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The major 85-kDa surface antigen of the mammalian-stage forms of *Trypanosoma cruzi* is a family of sialidases

(host–parasite interaction/cell adhesion molecules/immune response/gene family/protein secretion)

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ABSTRACT **_Trypanosoma cruzi_, an intracellular protozoan parasite infecting a wide variety of vertebrates, is the agent responsible for Chagas disease in humans. An estimated 15–20 million people in South and Central America are infected with the parasite. Chagas disease often results in severe autoimmune and inflammatory pathology and is the major cause of heart failure in endemic areas. Nevertheless, little is known about the host–parasite interactions that lead to this pathology. We have previously cloned several members of a large gene family (SA85-1) and shown that these genes encode 85-kDa *T. cruzi*, mammalian-stage-specific, surface antigens. Here we report that members of the SA85-1 family possess sialidase activity and are shed by the parasite. We suggest that the sialidases may contribute to the pathology during *T. cruzi* infection by cleaving sialic acid from cells of the immune system.**

Trypanosoma cruzi is the causative agent of Chagas disease, affecting 15–20 million people. Approximately 10% of cases are fatal, generally as a result of heart failure, megacolon, or megaesophagus. The parasites grow as epimastigotes in reduviid bugs and are transmitted to vertebrates in the feces of the insects. In the vertebrate, the parasites replicate intracellularly as amastigotes and circulate as trypomastigotes (1). *T. cruzi* can infect a wide range of mammalian species and proliferates in the cytoplasm of many different cell types (2). Acute infection with *T. cruzi* results in severe immunodepression (3). The lifelong chronic phase that follows is characterized by autoimmune pathology (3). It is not known how the parasites gain entry into their various host cells. Nor is it known how they evade the host immune system during the chronic infection. Finally, the mechanisms involved in immunodepression and autoimmunity have not been determined.

Several proteins have been characterized on the surface of *T. cruzi* mammalian-stage forms (4–12). Among these surface antigens, a large family of closely related 85-kDa proteins appear to be the predominant species (13–16). We have examined 10 members of the SA85-1 (surface antigen, 85 kDa) gene family from the CL strain and shown that at least three of these antigens are simultaneously expressed on the surface of each trypomastigote and amastigote in the population (13). The SA85-1 surface antigens, which are only present on parasites during the mammalian stages of their life cycle, are encoded by >100 genes with ≈80% homology to one another (13). We have characterized subsets of the SA85-1 family with specific oligonucleotide probes and antibodies (13). The SA85-1.1 gene is present in two telomere-linked copies and the SA85-1.2 gene exists as a single non-telomere-linked copy (13). We report here that the SA85-1 genes contain significant sequence homology to bacterial and viral sialidases and that antibodies purified on recombinant SA85-1 affinity columns precipitate the majority of cell-bound and shed sialidase activity from *T. cruzi*.§

MATERIALS AND METHODS

***T. cruzi*.** *T. cruzi* CL strain cultivation was as described (13).

DNA and RNA Analysis. Nucleic acid preparations and hybridizations were as described (13). Oligonucleotide hybridization to DNA was performed in partially dried gels; DNA fragment hybridization to DNA was performed on nitrocellulose membranes (13).

Cloning and Expression. A genomic library was made in the Bluescript SK(−) plasmid from *T. cruzi* CL strain clone 3 DNA digested with *Sal* I and *Pst* I. Screening for different SA85-1 genes was performed with specific oligonucleotides as described in the text. For expression of the SA85-1.1 gene in *Escherichia coli*, the genomic fragment was placed under the control of the phage T7 promoter as described (17). SA85-1.1 antigen was purified from inclusion bodies by the method of Nagai and Thogersen (18).

DNA Sequencing. DNA sequencing was performed as described (13). Sequences were analyzed with GENEPRO version 4.1 (Riverside Scientific, Seattle) MULTALIN (19), and PATMAT (20).

Antibodies. The SA85-1.1 antigen purified from inclusion bodies was coupled to Sepharose 4B (Pharmacia) as described (13), and anti-SA85-1.1 antibodies (anti-1.1 antibodies) were affinity purified from *T. cruzi* chronically infected mice as described (13). Anti-1.1A and anti-1.2A antibodies (anti-peptide antibodies specific for SA85-1.1 and SA85-1.2, respectively) were affinity purified as described (13). Anti-tubulin antibodies were purchased from Chemicon.

