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# Identification of the Gene Family Encoding the 160-Kilodalton *Trypanosoma cruzi* Complement Regulatory Protein

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***Trypanosoma cruzi* trypomastigotes are exquisitely resistant to the lytic effects of vertebrate complement, and this characteristic contributes to the survival of the parasites in the host bloodstream. Trypomastigotes avoid complement-mediated lysis by the production of a surface glycoprotein that inhibits the formation of the alternative and classical C3 convertase, thus preventing activation and amplification of the complement cascade at the parasite surface. We have developed a monoclonal antibody to the 160-kDa *T. cruzi* complement regulatory protein (CRP) and describe a one-step immunoaffinity purification procedure. The CRP was purified to homogeneity and subjected to amino-terminal peptide sequence analysis. Based on the protein sequence obtained, the CRP was identified as a member of a large family of trypomastigote-specific genes, and a complete cDNA was isolated and sequenced. The complete coding sequence was cloned in *Escherichia coli*, and antibodies raised against the full-length recombinant protein reacted specifically with a 160-kDa protein in trypomastigote membrane protein preparations as well as with native, purified CRP. Indirect immunofluorescence revealed that the protein is uniformly expressed at the cell surfaces of trypomastigotes.**

*Trypanosoma cruzi*, the causative agent of Chagas' disease, is a protozoan parasite which is transmitted to the vertebrate host by insect vectors. As with many pathogens, especially those which cause chronic diseases, *T. cruzi* has developed multiple means of avoiding host defenses. In this regard, it has been well established that infectious trypomastigotes are resistant to the lytic effects of normal human serum, whereas insect-stage parasites (epimastigotes) are rapidly killed by the alternative complement pathway (1, 6, 9). The robust complement resistance of *T. cruzi* trypomastigotes has been attributed to developmentally regulated surface glycoproteins which restrict complement activation on the parasite surface (4, 10, 17). In previous studies, we have characterized one such protein, a 160-kDa complement regulatory protein (CRP), as an inhibitor of C3 convertase, the central, multisubunit enzyme of the complement cascade (10, 14). Similar to the family of mammalian regulators of complement activation, the CRP has binding affinity for the complement components C3b and C4b, although no cofactor activity for factor I-mediated cleavage of C3b or C4b has been detected (14). Additionally, we have demonstrated that the CRP is specifically expressed by infectious trypomastigotes and is not detectable in the epimastigotes or intracellular amastigotes (11).

The purpose of the present study was to identify the gene or gene family encoding the 160-kDa CRP. Molecular characterization of the gene encoding the CRP has been hindered by the complexity of several related *T. cruzi* genes encoding surface glycoproteins which are members of a heterogeneous family related to the *T. cruzi trans*-sialidase protein. The family members encode proteins ranging in size from 80 to >200 kDa. The genes have been grouped into four subfamilies based on sequence similarities and the presence of various motifs shared with the *T. cruzi trans*-sialidase gene (18). Several *T. cruzi* that encode proteins of approximately 160 kDa have been reported, including the *trans*-sialidase, which can range in size from 160 to >220 kDa, as well as several proteins of unknown function (5, 15, 16, 22). A monoclonal antibody, 3H7, which specifically reacts with the CRP was developed, and a one-step affinity purification of CRP protein is described. Amino-terminal sequencing of the immunoaffinity-purified CRP revealed that it is most closely related to the family of trypomastigote-specific genes described by Van Voorhis et al. and Jazin et al. (5, 22) and is distinct from the active *trans*-sialidase gene. Based on the carboxy-terminal sequence of known gene family members, a full-length cDNA has been cloned by reverse transcription (RT)-PCR and the DNA sequenced has been determined.

## MATERIALS AND METHODS

**Media, buffers, and reagents.** The following media and buffers were used throughout these experiments. Complete Dulbecco's minimal essential medium (complete DMEM; GIBCO, Grand Island, N.Y.) was buffered with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4), and supplemented with 5% fetal calf serum, 5 mM glutamine, 200 μM sodium pyruvate, and 50 μg of gentamicin per ml (all from GIBCO). Complete RPMI medium was RPMI 1640 (GIBCO) supplemented with 15% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, and 50 μg of gentamicin per ml. HAT medium consisted of complete RPMI medium supplemented with 100 μM sodium hypoxanthine, 400 nM aminopterin, and 16 μM thymidine (all from GIBCO). HT medium was complete RPMI medium supplemented with 100 mM sodium hypoxanthine and 16 nM thymidine. Lysis buffer contained 2% Triton X-114 (Pierce Chemicals, Rockford, Ill.) in 50 mM Tris (pH 7.4)–150 mM NaCl. Labeling medium was DMEM without cysteine or methionine (ICN Biochemicals, Costa Mesa, Calif.) but supplemented with 100 μg of ovalbumin per ml, 10 mM HEPES (pH 7.4), and 2 mM glutamine. Tris-buffered saline (TBS) consisted of 50 mM Tris base (pH 7.5) and 150 mM NaCl. Blocking buffer was TBS containing 5% nonfat powdered milk. Transfer buffer was 50 mM Tris (pH 8.3)–380 mM glycine–0.1% sodium dodecyl sulfate (SDS)–20% methanol. Protease inhibitors (leupeptin, aprotinin, and E-64; all from Sigma Chemical Co., St. Louis, Mo.) were added as indicated at a final concentration of 1 μg/ml each. Monoclonal antibody, UPC10 (Cappel, Durham, N.C.) was used as a negative control for immunoprecipitations and immunofluorescence assays at the concentrations indicated in the text.

**Parasites.** *T. cruzi* trypomastigotes (strain Y) were passaged in BALB/c mice, and 7 days later bloodstream trypomastigotes were recovered and used to initiate infections of NIH 3T3 cells at a multiplicity of infection of 5 in DMEM buffered with 10 mM HEPES (pH 7.4) and supplemented with 5% fetal bovine serum,

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L-glutamine (5 mM), sodium pyruvate (0.2 mM), and gentamicin (50 μg/ml), all from GIBCO. Tissue culture-derived trypomastigotes were recovered after 7 to 9 days from supernatants of infected cultures and were used immediately for labeling or frozen at −80°C. Epimastigotes were maintained in complete brain heart infusion-liver medium at 28°C. The base medium was prepared from brain heart infusion and Bacto Liver Extract (Difco) as described in the manufacturer's directions and was supplemented with 10% fetal bovine serum, 1× BME vitamin mix, L-glutamine (5 mM), glucose (0.3%), and gentamicin (50 μg/ml), all from GIBCO.

