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A Partial cDNA Clone of Trypomastigote Decay-Accelerating Factor (T-DAF), a Developmentally Regulated Complement Inhibitor of *Trypanosoma cruzi*, Has Genetic and Functional Similarities to the Human Complement Inhibitor DAF

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Resistance to complement-mediated lysis in *Trypanosoma cruzi* is due to the expression of complement-regulatory factors by the virulent developmental forms of this protozoan parasite. An 87- to 93-kDa molecule, which we have termed T-DAF (trypomastigote decay-accelerating factor), is present on the surface of the parasite and inhibits complement activation in a manner functionally similar to the mammalian complement regulatory component, decay-accelerating factor. In this report, we characterized monospecific polyclonal and monoclonal antibodies which were obtained from mice and rabbits immunized with fast protein liquid chromatography-purified T-DAF. These polyclonal antibodies were shown to inhibit T-DAF activity and were capable of inducing lysis of the parasites. Both the polyclonal and monoclonal antibodies were used to screen a cDNA expression library prepared from *T. cruzi* trypomastigote mRNA. From this library, we obtained a partial λgt11 cDNA clone which showed genetic and functional similarity to the human C3 convertase inhibitor DAF (A. Nicholson-Weller, J. Burge, D. T. Fearon, P. F. Weller, and K. F. Austen, J. Immunol. 129:184–189, 1982).

Resistance to complement-mediated killing is an important developmentally regulated property of the protozoan parasite *Trypanosoma cruzi*, the causative agent of Chagas' disease. Infectious metacyclic trypomastigotes (MT), which develop in the hindgut of the insect vector, bloodstream trypomastigotes (BT), which differentiate in the vertebrate host, and tissue culture-derived trypomastigotes (TCT) are resistant to the lysis mediated by the alternative complement pathway, whereas the noninfective epimastigote (Epi) stages, which develop in the gut of the insect vector, are highly sensitive to alternative complement pathway-mediated lysis (13, 14, 24, 26).

We have previously shown that BT, TCT, and MT produce surface-associated molecules which interfere with the efficient assembly of the C3 convertases of both classical and alternative pathways. This C3 convertase inhibitory activity was detected in supernatants recovered from parasites incubated for 10 min at 45°C or for 4 h at 37°C (16, 33). Further analysis showed that these supernatants accelerated the decay of the C3 convertases but did not have factor H-like cofactor activity for factor I-mediated cleavage of C3b to iC3b. Therefore, a surface component of trypomastigotes was shed into the supernatant and was functionally analogous to human decay-accelerating factor (DAF), a complement regulatory surface membrane glycoprotein, which limits complement activation and lysis of cells by complement components from homologous species (25). We named the complement regulatory component(s) derived from trypomastigotes T-DAF since it was functionally analogous to human DAF. The C3 convertase inhibitory activity obtained from trypomastigotes copurified with an 87- to 93-kDa band on a fast protein liquid chromatofocusing column. The activity of T-DAF was retained on a concanavalin A-Sepharose column and destroyed by papain. This suggested that the molecule responsible for T-DAF activity was a glycoprotein (10). Furthermore, sera from patients chronically infected with *T. cruzi* recognized the 87- to 93-kDa components in enzyme-linked immunosorbent assays (ELISA) and immunoprecipitation assays (10, 40). In terms of molecular mass and other characteristics, T-DAF differs from two trypomastigote-specific glycoproteins with complement regulatory activity: gp160, which has recently been shown to have genetic and functional similarities to human DAF (28), and a 58- to 68-kDa glycoprotein which blocks assembly of the alternative pathway C3 convertase (7). Clear differences distinguish T-DAF from these proteins, and their relatedness, if any, is unknown.

In the present study, we describe the isolation and characterization of monospecific polyclonal and monoclonal anti-T-DAF antibodies. These antibodies were used in the isolation of a partial cDNA clone from an expression library. The nucleic acid sequence and the deduced protein sequence of this cloned fragment show partial homology to the sequences of human DAF, and the recombinant fusion protein had DAF activity.

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MATERIALS AND METHODS

Parasites. Culture metacyclic trypomastigotes (CMT), MT, TCT, and Epi of the Miranda 88 clone or the Y strain of *T. cruzi* were maintained as described previously (10).

Intrinsic labeling of TCT and preparation of parasite lysates and supernatants. TCT were intrinsically labeled with [^{35}S]methionine as described previously (10). After being labeled, the parasites were washed three times (2,000 × *g*, 15 min, 4°C) in Dulbecco's minimal essential medium (Sigma) and pelleted. For analysis of the total parasite extract, the pellets obtained from 10^8 parasites per ml were solubilized in lysis buffer containing 0.05 M Tris, 150 mM NaCl, 2% Nonidet P-40, 0.02% NaN_3, 10 mM EDTA, and protease inhibitors (25 μM *p*-nitrophenyl-*p'*-guanidino benzoate, 10 μg of pepstatin per ml, 10 μg of leupeptin per ml, and 1 mM iodoacetamide) for 2 h at 4°C. For analysis of the components released into the culture medium, the pellets were resuspended in Dulbecco's minimal essential medium and incubated for 10 min at 45°C prior to centrifugation at 10,000 × *g* for 15 min at 4°C. Supernatants were collected, and protease inhibitors were added.

