gp72, THE 72 KILODALTON GLYCOPROTEIN, IS THE MEMBRANE ACCEPTOR SITE FOR C3 ON *TRYPANOSOMA CRUZI* EPIMASTIGOTES

BY KEITH JOINER,* SARA HIENY, LOUIS V. KIRCHHOFF, AND ALAN SHER

*From the *Laboratory of Clinical Investigation and the Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205*

Trypanosoma cruzi, the protozoan parasite which causes Chagas' disease, has a complex life cycle that involves both insect and mammalian hosts. The epimastigote (Epi)[1] stage of *T. cruzi,* which multiplies in the gut of the vector, is rapidly lysed in human serum by means of alternative complement pathway activation (1). In contrast, the infective metacyclic trypomastigote stage, the amastigote stage, which multiplies intracellularly in the vertebrate host, and the bloodstream trypomastigote form are not lysed when incubated in human serum (1, 2). This type of adaptation to the mammalian host is commonly observed with protozoan parasites, but little is known about the mechanism by which these parasites are transformed from activators to nonactivators of the alternative pathway. The membrane molecules on the Epi stage of *T. cruzi* that activate the alternative complement pathway have not been identified. Similarly, the membrane molecules on vertebrate forms that prevent alternative complement pathway activation are not characterized. Although a trypsin-sensitive surface protein or glycoprotein apparently blocks alternative complement pathway activation in bloodstream trypomastigotes (2), the identity of this molecule(s) is unknown. By studying the interaction of complement proteins with the surface of *T. cruzi,* we hope to elucidate the mechanism by which vertebrate life cycle forms evade lysis by the alternative complement pathway.

Complement component C3 is a pivotal molecule in complement activation and complement biology. C3 is cleaved during both classical and alternative pathway activation, generating the fragments C3a and C3b. The larger C3b fragment binds to the activating surface, and in so doing may serve as an opsonic molecule for cells bearing C3b receptors. Alternatively, C3b may function with

Address correspondence to K. Joiner, at the Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Building 10, Room 11N-208, Bethesda, MD 20205.

[1] *Abbreviations used in this paper:* CMT, metacyclic trypomastigote; C8D, deficient in C8; Epi, epimastigote; DPBS, Dulbecco's phosphate-buffered saline; Δ56 C8D, C8D serum, heated to 56°C for 30 min; HBSS, Hanks' balanced salt solution with 0.15 mM $CaCl_2$ and 1.0 mM $MgCl_2$; LB, lysis buffer; LIT, liver-infusion tryptose broth; NHS, normal human serum; NPGB, nitrophenyl guanidino benzoate; PAGE, polyacrylamide gel electrophoresis; RT, room temperature; SDS, sodium dodecyl sulfate.

earlier components of the cascade to form the classical or alternative pathway C5 convertase (3, 4), a necessary step for the generation of C5b and the lytic C5b-9 complex. Finally, depending on the nature of the activating surface, C3b may be cleaved further to iC3b or C3dg, fragments that can no longer participate in C5 convertase formation. Thus, it is apparent that C3 plays a central role in determining the outcome of the interaction between complement and activating particles. In this regard, determination of the molecular site of C3b deposition (the C3 acceptor) and the nature of the bond between C3b and the activating surface may provide important clues to the fate of particles bearing C3b.

We therefore sought to identify those surface molecules on the complement-activating stage of *T. cruzi* to which C3b attaches during incubation in fresh serum. Using a recently developed (5) method for determining the membrane acceptor site for C3 deposition, we show here that gp72, a major developmentally regulated surface glycoprotein (6–12), is the preferential acceptor for C3 deposition on *T. cruzi* Epi during alternative pathway activation in nonimmune serum. In contrast, we find that minimal deposition of C3 occurs when metacyclic trypomastigotes (CMT) produced in vitro are incubated in serum, and that gp72 on CMT is a poor acceptor for C3. This is the first study to elucidate the C3 acceptor molecule on a protozoan parasite.

Materials and Methods

Buffers. The following buffers were used in these experiments: Hanks' balanced salts solution containing 0.15 mM $CaCl_2$ and 1.0 mM $MgCl_2$ (HBSS); lysis buffer (LB), containing 0.05 M Tris, 100 mM NaCl, 2% Nonidet P-40, and 0.5% sodium deoxycholate (pH 8.2); low ionic strength ($\mu = 0.060$) Veronal-buffered saline containing 0.1% gelatin, 5% dextrose, 0.15 mM $CaCl_2$, and 1.0 mM $MgCl_2$; Eagle's minimal essential medium; and Dulbecco's phosphate-buffered saline (DPBS), pH 7.2.

T. cruzi Strains. The Tulahuen strain (13) and the Miranda 88(M88) clone (14) of *T. cruzi* were obtained from J. Dvorak (NIH, Bethesda, MD). Epi of these isolates were maintained by serial passage in liver-infusion tryptose broth (Oxoid, Basingstoke, Hants, Great Britain) containing 10% (vol/vol) fetal calf serum, 0.02 mg/ml hemin, 100 U/ml penicillin, and 100 μg/ml streptomycin (LIT medium) (15). Parasites were grown at 26°C and passaged every 7–10 d. Epi in the logarithmic growth phase ($<2 \times 10^7$ Epi/ml) were used in all experiments.

CMT were produced in vitro using a modification of the method of Sullivan (16). Briefly, Epi of the M88 clone were grown in LIT medium and harvested at concentrations $<7 \times 10^7$ Epi/ml. The Epi were washed twice in DPBS and resuspended at 7×10^6 Epi/ml in Grace's insect medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum and 25 mM Hepes, and adjusted to pH 6.6. 40-ml volumes of the parasites were then maintained at 28°C in flasks with 75-cm^2 surface area for 6–8 d, at which time 30–55% CMT were observed.

CMT were separated from Epi using an adaptation of the procedure of Lanham and Godfrey (17). The parasite mixture was washed three times in a phosphate-buffered saline solution containing 5.4% glucose (ionic strength 0.217 osM, pH 8.0), and passed through a DE52 column (Whatman, Inc., Clifton, NJ) 1 cm high, set in the base of a 10-ml glass pipette. ~4 ml of the washed parasites, at 10^8 parasites/ml were passed through each of these columns, and typically 5–20% of input CMT were recovered in a solution containing 95% CMT.

