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ABSTRACT

Two subclones of rat XC cell line characterized by different morphology exhibited quite different adenylyl cyclase response upon various stimulations. Upon treatment with cholera toxin, clone RK1 accumulated high level of intracellular cAMP thereby changing its polygonal morphology to elongated morphology, while the other clone LK1 with fibroblastic morphology failed to increase the intracellular cAMP and remained morphologically unchanged. When membrane fractions derived from these two clones were stimulated with 10 μM forskolin, 10 μM GTPγS, or 10 mM NaF, five to twenty-fold more cAMP was accumulated in RK1-derived membranes than in LK1-derived membranes. With the same membrane fractions, upon treatment with Mn^{++} which directly stimulates the catalytic unit, high level of cAMP was accumulated both in RK1 and LK1, indicating that the catalytic function inducible by Mn^{++} was similar in the both clones. There was no significant difference in the level of expression of G protein α_s, α_i (at least α_{i1} and α_{i2}) and β subunits between LK1 and RK1. Cholate extracts of the membrane proteins of LK1 and RK1 reconstituted the adenylyl cyclase activity of cyc^- variant of S49 lymphoma cells to the same level. Therefore, it is inferred that the defect in LK1 resides in the interaction of stimulatory G proteins and the actual catalyst.

INTRODUCTION

The adenylyl cyclase system is composed of three major components: receptor for the ligand, guanine nucleotide-binding proteins (G proteins) and actual catalyst (Gilman, 1984). The activity of the catalytic unit is regulated by stimulatory (G_S) or inhibitory (G_I) G proteins. Both G_S and G_I are heterotrimers consisting of α, β and γ subunits. Common β and γ subunits are probably shared between G_S and G_I, however, the α subunit (α_S) of G_S is different from that (α_I) of G_I (Gilman, 1987). Although biochemical analysis of these components are well advanced (Ross and Gilman, 1980; Gilman, 1987) and genes encoding β-adrenergic receptor (Dixon et al., 1986), α_S (Itoh et al., 1986; Nukada et al., 1986a; Robishaw et al., 1986; Sullivan et al., 1986), α_I (Itoh et al., 1986; Nukada et al., 1986b; Sullivan et al., 1986; Jones and Reed, 1987; Itoh et al., 1988), β (Sugimoto et al., 1985; Gao et al., 1987) and γ (Hurley et al., 1984) subunits of G proteins and the catalytic unit (Krupinski et al., 1989) have been molecularly cloned, mode of interaction between the G proteins and the catalytic unit has remained relatively obscure. For this analysis, cell mutants defective in the interaction will be of value.

XC is a cultured cell line derived from a tumor obtained by injecting Prague strain of Rous sarcoma virus (RSV) to a Wister rat (Svoboda, 1961). At least two morphologically different cell types are present in XC cell line. One type had a fibroblastic shape while the other had a polygonal shape. I was able to establish relatively stable clones of each type. Interestingly these clones had very different adenylyl cyclase activity upon stimulation with cholera toxin, forskolin and other agents. The difference appeared to reside in the step of interaction between α_S and the catalytic unit. Characterization of the abnormality will greatly contribute to the elucidation of the mechanism of the interaction.

MATERIALS AND METHODS

Cell culture

XC cell line used in this study was kindly provided by late Dr. W. P. Rowe (National Institute of Allergy and Infectious Diseases, National Institutes of Health), cyc^- variant (Bourne et al., 1975) and wild type S49 mouse lymphoma cell lines by Dr. H. R. Bourne (University of California at San Francisco). XC was grown in Dulbecco's modified Eagle's minimal essential medium supplemented with 7 % heat-inactivated fetal calf serum and kanamycin. S49 lymphoma cells were grown in Dulbecco's modified minimal essential medium supplemented with 10 % heat-inactivated horse serum, 4.5 g/l d-glucose and kanamycin. Cells were maintained under an atmosphere of 95 % air and 5 % CO_2 in a humidified chamber. XC cells were cloned by the cylinder method (Yoshikura et al, 1979) according to the morphology. Since the clones were not permanently stable in their morphology, frozen aliquots were made soon after cloning. The clones used in the present study were stable at least for three months and the cells were thawed every three months for experimental use.

Membrane preparations

Crude plasma membrane fractions were prepared essentially as described (Ross et al.,1977b). XC cells were collected near or at confluency and S49 cells at 1-3 x 10^6 cells/ml. The following procedures were performed at 4 ^oC. Cells were rinsed three times with cold PBS containing 1 mM $MgCl_2$. They were homogenized in 25 mM Tris HCl, pH 7.5, 5 mM $MgCl_2$, 1mM EGTA, 1 mM DTT and 100 kallikrein inactivator units/ml aprotinin by passing 20 strokes through 21 G needle. Nuclei and cell debris were removed by centrifugation for 5 minutes at 600 x g. The supernatant fraction was centrifuged for 30 minutes at 20,000 x g. The pellets were resuspended in 75 mM Tris HCl, pH

7.5, 100 kallikrein inactivator units/ml aprotinin and 2.5 mM $MgCl_2$ at protein concentration of 10-30 mg/ml. The protein concentration was measured by the method of Bradford (1976) using a kit (BioRad). The membrane preparations were quickly frozen and kept in liquid nitrogen until use.