Radioimmunoprecipitation. Radiomethionine labeling of mammalian-stage parasites was performed as follows. Trypomastigotes and amastigotes were grown in rat 3T3 cells in the presence of 1 mCi of [^{35}S]methionine (41.8 TBq/mmol; Amersham) in methionine-free medium and dialyzed fetal calf serum (10%) for 12 hr. The parasites (1×10^8) were washed three times with nonradioactive medium. Radiomethionine-labeled parasites were lysed for radioimmunoprecipitation in a final vol of 1.0 ml as described (13). Radiomethionine-labeled shed antigens were prepared as follows. Trypomastigotes and amastigotes were labeled with [^{35}S]methionine as described above. The parasites (5×10^8) were washed and then incubated in 5 ml of nonradioactive medium for 2 hr at 37°C. Parasites were removed by centrifugation.

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§The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M62735 and M62736).

All immunoprecipitations were accomplished with 4 μg of antibody per ml, followed by protein A-Sepharose 4B (Pharmacia) as described (13).

Sialidase Assays. Sialidase assays were run by incubating cell lysates, supernatants, or immunoprecipitates in a final vol of 400 μl of 100 nM sodium acetate (pH 5.6) for 2–22 hr with 100 nM 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid at 37°C as described (21). Reactions were stopped by adding 700 μl of 0.1 M glycine (pH 10.4) and the fluorescence was read with a Perkin–Elmer 650-10S fluorescence spectrophotometer (excitation wavelength, 360 nm; emission wavelength, 440 nm). One unit is equal to 1 nmol of 4-methylumbelliferone produced per min at 37°C.

RESULTS

Previously, we characterized three cDNA clones corresponding to the SA85-1 family (13). Although they shared considerable homology, each had unique sequences, so that specific oligonucleotides could be used for genomic mapping and cloning. An oligonucleotide specific for SA85-1.1 recognized two telomere-linked fragments that differ only by the telomere length distal to the open reading frame. Using this SA85-1.1-specific oligonucleotide as probe, we have cloned the 4.5 kilobase (kb) *Sal* I/*Pst* I genomic fragment containing the gene. Similarly, using an oligonucleotide specific for the unique SA85-1.2 gene, we have isolated a 5.0-kb *Sal* I/*Sal* I genomic fragment containing all of the open reading frame except the 5′ N-terminal signal sequence (13). The DNA sequences of the two open reading frames are shown in Fig. 1. Sequence analysis indicated 80% homology at the nucleic acid and amino acid level to a previously cloned 85-kDa *T. cruzi* mammalian-stage specific surface antigen, pTt34 (16). The predicted amino acid sequences were compared with a published sequence in the Protein Identification Resource data base (version 26). Interestingly, all three genes share a region of significant homology with bacterial and viral sialidases (22–36). This region of the deduced *T. cruzi* proteins is compared to the homologous region of the *Clostridium perfringens* sialidase in Fig. 2*A*. The logarithm of odds (lod) score for homology (alignment window of 30 amino acids) between the predicted *T. cruzi* protein sequences and *Clostridium* sialidases ranged between 106 and 110 (38).

Roggentin *et al.* (37) have defined a highly conserved sequence termed the Asp block (SXDXGXTW) by comparing several bacterial sialidases. Four Asp blocks are present in the prokaryote enzymes, and a single similar sequence is present in influenza A sialidase (37). Fig. 2*A* shows an alignment of the conserved regions in the deduced *T. cruzi* 85-kDa antigen sequences and regions containing Asp blocks III and IV of the *C. perfringens* sialidase (22). There are two perfect Asp blocks in SA85-1.1 and pTt34. Furthermore, all of the sequences are very similar for an additional 6 amino acids adjacent to Asp block III. Another block of homology is found upstream of Asp block III (MXDGTIV). Using the PATMAT program, we searched the data base for homologies to a 20-amino acid consensus sequence beginning and ending 6 amino acids on either side of Asp block III (Fig. 2*B*) (20). The lysine in position 2 of Asp block III is conserved in all the

