Restoration of guanine nucleotide- and fluoride-stimulated activity to an adenylate cyclase-deficient cell line with affinity-purified guanine nucleotide regulatory protein

Thor B. NIELSEN,* Robert W. DOWNS, Jr.† and Allen M. SPIEGEL†

*Laboratory of Nutrition and Endocrinology, and †Metabolic Diseases Branch, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, MD 20205, U.S.A.

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A guanine nucleotide-binding protein purified from turkey erythrocytes by affinity chromatography confers both F^- - and guanine nucleotide-stimulation of adenylate cyclase to membranes from CYC^- cells, a mutant cell line deficient in these responses. Interaction of turkey erythrocyte membranes with β -adrenergic agonists before affinity chromatography, which is essential for binding of the guanine nucleotide regulatory protein to the affinity matrix, was also required for recovery of F^- -stimulation restoring activity in the affinity eluate.

Adenylate cyclase (EC 4.6.1.1) in eukaryotic cell plasma membranes consists of at least three functionally separable components: hormone receptor, guanine nucleotide binding protein (N protein), and the catalytic unit (Maguire *et al.*, 1977). Hormonal stimulation of adenylate cyclase involves all three components (Maguire *et al.*, 1977). However, the site of action of F^- , a universal stimulator of adenylate cyclase activity, is unknown. F^- had long been thought to act directly on the catalytic unit. However, it does not stimulate adenylate cyclase in membranes from a mutant S49 lymphoma cell line (CYC^-) that contains a catalytic unit but lacks hormone- and guanine nucleotide-stimulated cyclase activity (Ross *et al.*, 1978). This suggests that the presence of the catalytic unit alone is insufficient to support F^- -stimulated cyclase activity. Ross *et al.* (1978) showed that addition of crude detergent extracts of wild-type S49 cell membranes to CYC^- cell membranes restores F^- - and guanine nucleotide-stimulated activity, but the number and identity of additional component(s) in these extracts required to restore activity remain unclear. Clarification of the site of F^- action on adenylate cyclase should help elucidate the component structure of the enzyme.

Abbreviations used: N protein, guanine nucleotide binding protein; p[NH]ppG, 5'-guanylyl [β -imidodiphosphate]; [S]pppG, guanosine 5'- γ -thiotriphosphate; [S]ppG, guanosine 5'- β -thiodiphosphate; CYC^- , S49 mouse-lymphoma-cell clone 94.15.1; GTP-agarose, GTP-*p*-phenylenediamine linked to ε -amino-*n*-hexanoate-agarose.

N protein has been isolated from detergent extracts of pigeon (Pfeuffer, 1977) and turkey (Spiegel *et al.*, 1979) erythrocyte membranes by affinity chromatography on GTP-linked agarose. The affinity-purified N protein restored guanine nucleotide-stimulated activity to detergent extracts from homologous membranes depleted of N protein by incubation with GTP-linked agarose (Pfeuffer, 1977; Spiegel *et al.*, 1979). In the present communication, we show that affinity-purified N protein restores not only guanine nucleotide-stimulated, but also F^- -stimulated, activity to CYC^- cell membranes.

Materials and methods

The 94.15.1 clone of S49 lymphoma cells was grown and harvested, and the plasma membranes isolated, as described elsewhere (Nielsen *et al.*, 1980). Preparation of turkey erythrocyte plasma membranes, detergent extracts, and affinity-purified N protein was as previously described (Spiegel *et al.*, 1979) except that several guanine nucleotides (as listed in the Tables and Figure) in addition to p[NH]ppG were used to elute N protein from the affinity matrix.

CYC^- membranes were combined with affinity-purified N protein (in the proportions specified in the Tables and Figure) and left at 4°C for 30 min before assay. Control preparations [CYC^- membranes + the buffer used to elute the affinity matrix (Spiegel *et al.*, 1979) and affinity-purified N protein + CYC^- buffer (Ross *et al.*, 1978)] were also assayed for

each experiment. Adenylate cyclase activity was assayed as described by Lad *et al.* (1980) and cyclic AMP was isolated and quantified by the method of Salomon *et al.* (1974). Results are expressed as pmol of cyclic AMP/20 min per 100 μ l of assay volume at 30°C unless otherwise specified and are the mean of triplicate determinations that agreed within $\pm 10\%$. The data are from representative experiments. Each experiment was performed at least three times with different CYC⁻ membrane and affinity-purified N protein preparations.

[S]ppG was obtained from Boehringer, Mannheim, Germany, and was purified as described (Downs *et al.*, 1980). The sources of all other materials have been cited in previous publications (Salomon *et al.*, 1974; Spiegel *et al.*, 1979; Downs *et al.*, 1980; Lad *et al.*, 1980; Nielsen *et al.*, 1980).