Epi and CMT were surface-iodinated with [^{125}I]Na (New England Nuclear, Boston, MA) and Iodogen (Pierce Chemical Co., Rockford, IL), using a modification of the method of Howard et al. (18), as previously described (6). After labelling, parasites were

noted to be intact and motile in all instances. Specific activity was generally 10^7 cpm for 10^8 parasites.

Complement Source. Normal human serum (NHS) serum was obtained from nine healthy adult volunteers, pooled, and frozen in multiple aliquots at −70°C. Serum was obtained from a patient with congenital deficiency of C8 (C8D). For some experiments, C8D serum was absorbed with Epi before use, to remove any natural anti–*T. cruzi* antibody. 4 ml C8D serum was incubated at 0°C for 30 min with 5×10^8 Tulahuen Epi, then the Epi were removed by centrifugation at 2,000 *g* for 15 min. C8D serum absorbed in this fashion lost <15% of starting C3 titer, as measured by hemolytic assay (see below). For some experiments, C8D serum was chelated with 10 mM EGTA and 2 mM $MgCl_2$, to limit complement activation to the alternative complement pathway. C8D serum for control tubes was heated at 56°C for 30 min (Δ56 C8D) to block complement activation.

Antibodies. Monoclonal antibody WIC 29.26 (IgG2a) was used for some immunoprecipitation experiments. WIC 29.26 recognizes an epitope within the carbohydrate portion of gp72 on Epi of all strains and clones of *T. cruzi* (7). WIC 29.26 was purified from ascites by absorption and elution on protein A–sepharose. Rabbit antiserum against gp72 was prepared by immunizing rabbits with purified gp72, as described previously (7).

Goat IgG anti–human C3 was prepared as previously described (19), and coupled to Sepharose 4B. Antibodies coupled to anti-C3-sepharose recognized determinants on native human C3, and on all membrane-bound breakdown products of C3 (iC3b and C3dg) generated during complement activation.

Purification and Radiolabelling of C3. C3 was purified to homogeneity using minor modifications of the method of Hammer et al. (19), as previously described (20). C3 was radiolabeled with [^{3}H]sodium borohydride (New England Nuclear), by the process of reductive methylation, to sp act 1.17×10^5 dpm/μg. The hemolytic titers of purified C3 and of C3 in serum were measured essentially as described by Gaither and Frank (21).

Complement-mediated Lysis Assay. Lysis of Epi and CMT by NHS was measured. Log-phase Epi and CMT, purified as described above, were washed, and suspended in Eagle's minimal essential medium. NHS was added to achieve a final concentration of 1.6–50%. The final parasite concentration was 5×10^6 Epi or CMT/ml. Mixtures were incubated for 60 min at 37°C. Motile organisms were then counted in a hemacytometer, and results were compared to the numbers found for cultures containing NHS that had been heated for 60 min at 56°C to block complement activation.

Preparation of C3 Acceptor from ^{125}I-Epi and ^{125}I-CMT. Surface-iodinated parasites were washed twice in DPBS and suspended at 10^8 parasites/ml in HBSS. Parasites were then mixed vol/vol with C8D serum or Δ56 C8D serum, and the mixtures were incubated at 37°C for 45 min with periodic agitation. Parasites were washed three times in HBSS containing 25 μM serine esterase inhibitor *p*-nitrophenylguanidino benzoate (NPGB) (Sigma Chemical Co., St. Louis, MO). The parasite pellet was solubilized either by boiling in 1.0% sodium dodecyl sulfate (SDS) at 100°C for 5 min, or by rotation for 16 h at 4°C in LB containing 25 μM NPGB. The detergent-insoluble pellet (10.2 ± 6.1% of total counts in six experiments) was removed by centrifugation at 12,500 *g* for 5 min. Supernatant samples in 1% SDS were diluted 1:25 in NPGB-LB to achieve a final SDS concentration of 0.04%.

Supernatant samples were precleared by rotation for 1 h at room temperature (RT) with 0.4 ml of packed Sepharose 4B per milliliter of supernatant. This resulted in removal of 4–7% of total ^{125}I counts. Precleared supernatants were then applied to anti-C3 coupled to Sepharose at 0.4 ml of packed resin per milliliter, and rotated at RT for 3 h. Anti-C3–sepharose was washed five times in NPGB-LB, or until wash supernatants contained <0.1% of total applied ^{125}I counts. In preliminary experiments, >89% of C3 in supernatant samples bound to anti-C3–sepharose under these conditions.

Anti-C3–sepharose bearing ^{125}I-Epi or -CMT protein was then handled in two separate ways. To determine the extent of covalent attachment of ^{125}I-labeled Epi or CMT protein to C3, the anti-C3–sepharose resin bearing C3 and bound ^{125}I was boiled in SDS sample buffer, and applied to a 7.5–15% SDS-polyacrylamide gel, as described below. To determine the identity of the C3 acceptor molecule by SDS–polyacrylamide gel electro-

phoresis (SDS-PAGE), it was necessary to cleave the bond between C3 and its acceptor molecule. Oxyester linkages were cleaved by incubation of anti-C3–sepharose bearing ^{125}I-Epi or -CMT protein in 1 M NH_2OH in NPGB-LB (pH 10.5) at 37°C for 30 min. The supernatant was dialyzed overnight at 4°C against absolute methanol to precipitate released protein. The precipitate was pelleted, suspended in SDS–sample buffer, and analyzed by SDS-PAGE.

Preparation of Antiserum Against the C3 Acceptor on Tulahuen Epi. Antiserum was raised against the C3 acceptor molecule on Tulahuen Epi. Unlabelled Epi (5×10^7 Epi/ml) were incubated in 50% C8D or Δ56 C8D exactly as described above. Washed parasites were solubilized in 1% SDS at 100°C for 5 min, diluted 1:25 in NPGB-LB, and rotated with anti-C3–sepharose, as outlined. Anti-C3–sepharose was washed five times in DPBS, then used for immunization of rabbits. The animals initially received subcutaneous injection of a 1:1 mixture of 50% Sepharose in PBS with complete Freund's adjuvant; they were boosted every 2 wk with a 1:1 mixture in incomplete Freund's adjuvant. The rabbits were bled 2 wk after the last immunization (yielding anti-acceptor serum). Control animals were immunized with anti-C3–sepharose that had previously been incubated with lysates from Epi incubated in Δ56 C8D serum (Δ56 anti-acceptor).

