Functional expression of cloned cDNA encoding the α-subunit of adenylate cyclase-stimulating G-protein

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Cloned cDNA encoding the α-subunit of the adenylate cyclase-stimulating G-protein (G_s), carried by a simian virus 40 vector, has been introduced into the cyc⁻ variant of S49 lymphoma cells by electroporation. In contrast to untransfected cys⁻ cells, clones transformed with the cDNA exhibit an increase in intracellular cyclic AMP concentration in response to a β-adrenergic agonist.

Adenylate cyclase-stimulating G-protein; cDNA; β-Adrenergic agonist; cyclic AMP; Cholera toxin dependence; ADP-ribosylation; (cyc⁻ S49 lymphoma cell)

1. INTRODUCTION

A family of membrane-associated G-proteins are essential for transducing signals generated at cell surface receptors into changes in cellular function and metabolism [1]. These proteins are composed of three subunits termed α, β and γ. The α-subunit is responsible for binding guanine nucleotides and is unique to each G-protein. The stimulatory G-protein (G_s) mediates hormonal stimulation of adenylate cyclase [1]. The α-subunit of G_s is the activator of the catalytic moiety of the adenylate cyclase complex and has a site for NAD-dependent ADP-ribosylation catalysed by cholera toxin. The primary structure of the G_s α-subunit has been deduced by cloning and sequencing the cDNA [2–7]. Here we report that the cloned cDNA encoding the G_s α-subunit, carried by an SV40 vector, is functionally expressed in the cyc⁻ variant [8] of S49 murine lymphoma cells, which is defective in the G_s α-subunit [9,10].

2. MATERIALS AND METHODS

2.1. Construction of the expression vector

The plasmid pGSα7 [2] was digested with NcoI and HindIII, treated with T_4 DNA polymerase with the four deoxyribonucleoside triphosphates (dNTPs), ligated to the HindIII linker dACAAGCTTGT and cleaved with HindIII. The resulting 1.4-kilobase-pair (kb) fragment containing the entire coding sequence for the G_s α-subunit was inserted into the HindIII site of the plasmid pKCRH2 [11] in the same orientation with respect to SV40 early gene transcription to generate the plasmid pKGSα. The plasmid pSV2-neo [12] was digested with AccI and AatII, treated with T_4 DNA polymerase with the four dNTPs, ligated to the SalI linker dGGTCGACC and cleaved with SalI. The resulting 3.6-kb fragment containing the neo transcription unit was inserted into the SalI site of pKGSα in the same orientation as the G_s α-subunit transcription unit to produce the plasmid pKGSαneo. pKGSαneo was partially digested with

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Abbreviations: G-protein, guanine nucleotide-binding regulatory protein; G_s, adenylate cyclase-stimulating G-protein; G_i, adenylate cyclase-inhibiting G-protein; SV40, simian virus 40; DMEM, Dulbecco's modified Eagle's medium; IBMX, 3-isobutyl-1-methylxanthine

HindIII, treated with T_4 DNA polymerase with the four dNTPs and ligated to yield the plasmid pKGSαneo2, in which the HindIII site in the neo transcription unit was deleted. The plasmid pGα28 [13] was digested with PstI and EcoRI, treated with T_4 DNA polymerase with the four dNTPs, ligated to the HindIII linker and cleaved with HindIII. The resulting 1.5-kb fragment carrying most of the 3′-noncoding sequence for the α-subunit of G_i was inserted into the HindIII site of pKGSαneo2, located between the 3′-noncoding sequence for the G_s α-subunit and the exonic sequence of the β-globin gene, in the same orientation with respect to SV40 early gene transcription to generate the plasmid pKGSαN.

2.2. Isolation of transformants

Cells were grown in DMEM containing 10% heat-inactivated horse serum as in [14]. Transfection by electroporation of cyc^- S49 lymphoma cells [8] with the plasmid pKGSαN or pSV2-neo was carried out essentially as in [15]. Cyc^- cells $(3-5 \times 10^7)$, 40 μg pKGSαN (or pSV2-neo) linearized with PvuI and 315 μg denatured Escherichia coli DNA (boiled for 15 min) in 1 ml ice-cold HBS [21 mM Hepes-NaOH buffer (pH 7.0), 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 and 5.5 mM glucose] were subjected to a single electric pulse by a power supply (model MS-3000, M&S Instruments Trading); the voltage was set at 2.0 kV, the resistance at minimum and the capacitance at 12 μF. Transformants were selected in culture medium supplemented with 800 μg/ml G418 (Gibco) [12]. Clones could be scored and recovered 3–4 weeks later. Each recovered clone was tested for sensitivity to a β-adrenergic agonist by growing it for up to 7 days in culture medium supplemented with 0.1 mM terbutaline sulphate (Fujisawa-Astra) and cyclic AMP phosphodiesterase inhibitors [0.1 mM IBMX and 0.03 mM RO20-1724 (Hoffman La Roche)].

