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Biochemical Analysis of the Membrane and Soluble Forms of the Complement Regulatory Protein of *Trypanosoma cruzi*

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A developmentally regulated, 160-kDa trypomastigote surface glycoprotein was previously shown to bind the third component of complement and to inhibit activation of the alternative complement pathway, thus providing the parasites a means of avoiding the lytic effects of complement. We now show that this complement regulatory protein (CRP) binds human C4b, a component of the classical pathway C3 convertase, and may therefore also act to restrict classical complement activation. Characterization of the extent of carbohydrate modification of the protein revealed extensive N-linked glycosylation and no apparent O-linked sugars. The CRP purified from parasites treated with an inhibitor of N-linked glycosylation exhibited a decreased binding affinity for C3b compared with that of the fully glycosylated protein. We have previously shown that the protein was anchored to the membrane via a glycosyl phosphatidylinositol linkage and was spontaneously shed from the parasite surface. The spontaneous release of CRP from the parasite surface may augment the protection of the parasites from complement-mediated lysis by the removal of complement-CRP complexes. The majority of the shed CRP had an apparent molecular mass of 160 kDa and lacked the glycolipid anchor, whereas the membrane form was recovered with the glycolipid anchor attached and had an apparent molecular mass of 185 kDa. Both the membrane form (185 kDa) and the soluble form (160 kDa) retained binding affinity for C3b. Evidence is presented to indicate that the conversion of the 185-kDa membrane form to the 160-kDa form is the result of cleavage by an endogenous phospholipase C.

The complex life cycle of protozoan parasites involves the developmental regulation of characteristics which allow for survival in diverse microenvironments in insect vectors and mammalian hosts. In order to establish and disseminate the infection, parasites have evolved means whereby they are resistant to the effects of intrinsic host defense mechanisms. A crucial step in the establishment of infection by bloodborne pathogens is the avoidance of direct killing by serum factors, such as the complement system. Several mechanisms of complement resistance by microorganisms have been elucidated (16), and more recently, the mimicry of host complement components and complement regulatory proteins (CRPs) by microorganisms has been described (9–11, 17, 19, 24–26).

In the case of *Trypanosoma cruzi*, the causative agent of Chagas' disease, the bloodstream trypomastigotes have evolved one or more means of avoiding complement-mediated killing (9, 17, 26). Within the insect vector, the conversion of the parasites from epimastigotes to infectious trypomastigotes coincides with the acquisition of resistance to lysis by the alternative complement pathway. We have previously described a trypomastigote-specific membrane glycoprotein which inhibits the formation and stability of the alternative pathway C3 convertase, the central, multisubunit enzyme of the complement cascade, and thereby contributes to the complement resistance of the parasite (26). The glycoprotein was initially recovered from proteins spontaneously shed by trypomastigotes and was characterized as having an apparent molecular mass of 160 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (26). The 160-kDa glycoprotein is biochemically and genetically related to a family of mammalian CRPs, which serve to prevent lysis of autologous cells by complement activation and amplification (26). These proteins, which include factor H, decay-accelerating factor (DAF), and CR1, share a binding affinity for components of the alternative and/or classical pathway C3 convertases, C3b and C4b, respectively (reviewed in reference 20). Although the precise mechanism of interference with convertase formation and stability is not known, these CRPs bind to C3b (or C4b in the classical complement pathway) and competitively inhibit the uptake of the subsequent components, thereby preventing convertase formation and lysis of the cell.

The *T. cruzi* 160-kDa glycoprotein, as well as several other parasite membrane proteins, is anchored in the surface membrane via a glycosyl phosphatidylinositol (GPI) linkage (26). GPI-anchored proteins are found throughout the eukaryotes (4), although they appear to be particularly important in some protozoa, in which most of the surface proteins are anchored in this manner. The structural features of the glycolipid anchor of one such protein of *T. cruzi*, 1G7, have recently been described and were found to be similar to those of the anchor of the variable surface glycoprotein of *Trypanosoma brucei* as well as to those of higher eukaryotes (13). The purpose of such extensive modification of membrane proteins in the protozoa is not clear, but it may be related to the observations that membrane proteins in *T. cruzi* are spontaneously and continuously shed by the infectious forms of the parasites.

We describe here the purification and characterization of the membrane form of the *T. cruzi* CRP, which retains the glycolipid anchor and has an apparent molecular mass of 185 kDa by SDS-PAGE. The protein is functional in both the soluble form (160 kDa) and the membrane form (185 kDa).

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Because of the differences in apparent molecular mass of the two forms of the protein, we will refer to it as the CRP, membrane or soluble form. We have determined that the *T. cruzi* CRP binds the classical complement pathway component C4b and may therefore restrict activation of this pathway as well. In order to further characterize the regulatory role of this protein, we examined the extent and nature of its carbohydrate modification and the significance of carbohydrate and the glycolipid anchor in C3b binding.

MATERIALS AND METHODS

Buffers and reagents. The following buffers were used throughout these experiments. Dulbecco's minimal essential medium (DMEM) was buffered with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.4, and supplemented with L-glutamine (5 mM) and ovalbumin (100 μg/ml) (DMEM-OG). Membrane solubilization buffer was 10 mM HEPES (GIBCO), pH 7.4, with 0.5% Nonidet P-40 (NP-40) (Pierce Biochemical, Rockford, Ill.). Labeling medium was DMEM without cysteine and methionine (ICN Biochemicals, Costa Mesa, Calif.)–10 mM HEPES–100 μg of ovalbumin per ml–5 mM L-glutamine. C3b and C4b affinity chromatography binding buffer was 10 mM HEPES (pH 7.4)–50 mM NaCl. Wash buffer was 10 mM HEPES (pH 7.4)–50 mM NaCl–0.05% NP-40. Elution buffer was 10 mM HEPES (pH 7.4)–1 M NaCl. All buffers contained the following fresh protease inhibitors (Sigma Chemical, St. Louis, Mo.): aprotinin, E-64, and leupeptin (all at 1 μg/ml) and phenylmethylsulfonyl fluoride (100 μg/ml).