Immunoprecipitation of Labeled T. cruzi Components. 5×10^7 ^{125}I-Tulahuen Epi were solubilized in 1 ml of lysis buffer at 4°C for 16 h. The detergent-insoluble residue was removed by centrifugation. After preadsorption with Sepharose 4B at 4°C for 1 h, either 10 μl of W1C 29.26, 10 μl of anti–C3 acceptor antiserum, or 10 μl of antiserum against gp72 were added per milliliter of supernatant. Controls for immunoprecipitation experiments included normal rabbit serum and Δ56 anti-acceptor serum. The mixtures were incubated for 2 h at 4°C, then 0.05 ml of protein A–sepharose was added per milliliter of supernatant. Incubation was continued for 1 h at 4°C, after which the protein A–sepharose was washed five times at RT in LB, and bound ^{125}I-labeled protein was released and prepared for SDS-PAGE by boiling in SDS sample buffer.

Immunoblotting of Tulahuen Epi. 4×10^8 Tulahuen Epi were solubilized at 100°C in 200 μl 1% SDS containing 25 μM NPGB. The detergent-insoluble residue was removed by centrifugation at 12,500 *g* for 3 min. Protein content of the soluble supernatant was determined (22) and 15 μg of protein was applied to each lane of a 10% SDS-polyacrylamide gel. Following electrophoresis, samples were transferred to nitrocellulose (Millipore, Beford, MA) as described previously (23). Nitrocellulose strips were incubated with 2% bovine serum albumin, then developed with a 1:200 dilution of either anti-acceptor, Δ56 anti-acceptor, anti-gp72, or an antiserum raised against whole Tulahuen Epi, kindly provided by Dr. Vera Bongartz. Finally, nitrocellulose strips were incubated with 2×10^5 cpm/ml ^{125}I–protein A (New England Nuclear), and autoradiography was performed on the dried strips.

SDS-PAGE. 7.5–15% SDS-polyacrylamide gels were prepared using Acrylade (FMC Corp., Rockland, ME) mounting, as previously described (7). Samples were electrophoresed at 30 mA/gel, until the tracking dye entered the gradient gel, then at 50 mA/gel thereafter. Gels were stained with Coomassie brilliant blue and dried overnight in a dry 37°C oven. Autoradiography was performed with XAR-5 film (Eastman Kodak Co., Rochester, NY) with Lightning Plus Intensifying Screens. Molecular mass markers (Bio-Rad Laboratories, Richmond, CA) were: myosin (200 kilodaltons [kD]), β-galactosidase (116.3 kD), phosphorylase B (97.4 kD), bovine serum albumin (66.2 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), soybean trypsin inhibitor (21.5 kD), and lysoszyme (14.4 kD).

Results

Consumption and Deposition of C3 by Epi and CMT During Incubation in Serum. In initial experiments, we confirmed previous observations that Epi are lysed during incubation in NHS, whereas CMT are resistant to lysis at all serum concentrations (Fig. 1). To determine whether this difference was a reflection of the extent of complement activation, we compared deposition of C3 on Epi and

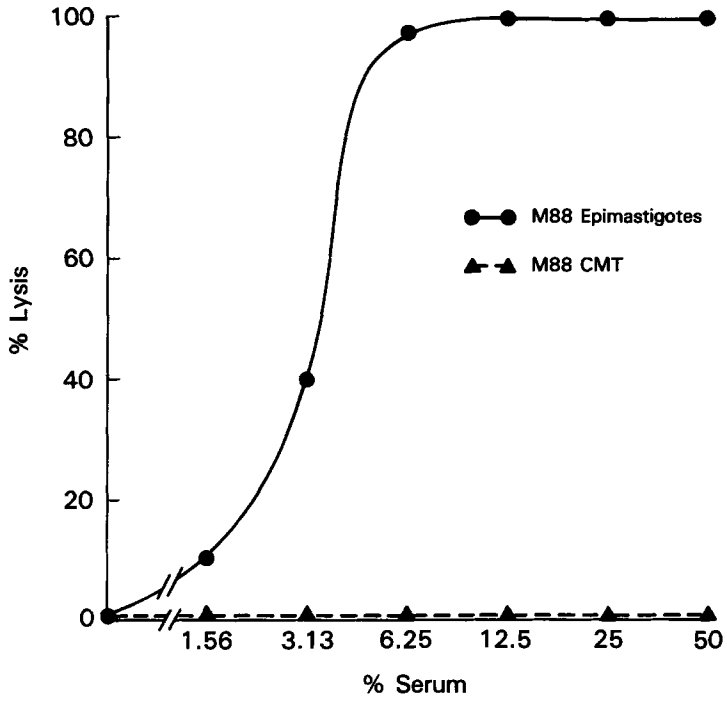

FIGURE 1. Epi, but not CMT, of M88 are lysed by NHS. Epi and DEAE-purified CMT of M88 were incubated at 5×10^6 parasites/ml in increasing concentrations of NHS for 60 min at 37°C. Lysis was assessed by visual microscopy of motile organisms.

CMT during incubation in C8D serum. Five- to six-fold more C3 was bound to M88 Epi than to M88 CMT (Fig. 2).

Binding of ^{125}I-labeled Epi and CMT Protein to Anti-C3–sepharose. Binding of ^{125}I-labeled Epi protein to anti-C3–sepharose after serum incubation was quantitated for two *T. cruzi* isolates (Table I). >15% of applied ^{125}I-Epi bound to anti-C3–sepharose, for both the Tulahuen and M88 strains, after incubation in 50% C8D serum at 2.5×10^7 parasites/ml. The binding was lower for parasites incubated in 50% C8D at 5×10^7 parasites/ml, possibly reflecting less total C3 bound per parasite at the higher concentration. Nonspecific binding of ^{125}I-Epi constituents to anti-C3–sepharose was significantly lower ($1/23$–$1/25$ of total binding) for parasites initially incubated at 5.0×10^7 parasites/ml than at 2.5×10^7 parasites/ml ($1/12$ of total binding). Therefore, a concentration of 5×10^7 parasites/ml was used for all subsequent experiments.