T-DAF purification. T-DAF was purified by batch absorption of unlabeled or ^{35}S-labeled TCT supernatants on Polybuffer Exchanger 94 chromatofocusing resin (Pharmacia LKB Biotechnology, Inc.) as described previously (10). The T-DAF biological activity (inhibition of classical C3 convertase assembly) was determined by procedures described by Kipnis et al. (16). Briefly, 100 μl of EAC14b cells at 1.5 × 10^8/ml in $DGVBS^{2+}$ (dextrose-gelatin Veronal-buffered saline containing 0.15 mM $CaCl_2$ and 1.0 mM $MgCl_2$) was incubated with the test samples or buffer (as control) in the presence of purified human C3 (2 site-forming units [SFU]; Diamedix Laboratories) for 15 min at 30°C. EAC14b2a sites were developed by the addition of 100 μl of a 1:20 dilution of normal human serum previously depleted of C3 and C4 components (R3 reagent) by treatment with a KBr solution (4), and the samples were then incubated at 37°C for 60 min. Two milliliters of cold saline was then added to each tube, and the extent of cell lysis was determined by measuring the A_{412}. The number of C3 convertase sites (Z) was calculated by using the formula $Z = -\log(1 - y)n$, where Y is the number of lysed cells and n is the total number of cells. The percent reduction of C3 convertase sites (%CR) was calculated by the following formula: %CR = [(Z control − Z experimental)/Z control] × 100.

Preparation of antibodies. Mouse and rabbit anti-T-DAF sera were produced by intrasplenic immunization with purified T-DAF. Mouse sera against the fusion protein or β-galactosidase were produced as described above. Monoclonal antibodies against T-DAF were developed as described by Lopes and Alves (20). To obtain serum from chronically infected mice, mice were infected by inoculation with 10^2 *T. cruzi* (Y strain) TCT, and serum (chronic mouse serum) was collected after 60 days of infection. The immunoglobulin G (IgG) fraction of the antisera was prepared by octanoic acid precipitation (38).

ELISA. The presence of anti-T-DAF antibodies in the sera of immunized animals was evaluated by ELISA. Briefly, ELISA plates were sensitized with 100 μl of purified T-DAF (1 μg/ml) per well and blocked with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). One hundred microliters of serum (1:100) diluted in PBS containing 1% BSA was incubated for 1 h at 37°C. After the plates were washed with PBS–0.05% Tween, 100 μl of peroxidase-conjugated goat anti-mouse IgG (Cappel, Malvern, Pa.) diluted 1:3,000 was added as a second antibody. The substrate *o*-phenylenediamine (Sigma Chemical Co., St. Louis, Mo.) and H_2O_2 were used to develop the reaction. Plates were read at 492 nm in a Titertek Multiskan ELISA reader.

Neutralization of T-DAF activity by antibodies. Neutralization of T-DAF activity during the classical C3 convertase assembly was determined after mixing 50 μl of different dilutions of normal or rabbit IgG anti-T-DAF (2.7 mg/ml) with 50 μl of $DGVBS^{2+}$ containing 1.2 U of purified T-DAF (150 μg/ml) for 60 min at 30°C (1 U of T-DAF was defined as the amount of the inhibitor that causes a 50% reduction in C3 convertase sites). The residual T-DAF activity was measured as described above.

Complement-promoting lysis in the trypanosome assay. The lytic activity of antisera on TCT of Y strain was tested by the procedures of Kipnis et al. (15).

Immunoprecipitation and immunoblotting. Immunoprecipitation assays were performed as described previously (10). For immunoblots, 10^7 parasites were washed and solubilized in sodium dodecyl sulfate (SDS)-Laemmli sample buffer (19). Electrophoresis and electrotransfer were carried out by using standard techniques (19, 41). After the addition of primary antibody, bound antibodies were detected by using peroxidase-conjugated goat anti-mouse IgG (1:3,000; Cappel).

Nucleic acid isolation and analysis. Parasite DNA was purified from 10^8 to 10^9 parasites. After being washed with cold PBS, the parasites were centrifuged at 14,000 × *g* for 2 min, and the cell pellet was extracted and DNA purified by anion-exchange chromatography by using ASAP columns as described by the manufacturer (Boehringer Mannheim, Indianapolis, Ind.).

Total RNA was purified from about 2 × 10^8 trypanosomes by using an extraction mixture containing 8 M guanidine-hydrochloride as described in published procedures (6). Poly(A)$^+$ RNA from CMT of the Miranda 88 clone of *T. cruzi* was purified from total RNA by using poly(U) Sephadex (Bethesda Research Laboratories, Gaithersburg, Md.) as described in the manufacturer's instructions.

For Southern blots, DNA was separated on 1% agarose gels in 0.25× TBE buffer (1× TBE is 100 mM Tris [pH 7.6], 100 mM boric acid, and 2 mM EDTA). The DNA was transferred to GeneScreen Plus nylon membranes (New England Nuclear, Boston, Mass.) by capillary action as described previously (3). DNA probes were labeled with [α-^{32}P]dCTP by using the random primer labeling kit of Boehringer Mannheim. Hybridization at 65°C was performed overnight, and the blots were usually washed at a final stringency of 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% SDS as described previously (22).

For Northern hybridization, about 8 μg of total RNA was separated on a 1.2% agarose-formaldehyde gel by using established procedures (34) and then transferred by capillary action to GeneScreen Plus membranes as described previously (42). The separation of the RNA and transfer to membrane were monitored by ethidium bromide staining. Hybridization of ^{32}P-labeled probes (described above) was conducted in 50% formamide overnight at 48°C as described previously (3). The blots were washed at a final stringency of 1× SSC plus 0.1% SDS at 60°C and autoradiographed at −70°C with a Cronex Lightning-Plus screen.