Metabolic labeling of trypomastigotes and protein preparations. *T. cruzi* (strain Y) tissue culture-derived trypomastigotes were recovered from culture in NIH 3T3 cells as described previously (26, 30). Parasites were harvested from tissue culture supernatant fluid by centrifugation, washed two times in phosphate-buffered saline (PBS) with 1% glucose, and resuspended in labeling medium at 10^8 cells per ml. [^{35}S]methionine (Trans-label; ICN) was added at 50 μCi/ml, and the cells were incubated for 1 h at 37°C. After being labeled, the cells were washed two times at 4°C in PBS with 1% glucose. Trypomastigote membrane proteins were prepared as follows. After metabolic labeling, parasites were resuspended in deionized H_2O at 4×10^8/ml with protease inhibitors and incubated for 5 min at 22°C; this was followed by centrifugation at 6,500 × *g* for 5 min. Pellets were resuspended in 10 mM HEPES (pH 7.4)–0.5% NP-40 in the original volume and incubated at 22°C for 15 min. The lysates were centrifuged at 13,000 × *g* for 15 min, and the supernatant fluid was collected.

Proteins were diluted in a 4× volume of sample buffer (8% SDS, 125 mM Tris-HCl [pH 6.8], 40% glycerol, 20% 2-mercaptoethanol, and 0.4% bromphenol blue), boiled for 3 min, and separated by SDS-PAGE in a discontinuous buffer system by the method of Laemmli (21). The gels were fixed in 40% methanol–10% acetic acid and then treated with Amplify as directed by the manufacturer (Amersham Corp., Arlington Heights, Ill.). Gels were dried and exposed to X-ray film at −70°C.

Affinity chromatography of *T. cruzi* CRP. Human C3b was prepared and coupled to cyanogen bromide-activated Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) as previously described (26) or to Affi-Gel 10 (Bio-Rad Laboratories, Richmond, Calif.) according to the manufacturer's directions in 0.1 M $NaHCO_3$ (pH 8.3)–0.5 M NaCl at 10 to 20 mg/ml of packed resin. Human C4b-Sepharose was a kind gift of Neal Cooper, Scripps Research Institute, La Jolla, Calif. For most experiments, 100 μl of NP-40-solubilized trypomastigote membrane preparations prepared as described above were diluted 1:10 in binding buffer to give a final detergent concentration of 0.05% NP-40. This preparation was loaded onto approximately 100 μl of packed C3b–Affi-Gel equilibrated in the same buffer. The columns were washed in 10 mM HEPES (pH 7.4)–50 mM NaCl–0.05% NP-40, and proteins were eluted in 10 mM HEPES (pH 7.4)–1 M NaCl.

Treatment of *T. cruzi* membrane preparations with *Bacillus thuringiensis* PIPLC. Detergent-solubilized, metabolically labeled membrane extracts were prepared as described above and treated as indicated below with purified *B. thuringiensis* phosphatidylinositol-specific phospholipase C (PIPLC) (a gift of Martin Low, Columbia University). Reactions were carried out at 22°C.

Phase separation of Triton X-114-solubilized membrane proteins. Separation of hydrophilic and amphiphilic proteins was carried out essentially as described previously (2). [^{35}S]methionine-labeled trypomastigote membrane proteins were prepared as described above. Forty-microliter samples were diluted 1:5 with 10 mM HEPES–50 mM NaCl. A 10% solution of Triton X-114 in 10 mM Tris (pH 7.4)–150 mM NaCl was precondensed as described previously (29) and used as the stock for subsequent extractions. Triton X-114 was added to the membrane preparations to a final concentration of 5%. The samples were mixed and incubated at 37°C for 3 min, and the phases were separated by centrifugation at 37°C in microcentrifuge for 3 min. Twenty microliters of the aqueous and detergent phases was diluted in sample buffer, separated by SDS-PAGE, and prepared for fluorography.

Metabolic labeling of *T. cruzi* trypomastigotes with [^{3}H] myristic acid. Tissue culture trypomastigotes were harvested and washed twice in PBS with 1% glucose. They were then resuspended at 2×10^8/ml in DMEM–10 mM HEPES–2 mM L-glutamine–0.1 mg of bovine serum albumin (fatty acid free; Sigma) per ml and incubated for 30 min at 37°C. [^{3}H]myristic acid in ethanol was then added at 50 μCi/ml (specific activity, 33.5 Ci/mmol; New England Nuclear). The cells were incubated at 37°C for 1 h and then centrifuged at 1,000 × *g* for 10 min. The cells were washed once in PBS with 1% glucose and frozen at −70°C. Whole-cell lysates were prepared by resuspending the pellet in SDS-PAGE sample buffer at 1.25×10^9/ml, boiling the lysate for 5 min, and pelleting the debris at 13,000 × *g* for 5 min. Detergent-solubilized membrane preparations of labeled parasites were prepared as described above.

Treatment of *T. cruzi* CRP with N- and O-linked glycosidases. Trypomastigote membrane proteins prepared as described above were treated with 1 U of PIPLC per ml for 30 min at 22°C. The CRP was C3b-affinity purified as described above, except that protein was eluted by boiling the beads for 10 min in 0.5% SDS–1% 2-mercaptoethanol for *N*-glycosidase treatment or in 0.1% SDS–0.2% 2-mercaptoethanol for *O*-glycosidase treatment. NP-40 and phosphate buffer, pH 7.5, were then added to the eluate to final concentrations of 1% and 50 mM, respectively. Three thousand units of PNGase F (E.C. 3.2.2.18; New England Biolabs, Beverly, Mass.) or 1 mU of *O*-glycosidase (E.C. 3.2.1.97; Boehringer-Mannheim) was added, and the reaction mixtures were incubated for 1 h at 37°C.