In contrast to ^{125}I-labeled Epi protein, only 2.64 ± 0.57% of applied ^{125}I-labeled CMT protein bound to anti-C3–sepharose for parasites incubated in 50% C8D serum. Nonspecific binding for control ^{125}I-CMT incubated in Δ56 C8D was 0.59 ± 0.31%. These results indicate that the fraction of ^{125}I-CMT proteins bearing C3 after serum incubation is only $1/4$–$1/5$ that of ^{125}I-Epi protein.

C3 Attaches to ^{125}I-labeled Surface Molecules During Antibody-independent Alternative Complement Pathway Activation. We next determined the complement and

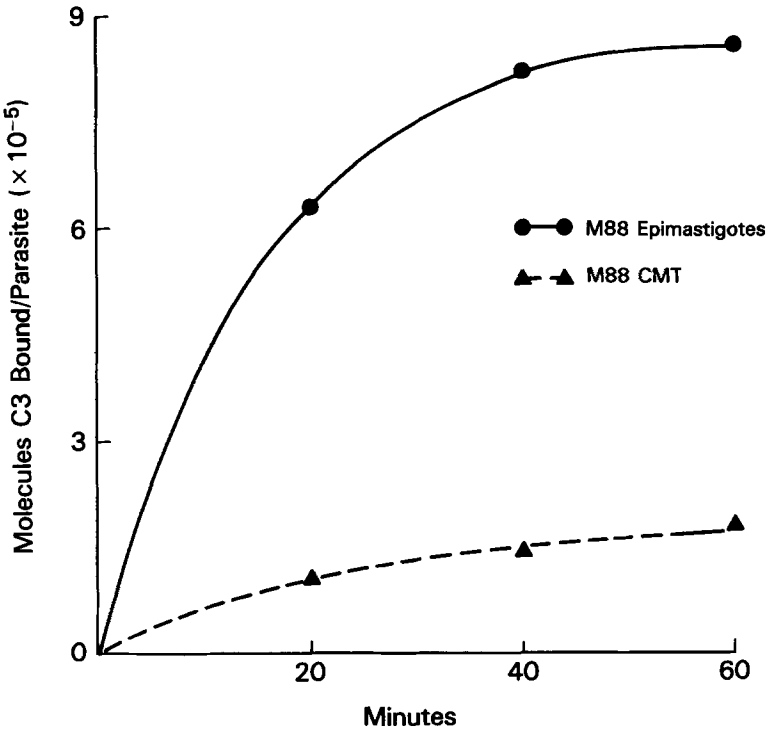

FIGURE 2. CMT bind C3 poorly in comparison to Epi during incubation in serum. Epi and CMT of M88 were incubated at 5×10^7 parasites/ml in 10% C8D containing [^{3}H]C3. Molecules of C3 specifically bound per parasite were determined as described in Materials and Methods.

antibody requirements for C3 deposition. ^{125}I-Epi were incubated in C8D serum that had been preabsorbed with Epi at 0°C to remove natural antibody, or in C8D serum chelated with Mg^{++} EGTA to limit complement activation to the alternative pathway. In both cases, specific binding of ^{125}I-Epi protein was equivalent to that in nonabsorbed, nonchelated C8D serum (Table I). This result indicates that attachment of C3 to ^{125}I-surface molecules occurs via the alternative complement pathway by an antibody-independent mechanism.

Covalent Binding of C3 to ^{125}I-labeled Tulahuen Epi. Surface-labeled Tulahuen Epi contain six major iodinated bands on SDS-PAGE, ranging from 37 kD to 118 kD, daltons (37 kD, 57 kD, 72 kD, 89 kD, 100 kD, and 118 kD) (Fig. 3). More heavily exposed autoradiograms revealed additional 20- and 210-kD bands. The identity of gp72 within native, surface-iodinated Epi is indicated by immunoprecipitation with WIC 29.26, revealing a single band that comigrates with a constituent in native Epi. ^{125}I-Epi protein that had bound specifically to anti-C3–sepharose migrated as a single broad band of 170–250 kD. Native ^{125}I-labeled Epi proteins were not observed. The increase in molecular weight of native ^{125}I-Epi proteins bound to anti-C3–sepharose presumably represents covalent attachment of the ^{125}I-labeled proteins to C3b (186 kD), iC3b (183 kD), or C3dg (41 kD). Negligible nonspecific binding of ^{125}I-Epi protein to anti-C3–sepharose was

TABLE I

Binding of ^{125}I-labeled Epi and CMT Protein to Anti-C3–sepharose After Serum Incubation

T. cruzi strain/clone	Parasites per milliliter of 50% serum	Binding to anti-C3–sepharose*	
		50% C8D	50% Δ56 C8D
		%	
Tulahuen Epi	5×10^7 (4)‡	10.24 ± 3.77	0.41 ± 0.17
	2.5×10^7 (3)	15.69 ± 3.17	1.34 ± 0.58
	5×10^7 (2)	11.13 ± 1.64§	0.52 ± 0.26
	5×10^7 (2)	9.06 ± 2.41‖	0.30 ± 0.25
M88 Epi	5×10^7 (3)	9.20 ± 0.82	0.40 ± 0.03
	2.5×10^7 (2)	15.24 ± 2.41	1.26 ± 0.43
M88 CMT	5×10^7 (3)	2.64 ± 0.57	0.59 ± 0.31

^{125}I-labeled Epi or CMT of the indicated strain were incubated in either 50% C8D or 50% Δ56 C8D serum for 45 min. The washed parasites were then solubilized in 1% SDS, and following a preclearing step on Sepharose 4B, the percentage of applied ^{125}I that bound to anti-C3–sepharose in NPGB-LB with 0.04% SDS was determined as described in Materials and Methods.

* Mean ± SD.

‡ Number of experiments.

§ 50% absorbed C8D.

‖ 50% Mg^{++} EGTA C8D.

observed, as indicated by the relative absence of bands from parasites incubated in Δ56 C8D serum, in which complement activation cannot occur.