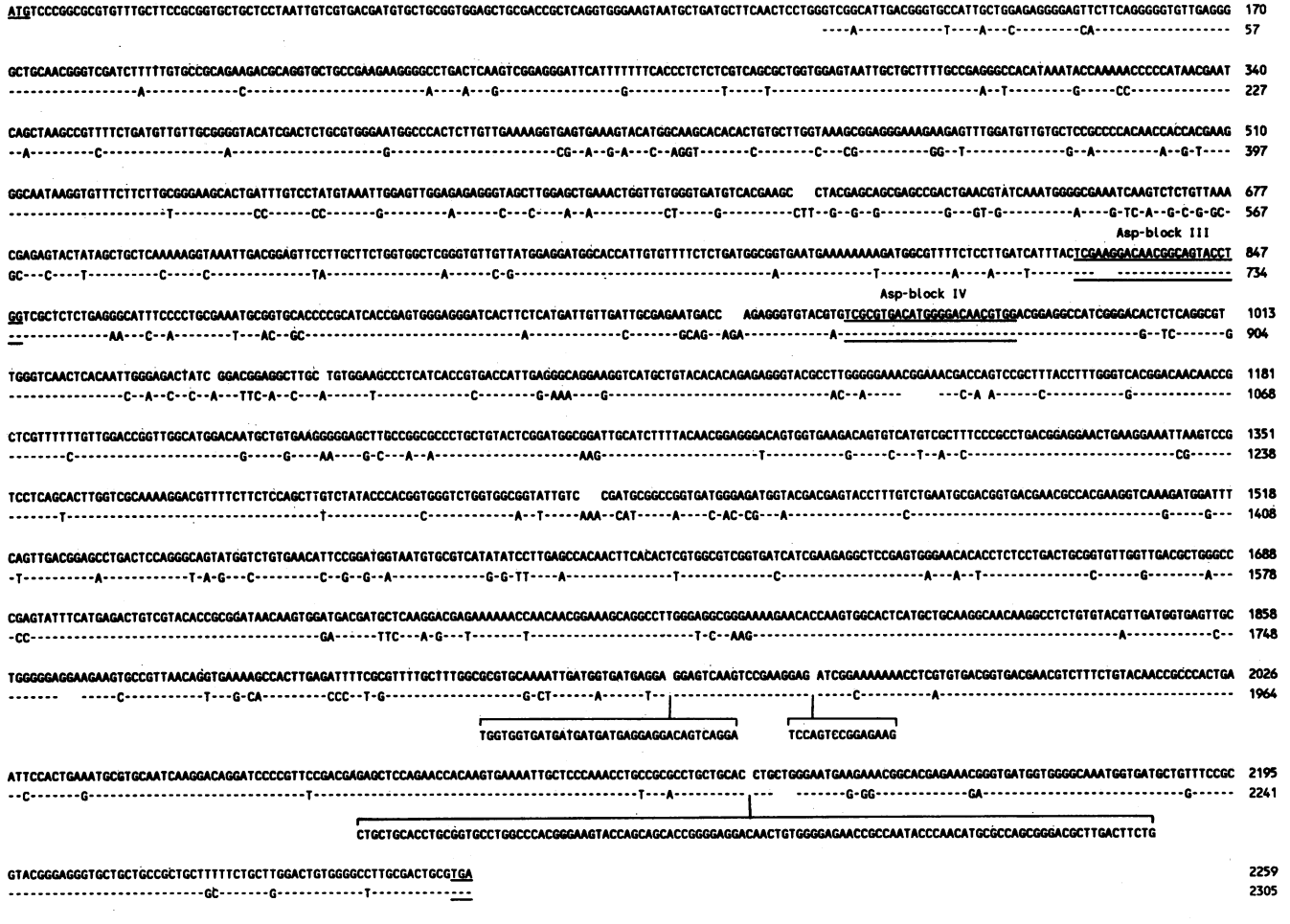

FIG. 1. Sequence comparison of SA85-1.1 and SA85-1.2. The sequence of SA85-1.1 is shown. Nucleotide identity of SA85-1.2 is marked by a dash. Nucleotide differences are indicated. The sequences are aligned to give the best homology. Predicted start codons and stop codons are underlined. The 24-base-pair sequences corresponding to Asp block III and Asp block IV homologous regions are indicated and underlined.