Measurement of intracellular cAMP accumulation

5 x 10^4 XC cells were plated in each well of 24 multi-well plates (Nunc). On the following day, medium was changed to serum free Eagle's minimal essential medium containing 20 mM HEPES, pH 7.4, 0.1 mM isobutylmethylxanthine (IBMX) and various concentrations of test reagents. After incubation at 37°C, the medium was discarded and cAMP was extracted three times with ethanol in a total volume of 2 ml. Ethanol was evaporated and the residual cAMP was dissolved in 50 mM sodium acetate buffer at pH 4.0 (Nissenson, 1985). cAMP was measured with cAMP radioimmunoassay kit (Yamasa shoyu, Co ltd.).

Measurement of cAMP accumulation in membrane fractions

The reaction mixture containing 20 μg of membrane protein was incubated with or without test reagents in 100 μl of reaction buffer (50 mM Tris HCl, pH 7.5, 1 mM ATP, 5mM $MgCl_2$, 0.1 mM EDTA, 0.25 mM IBMX, 20 mM creatine phosphate and 50 units/ml creatine phosphokinase)(Kimura and Shimada, 1983). Reactions were carried out at 37°C for 5 minutes and stopped by boiling. cAMP was assayed with a radioimmunoassay kit. Linear increase of cAMP during 30 minutes incubation was confirmed.

Cholera toxin-mediated ADP-ribosylation

500 μg/ml of cholera toxin was activated by incubation at 37°C for 20 minutes in a buffer containing 25 mM potassium phosphate, pH 8.0, and 20 mM

DTT. Membrane proteins were incubated at 30°C for 45 minutes with 50 μg/ml of activated cholera toxin in 50 μl potassium phosphate buffer (100 mM potassium phosphate, pH8.0, 12 mM thymidine, 1 mM ATP, 0.1 mM GTP, 1 mM $MgCl_2$, 0.5 mM EDTA, 5 mM HEPES and 7 μM [^{32}P]NAD)(Ribeiro-Neto et al., 1985). 15 μl of the reaction was mixed with 15 μl of 2 x Laemmli's sample buffer, boiled for 2 minutes and 20 μl (1/5 of each reaction) was separated through 10 % SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). The gels were fixed, stained, dried and exposed to a Fuji RX film with an intensifying screen.

Pertussis toxin-mediated ADP-ribosylation

200 μg/ml of pertussis toxin was activated at 30°C for 30 minutes in the presence of 100mM DTT and 100 mM ATP. The reaction was carried out in the similar conditions as described for cholera toxin-mediated ADP-ribosylation except that 100 mM Tris HCl, pH7.5 was substituted for potassium phosphate buffer and that the incubation time was 20 minutes (Ribeiro-Neto, 1985). The final concentration of pertussis toxin was 20 μg/ml.

Reconstitution of adenylyl cyclase system

Reconstitution of adenylyl cyclase system was performed as described (Rich et al., 1984) with minor modifications. Equal volume of suspension buffer containing 2 % sodium cholate was added to crude membrane fractions of XC cells. The mixtures were stirred for 1 hour at 4°C and centrifuged at 100,000 x g for 60 minutes at 4°C. The supernatants (cholate extracts) were incubated for 20 minutes at 30°C to inactivate the catalytic component. Membrane fractions of cyc$^-$ S49 lymphoma cells and cholate extracts were mixed at various concentrations and kept at 32°C for 20 minutes for preincubation in 50 μl containing 50 mM Tris HCl, pH 7.5, 0.5 mM ATP, 0.1 mM

GTP, 1 mM EDTA, 2 mM $MgCl_2$ and 0.2 % sodium cholate. After preincubation 50 μl of assay solution was added to get the final concentration of 50 mM Tris HCl, pH7.5, 1 mM ATP, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.25 mM IBMX, 10 mM NaF and 20 μM $AlCl_3$. Incubation was for 5 minutes at 37°C. To exclude the influence of the detergent on the adenylyl cyclase activity, the concentration of sodium cholate was fixed to 0.1 %. The reaction was stopped by boiling. cAMP was measured as described above. Fluoride stimulated adenylyl cyclase activities of cyc^- membrane and the heat-inactivated cholate extracts were also monitored.