Identification of C3 Acceptor Molecule. The covalent linkage between C3 and its acceptor molecule on ^{125}I-labeled Epi obviates direct identification of the acceptor molecule by SDS-PAGE. The bond between C3 and its acceptor molecule is frequently an ester linkage that can be cleaved by hydroxylamine at alkaline pH (24–26). Therefore, we treated anti-C3–sepharose bearing ^{125}I-labeled Epi protein with 1 M hydroxylamine at pH 10.5. ^{125}I-Epi protein released by hydroxylamine was analyzed by SDS-PAGE. Results from three representative experiments, out of a total of seven performed, are shown in Fig. 4. In each case, five or six major bands are apparent for native Epi, and there is marked enrichment of gp72 when the hydroxylamine eluates from anti-C3–sepharose are compared with Epi lysates. Densitometric scans (Fig. 5) showed that gp72 comprised 28 ± 10% (mean ±SD) of total density in native Epi extracts, but in lanes containing hydroxylamine-treated eluates from anti-C3–sepharose, gp72 showed 85 ± 9% of the total density. As expected, very little material was seen from hydroxylamine eluates from control samples incubated in Δ56 C8D serum. Selective enrichment of gp72 was observed when Epi were incubated in absorbed C8D serum or in Mg^{++} EGTA C8D serum, indicating that gp72 is the preferential acceptor for C3 deposited by the alternative pathway in the absence of antibody. These experiments indicate that C3 attaches preferentially to gp72 during complement activation by Tulahuen Epi. The absence of significant high molecular weight material in experimental samples after hydroxylamine treatment indicates nearly complete cleavage of the covalent bond between C3 and its

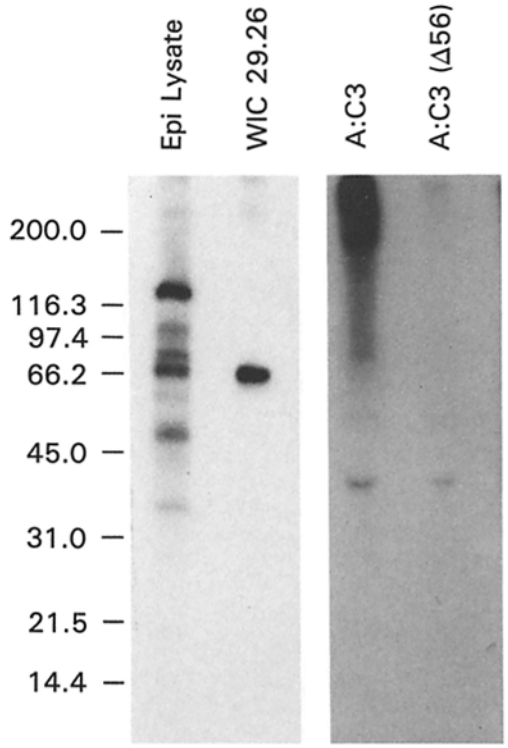

FIGURE 3. Autoradiogram of 7.5–15% SDS-polyacrylamide gel showing that C3b binds covalently to ^{125}I-labeled Tulahuen Epi protein during incubation in serum. ^{125}I-labeled Tulahuen Epi at 5×10^7 parasites/ml were incubated in either 50% C8D or 50% Δ56 C8D for 45 min at 37°C. Detergent lysates of washed parasites were applied to anti-C3–sepharose. ^{125}I-labeled Epi protein bound to anti-C3–sepharose was analyzed by SDS-PAGE autoradiography. Parasites incubated in 50% C8D are shown in the lane designated *A:C3*, those incubated with Δ56 C8D in the lane designated *A:C3* (*Δ56*). Also shown is the detergent lysate from intact ^{125}-labeled Epi (*Epi Lysate*) and the material immunoprecipitated from detergent lysates with monoclonal antibody WIC 29.26, which recognizes gp72.

acceptor molecule, and is most compatible with the hypothesis of a C3–acceptor ester linkage.

Selective enrichment of gp72 in experimental samples was not an artifact of sample processing. No such enrichment was seen when large samples of nonspecifically bound material, from either native Epi or Epi incubated in Δ56 C8D serum, were eluted from anti-C3–sepharose with hydroxylamine and examined by SDS-PAGE autoradiography. Furthermore, an average of 88.7 ± 3.1% of the ^{125}I that bound to anti-C3–sepharose was released with hydroxylamine, and >60% of released counts were recovered after dialysis against methanol.

Antiserum Against C3 Acceptor Immunoprecipitates and Immunoblots gp72. We wished to confirm, by another method, that gp72 was the acceptor molecule for C3 deposited on Epi. Antiserum against the C3 acceptor molecule was prepared as described in Materials and Methods, and was used for immunoprecipitation of ^{125}I-labeled Tulahuen Epi surface protein. This antiserum precipitated exclusively gp72 from detergent lysates of ^{125}I-labeled Tulahuen Epi (Fig. 6). In

