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Characterization of a G_i-protein from *Trypanosoma cruzi* epimastigote membranes

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A guanosine 5'-[γ-[^{35}S]thio]triphosphate-binding activity was detergent-extracted from *Trypanosoma cruzi* membranes. This binding activity was co-eluted from gel-filtration columns with a factor which, in a heterologous reconstitution system, blocks glucagon stimulation of adenylate cyclase activity in liver membranes. ADP-ribosylation of these membranes by pertussis toxin eliminated this blocking capacity. Incubation of *T. cruzi* membranes with activated pertussis toxin and [*adenylate*-^{32}P]NAD$^+$ led to the incorporation of radioactivity into a labelled product with an apparent M_r of approx. 43000. Crude membranes were electrophoresed on SDS/polyacrylamide gels and analysed, by Western blotting, with GA/1 anti-α_{common}, AS/7 anti-α_t, anti-α_{i1} and anti-α_{i2} polyclonal antibodies. These procedures led to the identification of a specific polypeptide band of about 43 kDa. Another polypeptide reacting with the SW/1 anti-β antibody, of about 30 kDa, was also detected in the membrane fraction.

INTRODUCTION

G-proteins involved in transmembrane signalling are heterotrimers composed of α, β and γ subunits, with molecular masses of 40–45, 35–36 and 5–8 kDa respectively. The α subunits bind and hydrolyse GTP; they are substrates for ADP-ribosylation by bacterial toxins, and they carry the specificity for receptors and effectors (Birnbaumer *et al.*, 1990).

There are several structural and functional differences between α subunits. The mammalian genome codes for several types of α subunits, including four α_s, three α_i, one α_o, one α_t and one α_{olf} (for review, see Birnbaumer *et al.*, 1990). Some of these subunits have well-defined functions: α_s couples stimulatory receptors to adenylate cyclase (Gilman, 1984) and Ca^{2+} channels (Yatani *et al.*, 1987), α_i may be involved in the inhibition of adenylate cyclase (Jakobs *et al.*, 1976) and in the opening of K^+ channels (Kurachi *et al.*, 1986), α_o acts in the closing of Ca^{2+} channels (Holz *et al.*, 1986), and α_t couples photolysed rhodopsin with the stimulation of cyclic GMP phosphodiesterase activity (Stryer, 1986).

Previous evidence from our laboratory indicates that *Trypanosoma cruzi* membranes have a 45 kDa polypeptide that can be ADP-ribosylated by cholera toxin. In addition, after electrofusion of *T. cruzi* and lymphoma S49 cyc$^-$ cells, a heterologous adenylate cyclase is reconstituted that is then activated by isoprenaline or fluoride (Eisenschlos *et al.*, 1986). This strongly suggests the existence in *T. cruzi* membranes of an α_s polypeptide.

The present paper reports evidence for the existence in *T. cruzi* membranes of a 43 kDa polypeptide that shows several functional and immunological properties that are characteristic of $G\alpha_i$ subunits. In addition, results presented here indicate the presence in these membranes of a 30 kDa polypeptide immunoreacting with an antiserum to Gβ-subunit.

MATERIALS AND METHODS

Materials

The sources of materials used in this work are given elsewhere (Eisenschlos *et al.*, 1986; Muschietti *et al.*, 1989).

Antisera raised against synthetic peptides that correspond to defined regions of G-protein subunits (Mumby *et al.*, 1986; Goldsmith *et al.*, 1988; Simonds *et al.*, 1989) were kindly provided by Dr. Allen M. Spiegel (National Institutes of Diabetes and Digestive and Kidney Diseases, N.I.H., Bethesda, MD, U.S.A.), Dr. Eduardo Lapetina and Dr. Daniel Altschuler (Wellcome Research Laboratories, Research Triangle Park, NC, U.S.A.), Dr. Lutz Birnbaumer (Baylor College of Medicine, Houston, TX, U.S.A.) and Dr. Alfred G. Gilman (University of Texas, Dallas, TX, U.S.A.). These antisera correspond to the following antigens: AS/7 (*C*-terminus of α_t, α_{i1}, α_{i2}); SW/1 (*C*-terminus of β-subunit); GA/1 (GTP-binding site; α_{common}); RM/1 (*N*-terminus of α_s).

