The Major 85-kD Surface Antigen of the Mammalian Form of *Trypanosoma cruzi* Is Encoded by a Large Heterogeneous Family of Simultaneously Expressed Genes

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Summary

Trypanosoma cruzi is an obligate intracellular protozoan parasite. The parasite mammalian stage surface antigens exhibit extensive antigenic diversity. We have characterized a family of *T. cruzi* genes that code for a polymorphic set of 85-kD surface antigens, the SA85-1 antigens. The family contains >100 genes and pseudogenes, of which a minimum of nine are transcribed. The gene family is expressed in the mammalian stage only. A subset of the gene family is present in two telomere-linked copies in the genome. Telomere linkage of other expressed SA85-1 genes has not been demonstrated. We have shown that at least three members of the SA85-1 gene family encode antigens at the surface of the mammalian stage of the parasite. Interestingly, these three antigens are expressed on all the trypanosomes examined. This suggests that *T. cruzi* simultaneously expresses a large repertoire of similar, but diverse antigens at its surface. Thus, *T. cruzi* exhibits extensive antigenic diversity in a system unique from that of African trypanosomes, perhaps reflecting its intracellular niche.

*T*rypanosoma cruzi, an obligate intracellular protozoan parasite of mammals, is the causative agent of Chagas Disease. During the acute phase of the disease, the parasites enter the circulation and disseminate throughout the body. A chronic phase ensues in which the parasites are difficult to detect, yet they persist in the tissues, and individuals remain infected throughout their lifetime (1). The mechanisms underlying this persistence are poorly understood. The ability of the parasites to remain intracellular might play a role.

T. cruzi epimastigotes develop in the gut of the blood-sucking reduviid bug. During feeding, metacyclic trypomastigotes are deposited in the feces and invade the mammalian host (1). *T. cruzi* can infect a wide range of mammalian species (2). During the mammalian stage, the parasites replicate as intracellular amastigotes, some of which transform into motile trypomastigotes (1).

Extensive genetic diversity in *T. cruzi* has been documented by comparative examination of antigens (3), enzymes (4, 5), and kinetoplast DNA (6). Surface antigen diversity has also been observed (7–14). There is no evidence that sexuality contributes to this genetic diversity (4, 15). In addition, extensive antigenic polymorphism of trypomastigote surface antigens has been noted between clones of the same strain (16), suggesting that the parasites are capable of some form of antigenic variation in the mammal.

Several trypomastigote surface antigens have been identified, including those with molecular masses of 90 kD (17), 160 kD (18), and the major 85 kD (9, 19, 20). A gene encoding an 85-kD surface antigen has been shown to be telomeric (21), and has given rise to speculation about the possibility of antigenic variation in *T. cruzi*, similar to variant surface glycoprotein (VSG)[1] switching in African trypanosomes (21).

We report here the characterization of a large (>100 members), previously undescribed gene family coding for a set of related, but diverse mammalian stage-specific 85-kD surface antigens. We have used antibodies specific for three members of the antigen family to examine the possibility of antigenic variation in a *T. cruzi* population. We do not see evidence of antigenic variation analogous to African trypanosomes. Rather, we find that the individual family members are expressed simultaneously on every amastigote and trypomastigote in the population. This suggests that the large repertoire of 85-kD surface antigens in *T. cruzi* is serving a function other than directly evading the host serologic immune response.

Materials and Methods

T. cruzi. *T. cruzi* CL strain was a recent subclone ("subclone three") (16). The Peru strain was obtained from Gerry E. Manning, University of California, Irvine. Epimastigotes were maintain in liver infusion/tryptone medium with 10% FCS. Trypomastigotes and amastigotes were obtained from culture supernatants of infected rat 3T3 cells. Radioiodine labeling of the mammalian stage parasite was performed as described (18).

cDNA Clones. Double-stranded cDNA was prepared from *T. cruzi* mammalian stage poly(A)$^+$ RNA as described by Gubler and Hoffman (22). After methylation of the EcoRI sites and addition of EcoRI adapters, the cDNA was ligated into the EcoRI site of the Glutagene expression vector (a generous gift of G.F. Michell, D.B. Smith, and the AMRAD Corp., Ltd., Abbotsville, Australia) (23). The plasmid expresses cloned genes as a fusion to *Schistosoma japonicum* glutathione S-transferase (GST). The ligation products were transformed into a mixture of pGEX-1, pGEX-2T, and pGEX-3X (23), and plated on selective media. Colonies were screened for expression of *T. cruzi* antigens, as described (24), using *Escherichia coli*–adsorbed chronically infected mouse serum (CIMS). Colonies were also screened by hybridization to specific DNA fragments. The original cDNA clones were subcloned into Bluescript plasmids (Stratagene Inc., La Jolla, CA) for sequence analysis.

Genomic Clones. A genomic library was made in the Bluescript SK(⁻) plasmid from *T. cruzi* CL strain clone 3 epimastigote DNA digested with SalI.

DNA and RNA Analysis. DNA was extracted from trypanosomes using standard techniques (24). RNA was extracted as described (18). Northern and Southern hybridization with DNA fragments were carried out at 42°C using standard techniques (24), and washed at high stringency (65°C, 0.2× SSPE). Oligonucleotide probes were synthesized on a DNA synthesizer (Applied Biosystems, Inc., Foster City, CA), and were phosphorylated with polynucleotide kinase (New England Bio Labs, Beverley, MA) and γ-[^{32}P]ATP (24). Oligonucleotide hybridization to DNA was performed in partially dried gels as described by Shatz et al. (25). The final wash was at 60°C, 0.5× SSC. DNA fragments were isolated from gels by standard techniques (24). Fragment 1.1 is the 5′ 500 bp of cDNA 1.1. Fragment 1.2-5′ is the 5′ 600 bp of the genomic 1.2 clone. DNA fragments were radiolabeled by random priming with α-[^{32}P]dCTP (24). Oligonucleotide hybridization to RNA was performed as described by Wahl et al. (26). Final washes were in 6× SSPE, 0.1% SDS. Exonuclease Bal31 (New England Bio Labs) digestion was performed as described (24). Samples were phenol extracted after the Bal31 digestion before addition of restriction endonucleases.