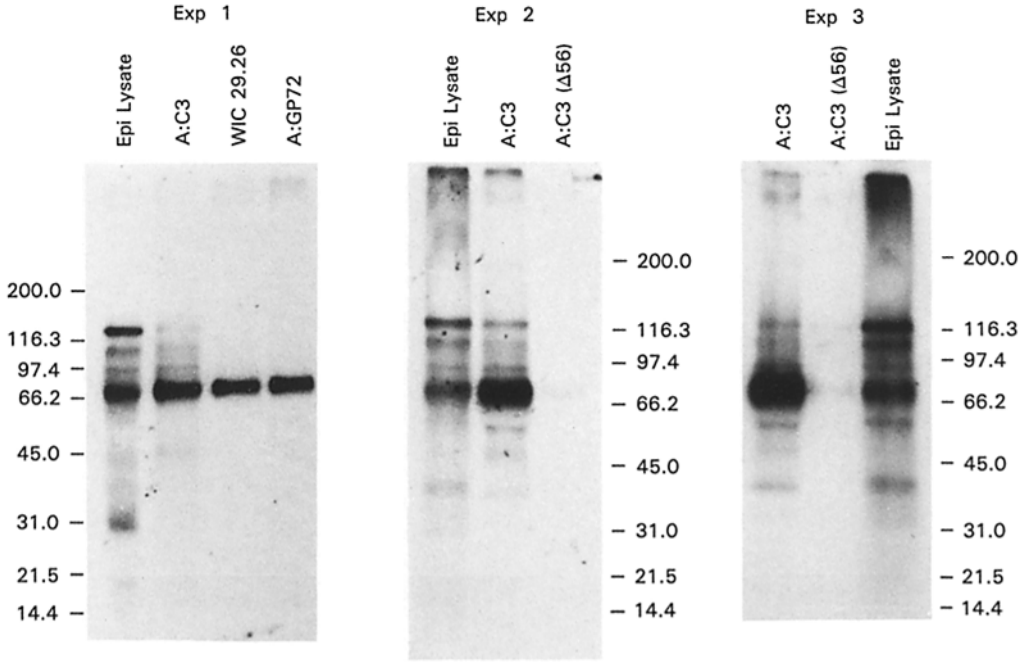

FIGURE 4. Autoradiogram of 7.5–15% SDS-polyacrylamide gel showing that C3b binds preferentially to gp72 on ^{125}I-labeled Tulahuen Epi during incubation in serum. Experimental conditions were as outlined in the legend of Fig. 3, except that anti-C3–sepharose bearing ^{125}I-labeled Epi protein was treated with 1 M NH_2OH (pH 10.5) for 30 min at 37°C to cleave ester linkages between C3 and acceptor molecules. Released ^{125}I-labeled Epi protein was then analyzed by 7.5–15% SDS-PAGE. Three separate experiments are shown comparing detergent lysates of intact Epi (*Epi Lysate*), and of material released from anti-C3–sepharose with hydroxylamine after incubation in 50% C8D (*A:C3*) or 50% Δ56 C8D [*A:C3* (*Δ56*)]. Also shown for exp. 1 is gp72 immunoprecipitated from detergent lysates with WIC 29.26, and with a rabbit antiserum against gp72 (*A:GP72*).

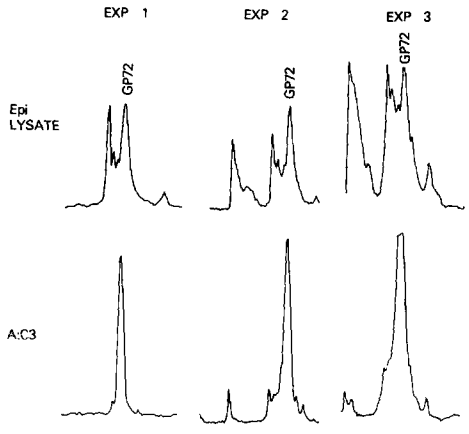

FIGURE 5. Densitometric scan of autoradiogram in Fig. 4.

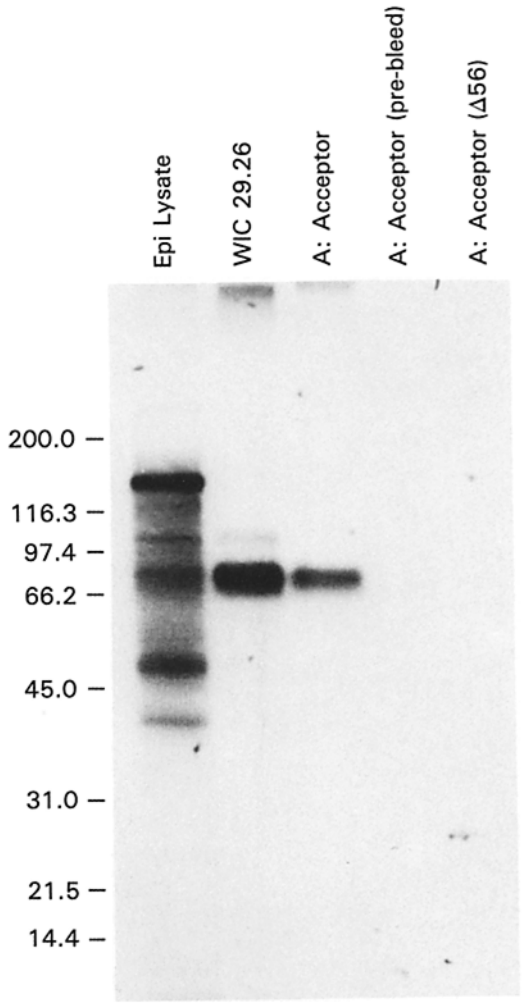

FIGURE 6. Autoradiogram of 7.5–15% SDS-polyacrylamide gel showing that antiserum against the C3 acceptor immunoprecipitates exclusively gp72. Antiserum was raised agianst the C3 acceptor on Tulahuen Epi as described in Materials and Methods. This antiserum was used (*A:Acceptor*) and compared with WIC 29.26 for immunoprecipitation of ^{125}I-labeled protein from detergent lysates of ^{125}I-labeled Epi (*Epi Lysate*).

contrast, antiserum raised against intact Tulahuen Epi immunoprecipitated at least five additional bands from detergent lysates of ^{125}I-Tulahuen Epi (not shown). No bands were observed using preimmunization serum from the rabbit subsequently used for preparation of anti–C3 acceptor antiserum. Similarly, no bands were immunoprecipitated using antiserum raised against Epi proteins that had bound nonspecifically to anti-C3–sepharose after incubation of Epi in Δ56 C8D serum.