Library construction and immunoscreening. Miranda 88 CMT cDNA was synthesized from a poly(A)$^+$ RNA template by random hexamer priming by using murine leukemia virus reverse transcriptase (Bethesda Research Laborato-

ries) as described previously (8, 29). After methylation of the *Eco*RI sites and addition of *Eco*RI linkers, the cDNA was ligated into the *Eco*RI site of the bacteriophage vector λgt11 by using standard procedures. Approximately 5×10^5 plaques from the CMT cDNA library were screened in duplicate after IPTG (isopropyl-β-D-thiogalactopyranoside) induction with the mouse and rabbit polyclonal sera and the monoclonal anti-T-DAF antibody. Positive clones were identified by using the ProtoBlot immunoscreening system as described in the manufacturer's directions (Promega, Madison, Wis.).

Subcloning and sequencing of cDNA inserts. Several λgt11 clones, positive by immunoscreening, were digested with *Eco*RI (Boehringer Mannheim) and separated on agarose gels as described above. The cloned inserts were recovered from the agarose and subcloned into *Eco*RI-digested and dephosphorylated Bluescript II plasmid (Stratagene, La Jolla, Calif.). Nucleotide sequences were determined on both DNA strands by using a modification of the chain termination method of Sanger et al. (35). Double-stranded DNA from recombinant pBluescript or λgt11 clones were sequenced by using commercially available oligonucleotide primers (Stratagene) and the T7 Sequenase-2 polymerase (United States Biochemical) as described previously (5). Sequence data were analyzed by programs developed by the Wisconsin Genetics Computer Group with a Vax 11/750 computer (37).

Purification of the β-galactosidase fusion protein. To analyze the protein sequence recognized by immunoscreening with anti-T-DAF sera, one of the positive λgt11 clones (designated λ8-4) was purified to homogeneity. A λ8-4 lysogen (in *Escherichia coli* Y1089) was induced for 1 h with IPTG, and a crude protein extract was prepared by sonication. The β-galactosidase fusion protein was purified by using a Protosorb immunoaffinity adsorbent column as described in the manufacturer's directions (Promega).

RESULTS

Production and initial characterization of immune sera and monoclonal antibodies raised against T-DAF. Monospecific polyclonal mouse and rabbit sera and mouse monoclonal antibody anti-T-DAF (monoclonal antibody E3G7) were produced after intrasplenic immunization with purified and biologically active T-DAF. These antibodies immunoprecipitated a single band of 87 to 93 kDa from detergent extracts of TCT labeled with [^{35}S]methionine (Fig. 1). These antibodies also recognized ^{35}S-T-DAF in immunoprecipitation assays or in an ELISA (data not shown). It was observed by immunoblot that the polyclonal anti-T-DAF sera recognized exclusively the 87- to 93-kDa band in extracts prepared from TCT but not Epi (Fig. 2). The polyclonal mouse sera recognized TCT but not Epi in an immunofluorescence assay (data not shown).

Polyclonal rabbit anti-T-DAF IgG blocks T-DAF activity. Purified rabbit anti-T-DAF IgG fraction or the IgG fraction from nonimmunized rabbits was incubated with T-DAF. As shown in Fig. 3, the anti-T-DAF antibody partially reversed, in a dose-dependent manner, the ability of T-DAF to inhibit the assembly of the classical C3 convertase. Inhibition of the assembly of the C3 convertase was unaffected by the presence of normal rabbit IgG fraction or the monoclonal anti-T-DAF antibody.

Isolation of cDNA clones by using anti-T-DAF serum. A recombinant λgt11 cDNA expression library, derived from poly(A)$^+$ RNA from MT of *T. cruzi* (Miranda 88 clone) was screened for the presence of clones expressing antigenic determinants recognized by the mouse polyclonal anti-T-DAF serum. Positive clones were subsequently screened for reactivity with monoclonal and rabbit polyclonal antibody to T-DAF. Thirteen clones reacting with all three antibody preparations were obtained, and of these, five were purified to homogeneity and subsequently shown, by DNA sequence analysis, to contain the identical partial cDNA insert. The uniformity of the clones might be explained in part by our screening criteria and our having had to amplify the cDNA library before immunoscreening. A clone designated λ8-4 was chosen for further characterization.

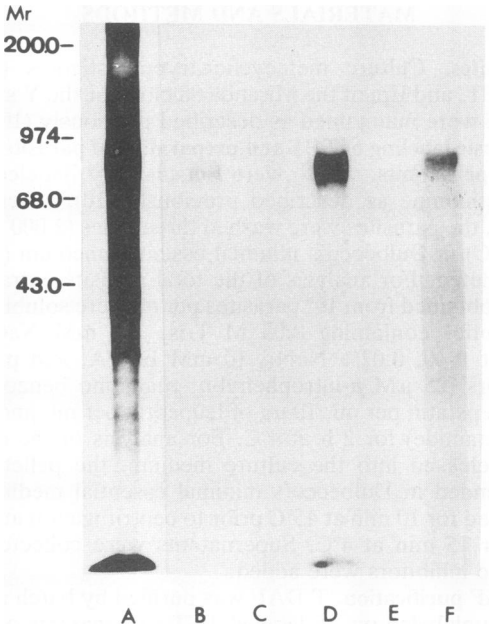

FIG. 1. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) profile of ^{35}S-labeled TCT immunoprecipitated with anti-T-DAF antibodies. TCT were labeled metabolically with [^{35}S]methionine, and the Nonidet P-40 extract was immunoprecipitated with chronically infected mouse anti-*T. cruzi* serum (A), polyclonal rabbit anti-T-DAF serum (B), normal rabbit serum (C), polyclonal mouse anti-T-DAF serum (D), normal mouse serum (E), and monoclonal antibody E3G7 (F) as indicated in the figure; SDS-PAGE and fluorography followed.

