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Viral *P21* Ki-RAS Protein: A Potent Intracellular Mitogen That Stimulates Adenylate Cyclase Activity in Early G_1 Phase of Cultured Rat Cells

Douglas J. Franks, James F. Whitfield, and Jon P. Durkin

Cell Physiology Group, Division of Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada K1A OR6 (D.J.F., J.F.W., J.P.D.), and Department of Pathology, Faculty of Health Sciences, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5 (D.J.F.)

Rat kidney (NRK) cells infected with a temperature-sensitive mutant of the Kirsten sarcoma virus were arrested in the G_0/G_1 phase of their cell cycle by incubation in serum-deficient medium at a *p21*-inactivating temperature of 41°C. These quiescent *ts* K-NRK cells were then stimulated to transit G_1 and initiate DNA replication by lowering the temperature to 36°C, which rapidly reactivated *p21*. Reactivating the viral Ki-RAS protein by temperature shift led to an increase in adenylate cyclase activity in early G_1 phase. The Ki-RAS protein increased the sensitivity of adenylate cyclase to guanyl nucleotides by a mechanism that seemed to involve inactivation of the enzyme's inhibitory G_I regulatory protein.

Key words: c-AMP, G-proteins, Ki-*ras* oncogene, proliferation, transformation

RAS genes may control the G_1 transit of the yeast *Saccharomyces cerevisiae* by increasing adenylate cyclase activity. Thus the yeast cell needs the product of at least one of its two RAS genes to transit G_1 [1,2]. Yeast cell proliferation is also dependent upon adenylate cyclase activity and cyclic AMP-dependent protein kinase [3–5]. These needs appear to be linked by the fact that both of the RAS gene products stimulate yeast adenylate cyclase, although the RAS2 (SC2) protein is by far the better stimulator [6]. Furthermore, the inhibition of G_1 transit in RAS^- mutants can be overcome by the *bcy*1 mutation that permanently raises cyclic AMP-dependent protein kinase activity by preventing the synthesis of the cyclic AMP-dependent protein kinase regulatory (R) subunit [6].

It would be reasonable to expect that the smaller, though still closely related, mammalian RAS proteins are also adenylate cyclase stimulators that may be responsible for the transient burst of adenylate cyclase activity that normal (but not all

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neoplastic) cells appear to require in order to complete G_1 transit and initiate DNA replication [7]. At first sight this possibility seems to have been ruled out by Beckner et al [8] and Broek et al [9] who showed that one mammalian *ras* gene product, the *p21* Ha-RAS protein, neither stimulates nor inhibits mammalian adenylate cyclase. However, extending these results obtained with the Ha-RAS protein to include the Ki-*ras* gene product is clearly premature because the two genes are structurally distinct [10], and their patterns of expression during the cell cycle are different [11]. Thus, the Ha-*ras* and Ki-*ras* genes may function differently. A relation between the Ki-RAS protein and mammalian adenylate cyclase is suggested by the facts that a burst of cellular Ki-*ras* gene transcription coincides with the burst of adenylate cyclase activity that occurs in both regenerating rat liver and BALB/c 3T3 cells as they near the G_1/S transition [7,11,12] and that an oncogenic viral Ki-*ras* protein does affect adenylate cyclase in BALB/c 3T3 cells [13].

Using a temperature-sensitive mutant of the Kirsten sarcoma virus we have shown recently that activation of the Ki-*ras* protein in quiescent, synchronous *ts* K-NRK cells leads to an early increase in adenylate cyclase activity [14]. Here we present further evidence suggesting that a stimulation of adenylate cyclase might be one of the early events in the G_1 transit of NRK cells that is triggered by the oncogenic viral Ki-RAS and that the mechanism of the adenylate cyclase stimulation may involve inactivation of the Gi regulatory protein (inhibitory G protein) of adenylate cyclase.

METHODS

Cell Lines

NRK cells and *ts* K-NRK cells were generous gifts from Dr. E.M. Scolnick (Merck, Sharpe and Dhome, West Point, PA). The *ts* K-NRK cells were derived by infecting normal NRK cells with a temperature-sensitive, transformation-defective mutant (ts 371) of Kirsten sarcoma virus that produces an abnormally thermolabile *p21* Ki-RAS protein [15].

Cell Culture

Cells were routinely cultured in a complete medium consisting of 85% Dulbecco's modification of Eagles medium (DMEM) containing gentamicin and 15% bovine serum (Colorado Serum Co., Denver, CO) and maintained at 36°C in a humidified atmosphere of 95% air and 5% CO_2. Before each experiment, cells were detached by a brief exposure to 0.25% trypsin in phosphate-buffered saline (PBS). They were then plated in 100-mm dishes at a density of 1.5×10^3 cells/cm^2 in 15 ml of complete medium and incubated at 40°C. After 48 hr the cells were arrested at G_0/G_1 by a further 48-hr incubation at 41°C in DMEM-F12 (1:1) medium containing 10 mM Hepes (pH 7.2) and 0.2% bovine serum [16].