**C3b preparation and affinity purification of CRP.** Human C3 was prepared from fresh plasma as described previously (10). C3b was prepared by treating 20 mg of C3 (at 2.5 mg/ml) with 200 μg of trypsin (Sigma) for 15 min at 37°C. Soybean trypsin inhibitor (Sigma; 400 μg) was added, and the reaction was continued for an additional 15 min at 37°C. C3b was coupled to Affi-Gel 10 (Bio-Rad Laboratories, Richmond, Calif.) at 10 mg of protein per ml of matrix, and CRP was affinity purified as described previously (14).

**Biosynthetic labeling and membrane protein preparation.** Tissue culture-derived trypomastigotes were recovered as described above, washed two times in phosphate-buffered saline (PBS)–1% glucose, and resuspended at $10^8$ cells per ml in labeling medium (ICN Biochemicals). [$^{35}$S]methionine (Trans-label; ICN Biochemicals) was added at 50 μCi/ml, and the cells were incubated for 1 h at 37°C. After labeling, the cells were washed two times at 4°C in PBS–1% glucose. Trypomastigote membrane extracts were prepared as follows. After biosynthetic labeling, parasites were resuspended at $4 \times 10^8$/ml in deionized water containing protease inhibitors and incubated at room temperature for 5 min. Membrane fragments were pelleted for 5 min at 5,000 × *g*. The pellet was solubilized in the original volume in 2% Triton X-114 in TBS with fresh protease inhibitors and incubated for 30 min on ice. Insoluble material was removed by microcentrifugation for 10 min at 4°C. To extract the detergent from the solubilized proteins, the supernatant was incubated at 37°C for 3 min and then microcentrifuged for 3 min at room temperature, and the aqueous phase was recovered. The protein concentration was measured with the Bio-Rad DC assay as directed by the manufacturer.

**Immunoprecipitation of membrane protein extracts and C3b-purified CRP.** For immunoprecipitation of membrane preparations, approximately 15 μg of protein was incubated with monoclonal antibodies (50 μg/ml) or antisera (1:400 dilution) for 1 h at room temperature. Fifty microliters of a 10% (wt/vol) suspension of *Staphylococcus aureus* Cowan strain I (Sigma) in TBS–0.05% Nonidet P-40 (NP-40) was added to the samples, and shaking was continued for 15 min. Mixtures were cleared by centrifugation for 3 min at 13,000 × *g*. Pellets were washed three times with TBS–0.05% NP-40 and resuspended in 20 μl of TBS. Samples were prepared for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography as described previously (10).

Immunoprecipitation of CRP was carried out as described previously except that 100 μl of the purified protein preparation was used (13). Monoclonal antibodies were used at 200 μg/ml, and antisera were used at 1:100 dilution.

**Monoclonal antibody production.** Six- to eight-week-old female BALB/c mice (Jackson Laboratory, Bar Harbor, Maine) were infected intraperitoneally with $10^5$ tissue culture-derived trypomastigotes. Serum antibody titers were monitored by immunoprecipitation of trypomastigote membrane extracts prepared as described above, and spleens were removed 141 days postinfection. Single-cell suspensions were prepared, and spleen cells were fused to mouse myeloma cells (P3X63-Ag8.653; American Type Culture Collection) as described previously (8). After fusion, the cells were resuspended in HAT medium that had been supplemented with 25 μg of allopurinol (Sigma) per ml to suppress parasite growth for 10 days (2). After 10 days, the cultures were grown in HT medium, and screening of culture supernatants was begun on day 11.

**Immunoblotting.** Trypomastigote membrane extracts were diluted to 10 μg/ml in TBS, and 200 μl was transferred to wells of a Bio-Rad dot blot apparatus and applied to a nitrocellulose membrane under gentle vacuum. The wells were blocked for 1 h with blocking buffer. Blocking buffer was removed, 100 μl of hybridoma supernatant fluid or diluted serum was added to the wells, and the wells were incubated for 30 min at room temperature. The wells were washed three times with TBS, and the filter was removed from the manifold and incubated with blocking buffer for 1 h. The filter was then incubated for 30 min with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (Cappel) diluted 1:15,000 in blocking buffer. The filter was washed three times in TBS and once in AP buffer (100 mM Tris [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$). Color development was carried out by incubation of the filter with 0.33 μg of nitroblue tetrazolium per ml and 0.165 μg of 5-bromo-1-chloro-3-indolyl phosphate per ml in AP buffer for 1 h, and the reaction was stopped with the addition of EDTA to a final concentration of 100 mM.

**Western blotting (immunoblotting).** Detergent-solubilized membrane extracts were prepared from tissue culture-derived trypomastigotes and axenically cultured epimastigotes as described above, and 7 mg of total protein was subjected to SDS-PAGE as described previously (14). The proteins were transferred to nitrocellulose in transfer buffer at 45 V for 16 h at 4°C. All incubations were carried out at room temperature. The filter was washed briefly in TBS, incubated in blocking buffer for 2 h, and then incubated in the primary antibodies (monoclonal antibody 3H7 or UPC10; 200 μg/ml) for 1 h. The filter was washed in TBS and incubated with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (Cappel) diluted 1:15,000 in blocking buffer for 1 h. The filter was washed in TBS and then in AP buffer, and the color was developed as described above for immunoblotting.

**Preparation of 3H7 immunoaffinity matrix and immunoaffinity purification of CRP.** Monoclonal antibody 3H7 was purified on protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J.) as described previously (8). Fifteen milligrams of purified 3H7 antibody was coupled to 3 ml of protein A-agarose by use of the Affinica antibody orientation kit (Schleicher & Schuell, Inc., Keene, N.H.) as described in the manufacturer's instructions, and the coupled matrix was washed with TBS–0.05% NP-40 prior to use. Membrane extracts were prepared from $10^{10}$ tissue culture-derived trypomastigotes as described above, except that parasites were solubilized at $10^9$/ml and, after removal of Triton X-114, NP-40 was added to a final concentration of 0.05%. The membrane preparation was incubated with 2.5 ml of 3H7-protein A-agarose for 1 h at 22°C. The resin was transferred to a column and washed with 10 bed volumes of TBS–0.05% NP-40. The protein was eluted with 2 bed volumes of 50 mM glycine (pH 2.3)–50 mM NaCl, and 0.5-ml fractions were collected into 50 μl of 1 M Tris (pH 8.0).