Northern blot analysis

Total RNA was isolated from cultured cells by the guanidine hydrochloride method (MacDonald et al., 1987). 10 μg of total cellular RNA was fractionated through 1 % agarose gels containing formaldehyde (Maniatis et al., 1982), blotted to Nitroplus 2000 membranes((Micron Separations Inc., MA), hybridized with DNA probes. Probes were mouse-derived α_s cDNA pGM13s and α_{i2} cDNA pGM1.3 (Sullivan et al., 1986) kindly provided by Dr. H. R. Bourne (University of California). The probes were [^{32}P]-labeled with the oligolabeling kit (Boehringer Mannheim).

Western blot analysis

Crude membrane fractions were fractionated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970), and electrophoretically blotted to Nitroplus 2000 membranes as described (Towbin et al., 1979). The membranes were processed as described by Handa et al. (1987). Anti-α_s rabbit serum was prepared by Dr. H. Uchiyama (Hamamatsu Medical School) by injecting oligopeptides consisting of 49 amino acid residues of bovine α_s according to the sequence reported by Nukada et al. (1986a). These 49 amino acids are com-

pletely conserved between bovine and rat (Itoh et al., 1986; Jones and Reed, 1987). Anti-α_1 rabbit serum was prepared by Dr. J. Kameyama (University of Tokyo) by injecting oligopeptides consisting of 10 C-terminal amino acid residues of bovine α_1 according to the sequence reported by Nukada et al. (1986b). These 10 residues (KNNLKDCGLF) are completely conserved in rat α_{i1} and α_{i2}, however, rat α_{i3} is different in 2 residues (KNNLKECGLY)(Jones and Reed, 1987; Itoh et al., 1988) and rat α_o is different in 4 residues (ANNLRGCGLY)(Itoh et al.,1986; Jones and Reed, 1987). These anti α_s and anti-α_1 antibodies were kindly provided by Dr. T. Haga (University of Tokyo). Affinity-purified rabbit anti-$\beta\gamma$ subunits antibody (Katada et al., 1987) was kindly provided by Dr. T. Katada (Tokyo Institute of Technology). Anti-rabbit IgG goat serum conjugated with horseradish peroxidase (Cappel Laboratories Inc.) was used as a secondary antibody.

RESULTS

Derivation of clones

XC cell line is derived from a rat Rous sarcoma (Svoboda, 1961). When the cells were plated in low numbers to form colonies, some colonies consisted of elongated fibroblastic cells, and some others composed of polygonal cells. By repeated subclonings, I established clones LK1 and RK1, consisting of pure population of elongated cells (Fig. 1A) and of polygonal cells (Fig. 1C), respectively. They were morphologically stable at least for three months. Southern blot analysis using Rous sarcoma virus LTR probe revealed that LK1 and RK1 clones were derived from the same cell, because the sites of integration of Rous sarcoma virus were the same in the two clones (note that the integration site of retroviruses is random, data not shown).

Different activation levels of adenylyl cyclase between two XC subclones in response to various agents

Cholera toxin is composed of A and B subunits. B subunit binds to the ganglioside GM1 on the cell surface (Craig and Cuatrecasas, 1975). The A subunit catalyzes mono-ADP-ribosylation of α subunit of the stimulatory G protein (Johnson et al., 1978). The ADP-ribosylated α_s irreversibly activates the catalyst of the adenylyl cyclase (Gill and Meren, 1978). The toxin is also known to elongate cultured mammalian cells such as Chinese hamster ovary cells (Guerrant et al., 1974).

I examined the effect of the toxin on the morphology and the cAMP level of the two XC clones. Cholera toxin at a dose of 100 ng/ml markedly elongated RK1 cells (Fig. 1D) but did not affect the morphology of LK1 cells (Fig. 1B). The basal level of cAMP was essentially the same for both LK1 and RK1 (Fig. 2). The amount of cAMP accumulated was plotted as a function of dose (Fig. 2A) and as a function of time (Fig. 2B). In RK1, the

treatment for 2 hours with the toxin at a dose of 1 ng/ml resulted in a detectable rise, and at 100 ng/ml 800-fold increase. The response of LK1 was remarkably different; increase was only 16-fold even at 100 ng/ml, and prolonged exposure to the toxin did not result in the significant rise in the cAMP level (Fig. 2B). With forskolin (Seamon and Daly, 1981), the same pattern was obtained (Figs. 2C and D).

Adenylyl cyclase activities of the membrane fractions.

Crude membrane fractions were prepared from LK1 and RK1, and were used to test the effect of forskolin, GTPγS, NaF and Mn^{++} on adenylyl cyclase (Table 1). Forskolin stimulates the catalytic unit directly in its low-affinity activation but requires α_s for its high-affinity activation (Seamon and Daly, 1981, 1986; Clark et al., 1982). GTPγS activates adenylyl cyclase as it exchanges with GDP but is not hydrolyzable to GDP. NaF is known to activate the enzyme by interacting with G_s (Howlett et al., 1979). Mn^{++} stimulates the catalyst directly (Ross et al., 1978).