2.3. Preparation of membranes and ADP-ribosylation by cholera toxin

Cells $(\sim 10^7)$ were centrifuged, suspended in ice-cold phosphate-buffered saline and centrifuged again. The pellet was resuspended in ~1 ml ice-cold 25 mM Tris-HCl buffer (pH 7.5) containing 2.5 mM $MgCl_2$ and 1 mM EDTA, allowed to stand at 0°C for 30 min, homogenized and centrifuged at $1000 \times g$ for 5 min. The supernatant, to which dithiothreitol was added (final concentration, 1 mM), was stored at $-70°C$. Immediately before use, it was thawed and centrifuged at $15\,000 \times g$ for 30 min, and the pellet suspended in 1 ml of 50 mM potassium phosphate buffer (pH 7.3). The membranes thus obtained were subjected to radiolabelling with cholera toxin and $[\alpha\text{-}^{32}P]NAD$ as in [16]; 0.1 mM ATP [17] was added to the reaction mixture. Lubrol PX extracts (15 μl each) from the membranes were analysed by electrophoresis on an SDS/10% polyacrylamide gel as in [16,18].

3. RESULTS AND DISCUSSION

An expression vector (pKGSαN) carrying the entire protein-coding sequence of the bovine cerebral G_s α-subunit cDNA [2] was constructed (fig.1). The 3′-noncoding region was extended by linking it with most of the 3′-noncoding sequence of the cDNA encoding the bovine cerebral G_i α-subunit [13] so that the resulting transcript would be distinguishable by size from the G_s α-subunit mRNA (~1800 nucleotides) present in wild-type S49 lymphoma cells (see below). The expression vector contained in addition the SV40 early gene promoter, donor and acceptor splice sites derived from the rabbit β-globin gene, polyadenylation sites derived from the rabbit β-globin and SV40 early genes and a second transcription unit to direct the expression of the neo marker gene (derived from the plasmid pSV2-neo [12]).

The plasmid pKGSαN was introduced by electroporation into cyc^- S49 lymphoma cells in culture. Twenty clones in $\sim 4 \times 10^7$ transfected cells were transformed to G418 resistance. Of these clones, nine were killed and seven were inhibited from growth by exposure for 4–5 days to medium containing a β-adrenergic agonist and cyclic AMP phosphodiesterase inhibitors. No growth inhibition was observed for untransfected cyc^- cells and for all eight tested clones that had been transformed to G418 resistance with pSV2-neo. From the nine clones that suffered cytolysis on exposure to the β-adrenergic agonist, three clones (CGSα1, CGSα4 and CGSα7) were randomly selected for further analysis.

Poly(A)$^+$ RNA was isolated from the three clones and subjected to blot hybridization analysis
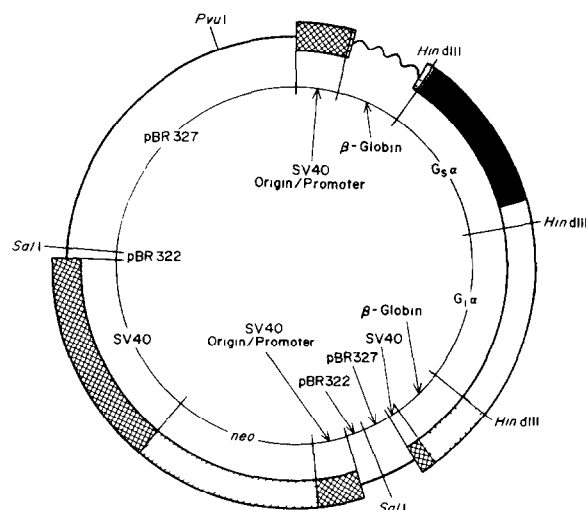
Fig.1. Schematic representation of the recombinant plasmid pKGSαN used for expression of the G_s α-subunit. The origins of the segments are shown on the inner circle. The protein-coding region of the bovine cerebral G_s α-subunit cDNA is indicated by a closed box, and the 5′- and 3′-noncoding regions of the G_s α-subunit cDNA and the 3′-noncoding region of the bovine cerebral G_i α-subunit cDNA by open boxes. The *neo* DNA segment is represented by a hatched box. The SV40 sequences are shown by cross-hatched boxes; the orientation of the two sets of the early gene promoter is such that transcription occurs clockwise. The exonic and 3′-flanking sequences of the rabbit β-globin gene are indicated by stippled boxes and its intronic sequence by a wavy line. The sequences of the plasmids pBR327 and pBR322 are represented by lines. Only the relevant restriction endonuclease sites are shown.