**Amino-terminal protein sequencing.** Fractions eluted from the 3H7 affinity column were analyzed by Western blotting to identify the CRP. Four fractions were concentrated approximately 16-fold and run in one lane of an SDS–8.5% polyacrylamide gel under reducing conditions as described previously (14). After electrophoresis, the gel was incubated in transfer buffer for 5 min at room temperature. Proteins were transferred to ProBlott polyvinylidene difluoride membranes (Applied Biosystems, Inc., Foster City, Calif.) in transfer buffer at 45 V for 16 h at 4°C. The membranes were rinsed in deionized water for 5 min and then in 100% methanol for 30 s. The protein band was visualized by staining the membrane in 0.1% Coomassie blue–1% acetic acid–40% methanol for 1 min at 22°C. Destaining was carried out in 50% methanol. The stained protein band was excised, and sequencing was carried out at Ariad Pharmaceuticals, Inc., Cambridge, Mass.

**Indirect immunofluorescence.** Tissue culture-derived trypomastigotes were washed in PBS–1% glucose, air dried on slides, and then fixed in 4% paraformaldehyde. All incubations were carried out at room temperature for 30 min. The slides were washed in PBS, blocked in 5% normal goat serum, and then incubated in the primary antiserum (normal mouse serum or mouse anti-recombinant CRP antiserum) at a dilution of 1:200. The slides were washed and incubated with Cy3 Fluorolink-conjugated goat anti-mouse immunoglobulin G (1:3,000 dilution; Jackson ImmunoResearch Laboratories, Westgrove, Pa.). Cells were evaluated for fluorescence with a Nikon Microphot microscope and Nomarski differential interference contrast optics.

**Neuraminidase assay.** Assays for neuraminidase activity were performed by the measurement of fluorescence following the hydrolysis of 2′-(4-methylumbelliferyl)-alpha-D-*N*-acetylneuraminic acid (MuNana) as described before (19). Trypomastigote membrane preparations were immunoprecipitated as described above, and the enzyme activity remaining in the supernatants was measured. Fluorescence was measured at 420 nm with excitation at 360 nm on a Perkin-Elmer LS 50 fluorimeter.

**RT-PCR and cloning of full-length CRP cDNA.** Total cellular RNA was prepared from tissue culture-derived trypomastigotes by use of Trizol reagent (GIBCO BRL) as described in the manufacturer's directions. For cDNA synthesis, 5 μg of trypomastigote RNA was reverse transcribed by use of the GIBCO BRL Superscript Preamplification System, as described in the manufacturer's directions. The RT primer (0.5 μM concentration; 5′-ATATTTGAGTGCGTA GTGCTG-3′) is the antisense sequence immediately downstream of the coding termination of the CEA-1 gene (5). PCR was carried out with 1/10 of the final volume of the cDNA reaction, a 0.2 μM concentration of a sense oligomer derived from the last 25 nucleotides of *T. cruzi* spliced leader sequence (ME-2 oligonucleotide sequence; 5′-ATCTGCAAGCTTAACGCTATTATTGATACA GTTTCTGTA-3′), and a 0.2 μM concentration of an antisense oligomer derived from the 3′ end of CEA-1 (5) (160-2 oligonucleotide sequence; 5′-CTTCTGCT CGAGTCACACCTCAGCAGAGAACCGCAGT-3′). A *Hin*dIII site was incorporated at the 5′ end of oligomer ME-2, and a *Xho*I site was included in oligomer 160-2 for subcloning. PCR was carried out for 30 cycles of 94°C for 1 min, 52°C for 2 min, and 72°C for 3 min. A 10-min extension incubation was carried out at 72°C at the end of the 30 cycles. Products were visualized by ethidium bromide staining of 1% agarose gels, and a 3.3-kb product was cut out and extracted with GeneClean (Bio 101, Inc., La Jolla, Calif.). The 3.3-kb DNA fragment was ligated into the plasmid pCRII (Invitrogen, San Diego, Calif.) at a 3:1 insert-to-vector ratio and incubated at 15°C for 18 h. One-tenth of the ligation reaction volume was then used to transform *Escherichia coli* competent cells (One-Shot; Invitrogen). One of the transformants (CRP-10) carrying a 3.3-kb insert was selected for further analysis.

**Nucleic acid sequencing.** DNA was prepared for sequencing reactions with plasmid DNA purification kits as directed by the manufacturer (Qiagen, Chatsworth, Calif.). Sequencing reactions were performed by the University of Pittsburgh Department of Molecular Genetics and Biochemistry Core Facility with the Dye Deoxy Terminator cycle sequencing kit (Applied Biosystems). Sequencing reactions contained 0.5 μg of template DNA and 3.4 pmol of primers. The sequence was confirmed on both strands. Automated sequencing was carried out with an Applied Biosystems PRISM DNA sequencer, and analysis of the DNA sequence was performed with the Genetics Computer Group sequence analysis software package from the University of Wisconsin, Madison (3).

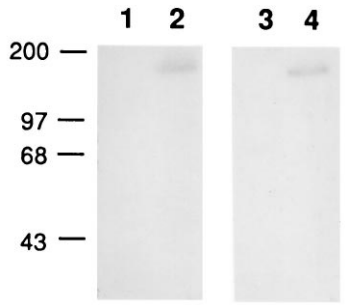


FIG. 1. Immunoprecipitation of metabolically labeled *T. cruzi* membrane protein preparations and native, C3b-purified CRP with the monoclonal antibody 3H7. *T. cruzi* membrane proteins precipitated with control monoclonal antibody UPC10 (lane 1) or 3H7 (lane 2). C3b-purified *T. cruzi* CRP immunoprecipitated with UPC10 (lane 3) or 3H7 (lane 4). Molecular mass standards (in kilodaltons) are indicated on the left.

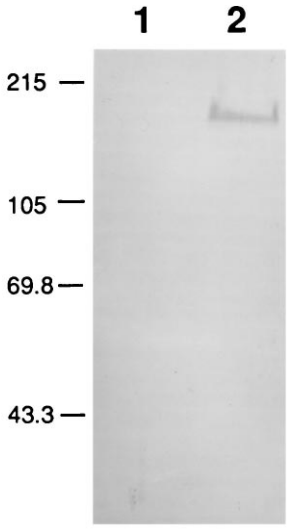


FIG. 2. Western blot analysis of detergent-solubilized membrane extracts of *T. cruzi* epimastigotes (lane 1) and trypomastigotes (lane 2) with monoclonal antibody 3H7. Molecular mass standards (in kilodaltons) are indicated on the left.

