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gp 58/68, a parasite component that contributes to the escape of the trypomastigote form of *T. cruzi* from damage by the human alternative complement pathway

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SUMMARY

A glycoprotein of apparent molecular weight 58,000 (unreduced)/68,000 (in its reduced form) (gp 58/68), which is one of the fibronectin/collagen receptors of *Trypanosoma cruzi*, was purified to homogeneity from the trypomastigote forms of the Tehuantepec and Y strains of the parasite. Purified gp 58/68 inhibited formation of cell-bound and fluid-phase alternative pathway C3 convertase in a dose-dependent fashion, as assessed using purified human complement components. Gp 58/68 differed from the human regulatory proteins H, DAF, MCP and CR1 and from previously reported regulatory proteins on the parasite membrane in that it was unable to enhance decay-dissociation of preformed alternative pathway C3 convertase sites, did not serve as a co-factor for I-mediated cleavage of C3b and had no inhibitory activity on the classical pathway convertases. The inhibitory effect of gp 58/68 was most likely dependent on an interaction of the protein with factor B rather than with C3b. Gp 58/68 provides trypomastigotes with an additional potential mechanism for escaping complement lysis by the human alternative pathway.

INTRODUCTION

Blood and tissue culture-derived trypomastigotes of *Trypanosoma cruzi*, the causative agent of Chagas' disease, are resistant to lysis mediated by human complement (Nogueira, Bianco & Cohen, 1975; Kipnis *et al.*, 1981) in contrast to the epimastigote forms (the major insect stage) of the parasite. The mechanisms by which trypomastigotes evade attack by the alternative complement pathway are not fully understood. Recent observations demonstrated that culture-generated metacyclic trypomastigotes (i.e. the infective stage which develops in the vector) and tissue culture-derived trypomastigotes produce factors that interfere with the function of classical and alternative pathway C3 convertases (Joiner *et al.*, 1987). These studies also indicated that the parasites shed into the culture medium a limited number of components ranging in MW from 87,000 to 155,000, the most prominent molecules being 87–93,000, which accelerate the intrinsic decay of the alternative and classical pathway C3 convertases. By exhibiting these functions, the parasite proteins behave in an analogous manner to the human membrane regulatory protein, decay-accelerating factor (DAF).

We have been studying the mechanisms of *T. cruzi* trypomastigote-host cell recognition and the implication of host cell fibronectin and collagen in parasite attachment. The parasite receptor for fibronectin/collagen consists of two molecules of MW 80–85,000 and 58/68,000 (Ouaissi, Cornette & Capron, 1986; Velge *et al.*, 1988). These proteins are expressed on the parasite surface and are released in the culture medium (M. A. Ouaissi, J. Cornette, A. Taibi, P. Velge and A. Capron, manuscript submitted for publication). The use of affinity chromatography on Ultrogel-gelatin or wheatgerm agglutinin (WGA), enabled us to purify to homogeneity the 58/68,000 MW protein from the culture and blood forms of *T. cruzi* trypomastigotes. We now report that the parasite glycoprotein gp 58/68 inhibits formation of the cell-bound and fluid-phase alternative pathway C3 convertases. The protein has no inhibitory effect on classical pathway C3 convertase activity. Thus, multiple-acquired surface components contribute to prevent complement-mediated elimination of the trypomastigote form of the parasite.

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Abbreviations: BSA, bovine serum albumin; CMT, culture-derived metacyclic trypomastigote form; DAF, decay-accelerating factor; $DGVB^{2+}$, half-isotonic GVB with 5% destrose containing 0·5 mM MgC12 and 0·15 mM CaC12; DGVB-Ni^{2+}, DGVB containing 0·05 mM NiC12; EPI, epimastigote form; E^s, sheep erythrocyte; E^sC3b, C3b bearing E^s; GVB, VBS containing 0·1% gelatin; GVB-EDTA, GVB containing 0·04 M EDTA; NP-40, Nonidet-P 40; NRS-EDTA, normal rat serum diluted in GVB-EDTA; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; VBS, veronal-buffered saline; WGA, wheatgerm agglutinin.

MATERIALS AND METHODS

Parasites

The Tehuantepec and Y strains of *T. cruzi* were used throughout

this work. Trypomastigotes were maintained in tissue culture by weekly infection of fibroblasts, as described elsewhere (Ouaissi *et al.*, 1986).