Results and discussion

The guanine nucleotide binding protein, N protein, can be partially purified from turkey erythrocyte membranes by incubating Lubrol extracts of the membranes with GTP-agarose and, after extensive washing of the affinity matrix with buffer, specific elution of N protein from the matrix with buffer containing guanine nucleotides (Pfeuffer, 1977; Spiegel *et al.*, 1979). Cholera toxin specifically labels a 42000- M_r (on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis) peptide that is a subunit of the N protein (Cassel & Pfeuffer, 1978). The ratio of [³²P]ADP-ribosylated 42000- M_r peptide to protein in membranes, detergent extracts and affinity eluate can be used to estimate N protein purification. We have recently developed a method for specifically labelling the 42000- M_r peptide in turkey erythrocyte membranes (R. W. Downs, Jr. &

A. M. Spiegel, unpublished work). Using this method, we find, in agreement with Cassel & Pfeuffer (1978), that affinity elution results in a 100-fold enrichment of N protein from detergent extracts.

For clarity in presentation the affinity eluate is referred to as N protein (guanine nucleotide), according to the guanine nucleotide used to eluate the protein from the matrix, e.g. N protein (p[NH]ppG), N protein (GTP), etc. Purification of N protein depends critically upon incubation of turkey erythrocyte membranes with β -adrenergic agonists and GMP before detergent extraction and affinity chromatography. This requirement arises from a special feature of avian erythrocyte N protein; guanine nucleotide stimulation of turkey erythrocyte adenylate cyclase is almost entirely dependent upon the presence of β -adrenergic agonists (Downs *et al.*, 1980; Lad *et al.*, 1980). Incubation of turkey erythrocyte membranes with isoproterenol and high concentrations of GMP removes tightly bound GDP (Cassel & Selinger, 1978; Downs *et al.*, 1980) from N protein and permits it (even after solubilization) to bind guanine nucleotide in a hormone-independent manner (Pfeuffer, 1977; Downs *et al.*, 1980; Lad *et al.*, 1980). Thus incubation of membranes with isoproterenol + GMP before Lubrol solubilization is required to enable N protein to bind to the affinity matrix and be recovered in the affinity eluate. The contaminating proteins in the affinity eluate, by contrast, do not display a requirement for β -adrenergic treatment of the membranes.

We tested the ability of affinity eluates to restore adenylate cyclase activity to CYC⁻ membranes. Soluble extracts from isoproterenol + GMP-treated and untreated (control) membranes were incubated

Table 1. Hormone dependence for restoration by affinity eluate of F⁻- and p[NH]ppG-activation of adenylate cyclase in CYC⁻ membranes

Turkey erythrocyte membranes were incubated with GMP (control) or isoproterenol + GMP, washed and solubilized as described in Spiegel *et al.* (1979); 3 ml of control or isoproterenol + GMP-treated soluble extracts were incubated with 0.5 ml of packed GTP-agarose and affinity eluates were prepared as described in Spiegel *et al.* (1979) except that 0.5 ml of buffer containing either 0.2 mM-p[NH]ppG or 0.2 mM-GTP was used to elute 0.5 ml of packed gel. Affinity eluates + CYC⁻ membranes (or buffer) were mixed in equal volumes and assayed for adenylate cyclase activity.

Incubation	Assay conditions	Adenylate cyclase activity (pmol/ 20 min per 100 μ l at 30°C)* with eluate prepared from:	
		Control extract	Isoproterenol + GMP extract
N protein (p[NH]ppG) + buffer	p(NH)ppG (50 μ M)	0.1	3.1
N protein (p[NH]ppG) + CYC ⁻ membranes	p(NH)ppG (50 μ M)	0.2	8.1
N protein (GTP) + buffer	NaF (10 mM) + GTP (50 μ M)	0.1	0.6
N protein (GTP) + CYC ⁻ membranes	NaF (10 mM) + GTP (50 μ M)	0.4	2.5

* CYC⁻ membranes alone showed undetectable activity (assayed with p[NH]ppG or NaF).

with GTP-agarose; after washing, the matrix was eluted with either p[NH]ppG or GTP. Addition of N protein (p[NH]ppG) derived from isoproterenol + GMP-treated membranes to CYC^- membranes resulted in significantly greater adenylate cyclase activity than could be accounted for by the intrinsic activity of N protein (p[NH]ppG) (Table 1). Addition of N protein (GTP) derived from isoproterenol + GMP-treated membranes to CYC^- membranes resulted in restoration of F^- -responsive adenylate cyclase activity. The N protein (p[NH]ppG) and N protein (GTP) derived from untreated membranes were devoid of activity when assayed by complementation with CYC^- membranes (Table 1). Note that (a) a factor that restores F^- -stimulated activity can be eluted with GTP and (b) binding of such a factor to the affinity matrix requires the same pretreatment (incubation of membranes with isoproterenol + GMP) necessary for binding of N protein to the matrix. Lack of activity in eluates prepared from control extracts argues strongly that N protein, and not other protein contaminants in the affinity eluate, is responsible for the restoration of the F^- response to CYC^- membranes.