**Production of CRP-histidine fusion protein and anti-CRP antiserum.** The CRP-10 cDNA clone was partially digested with *Pst*I, and a fragment from nucleotides 295 to 3265 was isolated by gel fractionation and GeneClean as described above. The fragment, which contained the complete coding sequence for the mature protein with 4 amino acids of the signal sequence, was cloned into pTrcHis(B) vector (Invitrogen) and used to transform the *E. coli* strain SURE (Stratagene, La Jolla, Calif.). An overnight culture was diluted 1:50 in 500 ml of Luria broth (Difco) with 100 μg of carbenicillin (Sigma) per ml, grown at 37°C to the log phase, and then induced with 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and growth was continued for an additional 2 h. The cells were pelleted at 4,000 × *g* for 10 min, and the pellet was frozen. The pellet was thawed and resuspended in 1/10 of the original volume of binding buffer (20 mM sodium phosphate [pH 7.8], 500 mM NaCl, 0.05% NP-40, protease inhibitor cocktail). Lysozyme was added to 100 μg/ml, and the cells were incubated for 15 min on ice. The cells were lysed by three cycles of freezing and thawing and sonication. Insoluble material was removed by centrifugation at 13,000 × *g* for 15 min at 4°C. The lysate was then incubated with 5 ml of ProBond $Ni^{2+}$ resin (Invitrogen) at room temperature for 1 h. The resin was transferred to a column and washed with 10 bed volumes of binding buffer and then with 20 bed volumes of wash buffer (20 mM sodium phosphate [pH 6.0], 500 mM NaCl, 0.05% NP-40, and protease inhibitors, as indicated above). The recombinant protein was eluted by applying a step gradient of 50 to 350 mM imidazole in wash buffer without NP-40 or protease inhibitors. Each fraction was run on an SDS–8.5% polyacrylamide gel, and the protein in the relevant fractions was quantitated (Bio-Rad). Female BALB/c mice were immunized intraperitoneally with 40 μg of protein emulsified in complete Freund's adjuvant, followed by two boosts of 40 μg of protein emulsified in incomplete Freund's adjuvant, each at 18-day intervals. Twelve days after the last immunization, the mice were bled and serum was collected.

**Nucleotide sequence accession number.** The nucleic acid sequence of the *T. cruzi* CRP-10 cDNA has been submitted to GenBank and assigned the accession number U59297.

## RESULTS

**Generation and characterization of monoclonal antibodies to the 160-kDa CRP.** Hybridomas were prepared from the spleens of chronically infected mice, and antibody-producing cell lines were initially tested with trypomastigote membrane proteins as the detecting antigens. Hybridomas producing antitrypomastigote antibodies were further screened by immunoprecipitation to determine the size of the target antigen. One monoclonal antibody, 3H7, specifically precipitated a protein of 160 kDa from trypomastigote membrane preparations (Fig. 1) and was chosen for further characterization. To determine if the reactivity of the 3H7 monoclonal antibodies was specific for the CRP, 3H7 was used to immunoprecipitate C3b affinity-purified CRP. In these experiments, metabolically labeled CRP was purified to homogeneity from detergent-solubilized trypomastigote membrane extracts on a human C3b affinity matrix as described previously (13). CRP was immunoprecipitated with 3H7 monoclonal antibodies, whereas no cross-reactivity was detected with an unrelated, isotype-matched monoclonal antibody (Fig. 1). In addition, metabolically labeled trypomastigote membrane extracts were bound to a 3H7 affinity matrix resin and the unbound proteins were collected and applied to the C3b affinity column. Little additional C3b-binding protein was recovered from the C3b affinity column (data not shown), indicating that the 3H7 affinity column bound the majority of the CRP in the membrane preparations.

The CRP is a developmentally regulated protein produced by the infectious trypomastigote stage but is not detectable in the insect-stage epimastigotes (11). This expression pattern is consistent with the complement resistance characteristics of *T. cruzi* in that insect-stage epimastigotes are exquisitely sensitive to lysis by the alternative complement pathway, whereas the blood-stage trypomastigotes are completely resistant to the complement-mediated lytic properties of normal serum. The reactivity of 3H7 monoclonal antibodies was tested by Western blotting with detergent-solubilized membrane proteins from tissue culture-derived trypomastigotes and axenically cultured epimastigotes (Fig. 2). The 3H7 monoclonal antibodies detected a single band migrating at 160 kDa in trypomastigote membrane extracts, consistent with the immunoprecipitation results, whereas no protein was detected in the epimastigote preparation.

**Immunoaffinity chromatography with 3H7 monoclonal antibodies and determination of amino acid protein sequence of CRP.** 3H7 monoclonal antibodies were used to prepare an immunoaffinity column for the purification of CRP from trypomastigote detergent-solubilized membrane preparations. In several runs, approximately 2 to 5 μg of purified protein was recovered from membrane extracts prepared from $10^{10}$ tissue culture-derived trypomastigotes. The purified protein was subjected to SDS-PAGE and electroblotted to a polyvinylidene difluoride membrane, and the stained band was excised and sequenced. Automated sequence analysis of purified CRP was performed on two separate preparations of purified protein through 16 cycles of Edman degradation. Protein sequence analysis revealed that the sequence of the protein eluted from the 3H7 column most closely conformed to the predicted amino acid sequence of the family of genes described by Van

TABLE 1. Amino-terminal protein sequence of affinity-purified 160-kDa *T. cruzi* CRP and the deduced amino acids of reported FL-160–CEA gene family members

| Source | Amino acid sequence[a] | Reference |
|---|---|---|
| Clone 18 | AAAVEG**N**SE**GTIIF**KG | 5 |
| Clone 44 | TYGKEG**N**SRNG**IIF**KG | 5 |
| CEA-1[b] | CG**IIFQV** | 5 |
| FL-160-2 | TYGKEG**N**SRNG**IIFQG** | 22 |
| FL-160-5[c] | A**PTEASN**SG**KKIIFQV** | |
| CRP-10 | TYGKEG**N**SRNG**IIF**EG | This study |
| CRP[d] | X**PDEASN**X**SKLIIFQV** | This study |

[a] Amino acids common to both the deduced sequences and the peptide sequence are indicated by boldface type. X indicates that amino acid identification could not be determined.
[b] The complete 5′ coding sequence was not obtained for this clone.
[c] Direct submission to GenBank (accession number X70947).
[d] Purified protein.