Tunicamycin treatment of trypomastigotes. Trypomastigotes were incubated for 3 h at 37°C in 5 μg of tunicamycin (Boehringer-Mannheim) per ml in DMEM-OG at 5×10^7 cells per ml. The parasites were then washed in PBS with 1%

glucose and resuspended in the original volume in labeling medium containing 5 μg of tunicamycin per ml. Cells were metabolically labeled as described above with [^{35}S]methionine. The cells were washed once in cold PBS with 1% glucose, and membrane proteins were prepared as described above, except that cells were resuspended at 1.2×10^9/ml in membrane solubilization buffer. Before this preparation was loaded onto C3b–Affi-Gel, the amount of radioactivity incorporated into trichloroacetic acid-precipitable protein was determined so that equal amounts of labeled control and tunicamycin-treated proteins were loaded on the affinity column. Fluorograms were scanned on a Bio-Rad laser densitometer.

RESULTS

Characterization of C3b-binding activity of detergent-solubilized trypomastigote membrane proteins. We previously identified a C3b-binding CRP of *T. cruzi* which was shed from tissue culture-derived trypomastigotes and had an apparent molecular mass of 160 kDa by reducing SDS-PAGE (26). The protein was purified to homogeneity in native form from culture supernatants and was found to exhibit complement regulatory activity at the level of C3 convertase formation and stability (26). In order to further characterize this protein and to improve recovery of the purified protein, we developed a purification scheme based on its affinity to human C3b, using detergent-solubilized trypomastigote membrane extracts. In order to minimize the effects of contaminating proteases which were present in the detergent-solubilized extracts, we examined the proteolytic profile of NP-40 membrane extracts on gelatin acrylamide gels as described previously (23). We observed serine and cysteine protease activities that were present at neutral pH and inhibitable by leupeptin and E64, respectively (25a). We therefore included excess levels of these protease inhibitors as well as phenylmethylsulfonyl fluoride and aprotinin in all membrane preparations and subsequent experiments. We examined the C3b-binding capacities of proteins from various detergent membrane extracts of metabolically labeled trypomastigotes, and the results with NP-40-solubilized proteins are shown in Fig. 1. The predominant proteins in the detergent-solubilized membrane extracts are a 185-kDa protein and a broad signal between 100 and 80 kDa. Solubilization with Tween 20, *n*-octylglucoside, and CHAPSO {3-[(3-cholamidopropyl)-dimethyl-ammonio]-2-hydroxy-1-propane sulfonate} gave similar results (not shown). While very little protein migrating at 160 kDa could be identified in the initial membrane preparation (Fig. 1, lane A), it was noted that such a protein appeared during incubation of the membrane preparation with C3b–Affi-Gel (Fig. 1, lane B). The NP-40-solubilized material was diluted and loaded directly onto C3b–Affi-Gel, and after washing, a protein with an apparent molecular mass of 160 kDa was specifically eluted in 1 M NaCl (Fig. 1, lane C). No other proteins were released by boiling of the eluted C3b–Affi-Gel beads in SDS-PAGE sample buffer (not shown). The eluted CRP still retained native function as determined by subsequent C3b binding (not shown).

In order to determine if the 160-kDa protein was generated as a result of interaction of the solubilized membrane extract with the C3b–Affi-Gel or chromatography conditions, we prepared NP-40 membrane extracts as described above (in the presence of fresh protease inhibitors as described in Materials and Methods) and divided them into six samples which were treated as follows. One sample was immediately boiled in SDS-PAGE sample buffer (Fig. 2, lane A) and contained a prominent band at 185 kDa as in Fig. 1. After a 60-min incubation in binding buffer with the addition of fresh protease inhibitor mixture, the signal at 185 kDa had decreased in intensity and there was a concomitant increase in

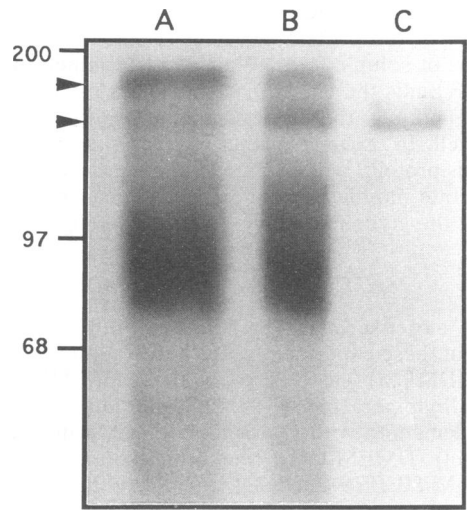

FIG. 1. Detergent solubilization of metabolically labeled *T. cruzi* trypomastigote membrane proteins and purification of the C3b-binding CRP. [^{35}S]methionine-labeled proteins were solubilized in NP-40 and separated on 8.5% acrylamide gels by SDS-PAGE; this was followed by fluorography. Lanes: A, total trypomastigote membrane proteins; B, membrane protein prepared as for lane A and then incubated at room temperature for 30 min prior to electrophoresis; C, C3b-affinity purified CRP from NP-40 detergent-solubilized membrane extracts. Numbers at the left are molecular masses in kilodaltons. Arrows indicate positions of the 185- and 160-kDa proteins.

