Volume 228, number 1, 37–41 FEB 05500 February 1988

Adenylate cyclase activity of *v-ras-k* transformed rat epithelial thyroid cells

Giulia Colletta, Daniela Corda*, Gennaro Schettini°, Anna Maria Cirafici, Leonard D. Kohn+ and Eduardo Consiglio

*Dipartimento di Biologia e Patologia Cellulare e Molecolare, II Facoltà di Medicina e Chirurgia and Centro di Endocrinologia ed Oncologia Sperimentale del CNR, Via S. Pansini 5, Naples, *Istituto di Ricerche Farmacologiche 'Mario Negri', Via Eritrea 62, Milan, +Istituto di Farmacologia, II Facoltà di Medicina e Chirurgia, Via S. Pansini 5, Naples, Italy* and *°Laboratory of Biochemistry and Metabolism, National Institute of Health, Bethesda, Maryland, USA*

Received 19 November 1987

The regulation of adenylate cyclase has been analyzed in normal rat thyroid cells as well as in the same cells transformed by the *v-ras-k* oncogene. In both cell types the adenylate cyclase complex consists of the two GTP-binding proteins, G_i and G_s, as demonstrated by the specific ADP-ribosylation induced by pertussis and cholera toxin, respectively. The response of adenylate cyclase of the transformed cells to forskolin, pertussis toxin and cholera toxin is attenuated with respect to the control cell line. The thyrotropic hormone (TSH), that acts on normal thyroid cells in culture as a growth factor by stimulating the adenylate cyclase activity, is not able to induce DNA synthesis nor does it stimulate adenylate cyclase in *v-ras-k* transformed cells.

v-ras-k oncogene; Thyrotropin; cyclic AMP; (Thyroid cell)

1. INTRODUCTION

FRTL-5 is a clonal cell line of differentiated rat thyroid cells that continue to divide when grown in the presence of thyrotropic hormone (TSH) [1]. Upon withdrawal of this hormone, the cells stop dividing, but maintain their viability and become hypersensitive to TSH with respect to cAMP production [2,3]. Thus TSH acts on these cells as a potent stimulator of adenylate cyclase activity and cAMP mediated thyroid functions such as iodide transport [4], growth [3] and protein synthesis [5].

It has been shown that these epithelial cells can be transformed by the Kirsten sarcoma virus [6–8]. The *v-ras-k* transformed rat thyroid cell lines used in this study (KiKi and KiMol cell lines), exhibit very high levels of KiMSV mRNA and p21 oncogene product [9]. Known biochemical activities associated with *ras* p21 proteins include GTP binding and GTPases activity [10], in addition, studies by Hurley et al. [11] have demonstrated sequence similarity between mammalian G-proteins and p21. These observations suggest that the p21 *ras* proteins may function as GTPases and that they might belong to a distinct group of GTP-binding proteins that regulated normal cellular functions. When infected by the Kirsten murine sarcoma virus, FRTL-5 cells become completely transformed. In particular, the transformed cells are independent of TSH for their growth [8]. It is possible that the transformation process interrupts the hormone-stimulated adenylate cyclase pathway. This lesion in the signaling pathway could occur at the level of the TSH receptor, of the G-protein or of the adenylate cyclase enzyme.

Since one function of G-proteins is to regulate adenylate cyclase in several cell systems [12], in the present work a series of experiments has been undertaken to study the effects of the *v-ras-k* gene product on adenylate cyclase in the rat thyroid cell system.

Correspondence address: G. Colletta, Dipartimento di Biologia e Patologia Cellulare e Molecolare, II Facoltà di Medicina e Chirurgia, Via S. Pansini 5, Naples, Italy

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

2. MATERIALS AND METHODS

The FRTL-5 cells are a strain of cloned normal rat thyroid cells, previously shown to maintain the functional characteristics of iodide uptake and thyroglobulin synthesis [1]. The KiMol cells are FRTL-5 cells infected with the Kirsten sarcoma virus (KiMSV-MoMuLV). The KiKi cells are a different cell line of FRTL-5 cells independently infected with the Kirsten sarcoma virus (KiMSV-KiMuLV), with a transformed phenotype identical to KiMol cells [6–8].