Using 20 μg of membrane proteins the zero-time cAMP levels were 9 and 10 pmol/mg protein in RK1 and LK1, respectively. Adenylyl cyclase activities elicited from five minutes incubation in the reaction buffer alone (5 mM $MgCl_2$) were 89 and 21 pmol/min/mg protein for RK1 and LK1, respectively. Stimulation with GTPγS raised the level up to 256 pmol/min/mg in RK1 but only to 47 pmol/min/mg in LK1. Stimulation with 10 mM NaF raised the level up to 309 pmol/min/mg in RK1 but only to 51 pmol/min/mg in LK1. There was thus about six-fold greater cAMP accumulation in RK1 than LK1. Forskolin (10 μM) elevated the level up to 731 pmol/min/mg in RK1 but only to 37 pmol/min/mg in LK1. Thus twenty-fold greater cAMP was accumulated in RK1 in conformity with the <u>in vivo</u> data (Figs. 2C and D). Upon stimulation with Mn^{++} which can activate the catalytic unit by itself, the membranes of

LK1 accumulated the high level of cAMP as those of RK1 (239 and 291 pmol/min/mg, respectively). The above experiments indicated that the catalytic unit in LK1 was functional as in RK1, but can not reach to the full stimulation when the interaction with G_s is required.

Expression of G protein subunits

Since the activity of the catalytic unit is regulated both positively and negatively by G proteins, it is pertinent to examine the expression of G protein subunits in XC cells. To examine the mRNA expression of α_s and α_i subunits, total cellular RNA was extracted from LK1 and RK1. 10 μg of total RNA was subjected to Northern blot analysis using α_s and α_{i2} specific cDNA probes. The amount of RNA loaded per lane was checked by staining with ethidium bromide (Fig. 3C). There was no significant difference in the level of α_s (Fig. 3A) or α_{i2} (Fig. 3B) transcripts between LK1 and RK1.

To examine the protein expression of G protein subunits, membrane proteins were separated through 10 % polyacrylamide gels and subjected to Western blot analysis.

Anti-α_s antibody detected 52 and 45 kDa proteins (indicated by arrowheads on the left, Fig. 4A). The two bands were present in wild type S49 mouse lymphoma but were absent in cyc^- variant which lacks α_s (Ross and Gilman, 1977a, data not shown) and the size of them fits the published molecular mass of α_s (Northup et al., 1980) which are translated from the two alternatively spliced mRNAs (Robishow et al., 1986). XC cells express more abundant 52 kDa than 45 kDa α_s. The blot shown in Fig. 4A shows slightly more 52 and 45 kDa forms of α_s in LK1 than in RK1, however, the results fluctuated slightly when the membrane proteins extracted on different occasions were used. It is unlikely that there is any significant difference in α_s protein expression between LK1 and RK1.

Anti-α_i antibody which recognizes α_{i1} and α_{i2} (Dr. T. Haga, personal communication) detected a band migrating around 41 kDa (indicated by an arrow, Fig. 4B). The molecular mass of α_{i1} and α_{i2} is 40.4 and 40.5 kDa, respectively (Gilman, 1987). Because the intensity of the band fluctuated slightly as in the case of α_s, there does not seem to be a significant difference in the expression between LK1 and RK1.

Affinity-purified anti-$\beta\gamma$ subunits antibody detected one major band around 35 kDa (indicated by a white arrowhead, Fig. 4C). The molecular mass correspond with that of β subunit (Katada et al., 1987). The amount was almost the same in LK1 and RK1. γ subunit was not detected in our condition. Therefore, LK1 and RK1 expressed similar levels of α_s, α_i (at least α_{i1} and α_{i2}) and β subunits of the G proteins.

Functional assay of α_s

The α_s subunit is released from the heterotrimeric form by association of GTP with α_s. Released α_s directly stimulate the catalytic unit (Gilman, 1987). Although the above Northern and Western blot analyses indicated that α_s expression was almost the same in RK1 and in LK1, it was possible that α_s in LK1 was functionally defective. In order to check this possibility, I compared the capability of the cholate extracts from LK1 or RK1 membranes to reconstitute the adenylyl cyclase activity in the membranes of cyc^- variant. Here it should be noted that cholate extraction and subsequent incubation at 30 ^oC inactivate the catalytic unit but not α_s subunit in LK1 or RK1 membranes. The cholate extracts from LK1 or RK1 showed quite similar dose-response activity in reconstituting cyc^- adenylyl cyclase (Fig. 5). Therefore, α_s in LK1 was as functional as that in RK1.