The response of turkey erythrocyte adenylate cyclase to guanine nucleotides (Downs *et al.*, 1980) was closely paralleled by the responses observed in CYC^- membranes upon complementation with

affinity-purified N protein eluted with various guanine nucleotides. N protein (p[NH]ppG) (Table 2) or N protein ([S]pppG) (results not shown) restored activity to CYC^- membranes without further addition to the adenylate cyclase assay medium. By contrast, N protein (GTP) or N protein (GDP) (Table 2) restored activity to CYC^- membranes only when F^- was added to the assay medium. [S]ppG, a thio analogue of GDP, is clearly capable of specifically binding to N protein, as shown by its ability to block p[NH]ppG activation in a β -adrenergic agonist-dependent manner (Eckstein *et al.*, 1979; Downs *et al.*, 1980). [S]ppG also elutes N protein, specifically labelled by cholera toxin, from GTP-agarose (R. W. Downs, Jr. & J. M. Spiegel, unpublished work). [S]ppG has recently been shown to prevent F^- stimulation (Eckstein *et al.*, 1979; Downs *et al.*, 1980); this is paralleled by the inability of N protein ([S]ppG) to restore the F^- response to CYC^- membranes (Table 2).

A single affinity eluate preparation, N protein (GMP), is capable of restoring either p[NH]ppG- or F^- -responsive adenylate cyclase activity to CYC^- membranes. GMP is readily displaced from the N protein binding site of avian erythrocyte adenylate cyclase by either p[NH]ppG or GTP (Pfeuffer, 1977; Cassel & Selinger, 1978; Downs *et al.*, 1980; Lad *et al.*, 1980). Addition of

Table 2. Effect of guanine nucleotide used to elute N protein from GTP-agarose on activation of adenylate cyclase in CYC^- membranes

Affinity eluates were prepared as described in the legend to Table 1, except that the 0.5 ml of buffer used to eluate 0.5 ml of packed gel contained one of the following guanine nucleotides: 0.2 mM-p[NH]ppG, -GTP, -GDP, -[S]ppG, or 2 mM-GMP. Affinity eluates + CYC^- membranes (or human erythrocyte membranes prepared by hypotonic lysis, or buffer) were mixed in equal volumes and assayed for adenylate cyclase activity.

Incubation	Assay conditions	Adenylate cyclase activity (pmol/20 min per 100 μ l at 30°C)
N protein (p[NH]ppG) + buffer	p[NH]ppG (50 μ M)	2.2
N protein (p[NH]ppG) + human erythrocyte membranes	p[NH]ppG (50 μ M)	2.1
N protein (p[NH]ppG) + CYC^- membranes	p[NH]ppG (50 μ M)	7.0
N protein (GTP) + buffer	NaF (10 mM) + GTP (50 μ M)	0.3
N protein (GTP) + CYC^- membranes	GTP (50 μ M)	0.2
N protein (GTP) + CYC^- membranes	NaF (10 mM) + GTP (50 μ M)	2.5
N protein (GDP) + buffer	NaF (10 mM) + GDP (50 μ M)	0.4
N protein (GDP) + CYC^- membranes	GDP (50 μ M)	0.4
N protein (GDP) + CYC^- membranes	NaF (10 mM) + GDP (50 μ M)	2.7
N protein ([S]ppG) + buffer	NaF (10 mM) + [S]ppG (50 μ M)	0.4
N protein ([S]ppG) + CYC^- membranes	[S]ppG (50 μ M)	0.3
N protein ([S]ppG) + CYC^- membranes	NaF (10 mM) + [S]ppG (50 μ M)	0.4
N protein (GMP) + buffer	GMP (0.5 mM) + p[NH]ppG (50 μ M)	0.3
N protein (GMP) + CYC^- membranes	GMP (0.5 mM)	0.1
N protein (GMP) + CYC^- membranes	GMP (0.5 mM) + p[NH]ppG (50 μ M)	3.0
N protein (GMP) + CYC^- membranes	GMP (0.5 mM) + GTP (50 μ M) + NaF (10 mM)	2.5

p[NH]ppG or GTP + F⁻ to the assay medium thus permits N protein (GMP) to restore p[NH]ppG- and F⁻-responsive adenylate cyclase activity respectively to CYC⁻ membranes (Table 2).