DNA synthesis was evaluated by measuring the [^{3}H]thymidine uptake as described [13].

The intracellular cAMP content of FRTL-5, KiMol, and KiKi cells was measured by a method previously reported using a RIA technique (Biomedical Technologies, Inc., Cambridge, MA) [14], 0.5 mM IBMX was included in all assays. The amount of DNA in the different experiments was approx. 8 μg per well in the transformed cells and 14/17 μg in FRTL-5 cells.

Adenylate cyclase activity in the cell homogenate was determined according to the method of Solomon et al. [15]. Adenylate cyclase activity was calculated from quadruplicate samples and reported as the mean pmol of [α-^{32}P]cAMP/mg of protein. Protein content was determined by the modified method of Lowry [16].

ADP-ribosylation activity was measured by following the incorporation of [^{32}P]ADP-ribose into membrane components, as described [14]. TSH used in ADP-ribosylation, cAMP, or other assays was a purified preparation [17]. Forskolin was obtained from Sigma; pertussis toxin from List Biological Laboratories, Campbell, CA; cholera toxin from Calbiochem-Behring, La Jolla, CA.

3. RESULTS

Rat thyroid cells grown in the absence of TSH for two days, become quiescent and incorporate background levels of [^{3}H]thymidine. Treatment with pure TSH allows the thyroid cells to enter the S phase [13]. It has been shown that TSH acts on FRTL-5 cells by inducing high levels of cAMP [3]. In order to verify whether forskolin (an adenylate cyclase stimulator [18]) was able to induce DNA synthesis in FRTL-5 cells, an [^{3}H]thymidine incorporation experiment was performed. Fig.1 shows that forskolin, at a concentration of 1×10^{-7} M, is able to stimulate DNA synthesis in the FRTL-5 cells at levels similar to those induced by TSH. In contrast, in KiKi cells, neither TSH nor forskolin are able to induce DNA synthesis; only a slight effect was observed with forskolin 1×10^{-7} M (fig.1). The KiKi cells are not quiescent, therefore their basal value is always higher than in the FRTL-5 cells. Yet one could expect an effect of forskolin and TSH since these cells can be stimulated, as is the case of 5% calf serum that induces a 5-fold increase in [^{3}H]thymidine uptake (not shown). KiMol cells displayed a similar behavior (not shown).