Guanosine 5'-[γ-[^{35}S]thio]triphosphate (GTP[^{35}S]) and [*adenylate*-^{32}P]NAD$^+$ were purchased from New England Nuclear (Boston, MA, U.S.A.), and pertussis toxin was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). A peptide with the *C*-terminal amino acid sequence of α_t, α_{i1} and α_{i2} (KENLKDCGLF) was obtained from Biodynamics (Martinez, Argentina).

Membrane preparation

Liver plasma membranes were prepared by the procedure of Neville (1968). *T. cruzi* membranes were prepared as described by Torruella *et al.* (1986).

Detergent extraction of *T. cruzi* membranes

Membranes were suspended (10 mg of protein/ml) in 50 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-β-mercaptoethanol and 1% (w/v) sodium cholate, left in ice for 2 h with magnetic stirring, and centrifuged for 60 min at 105000 *g*. The detergent concentration in the extract was lowered by filtration through Ultrogel AcA34. The column (1.4 cm × 40 cm), equilibrated with 50 mM-Tris/HCl buffer, pH 7.5, containing 0.1% sodium cholate, was loaded with 1.5 ml of the supernatant (9 mg of protein). Elution (0.5 ml/min) was performed at 4 °C; 1.5 ml fractions were collected. Fractions with the highest GTP[S]-binding activity were pooled and stored at 4 °C (giving the 'Ultrogel fraction').

GTP-binding assay

Binding of GTP[^{35}S] to purified protein fractions was carried

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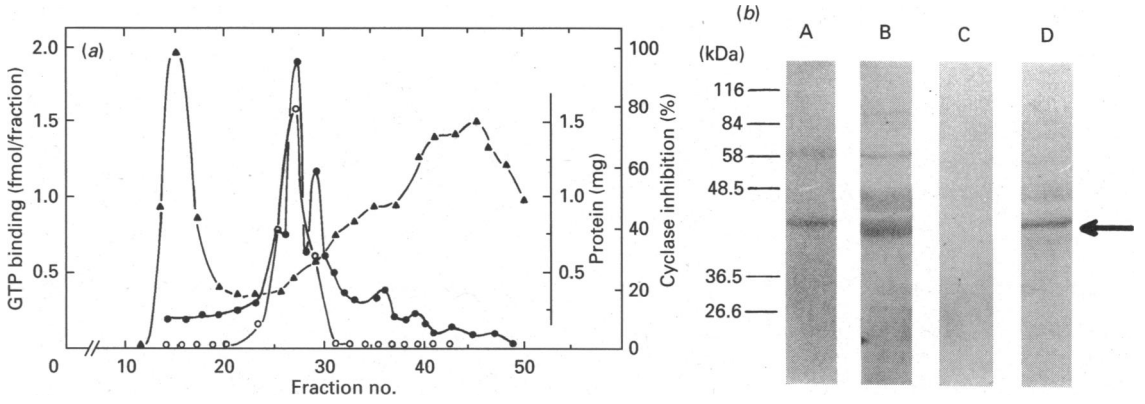

Fig. 1. Gel filtration on Ultrogel AcA34 of a detergent extract of *T. cruzi* membranes

(*a*) ●, GTP binding; ○, inhibition of liver membrane adenylate cyclase activity; ▲, protein. (*b*) Immunoreaction of *T. cruzi* polypeptides from membranes (A), total epimastigote extract (B) and Ultrogel fractions 44 (C) and 27 (D) with the antiserum AS/7. Conditions were described in the Materials and methods section.

