Interaction of *Trypanosoma cruzi* adenylate cyclase with liver regulatory factors

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*Trypanosoma cruzi* adenylate cyclase catalytic subunits may interact with regulatory factors from rat liver membranes, reconstituting heterologous systems which are catalytically active in assay mixtures containing MgATP. The systems show stimulatory responses to glucagon and guanosine 5′-[(βγ-imido)triphosphate (p[NH]ppG) or fluoride. Reconstitution was obtained by three different methods: (1) fusion of rat liver membranes (pretreated with N-ethylmaleimide) to *T. cruzi* membranes; (2) interaction of detergent extracts of rat liver membranes with *T. cruzi* membranes; or (3) interaction of purified preparations of *T. cruzi* adenylate cyclase and of liver membrane factors in phospholipid vesicles. The liver factors responsible for the guanine nucleotide effect were characterized as the N₆ protein. Data also indicate that reconstitution requires the presence of a membrane substrate.

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INTRODUCTION

Hormonal control of adenylate cyclase systems is a phenomenon which results from the interaction of at least four protein entities: hormone receptors, two guanine-nucleotide-binding proteins, N₆ and N₇ (for enzyme stimulation and inhibition respectively), and adenylate cyclase catalytic subunits. Important progress in the understanding of the regulatory mechanism of this complex system was achieved through two combined approaches: purification of the different components of the adenylate cyclase system and analysis of the interaction of these components in detergent-containing mixtures (see Gilman, 1984).

Basic requirements in such mixtures are the adenylate cyclase catalytic subunits which may be obtained either from the cys′-S49 lymphoma cell variant or from a lower eukaryotic organism such as *Neurospora*. Both enzymes are almost inactive with MgATP as substrate, fully active with MnATP and insensitive to fluoride (Ross et al., 1978; Flawiá & Torres, 1972).

The case of *Neurospora* adenylate cyclase is particularly interesting, since this enzyme has been reasonably well purified and characterized biochemically and genetically (Reig et al., 1982; Flawiá et al., 1977). Moreover, it may interact with avian erythrocyte regulatory factors, reconstituting an heterologous system which shows full responsiveness to p[NH]ppG and isoprenaline or fluoride (Flawiá et al., 1983).

Work on adenylate cyclase by our laboratory was focused on another eukaryotic organism, the parasite *Trypanosoma cruzi*, which is the etiologic agent of Chagas's disease (Torruella et al., 1986) The purpose of the present paper is to describe studies on *T. cruzi* adenylate cyclase that extend the information obtained in *Neurospora*. The approach used in these studies combines the use of different preparations of *T. cruzi* adenylate cyclase and regulatory factors from liver membranes.

Evidence is provided indicating that heterologous systems with responses to glucagon, p[NH]ppG or fluoride may be reconstituted by a variety of procedures, such as membrane fusion, interaction between membranes and factors in the detergent extract, or by incubation of detergent-solubilized preparations with phospholipid vesicles.

EXPERIMENTAL

Materials

The origin of the materials and some general procedures used in these experiments have been described elsewhere (Reig et al., 1982; Torruella et al., 1986).

Liver membranes

Rat liver membranes were purified by the procedure of Neville (1968). Membrane pellets were stored at −20 °C.

Treatment of liver membranes with N-ethylmaleimide

Liver membranes (32 mg of protein) were adjusted to a total volume of 10 ml with 1 mm-NaHCO₃ and centrifuged for 60 min at 105 000 g. Membrane pellets were resuspended in 1 mm-NaHCO₃ (6 mg of protein/ml) and incubated for 30 min at 37 °C in the presence of 1 mm-N-ethylmaleimide. Reaction was stopped by addition of 6 vol. of 5 mm-dithiothreitol/1 mm-NaHCO₃, after which membranes were recovered by centrifugation at 105 000 g for 60 min.

Detergent extraction of liver membranes

Membranes (not treated with N-ethylmaleimide) were suspended (10 mg of protein/ml) in 50 mm-Tris/HCl buffer, pH 7.5, containing 1 mm-β-mercaptoethanol and 1% sodium cholate, left in ice for 2 h with magnetic stirring, and centrifuged for 60 min at 105 000 g. After
that, the upper portion of the supernatant (one-tenth of total volume) was removed by suction and discarded. Three-quarters of the remaining supernatant fluid were carefully pipetted off and incubated at 37 °C for 30 min to inactive adenylyl cyclase. Detergent concentration in the extract was lowered by filtration through Ultrogel Ac34. 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 incubated supernatant (8.7 mg of protein). Elution (0.5 ml/min) was performed at 2–4 °C; 1.4 ml fractions were collected. Fractions corresponding to the protein peak were pooled and stored in the cold for no more than 2 days (giving ‘Ultrogel fraction’).