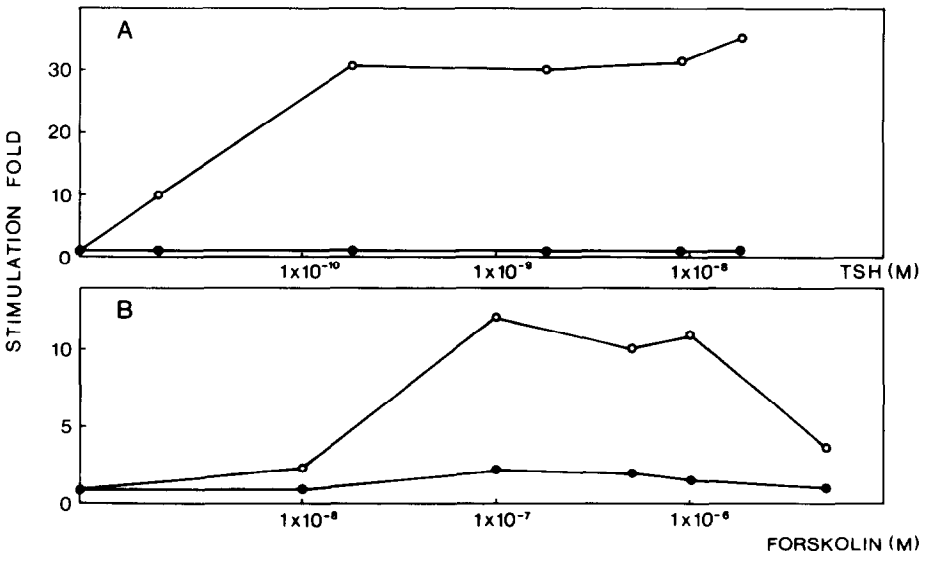

Fig.1. Incorporation of [^{3}H]thymidine in (A) FRTL-5 (○) and KiKi (●) cells after TSH treatment. (B) FRTL-5 (○) and KiKi (●) cells after forskolin treatment. The stimulation fold indicates the ratio of incorporation into trichloroacetic acid-precipitable material for the stimulated culture relative to incorporation into unstimulated cells.

Table 1

Levels of cAMP in KiKi, KiMol and FRTL5 cells stimulated with various compounds

	KiKi	KiMol	FRTL5
Control[a]	0.7 ± 0.1 (5)	0.5 ± 0.1 (5)	0.85 ± 0.2 (4)
Cholera toxin (10^{-8} M)	9.9 ± 0.5 (3)	6.5 ± 0.8 (3)	26 ± 4 (3)
Pertussis toxin (10^{-8} M)	0.9 ± 0.1 (3)	0.5 ± 0.2 (3)	2 ± 0.2 (3)
Forskolin (10^{-6} M)	7.6 ± 0.7 (4)	7.3 ± 0.4 (3)	12 ± 1.7 (3)
TSH (10^{-6} M)	2 ± 0.4 (5)	0.7 ± 0.1 (4)	21 ± 1 (3)

[a] cAMP (pmol/well). The cAMP assay was performed as described [30]. The incubation with the various compounds was for 1 h at 37°C. In parentheses are the number of experiments, each performed in duplicate. The data are presented as mean ± SE. FRTL5 cells were maintained without TSH in the medium for 5 days prior to the cAMP determination

To study the possible role that *ras* proteins could play in the regulation of the adenylate cyclase in *v-ras* transformed cells, we first examined the cAMP content in the presence and in the absence of TSH. The intracellular levels of cAMP were slightly affected in the two *v-ras-k* transformed cell lines, when compared to the pronounced effect observed in the FRTL-5 cells (see table 1). In fact a slight increase (about 2-fold) was present in the KiKi cell line stimulated by TSH, whereas no effect was detected in the KiMol cells. The basal intracellular cAMP content in FRTL-5 cells was similar to that found in the two transformed thyroid cell lines.

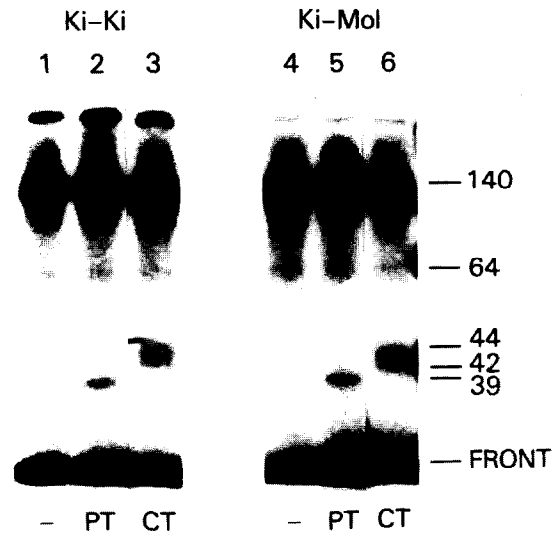

Fig.2. Autoradiographic analysis of ADP-ribosylated proteins in KiKi and KiMol cell membranes which were incubated with pertussis toxin (PT, 1×10^{-7} M) and cholera toxin (CT) in the assay mixture (see section 2). The molecular masses of the various bands are indicated.

Table 1 shows that cholera toxin and forskolin increase cAMP levels in normal and in the two transformed cell lines. However, the extent of this increase is higher in the normal cell when compared to the transformed ones. Unlike the normal cells pertussis toxin does not increase the cAMP level in the two transformed cell lines (table 1).