Voorhis et al. (23) and Jazin et al. (5) (Table 1). This gene family encodes variable but highly homologous proteins of unknown function, reported as FL-160 and CEA (chronic exoantigen). The deduced amino acid sequence of five gene family members and the protein sequence of the immunoaffinity-purified CRP all contain an asparagine at position 7 and isoleucine-isoleucine-phenylalanine at positions 12 to 14 (Table 1). The amino-terminal sequence obtained from the purified CRP did not have similarity with any other reported *T. cruzi* protein sequence nor were any significant homologies revealed in a search of the databases translated in all six reading frames.

**Cloning of the full-length CRP cDNA.** Although the sequences of a number of gene family members have been reported, no full-length cDNA has been isolated to date (5, 22, 23). To further characterize the gene(s) encoding the CRP, we isolated a full-length cDNA by RT-PCR of trypomastigote RNA. A 3′ antisense primer downstream of the termination sequence of CEA-1 (5) was used for RT of trypomastigote mRNA. An antisense primer spanning the translational termination sequence of CEA-1 and a sense primer corresponding to the spliced leader sequence at the 5′ end of the mRNA were used for the PCR. The spliced leader sequence is a 39-nucleotide sequence which is *trans*-spliced onto the 5′ end of all *T. cruzi* mRNAs as part of the maturation process. The PCR produced a 3.3-kb product that was cloned and sequenced (Fig. 3). The CRP-10 cDNA contained 27 nucleotides of the *T. cruzi* spliced leader sequence at the 5′ end, confirming that the clone was derived from mRNA. The 5′-upstream region contained three in-frame ATGs, although only the third ATG is in the context of the Kozak consensus sequence (7) and is followed by a potential signal sequence. The protein sequence obtained from the purified CRP confirms the start of the mature protein at nucleotide 307, consistent with translational start at the third ATG at nucleotide 235. The predicted amino acid sequence of the carboxy-terminal end of CRP-10 contains a stretch of hydrophobic residues and a potential GPI anchor addition site (Gly-Asp-Ser) at position 3175.

Analysis of the predicted amino acid sequence of the clone CRP-10 revealed >80% overall homology with the reported FL-160 genes and approximately 60% homology with the CEA genes (5, 22). Multiple sequence alignment of four gene family members reveals a high degree of homology at the amino-terminal and central regions of the predicted amino acid sequence (Fig. 4). The predicted amino acid sequence of the CEA-1 gene (5) has a high degree of homology with the family members through this region but diverges significantly at the carboxy-terminal end.

Based on the amino-terminal protein sequence derived from purified CRP, we conclude that the CRP is encoded by the FL-160–CEA gene family. To support this conclusion, we tested the reactivity of polyclonal antibodies to a recombinant, full-length CRP-10 fusion protein with trypomastigote membrane preparations as well as with purified CRP (Fig. 5). In these experiments, the anti-recombinant CRP antibodies precipitated a protein from trypomastigote membrane preparations which migrated at approximately 160 kDa on reducing SDS-PAGE (Fig. 5, lane 2). In addition, C3b-purified CRP was immunoprecipitated with the antirecombinant antibodies, supporting the conclusion that the CRP-10 cDNA encodes the CRP protein (Fig. 5, lane 4).

Since the CRP gene has limited homology (~25%) with the *T. cruzi trans*-sialidase, we asked whether the immunologic cross-reactivity of antiserum to recombinant CRP or 3H7 extended to the *trans*-sialidase. In these experiments, trypomastigote membrane extracts containing *trans*-sialidase were prepared and subjected to immunoprecipitation with either anti-recombinant CRP antiserum, 3H7 monoclonal antibodies, or sera from mice chronically infected with *T. cruzi*. Isotype-matched monoclonal antibodies (UPC10) or normal mouse serum was used as the control. Neither the anti-recombinant CRP antiserum nor the 3H7 monoclonal antibodies precipitated sialidase activity, whereas antisera from chronically infected mice precipitated between 40 and 80% of the activity present in the membrane preparations (data not shown).

**Immunofluorescence and surface localization of CRP.** Anti-recombinant CRP antiserum was used in indirect immunofluorescence assays of trypomastigotes to characterize the surface localization of the CRP protein. Uniform surface staining of the parasites with the anti-recombinant CRP antibodies was observed (Fig. 6), supporting previous results with antibodies to parasite-derived CRP (11).

## DISCUSSION

We have developed an immunoaffinity purification procedure for the recovery of the 160-kDa *T. cruzi* CRP. This protein has been shown previously to restrict the assembly and stability of human complement C3 convertases (10, 14) and therefore provides trypomastigotes with a means of avoiding clearance by the complement cascade. The 3H7 monoclonal antibody specifically recognizes the C3b-purified CRP and does not cross-react with other trypomastigote or epimastigote membrane proteins, as determined by immunoprecipitation and Western blotting. Absorption of trypomastigote membrane preparations on a 3H7 immunoaffinity matrix removed all detectable C3b-binding protein, and 3H7 antibodies did not precipitate functional *trans*-sialidase, indicating that these two activities are distinct. The 3H7 affinity column provides an additional means of protein purification, which had only been possible by affinity chromatography with human C3b (10, 14). Although both immunoaffinity and C3b affinity chromatography yield comparable amounts of protein, use of the 3H7 monoclonal antibody reduces the need for large amounts of highly purified human C3b for larger-scale preparations of CRP.

Protein sequencing of the affinity-purified CRP reveals that it is encoded by a member of a large gene family previously described by Van Voorhis (22) and Jazin (5). Van Voorhis and colleagues described the FL-160 gene family which encoded trypomastigote-specific proteins of 160 kDa with at least five different members actively transcribed, and over 750 genomic

