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MODULATION OF ADENYLATE CYCLASE BY GUANINE NUCLEOTIDES AND KIRSTEN SARCOMA VIRUS MEDIATED TRANSFORMATION

Danièle SALTARELLI*, Sigmund FISCHER & Gérard GACON

Institut de Pathologie et de Biologie Cellulaires et Moléculaires
INSERM U.15
24 rue de Faubourg Saint-Jacques 75014 PARIS, France

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Certain tumour cells contain activated ras genes that code for 21 000 dalton proteins (p21). These proteins associate with the inner face of the plasma membrane and bind guanine nucleotides specifically. In order to determine whether p21s have functions similar to other GTP binding proteins, we investigated the regulation, by guanine nucleotides, of adenylate cyclase (AC) activity in membrane preparations isolated from fibroblasts (C127) transformed by a temperature sensitive mutant of Kirsten sarcoma virus (Ts 371). The degree of AC stimulation by GMP P(NH)P increased when these cells were shifted from the permissive temperature (33°C) to the non-permissive temperature (39°C). This effect was more pronounced at low Mg^{++} and low GMP P(NH)P concentrations. AC stimulation remained unchanged in rat fibroblasts infected with a temperature sensitive mutant of Rous Sarcoma virus. AC activity was depressed in C127 cells infected with wild type KiMSV. Our data illustrate the feasibility of correlating alterations in the AC system with ras gene expression and using such experimental approaches to elucidate the physiological functions of the p21 proteins.

Kirsten and Harvey murine sarcoma virus (Ki and Ha MSV) contain the ras oncogene which codes for the 21 000 dalton transforming protein p21 (1). A number of human tumours and chemically induced animal tumours have been shown, by transfection experiments, to contain activated ras genes that can induce tumorigenic and morphological transformation of NIH 3T3 fibroblasts (2-3). As a rule, these genes differ from the normal protooncogene c-ras by no more than a single base pair substitution (4-7). It has also been shown that a high level of expression of the normal c-ras gene can induce transformation (8).

Although the precise mechanism by which p21 proteins cause transformation is unknown, subcellular fractionation and electron microscopy have indicated that, after post translational modification, the mature proteins become associated with the inner surface of the plasma membrane (9-10) and it is known that all forms of the ras proteins bind non-covalently and with high affinity

*Present address: Department of Clinical Science and Immunology
Medical school, Observatory,
CAPE TOWN, South Africa 7925

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to guanine nucleotides (11). Furthermore, the v-Ha-ras protein may be autophosphorylated using GTP as the phosphate donor(12). Normal cellular P21 is not autophosphorylated - presumably because the c-ras gene product lacks a phosphorylatable threonine residue at position 59 (13). These observations suggested that the p21 proteins may function as latent phosphotransferases or GTPases and that they might belong to a distinct group of GTP-binding proteins that regulate normal cellular functions. It is known that GDP/GTP exchange and the hydrolysis of GTP to GDP are involved in a variety of membrane-mediated intracellular phenomena such as tubulin assembly (14-16), adenylate cyclase responses to hormonal stimulation, (17-18) light-induced activation of cGMP phosphodiesterase in the retina (19,20) or protein synthesis through initiation factor eIF (21). In all of these processes the binding of GTP to specific binding proteins induces a confirmational change that promotes the interaction with an effector protein.

To investigate the possible involvement of p21 proteins in the transduction of signals that lead to changes in the concentration of cAMP we have studied the effects of guanyl nucleotides on adenylate cyclase (AC) activity in membrane preparations isolated from murine fibroblasts transformed by infection with a temperature sensitive mutant of Kirsten murine sarcoma virus (Ts371) cultured at the permissive and non permissive temperatures (22).

MATERIALS AND METHODS

Reagents

^{3}H cAMP and $\alpha^{32}P$ ATP were obtained from Amersham. Creatine kinase, GTP and GMP P(NH)P were from Boehringer-Mannheim. Forskolin was obtained from Calbiochem-Behring and kept at -20°C as a 10 mM stock solution in ethanol. Other reagents were obtained from Sigma.

Cells and cell cultures

Cells used in this study were obtained from the following sources:- Mouse C127 cells : Centre de Biochimie, Universite de Nice (Prof. Cuzin). Ts 371 C127 cells and wild type KiMSV C127 cells (22): Hopital St-Louis, Paris (Prof. P.R. Boiron). Fisher rat 3T3 cells and the same cells infected with a thermosensitive mutant (PA1) of Rous sarcoma virus: Institut Curie,Orsay (Dr.Calothy). NIH 3T3 cells and the same cells transfected with human myc gene: Institut de la Recherche sur le Cancer, Villejuif (Dr. D'Autry). Cells were grown and maintained as monolayer cultures in a humid atmosphere of 5% CO in air in Dulbecco's modified Eagle's medium (Flow laboratories) supplemented with 10% foetal bovine serum and 2mM glutamine.