DNA Sequencing. Nucleotide sequences were determined by the dideoxynucleotide chain termination method of Sanger et al. (27). Single-stranded templates of the appropriate cDNA clones and commercially available M13 primers were used. The primers were labeled with fluorescent dyes before sequencing reactions. The reactions were analyzed on a DNA sequencer (370A; Applied Biosystems, Inc.). Double-stranded templates were prepared according to Hsu (28), and sequenced with oligonucleotide primers phosphorylated with γ-[^{32}P]ATP. The sequences were analyzed and compared using Genepro sequence analysis software version 4.1 (Riverside Scientific Enterprises, Seattle, WA).

Antibodies. 6-wk-old female C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, ME) were infected with 10⁵ tissue culture–grown trypomastigotes. Mice were considered chronically infected at 3 mo after infection. CIMS were adsorbed with 10% (vol/vol) formalin-fixed *E. coli*. Recombinant fusion proteins were purified from isopropylthiogalactoside (Sigma Chemical Co., St. Louis, MO)-induced bacterial lysates as described (24). Anti-GST and anti-1.1 antibodies were affinity purified by passing CIMS sequentially over a 2-ml column of GST-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) and then a SA85-1.1-GST fusion protein column linked to Sepharose 4B, as previously described (18). Peptides were synthesized on a peptide synthesizer (430A; Applied Biosystems, Inc.). Peptides were coupled to BSA according to the procedure of Bassin et al. (29). BSA and peptides linked to BSA were coupled to CNBr-Sepharose 4B, and these columns were used to purify peptide-specific antibodies from CIMS. The following peptides were used: 1.1A, KIDGDEEESSPKEIGK; 1.2B, HGKY-QQHRGGQLWGEPP; 1.3A, GACGEENPSQES. ELISAs, Western blots, and immunofluorescent stainings were performed as previously described (18). Parasites were surface labeled with ^{125}I as described (18). All radioimmunoprecipitations were accomplished by incubation with 2 µg/ml antibody, followed by protein A-Sepharose 4B (Pharmacia Fine Chemicals). Immunofluorescent images were visualized on a MRC-500 Laser Scanning Confocal Microscope (Bio-Rad Microscience, Cambridge, MA) with a 60× objective.

Results

Characterization of the SA85-1 Gene and Antigen. A clone (1.1) that reacted with CIMS was selected from a *T. cruzi* CL strain mammalian stage cDNA expression library in the Glutagene plasmid (22). The clone contains the *T. cruzi* DNA fused to the GST gene. GST and the 1.1-GST fusion protein were purified on glutathione-agarose, and coupled to sepharose beads. We purified anti-1.1 antibodies from CIMS by affinity chromatography on 1.1-GST. In Western blot (Fig. 1 *A*) and ELISA (not shown), the 1.1-purified antibodies specifically recognized the GST-1.1 fusion protein, but not GST.

The purified anti-1.1 antibodies specifically recognized an 85-kD band in a Western blot of *T. cruzi* trypomastigote proteins (Fig. 1 *A*). To determine whether this represented the major 85-kD surface antigen, we used the anti-1.1 antibodies to immunoprecipitate ^{125}I-labeled surface proteins from trypomastigote lysates (Fig. 1). A protein of 85 kD was precipitated, indicating that 1.1 indeed codes for the 85-kD surface antigen. The 1.1 cDNA was sequenced (Fig. 2) and no significant homology was detected between this sequence and those of previously described *T. cruzi* genes encoding 85-kD surface antigens (19–21). Thus, the 1.1 cDNA clone defines a new *T. cruzi* 85-kD surface antigen, SA85-1.1 (surface antigen, 85 kD).

The SA85-1 Genes Make up a Large Family. In the initial characterization of other SA85-1.1-related cDNA clones, we noted heterogeneity in the restriction maps, suggesting polymorphism or a family of genes. To examine these possibilities, two other cDNA clones, SA85-1.2 and SA85-1.3, were sequenced. These sequences are compared with that of SA85-1.1 in Fig. 2. The three clones are ~80% homologous at the DNA level, and the predicted amino acid sequences have a similar degree of homology. Differences between the three clones are consistent with point mutations, insertions, and deletions. Furthermore, the presence of clustered differences suggests that gene conversion and/or recombination occurs between individual genes.
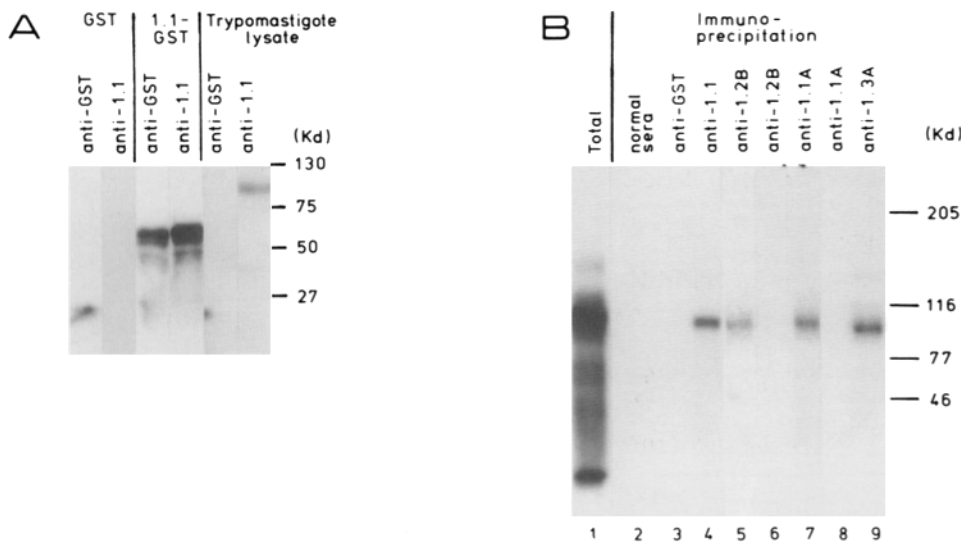
Figure 1. Detection of SA85-1 antigens by Western blot and immunoprecipitation of trypomastigote lysates. (A) Western blot on GST (4 μg/ lane), SA85-1.1-GST (4 μg/ lane), and trypomastigote lysate (25 μl of 2 × 10⁸ cells/ml) reacted with anti-GST and anti-SA85-1.1 antibodies (10 μg/ml). Antigens were separated on a 4–12% gradient SDS-PAGE, transferred to nitrocellulose, and reacted with antibodies and ^{125}I protein A. (B) Immunoprecipitation of ^{125}I surface-labeled trypomastigote lysate. 5 × 10⁸ cells were radioiodinated, washed, and lysed in 2.0 ml of lysis buffer, as described (18). (Lane 1) 2 μl of total lysate. Lanes 2–4, immunoprecipitation from 200 μl of lysate: (lane 2) normal mouse serum (1:100 dilution); (lane 3) anti-GST; (lane 4) anti-1.1. Lanes 5–9, sequential immunodepletion of SA85-1 antigens. 200 μl ^{125}I surface-labeled trypanosome lysate were exhausted of 1.2B-reactive species by incubation with 0.4 μg anti-1.2B and protein A-Sepharose. After centrifugation, the bound antigen was examined by SDS-PAGE (lane 5). The lysate was re-examined for 1.2B-reactive species by re-addition of anti-1.2B (lane 6). The 1.2B-depleted lysate was then depleted of 1.1A-reactive species by addition of 1.6 μg anti-1.1A, as described above. The adsorbed material is shown (lane 7). The lysate was examined for remaining 1.1A-reactive species by re-addition of anti-1.1A (lane 8). The doubly depleted lysate was examined for the presence of 1.3A-reactive species by addition of anti-1.3A (lane 9).