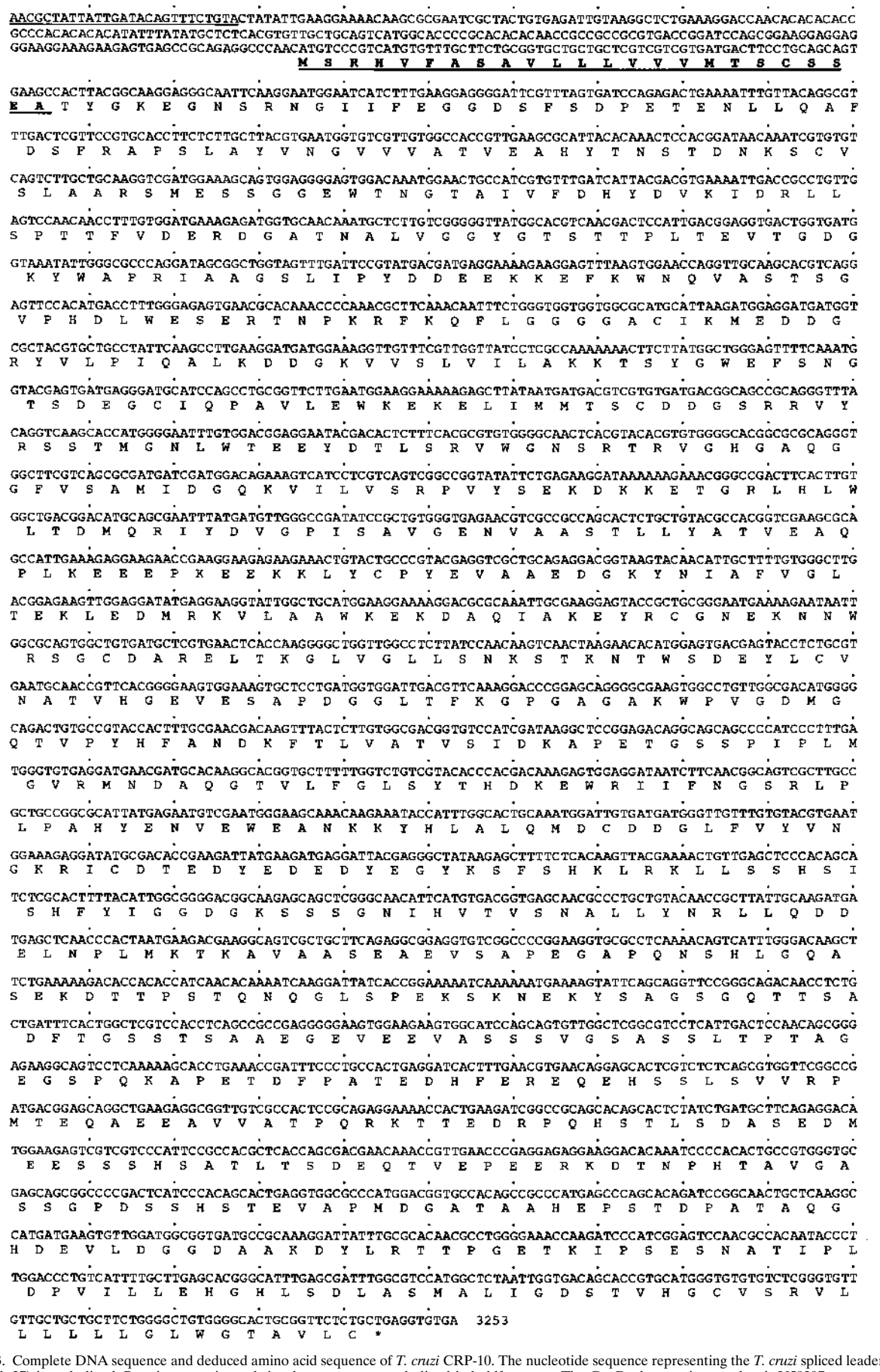

FIG. 3. Complete DNA sequence and deduced amino acid sequence of *T. cruzi* CRP-10. The nucleotide sequence representing the *T. cruzi* spliced leader sequence (1 through 27) is underlined. Putative start site and signal sequence are underlined in boldface type. The GenBank accession number is U59297.