Characterization of the cDNA clone λ8-4. The λ8-4 clone was cut with *Eco*RI, and the cDNA insert was subcloned into the Bluescript II plasmid. DNA sequencing revealed that the cloned *T. cruzi* cDNA insert was 285 bp in length. The complete nucleotide sequence and deduced amino acid sequence are shown in Fig. 4. The cloned insert contains a single open reading frame spanning its entire length. Stop codons are present in the other two reading frames. A comparison of the nucleotide sequences of the λ8-4 insert and the coding region of human DAF (2), by using the Gap sequence comparison program of the Wisconsin Genetics Computer Group software package, revealed an average homology of about 40% between the two DNA sequences (see Fig. 9). This region of similarity occurs near the amino terminus of human DAF and extends over a region which includes most of the 3′ half of short consensus repeat 1 (SCR-1) and continues almost to the end of SCR-2 (nucleotides 237 to 520). When aligned with the amino acid sequence of human DAF (by using conserved replacements),

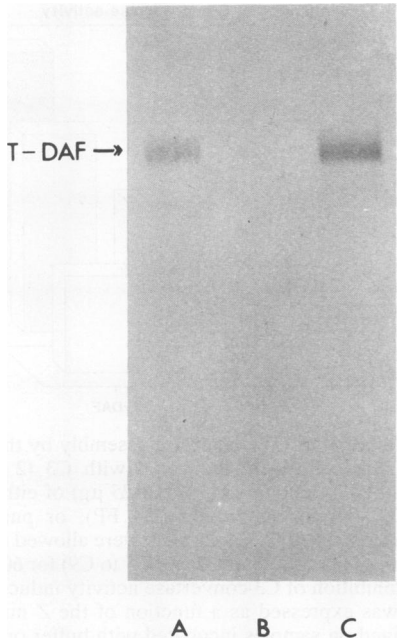

FIG. 2. Recognition of T-DAF in different developmental forms of *T. cruzi* as analyzed by immunoblotting. Samples of purified T-DAF (A), Epis (B) or TCT (C) of *T. cruzi* (Y strain) were solubilized in SDS-Laemmli sample buffer and electrophoresed on an SDS-10% polyacrylamide gel. Immunoblotting was performed by electrotransferring the SDS-polyacrylamide gel electrophoresis-separated proteins to nitrocellulose membranes and then incubating them with polyclonal mouse anti-T-DAF serum (diluted 1:500).

the λ8-4 cDNA insert was found to have an average similarity of 27% to a 93-amino-acid stretch of human DAF (see Fig. 9). This region of homology to DAF (amino acids 45 to 138) includes the last 17 amino acids of SCR-1, all of SCR-2,

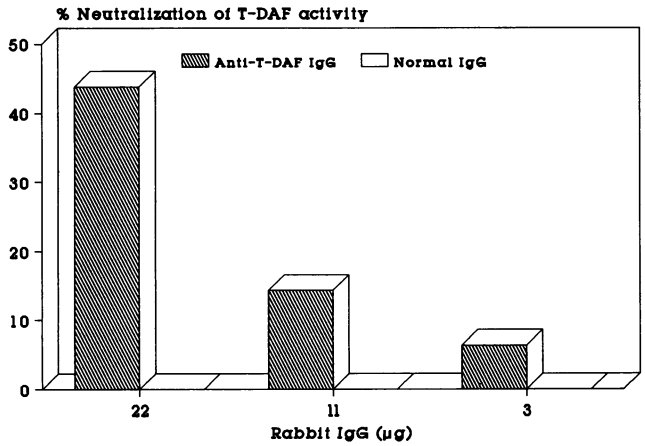

FIG. 3. Neutralization of the inhibitory effect of T-DAF on C3 convertase assembly by monospecific rabbit polyclonal anti-T-DAF antibodies. Different amounts of normal or anti-T-DAF rabbit IgG were preincubated with 1.2 U of T-DAF or buffer at 30°C for 60 min before addition of EAC14b cells and C3. The cells were further incubated for 15 min at 30°C, and C4b2a sites were developed by the addition of R3 (as source of C2 and C5 to C9) for 60 min at 37°C. The percent neutralization of T-DAF activity was expressed as a function of the *Z* number (C4b2a sites) determined for samples coincubated with buffer plus antibodies and T-DAF plus buffer.

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1/1
GAA TTC GGC ATC CTG GAG GAC GGT GAA ACA ATC TTT GTG TAC GTG
Glu Phe Gly Ile Leu Glu Asp Gly Glu Thr Ile Phe Val Tyr Val>

46/16
AAT GGC CAG ATG CTG GGG AGC TCG AAA ACG ATG CCG ACT GGT AAG
Asn Gly Gln Met Leu Gly Ser Ser Lys Thr Met Pro Thr Gly Lys>

91/31
GAA CGG CTA CTC GAT ATC TCG CAC TTC TAC TTT GGC GGT GAC AAC
Glu Arg Leu Leu Asp Ile Ser His Phe Tyr Phe Gly Gly Asp Asn>

136/46
GGC GAA GAA AAG GGA AAC CGC CAT GTG AAA GTG AGG AAC GTA TTG
Gly Glu Glu Lys Gly Asn Arg His Val Lys Val Arg Asn Val Leu>