Radioactive labelling of T. cruzi trypomastigotes

Parasites were surface-labelled with ^{125}I using the Iodogen procedure (Pierce Chemicals, Rockford, IL). Two times 10^8 washed viable trypomastigotes were incubated in 0·5 ml phosphate-buffered saline (PBS) (150 mM NaCl, 10 mM PO_4^{2-}, pH 7·2) with 11·1 MBq (300 μCi) Na ^{125}I and 200 μg Iodogen for 15 min at 4°. Labelled parasites were washed three times in 10 ml PBS containing 5 mM NaI and five times in PBS, then pelleted by centrifugation at 2000 *g* for 15 min. Trypomastigotes lysates were prepared by resuspending the parasites in 1 ml of 10 mM Tris HCl, pH 6·8, containing 1% Nonidet P40 (NP-40), 100 UK ml^{-1} aprotinin (Sigma, St Louis, MO) and 2 phenyl methylsulphonyl fluoride (PMSF) (Sigma) and rotating overnight at 4°. The insoluble material was removed by centrifugation at 3000 *g* for 15 min.

Affinity chromatography on Sepharose-bound wheatgerm agglutinin

A column (10 ml) of Sepharose-WGA (Pharmacia, Uppsala, Sweden) was equilibrated at 4° with PBS. NP-40 lysates from *T. cruzi* were loaded on the column overnight at 4°. The column was extensively washed with PBS and eluted with PBS containing 0·2 M *N*-acetyl-D-glucosamine. The radioactivity was determined in 1·0 ml fractions. Eluted proteins were dialysed, concentrated and analysed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were stained with silver (Morrissey, 1981). Molecular weight marker proteins were from Sigma Chemical Co.

Complement components

Human C3 (Tack & Prahl, 1976), B (Hunsicker, Ruddy & Austen, 1979), D (Fearon & Austen, 1975), P (Fearon & Austen, 1977), C5 (Fischer & Kazatchkine, 1983), H (Jouvin *et al.*, 1984) and I (Fearon, 1977) were purified to homogeneity as assessed by SDS–PAGE. Guinea-pig C1 (C1 gp) was partially purified as described elsewhere (Nelson *et al.*, 1966). Human C2 was purchased from Cordis Laboratories Inc. (Miami, FL). The IgG fraction of a serum containing C4 Nef activity was a kind gift from Dr L. Halbwachs (Hôpital Necker, Paris). Normal rat serum was depleted in C3 and C5 activities (C6–9 reagent) using 1 M KSCN and 0·015 M hydrazine (Fischer & Kazatchkine, 1983).

Cellular intermediates and complement assays

Veronal-buffered saline (VBS) containing 0·1% gelatin (GVB), half isotonic GVB with 2·5% dextrose containing 0·15 mM Ca^{2+} and 0·5 mM Mg^{2+} ($DGVB^{2+}$) or 0·05 mM nickel ($DGVB\ Ni^{2+}$), GVB containing 0·04 M EDTA (GVB-EDTA) were prepared as described elsewhere (Nelson *et al.*, 1966).

EA and EAC1 were prepared using sheep erythrocytes (E^s) and rabbit anti-sheep erythrocyte antibodies (Institute Pasteur Production, Marnes la Coquette) and C1gp and were converted to EAC4b using citrated human plasma as a source of C4. To form EAC1,4b-bearing haemolytically active C1, EAC4b were reacted with C1gp. EAC4b, 3b were prepared by interacting EA with zymosan-treated human serum in the presence of antrypol (Bayer, Sens). E^s-bearing C3b (E^sC3b) were prepared by sequential deposition of C3b by fluid-phase and cell-bound amplification C3 convertases using purified human alternative pathway proteins as described previously (Fischer *et al.*, 1986).