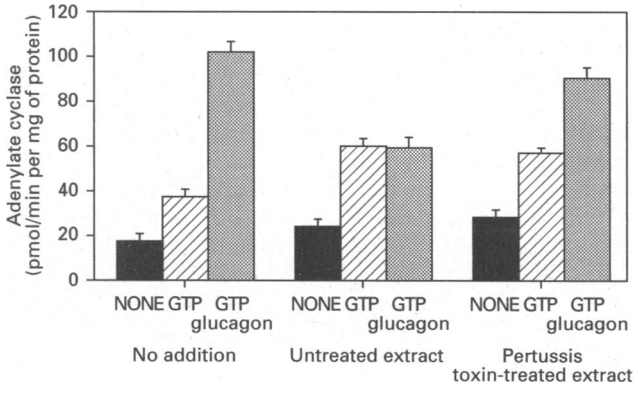

Fig. 2. Effect of detergent extract from *T. cruzi* membranes on the activation by glucagon of liver membrane adenylate cyclase activity

Glucagon and GTP concentrations in the mixtures were 10 nM and 1 μM respectively. The experiment was performed in triplicate samples. Bars indicate S.D. Other conditions are described in the Materials and methods section.

out by the procedure of Northup *et al.* (1982) with the modifications indicated by Waldo *et al.* (1987). Under the conditions for the assay procedure, GTP binding was proportional to the protein concentration.

NAD^+ and pertussis-toxin treatment of membrane proteins

Very fresh *T. cruzi* membranes or bovine rod-outer-segment membranes (0.1 mg of protein/assay) were incubated for 30 min at 30 °C in 50 mM-Tris/HCl buffer, pH 7.4, containing 2 mM-$MgCl_2$, 1 mM-EDTA, 10 mM-dithiothreitol, 0.01 % (v/v) Lubrol, 1 mM-ATP, 0.2 mM-GTP, 1 μM-[*adenylate*-^{32}P]NAD^+ (sp. radioactivity 40000 c.p.m./pmol) and 12 μg of activated pertussis toxin (preincubated at a concentration of 0.4 mg/ml, in the presence of 10 mM-dithiothreitol). Total volume was 0.06 ml. After incubation, a 0.025 ml sample was diluted in 0.5 ml of 3 % (w/v) SDS and precipitated by addition of 0.5 ml of cold 30 % (w/v) trichloroacetic acid. The precipitate was collected on nitrocellulose filters, washed five times with 6 % trichloroacetic acid and counted for radioactivity. The rest of the incubation mixture was adjusted to final concentrations of ATP and NAD^+ of 10 mM and 5 mM respectively and then subjected to SDS/PAGE. The resolved polypeptides were transferred from the gel to a nitrocellulose membrane, which was further exposed to a radioautographic film.

In the reconstitution experiments, membranes were ADP-ribosylated as indicated above, except that 10 μM unlabelled NAD^+ was used instead of [^{32}P]NAD^+. After incubation, the mixtures were dialysed for 3 h at 4 °C against 50 mM-Tris/HCl buffer, pH 7.5, containing 0.1 % sodium cholate. If, after toxin pretreatment in the presence of unlabelled NAD^+, the incubation mixture was further incubated in the presence of [^{32}P]NAD^+, there was no incorporation of radioactivity in the trichloroacetic acid precipitate.

Heterologous reconstitution by using membranes and detergent extracts

Samples (20 μl) of *T. cruzi* 'Ultrogel fraction' or ADP-ribosylated membranes (150 μg of protein) were preincubated for 15 min at 37 °C with 5 μl of liver membranes (100 μg of protein). After that, the mixtures were immediately assayed for adenylate cyclase activity.

Analytical methods

Adenylate cyclase assays were performed as described elsewhere (Flawiá *et al.*, 1983; Eisenschlos *et al.*, 1986). Protein was determined by the method of Lowry *et al.* (1951). Procedures for SDS/PAGE of protein samples were described elsewhere (Kornblihtt *et al.*, 1981).

Polypeptide transfer from polyacrylamide gels to nitrocellulose membranes was carried out by electrotransference (Khyse-Andersen, 1984). For reaction with antibodies, the membranes after the transfer were blocked with a suspension of non-fat milk (Johnson *et al.*, 1984). After reaction with the corresponding antibody, detection was carried out with the Vectastain ABC-AP kit (Vector Laboratories, Burlingame, CA, U.S.A.), by following the instructions of the manufacturer. Controls were performed either with pre-immune rabbit serum or with 200 μM specific peptide. In this latter case conditions were those described by Burstein & Macara (1989).