181/61
TTG TAC AAC CGA GTG TTG AGT GCC AGT GAA CTG CAA TGC CGT CTT
Leu Tyr Asn Arg Val Leu Ser Ala Ser Glu Leu Gln Cys Arg Leu>

226/76
CCT GAA GAA GTT GTG CAA AAG CCA CAG AGT GCG TCA CCC ACT TAC
Pro Glu Glu Val Val Gln Lys Pro Gln Ser Ala Ser Pro Thr Tyr>

271/91
CTA AAA GCA CGA ATT
Leu Lys Ala Arg Ile
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FIG. 4. Nucleotide and deduced amino acid sequences of the λ8-4 cDNA insert recognized by anti-T-DAF antibodies. The complete nucleotide sequence of the λ8-4 cDNA insert is shown with the corresponding predicted amino acid residues indicated below. The sequencing is described in Materials and Methods.

and the first 13 amino acids of SCR-3 (2). The λ8-4 insert is also about 18 to 23% homologous with the amino acid sequences of each of the other three SCR domains of human DAF (data not shown). A search of the GenBank/EMBL and Swiss protein data banks indicated that the λ8-4 insert sequence showed some similarity to the sequence of an 85-kDa TCT-specific surface antigen of *T. cruzi* (11, 12): a stretch of 65 amino acids in the λ8-4 insert sequence was found to be about 34% homologous to the sequence of this major surface antigen of *T. cruzi* (data not shown).

The λ8-4 cDNA insert hybridizes to a developmentally expressed mRNA and does not hybridize to a large family of genes. To confirm that the λ8-4 cDNA insert was derived from a developmentally regulated gene, Northern blot analysis was carried out. Total RNA from TCT and Epi stages of *T. cruzi* was probed with the purified λ8-4 cDNA insert. The probe hybridized to a 4.0-kb TCT-specific RNA (Fig. 5). Hybridization of the λ8-4 insert to Epi RNA was not detected even after prolonged exposure (7 days).

Southern blots of genomic *T. cruzi* (Y) DNA digested with a variety of restriction enzymes (none of which cut within the probe sequence itself) and probed with the λ8-4 insert produce a simple hybridization pattern consistent with the presence of one or two copies of this sequence within the parasite genome (Fig. 6). This result does not exclude the possibility that the λ8-4 insert is a nearly unique sequence found within a very few members of a larger family of genes.

The λ8-4 fusion protein is functionally and antigenically related to T-DAF. To establish that the λ8-4 cDNA insert is a partial clone of T-DAF, the λ8-4 β-galactosidase fusion protein was purified and assayed for the presence of T-DAF activity. The capacity of the fusion protein to inhibit C4b2a (C3 convertase) sites was measured by incubating EAC14b cells with different amounts of fusion protein or β-galactosidase (as a control). C3 convertase sites were determined. As shown in Fig. 7, the purified fusion protein was capable of partially blocking the formation or activity of the C4b2a sites in a dose-dependent fashion, while the control had no noticeable effect.

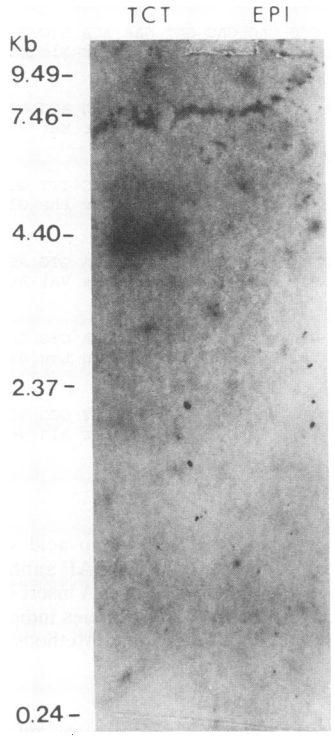

FIG. 5. Northern blot analysis of total RNA from *T. cruzi* (Y strain) TCT and Epi. About 8 μg of total RNA from each developmental form of *T. cruzi* was separated on a 1.2% formaldehyde gel, transferred to a nylon membrane, and probed with a ^{32}P-labeled λ8-4 insert as described in Materials and Methods. The sizes of the RNA standards (Bethesda Research Laboratories) are indicated (in kilobases).

Mouse antiserum prepared against the λ8-4 β-galactosidase fusion protein, but not mouse anti-β-galactosidase sera, specifically recognized the purified T-DAF in an ELISA (Fig. 8). Thus, the fusion protein and T-DAF have one or more antigenic epitopes in common.

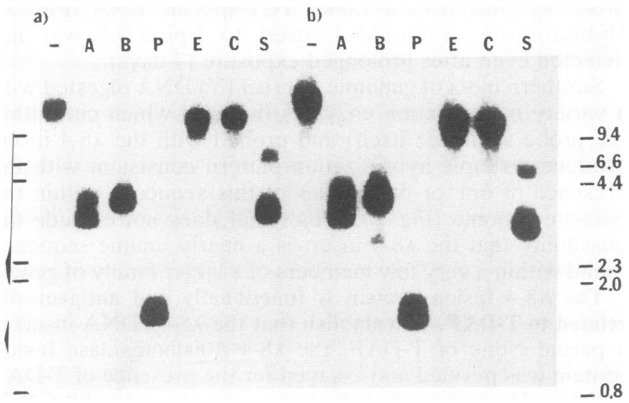

FIG. 6. Southern blot analysis of *T. cruzi* genomic DNA isolated from Epi (a) and trypomastigote (b) forms of the parasite. About 3 μg of genomic *T. cruzi* (Y strain) DNA was used in each of several separate restriction digests. Abbreviations: A, *Ava*I; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; P, *Pst*I; S, *Sca*I. The restriction-digested DNA was run on a 1% agarose gel, transferred to a nylon membrane, and probed with a ^{32}P-labeled λ8-4 insert as described in Materials and Methods.