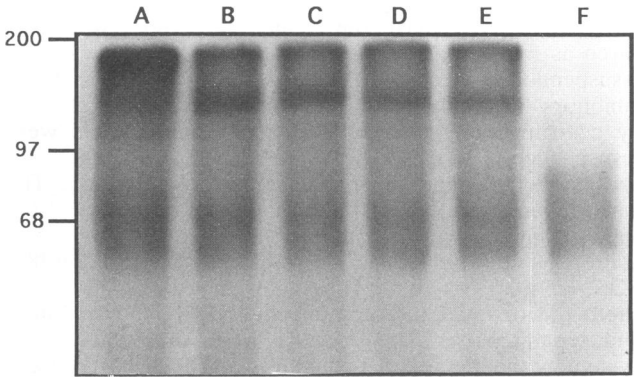

FIG. 2. Time-dependent generation of CRP in NP-40 detergent-solubilized membrane extracts. [^{35}S]methionine-labeled membrane proteins were prepared by NP-40 detergent solubilization and were immediately boiled in SDS sample buffer (lane A) or incubated for 60 min at room temperature prior to boiling in sample buffer (lane B) and then separated by SDS-PAGE. Samples were prepared as for lane A and incubated for 60 min at room temperature with C3b–Affi-Gel (lane C), C3b–Affi-Gel with an additional protease inhibitor (50 μg of TPCK per ml) added prior to the incubation (lane D), or blocked Affi-Gel beads alone (lane E), or were treated with trypsin (lane F), prior to boiling in sample buffer and separation by SDS-PAGE. The gel was prepared for fluorography as described in Materials and Methods. Numbers are molecular masses in kilodaltons.

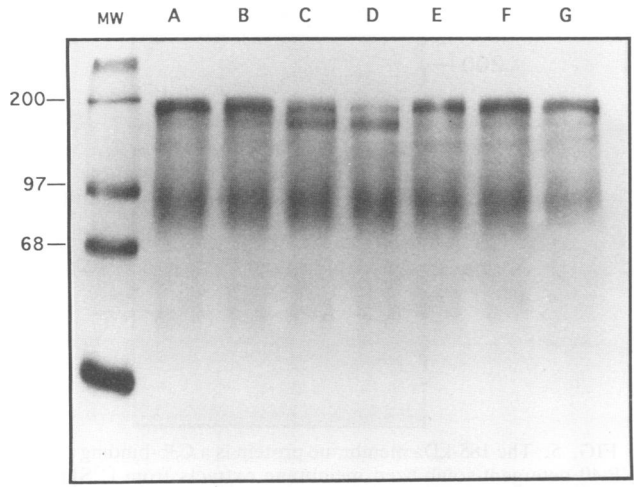

FIG. 3. Conversion from the 185-kDa form to the 160-kDa form is inhibitable by zinc. [^{35}S]methionine-labeled membrane proteins were prepared as described in Materials and Methods and incubated at room temperature, and aliquots were examined at various times by SDS-PAGE and fluorography. Lanes: A, detergent-solubilized membrane preparation boiled in SDS-PAGE sample buffer (time zero); B, 30-min incubation; C, 60-min incubation; D, 90-min incubation; E, F, and G, aliquots taken at 30, 60, and 90 min of incubation, respectively, to which $ZnCl_2$ was added to a concentration of 10 mM. Numbers are molecular masses in kilodaltons.

the 160-kDa signal (Fig. 2, lanes A and B). This conversion occurred in binding buffer alone and was independent of incubation with C3b–Affi-Gel, the presence of an additional irreversible serine protease inhibitor, or the interaction of the proteins with Affi-Gel itself (Fig. 2, lanes C, D, and E). Since the C3b was prepared by treatment of purified C3 with trypsin to generate the C3b cleavage product (26), we also tested the possibility that the reduction in molecular mass from 185 to 160 kDa may have been the result of trypsin contamination of the column. We examined this possibility in two ways. First, in addition to the protease inhibitors already present in the membrane preparation and binding buffer, we added TLCK (*Nα-p*-tosyl-L-lysine chloromethyl ketone) (50 μg/ml) to the preparation during incubation with the C3b–Affi-Gel (Fig. 2, lane D). TLCK is an irreversible trypsin inhibitor and at this level is in considerable excess of any trypsin which may have contaminated the column. This treatment had no observable effect on the molecular mass conversion from 185 to 160 kDa. In addition, we added 1 μg of trypsin to 30 μl of the membrane preparation in binding buffer prior to SDS-PAGE and fluorography. Under these conditions, both signals at 185 and 160 kDa were decreased (Fig. 2, lane F), indicating that the 160-kDa product was not the result of tryptic digestion of the 185-kDa protein.

Conversion from high to low molecular mass is inhibitable by zinc. In order to determine if the protein migrating at 185 kDa in the detergent-solubilized membrane extracts was the source of the 160-kDa CRP, a [^{35}S]methionine-labeled membrane preparation was incubated at room temperature, and aliquots were removed at various time points and analyzed by SDS-PAGE and fluorography. By 90 min most of the 185-kDa protein had disappeared and there was a simultaneous increase in the intensity of a signal at 160 kDa (Fig. 3, lanes A to D). A similar conversion pattern occurred in less than 60 min when the membrane preparation was incubated at 37°C (not shown). The loss of the 185-kDa protein with the simultaneous increase in the presence of the 160-kDa protein in a time- and temperature-dependent manner suggested that the two proteins were related.

We have previously demonstrated that the CRP was spontaneously shed from the parasite surface and that this release was augmented by the addition of exogenous bacterial PIPLC, indicating that the protein was anchored in membrane via a GPI linkage (26). In addition, because the conversion of the 185-kDa protein to the 160-kDa protein did not appear to be affected by excess protease inhibitors, we investigated the possibility that the action of an endogenous PIPLC present in the membrane preparation on the 185-kDa form was responsible for the molecular mass shift. The activity of *T. brucei* and *B. cereus* PIPLC is sensitive to the presence of zinc ions (2), and we observed that the molecular mass shift of the 185-kDa protein was completely inhibited by the presence of 10 mM zinc (Fig. 3, lanes E, F, and G).