ADP-ribosylation of α_s and α_i

Cholera toxin or pertussis toxin catalyzes the incorporation of [^{32}P]ADP-ribose from [^{32}P]NAD into α_s or α_i, respectively. Thus, ADP-ribosylated α_s or α_i can be visualized by incubating membrane proteins in the presence of [^{32}P]NAD and cholera toxin or pertussis toxin, respectively, and by subsequent SDS-polyacrylamide gel electrophoresis (Ribeiro-Neto et al., 1985).

Fig. 6A and B show the cholera toxin-mediated ADP-ribosylation. Black arrowheads show the ADP-ribosylated α_s proteins while white arrowheads show the ADP-ribosylated A_1 subunit of cholera toxin, the only toxin component heavy enough to be retarded by the gel (Lester et al., 1982). The bands migrating around 97.4 kDa molecular size marker were ADP-ribosylated in the absence of cholera toxin (data not shown). When 100 μg of membrane proteins were used, much greater amount of radiolabel was incorporated into proteins in LK1 than in RK1 (compare lane 1 in Fig. 6A with lane 1 in B). There was a discrepancy in the apparent amount of α_s protein in the cholera toxin-mediated ADP-ribosylation and the Western blot analysis shown above. To examine the possibility of the involvement of some inhibitor(s) of ADP-ribosylation, serially diluted membrane proteins were subjected to cholera toxin-mediated ADP-ribosylation (Fig. 6A and B). In LK1 radiolabel incorporation into α_s proteins decreased in a dose dependent manner as the input membrane proteins decreased (Fig. 6A), however, the pattern of the incorporation was drastically different in RK1 (Fig. 6B). The highest incorporation was observed when 25-6 μg of membrane protein was used (lanes 3-5, Fig. 6B). When larger amounts of membrane protein were used, the radiolabel incorporation paradoxically decreased. When smaller amounts of membrane protein were used, the difference in radiolabel incorporation between LK1 and RK1 diminished (compare lanes 5-7, Fig. 6A and B). Similar result was obtained in pertussis toxin-mediated ADP-ribosylation (Fig. 6C

and D). Therefore, it can be concluded that membranes of RK1 contained high activity that inhibits ADP-ribosylation.

DISCUSSION

XC cells gave rise to subclones with different adenylyl cyclase activity in response to cholera toxin, forskolin, GTPγS and NaF. One clone named RK1 was responsive while another clone LK1 was refractory. What brings about this clonal variation in adenylyl cyclase?

Intracellular cAMP level can be increased by either arrest of its decay or increase of its generation. It must be the latter mechanism which operates here because IBMX, an inhibitor of phosphodiesterase, was always included in the assay, and in experiments with Mn^{++}, the cAMP level in LK1 could reach the level of RK1. Thus, it must be the step in the generation of cAMP which was defective in LK1.

The adenylyl cyclase activity could be impaired by an abnormality in the catalytic unit or in the regulatory G proteins. As membrane proteins of LK1 accumulated the same level of cAMP as those of RK1 upon stimulation with Mn^{++}, the enzymatic function of the catalytic unit was not impaired in LK1. The catalytic unit is controlled positively by G_S and negatively by G_I. The positive control by G_S is exerted by GTP-α_S released from GDP-$\alpha_S\beta\gamma$ heterotrimer (Gilman, 1987). LK1 and RK1 expressed almost equal amount of α_S and the cholate extracts of the membrane proteins reconstituted the adenylyl cyclase activity of cyc^- cells equally well. Therefore, α_S in LK1 is similar to that in RK1 both quantitatively and qualitatively. LK1 expressed almost the same amount of α_I (at least α_{I1} and α_{I2}) and β subunits as RK1. Although the possible contribution of α_{I3} to the unresponsive phenotype of LK1 has not been ruled out, the role of α_{I3} in the adenylyl cyclase system has not been clarified and the generally accepted model of the negative control by G_I is that $\beta\gamma$ subunits interfere with G_S dissociation and inhibit the stimulatory function of G_S (Gilman, 1987). Therefore, it is unlikely that the catalytic unit in LK1 is inhibited by G_I. From the

above discussion and by exclusion of implausible explanations it is inferred that the defect in LK1 is in the step of interaction between α_s and the catalytic unit. As a cause of impaired interaction the catalytic unit of LK1 might have a defect at the coupling site with α_s keeping the catalytic domain intact. Alternatively, meeting of normal catalytic unit and α_s might be impaired by some properties in or around the membranes of LK1 (Neer and Clapham, 1988).