A

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                                                               III                                                   IV
                                                            SXDXGXTW                                              SXDXGXTW
Clostridium perfringens         GSGIVMDDGTIVMPAQISLRENNENNYYSLIIYSKDNGETWTMGNKVPNSNTSENMVIELDGALIMSTRYDYSGYRAAYISHDLGTTW EIYEPLNG
Trypanosoma cruzi SA85-1.1      GSG VM DGTIV                  SLIIYSKDNG TW                   E  G L M            Y S D GTTW E    L G
Trypanosoma cruzi pTt34         G G  M DGTIV        E       YS IIYSKD G TW   N V              E  G L M            Y S D GTTW E    L G
Trypanosoma cruzi SA85-1.2      GSG  M DGTIV          N     YS IIYS DNG TW          N         E  G LIM            Y S D GTTW E    L G

Trypanosoma cruzi SA85-1.1      GSGVVMEDGTIVFSLMAVNEKKDG  VFSLIIYSKDNGSTWSLSEGISPAKCGAPRITEWEGSLLMIVDCENDQ  RVYVSRDMGTTWTEAIGTLSG
Trypanosoma cruzi SA85-1.2      GSGVLMEDGTIVFSLMAMNEKNDG  VYSMIIYS DNGSTWSLSENISPANCTGPRITEWEGSLIMIVDCESSQ KRVYESRDMGTTWTEAIGTLPG
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B

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                                              SXDXGXTW                                                            SXDXGXTW
Clostridium perfringens                 YSLIIYSKDNGETWTMGNKV    Influenza B (stain B/Lee/40)                 MGMELYVKYDGDPWTDSDAL
Clostridium sordellii                   YSSVIYSKDNGETWTMGNKV    Influenza B (strain B/Hong Kong/8/73)        MGMELYVKYDGDPWTDSDAL
                                                                Influenza B (strain B/Leningrad/179/86)      MGMELYVKYDGDPWTDSDAL
Bactoroides fragilis                    NAGIMYSKDGGKNWKMHNYA    Influenza B (strain B/Maryland/59)           MGMELYVKYDGDPWTDSDAL
                                                                Influenza B (strain B/Memphis/3/89)          MGMELYVKYDGDPWTDSDAL
Trypanosoma cruzi pTt34                 YSMIIYSKDDGSTWALSNSV    Influenza B (strain B/Oregon/5/80)           MGMELYVKYDGDPWTDSDAL
Trypanosoma cruzi SA85-1.1              FSLIIYSKDNGSTWSLSEGI    Influenza B (strain B/USSR/100/83)           MGMELYVKYDGDPWTDSDAL
Trypanosoma cruzi SA85-1.2              YSMIIYS■DNGSTWSLSENI    Influenza B (strain B/Victoria/3/85)         MGMELYVKYDGDPWTDSDAL
                                                                Influenza B (strain B/Singapore/222/79)      MGMELYVKYDGDPWTDSEAL
Influenza A (strain A/PR/8/34)          RGWAIYSKDNSIRIGSKGDV
Influenza A (strain A/USSR/90/77)       RGWAIYSKDNSIRIGSKGDV
Influenza A (strain A/WSN/33)           RGWAIHSKDNGIRIGSKGDV
Influenza A (stain A/tern/Austalia)     NSWHIYGKDNAVRIGEDSDV
Influenza A (stain A/whale/Maine)       NSWHIYGKDNAVRIGEDSDV
Influenza A (strain A/parrot/Ulster)    SGWAIYSKDNGIRIGSRGDV
Influenza A (strain A/Chile/1/83)       RGWAIYSKDNSIRIGSKGDV
Influenza A (strain A/shearwater/72)    SGFAIVSKDNGIRIGSRGHI
Influenza A (strain A/Kenya/1/81)       QGFAPFSKDNGIRIGSRGHV
Influenza A (strain A/NJ/8/769)         SGWAIYSKDNSIRIGSKGDI
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FIG. 2. Sequence comparison of *T. cruzi* 85-kDa antigens and sialidases. (*A*) Protein alignments were made to maximize homology (19). *T. cruzi* amino acids identical to the *C. perfringens* sequence are shown. Asp blocks III and IV are indicated (37). The deduced amino acid sequences of SA85-1.1 and SA85-1.2 in this region appear at the bottom of the figure. (*B*) The 20-amino acid sialidase consensus sequence was used to search the protein data base (20). Solid block indicates deletion. Shading highlights the conserved lysine.

sialidase sequences except SA85-1.2, where it has been deleted.