RESULTS AND DISCUSSION

Extraction and functional characterization

Treatment of *T. cruzi* membranes with a buffer containing 1 % sodium cholate led to the extraction of a GTP[^{35}S]-binding activity. Upon gel filtration on an Ultrogel AcA34 column this

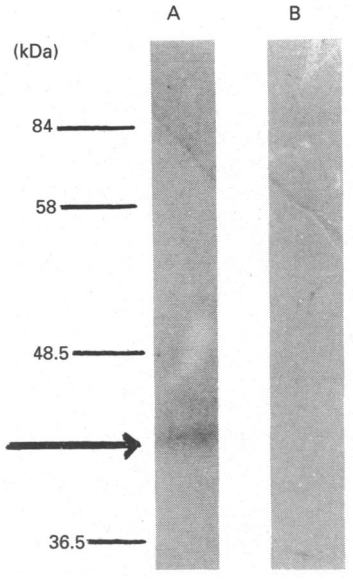

Fig. 3. ADP-ribosylation of *T. cruzi* membranes in the presence of [*adenylate*-^{32}P]NAD$^+$

SDS/PAGE of *T. cruzi* membranes treated with (A) or without (B) pertussis toxin in the presence of [*adenylate*-^{32}P]NAD$^+$. Conditions are described in the Materials and methods section.

binding activity co-eluted with a factor which, in a heterologous reconstitution system, blocked glucagon stimulation of adenylate cyclase activity in liver membranes (Fig. 1*a*). This capacity to block hormone stimulation of liver adenylate cyclase might be the consequence of a putative G_i-protein extracted from *T. cruzi* membranes. As shown in Fig. 1(*b*), in crude extracts, membranes and the binding-activity peak fractions a 43 kDa polypeptide band was identified that immunoreacted with the AS/7 anti-α_i antibody. This band was not observed in column fractions devoid of GTP[S]-binding activity.

ADP-ribosylation of $G\alpha_i$ polypeptides by pertussis toxin decreases their ability to block adenylate cyclase activation (Gilman, 1984). For this reason, the blocking capacity of cholate extracts from *T. cruzi* membranes treated with pertussis toxin and NAD$^+$ was also examined. Under the selected conditions, the putative polypeptide acceptor seemed to be fully ADP-ribosylated. As shown in Fig. 2, extracts from untreated membranes, but not from toxin-treated membranes, blocked glucagon stimulation of adenylate cyclase activity in liver membranes. This strongly suggests the involvement of a *T. cruzi* $G_{\alpha i}$ protein in this effect.

ADP-ribosylation with pertussis toxin

ADP-ribosylation catalysed by *Bordetella pertussis* toxin has been used for specific identification of $G\alpha_i$, $G\alpha_t$ and $G\alpha_o$ subunits in crude membranes (Gilman, 1984). Consequently, *T. cruzi* membranes were incubated with activated pertussis toxin plus [*adenylate*-^{32}P]NAD$^+$, and the incorporation of radioactivity into a trichloroacetic acid-insoluble product was determined. As shown in Fig. 3, after resolution of membrane polypeptides by SDS/PAGE a labelled product was detected with an electrophoretic mobility slightly slower than that of ADP-ribosylated transducin. The apparent molecular mass of this product was approx. 43 kDa.

On the other hand, the incorporation was dependent on the presence of this toxin and could be displaced by addition of unlabelled NAD$^+$ (results not shown).

Characterization with specific antisera

Further characterization of the putative *T. cruzi* G_i protein was performed by analysis of membrane polypeptides by Western blotting and further reaction with antisera raised against synthetic peptides corresponding to conserved sequences of G-protein subunits. Reaction with GA/1 (anti-α_{common}) or with AS/7 (anti-α_t, anti-α_{i1} and anti-α_{i2}) polyclonal antibodies led to the identification of specific polypeptide bands with mobilities corresponding to about 43 kDa (Figs. 4*a*, 4*b* and 4*c*). In contrast, the RM/1 antibody (anti-α_s) revealed a polypeptide band of about 46 kDa (Fig. 4*d*), which is similar to that previously reported by this laboratory for the cholera-toxin ADP-ribosylated polypeptide (Eisenschlos *et al.*, 1986).