It has recently been shown that G_i and G_s can be demonstrated in FRTL-5 cells by their specific

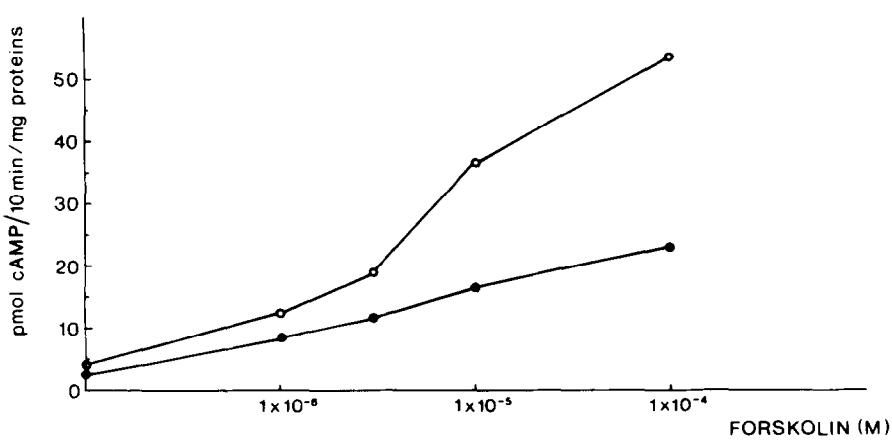

Fig.3. Adenylate cyclase activity in normal FRTL-5 (○) and transformed KiMol cells (●) after forskolin treatment. The data represent the mean of quadruplicate determination, in all cases the SD was <5% of the mean.

ADP-ribosylation produced by pertussis and cholera toxin, respectively [14]. We have performed similar experiments in the transformed cell lines and shown that the peptide identifiable as α_i (40 kDa) and α_s (42 kDa and 45 kDa) observed in FRTL-5 cells are also present in the KiKi and KiMol cells (fig.2).

It was found that the activity of adenylate cyclase varies with the rate or phase of cell growth [19]. We have therefore measured forskolin-stimulated adenylate cyclase activity in membranes isolated from normal and transformed cell lines grown exponentially. Fig.3 shows that forskolin stimulates the adenylate cyclase activity in normal, as well as in KiMol cell lines. The levels of stimulation are lower in the transformed cells. Ions such as magnesium and manganese which activate directly G-proteins, increase the cAMP level in normal and transformed cells (not shown). Membranes from normal and transformed cells were then studied for their ability to catalyze the formation of cAMP in the presence of different concentrations of the non-hydrolyzable analog of GTP, GPP(NH)P, which leads to a persistent 'activated state' of the enzyme. Fig.4 shows that the degree of adenylate cyclase stimulation is higher in normal cells with respect to the transformed ones. The GPP(NH)P stimulation of transformed rat thyroid cells in the presence of TSH (10^{-7} M) remain unchanged (not shown), suggesting a lack of interaction between the TSH receptor and the G_s protein.