Up to now several mutants which have defects in adenylyl cyclase have been isolated. Two among them merit close comparison with LK1. Schimmer et al. (1984) reported forskolin-resistant mutants of Y1 adrenocortical tumor cells. The mutants had decreased adenylyl cyclase activity against forskolin and corticotropin compared to the wild type but retained fluoride-stimulated activity. As cholera toxin-mediated incorporation of [^{32}P]NAD to α_s was reduced as much as 70 %, a defect in α_s was suggested (Schimmer et al., 1987). Thus, their mutants are different from LK1. H21a (Salomon and Bourne, 1981) derived from S49 lymphoma has a character shared by LK1. It failed to respond to cholera toxin and GTPγS, but the amount of cholera toxin-mediated ADP-ribosylation of α_s was similar in the mutant and the wild type. The major difference from LK1 is that detergent extracts of H21a membranes failed to reconstitute the adenylyl cyclase activity of cyc$^-$. Therefore, the abnormality in LK1 appears to be unique.

It has been known that toxin-mediated ADP-ribosylation cannot be used to quantitate the amount the α subunits of G proteins (Ribeiro-Neto et al., 1985). The difference in ADP-ribosylation between LK1 and RK1 was striking when relatively high amount of membrane proteins were used; ADP-ribosylation in RK1 was remarkably inhibited. Since ADP-rybosylation of proteins both specific (α_s and α_i) and nonspecific to the toxins (bands around 97.4 kDa molecular marker in Fig. 6) was inhibited, it is likely that the inhibi-

tion was caused by the consumption of NAD, the cosubstrate of ADP-ribosylation. NAD is a substrate of NAD splitting enzyme, NAD glycohydrolase (Hayaishi and Ueda, 1977). It is inferred that RK1 might contain higher NAD glycohydrolase activity. NAD glycohydrolase is high in macrophages (Artman and Seeley, 1978) and induced by differentiation in HL-60 human leukemic cells (Borelli et al., 1985). LK1 and RK1 are different not only in morphology but also in adenylyl cyclase and possibly in NAD glycohydrolase activities. It must be interesting to study how these heterogeneous differences are produced.

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Fig. 1. **Phase contrast microscopy of subclones of XC, LK1 (A, B) and RK1 (C, D) with (B, D) or without (A, C) cholera toxin (CTX).** 5 x 10^4 cells were plated in 12 well titer plates. On the following day the photographs were taken before (**A, C**) and 18 hours after addition of 0.1 μg/ml of cholera toxin (**B, D**). Bar, 30 μm.

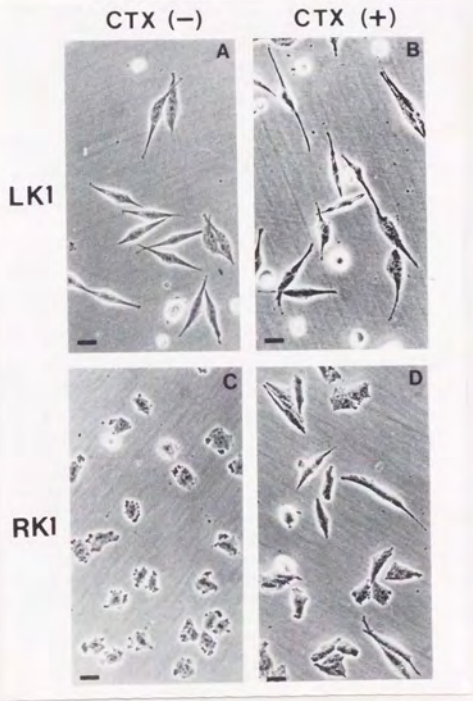


Fig. 2. **Effect of cholera toxin (CTX) and forskolin on LK1 and RK1.** Dose-response for cholera toxin-stimulated (A) or forskolin-stimulated (C) cAMP accumulation. Incubation time was two hours (cholera toxin) or one hour (forskolin). Time course of cAMP accumulation after stimulation with 10 μg/ml of cholera toxin (B) or 50 μM of forskolin (D). ●, RK1 ; ○, LK1.

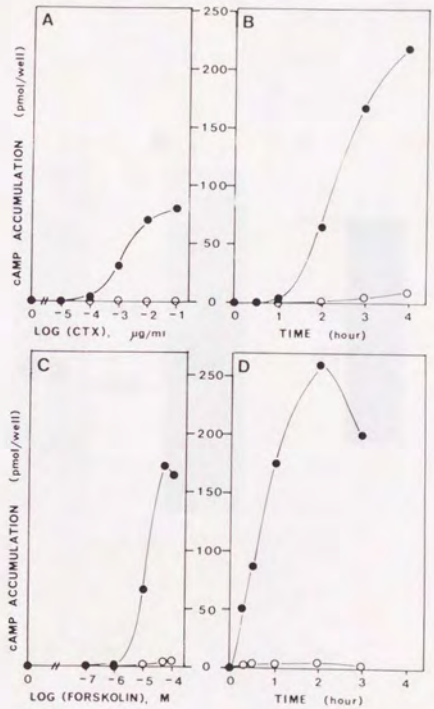


Fig. 3. **Expression of α_s and α_{i2} mRNAs.**

A: Northern blot analysis of α_s. **B:**Northern blot analysis of α_{i2}. **C:**Ethidium bromide staining of the gel to show the amount of total RNA loaded per lane. Lane 1: LK1. Lane 2: RK1.