Identification of the GPI-anchored membrane form of CRP. Although the inclusion of excess specific protease inhibitors in the detergent preparations did not alter the conversion of the 185-kDa protein to the 160-kDa protein, we could not exclude the possibility that the spontaneous conversion was the result of proteolysis rather than cleavage by an endogenous PIPLC. To examine this question further, we tested whether the addition of exogenous PIPLC to the membrane preparations would mediate the conversion. Cleavage and release of membrane proteins by bacterial PIPLC have become an established and readily testable means of identifying GPI-linked proteins (29). These PIPLC preparations have been shown to be free of contaminating protease activity and highly specific for GPI anchors. The results of cleavage of membrane proteins by PIPLC may be observed by mobility shifts on SDS-PAGE or native gels, as well as by separation of amphiphilic and hydrophilic forms of the proteins by phase partitioning in Triton X-114 (29).

In these experiments, treatment of detergent-solubilized membrane extracts with exogenous bacterial PIPLC resulted in a complete loss of the 185-kDa form and a concomitant increase in the amount of 160-kDa form (Fig. 4). As shown in previous experiments, the membrane preparation contains no detectable protein migrating at 160 kDa (Fig. 4, lane A). After 30 min of incubation at room temperature (without exogenous PIPLC) the 185-kDa protein and the 160-kDa protein appear in relatively equal amounts. With the addition of 1 U of exogenous bacterial PIPLC per ml, all detectable protein migrating at 185 kDa was converted to the 160-kDa protein (Fig. 4, lanes C and D).

To further support the conclusion that the conversion of the 185-kDa protein to one with an apparent molecular mass of 160 kDa was coincident with loss of the glycolipid anchor, we metabolically labeled trypomastigotes with [^{3}H]myristic acid, the fatty acid component of GPI anchors in trypanosomes. Examination of whole-cell lysates and NP-40 detergent-solubilized membrane extracts by SDS-PAGE and fluorography revealed a labeled protein migrating at 185 kDa which was eliminated by prior treatment of the samples with exogenous PIPLC (not shown). No ^{3}H-labeled protein migrating at 160 kDa was detected in either the whole-cell lysates or the membrane preparations (not shown). In addition, phase separation of [^{35}S]methionine-labeled proteins in Triton X-114 confirmed that the 160-kDa form is hydrophilic and that the 185-kDa form is amphiphilic. Treatment of these preparations with exogenous PIPLC prior to Triton X-114 phase separation completely eliminates the 185-kDa protein from the detergent phase, with a concomitant increase in the 160-kDa form present in the aqueous phase (not shown).

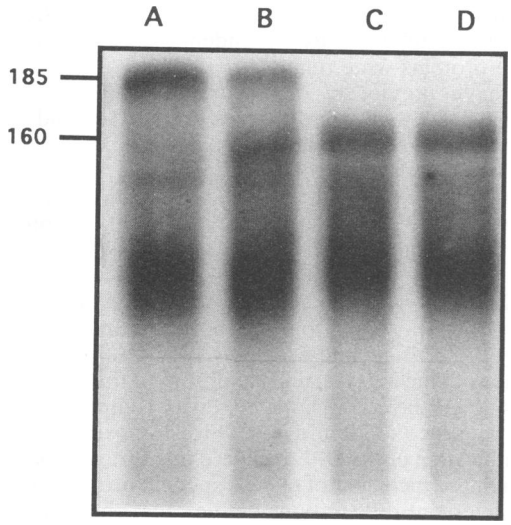

FIG. 4. Exogenous PIPLC promotes conversion of 185-kDa protein to 160 kDa. [^{35}S]methionine-labeled membrane proteins were prepared as described in Materials and Methods, and aliquots containing various amounts of *B. thuringiensis* PIPLC were incubated at room temperature for 30 min prior to SDS-PAGE and fluorography. Lanes: A, detergent-solubilized membrane preparation boiled in SDS-PAGE sample buffer (time zero); B, detergent membrane extract after 30 min of incubation at 22°C; C and D, detergent-solubilized membrane preparations treated with 1 and 10 U of PIPLC per ml, respectively. Numbers are molecular masses in kilodaltons.

The 185-kDa protein is a C3b-binding protein. In order to confirm that the CRP was derived from the 185-kDa protein and is functionally related, we prepared membrane extracts from metabolically labeled trypomastigotes in the presence or absence of 10 mM zinc (as described above) and carried out C3b-affinity chromatography. Although the binding and elution of proteins in the presence of zinc are somewhat reduced, both the 160- and 185-kDa proteins were specifically bound by C3b (Fig. 5, lanes B and C). These results support that the conclusion that the 185-kDa protein is the functional membrane form of the shed 160-kDa protein.

Effect of N- and O-linked carbohydrates on binding of CRP to C3b. In these experiments, C3b-affinity-purified CRP (soluble form) was treated with either PNGase F or O-glycosidase to determine the extent of glycosylation of the protein. The CRP was deglycosylated with PNGase F with a reduction in apparent molecular mass from 160 to 145 kDa (Fig. 6, lanes B and C), whereas treatment with *O*-glycosidase did not alter the migration of the 160-kDa protein (Fig. 6, lanes D and E). Treatment of the trypomastigote membrane proteins with *O*-glycosidase reduced the migration of some of the membrane proteins in the 80- to 100-kDa range, indicating that the enzyme was active and not inhibited by any components present in the preparation.