NAD+ and cholera-toxin treatment of N₄ protein associated with liver membranes

Rat liver membranes were resuspended in 1 mM-NaHCO₃. The suspension (10 mg of protein) was incubated for 30 min at 37 °C in 20 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-ATP, 10 μM-GTP, 2 mM-MgCl₂, 1 mM-dithiothreitol, 1 mM-NAD+ and 20 μg of activated chola toxin (preincubated at a concentration of 100 μg/ml, in the presence of 20 mM-dithiothreitol). Total volume was 2 ml. After incubation the mixture was diluted with 8 ml of 1 mM-NaHCO₃ and centrifuged for 60 min at 105 000 g. Membranes were then resuspended and centrifuged twice by the same procedure. Finally the membrane pellet was extracted with 2 ml of 1% sodium cholate for 2 h at 0 °C with magnetic stirring. After centrifugation for 60 min at 105 000 g, a sample of the supernatant fluid (0.5 ml) was filtered through Ultrogel Ac34 as described above.

T. cruzi membranes and detergent extraction

The procedure for the preparation of these membranes was previously described (Torrue la et al., 1986). Detergent extraction was performed as indicated for liver membranes, up to the step of centrifugation at 105 000 g. When necessary, adenylyl cyclase in T. cruzi preparations was inactivated by heating at 50 °C for 30 min.

Purification of T. cruzi adenylyl cyclase activity

Enzyme activity was purified from detergent-extracted membranes up to the step of hexylamino-Sepharose column chromatography (Torrue la et al., 1986) to obtain the ‘hexylamino-Sepharose’ fraction.

Neurospora crassa membranes

Cultures of the Fungal Genetic Stock Center 1118 slime strain (os-fz-sg) were grown in Vogel’s (1956) medium containing 2.5% (w/v) sucrose, 0.75% nutrient broth, 0.75% yeast extract and 2.5 μg of biotin/ml. Growth was carried out at 28 °C for 48 h by shaking (100 rev./min) in 500 ml Erlenmeyer flasks containing 125 ml of medium. Cells were collected by centrifugation at 3000 g for 10 min, and lysed by hypo-osmotic shock by resuspension in chilled 1 mM-NaHCO₃ (20 times the volume of the cell pellets). The preparation was left in ice for 60 min and then centrifuged at 10 000 g for 10 min. This treatment generates Neurospora ‘ghosts’, the structure of which has been previously described (Flawia & Torres, 1972).

Reconstitution by polyethylene glycol-mediated membrane fusion

Membrane pellets from liver and T. cruzi (or Neurospora) were resuspended in 1 mM-NaHCO₃. Various amounts of this membrane suspension were mixed to give a total protein concentration of 8 mg/ml. Samples (0.5 ml) of these mixtures were incubated at 37 °C with magnetic stirring. After 20 min, 0.5 ml of a polyethylene glycol-6000 solution (520 mg in 0.48 ml in 1 mM-NaHCO₃) was added and the incubation continued for another 2 min; then 7 ml of 1 mM-NaHCO₃ was slowly added. Thereafter the mixture was chilled and centrifuged at 10 500 g for 60 min.

Reconstitution by using membranes and detergent extracts

Reconstitution by using Ultrogel fractions and T. cruzi membranes was performed as follows: samples of the fractions (20 μl) were preincubated for 60 min at 0 °C in the presence of different additions (1 μM-p[NH]ppG, 1 μM-glucagon or 10 mM-NaF) as indicated in each case (25 μl total volume). After addition of 5 μl of a suspension of T. cruzi membranes (70 μg of protein), the preincubation was continued for another 20 min. The components for the adenylyl cyclase assay reaction were added after this preincubation.