The finding that the three sequenced SA85-1 genes all have unique regions (Fig. 2) permitted us to synthesize oligonucleotide probes specific for each. Oligonucleotides 1.1A, 1.2A, and 1.3A, corresponding to unique regions of SA85-1.1, 1.2, and 1.3, respectively, were used for genomic mapping. The Southern blot in Fig. 3 *A* shows *T. cruzi* genomic DNA digested with several restriction enzymes and probed with oligonucleotide 1.1A. Depending on the enzyme used, one or two fragments were generated. Restriction analysis (Fig. 3 *C*) revealed a universal restriction site, suggesting a chromosome end. Furthermore, some of the enzymes, such as ApaI, generated two diffuse bands, also consistent with a telomeric location of the SA85-1.1 genes. We treated genomic DNA with exonuclease BAl31, which has been shown to specifically digest genomic DNA from telomeres. In Fig. 3 *B*, it can be seen that Bal31 treatment resulted in shortening of both AvaI-generated bands, thus confirming the telomeric localization of the two copies of the SA85-1.1 genes. Rehybridization with oligonucleotide 1.3A (not shown) detected five bands generated by AvaI, none of which were shortened, thereby controlling for the Bal31 specificity.

Southern blots of genomic DNA digested with several restriction enzymes, and probed with oligonucleotides 1.2A and 1.3A, are presented in Fig. 3 *A*. Oligonucleotide 1.2A hybridized to one major fragment in each digest. Oligonucleotide 1.3A hybridized to five fragments. The bands do not appear diffuse, and Bal31 sensitivity could not be demonstrated (not shown). By comparing hybridization patterns of oligonucleotides 1.1A, 1.2A, and 1.3A after SalI digestion, it appears that the three oligonucleotides recognize independent, nonoverlapping subsets of this gene family. One subset is telomeric, and the others are not.

To examine the transcription products of the different SA85-1 gene subsets, we sequentially hybridized a Northern blot with cDNA 1.2, fragment 1.1 (the 5′ 500 bp of cDNA 1.1), and oligonucleotides 1.1A and 1.3A (Fig. 4). All probes hybridized to a mammalian stage–specific RNA of 3.9 kb. The same results were obtained when independent Northern blots were used (not shown). Therefore, the composition of this 3.9-kb band is heterogeneous, and represents transcripts of multiple members of the gene family.

The sequence data suggested that a family of homologous but nonidentical genes encodes the SA85-1 antigens. A Southern blot of genomic DNA digested with several restriction enzymes and hybridized with fragment 1.1 is indicative of a large gene family (Fig. 5 *A*). Each digestion revealed a complex hybridization pattern. The pattern of hybridization seen with DNA from the CL strain was also seen with DNA of the geographically distant Peru strain (Fig. 5 *A*). Fragment 1.1 encompasses the 3′ end of the predicted open reading frame (ORF). We isolated a 5.0-kb genomic SalI fragment (Fig. 3 *A*) that encodes SA85-1.2. A restriction map of this genomic clone is presented (Fig. 3 *C*). The 5′ SalI to BstXI fragment (fragment 1.2-5′) encompasses the 5′ end of the predicted ORF. Fragment 1.2-5′ was hybridized to Southern blots of genomic DNA (Fig. 5 *B*). The Southern blots hybridized with fragments 1.1 and 1.2-5′ appear to be identical, confirming that the homology within the members of the gene family extends from the 5′ to the 3′ ends of the predicted ORF.

To investigate the number of SA85-1-related sequences present in the genome, we compared the hybridization intensities of 2 μg of *T. cruzi* DNA (4 × 10⁷ genomes), with 330 ng of the SA85-1.2 genomic plasmid clone (4 × 10¹⁰

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1.3   ATCTTTGCCTGAATGCGACGGTGAGGAACGCCACGAAGGTCAAAGATGGGTTTCAGTTGACGGAGCCTGACTCCGGGGTAATGTGGCCTGT   91

1.3   -------------TA----AA--------G-G-TT----A---------T----T------------CA-----------C-------------T---   191
1.1   GAACATTCCGGATGGTAATGTGCGTCATATATCCTTGAGCCACAACTTCACACTCGTGGCGTCGGTGATCATCGAAGAGGCTCCGAGTGGGAACACACCT   100
1.2   ------C- ---G--A-----------G-G-TT----A---------------T------------C-------------------A---A--T----   99