The enzymatic deglycosylation of CRP required denaturation of the protein; therefore, in order to assess the effect of carbohydrates on the binding interaction with C3b, CRP was purified from trypomastigotes which had been incubated in the presence of tunicamycin, an inhibitor of N glycosylation. Membrane preparations from parasites treated with tunicamycin showed a similar conversion of the 160-kDa form to the 145-kDa form, in agreement with the results of the PNGase F treatment. In comparing the C3b-binding affinity

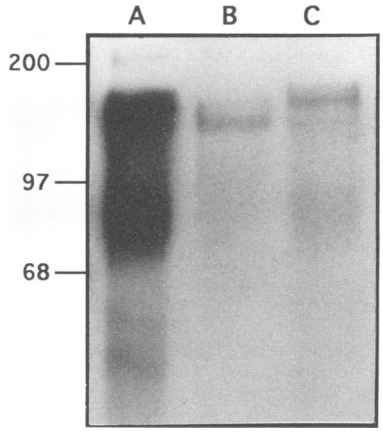

FIG. 5. The 185-kDa membrane protein is a C3b-binding protein. NP-40 detergent-solubilized membrane extracts from [^{35}S]methionine-labeled trypomastigotes were prepared in the presence or absence of 10 mM $ZnCl_2$; this was followed by C3b-affinity chromatography. Lanes: A, detergent-solubilized membrane extract; B, C3b-affinity-purified CRP under standard conditions; C, C3b-affinity-purified CRP in the presence of Zn^{2+}. The gel was prepared for fluorography as described in Materials and Methods. Numbers are molecular masses in kilodaltons.

of the 160- and 145-kDa forms, we observed a marked reduction in the amount of binding by the 145-kDa form (Fig. 7). The reduction in binding capacity of the deglycosylated CRP was estimated to be 60 to 70% by densitometric analysis (not shown).

Binding of *T. cruzi* CRP to C4b–Affi-Gel. Several mammalian CRPs express inhibitory activity on both the alternative

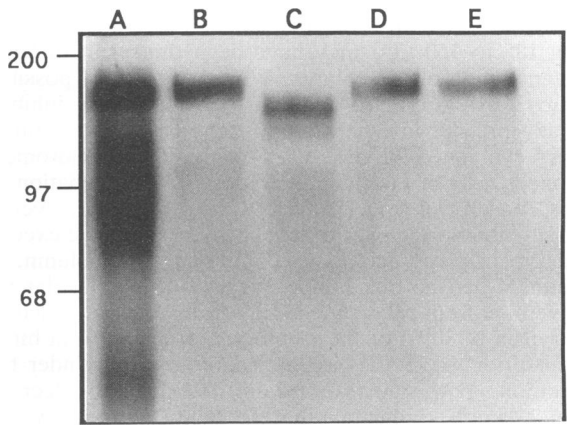

FIG. 6. Treatment of CRP with N- and O-linked glycosidases. [^{35}S]methionine-labeled membrane proteins were prepared as described in Materials and Methods, treated with 1 U of PIPLC per ml, and incubated for 30 min at room temperature (lane A). The CRP was purified by C3b-affinity chromatography as described in Materials and Methods, except that the protein was eluted by boiling the C3b–Affi-Gel beads for 10 min in either 0.5% SDS–1% 2-mercaptoethanol for PNGase treatment or 0.5% SDS–0.2% 2-mercaptoethanol for *O*-glycosidase treatment. Lanes B and C, C3b-affinity purified CRP, untreated and PNGase treated, respectively. Lanes D and E, C3b-affinity purified CRP, untreated and *O*-glycosidase treated, respectively. The gel was prepared for fluorography as described in Materials and Methods. Numbers are molecular masses in kilodaltons.

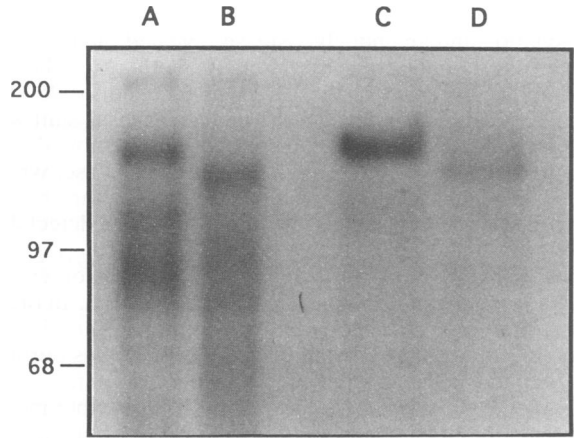

FIG. 7. Effect of N-linked carbohydrates on the CRP-C3b binding interaction. Trypomastigotes were incubated in the presence or absence of tunicamycin and then labeled with [^{35}S]methionine, and detergent-solubilized membrane extracts were prepared. Equal amounts of trichloroacetic acid-precipitable counts from the tunicamycin-treated and untreated membrane preparations were bound to equal amounts of C3b–Affi-Gel, and bound proteins were eluted by boiling the beads in sample buffer. Lanes A and B, detergent-solubilized membrane proteins from untreated and tunicamycin-treated trypomastigotes, respectively. Lanes C and D, C3b-affinity-purified CRP from untreated or tunicamycin-treated trypomastigotes, respectively. The gel was prepared for fluorography as described in Materials and Methods. Numbers are molecular masses in kilodaltons.