The adenylate cyclase activity of CYC⁻ membrane + affinity-purified N protein was linear for at least 20 min at 30°C (results not shown). Activity of the N protein (p[NH]ppG) + CYC⁻ combination rose linearly as a function of the amount of added CYC⁻ membranes (Fig. 1a). F⁻-stimulated activity of the N protein (GTP) + CYC⁻ combination also increased but was lower than that with N protein (p[NH]ppG) at all concentrations of added CYC⁻ membranes. In addition, N protein (GTP) + CYC⁻ membranes showed a tendency to saturation at high concentrations of CYC⁻ membranes (Fig. 1a). Activity also rose as a function of increasing N protein (GTP) or N protein (p[NH]ppG) if the amount of CYC⁻ membranes was held constant, but, at low CYC⁻ membrane concentration, saturation was achieved with increasing N protein (p[NH]ppG) (Fig. 1b).

Both of these saturation effects would suggest that the amount of N protein capable of reconstituting CYC⁻ adenylate cyclase activity was less in N protein (GTP) than in N protein (p[NH]ppG). Such a difference in activity could in theory result either from a difference in the efficiency of GTP versus p[NH]ppG in eluting N protein from the affinity matrix or from a difference in the ability

of N protein (GTP) versus N protein (p[NH]ppG) to activate the catalytic unit in CYC⁻ membranes. Pfeuffer (1979) has reported that GDP and [S]pppG have similar elution efficiency, based on recovery of [³²P]ADP-ribose-labelled affinity-purified N protein from pigeon erythrocyte membranes. Likewise, in turkey erythrocyte membranes, equivalent amounts of labelled N protein were eluted by (p[NH]ppG) GDP, GMP and [S]pppG (R. W. Downs, Jr. & A. M. Spiegel, unpublished work). Thus the differences in the activity of the affinity eluates observed upon complementation with CYC⁻ do not result from differences in the efficiency of elution. Rather, the nucleotides used in elution must differentially affect the activation of the catalytic unit by N protein.

Our data, in agreement with those of Pfeuffer (1977), suggest that some active catalytic unit is still present in affinity-purified N protein preparations. As N protein and the catalytic unit must exhibit a finite affinity for each other, one may suppose that some catalytic unit may remain bound to N protein even after washing the affinity matrix. The increase in adenylate cyclase activity observed upon addition of affinity-purified N protein to CYC⁻ membranes, however, cannot be attributed to non-specific effects of membrane proteins or lipids on the intrinsic catalytic activity of affinity-purified N protein; addition of N protein (p[NH]ppG) to human erythrocyte membranes, which contain little

Fig. 1. Effect of increasing amounts of CYC⁻ membranes or affinity eluate on F⁻- and p[NH]ppG-responsive adenylate cyclase activity

Affinity eluates were prepared as described in the legend to Table 1. In (a) increasing amounts of CYC⁻ membranes were added to 12 μl of N protein (p[NH]ppG) (Δ) or N protein (GTP) (●). In (b) increasing amounts of N protein (p[NH]ppG) (Δ, ▲) or N protein (GTP) (O, ●) were added to either 20 μg (▲, ●) or 10 μg (Δ, O) of CYC⁻ membranes. Non-protein constituents were kept constant by adding the appropriate buffers used in the preparation of affinity eluate or CYC⁻ membranes. N protein (p[NH]ppG) + CYC⁻ membranes were assayed without F⁻, final concentration of p[NH]ppG 50 μM; N protein (GTP) + CYC⁻ membranes were assayed with 0.01 M-NaF, final GTP concentration 50 μM. Activity of eluate + buffer controls was subtracted from combined eluate + CYC⁻ membrane activity.

or no functional catalytic unit (Kaslow *et al.*, 1979; Nielsen *et al.*, 1980), did not result in increased adenylate cyclase activity (Table 2).

Several authors (Ross & Gilman, 1977; Hebdon *et al.*, 1978) have suggested that distinct regulatory components mediate guanine nucleotide- and F^- -responsive activity. Pfeuffer's studies (Pfeuffer, 1977; Cassel & Pfeuffer, 1978) and our previous observations (Spiegel *et al.*, 1979; Downs *et al.*, 1980; Nielsen *et al.*, 1980) suggested that N protein was involved in the stimulation of the enzyme by F^- . Pfeuffer (1977) showed that affinity-purified N protein (GTP) increased F^- -stimulated activity of a solubilized pigeon erythrocyte membrane preparation. The latter had been depleted of about 50% of its F^- -stimulated activity by treatment with GTP-agarose. Our data show that a single affinity-purified preparation of N protein restores stimulation by both F^- and guanine nucleotide to CYC^- membranes, which are genetically completely deficient in F^- and guanine nucleotide-stimulated activity. Interaction of catecholamine agonists with the β -adrenergic receptor before affinity chromatography was essential for the recovery of both F^- and guanine nucleotide-stimulation-restoring activity. N protein eluted with [S]ppG failed to restore F^- -stimulated activity. Thus we have provided direct evidence that the guanine nucleotide-binding protein mediates stimulation of adenylate cyclase by F^- .

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