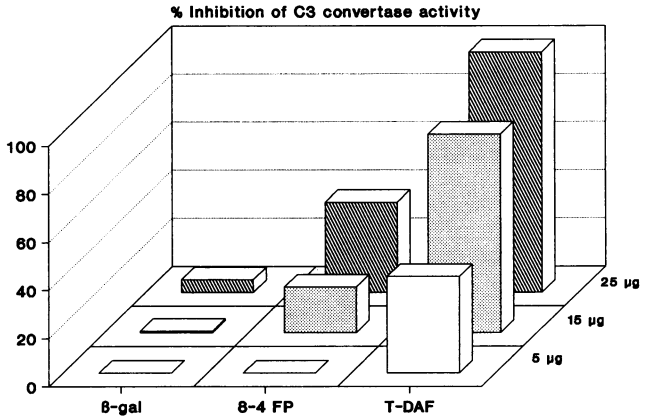

FIG. 7. Inhibition of C3 convertase assembly by the λ8-4 fusion protein. EAC14b cells were incubated with C3 (2 SFU) in the presence of different amounts (5, 15, or 25 μg) of either β-galactosidase (β-gal), λ8-4 fusion protein (8-4 FP), or purified T-DAF (T-DAF) for 15 min at 30°C. C4b2a sites were allowed to develop by the addition of R3 (as source of C2 and C5 to C9) for 60 min at 37°C. The percent inhibition of C3 convertase activity induced by each of the proteins was expressed as a function of the *Z* number (C4b2a sites) determined on samples incubated with buffer only.

Both anti-T-DAF and anti-λ8-4 fusion protein sera are lytic for *T. cruzi* trypomastigotes. The lytic activity of the anti-T-DAF serum and the anti-fusion protein serum on TCT was tested. As shown in Table 1, these immune sera were equally efficient in mediating lysis of trypanosomes in the presence of normal human serum as the source of complement. Under the same conditions, the use of neither normal mouse serum, rabbit serum, nor mouse anti-β-galactosidase serum resulted in significant lysis of the parasites.

DISCUSSION

We have shown previously that BT, TCT, and MT of *T. cruzi* exhibit a biological activity which both interferes with the formation and accelerates the decay of classical and

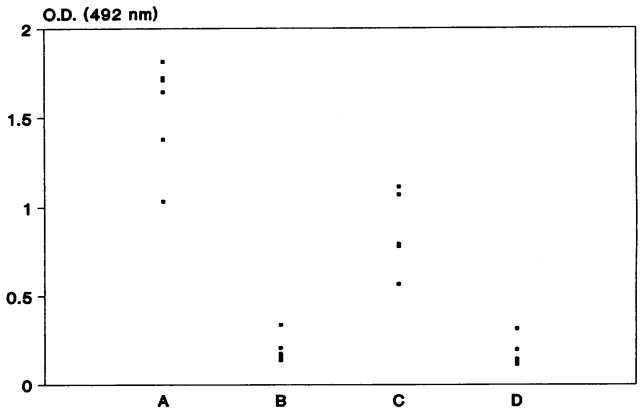

FIG. 8. Recognition of T-DAF by anti-λ8-4 fusion protein antibodies. BALB/c mice sera (six animals per group) were analyzed by ELISA for the presence of anti-T-DAF antibodies by using purified T-DAF as the antigen. BALB/c mice were immunized with purified T-DAF (A), β-galactosidase (B), λ8-4 fusion protein (C), or saline (D). Each point represents the mean optical density (at 492 nm) values for duplicate samples of individual sera (diluted 1:100).

TABLE 1. Complement-mediated lysis of *T. cruzi* (Y strain) TCT by anti-T-DAF antibodies

Treatment	% Lysis
Rabbit serum anti-T-DAF	49.3
Normal rabbit serum	2.8
Mouse serum anti-λ8-4 FP	41.7
Normal mouse serum	5.3
Mouse serum anti-β-gal	6.7
Monoclonal antibody E3G7	25.8
Chronic mouse serum anti-*T. cruzi*	73.1

alternative pathway C3 convertases (16, 33). This factor, which is spontaneously and selectively shed by the infective forms (but not Epi) of *T. cruzi* during incubation in medium, was isolated and biochemically characterized as an 87- to 93-kDa glycoprotein (10). We now describe the production of specific antibodies to this trypomastigote complement inhibitor (T-DAF) and the isolation of a partial cDNA clone with biochemical and functional relatedness to T-DAF.

Antisera and monoclonal antibodies specific for T-DAF were desired to further characterize T-DAF and to isolate cDNA clones expressing the T-DAF gene product. We have shown that polyclonal sera obtained from mice and rabbits after immunization with purified T-DAF were monospecific as judged by immunoprecipitation and Western blot assays (Fig. 1 and 2). Moreover, IgG purified from the rabbit anti-T-DAF serum was shown to partially neutralize the ability of T-DAF preparations to block C3 convertase activity. The monoclonal antibody we produced, although specific for T-DAF, was not able to block T-DAF function (data not shown).

With the availability of the specific antisera, we were able to address the question of whether Epi supernatants, which do not possess T-DAF activity (10, 16, 33), contain a biologically inactive form of T-DAF. By performing immunoblots with polyclonal anti-T-DAF sera, we were unable to detect immunoreactive material in Epi lysates (Fig. 2), suggesting that Epis do not synthesize a biologically inactive form of T-DAF.