To determine whether Asp block III-like sequences were a common feature of the SA85-1 gene family, a Southern blot of *T. cruzi* genomic DNA digested with several restriction enzymes was hybridized with an oligonucleotide corresponding to the Asp block III region from the SA85-1.1. This revealed multiple DNA fragments that are distributed similarly to those that hybridize with the SA85-1.1 cDNA (Fig. 3*A*) (13). A Northern blot of total *T. cruzi* RNA hybridized with the Asp block probe revealed a single 3.9-kb mammalian-stage specific transcript (Fig. 3*B*). The size and pattern of expression are the same as that identified with all SA85-1 probes (Fig. 3*B*) (13). The results are consistent with the presence of Asp blocks in at least a subset of the SA85-1 gene family.

We used affinity-purified anti-SA85-1 antibodies, isolated from chronic *T. cruzi*-infected mouse serum, to immunoprecipitate the SA85-1 antigens from [^{35}S]methionine-labeled *T. cruzi* extracts (Fig. 4), and sialidase activity was measured in the precipitates (Table 1). While normal mouse serum did not precipitate either 85-kDa antigen or sialidase activity, the anti-SA85-1.1 antibodies brought down both antigen (Fig. 4*A*) and 73% of the total sialidase activity in the extracts (Table 1), strongly suggesting that the SA85-1 antigens encode sialidase activity.

It has been shown that *T. cruzi* sialidase activity is shed into the medium by trypomastigotes in culture (39). We therefore examined supernatants of trypomastigote cultures for the presence of SA85-1 antigens and immunoprecipitable sialidase activity. [^{35}S]Methionine-labeled mammalian-stage parasites were incubated for 2 hr in nonradioactive, serum-free medium, after which the cells and their supernatants were examined for the presence of SA85-1 antigens by radioimmunoprecipitation. The shed proteins comprise a subset of the total (Fig. 4) and correspond to all the surface proteins (ref. 13; unpublished results). As with cell extracts, a single band of 85 kDa was immunoprecipitated with anti-SA85-1.1, indicating that the SA85-1 antigens are shed (Fig. 4*B*). To control for cell lysis, we examined the labeled culture supernatants for the presence of tubulin, the major ^{35}S-labeled band in the cell extract, and none was detected by immunoprecipitation (Fig. 4*B*). Immunoprecipitates were also examined for sialidase activity. It can be seen in Table 1 that ≈70% of the amount of activity found in cell extracts was present in the culture medium after the 2-hr incubation. Of this, 79% was immunoprecipitable by anti-SA85-1.1 antibodies.

We also performed immunoprecipitations with antibodies specific for individual SA85-1 family members SA85-1.1 (anti-1.1A) and SA85-1.2 (anti-1.2A) (Fig. 4*B*). Table 1 shows that the material immunoprecipitated by anti-1.2A had no sialidase activity, whereas anti-1.1A immunoprecipitated material with sialidase activity. As shown above, SA85-1.1 has two perfect Asp blocks, while SA85-1.2 is deleted for the lysine residue at position 2 of Asp block III (Fig. 2). Since Asp blocks are highly conserved (37) the deletion in SA85-1.2 may inactivate the enzyme for the substrate we used. It is unlikely that the anti-1.2A antibodies are inactivating the sialidase since the anti-SA85-1.1 antibodies purified from the same chronic infected mouse sera are directed to the complete molecule and do precipitate activity (13).

DISCUSSION

We have previously demonstrated that the SA85-1 genes encode a diverse family of surface antigens (13). Here we have shown that the family of antigens is shed from the cell surface. Our data strongly suggest that the SA85-1 antigens are sialidases. The homology between the translated products of the SA85-1 genes and published sialidase sequences is compelling. The lod scores in the regions of the Asp blocks are at least an order of magnitude above background. Furthermore, antibodies that have been affinity purified on recombinant SA85-1 antigens and precipitate 70–80% of the cellular and shed sialidase activity of the parasite also precipitate only a single 85-kDa band from both total cell extracts