1.3   --A----T-----------CGA--A---A----AC-C--AC-------A-CCT---T-------------------------------------   291
1.1   CTCCTGACTGCGGTGTTGGTTGACGCTGGGCCCGAGTATTTCATGAGACTGTCGTACACCGCGGATAACAAGTGGATGACGATGCTCAAGGACGAGAAAA   200
1.2   ------------C------G--------A----CC-------------------------------GA------TTC---A-G---T----   199

                                          Oligo 1.0 A
1.3   ----------------TG-CA---------C--AAG----------------A-----↓------------   391
1.1   AACCAACAACGGAAAGCAGGCCTTGGGAGGCGGGAAAAGAACACCAAGTGGCACTCATGCTGCAA<u>GGCAACAAGGCCTCTGTG</u>TACGTTGATGGTGAGTT   300
1.2   ---T----------------------T-C--AAG--------------------------------A-----------C   299

                                             Oligo 1.1 A
1.3   --------------------------------G-----------C------------------   ↓   -G------ <u>-A-CC-</u>   478
1.1   GCTGGGGGAGGAAGAAGTGCCGTTAACAGGTGAAAAGCCACTTGAGATTTTCGCGTTTTGCTTTGGCGCGTGC<u>AAAATTGATGGTGATGAGGA</u> GGAGTC   399
1.2   --------- -----C-----------T---G-CA---------CCC--T-G----------------G-CT------A------T-*------   431

1.2   Oligo 1.3 A   Oligo 1.2A ⟶ <u>TGGTGGTGATGATGATGATGAGGAGGACAGTCAGG</u>A   425

1.3   C--↓--A-GA-T   -- CA--------- ┌-------------T-----T---┐   527
1.1   AAGTCCGAAGGAG ATCGGAAAAAAACCTCGTGTGACGGT │ACGAACGTCTTTCTGTACAACCGCCCACTGA │TTCCACTGAAATGCGTGCAATCAAGGA   498
1.2   ------------------C----------A-------- │-------------------------------│C-------G----------------   545

1.2   TCCAGTCCGGAGAAG   459

1.1   CAGGATCCCCGTTCCGACGAGAGCTCCAGAACCACAAGTGAAAATTGCTCCCAAACCTGCCGCGCCTGCTGCAC CTGCTGGGAATGAAGAAACGGCACG   597
1.2   ------------------T------------------------------------T---A-------*---- -------G-GG--------   751

1.2   ┌<u>CTGGCTCGCTGCGGTGCCTGGCCCAC</u>GGGAAGTACCAGCAGCACCGGGGAGGACA┐   674
1.2   Oligo 1.2B ⟶ │<u>ACTGTGGGGAGAACCGCCA</u>ATACCCAACATGCGCCAGCGGGACGCTTGACTTCTG│   729

1.1   AGAAACGGGTGATGGTGGGGCAAATGGTGATGCTGTTTCCGCGTACGGGAGGGTGCTGCTGCCGCTGCTTTTTCTGCTTGGACTGTGGGGCCTTGCGACT   697
1.2   -----GA----------------------------G-----------------------GC------G------------T--------   851

1.1   GCC<u>TGAGGAG</u>TGTGCGGCCTCAGTGTGGAGTATTTGCGCCCTCTTCCACACCGACACTCACACACTCCGCTCGGAACAAAGTTCACCTTGCAAATAACAA   797
1.2   ------------------------GAC-C-------A--CC----------G-A-G   -T--TG--T-A-T-TCC-   933

1.1   TTTCTTTTTGCTTTTAGTACCTTCCTTCTTTTGCCTTTTAATTTAGGGCAACCACTATGGACATTTTTTTCTTCGGCTTTTAATTTTGTTTTCTTTTCTG   897
1.1   TTTGGAGTATAATTTCTCATGTTTTTTTATGTTTGTTTTTAATTATCTTGGTTTGCTTTTTTATAAAAATTTTTATATCTGTTTTTTCTTTTATGCTGCA   997
1.1   CTTCCTGCGAGGGGTGCGTGTGTTTCCCGCCCAACTGCTCCACTCGCACACCACCGACACGCTCATGACGGCCCTGTGTGCTCCACTCGCACACCACCGA   1097
1.1   CACGCTCATGACGACGGCCCTGTGCGCCTCGCCTGCTGCACGCATGAGGAGGGGCCGCGACGAACGGCGCGCTGAGGGAGCCGTGACGTGTGGGCGTGCG   1197
1.1   ACGAGGGCCTGTGGGGCGGCGCACACATTTCTTCCCTCTTGTTTTGTTTTCTTGCGGCCAGGGCGGGCAGACACCCGCAGCGCCACGCACACACACACAG   1297
1.1   CCACACCCTGCCGCCGTGTTTTTTTTTTTTTTCTCACTCTCCCCGATGGAGGAGACGCGACTCCCGTCATGTCGCCGTGCTTCTCTCAGTCCACACACAC   1397
1.1   GAGTGACTGTGCTCTGCGGGCGGCACTCGCGCCGATGAGAGGGCTGAGGGGAGCAGCAACAACGCAGACTGACCCATGAAATATAAACGATGAGCGGACAC   1497
1.1   ACGGAGGGTCGGACGGTTGAAGCACACGTAATTATTTAACTTTTCTTTTGCGGCAAAATATACAAACAGTTTCTGGAGCAACATGGTGGTTAGGTGCTTT   1597
1.1   TTTTGTGTGGTTTTTCTTATCATGATTTGTGTGTATCCGTAATAGAGGACGACATTTTCTTTTTTCTTGTTTTTTCTTGTTTTTTCTTGTTTTTCCTTTT   1697
1.1   TTTTTCTTTCATTTTTTTCCCCAAAGTGTGAGGTGTGGAATGGTGAAGATGAATTTTTTAAAATTTTAATAGTTTTTTATGAAAAAAAAAAAAAAAAAAA   1796