Reconstitution by using phospholipid vesicles

A mixture of egg phosphatidylcholine (21 mg/ml), lysophosphatidylinholine (1 mg/ml) and cetyltrimethylammonium bromide (1.9 mg/ml) in 50 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-β-mercaptoethanol was dispersed by sonication at 0 °C under N₂ for 5 min. Samples of the phospholipid suspension (10 μl) were preincubated for 20 min at 21 °C in the presence of different additions (1 μM-p[NH]ppG, 1 μM-glucagon or 10 mM-NaF) as indicated in each case, plus the Ultrogel fraction (20 μg of protein), and ‘hexylamino-Sepharose’ fraction (100 μg of protein). Thereafter, the components for the adenylyl cyclase assay reaction were added.

Assays

The adenylyl cyclase activity was as described elsewhere (Flawia et al., 1983). N₄ was assayed by the capacity to restore adenylyl cyclase activity (MgATP as substrate) in membranes from cyc⁻ lymphoma S49 cells (Ross et al., 1978).

RESULTS

Adenylyl cyclase activities in liver, T. cruzi and N. crassa membrane preparations and in the corresponding fusion products

Fig. 1 shows time courses of adenylyl cyclase activity corresponding to rat liver, T. cruzi and N. crassa membranes before and after fusion. Liver membranes show the usual responses to glucagon and p[NH]ppG or fluoride in assays containing MgATP. Specific activities were similar to those reported by Pohl et al. (1971). After treatment with N-ethylmaleimide all the activities were abolished (Fig. 1a).

Adenylyl cyclase activity in T. cruzi membranes was 10 times higher in assays containing ManATP than in those containing MgATP, and the effects of glucagon and p[NH]ppG or fluoride were negligible (Fig. 1b). Occasionally in some membrane preparations a slight
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Fig. 1. Adenylate cyclase activities in liver, T. cruzi, N. crassa and fused membranes

(a) Liver membranes (200 μg of protein) assayed with MgATP in the presence of the following additions: none (○), 1 μM-glucagon and 1 μM-p[NH]ppG (■), or 10 mM-NaF (▲); N-ethylmaleimide-treated membranes (●). (b) T. cruzi membranes (200 μg of protein) assayed with MgATP (○) or MnATP (□). (c) T. cruzi membranes (60 μg of protein) fused to N-ethylmaleimide-treated liver membranes, pretreated (○) or not (○, ■ and ▲) with cholera toxin. Assays were performed with MgATP in the presence of the following additions: none (○ and ○), 1 μM-glucagon and 1 μM-p[NH]ppG (■), or 10 mM-NaF (▲). (d) and (e), as (b) and (c), except that Neurospora membranes (30 μg of protein) were used instead of T. cruzi membranes. Other conditions were given in the Experimental section.

activation by fluoride in the presence of MgATP was detected (results not shown).

After fusion of liver membranes (treated with N-ethylmaleimide) with T. cruzi membranes, a clear increase in basal activities was noted in assays performed with MgATP. Under these conditions a 3–4-fold stimulation by glucagon plus p[NH]ppG or fluoride was observed (Fig. 1c). It is evident that, at the maximum adenylate cyclase stimulation, specific activities were equivalent to those measured with MnATP in T. cruzi membranes (compare Figs. 1c and 1b). In addition, when liver membranes were first treated with cholera toxin and thereafter with N-ethylmaleimide, basal activities in the fusion product were significantly higher than in the product from liver membranes not treated with the toxin (Fig. 1c).

For comparison, a similar experiment performed with N. crassa membranes and the corresponding membrane fusion product was included (Figs. 1d and 1e). Basal activities measured with MgATP in Neurospora membranes were negligible (Fig. 1d), and consequently the dependence on MnATP was much higher than that observed in T. cruzi membranes (compare Figs. 1d and 1b). In other aspects, the behaviour of the fusion product was identical with that derived from T. cruzi membranes (compare Figs. 1e and 1c).

After fusion of T. cruzi membranes with liver membranes (treated with N-ethylmaleimide), the heterologous reconstituted adenylate cyclase system showed a dose-dependence on glucagon concentration (Fig. 2a). In the presence of 1 μM-p[NH]ppG, stimulation resulted in a saturable phenomenon reaching a plateau at 0.1 μM-
glucagon. The curve shows an hormone requirement for half-maximal enzyme stimulation which was slightly higher than in fresh liver membranes (Pohl et al., 1971). On the other hand, dependence on fluoride concentration for enzyme activation was identical with that found in liver (Pohl et al., 1971).