Cell-bound classical pathway C3 convertase sites C4b, 2a were formed by incubating EAC1, 4b with human C2 (0·12 U/10^7 cells) in $DGVB^{2+}$ for 7 min at 30°. Haemolytic sites were revealed by addition of 0·3 ml of a 1:20 dilution of normal rat serum in GVB-EDTA (NRS-EDTA) and incubation for 1 hr at 37°. C4 Nef-stabilized classical pathway C5 convertase sites C4Nef, C4b, 2a, 3b, were generated by first incubating 1×10^8 EAC1, 4b with 10 U of C2 and 1·15 mg of C4 Nef-containing IgG in 3·0 ml $DGVB^{2+}$ for 7 min at 30° and then incubating the C3 convertase-bearing cells with 14 μg of C3 for 15 min at 37°. The cells were washed in GVB-EDTA and resuspended at 1×10^8 L/ml in this buffer. C5 convertase sites were revealed by incubating 1×10^7 C4Nef EAC4b, 2a, 3b with 0·2 μg of purified C5 in 0·2 ml GVB-EDTA for 4 min at 30° before addition of 0·3 ml of a 1:10 dilution of C6–9 reagent in GVB-EDTA for 1 hr at 37°.

P-stabilized C3 convertase sites, C3b, Bb, P were formed by incubating 1×10^7 EAC4b, 3b with 0·4 ng B, excess D and P in 0·2 ml of $DGVB^{2+}$ for 30 min at 30°. Ni-stabilized C3b, Bb, Ni^{2+} sites were formed by incubating 1×10^7 EAC4b, 3b with 1·4 ng B and excess D in 0·2 ml of DGVB Ni^{2+} for 30 min at 30°. C3b, Bb sites were formed by incubating 1×10^7 EAC4b, 3b with 4·4 ng B and excess D in 0·2 ml of $DGVB^{2+}$ for 30 min at 30°. C3 convertase sites were revealed by addition of NRS-EDTA and further incubation for 1 hr at 37°. B haemolytic assays were performed as described elsewhere (Hunsicker *et al.*, 1973).

Binding experiments

The purified gp 58/68 membrane protein from *T. cruzi* was radiolabelled with ^{125}I (Amersham, Les Ulis) using the Iodogen method to a specific activity of 21,000 c.p.m./μg. For binding studies 100 μl PBS with 0·2% bovine serum albumin (BSA) containing 2×10^7 E^s or E^sC3b were incubated with 100 μl of the same buffer containing 1·0–25 μg of ^{125}I-gp 58/68 for 30 min at 30°. Duplicate samples of the reaction mixture were layered over 0·3 ml of dinonyl-dibutylphtalate (3/7 v/v) and centrifuged at 8000 *g* for 1 min. The tubes were cut and the radioactivity in the pellets was assessed using an LKB minigamma counter (LKB, Les Ulis).

RESULTS

Identification and isolation of the trypomastigote glycoprotein gp 58/68 by affinity chromatography

The supernatant of solubilized extracts from labelled trypomastigote culture forms (Tehuantepec strain) was chromatographed on WGA–Sepharose; 0·2–0·5% of the radioactivity that had been applied to the column was eluted from the immunoabsorbent in the presence of *N*-acetyl-D-glucosamine. The protein and radioactivity elution pattern is shown in Fig. 1. Analysis of the eluate by SDS–PAGE under non-reducing conditions demonstrated the presence of a protein with an apparent molecular weight of 58,000, as revealed by silver staining of the gel (Fig. 1). Upon reduction the protein band migrated to a position of approximately 68,000 MW (Fig. 1). The parasite surface protein gp 58/68 was purified to homogeneity following this procedure, dialysed and lyophilized. The protein was resuspended in appropriate buffer for use in complement assays.