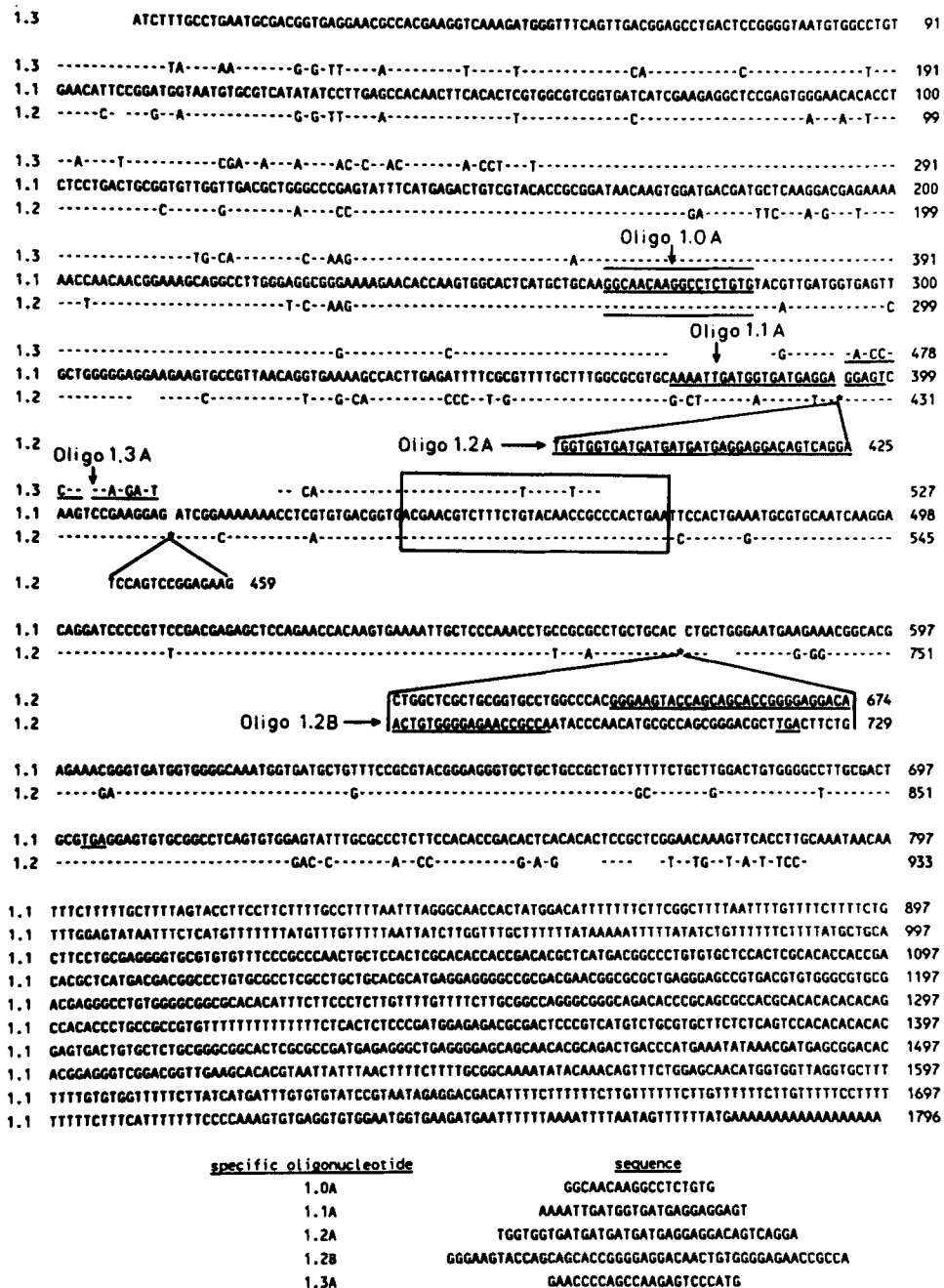| specific oligonucleotide	sequence
1.0A	GGCAACAAGGCCTCTGTG
1.1A	AAAATTGATGGTGATGAGGAGGAGT
1.2A	TGGTGGTGATGATGATGATGAGGAGGACAGTCAGGA
1.2B	GGGAAGTACCAGCAGCACCGGGGAGGACAACTGTGGGGAGAACCGCCA
1.3A	GAACCCCAGCCAAGAGTCCCATG

Figure 2. Sequence comparison of three SA85-1 cDNA clones. The sequence of cDNA 1.1 is shown. Nucleotide identity is marked by a dash. Nucleotide differences are indicated. The sequences are aligned to give the best homology. Asterisks indicate positions of insertions. Oligonucleotide sequences are underlined and indicated in the figure. Oligonucleotides are as follows: 1.1A, 374–398; 1.2A, 390–425; 1.2B, 646–693; 1.3A, 473–489; and 1.0A, 266–283 of the SA85-1.1 sequence. Predicted stop codons in SA85-1.1 (bp 702–704) and SA85-1.2 (bp 721–723) are underlined. The 32-bp region of homology between SA85-1.1, SA85-1.2, F1-160 (18), and the 85-kD surface antigen gene cloned by Peterson et al. (19) is boxed. These sequence data have been submitted to the EMBL/GenBank Data Libraries and have been assigned the accession numbers X53545, X53546, and X53547.

genomic equivalents) (Fig. 5 *B*). The relative hybridization intensity of the digested genomic DNA suggests that >100 copies of genes and pseudogenes belonging to the SA85-1 family are present in the genome. Additional experiments using quantitative densitometry estimate 150 SA85-1-related sequences within the genome (not shown).

To further assess the expression of diversity of the SA85-1 family, we selected additional cDNA clones with fragment 1.1, and sequenced a homologous portion of each for comparison. A consensus oligonucleotide, 1.0A, was derived from the sequences of SA85-1.1, 1.2, and 1.3, (Fig. 2), and was used to prime the sequencing reactions. Oligonucleotide 1.0A primed the sequencing of seven additional clones. In Table

1, the comparative sequence analysis of the 10 independently isolated clones is presented. The DNA and predicted amino acid sequence of each clone is unique, except for SA85-1.1 and 1.10, indicating that a minimum of nine genes of the SA85-1 family are expressed.