The results of these experiments suggest that the catalytic subunit of adenylate cyclase in T. cruzi is capable of interacting with mammalian regulatory factors in a hybrid membrane. Since T. cruzi membranes did not show any specific binding of glucagon (results not shown), the receptor entities have to be provided by the liver membranes. In terms of the involvement of a putative Nₐ guanine-nucleotide-binding protein in this type of reconstitution, the stimulation observed with cholera toxin indicates that liver membranes are also providing this regulatory component to the fusion product.

**Adenylate cyclase in mixtures of membranes and detergent extracts**

A heterologous hormone-dependent system was also reconstituted from T. cruzi membranes and detergent extracts of liver membranes. To achieve this reconstitution, it was important to maintain a relatively low detergent concentration in the mixtures (not higher than 0.1%). Fig. 3(a) shows basal adenylate cyclase activities in T. membranes assayed with Mg²⁺ and the extent of stimulation by glucagon plus p[NH]ppG or by fluoride as a function of detergent extract concentration (Ultrogel fraction). With increasing amounts of the cholate extract the system showed a tendency to saturate. This extract, in turn, did not show any detectable adenylate cyclase activity in assays performed in the absence of T. cruzi membranes, supplemented or not with glucagon plus p[NH]ppG or fluoride.

Fig. 3(b) shows the results of an experiment in which adenylate cyclase activity was determined as a function of T. cruzi membrane concentration. In the absence of detergent extract the activity was roughly proportional to the amount of added membranes, but in its presence basal activities reached a plateau with increasing concentrations of membranes. At the highest concentration, the effect of the detergent extract disappeared. Activities of mixtures containing detergent extract and assayed in the presence of glucagon plus p[NH]ppG or fluoride also showed a saturable dependence on membrane concentration. On the other hand, there was no activity in mixtures containing heat-treated membranes. This may suggest that reconstitution is a consequence of the interaction between T. cruzi cyclase and regulatory factors in the liver membrane extract, and not a re-activation of enzyme activity in the latter extract, caused, for example, by membrane lipids. In addition, studies on the dose-dependence for glucagon or fluoride in these reconstituted mixtures showed relationships similar to those described above (results not shown).

Evidence on the participation of the liver Nₐ protein in the heterologous reconstituted system was obtained from the experiment shown in Fig. 4. Liver membranes were treated with cholera toxin in the presence of NAD⁺ and GTP and thereafter extracted with a sodium cholate-containing buffer. The supernatant was then filtered through an Ultrogel Ac34 column pre-calibrated with protein standards.

The capacity to stimulate basal adenylate cyclase activity in cyc⁻ lymphoma S49 cells of T. cruzi membranes (MgATP as substrate) was measured in all the fractions. As shown in Fig. 4, there is a coincidence between the elution peaks with the capacity to stimulate T. cruzi or lymphoma adenylate cyclase activity. Peak maxima corresponded to a Rₑ of about 3.2 nm. This value is similar to that reported for the α subunit of the Nₐ guanine-nucleotide-binding protein (Sternweiss et al., 1981). It is important to emphasize that membranes from the lymphoma S49 cyc⁻ variant provide the best system for the assay of this guanine-nucleotide-binding protein (Ross et al., 1978).
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Fig. 3. Adenylate cyclase activities in the reconstituted system containing *T. cruzi* membranes and liver 'Ultrogel fraction'
(a) Activities assayed as a function of the amount of 'Ultrogel fraction', in the presence of *T. cruzi* membranes (70 μg of protein); assay mixtures contained the following additions: none (●), 1 μM-glucagon and 1 μM-p[NH]ppG (■), or 10 mM-NaF (▲). Activities were also assayed in the absence of *T. cruzi* membranes (○). (b) Activities assayed as a function of the amount of *T. cruzi* membranes in the presence of a constant amount of liver 'Ultrogel fraction' (12 μg of protein); assay mixtures contained the following additions: none (●), 1 μM-glucagon and 1 μM-p[NH]ppG (■) or 10 mM-NaF (▲). Activities were also assayed in the absence of the 'Ultrogel fraction' (○) or in the mixtures containing heat-inactivated (100 °C; 2 min) *T. cruzi* membranes instead of active membranes (▲). All the assays were performed with MgATP. Other conditions were given in the Experimental section.