On the other hand, the reaction of membrane polypeptides with the SW/1 (anti-β) antibody revealed a specific polypeptide band with a mobility corresponding to about 30 kDa (Fig. 4*e*). The size of this Gβ-like polypeptide is smaller than those of the two types of β subunits identified in higher eukaryotic organisms, with molecular masses of approx. 35–36 kDa (Birnbaumer *et al.*, 1990).

Results in the present paper indicate the presence in *T. cruzi*

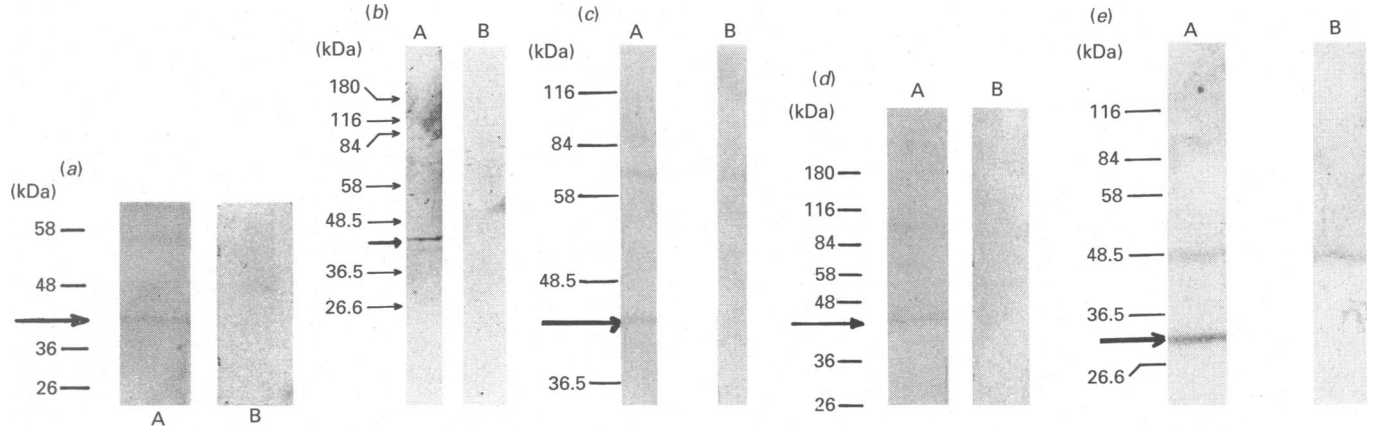

Fig. 4. Immunoreaction of *T. cruzi* membrane polypeptides with antisera directed against G-protein subunits: (*a*) GA/1; (*b*) and (*c*) AS/7; (*d*) RM/1; (*e*) SW/1

Lanes A and B respectively show reaction with the specific antisera and the result of control reactions performed with preimmune serum (*a*, *b*, *d* and *e*); in *c*, the control was performed by blockage of the antiserum with the specific peptide. Conditions for gel electrophoresis, blotting and competition with the peptide are given in the Materials and methods section.

membranes of a polypeptide of the $\alpha_i/\alpha_{i1,2}$ type which can be ADP-ribosylated in the presence of NAD^+ and preactivated pertussis toxin. This polypeptide may be a part of a hetero-multimeric protein of the G-family, since the presence in the same membrane fraction of a polypeptide having the epitope characteristic of $G\beta$ subunit was also detected. As occurs with mammalian G_i-proteins, this *Trypanosomatidae* membrane component was able to block hormone stimulation of liver membrane adenylate cyclase activity in heterologous reconstituted systems.

These results provide the first indication of the existence of G_i-like proteins in lower eukaryotic organisms. This fact, together with the previous description of an α_s-like polypeptide in *T. cruzi* membranes (Eisenschlos *et al.*, 1986) indicates that *Trypanosomatidae*, like animal tissues, have a well-developed set of G-proteins that may be associated with membrane transduction mechanisms. *In vivo*, this transduction mechanism may be precisely the regulation of adenylate cyclase activity by a receptor whose hormone has not yet been identified.

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