Antigens Encoded by the SA85-1 Gene Family. We used antibodies specific for SA85-1.1, 1.2, and 1.3 to detect the corresponding antigens on the parasite surface. Peptides corresponding to the sequences predicted from the specific oligonucleotides (oligonucleotides 1.1A, 1.2B, and 1.3A; see Fig. 2) were synthesized. Antibodies to these peptides were affinity purified from CIMS (antibodies 1.1A, 1.2B, and 1.3A). These antibodies specifically recognized their respective pep-
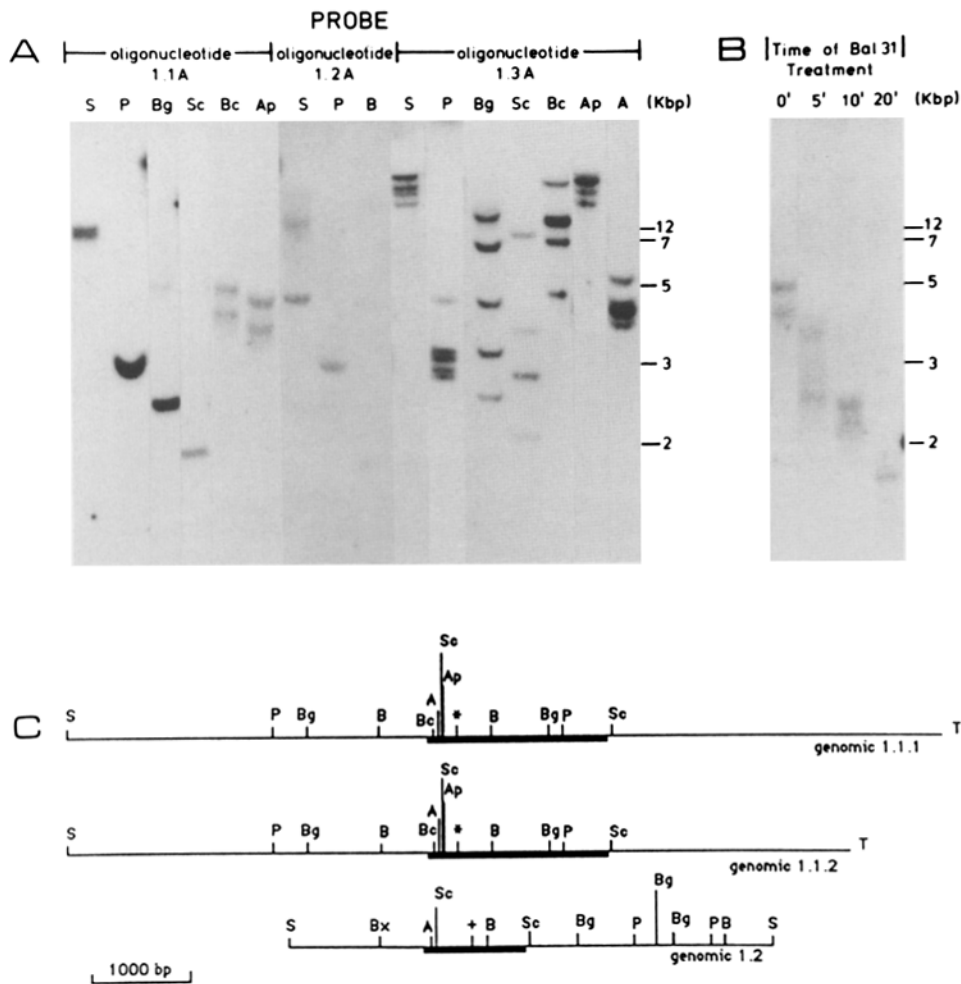
Figure 3. (A) Genomic mapping of SA85-1 gene subsets with oligonucleotide probes. SalI (S); PstI (P); BglI (Bg); SacI (Sc); BclI (Bc); ApaI (Ap); AvaI (A); BamHI (B). (B) Exonuclease Bal31 sensitivity of genomic DNA with homology to oligonucleotide 1.1A. 12 μg of *T. cruzi* DNA was digested with exonuclease Bal31 (3U), and at increasing times, 3-μg aliquots were restricted with AvaI, subjected to electrophoresis on an agarose gel that was partially dried, and probed with the ^{32}P-labeled oligonucleotide 1.1A. (C) Genomic maps of three SA85-1 genes. Restriction sites: AvaI (A); ApaI (Ap); BamHI (B); BglI (Bg); BstXI (Bx); PstI (P); SalI (S); SacII (Sc). Homology to oligonucleotide 1.1A, (*); homology to oligonucleotide 1.2A, (+). Thick lines indicate homology to corresponding cDNA clone. (T) A cluster of restriction sites corresponding to a telomere.

tide in ELISA (not shown). The surface of CL strain trypomastigotes was labeled with ^{125}I, and antigens recognized by each specific antibody preparation were successively depleted by exhaustive immunoprecipitation. In each case, the immunoprecipitated antigen was subsequently examined by SDS-PAGE (Fig. 1 B). After removal of all antigens recognized by anti-1.2B antibodies, immunoprecipitable 1.1A-specific antigen still remained. Similarly, exhaustion of the 1.1A-specific antigens did not remove those antigens recognized by the anti-1.3A antibodies. This demonstrates the specificity of the three antibody preparations used. Furthermore, epitopes specific to SA1.1, 1.2, and 1.3 are all expressed at the surface of the trypomastigotes on separate 85-kD molecules.

The immunoprecipitation experiment described above did not distinguish between polymorphism in the population of parasites, or simultaneous expression of the three genes by each cell. To distinguish these possibilities, we used indirect immunofluorescence with the specific antibodies. The immunofluorescence displayed in Fig. 6 demonstrates that each of the three antigens are present on the trypanosomes. For each specific antibody, we examined 2,000 mammalian stage cells, and all were stained (not shown). Therefore, these three antigens are expressed simultaneously on each amastigote and

trypomastigote. No antigen was detected on epimastigotes with either anti-1.1 antibodies (Fig. 6 E), or anti-peptide antibodies (not shown).