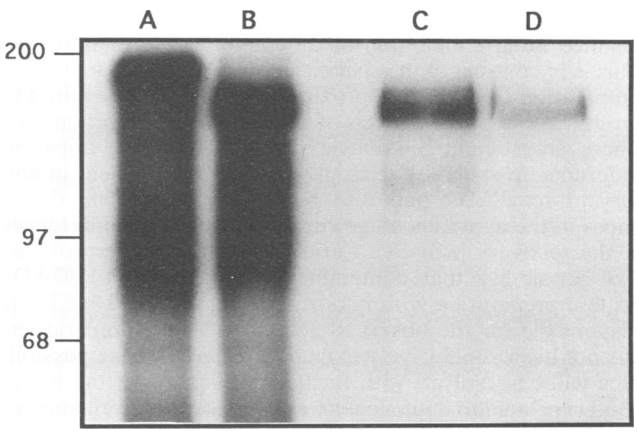

FIG. 8. *T. cruzi* CRP is a C4b-binding protein. NP-40 detergent-solubilized membrane extracts from [^{35}S]methionine-labeled trypomastigotes were prepared (lane A) and treated with 1 U of PIPLC per ml (lane B); this was followed by C3b-affinity chromatography (lane C). C3b-affinity purified CRP was diluted 1:10 in 10 mM HEPES (pH 7.4), applied to C4b-Sepharose, and eluted in 10 mM HEPES (pH 7.4)–1 M NaCl. The eluate was diluted 1:3 in 10 mM HEPES (pH 7.4) prior to SDS-PAGE and fluorography (lane D).

and classical pathway C3 convertases. Those proteins which regulate both pathways also share binding affinity for C3b as well as C4b, a component of the classical pathway C3 convertase. In addition to inhibition of the alternative complement pathway, we and others have previously observed inhibition of the classical complement pathway by proteins present in culture supernatant fluids recovered from *T. cruzi* trypomastigotes (25b, 28). The protein(s) responsible for this activity has not been conclusively identified or purified. In order to determine if the *T. cruzi* CRP was also capable of binding human C4b, the protein was purified by C3b-affinity chromatography and then subjected to chromatography through C4b–Affi-Gel. The CRP was bound to and efficiently eluted from C4b, as shown in Fig. 8. In addition, a protein which comigrated with the CRP was also eluted from C4b–Affi-Gel after binding of [^{35}S]methionine-labeled trypomastigote membrane preparations (not shown).

DISCUSSION

We have characterized a *T. cruzi* membrane glycoprotein which functions to restrict complement activation on the surface of the infectious forms of the parasite (26). In our initial studies of its complement regulatory activity, we observed that this protein, along with most of the other surface proteins of trypomastigotes, was spontaneously released from the parasite surface. The CRP was purified from spontaneously released proteins and had an apparent molecular mass of 160 kDa (26). Initial biochemical characterizations of the protein and analysis of a partial genomic clone of the gene revealed striking similarities with a family of mammalian CRPs (26). These mammalian proteins are members of the family of regulators of complement activation and restrict complement activation on autologous cells through binding interactions with C3b and/or C4b (reviewed in reference 20). Several of these proteins have multiple functions which affect the formation and stability of the C3 convertase as it assembles on the surface of complement activators. Among these mammalian complement regulatory elements, the *T. cruzi* CRP most closely resembles DAF (26). DAF is a membrane glycoprotein with widespread cell distribution and is anchored in the cell membrane via a GPI linkage (7). Similar to the *T. cruzi* CRP, DAF binds C3b and C4b, thus regulating both alternative and classical complement pathways, although it does not permanently alter or destroy the complement components (18). Unlike other C3b-binding proteins, such as factor H and CR1, neither DAF nor *T. cruzi* CRP can serve as a cofactor for factor I-mediated proteolytic cleavage of C3b or C4b (18, 25b). In addition, we have previously demonstrated that a 1.9-kb genomic clone of the *T. cruzi* CRP gene has significant homology by Southern hybridization with the DAF cDNA clone (26). We now demonstrate that the *T. cruzi* CRP binds C4b in addition to C3b and thus may restrict classical pathway activation as well.

A C3 convertase decay-accelerating activity of *T. cruzi* was first identified in shed supernatant proteins of trypomastigotes which contained proteins with apparent molecular masses in the 80- to 160-kDa range (28). Subsequently, Joiner and coworkers reported the partial purification of C3 convertase decay-accelerating activity from trypomastigote shed supernatants in which proteins with apparent molecular masses of between 87 and 93 kDa were predominant. However, since other proteins were present in the partially purified preparations, most notably in the 160-kDa range, it was not possible to definitively assign the activity to a specific protein (17). Because a 160-kDa protein copurifies with the 87- to 93-kDa preparation, it has not been determined whether these represent distinct proteins with similar activities or whether the 160-kDa protein present in the preparations is responsible for the decay-accelerating activity observed. We have previously reported that the purified 160-kDa CRP has a specific activity comparable to that of

human factor H; i.e., less than 0.5 μg of the 160-kDa protein resulted in 80% inhibition of C3 convertase formation (26). This is in contrast to the specific activity of the 87- to 93-kDa preparations, of which approximately 20 μg was required to produced comparable levels of C3 convertase inhibition (31). There are several possible explanations for the apparent difference in specific activities of these complement inhibitory proteins. The activities may reflect significant differences in the activities of two distinct proteins or differences in the recovery of native forms of the respective proteins. The possibility that contamination of the 87- to 93-kDa protein preparation with minor amounts of 160-kDa CRP is responsible for the observed activity of these preparations has not been conclusively excluded (17, 31). These possibilities will be resolved with further purification of the 87- to 93-kDa protein to homogeneity as well as characterization of the respective cDNA clones.