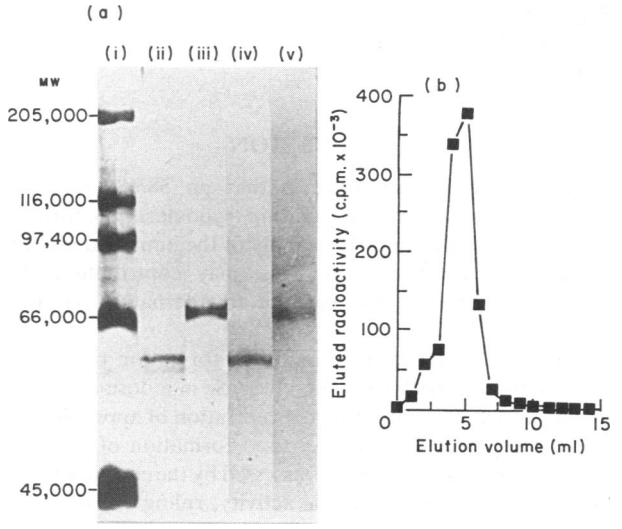

Figure 1. (a) Silver-stained SDS–PAGE of the material eluted with *N*-acetyl-D-glucosamine upon affinity chromatography on WGA–Sepharose of NP-40-solubilized membrane proteins from *T. cruzi*. NP-40 lysates from *T. cruzi* trypomastigote culture forms of the Tehuantepec strain (ii, iii) and from trypomastigote culture forms of the Y strain (iv, v); lanes (i), molecular weight markers; lanes (ii) and (iv), electrophoresis under non-reducing conditions; lanes (iii) and (v), electrophoresis under reducing conditions. (b) Pattern of elution of ^{125}I-labelled solubilized *T. cruzi* membranes upon affinity chromatography on WGA–Sepharose.

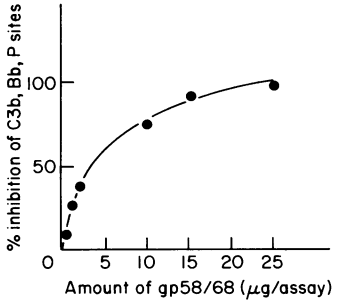

Figure 2. Inhibition by purified gp 58/68 of formation of cell-bound alternative pathway C3 convertase sites C3b, Bb, P. One times 10^7 EAC4b, 3b cells were incubated with appropriate amounts of B, P and D in the absence or presence of increasing amounts of gp 58/68 before convertase sites were revealed with NRS-EDTA.

Affinity chromatography on Sepharose–WGA of lysates from trypomastigote culture forms of the Y strain also yielded a protein of molecular weight 58,000, as assessed by SDS–PAGE under non-reducing conditions and 68,000 MW under reducing conditions (Fig. 1).

Inhibition of cell-bound alternative pathway C3 convertase sites by gp 58/68

The effect of purified gp 58/68 on formation of cell-bound alternative pathway C3 convertase sites was examined by incubating 1×10^7 EAC4b, 3b with 0·4 ng of B and excess P and

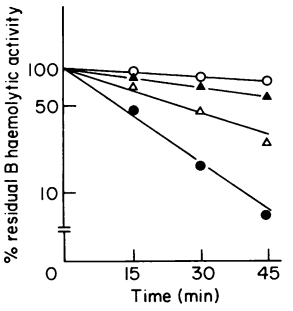

Figure 3. Inhibition by purified gp 58/68 of formation of fluid-phase alternative pathway C3 convertase sites C3b, Bb. C3, B and D were incubated in buffer alone (●) or containing 7·5 μg (△) and 15 μg (▲) of gp 58/68 at 37°. Formation of fluid-phase C3b, Bb was determined by measuring the consumption of haemolytic B in the reaction mixture. A sample containing B and D and no C3 served as a control (○).

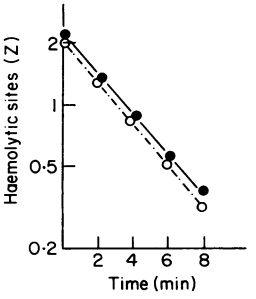

Figure 4. Lack of effect of gp 58/68 on the decay of preformed C3b, Bb sites. Five times 10^7 EAC4b cells bearing 2Z were incubated in 0·5 ml of GVB-EDTA alone (●) or containing 50 μg of gp 58/68 (○) at 30°. At various times convertase sites were revealed by addition of samples of the reaction mixture to NRS-EDTA.

D in the presence of increasing amounts of gp 58/68 (0·5–25 μg/assay) for 30 min at 30°. C3b, Bb, P sites were revealed by addition of NRS-EDTA. Gp 58/68 inhibited formation of C3b, Bb, P in a dose-dependent fashion (Fig. 2). The 50% inhibitory input of gp 58/68 was 4 μg/assay. No inhibition was observed when gp 58/68 was added to NRS-EDTA, indicating that this protein does not interfere with the function of the terminal components. Pre-incubation for 30 min at 30° of 1×10^7 EAC4b, 3b with 10 μg of gp 58/68 followed by two washes in DGVB^{2+} did not alter the capacity of C3b molecules to react with B and form C3b, Bb, P sites. A dose-dependent inhibition of C3 convertase formation by gp 58/68 was also observed when Ni^{2+}-stabilized or unstabilized alternative sites was studied. Fourteen micrograms of gp 58/68 were required to inhibit 50% of C3b, Bb, Ni^{2+} sites formed with 1·4 ng of B/assay. Thirty micrograms of gp 58/68 were sufficient to inhibit 50% of non-stabilized C3b, Bb sites formed with 44 ng of B/assay.