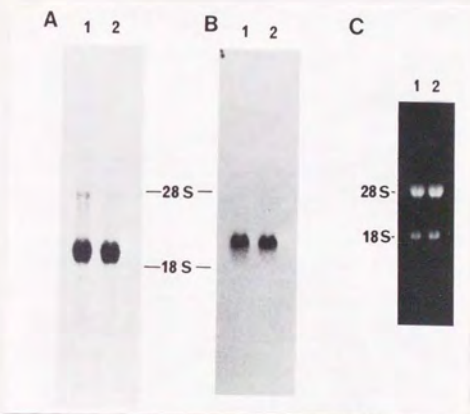


Fig. 4. **Expression of α_S, α_I and β proteins.** **A**: Western blot analysis using anti-α_S antibody. Black arrowheads indicate α_S proteins. **B**: Western blot analysis using anti-α_I antibody. An arrow indicates α_I proteins. **C**: Western blot analysis using anti-$\beta\gamma$ subunit antibody. A white arrowhead indicates β subunit proteins. Lane 1: LK1. Lane 2: RK1.

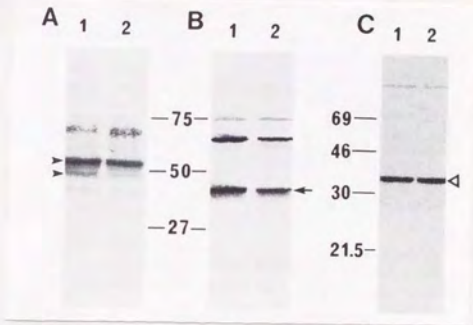


Fig. 5. **Reconstitution of adenylate cyclase system in cyc⁻ S49 lymphoma cells.** A: The amount of cyc⁻ membranes was constantly 100 μg per reaction and cholate extracts from LK1 (O) and RK1 (●) were varied. B: The amount of cholate extracts from LK1 (○) or RK1 (●) was constantly 10 μg and the amount of cyc⁻ membrane was varied.

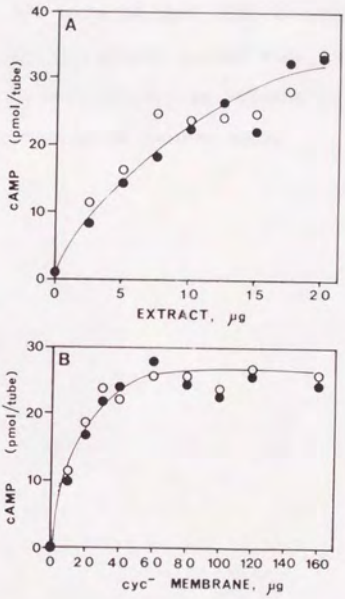


Fig. 6. **ADP-ribosylation of α_s or α_i with activated cholera toxin or pertussis toxin.** Membrane proteins of LK1 (**A,C**) or RK1 (**B,D**) were ADP-ribosylated with [^{32}P]NAD and activated cholera toxin (**A,B**) or pertussis toxin (**C,D**). Serial two-fold dilution of the membrane proteins were subjected to toxin-mediated ADP-ribosylation and 1/5 of each reaction mixtures was loaded per lane. The amounts of the proteins in the reaction were: Lane 1: 100 μg. Lane 2: 50 μg. Lane 3: 25 μg. Lane 4: 12 μg. Lane 5: 6 μg. Lane 6: 3 μg. Lane 7: 1.5 μg. Figures marked with lower case letters are gels stained with Coomassie brilliant-blue and correspond to each autoradiography marked with upper case letters. Black arrowheads indicate α_s proteins. Arrows indicate α_i proteins. White arrowheads indicate the A_1 subunit of cholera toxin.