We then examined the possibility that membrane molecules other than those labelled by the Iodogen procedure also function as C3 acceptors. These molecules would not have been detected with the methods employed above. We therefore

tested the reactivity of anti-acceptor sera for Tulahuen Epi by immunoblotting, an assay which does not depend on surface labelling. Results were compared with control antisera, and with antibodies against gp72, and against Tulahuen Epi. A doublet of bands (72 and 61 kD) was recognized by both anti-acceptor antisera and anti-gp72 antisera (Fig. 7). It is likely, therefore, that the 61 kD component, which may or may not be surface exposed, represents a breakdown product of gp72. An additional band of 44 kD was detected in the immunoblot with anti-acceptor antisera, but was present with similar intensity when control antisera was used. As expected, a variety of additional constituents were detected with the polyspecific anti-Epi antisera. These results confirm the findings based

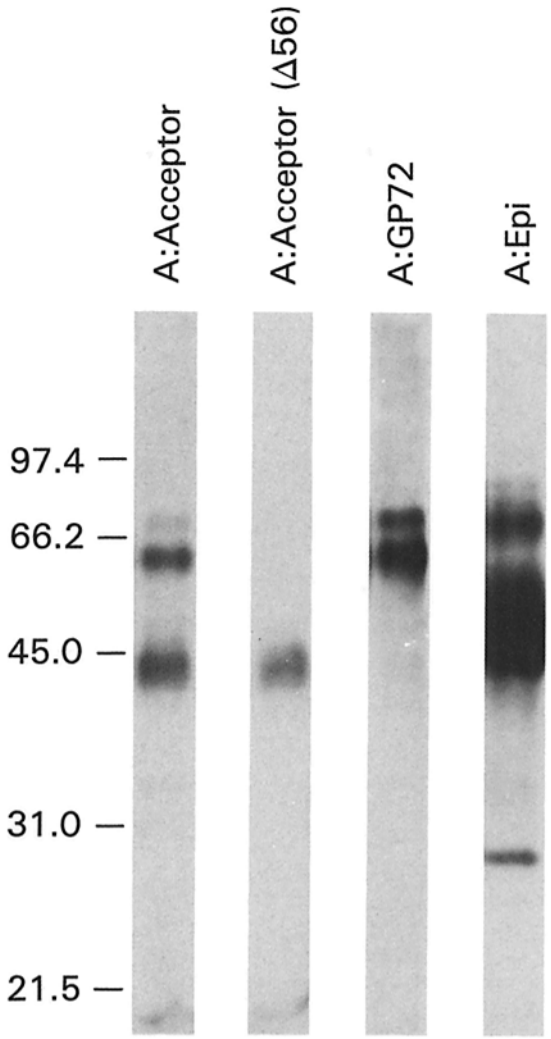

FIGURE 7. Autoradiogram of immunoblot showing that antiserum against the C3 acceptor (*A:Acceptor*) specifically recognizes only gp72 on Tulahuen Epi. This antiserum was compared with control antiserum (*A:Acceptor* Δ*56*), antiserum against gp72 (*A:GP72*), and antiserum against whole Tulahuen Epi (*A:Epi*).

on immunoprecipitation of surface-labeled proteins, and argue that gp72 is the only membrane acceptor molecule in Epi.

Comparison of C3 Acceptor on Epi and CMT of M88. gp72 is present on both Epi and CMT forms of *T. cruzi*. The above experiments (Fig. 2 and Table I) demonstrate that CMT inefficiently activate complement in comparison to Epi, for which gp72 is the acceptor molecule for C3 deposition. We therefore wished to identify the acceptor molecule for C3 deposition on CMT. gp72 on M88 Epi grown in LIT medium gives a double band containing components of 72 kD and 59 kD when immunoprecipitated with either polyclonal anti-gp72 antiserum (Fig. 8) or with WIC 29.26 (not shown).

gp72 is the principle C3 acceptor molecule on M88 Epi during incubation in serum. There is marked enrichment of the gp72 double band in the anti-C3–sepharose sample in comparison to Epi lysates (Fig. 8). CMT of M88 demonstrate a simpler surface iodination pattern than Epi, with gp72 as the major constituent. The molecule in CMT comigrates with the upper band from ^{125}I-labeled M88 Epi, and can be immunoprecipitated with WIC 29.26 and anti-gp72 antiserum (not shown), suggesting that it is similar if not identical to gp72. However, gp72 of CMT does not serve as an efficient acceptor molecule for C3 deposition. Not only is total C3 deposition on CMT only ⅕ of that on Epi (Fig. 2), but only a faint band at 72,000 daltons is observed in the C3 acceptor preparation from M88 CMT.

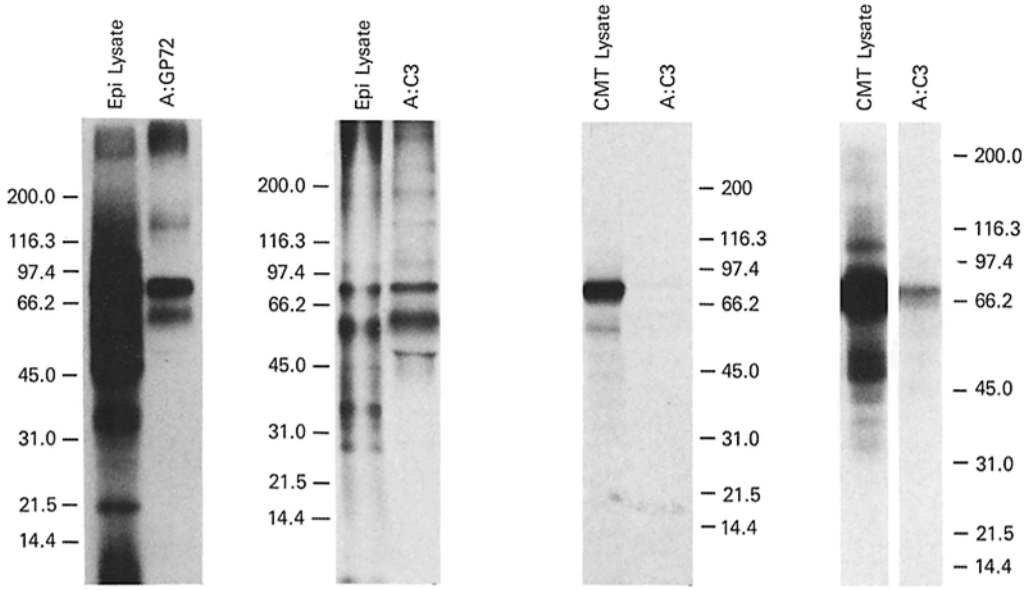

FIGURE 8. Autoradiogram of 7.5–15% SDS-polyacrylamide gel showing that gp72 on CMT of M88 is a poor acceptor for C3 deposition during incubation in serum. Experimental conditions were as outlined in the legend of Fig. 4, except that Epi and CMT of strain M88 were used. Immunoprecipitation of gp72 from M88 Epi using rabbit antisera shows a double band with 72- and 59-kD components.