Fig. 4. Ultrogel Ac34 column chromatography of a detergent extract from liver membranes treated with cholera toxin and NAD⁺
●, Reconstitution with *T. cruzi* membranes; ○, reconstitution with lymphoma S49 cyc⁻ membranes. BD, C, M and Cc indicate the elution positions of Blue Dextran, catalase, malate dehydrogenase and cytochrome c respectively. The inset shows the relationships between *Rf* and fraction number corresponding to protein standards (○) and the peak maxima (●).
Reconstitution with phospholipid vesicles

The requirement of a lipid substrate for the function of the heterologous cyclase system was explored. The complete mixture contained: (1) a preparation of *T. cruzi* adenylate cyclase purified up to the step of hexylamino-Sepharose column chromatography as source of catalytic subunits; (2) the fraction having the highest reconstitution activity (apparent $R_a$ about 3.2 nm; see Fig. 4) from the Ultrogel Ac34 column chromatography; and (3) phospholipid vesicles. As shown in Table 1, the presence of these three components was required to reconstitute a system stimulated by fluoride or p[NH]ppG. Stimulation by glucagon was not observed, because an Ultrogel fraction was used corresponding to a molecular size much smaller than that of the receptor–$N_a$ complex.

This type of reconstitution may be considered as an improvement over the other procedures used throughout this work, particularly because its efficiency was excellent in terms of the maximal enzyme activity measured (MgATP and fluoride), compared with that found in assays containing MnATP.

DISCUSSION

Results reported in this paper indicate that adenylate cyclase subunits from the protozoan *Trypanosoma cruzi* are capable of interacting with the guanine-nucleotide-binding protein $N_a$ from rat liver. Evidence obtained in the ascomycete fungus *Neurospora crassa* also indicates the existence of adenylate cyclase catalytic subunits capable of interacting with regulatory factors from avian erythrocytes (Flavía *et al.*, 1983) or from rat liver (the present paper).

These characteristics have important implications in terms of the evolutionary aspects of hormone-regulated systems since the Metazoa Kingdom branch, which includes vertebrates, diverged from the Fungi and Protoctista Kingdom branches 750 million years ago (Whittaker & Margulis, 1987; Margulis, 1981). This may support the view that the appearance of the eukaryotic cell structure was accompanied by the occurrence of an adenylate cyclase catalytic entity which was functionally similar to that found in vertebrates today.

The next step in evolution is the occurrence of a guanine-nucleotide-binding protein of the N type. Evidence from other laboratories indicates that guanine nucleotides activate adenylate cyclase in crude extracts of *Neurospora* or *Saccharomyces* (Rosenberg & Pall, 1983; Casperson *et al.*, 1983). In our experiments we occasionally found some stimulation by fluoride of adenylate cyclase in *T. cruzi* membranes (see, e.g., Table 1). In addition, accepting that the magnitude of basal enzyme activities (MgATP as substrate) in crude membranes may reflect the amount of $N_a$ protein interacting with adenylate cyclase catalytic subunits, it is clear that *T. cruzi* should have higher amounts of this protein than *Neurospora* (cf. Figs. 1b and 1d). However, there is no unambiguous demonstration of the existence

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<th>Preparation</th>
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of a N protein in these organisms, according to accepted criteria such as ADP-ribosyl acceptor capacity or capability to reconstitute adenylate cyclase activity in membranes from lymphoma S49 cye- cells (MgATP as substrate). Thus it is our opinion that the existence in the above-mentioned organism of a N protein regulating adenylate cyclase activity has yet to be proved.

The last point worthy of comment is the role played by oncogenic ras proteins in the control of adenylate cyclase and the corresponding evolutionary implications. In vertebrates, a ras gene product has GTPase activity (Scolnick et al., 1978; Sweet et al., 1984; McGrath et al., 1984), but there is no evidence on its physiological function except that, upon a specific mutation, the ras gene acquires an oncogenic capacity (Capon et al., 1983; Yuasa et al., 1983). This mutation abolishes the GTPase activity in the corresponding product (McGrath et al., 1984).

In Saccharomyces there are two genes, RAS1 and RAS2, closely related to the vertebrate ras family (Powers et al., 1984; Kataoka et al., 1984), and at least the protein product of one of these genes is responsible for the stimulation of yeast adenylate cyclase activity by guanine nucleotides (Toda et al., 1985). A gene encoding a protein of unknown function and homologous to ras vertebrate proteins has also been described in Dictyostelium (Reymond et al., 1984).

This evidence may indicate that, at least in fungi, ras-gene products may play a role equivalent to that of N proteins in vertebrates. This, of course, did not eliminate the existence of such proteins in Fungi or Protocotista.

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