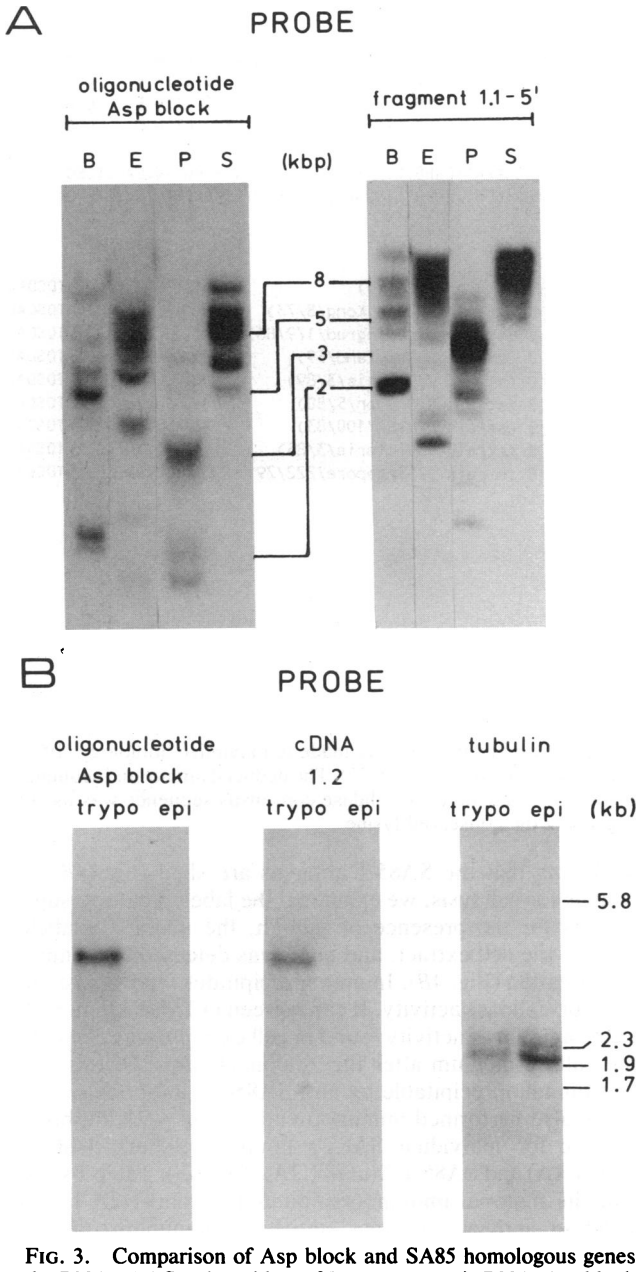

FIG. 3. Comparison of Asp block and SA85 homologous genes and mRNAs. (*A*) Southern blots of *T. cruzi* genomic DNA. Asp block oligonucleotide probe is TCCTTCGAGTAAATGATC. Fragment 1.1-5′ is the 5′ 500 base pairs of genomic clone SA85-1.1 (13). Three micrograms of DNA was loaded in each lane. B, *Bam*HI; E, *Eco*RI; P, *Pst* I; S, *Sal* I. Blots were prepared as described (13). kbp, Kilobase pairs. (*B*) Northern blots of trypomastigote poly(A)$^+$ RNA (1.5 μg) and total epimastigote RNA (5 μg) hybridized with different probes. cDNA probe is SA85-1.2 (13).

and culture supernatants. Thus, it is unlikely that the observed sialidase activity is a contaminant in the immunoprecipitations. In support of this, antibodies to SA85-1.2 did not precipitate sialidase activity.

Previous reports of sialidase activity from *T. cruzi* have suggested that there are multiple enzymes and have given conflicting estimates of the molecular mass of the major species (40–44). Our data do not agree with either estimate. Prioli *et al.* (43) report activity ranging in size from 121 to 203 kDa (43). However, none of the species has been purified. Harth *et al.* (41) report a molecular mass of 60 kDa (41). However, their purified protein lacked enzymatic activity and could be a degradation product. A unique sialidase in *T. cruzi* capable of transferring sialic acid from fetuin to parasite