Discussion

We have shown in this paper that the 72 kD glycoprotein of *T. cruzi* Epi (gp72) is the major acceptor for C3 deposition during alternative pathway activation in serum. This was demonstrated by affinity purification, using anti-C3–sepharose, of C3 and its accompanying acceptor molecule from detergent lysates of serum-incubated ^{125}I-labeled Epi. In addition, antiserum raised against the affinity-purified C3 acceptor molecule immunoprecipitated only gp72 from complete lysates of ^{125}I-labeled Epi and specifically immunoblotted only gp72 from unlabelled Epi. Deposition of C3 on gp72 occurs via the alternative pathway, and is not dependent upon specific antibody. Deposition of C3 on CMT of *T. cruzi* is inefficient, since only 1/5 as much C3 binds to CMT as to Epi during a 1-h incubation in serum. Furthermore, gp72 on CMT is not an efficient acceptor for C3 deposition, despite being the major surface-labelled constituent.

Active C3 in serum contains an internal thioester linkage (25, 27). Cleavage of C3 to C3b results in covalent attachment of C3b to acceptor molecules via a transesterification reaction (25). This reaction can occur at exposed hydroxyl groups of mono-, oligo-, and polysaccharides (28, 29). Transesterification with C3b can also occur with proteins. For example, C3b can bind to the carbohydrate-free Fd portion of IgG via a hydroxylamine-sensitive linkage, presumably by a transesterification reaction involving hydroxyl groups on tyrosine, serine, or threonine (30). Finally, C3 can attach to amino acids or proteins by a hydroxylamine-resistant linkage, most likely an amide bond (28, 31, 32). Our experiments indicate that C3b attaches covalently to Epi surface glycoprotein by an ester linkage, since the bond can be cleaved with hydroxylamine.

gp72 is heavily glycosylated, containing 49% carbohydrate by weight (33). Two classes of carbohydrate chains are present. One class constitutes 15% of the total carbohydrate in gp72 and contains mostly mannose, with smaller amounts of galactose and glucosamine. The other class constitutes 85% of the total carbohydrate, and contains a large amount of phosphate, as well as galactose, mannose, fucose, xylose, ribose, and glucosamine. WIC 29.26 binds to an epitope contained within this pentose- and phosphate-rich fraction. Sialic acid, a molecule that, in some systems, abrogates alternative pathway activation (34), is not present. Thus, the carbohydrate moiety of gp72 may serve as the acceptor for C3 deposition on Epi. Although our experiments do not allow us to distinguish whether C3 forms covalent ester bonds predominantly with the carbohydrate or the protein portion of gp72, they do indicate that hydroxylamine-resistant amide bonds between C3b and gp72 are not formed.

Of interest, Venkatesh et al. (35) have recently published evidence for spontaneous time-, temperature-, and pH-dependent cleavage of the ester linkage between C3 and acceptor molecules. This spontaneous release can be prevented by SDS. In our early experiments, we alternatively solubilized serum-incubated Epi in NPGB-LB at 4°C for 16 h, or by boiling in 1% SDS for 5 min. For SDS-solubilized parasites, the results of SDS-PAGE suggested complete covalent attachment of ^{125}I-gp72 to C3. For Epi solubilized in NPGB-LB only, SDS-PAGE of ^{125}I-labeled Epi protein bound to anti-C3–sepharose revealed a prominent gp72 band as well as a band at 170–250 kD. These results suggest that spontaneous cleavage of the ester linkage between C3b and gp72 occurred in the

absence of SDS, and prompted us to use SDS solubilization of serum-incubated parasites for all subsequent experiments.

gp72 is present on the surface of Epi from all strains and clones of *T. cruzi* (7). Vaccination of mice with purified gp72 decreased the severity of infection caused by challenge with CMT (36), raising the possibility that this antigen may be of value as an immunogen. Furthermore, WIC 29.26 inhibits transformation of Epi into trypomastigotes in vitro (37), suggesting that gp72 may be a receptor involved in the control of differentiation. Our finding that gp72 is the preferential acceptor for C3 on Epi, yet fails to bind C3 efficiently on CMT suggests that membrane molecules other than gp72 may be important in the regulation of complement activation. In particular, other major surface molecules (38–41) may be important in complement activation by *T. cruzi* Epi, possibly by influencing the interaction of regulatory proteins H and I with bound C3 (34, 42). We are currently measuring the ability of purified gp72 to directly activate the complement cascade, and are evaluating the role of other membrane components as control elements in complement activation.

This is the first study to elucidate the role of the acceptor molecule in C3 deposition on the surface of a protozoan parasite. The approach we have used is widely applicable to the study of complement–parasite interactions. Evaluating the surface molecules that serve as activators, acceptors or regulators for the biologically critical C3b moiety provides a means for understanding how parasites at different developmental stages may evade destruction by the complement system.

Summary

We examined the interaction of complement component C3 with surface molecules on *Trypanosoma cruzi*. Five- to six-fold more C3 was bound to epimastigotes (Epi) than to metacyclic trypomastigotes (CMT) of strain M88. Epi and CMT were surface iodinated, then incubated in C8-deficient serum, and detergent lysates were applied to anti-C3 antibody that had been coupled to Sepharose. We found that 9.20–10.24% of applied ^{125}I-Epi protein bound to anti-C3–sepharose, compared to 2.64% binding of ^{125}I-CMT protein. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed that C3 was attached to ^{125}I-Epi protein by a covalent bond. Samples eluted from anti-C3–sepharose with hydroxylamine revealed a single, major, 72 kD band, suggesting that C3b attaches almost exclusively to the 72 kD glycoprotein of Epi by a hydroxylamine-susceptible ester bond. An antiserum was prepared from lysates of serum-treated Epi that had been affinity-purified on anti-C3–sepharose. This antiserum immunoprecipitated a single 72 kD component (gp72) from surface-iodinated Epi, and specifically recognized only gp72 from Epi in immunoblots. In contrast to the results with Epi, gp72 on CMT was not found to be an efficient acceptor molecule for C3 deposition. The results are the first to evaluate the acceptor site for C3 deposition on a parasite, and they show that gp72 on Epi, but not gp72 on CMT, serves as the preferential acceptor for C3 during antibody-independent alternative complement pathway activation.

Received for publication 10 December 1984.

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