Membrane preparation

Monolayers were washed three times with PBS and then incubated for 5 minutes in ice cold 1 mM $NaHCO_3$ containing 1 mM dithiothreitol. The dishes were then scraped with a rubber policeman, the detached cells were homogenized at 4°C with 8 strokes of a teflon-glass potter homogenizer, and the homogenates were centrifuged for 45 min at 35 000g. The pellet was resuspended in a minimum volume of fresh bicarbonate medium and stored under liquid nitrogen.

Adenylate cyclase assay

The assay was initiated by the addition of 20 1 of membrane suspension (50-100 µg of protein) to 40 µl of assay medium to give final concentrations of 0.3 mM $\alpha^{32}P$ ATP ($2x10^{6}$ cpm), 1 mM cAMP, 50 mM Tris HCl pH 7.6, 25 mM

phosphocreatine and 1 mg/ml creatine phosphokinase. Duplicate samples were incubated at 32°C in a shaking waterbath. Assays were stopped by the addition of 0.2 ml of 0.5N HCl and boiling for 6 min. followed by the addition of 0.2 ml of 1.5 M imidazole pH 7.6. Samples were then added to a column of alumina using a modification using the method of White (23) and cAMP was eluted with 3 ml of 10 mM imidazole-HCl pH 7.6. Blank values obtained by this procedure were always less than 0.005% of the total radioactivity applied. The yield was calculated from the recovery of an added ^{3}H cAMP "spike". The protein content was determined by the method of Lowry et al (24) using crystallized bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Changes in AC activity in mouse C127 cells infected with the thermosensitive KiMSV mutant, Ts 371, were studied by seeding and culturing the cells at 33°C. When semi-confluent, some of the cultures were shifted to 39°C for 48 hours while the remainder of the cells were kept at 33°C. This protocol avoided the effects of cell density on AC activity (25) and allowed sufficient time after the temperature shift for the transformed phenotype to become expressed (22).

Membrane preparations from the cells were then studied for their ability to catalyze the synthesis of cAMP in the presence of different concentrations of Mg++ and the unhydrolyzable analog of GTP, GMP P(NH)P. This analog was used since $\gamma^{32}P$ GTP is rapidly hydrolyzed by the membrane protein and for the reason that the analog leads to the formation of a persistently "activated" state of the enzyme since the nucleotide binding site of the regulatory protein is "non exchangeable" in the presence of Mg++. The effect of GMP P (NH)P and Mg++ on membranes from Ts 371 C127 cells cultured at permissive and restricted temperatures were demonstrated in the experiments summarized in Fig. 1. As can be seen, the degree of AC stimulation by GMP P(NH)P increased when cells were shifted to the non-permissive temperature. This effect was more pronounced at low Mg++ and low GMP P(NH)P concentrations. It is noticeable that the activation of AC by GMP P(NH)P occurs with a higher EC_{50} (50% effective concentration) in cells cultured at the permissive temperature than in cells cultured at 39.5 C (inset Fig. 1). Control experiments showed no significant differences in AC activity or in the relative stimulation by GMP P(NH)P in Ts 371 C127 cells during the course of their exponential growth at 33°C nor in parental C127 cells or Fischer rat 3T3 cells cultured at 33°C or 39°C.

Cellular transformation is known to be associated with changes in enzyme activity, membrane lipids, distribution of cytoskeletal proteins and other functions. It was possible, therefore, that the changes in AC activity that we observed were not primarily attributable to v-ras gene expression but secondary to changes in the environment of the AC complex resulting from the effect of transformation on the membrane or the cytoskeleton. With this possibility in