Fig.2. Blot hybridization analysis of poly(A)$^+$ RNA from wild-type S49 (lane 1), CGSα7 (lane 2), CGSα4 (lane 3), CGSα1 (lane 4) and cyc$^-$ S49 cells (lane 5) using a bovine cerebral G_s α-subunit cDNA probe. Analysis was carried out according to [19,20]; the procedure used has been described [21]. The amount of each poly(A)$^+$ RNA sample used was 10 μg. The hybridization probe (spec. act. 4×10^7 cpm/μg) was the PvuII(88)/PvuII(511) fragment (numbers indicating the 5′-terminal nucleotide generated by cleavage; for nucleotide numbers, see [2]) excised from clone pGSα7 [2] and labelled by nick-translation with [α-^{32}P]dCTP [22]. Autoradiography was performed at −70°C for 6 days with an intensifying screen. The size markers were murine and *E. coli* rRNAs [23].

using a bovine cerebral G_s α-subunit cDNA probe. Two hybridizable RNA species with estimated sizes of ∼3100 and ∼3500 nucleotides were observed (fig.2, lanes 2–4). S_1 nuclease mapping [24] of the total RNA isolated from the three clones indicated that the 5′-end of the G_s α-subunit-specific mRNA produced by expression of pKGSαN corresponded to the heterogeneous start sites of the transcripts formed under the control of the SV40 early gene promoter [25–28] and that the intronic sequence of the rabbit β-globin gene was eliminated. Thus, the sizes of the two hybridizable mRNA species agree with those expected if polyadenylation occurs at both the polyadenylation sites derived from the β-globin and SV40 early genes. No hybridizable RNA species was detected in the poly(A)$^+$ RNA
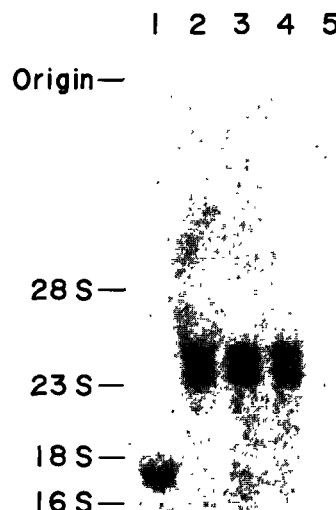
from untransfected cyc$^-$ cells (fig.2, lane 5) and in the total RNA from all four tested clones that had been transformed with pSV2-*neo* (not shown). These results, together with the fact that the clones transformed with pKGSαN did not contain the hybridizable mRNA species of ∼1800 nucleotides observed in wild-type S49 lymphoma cells [10] (fig.2, lane 1), exclude the possibility that cyc$^-$
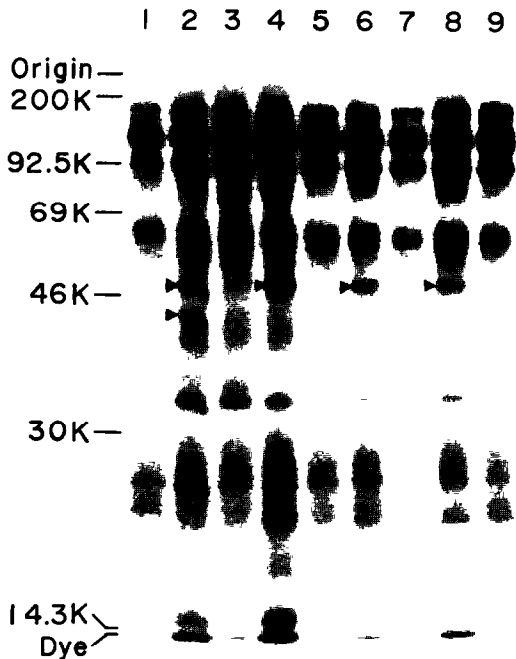
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Fig.3. SDS-polyacrylamide gel electrophoresis of polypeptides ADP-ribosylated by cholera toxin. Cultures of cyc⁻ S49 (lane 1), wild-type S49 (lanes 2,3), CGSα1 (lanes 4,5), CGSα4 (lanes 6,7) and CGSα7 cells (lanes 8,9) were divided into two equal portions, which were incubated at 37°C for 17 h in the presence (lanes 3,5,7,9) and absence (lanes 1,2,4,6,8) of 0.8 μg/ml cholera toxin. Membranes were then prepared from each sample and subjected to ADP-ribosylation using cholera toxin and [α-^{32}P]NAD. The labelled products were analysed electrophoretically. Autoradiography was performed at −70°C for 40 h with an intensifying screen. The polypeptides ADP-ribosylated specifically by cholera toxin are indicated by arrowheads. The size markers used were [^{14}C]methylated rabbit muscle myosin (200 kDa), rabbit muscle phosphorylase b (92.5 kDa), bovine serum albumin (69 kDa), chicken ovalbumin (46 kDa), bovine erythrocyte carbonic anhydrase (30 kDa) and chicken egg white lysozyme (14.3 kDa) (Amersham). K, kDa.