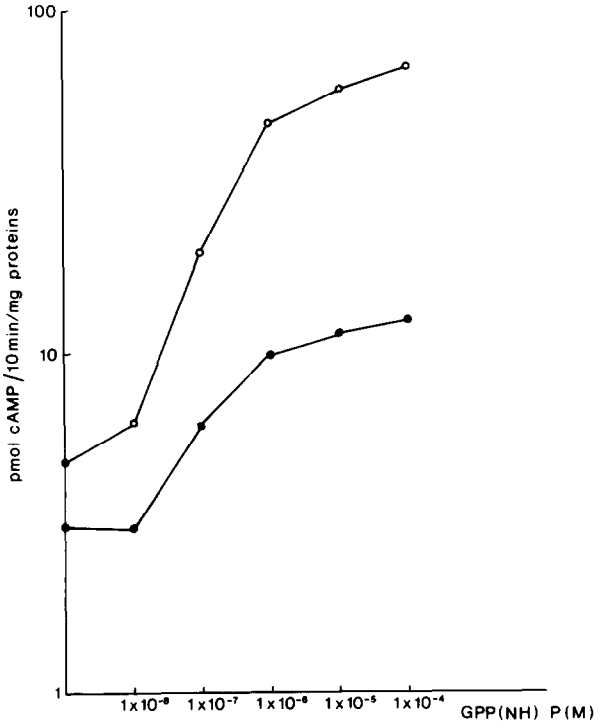

Fig.4. Adenylate cyclase activity in normal FRTL-5 (○) and transformed KiMol cells (●) after GPP(NH)P treatment. The data represent the mean of quadruplicate determinations, in all cases the SD was <5% of the mean.

4. DISCUSSION

It has been shown that TSH acts in FRTL-5 cells as a growth factor by inducing thymidine uptake and cell proliferation [5,13]. In the *v-ras-k* transformed cells TSH is neither able to induce cell proliferation nor thymidine uptake, and it is not able to stimulate the adenylate cyclase as demonstrated both in intact cells and in isolated membranes. A small effect of the hormone was demonstrated in the KiKi cell line (table 1) at a concentration 1000-fold higher than that effective in normal cells [3,14,20]. A possible alteration present at the TSH receptor level could be the cause of the insensitivity of these transformed cells to the hormone. Preliminary evidence, however, suggests that the TSH receptor is present in these *v-ras-k* transformed cells [21].

Forskolin and cholera toxin stimulate adenylate cyclase in the two transformed cell lines analyzed here; this stimulation however is smaller than that shown in normal cells. This is in agreement with previous studies on a different strain of rat thyroid cells [22] and on mammalian *ras*-transfected fibroblasts [23,24]. The decreased stimulation caused by cholera toxin (30-fold in control cells, 14-fold in the transformed ones) could be due either to a decreased amount of G_s present in the transformed cells and/or inactivated G_s or to an attenuated adenylate cyclase. From data obtained in the ADP-ribosylation experiments there is no apparent difference in the amount of G_s protein in normal and transformed cells.

Pertussis toxin did not affect adenylate cyclase activity in the transformed cell lines. The effect of the toxin is indeed small also in normal thyroid cells (2-fold increase). If, as shown for the other stimulators, we assume a smaller effect of the toxin in the transformed cells than in the normal cells, it

would have been difficult to detect such an effect under our experimental conditions. Another possibility is that G_i is not inhibiting the adenylate cyclase complex in the transformed cells, therefore the effect of the toxin at the G_i level is not translated into an increase in cAMP. The proof of this hypothesis would require however the stimulation of inhibitory receptors coupled to G_i, which have not yet been identified in these cells.

In summary, our results are consistent with previous studies suggesting a decreased stimulation of adenylate cyclase activity in cells transformed by a variety of viral oncogenes [23]. G-proteins are present in the two independently infected thyroid cells and the stimulation of cyclase by GTP analogs indicates a role of G-proteins in the regulation of the enzyme. Probably the differences in the response to the TSH hormone between *v-ras-k* transformed and normal cells may be due to an inactive receptor or to a lack of receptor-G-protein interaction. Forskolin which is able to increase cAMP levels also in transformed cells induces only a slight increase in the [^{3}H]thymidine uptake. This suggests that cAMP per se is not sufficient to stimulate growth in the transformed cells and that probably endocrine factors, that activate different signals and pathways, may be important in this regulation.

Acknowledgements: This work was supported by contract 84.009.15.51, from the Consiglio Nazionale delle Ricerche, Progetto Finalizzato CNR Ingegneria Genetica e Basi Molecolari delle Malattie Ereditarie, by contract 87.00032.04 from the Consiglio Nazionale delle Ricerche, and by FIDIA spa. We thank Giancarlo Vecchio for critical reading of the manuscript.