Inhibition of fluid-phase alternative C3 convertase sites by gp 58/68

In order to examine the effect of gp 58/68 on the formation of a fluid-phase alternative pathway C3 convertase, 7 μg of B, 2·6 μg

Table 1. Lack of effect of gp 58/68 on formation and function of cell-bound classical pathway C3 and C5 convertases

gp 58/68 μg/assay	C4b, 2a haemolytic sites (Z)	C4b, 2a, 3b haemolytic sites (Z)
—	0·93	1·31
0·4	0·90	1·28
1	0·87	1·19
2	0·92	1·29
5	0·90	1·26
10	0·83	1·13

of C3 and 10 ng of D were interacted in 360 μl of $DGVB^{2+}$ at 37° in the absence or presence of 7·5 and 15 μg of gp 58/68. At various times, samples of the reaction mixtures were diluted 200-fold in ice-cold $DGVB^{2+}$ and assayed for B haemolytic activity. Gp 58/68 inhibited formation of fluid phase C3b, Bb sites (Fig. 3).

Lack of enhancement of decay of Bb from C3b, Bb by gp 58/68

The capacity of purified gp 58/68 to accelerate decay-dissociation of Bb from a preformed cell-bound C3 convertase was assessed by incubating 5×10^7 EAC4b, 3b, Bb with 0·5 ml GVB-EDTA alone or containing 50 μg of gp 58/68 at 30°. At various times, 0·2 ml of the reaction mixture was added to 0·3 ml of NRS-EDTA and incubation was continued for 60 min at 37°. As shown in Fig. 4, the kinetics of dissociation of Bb from C3b, Bb were similar in the presence and in the absence of gp 58/68, suggesting that gp 58/68 does not inhibit formation of C3b, Bb by binding to cell-bound C3b.

Further evidence for a lack of binding of the protein to C3b was obtained in binding experiments of ^{125}I-labelled gp 58/68 (1–25 μg) to $E^{s}C3b$ bearing 10,000 molecules of C3b/cell. No evidence for specific binding of gp 58/68 was obtained (data not shown).

Lack of co-factor activity of gp 58/68 on I-mediated cleavage of cell-bound C3b.

Five times 10^7 EAC4b, 3b were incubated in 0·5 ml of DGVB containing 1·5 μg of purified I alone or I with gp 58/68 (25–50 μg/assay) for 45 min at 37°. The percentage of inactivated C3b molecules was assessed by measuring the consumption of B in the presence of C3b-bearing cells. Inactivation of C3b was similar on cells incubated with I alone or with I and gp 58/68. In contrast, C3b on EAC4b, 3b treated with purified I and H was totally inactivated under the same experimental conditions (data not shown).

Lack of effect of gp 58/68 on formation and function of the classical pathway C3 and C5 convertases

In order to determine whether gp 58/68 interferred with the formation and function of the classical C3 convertase C4b, 2a and the classical C5 convertase C4b, 2a, 3b, 0·4–10 μg of gp 58/68 were added to 1×10^7 EAC1, 4b together with 0·12 U of C2 or added to C4 Nef, EAC14b, 2a, 3b together with 0·12 μg of C5 before addition of either NRS-EDTA or C6–9 reagent. As shown in Table 1, gp 58/68 had no inhibitory effect on classical C3 and C5 convertase sites.

DISCUSSION

The present study demonstrates that gp 58/68, a surface glycoprotein purified from the culture trypomastigote forms of *T. cruzi*, specifically inhibits assembly of the human alternative pathway C3 convertase and, thus, may contribute to the acquired resistance of the parasite to alternative pathway-mediated lysis.

The parasite glycoprotein inhibited formation of the cell-bound alternative pathway C3 convertase in a dose-dependent manner, with a 50% inhibitory concentration of approximately $0{\cdot}4 \times 10^{-6}$M. Gp 58/68 also decreased formation of the fluid-phase C3 convertase C3b, Bb, as assessed by the consumption of fluid-phase factor B haemolytic activity, ruling out the possibility of a proteolytic effect of the parasite protein on human factor B. Gp 58/68 inhibited formation of cell-bound unstabilized as well as Ni^{2+}- and P-stabilized C3b, Bb convertase sites. The 50% inhibitory concentration of gp 58/68 on cell-bound convertase formation decreased when the affinity of the interaction between C3b and B increased, suggesting that gp 58/68 acted on factor B to prevent its binding to surface-fixed C3b.