cells have undergone reverse mutation to the wild type.

To identify the translation product encoded by the mRNA generated by expression of pKGSαN, we examined membranes prepared from the three transformants for ADP-ribosylation arising from incubation with cholera toxin and [α-^{32}P]NAD. The toxin effected radiolabelling of a polypeptide of M_r ~52000 (fig.3, lanes 4,6,8), which cor-

responded to the larger of the two polypeptides (M_r ~52000 and M_r ~45000) known to be ADP-ribosylated in a toxin-dependent manner in membranes of wild-type S49 cells [16] (fig.3, lanes 2,3). Pretreatment of intact cells with cholera toxin prevented subsequent toxin-dependent radiolabelling of the polypeptide of M_r ~52000 in membranes prepared from the pretreated cells (fig.3, lanes 5,7,9). This indicates that the translation product of M_r ~52000 is a cholera toxin-specific substrate. No ADP-ribosylation product of M_r ~52000 was observed in membranes from untransfected cyc⁻ cells (fig.3, lane 1).

Table 1 shows the effect of the β-adrenergic agonist isoproterenol on the cellular content of cyclic AMP in the three pKGSαN-transformed clones. These clones, like wild-type S49 cells, exhibited a large increase in cyclic AMP content in response to isoproterenol, whereas untransfected cyc⁻ cells and pSV2-neo-transformed clones failed to respond to the agonist. These data indicate that the sensitivity of the pKGSαN-transformed clones

Table 1

Effect of isoproterenol on the cellular content of cyclic AMP

Vector	Cell line / Transformant	Cyclic AMP (pmol/10^7 cells)	
		Control	+ isoproterenol
	wild-type S49	71 ± 43	4979 ± 2193
	cyc⁻ S49	3 ± 1	6 ± 1
pKGSαN	CGSα1	54 ± 31	1541 ± 885
	CGSα4	22 ± 12	1475 ± 719
	CGSα7	26 ± 10	1305 ± 424
pSV2-neo	CN1, CN2 and CN3	15 ± 3	15 ± 3

Cells (~10^6) were incubated at 37°C for 10 min in 0.5 ml of DMEM containing 0.2 mM IBMX in the presence and absence of 10 μM isoproterenol. Cellular cyclic AMP was then determined using a radioimmunoassay kit (Yamasa Shoyu) according to the procedure described by the vendor. Data are given as means ± SD from three experiments, except that the values obtained with the three different pSV2-neo-transformed clones (one experiment each) are averaged

to a β-adrenergic agonist is due to an elevated intracellular cyclic AMP concentration [14]. Thus our results demonstrate that the G_s α-subunit expressed from the cloned cDNA is functional in vivo.

Most cells, including the wild-type S49 lymphoma cell, contain two forms of the G_s α-subunit (52 kDa and 45 kDa forms) [1]; the 45 kDa form has a deletion of 14 consecutive amino acid residues and two amino acid substitutions as compared with that of 52 kDa [5]. It should be noted that the isoproterenol-dependent increase in cyclic AMP content observed for the pKGSαN-transformed clones is smaller than that observed for wild-type S49 cells (see table 1). This may be accounted for at least partly by the difference between the content of the 52 kDa form in the transformants and the cumulative content of both the 52 kDa and 45 kDa forms in wild-type cells (see fig.3).

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