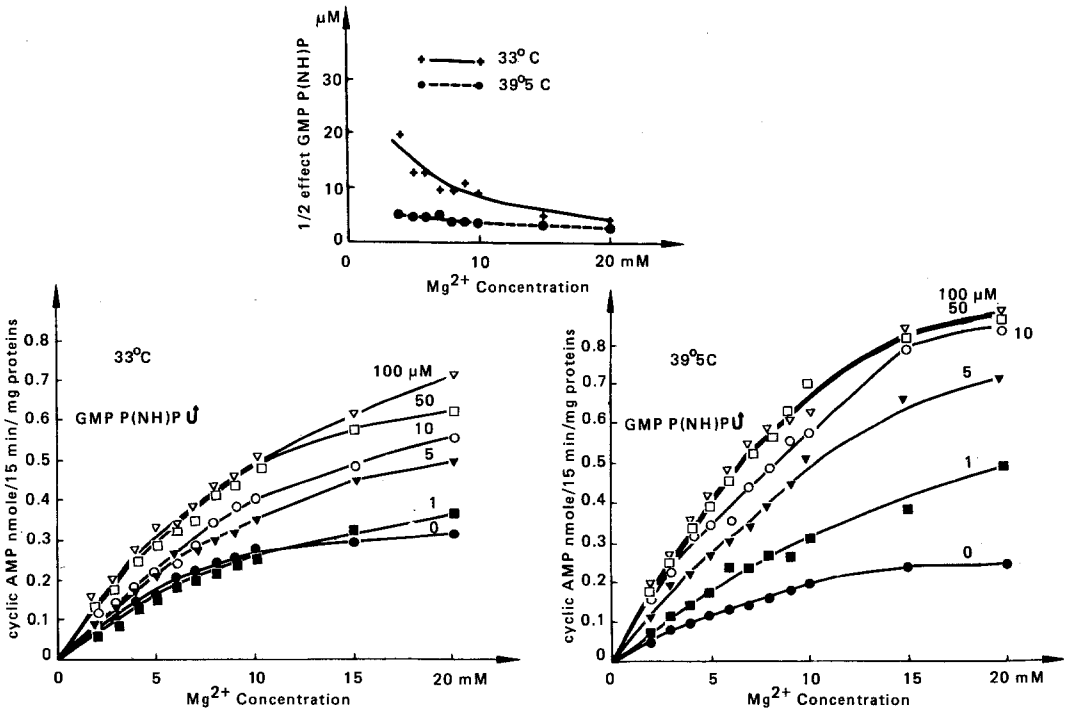

Figure 1

cAMP synthesis catalyzed by membranes isolated from C127 cells infected with thermosensitive mutant of Kirsten SarcomaVirus Ts371) as function of Mg concentration and GMP P(NH)Pconcentration.

Left panel: cells were grown at permissive temperature (33.0°C)

Right panel: cells cultured at 33 C were shifted to nonpermissive temperature (39.5°C) for 48 hours.

Inset : variation of EC_{50} (1/2 effective effect) for activation of adenylate cyclase activity by GMP P(NH)P

mind we have measured basal AC activity and AC responsiveness to GMP P(NH)P stimulation in membranes isolated from Fisher rat 3T3 cells infected with a temperature sensitive mutant of Rous sarcoma virus (RSV). Cells cultured at the permissive temperature (33°C) were no different from cells cultured at the non-permissive temperature (39.5°C) in this regard.

Transformation induced by RSV is associated with the other cellular features of transformation and involves the expression of an onc gene product (src pp60) that is similar to p21 in its tendency to associate with the cytoplasmic surface of the plasma membrane. The fact, therefore, that transformation with RSV does not affect AC activity whereas ras gene expression does, supports the contention that p21 proteins are specific in this regard.

Cellular AC activity may be subject to simultaneous inhibitory and stimulatory control by regulatory proteins that share with p21, the ability to bind guanine nucleotides. The stimulatory and inhibitory proteins are heterodimers that contain different GTP-binding subunits (αi:MW 41 000 αs: MW 42 000) in association with the same β (MW 35 000) subunit. The "subunit dissociation"

model (27) for the regulatory action of the G proteins implies that persistent stimulation or inhibition of AC activity by GTP analog depends upon the relative concentrations of α_s α_i and β subunits; the affinities and exchange rates of the nucleotide-binding sites; and the concentration of Mg++. Moreover it has been suggested by Gilman and his coworkers (28) that the β subunit of the inhibitory GTP binding protein exerts its inhibitory activity by associating with the α_s subunit and inactivating the stimulatory guanine nucleotide-binding complex. This inhibitory association is favoured by the relative absence of Mg++ or guanine nucleotides. These considerations suggested that p21 proteins might exert their effects by interacting with the G protein subunits or, directly, with the catalytic unit of AC. Nevertheless, to the best of our knowledge, no inhibitory process mediated by inhibitory GTP binding proteins has previously been documented in cultured fibroblasts.

