detected [2]. A mRNA of length 1250 nucleotides can at most encode a polypeptide of 417 amino acids, and considering the presence of both a poly(A) sequence on the 3' terminus of the mRNA as well as possible 5' and 3' nontranslated nucleotides, the mRNA likely encodes a polypeptide considerably smaller than 417 amino acids. It is likely, therefore, that the polypeptide portion of this surface antigen represents less than half of its apparent molecular weight. Also, the single uniform size of the mRNA in the different strains suggests that the difference observed in the apparent molecular weights of the surface proteins recognized by monoclonal 4.2 may not be due to differences in the size of the polypeptide portion of the surface antigen, but more likely is due to differences in subsequent addition modifications to the polypeptide.

The collective observations that the 90 kDa surface antigen is strain specific and that some, but not all, Chagasic patients have serum antibodies

that recognize the Tcc-20 fusion protein, suggested the possibility that a correlation may exist between the clinical symptoms (i.e. cardiomyopathy vs. digestive tract disorders) of the patients and the presence, or absence, of serum antibodies against the fusion protein. We have, however, found no such correlation. It is also worth noting that no correlation exists between the zymodeme of parasites cloned from the 16 Chagasic patients and the presence of serum antibodies against the Tcc-20 fusion protein.

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