Characterization of a G1-protein from Trypanosoma cruzi epimastigote membranes

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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 αt, three αo, one αv, one αi and one αot (for review, see Birnbaumer et al., 1990). Some of these subunits have well-defined functions: αt couples stimulatory receptors to adenylate cyclase (Gilman, 1984) and Ca2+ channels (Yatani et al., 1987). αi may be involved in the inhibition of adenylate cyclase (Jakobs et al., 1976) and in the opening of K+ channels (Kurachi et al., 1986). αi acts in the closing of Ca2+ channels (Holz et al., 1986), and αo couples photolyzed 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 cec-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 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 Ga subunits. In addition, results presented here indicate the presence in these membranes of a 30 kDa polypeptide immunoreacting with an antisemur to Gß-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, 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 αt, αi, αo); SW/1 (C-terminus of β-subunit); GA/1 (GTP-binding site; αcommon); RM/1 (N-terminus of αi).

Guanosine 5′-[γ-32S]thiophosphate (GTP[32S]) and [adenylate-32P]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 αt, αi, and αo (KENLKDCLGF) 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 Ultragel AcA34. The column (1.4 cm x 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 'Ultragel fraction').

GTP-binding assay

Binding of GTP[32S] to purified protein fractions was carried out as described by Spiegel et al. (1989).
After was a mixture of 10\% protein and nitrocellulose at a concentration of 0.1 mg/ml. SDS was precipitated by addition of 0.5 ml of cold 30\% (v/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 mM unlabelled NAD\(^+\) was used instead of \[^{32}\text{P}\]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 \[^{32}\text{P}\]NAD\(^+\), there was no incorporation of radioactivity in the trichloroacetic acid precipitate.

Heterologous reconstitution by using membranes and detergent extracts

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

Analytical methods

Adenylate cyclase assays were performed as described elsewhere (Flawià 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 (Kornblihlt et al., 1981).

Polypeptide transfer from polyacrylamide gels to nitrocellulose membranes was carried out by electrotransference (Khys-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 \(\mu\)M 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\(^{[38]}\)-binding activity. Upon gel filtration on an Ultrogel AcA34 column this

![Fig. 1. Gel filtration on Ultrogel AcA34 of a detergent extract of \(T. cruzi\) membranes](image1)

(a) GTP binding; (b) 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](image2)

Glucagon and GTP concentrations in the mixtures were 10 nm and 1 \(\mu\)M respectively. The experiment was performed in triplicate samples. Bars indicate s.d. Other conditions are described in the Materials and methods section.
Trypanosoma cruzi G-protein

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

SDS/PAGE of T. cruzi membranes treated with (A) or without (B) pertussis toxin in the presence of [adenylate-32P]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. 1a). This capacity to block hormone stimulation of liver adenylate cyclase might be the consequence of a putative Gβ-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-α1 antibody. This band was not observed in column fractions devoid of GTP[S]-binding activity.

ADP-ribosylation of Gα 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 Gα protein in this effect.

ADP-ribosylation with pertussis toxin

ADP-ribosylation catalysed by Bordetella pertussis toxin has been used for specific identification of Gα1, Gα7 and Gα9 subunits in crude membranes (Gilman, 1984). Consequently, T. cruzi membranes were incubated with activated pertussis toxin plus [adenylate-32P]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 Gβ 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-α7comm) or with AS/7 (anti-α1, anti-α7, and anti-α9) polyclonal antibodies led to the identification of specific polypeptide bands with mobilities corresponding to about 43 kDa (Figs. 4a, 4b and 4c). In contrast, the RM/1 antibody (anti-α7) revealed a polypeptide band of about 46 kDa (Fig. 4d), 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. 4e). 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...
membranes of a polypeptide of the $\alpha_i/\alpha_{1,2}$ type which can be ADP-ribosylated in the presence of NAD$^+$ and preactivated pertussis toxin. This polypeptide may be a part of a heteromultimeric 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 $\alpha_i$-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.

H. N. T. and M. M. F. are career members of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET); J. P. M., A. D. A. and O. A. C. are fellows of CONICET, University of Buenos Aires and Comisión de Investigaciones Científicas, Provincia de Buenos Aires (Argentina) respectively. This work was partially supported by the TDR Programme, World Health Organization and by Fundación Antorchas (Argentina).

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Received 28 February 1992/27 April 1992; accepted 30 April 1992