We therefore performed a series of experiments in which membranes were prepared from non-infected C127 cells, C127 cells infected with wild type Kirsten sarcoma virus (wt-Ki MSV) and cells that were infected with Ts 371 KiMSV and cultured at the permissive temperature. The effect, on AC activity, of incubating the membranes with varying concentrations of GMP.P(NH)P in the presence or absence of 10 μM forskolin are shown graphically in Fig. 2.

The results of these experiments are noteworthy in the following respects. Firstly, basal AC activity was moderately increased by infection of C127 cells with thermosensitive KiMSV whereas it was profoundly depressed by infection of the cells with the wild type virus. Secondly, AC activity in membranes isolated from non infected cells and Ki C127 cells were responsive to GMP P(NH)P whereas the responses of membranes from Ts-371 cells were attenuated.

Forskolin has been shown to increase AC activity both in intact cells and purified membranes (29). Although its site of action has not been elucidated, it has been suggested that there are two components that mediate the action of forskolin on AC: one requires that G proteins are occupied by GTP whereas the other does not (30,31). In our experiments forskolin (10 mM) caused a 16-fold rise in AC activity in cells infected with KiMSV, but only a 2-fold rise in cells infected with Ts 371. It is noticable that unlike Ts 371 C127 cells, forskolin potentiated the stimulation by GMP P(NH)P in C 127 cells and Ki MSV C 127 cells.

The depressed levels of AC activity in membranes derived from cells transformed with wt KiMSV is of interest since we were unable to demonstrate a similar effect (or any effect on GMP P(NH)P responsiveness) in NIH 3T3 cells transformed by transfection with a human myc gene. Changes in AC activity therefore are not a general feature of transformation. While this work was in progress, Beckner (32) described depressed AC activity in MDCK cells transformed with Harvey sarcoma virus, in Ki MSV-transformed NRK cells and in rat

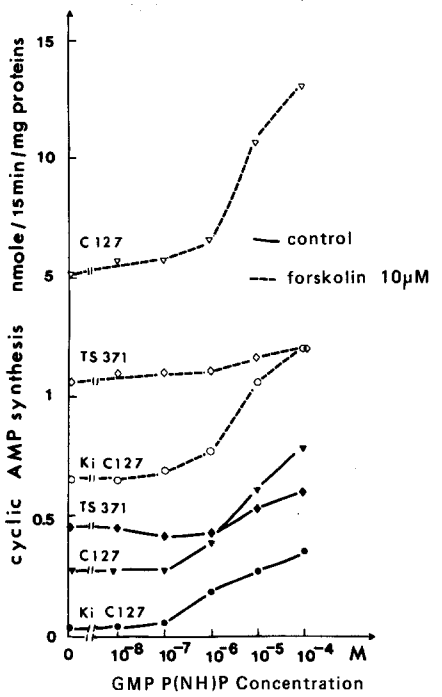

Figure 2

cAMP Synthesis as a function of GMP P(NH)P concentration;
without (full lines) and with 10 M forskolin (dotted lines).
(▼—— ▼) C127 cells
(▽ - - - ▽)
(● —— ●) C127 cells infected with wild type KiMSV
(○ _ _ _ ○)
(◆ —— ◆) C127 cells infected with Ts371 KiMSV
(◇ _ _ _ ◇)
Mg++ concentration : 10 mM
Cells were cultured at 33°C.

fibroblasts transformed with middle T. Our results are consistent with their observations in this respect. The failure of c-myc expression to alter AC activity may reflect the fact that, unlike p21 proteins, the c-myc gene products are localized in the nucleus of cells where they appear to function as DNA-binding proteins (33,34).

We have no satisfactory explanation for the interesting differences we observed between AC activity of membranes from cells infected with wt KiMSV and from cells cultured at the permissive temperature, after infection with Ts-371 KiMSV. Shih et al (22) have shown structural differences between p21's from Ts-371 KiMSV and that from cells infected with the wild type virus. Furthermore, p21 from wt KiMSV cells is phosphorylatable in vivo whereas that from Ts-371 KiMSV greaded at permissive temperature is not. It would prove most interesting if the striking differences we have observed with regard to AC activity

could be attributed to the chemical differences that have been described in the structure of the ras gene products.

The results reported in this paper show that p21 proteins have effects upon the AC system. These effects may be involved in mediating transformation and thus provide a convenient experimental system for quantitating transforming gene expression. It would be of interest to examine p21's ability to modulate AC activity in reconstituted systems such as those that have been described with tubulin (35,36).