In the present studies, we sought to characterize the membrane form of the CRP. We observed that detergent solubilization of trypomastigote membranes did not produce a protein at 160 kDa but rather a protein with an apparent molecular mass of 185 kDa, which under certain conditions appeared to convert to 160 kDa. The conversion of the protein from high to low molecular mass in the presence of the other solubilized proteins was inhibited by boiling in SDS or by the addition of zinc ions. The conversion was not affected by protease inhibitors, although it was time and temperature dependent, indicating that the generation of the low-molecular-mass form was a dynamic process. Since the CRP, as well as most other surface glycoproteins of *T. cruzi* trypomastigotes, is anchored in the parasite membrane by a GPI anchor, we tested the possibility that the differences in electrophoretic mobilities of the two forms were the result of loss of the glycolipid anchor by an endogenous GPI-specific phospholipase present in the preparation. We observed that the 185-kDa protein is labeled with [^{3}H]myristic acid and that the label is released by the addition of PIPLC, indicating that it is incorporated as a component of the GPI membrane anchor. Furthermore, we did not detect any protein label at 160 kDa in these experiments, even after prolonged exposure of the fluorogram (not shown). In addition, we demonstrated that the addition of exogenous bacterial PIPLC to [^{35}S]methionine-labeled membrane preparations rapidly and completely converts all detectable 185-kDa protein to 160 kDa, indicating that the difference in mobility is the result of loss of the glycolipid anchor (Fig. 4). That the 160-kDa protein is generated by the action of an endogenous PIPLC is further supported by the observation that 160-kDa protein was detectable with *T. brucei* anti-cross-reacting determinant antibodies (26). These antibodies specifically recognize a C-terminal epitope on GPI-anchored proteins which can be detected only after cleavage with PIPLC (14). This conclusion was further supported by the observation that conversion from high (185-kDa) to low (160-kDa)-molecular-mass forms coincided with the conversion from an amphiphilic to a hydrophilic state as determined by phase separation in Triton X-114. Similar to the *T. brucei* PIPLC, the *T. cruzi* enzyme responsible for the release of the 160-kDa CRP was found to be inhibitable by zinc (Fig. 3). We took advantage of this observation to purify the protein migrating at 185 kDa by the addition of zinc in the initial solubilization buffer and during C3b-affinity chromatography, thus confirming that the 160-kDa protein and the 185-kDa protein were functionally the same. Taken together, these results support the conclusion that the 185-kDa membrane protein is the GPI-anchored form of the 160-kDa CRP.

Further analysis of CRP by using an inhibitor of N-linked glycosylation and an endoglycosidase revealed the presence of extensive N-linked glycosylation. In both cases the apparent molecular mass of the CRP was reduced to 145 kDa by SDS-PAGE (Fig. 6 and 7). The molecular mass shift was not detectable unless the GPI anchor was first removed with PIPLC. Treatment of the protein with *O*-glycosidase, which specifically cleaves Galβ(1-3)GalNAc from *O*-glycans bound to either serine or threonine did not result in a detectable alteration in electrophoretic mobility of the CRP (Fig. 6), although other proteins in the membrane preparation exhibited increased mobility by SDS-PAGE (not shown). In order to assess the role of carbohydrate structures in the binding interactions between CRP and C3b, the protein was purified from metabolically labeled trypomastigotes which were grown in the presence of tunicamycin. These experiments show that although the deglycosylated form was able to bind C3b, the amount of binding was significantly diminished relative to that with native CRP (Fig. 7). This is in contrast to the results obtained with the herpes simplex virus I gC CRP, deglycosylation of which did not affect C3b binding (8, 15). A detailed determination of the binding interactions of CRP and C3b at a molecular level is required before these results can be fully interpreted.

Mimicry of host molecules by pathogenic microorganisms is emerging as an important means of immune evasion, where functional mimicry of host immune molecules prevents recognition or clearance of the microbe. Although not necessarily antigenically or genetically related to the analogous host molecule, molecules which are functionally related to host immune regulatory molecules have evolved in microbial pathogens, thus damping innate or acquired immune mechanisms. Such examples have been identified among viral, prokaryotic, and eukaryotic pathogens (for a review, see reference 3). In some cases, such as the vaccinia virus gp35 and the herpesvirus saimiri complement control protein, there is striking similarity at the genetic and protein structure levels with the family of C3b-C4b mammalian CRPs (1, 19). In other cases, genetic and structural similarities are lacking. This is true of the herpes simplex virus I gC protein, which has complement-binding and regulatory functions yet does not appear to share the common structural motif found in the mammalian family (15).

The *T. cruzi* CRP is most likely in between these two examples in that it appears to be genetically and functionally related to human DAF (26) although not to the extent found between the vaccinia virus protein and human C4-binding protein, for example (19). While neither the *T. cruzi* CRP nor human DAF has cofactor activity for the proteolytic inactivation of C3b or C4b by factor I (27), they both interact with these components of the alternative and classical pathway convertases and restrict C3 convertase formation and accelerate its decay (22, 26, 27). We have shown that in addition to genetic and functional similarities, human DAF and *T. cruzi* CRP are structurally related in that both are anchored in the membrane by GPI anchors and in that release of both proteins with PIPLC exposes the same *T. brucei* anti-cross-reacting determinant epitope (5, 26). Similar to the *T. cruzi* CRP, human DAF is spontaneously released from the cell surface, most likely by a plasma-derived GPI-specific lipase (PIPLD) (6), although apparently not by the actions of endogenous PIPLC (32). The release may enable these proteins to enhance their complement-restricting activity by removing bound complement components from the cell surface. The nature of the spontaneous release of the *T. cruzi* membrane proteins has not be fully examined, although

Gonçalves et al. have evidence that membrane blebbing is responsible for a significant amount of the released protein (12). Whether the release of membrane proteins is augmented by ligand binding to the CRP has not been investigated, but this may provide the parasites with a rapid means of eliminating active complement components from their surface. This may not only diminish the lytic effects of complement but also reduce the efficiency of complement-mediated opsonization and clearance of the parasites. A more complete analysis of the mechanism of the release of CRP may address this question.

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