Having established that our antisera were monospecific for T-DAF and capable of inhibiting T-DAF activity, we proceeded to use these reagents to isolate T-DAF-positive clones from a λgt11 expression library constructed from *T. cruzi* MT poly(A)$^+$ RNA. In our initial immunoscreening of the cDNA expression library, we isolated 13 clones which reacted positively to our three anti-T-DAF antibody preparations. Five of the 13 positive clones were shown to be identical by DNA sequencing, and one of the clones (designated λ8-4) was used exclusively in the remaining experiments.

As mentioned above, T-DAF is functionally related to human DAF, a member of a large family of genes with functional and structural relatedness (32). DAF is a 70-kDa glycoprotein which is capable of protecting host cells from lysis by homologous sera by preventing the assembly of C3 convertases, as well as disrupting preformed C3 convertases (25). The cDNA for DAF has been cloned and sequenced (2, 23), and the encoded protein is constructed of four homologous repeats, designated SCR, arranged in tandem at the amino terminus of the protein. Each SCR has a general consensus sequence of about 60 amino acids. The nucleotide sequences of these SCRs show little in common, and at the amino acid level each of the DAF SCRs is about 25% homologous to the others (2). However, SCRs are characterized by having a framework of highly conserved amino acids at invariant positions within the 60-amino-acid SCR domains (2, 23). The presence of tandemly repeated SCRs is a common feature of a number of C3b/C4b-binding proteins including DAF, CR1, MCP, C4bp, and factor H, all of which function in down-regulating the complement system at the critical level of the C3 convertase (1, 31). Further, several viral proteins which interact with C3b or C4b possess SCR-like motifs and probably function as virulence factors through their ability to regulate the complement system (17, 36).

A)

```
                  *         *         *         *         *
DAF     237  GATACTGTAATAACGTACAAATGTGAAGAAAGCTTTGTGAAAATTCC.TG
             GA   G AT   G A  A  GTGAA  AA CTTTGTG A   T   TG
T-DAF     1  GAATTCGGCATCCTGGAGGACGGTGAAACAATCTTTGTGTACGGTGAATG

                  *         *         *         *         *
DAF     286  GCGAGAAG..GACTCAGTGATCTGCCTTAAGGGCAGTCAATGGTCAGATA
             GC AGA G  G      T      C  T   G C G  AA G  C G TA
T-DAF    51  GCCAGATGCTGGGGAGCTCGAAAACGATGCCGACTGGTAAGGAACGGCTA

                  *         *         *         *         *
DAF     334  TTGAAGAGTTCTGCAATCGTAGCTGCGAGGTGCCAACAAGGCTAAATTCT
              T  A  A  TC GCA T  TA  T  G GGTG CAAC   G   AA
T-DAF   101  CTCGATATCTC.GCACTTCTACTTTGGCGGTGACAACGGCGAAGAAAAGG

                  *         *         *         *         *
DAF     384  GCATCCCTCAAACAGCCTTATATCACTCAGAATTATTTTCCAGTCGGTAC
             G A CC  CA        T     AC   G ATT TT T CA  CG
T-DAF   150  GAAACCGCCATGTGAAAGTGAGGAAC...GTATTGTTGTACAACCGAGTG

                  *         *         *         *         *
DAF     434  .TGTTGTGGAA.TATGAGTGCCGT.CCAGGTTACAGAAGAGAACCTTCTC
              TG  GTG  A TAT    GC  T CC   TT C GAAGA     T C
T-DAF   197  TTGA.GTGCCAGTGAACT.GCAATGCCGTCTTCCTGAAGAAGTTGTGCAA

                  *         *         *         *
DAF     481  TATCACCAAAACTAACTTGCCTTCAGAATTTAAAATGGT
              A   CCA A     C T  C      A  TAAAA
T-DAF   245  AAG..CCACAGAGTGCGTCACCCACTTACCTAAAAGCAC
```

B)

```
                  *         *         *         *         *
T-DAF     1  EFGILEDGETIFVYVNGQMLGSSKTMPTGKERLLDISHFYFGGDNGEEKG
             . :    : . .  ..  .:  : ::  : . ::. :::.  . :
DAF      45  VICLKGSQWSDIEEFCNRSCEVPTRLNSASLKQPVITQNYFPVGTVVEYE

                  *         *         *         *
T-DAF    51  NRHVKVRNVLLYNRVLSASELQCRLPEEVVQKPQSASPTYLKARI
             : .  ::.:. ::. . ::.  .:   . : .: : :.   ..
DAF      95  CRPGYRREPSLSPKLTCLQNLK-WSTAVEFCKKKSCPNPGEIRNG
```

FIG. 9. (A) DNA sequence alignment of the λ8-4 cDNA insert with human DAF. Regions of sequence identity are indicated by the letters separating the two sequences. The λ8-4 sequence (T-DAF) is numbered from the first (5′) nucleotide of the cDNA insert. The human DAF sequence (DAF) (2) is numbered consecutively from the first nucleotide (nucleotide 237) of the aligned sequence. Asterisks are used as sequence reference marks, and periods indicate gaps in the aligned sequence. (B) Amino acid alignment of the deduced amino acid sequence of the λ8-4 cDNA clone and the amino acid sequence of human DAF. The λ8-4 (T-DAF) sequence is numbered from the first (5′) amino acid in the cDNA insert. The human DAF sequence (DAF) is numbered consecutively from the first amino acid (+45) of the aligned sequence (2). Asterisks are used as sequence reference marks; dashes indicate gaps introduced into the sequence; colons indicate a match between identical amino acids or a conserved amino acid replacement; periods indicate positions where the two aligned amino acids are hydrophilic or hydrophobic.