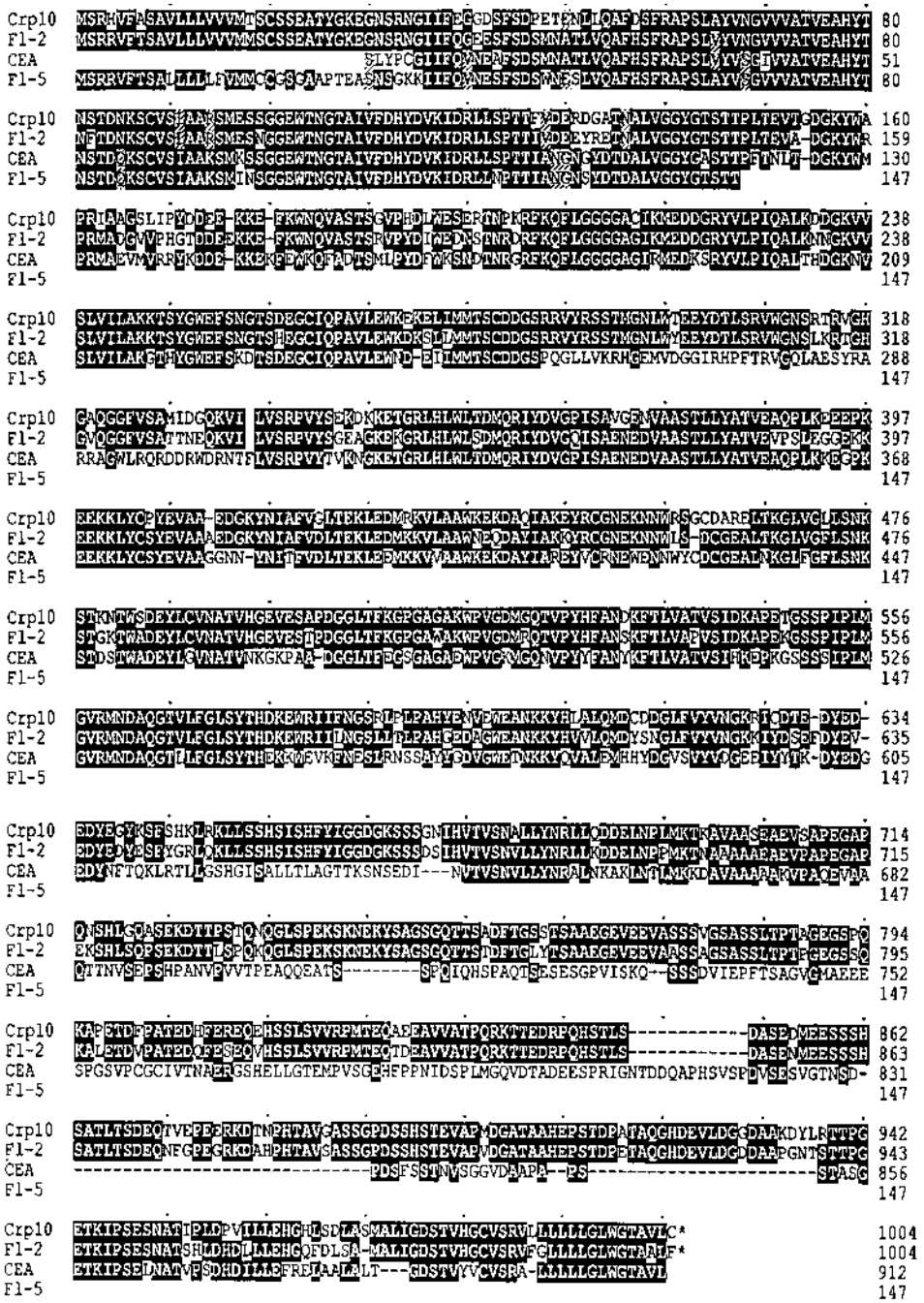


FIG. 4. Multiple sequence alignment of deduced amino acid sequences of CRP-10 and members of the FL-160–CEA gene family, i.e., FL-2 and FL-5 (FL-160-2 and FL-160-5) (22) and CEA (5). Spaces were added to maintain the best alignment. Alignment was performed with the Multiple Alignment Editor (version 1.64).

copies, although the function of the protein was not determined (22). The deduced amino acid sequences of FL-160 contained a conserved carboxy-terminal 12-amino-acid motif which elicited antibodies that were cross-reactive with neural tissue, indicating a possible role in the induction of autoantibodies in Chagas' disease (23). Jazin et al. identified a related gene, CEA-1, with approximately 60% overall homology to the FL-160 gene family at the amino acid level and highly conserved amino-terminal and central regions but a divergent carboxy-terminal end (5). The deduced amino acid sequence of the carboxy-terminal region of CEA-1 did not contain the 12-amino-acid motif associated with immunologic cross-reactivity to neural tissue present in the FL-160 genes (23) and thus may represent a related subfamily. Since the CEA-1 gene fragment was isolated from a genomic library, it remains to be determined whether the CEA clones represent transcribed copies.

Although a number of cDNA and genomic copies of FL-

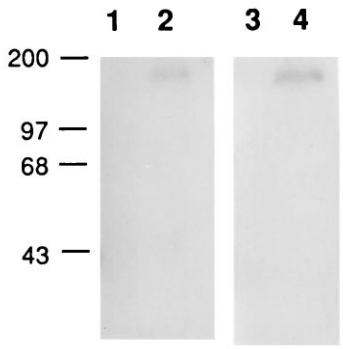


FIG. 5. Immunoprecipitation of metabolically labeled *T. cruzi* membrane protein preparations and native, C3b-purified CRP with anti-recombinant CRP antiserum. *T. cruzi* membrane proteins precipitated with normal mouse serum (lane 1) or anti-recombinant CRP antiserum (lane 2). C3b-purified *T. cruzi* CRP immunoprecipitated with normal mouse serum (lane 3) or anti-recombinant CRP serum (lane 4). Molecular mass standards (in kilodaltons) are shown on the left.

160–CEA gene family members have been characterized, no full-length cDNA has been isolated previously (5, 22, 23). By use of a conserved 3′ sequence of known family members and the *T. cruzi* 5′-spliced leader sequence, we generated a full-length cDNA clone by RT-PCR. Antibodies raised against the recombinant CRP-10 protein cross-reacted with a 160-kDa trypomastigote membrane protein as well as with native, parasite-derived CRP (Fig. 5). A comparison of the amino-terminal peptide sequences derived from purified CRP and the deduced peptide sequence of the CRP-10, FL-160, and CEA-1 clones (Table 1) indicates high variability through the first 16 amino acids of the mature protein, although one clone, FL 160-5, had 11 amino acids in common with the sequence derived from the purified protein. These results indicate that trypomastigotes may express multiple isoforms of CRP. The multiple sequence alignment of CRP-10, CEA-1, and two members of the FL-160 family shows very high levels of amino acid identity over the lengths of the clones, supporting the conclusion that they represent multiple variants of a single gene family (Fig. 4). Taken together, the immunologic cross-reactivity of antibodies to native CRP and the peptide and nucleic acid sequence data support the conclusion that the CRP-10 clone encodes a variant of the trypomastigote CRP.

The amino-terminal peptide sequence obtained from the purified protein identifies the mature protein as beginning at nucleotide 307 of CRP-10. The predicted translation from the