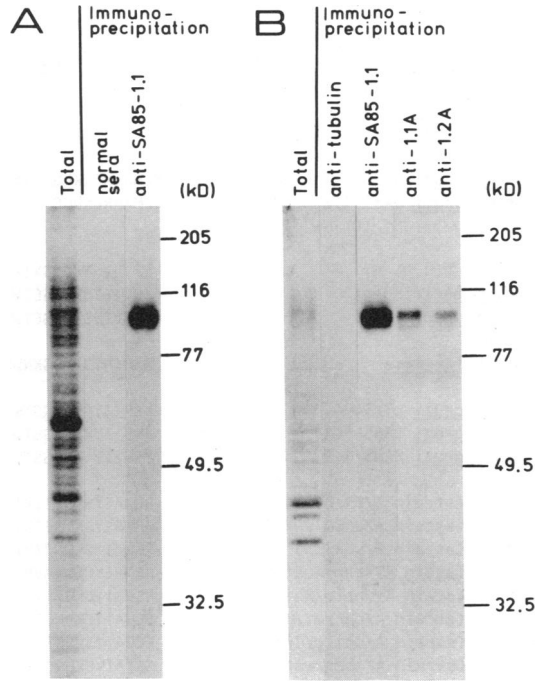

FIG. 4. Detection of SA85-1 antigens by immunoprecipitation of mammalian stage lysates and supernatants. (*A*) Trypomastigotes and amastigotes were radiolabeled with [^{35}S]methionine. The parasites were washed and lysed. Samples were analyzed by SDS/PAGE. Lanes: 1, 1 μl of total lysate (50,000 cpm); 2 and 3, immunoprecipitation from 0.1 ml of lysate with normal mouse serum (1:10 dilution) (lane 2) or anti-SA85-1.1 antibodies (lane 3). (*B*) Immunoprecipitations were performed on 0.1-ml aliquots of shed antigens (supernatant) followed by SDS/PAGE. Lanes: 1, 50,000 cpm of total supernatant; 2–5, immunoprecipitation with anti-tubulin antibodies (lane 2), anti-SA85-1.1 antibodies (lane 3), anti-1.1A antibodies (lane 4), or anti-1.2A antibodies (lane 5). Throughout the procedure, parasite viability was >99%.

glycolipids has been demonstrated (45). It is possible that the SA85-1 family includes this enzyme.

The finding that the *T. cruzi* surface antigens are shed suggests an important mechanism for evasion of host antibodies. Surface antigens bound by antibodies could be shed as a complex by the extracellular parasites.

Mice chronically infected with *T. cruzi* produce relatively large amounts of anti-sialidase (anti-SA85-1.1) antibodies. This is not unexpected since it is shed by the parasite. In addition, our anti-SA85-1.1 antibodies do not inactivate the sialidase activity, suggesting that the protein is structured such that antibodies produced during an infection do not destroy its enzymatic function.

Table 1. Immunoprecipitation of sialidase activity

Sample	Activity per 10^7 cells	%
Cell lysates		
Total	17,000	100
NMS	0	0
Anti-SA85-1.1	12,410	73
Cell supernatants		
Total	12,000	100
NMS	0	0
Anti-SA85-1.1	9,480	79
Anti-1.1A	1,200	10
Anti-1.2A	0	0

Immunoprecipitations were performed as described in Fig. 4. Results are averages of duplicate experiments and are expressed as units ($\times 10^{-6}$). NMS, normal mouse serum.

Sialic acid is found on the surface of many eukaryotic cells and plays several critical roles in cell–cell interactions and cell–ligand recognition (46). Sialidases alter cell–cell interactions by cleaving sialic acid and decreasing the negative charge on the cell surface (46). Sialic acid removal has been shown to affect lymphocyte homing (46), antigen presentation (46), and lymphocyte activation (46). Recently, sialic acids have been shown to be critical components of the ligands for LECCAM1 and LECCAM2 (47). Sialidase treatment of lymph nodes and leukocytes has been shown to destroy these ligands and disrupt their binding to LECCAM1 (48) and LECCAM2 (49, 50). Thus sialidase shedding may contribute to the immune dysfunction seen after *T. cruzi* infection (3, 51).

Sialidase shed by intracellular *T. cruzi* could also alter the host cell's surface glycoproteins. We have shown that SA85-1 antigens are shed intracellularly (unpublished data). Since *T. cruzi* grows in the cytoplasm rather than in vacuoles and sheds its surface antigens, it is possible that the sialidase can somehow enter the Golgi apparatus and cleave sialic acid from nascent glycoproteins. Alternatively, it could be targeted to the cell surface where, by cleaving sialic acid from glycoproteins and/or glycolipids, it might drastically alter the susceptibility of the infected cells to immune functions and other external effectors. In addition, the sialidase may contribute to the parasite's ability to penetrate host cells by altering the charge and allowing binding.

We do not know what role the sialidases play in the life cycle of *T. cruzi*. It has been suggested that antibodies to sialidase enhance infection (42). However, our finding that the sialidases are on the surface of the parasites raises the possibility that the enhanced infection was through opsinization.

We have suggested that the diversity of the SA85-1 antigens plays a role in generating the broad host range and cell-type specificity of *T. cruzi* (13). Our finding that the antigen has sialidase activity is consistent with this role. In this case, the different sialidase species could have different substrate specificities.

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