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REFERENCES

1. Ellis, R.W., Lowry, D.R., Scolnick, F.M. (1982) Adv. Viral Oncol. 1, 107-126.
2. Parada, L., Tabin, C., Shih, C., Weinberg R (1982) Nature 297, 474-478.
3. Balmain, A., Pragnell, I.B. (1983) Nature 303, 72-74.
4. Tabin, G.J., Bradley, S.M., Bargman, C.L., Weinberg R.A., Papageorge,A.G., Scolnick, E.M., Dhar, R., Lowy, D.R.,Chang E.H. (1982) Nature 300, 143-149
5. Reddy, E.P., Reynolds, R.K., Santos, E., Barbacid, M. (1982) Nature 300, 149-152.
6. Capon, D.J., Seeburg, P.F., McGrath, J.P., Hayflick, J.S., Edman, V., Levinson, A.D., Goeddel, D.V. (1983) Nature 304, 507-513.
7. Yuasa, Y., Scrivastava, C., Dunn, C., Rlum, C., Reddy, J.(1983) Nature 303, 775-779.
8. Chang, E., Furth, M., Scolnick, E., Lowy, D. (1982) Nature 297, 479-483.
9. Willingham, M.C., Pastan, I., Shih, T.Y., Scolnick, E.M.(1980) 19, 1005-1014.
10. Furth, M.E., Davis, L.J., Fleurdelys, B., Scolnick, E.M. (1982) J. Virol. 43, 294-304.
11. Papageorge, A., Lowy, D., Scolnick, E.J. (1982) J. Virol. 44, 509-519.
12. Shih, T.Y., Papageorge, A.G., Stockes, P.E., Week, M.O., Scolnick, E.M. (1980) 287, 686-691.
13. Capon, D.J., Chen, E.Y., Jerrinson, A.D., Seeburg P.H., Goeddel, D.V. (1983) Nature 303, 33-37.
14. Carlier, M.F. (1982) Mol. Cell Biochem. 47, 97-113.
15. David Pfeuty, T., Erickson, H.P., Pantaloni, D. (1977) Proc.Natl. Acad. Sci. USA 77, 5372-5375.
16. Saltarelli, D., Pantaloni D. (1983) Biochemistry 22, 4607-4614.
17. Ross, E.M., Gilman, A.G. (1980) Ann. Rev. Biochem. 49, 533-564.
18. Cooper, D.M.F. (1982) Febs Letters 138, 157-163.
19. Wheeler, G.L., Bitensky, H.W. (1977) Proc. Natl. Acad. Sci. USA, 74, 4238-4244.
20. Keung fung, B., Hurley, J.B. and Stryer, L. (1981) Proc. Natl. Acad. Sci. USA 78, 152-155.
21. Lagus, R., Anderson, M.F., and Safer, B. (1981) Prog. Nucl.Acid Res. & Mol. Biol. 25, 127-185.
22. Shih, T.Y., Weeks M.O., Young, H.A. and Scolnick, E.M. (1979) J. of Virology 31, 546-556.
23. White, A.A. In "Methods in Enzymology" (Hardman J.G., O'Malley B.W. Eds) Academic Press New York XXXVIII p.41-46.

24. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randal, R.D. (1951) J.Biol. Chem. 193, 265-275.
25. Pastan, I.H., Johnson, G.S. and Anderson, W.B. (1975) Ann. Rev. Biochem. 44, 491-522.
26. Krueger, J.G., Garber, E.A., Goldberg, A.R. and Hanafusa, H. (1982) Cell 28, 889-896.
27. Gilman, A.G. (1984) J. of Clinical Invest. 73, 1-4
28. Katada, T., Bokoch, G.M., Smigel, M.D., Ui M. and Gilman A.G. (1984) J. Biol. Chem. 259, 3586-3595.
29. Seamon, K.B. and Daly, J.W. (1981) J. Cyclic Nucl. Res. 7, 201-202.
30. Award J.A., Johnson, R.A., Jakobsand, K.H., Schultz, G.(1983) J. Biol. Chem. 258, 2960-2965.
31. Insel, P.A., Stengel, D., Ferry, N., Hanoune J. (1982) J.Biol. Chem. 257, 7485-7490.
32. Beckner, S.K.(1984) Febs letters 166, 170-174.
33. Hann, S.R., Abrams, H.D., Rohrschneder, L.R., Eiseman, R.N.(1983) Cell 34, 789-796.
34. Alitalo, K. (1983) Nature 306, 274-277.
35. Deanin, G., Gordon, M.W., Berlin, R.D. (1983) J. Cell Biol. 97, 209a.
36. Simonin, G., Zachowski, A., Huitorel, P., Paraf, A., Pantaloni, D. (1981) Eur. J. Biochem. 118, 515-519.