Discussion

We describe here a large *T. cruzi* gene family, SA85-1, that encodes antigenically similar, but diverse, 85-kD surface proteins on mammalian stage parasites. The gene family contains >100 members, and a minimum of nine are transcribed. Three randomly selected SA85-1 cDNAs were shown to encode proteins that are expressed simultaneously, suggesting that many or all of the transcribed members are expressed simultaneously on each amastigote and trypomastigote surface.

The individual members of the SA85-1 gene family share a high degree of homology. They probably arose by gene duplication and are diversifying by mutation. Our sequence data indicate the presence of point mutations, deletions, and insertions. In addition, the presence of clustered differences suggests that gene conversion and recombination contribute significantly to the diversity of the gene family. Thus, it is likely that the gene family is evolving rapidly. This rapid diversification may offer an explanation for the past studies
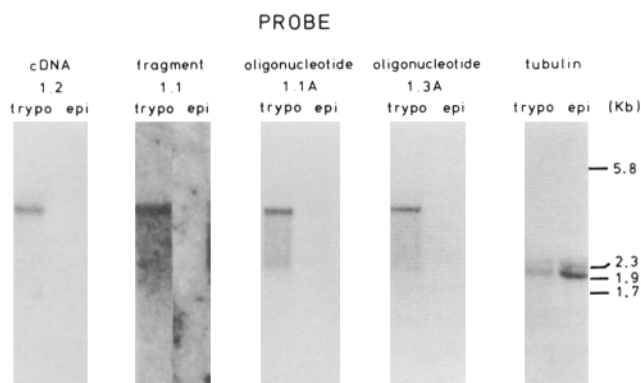
Figure 4. Expression pattern of SA85-1 mRNA. A Northern blot of trypomastigote poly(A)$^+$ RNA (1.5 μg) and total epimastigote RNA (5 μg) was hybridized sequentially with different probes as indicated.

on *T. cruzi* that have demonstrated significant surface antigen diversity (7–14, 16).

Other *T. cruzi* 85-kD surface antigen coding genes have been cloned, and like the SA85-1 genes, they belong to large gene families (19–21). These cloned genes do not share significant homology with each other or with SA85-1, indicating that multiple 85-kD families exist, which would further explain the observed surface antigen diversity.

Telomeric expression sites are the targets for the gene conversions associated with antigenic variation in the African trypanosomes (30). Peterson et al. (21) demonstrated that the *T. cruzi* 85-kD surface antigen coding gene that they cloned is located at a telomere and is the predominantly transcribed member of a large gene family. Based on this, they speculate that antigenic variation may be occurring in *T. cruzi* by a mechanism similar to that in African trypanosomes. Although two members of the SA85-1 family are located near telomeres, they are not the only transcribed members. Furthermore,
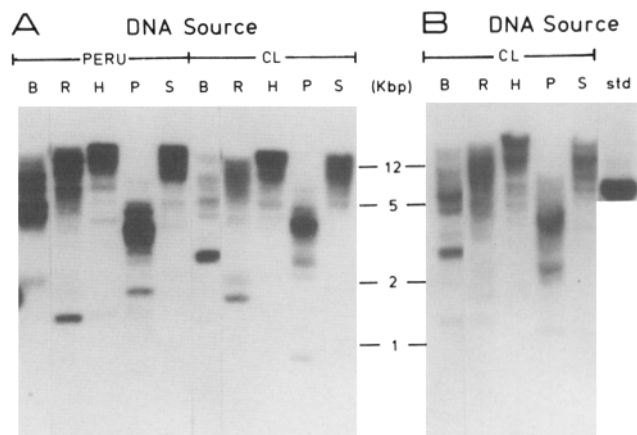
Figure 5. Genomic Southern blots reveal the SA85-1 gene family. (*A*) Southern blots of Peru strain and CL strain DNA digested with several restriction enzymes, and probed with fragment 1.1 (5' 500 bp of cDNA 1.1). 3 μg of DNA was loaded in each lane. BamHI (B); EcoRI (R); HindIII (C); Pst I (P); SalI (S). (*B*) Southern blot of CL strain DNA digested with several restriction enzymes, and probed with fragment 1.2-5' (5' 600 bp of the genomic clone 1.2). BamHI (B); EcoRI (R); HindIII (H); PstI (P); SalI (E). 2 μg of genomic DNA (4 × 10^7 genomes) was loaded in each lane after incubation with the appropriate restriction enzyme. The standard (std) lane contained 330 ng of the SalI-digested genomic 1.2 clone (4 × 10^{10} genomic equivalents).

Northern blot analysis using gene-specific oligonucleotide probes did not show a predominance of transcripts coming from the telomere-linked genes. Therefore, the SA85-1 genes of *T. cruzi* do not appear to be switching like the VSG genes of the African trypanosomes. We do not know the significance of the telomeric linkage of the two SA85-1.1 genes. It is possible that they are preferential targets for gene conversion events in the generation of diversity of the family. We are presently investigating this possibility.

Table 1. *Comparative DNA Sequence Analysis (Primed with Oligonucleotide 1.0A)*

cDNA clone	Sequence
1.1	AACCAACAACGGAAAGCAGGCCTTGGGAGGCGGGAAAAGAACACCAAGTG
1.2	- - - T - - - - - - - - - - - - - - - - - - - T - C - - AAG - - - - - - - - - - - - - -
1.3	- - - - - - - - - - - - - - - TG - CA - - - - - - - - C - - AAG - - - - - - - - - - - - -
1.4	- CAAC - AC - - - - - GCAGC - CTTGG - A - CC - A - CA - - GGAC - ACG - C - A - T
1.5	- GAA - - - C - AAA - GT - GCACTTGG - A - CC - AA - A - - G - A - TA - - - - - - -
1.6	- GAAG - A - C - AA - - - - TG - C - - - - - - - - - CTTAAG - - - - - T - - - - - - - -
1.7	- - GAC - AT - AAA - GT - T - ACG - - - - - - - CTTAAG - - - - - T - - - - - - - -
1.8	*CCAAA - AAGGG - T - C - GAGTG - CA
1.9	* - TC - AA
1.10	- -