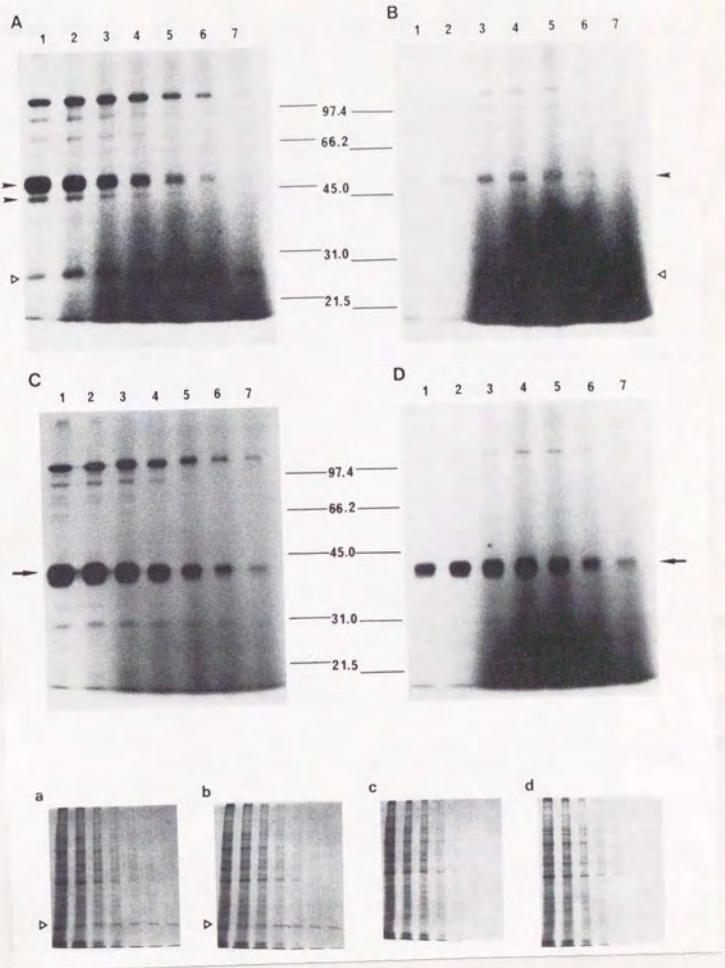
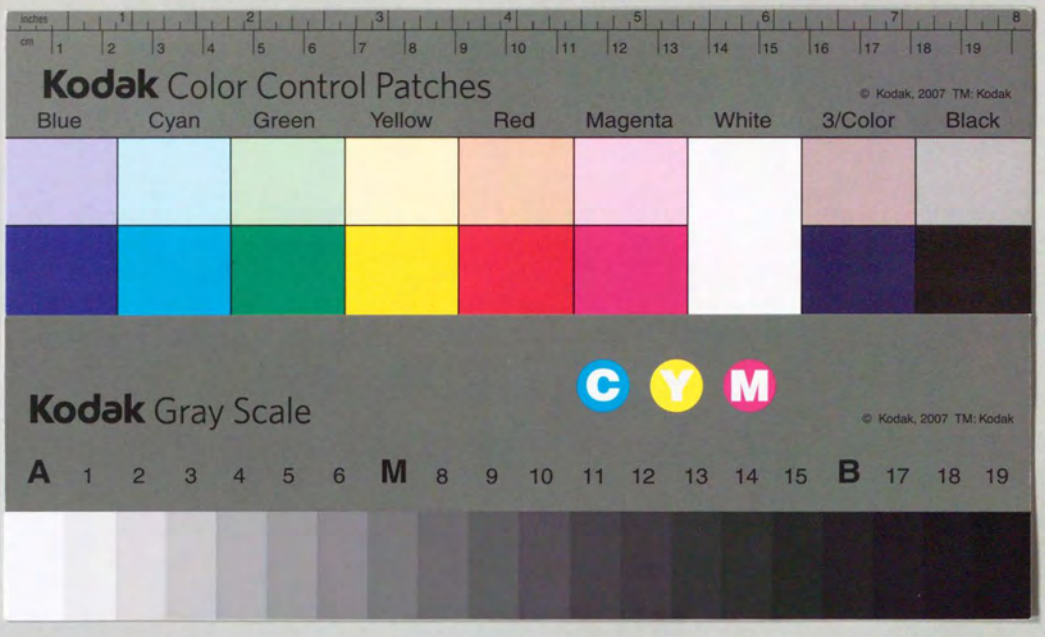

A
1 2 3 4 5 6 7
97.4
66.2
45.0
31.0
21.5
B
1 2 3 4 5 6 7
C
1 2 3 4 5 6 7
97.4
66.2
45.0
31.0
21.5
D
1 2 3 4 5 6 7
a
b
c
d

Table 1.

Adenylate cyclase activity of membrane fractions

	LK1	RK1
No incubation [a]	10 ± 4	9 ± 1
Basal activity [b]	21 ± 2	89 ± 8
10 μM GTPγS	47 ± 3	256 ± 45
10 mM NaF	51 ± 1	309 ± 23
10 μM forskolin	37 ± 1	731 ± 87
10 mM $MnCl_2$	239 ± 33	291 ± 18

Figures indicate amount of cAMP (mean ± S.E. derived from three determinations, pmol/min/mg generated by 20 μg of membrane fractions incubated for five minutes with or without stimulating agents. [a] immediately after dissolving membranes into the reaction buffer. [b] incubation without any reagents.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Cyan
Green
Yellow
Red
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White
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Black
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A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19