With this information, we sought to compare the nucleotide sequence of the λ8-4 cDNA insert with that of human DAF. The insert DNA sequence was found to be about 40% homologous with a portion of the coding region for human DAF (Fig. 9). Given the limited amount of sequence identity

among mammalian SCRs, and the fact that we were comparing protozoan and human gene sequences, we considered this level of homology to be significant. The regional similarity between DAF and the λ8-4 insert sequence, as generated by the Gap computer program, starts about halfway through SCR-1 and continues until nearly the end of SCR-2. Thus, the λ8-4 insert spans about 1.5 SCRs (two SCRs have been shown to be the minimum number required for C3b/C4b binding [18]).

The functional activity of human DAF resides in its four SCR domains, as mentioned above. When aligned with the human DAF sequence, the derived λ8-4 amino acid sequence completely spans a region which includes SCR-2 (i.e., amino acids 45 to 138 in human DAF; Fig. 9). When conservative amino acid substitutions are included, the average homology between λ8-4 and DAF in this region is about 27%. However, conspicuously absent from the cDNA sequence are the four cysteine residues usually present in SCRs as part of the consensus sequence. Thus, we have not been able to discern in the cDNA clone the framework of conserved amino acids seen in most SCRs (2, 23, 31).

It is difficult to assess the significance of these data regarding the similarity of T-DAF sequences with those of human DAF. This is especially true given that important functional differences do exist between human DAF and T-DAF, namely, that DAF function is species restricted (i.e., human DAF confers protection primarily against human complement activation but not against complement from other species [21]), while T-DAF appears capable of protecting parasites from lysis by sera from a number of different species. This may be an evolutionary adaptation, allowing *T. cruzi* to successfully infect a variety of mammalian hosts. Given this difference, a functional similarity between T-DAF and DAF does not necessitate a priori that there be strict agreement at the sequence level. Indeed, this is exemplified in the case of herpes simplex virus glycoproteins C (gCs). These viral proteins (gC-1 and gC-2) have C3b binding activity, and gC-1 has C3 convertase decay-accelerating activity as well. Each of these glycoproteins has domains (containing four cysteines) which were thought to be structurally related to SCRs (36). However, recently it was shown that these conserved cysteines may be eliminated without affecting C3b binding activity (9). The λ8-4 cDNA clone, and by extension T-DAF, may be another example of a protein possessing complement regulatory activity in the absence of a recognizable SCR.

The Northern blot analysis, using the λ8-4 insert as a probe, confirmed that T-DAF expression is regulated at the transcriptional level (Fig. 5). The 4.0-kb molecule recognized in trypomastigote RNA preparations is similar in size to mRNAs which encode developmentally regulated 85-kDa surface proteins of trypomastigotes described by others (12, 30, 39). These investigators have demonstrated that the 85-kDa proteins are encoded by members of a large gene family consisting of more than 100 genes and pseudogenes. T-DAF is also an 85-kDa surface protein of trypomastigotes, and we have found that a portion of the λ8-4 insert-derived amino acid sequence is 34% homologous to a member of this gene family (data not shown). Our Southern hybridization data, however, indicate that the λ8-4 clone does not recognize more than one or two fragments in a genomic digest of *T. cruzi* DNA (Fig. 6), although the λ8-4 sequence could be unique to a very few members of this larger gene family. It would be of interest to know if T-DAF is a member of the 85-kDa gene family, but further study will be required before we can reach any conclusion regarding this possibility.

Our genetic analysis of the λ8-4 cDNA insert suggested that this cloned sequence was related to DAF, so we next analyzed the λ8-4 fusion protein for the presence of T-DAF activity. The ability of the fusion protein to partially block C3 convertase assembly (Fig. 7) suggests that we have cloned a functionally active portion of T-DAF. Moreover, additional evidence that our cloned fusion protein is part of T-DAF is provided by our demonstration that antisera raised against the fusion protein recognize the purified T-DAF molecule in an ELISA (Fig. 8).

Antibodies directed against complement-regulatory molecules have been termed lytic because they can prevent the target molecule from protecting cells from complement lysis. We observed that the anti-fusion protein and anti-T-DAF sera were capable of promoting the lysis of trypomastigotes in the presence of complement (Table 1). This result is further evidence that we have cloned a portion of the T-DAF gene, and it is similar to the findings of others (27). Antibodies to gp160, a 160-kDa molecule isolated from trypomastigotes, were found to be lytic. This molecule, like T-DAF, is endowed with complement-regulatory activity since it is able to bind C3 and inhibit C3 convertase assembly (28). Another complement-regulatory protein, the 58- to 68-kDa glycoprotein isolated from trypomastigotes of *T. cruzi*, has been shown to block only the alternative pathway C3 convertase assembly (7). The precise relationship between T-DAF and these other trypomastigote-specific complement inhibitors needs to be investigated. However, the biochemical, functional, and antigenic properties of T-DAF suggest that it is distinct from other complement-regulatory proteins of trypomastigotes.

In this study, we report the isolation and characterization of a partial cDNA clone possessing antigenic and functional relatedness to the *T. cruzi* complement-regulatory molecule T-DAF as well as functional and genetic relatedness to its mammalian counterpart DAF. Our isolation of a cloned functional domain of T-DAF may allow the molecular dissection of the structural components necessary for the complement-regulatory activity of T-DAF.

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