50 bases of cDNA 1.1 are shown. cDNA 1.2-1.10 are aligned sequentially, 15 bases 5' of oligonucleotide 1.0A. Nucleotide identity of the other cDNAs is marked by a dash. Nucleotide differences are shown.
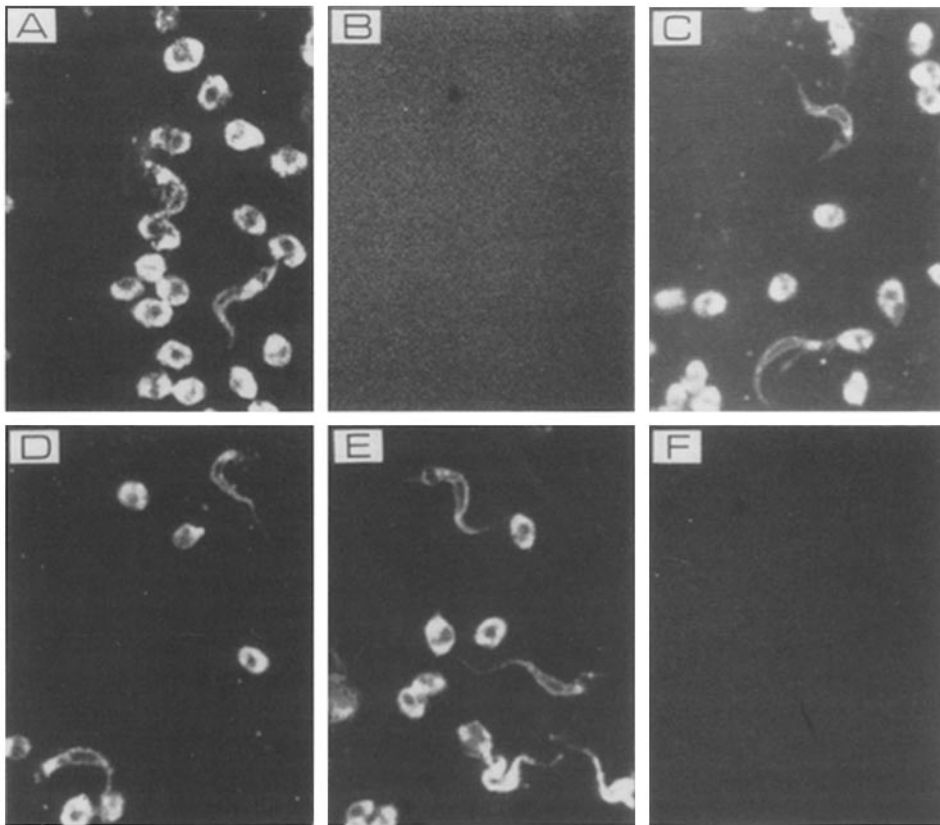* 5' end of cDNA clone.

Figure 6. Immunofluorescence with SA85-1 anti-1.1 antibodies and antipeptide-specific antibodies. Parasites were fixed and stained with 10 μg/ml antibodies and FITC-F(ab')$_2$ anti-mouse IgG as described (17). (*A-E*) Trypomastigotes and amastigotes stained with: *A*, Anti-1.1; *B*, normal mouse sera; *C*, anti-1.1A; *D*, anti-1.2B; *E*, anti-1.3A; *F*, epimastigotes stained with anti-1.1.

Although there is no extensive sequence homology between the SA85-1 gene family and other sequenced *T. cruzi* genes, we have found a limited region of homology with other genes. A 32-bp sequence common to SA85-1.1 and SA85-1.2 is present in two other *T. cruzi* surface antigens, the 85-kD gene reported by Peterson et al. (19), and the FL-160 gene (18). The common 32-bp sequence is present in the four genes in the same reading frame, near the 3' end of the ORF (Fig. 2), suggesting that it plays a functional role.

The SA85-1.2 gene also contains a limited region of significant DNA homology with tubulin genes (manuscript in preparation). This region surrounds the 1.2A oligonucleotide, which translates into a peptide rich in aspartate and glutamate (see Fig. 2). Similar regions are found at the COOH termini of α and β tubulin (31). Furthermore, the acidic amino acid regions of SA85-1.2 and tubulin are immunologically crossreactive (manuscript in preparation). The acidic region of tubulin is involved in both tubulin-tubulin interactions (32), and in the binding of microtubule-associated proteins (33). Also, areas rich in acidic amino acids are present in actin-binding proteins (34). This suggests that the amastigote and trypomastigote surface may be able to interact with the host cell cytoskeleton. Two recent reports indicate that other intracellular pathogens may interact with host cell cytoskeleton. *Listeria monocytogenes* has been shown to associate with the macrophage cytoskeleton (35). Also, the E1B 19-kD protein of adenovirus, which has a COOH termini rich in acidic amino acids (36), has been shown to associate with the cytoskeleton (37). This suggests that cytoskeletal interactions, promoted by regions of acidic amino acids, may be a common tactic of intracellular pathogens.

African trypanosomes, which are extracellular parasites, express only one or a few variant surface antigens at a time. Their ability to periodically replace the currently expressed VSG antigen by an immunologically unrelated VSG forces the host to mount a new immune response and allows the parasites to escape antibodies. In contrast, in the case of *T. cruzi*, many or all of the antigens in the SA85-1 family are simultaneously expressed at the surface of every trypanosome in the population. Furthermore, all the members of the SA85-1 family that we examined were antigenic in the chronically infected mice. We have also found antibodies to the SA85-1 antigen family in most sera examined from chronically infected humans (manuscript in preparation). It is thus unlikely that the diversity generated in the SA85-1 antigens is used for immunoevasion in the same sense that antigenic variation in the African trypanosomes is used.

We do not know the function of the SA85-1 antigens. Unlike the African trypanosomes, *T. cruzi* is an intracellular parasite. It has a wide host range among mammals (1) and can replicate in many cell types (2). It is possible that the diversity of the SA85-1 antigens is important in maintaining these broad possibilities. It could, for example, be involved in host cell invasion. An 85-kD protein has been implicated in *T. cruzi* binding to mammalian cells (38). It is also possible that the receptor for *T. cruzi* is itself a molecule that is polymorphic in the population, and is present on the majority of cells in the population.

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