REFERENCES

[1] Ambesi-Impiombato, F.S., Parks, L.A.M. and Coons, H.G. (1980) Proc. Natl. Acad. Sci. USA 79, 6680–6685.
[2] Mandato, E., Catapano, R., Ambesi-Impiombato, F.S. and Macchia, V. (1981) Biochim. Biophys. Acta 676, 91–100.
[3] Valente, W.A., Vitti, P., Kohn, L., Brandi, M.L., Rotella, C., Toccafondi, R., Tramontano, D., Aloj, S.M. and Ambesi-Impiombato, F.S. (1983) Endocrinology 112, 71–79.
[4] Weiss, S.J., Philp, N.J., Ambesi-Impiombato, F.S. and Grollman, E.F. (1984) Endocrinology 144, 1099–1107.
[5] Khon, L.D., Aloj, S.M., Tombaccini, D., Rotella, C.M., Toccafondi, R., Marcocci, C., Corda, D. and Grollman, E.F. (1985) in: The Thyrotropin Receptor Biochemical Action of Hormones, vol.XII, chapt. 14, pp.457–511.
[6] Fusco, A., Pinto, A., Ambesi-Impiombato, F.S., Vecchio, G. and Tsuchida, N. (1981) Int. J. Cancer 28, 655–662.
[7] Fusco, A., Pinto, A., Tramontano, D., Tajana, G., Vecchio, G. and Tsuchida, N. (1982) Cancer Res. 42, 618–626.
[8] Colletta, G., Pinto, A., Di Fiore, P.P., Fusco, A., Ferrentino, M., Avvedimento, V.E., Tsuchida, N. and Vecchio, G. (1983) Mol. Cell. Biol. 3, 2099–2109.
[9] Ferrentino, M., Di Fiore, P.P., Fusco, A., Colletta, G., Pinto, A. and Vecchio, G. (1984) J. Gen. Virol. 65, 1955–1961.
[10] Scolnick, E.M., Papageorge, A.G. and Shih, T.Y. (1979) Proc. Natl. Acad. Sci. USA 76, 5355–5359.
[11] Hurley, J.B., Simon, M.I., Teplow, D.B., Robishaw, J.D. and Gilman, A.G. (1984) Science 226, 860–862.
[12] Gilman, A. (1984) Cell 36, 577–579.
[13] Colletta, G., Cirafici, A.M. and Vecchio, G. (1986) Science 233, 458–481.
[14] Corda, D., Sekura, R.D. and Kohn, L.D. (1987) Eur. J. Biochem. 166, 465–481.
[15] Solomon, Y., Londos, C. and Rodbell, M. (1974) Anal. Biochem. 58, 541–548.
[16] Markwell, M.A.K., Hass, S.M., Bieber, L.L. and Tolbert, N.E. (1974) Anal. Biochem. 87, 206–210.
[17] McKenzie, J.M. (1958) Endocrinology 63, 372–384.
[18] Seamon, K.B., Padgett, W. and Daly, J.W. (1981) Proc. Natl. Acad. Sci. USA 78, 3363–3367.
[19] Anderson, W.B., Russel, T.R., Carchman, R.A. and Pastan, I. (1973) Proc. Natl. Acad. Sci. USA 70, 3502–3505.
[20] Corda, D. and Khon, L.D. (1985) Proc. Natl. Acad. Sci. USA 82, 8677–8680.
[21] Colletta, G., Pinto, A., Schettini, G., Cirafici, A.M., Marinaccio, M. and Consiglio, E. (1985) in: Cell Membranes and Cancer (Galeotti, T. et al. eds) pp.101–106, Elsevier, Amsterdam, New York.
[22] Spina, A., Di Donato, A., Colella, G., Illiano, G., Berlingieri, M.T., Fusco, A. and Grieco, M. (1987) Biochem. Biophys. Res. Commun. 142, 527–535.
[23] Tarplay, W.G., Hopkins, N.K. and Gorman, R.R. (1986) Proc. Natl. Acad. Sci. USA 83, 3703–3707.
[24] Levitzki, A., Ruddick, J., Pastan, I., Vass, W.C. and Lowy, D.R. (1986) FEBS Lett. 197, 134–138.