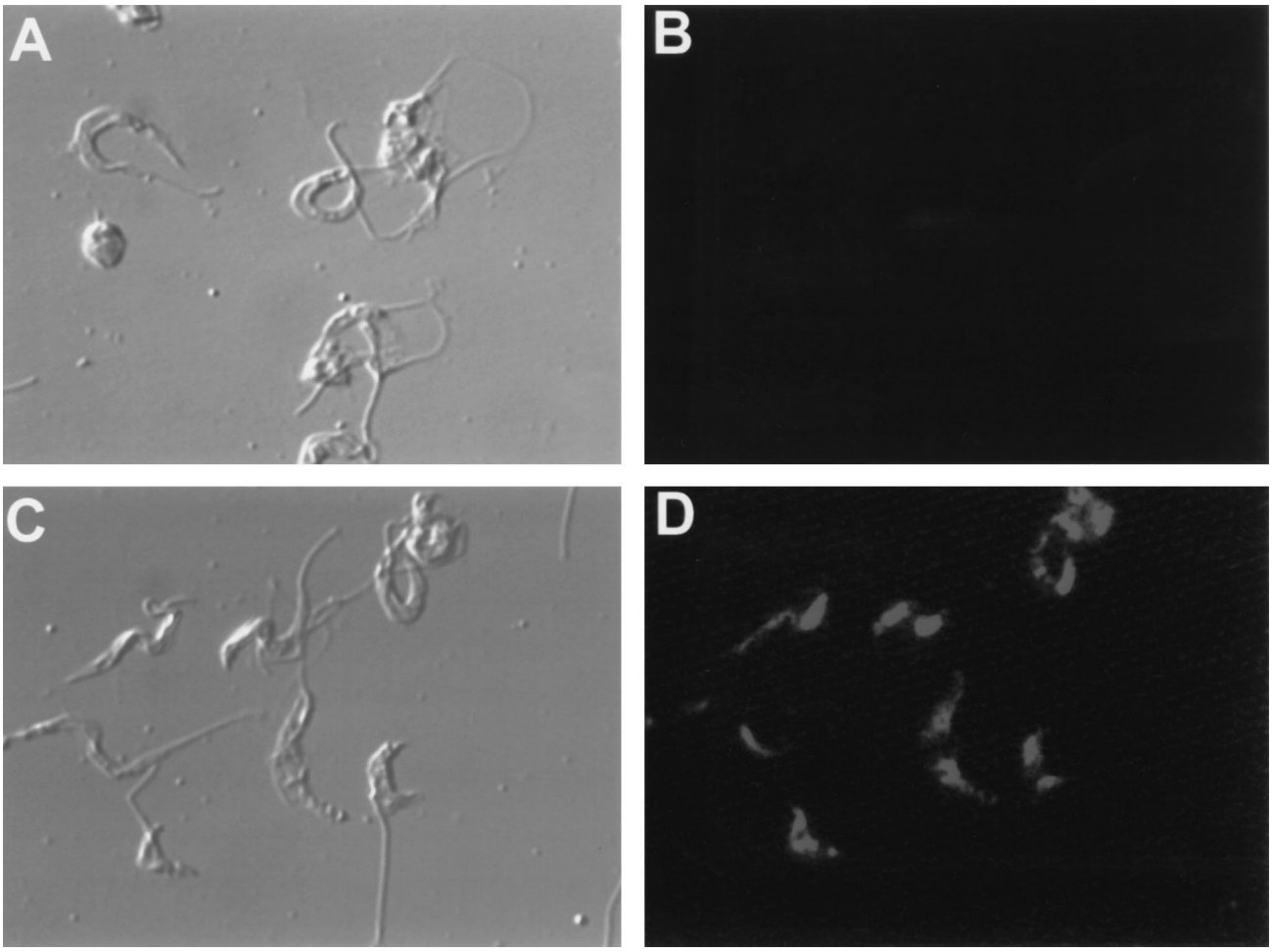


FIG. 6. Indirect immunofluorescence and cell surface localization of *T. cruzi* CRP. Tissue culture-derived trypomastigotes were fixed in paraformaldehyde and incubated with either normal mouse serum (A and B) or anti-recombinant CRP antiserum (C and D) (1:200) and Cy-3 Fluorolink-conjugated goat anti-mouse immunoglobulin G (1:3,000). (A and C) Nomarski differential interference contrast images; (B and D) fluorescence images corresponding to panels A and C, respectively.

third ATG (at nucleotide 235) identified a putative 24-amino-acid signal sequence which conforms to eukaryotic consensus sequences and is consistent with the peptide sequence data regarding the amino-terminal end of the mature protein. This ATG is also the only one in the context of the Kozak consensus sequence (7). The predicted translation from the first in-frame ATG would encode 38 amino acids which do not conform to any known signal sequence consensus. In addition, in contrast to the third ATG, the sequence of the nucleotides surrounding the first ATG does not conform to the Kozak consensus sequence. These results suggest that the third in-frame ATG at nucleotide 235 is most likely the translation initiation site.

Similar to other trypanosome genes characterized to date, the predicted carboxy-terminal portion of the protein contains a hydrophobic region, with a putative glycolipid anchor attachment sequence, most likely Gly-Asp-Ser, 27 amino acids from the carboxy-terminal end. This is consistent with our previous demonstration that CRP is anchored to the parasite membrane via a GPI anchor (10, 14). The open reading frame of the CRP-10 corresponding to the mature protein, beginning at nucleotide 307, predicts a protein of 979 amino acids with a molecular mass of 106,039 kDa. We have previously shown that deglycosylated CRP devoid of the GPI anchor has an apparent molecular mass of 145 kDa on SDS-PAGE reducing gels (14). This disparity in predicted size and apparent size based on migration in SDS gels was also seen with the full-length *E. coli* CRP fusion protein, which has a predicted size of 106,584 kDa and an apparent molecular weight of 145,000 based on migration in SDS-PAGE gels (data not shown).

The CRP gene and members of this family have limited homology with the family of *T. cruzi* genes encoding a *trans*-sialidase. The *trans*-sialidase exhibits size heterogeneity based on the number of carboxy-terminal amino acid repeats and can range in apparent molecular mass between 100 and >220 kDa (18). The deduced amino acid sequence of the CRP gene and FL-160 gene family members have ~25% homology with the *trans*-sialidase, although no immunologic cross-reactivity was detected with either the 3H7 monoclonal antibodies or the anti-recombinant CRP antiserum. These results confirm that the CRP and the *trans*-sialidase are distinct glycoproteins.

Indirect immunofluorescence of fixed, nonpermeabilized trypomastigotes with anti-recombinant CRP antiserum revealed uniform surface staining (Fig. 6). These results are consistent with its role in restriction of complement activation. Surface localization of the CRP has been demonstrated previously with antibodies to parasite-derived CRP (11), and these results are consistent with those of Jazin et al., who demonstrated uniform surface labeling with anti-recombinant CEA antibodies (5). In contrast, Van Voorhis and colleagues reported that the FL-160 protein was localized to the flagellum and flagellar pocket of trypomastigotes (22). In those studies, immunofluorescence assays were carried out on live parasites and the staining pattern observed may reflect extensive patching and capping, as has been described previously for *T. cruzi* (20, 21). In the present study, parasites were fixed prior to incubation with antiserum.

The activation of classical and alternative complement pathways by microorganisms is an important immune effector mechanism which contributes to the elicitation of the inflammatory response, enhanced phagocytosis, and the assembly of cytolytic components on microbial surfaces. Complement activation is regulated in the host by several membrane-bound and soluble plasma glycoproteins, thereby limiting local damage to autologous cells due to an inflammatory response. One subset of host complement regulatory glycoproteins noncovalently binds C3b and C4b, thus preventing the proper assembly and stability of the alternative and classical pathway convertases. In addition to the *T. cruzi* CRP, a number of viral and eukaryotic pathogens have been shown to restrict complement activation by the production of complement regulatory molecules, which function in a similar manner as the host counterparts (12). These molecules likely play an important role in the overall immune evasion strategies of these pathogens and thus may represent useful targets for vaccine development. The identification of the gene family encoding the *T. cruzi* CRP and the isolation of a full-length cDNA should allow further investigation into the precise nature of the interaction of CRP with the complement components C3b and C4b, thus providing a more complete understanding of the mechanism of complement restriction by the trypomastigotes.

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