Extracts and culture supernatants from trypomastigotes accelerate the decay of the classical and alternative pathway C3 convertases on inert particles and on epimastigote targets. The latter 'DAF-like' activity was assigned to a 87–93,000 MW parasite glycoprotein, which exhibited affinity for Con A Sepharose (Joiner *et al.*, 1987). Gp 58/68 differs from this (these) parasite component(s) by its molecular weight and affinity for WGA. Furthermore, the mechanism of the inhibitory action of gp 58/68 differs from that of human DAF (Nicholson-Weller *et al.*, 1982; Medof, Kinoshita & Nussenzweig, 1984) in several ways: (i) the parasite protein did not accelerate decay-dissociation of preformed C3b, Bb and C3b, Bb, P sites; (ii) it did not inhibit formation of classical pathway C4b, 2a convertase sites; (iii) it did not incorporate into membranes of sheep red cells as assessed by the lack of inhibition of convertase formation when C3b-bearing cells were pre-incubated with gp 58/68 and washed before addition of factors B and D.gp58/68 also differed in its mechanism of action from the plasma regulatory protein factor H and from C3b receptor, CR1, which both inhibit formation of and actively decay the alternative pathway C3 and C5 convertases by strongly binding to C3b (Fearon, 1978; Fischer *et al.*, 1983). CR1 also inhibits the activity of classical pathway C3 and C5 convertases (Iida & Nussenzweig, 1981) whereas gp 58/68 had no inhibitory effect on formation of the classical pathway C3 convertase. In addition, gp 58/68 differed from other human C3b-binding membrane regulatory proteins such as MCP (Seya, Turner & Atkinson, 1986) in that it had no co-factor activity for I-mediated cleavage of cell-bound C3b. No evidence of a specific binding of the protein to cell-bound C3b could be obtained in binding experiments. As mentioned above, functional experiments favoured the hypothesis that the inhibitory effect of gp 58/68 is mediated by an interaction with factor B rather than with C3b.

The presence of gp 58/68 molecules either on the surface of the parasite or shed in the bloodstream could prevent the efficient binding of factor B to C3b molecules and thus

contribute to decreased formation of C3b, Bb complexes on the trypomastigotes forms of *T. cruzi* (Joiner *et al.*, 1986). The present study agrees with the notion of developmentally regulated mechanisms acquired by *T. cruzi* by which the trypomastigote stage of the parasite becomes resistant to complement activation. Several surface proteins expressed by the culture-derived metacyclic trypomastigote stages (CMT) of the parasite have been implicated in the control of alternative pathway activation. Thus, upon incubation with human serum, a five-fold higher amount of C3 becomes covalently linked to the epimastigote (EPI) forms than to the CMT forms (Joiner *et al.*, 1985). The C3b-acceptor proteins differ between the two stages of the parasite (Joiner *et al.*, 1985), C3bi molecules have predominantly been found on the CMT forms whereas C3b and C9 are deposited on EPI forms (Joiner *et al.*, 1986). Digestion of a 90–115,000 MW glycoprotein with pronase allows deposition of haemolytically active C3b on CMT (Sher, Hieny & Joiner, 1986). Efficiency of C3b, Bb formation is decreased on C3b-bearing CMT compared with C3b-bearing EPI, either because of a lower affinity of B for C3b compared with that of H (Joiner *et al.*, 1986) or because of higher affinity of H for C3b over that of B (Schenkman, Guther & Yoshida, 1986). As mentioned earlier, surface components of CMT have been identified with decay-preformed alternative and classical convertases (Joiner *et al.*, 1987; Kipnis *et al.*, 1986). In the present study, we report functional properties of gp 58/68, a parasite surface protein which is only expressed on trypomastigote stage since specific polyclonal mouse antibodies against the protein do not react with epimastigote forms of *T. cruzi* (Velge *et al.*, 1988). This newly characterized component provides trypomastigotes with an additional potential mechanism for escaping complement activation by the alternative pathway.

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