Adenylate Cyclase Assay

Cells were washed twice with PBS and scraped off the dish in 2 ml of PBS using a rubber policeman. The cells were sedimented by centrifugation at 500 g for 3 min and washed once with 5 ml of PBS. After centrifugation, the cell pellet was frozen at −90°C. The thawed cell pellets were homogenized in 0.5 ml of a buffer consisting of 50 mM Tris (pH 7.4), 330 mM sucrose, 1 mM $MgCl_2$, and 1 mM dithiothreitol (DTT). Cells were homogenized in a motor-driven teflon/glass homog-

enizer (10 strokes, 10,000 rpm). The homogenizer was rinsed with a further 0.5 ml of homogenizing buffer, and the combined homogenate (1 ml) was centrifuged at 20,000g for 20 min. The supernatant fluid was discarded, and the pellet was dispersed in a small volume of homogenizing buffer using a small Dounce homogenizer. This dispersed pellet was the source of adenylate cyclase.

Adenylate cyclase activity was determined by measuring the conversion of α [^{32}P]-ATP to [^{32}P]-cyclic AMP as previously described [17]. The reaction mixture (0.1 ml) contained 50 mM Tris buffer (pH 7.4), 10 mM $MgCl_2$, 2 mM DTT, 2 mM cyclic AMP + 0.01 μCi [^{3}H]-cyclic AMP, 0.5 mM ATP-Mg + 0.5 μCi α [^{32}P]-ATP, 0.15% bovine serum albumin, an ATP regenerating system consisting of 5 mM phosphocreatine and 0.4 mg/ml phosphocreatine kinase, and 20–50 μg of enzyme protein in a total volume of 0.1 ml. The reaction was started by adding the enzyme preparation, and it was allowed to proceed for 10 min at 37°C. The reaction was stopped by adding 0.1 ml of 10 mM ATP and diluting the reaction mixture to 0.6 ml with water. Cyclic AMP was isolated by sequential Dowex and alumina chromatography as described previously [17]. The reaction was linear with time and protein concentration under the conditions used. All assays were performed in triplicate. Protein concentration was determined by the method of Bradford [18].

DNA Synthesis

DNA synthetic activity was assessed autoradiographically by exposing cells, which had been grown on 25-mm round plastic coverslips, to [^{3}H] thymidine (5 μCi/ml of medium: Sp Act. 20 Ci/mole: New England Nuclear, Boston, MA), immediately after lowering the incubation temperature to 36°C. The coverslips were processed as previously described [19], and the percentage of labeled nuclei was determined by examining at least 1,000 cells.

RESULTS

Incubation at 41°C inactivated the *p21* product of the Ki-ras gene in *ts* K-NRK cells and caused the cells to become proliferatively quiescent in a G_0/G_1 state in medium containing 0.2% serum instead of the normal 15% serum [16]. Reactivating *p21* by dropping the temperature to 36°C caused the cells to transit G_1 and initiate DNA replication, despite the serum deficiency (Fig. 1). Uninfected NRK cells were also arrested in a G_0/G_1 state by incubation in the serum-deficient media at 41°C, but they were not stimulated to transit G_1 by dropping the temperature to 36°C (data not shown).

The *p21*-activating 41°C to 36°C temperature shift also caused a GTP-dependent increase in adenylate cyclase activity in crude membrane preparations from *ts* K-NRK cells that became significant ($P < .001$) 5 hr after the *p21*-activating temperature shift (Fig. 1). By contrast, dropping the incubation temperature from 41°C to 36°C slightly (15%) decreased the adenylate cyclase activity in crude membrane preparations from uninfected NRK cells (data not shown). Moreover, concentrations of the nonhydrolysable GTP analogue GMPPNHP between 0.01 and 100 μM stimulated adenylate cyclase activity in crude membrane preparations obtained from *ts* K-NRK cells 5 hours after a 41°C to 36°C shift to a significantly greater extent than the enzyme in membranes from *ts* K-NRK cells held at 41°C (Fig.

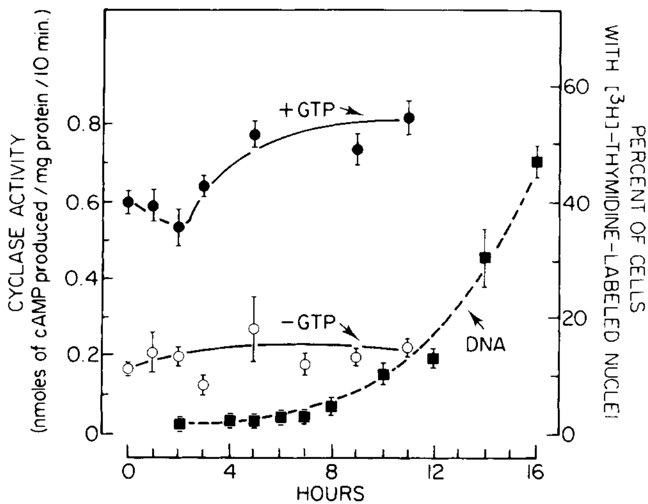

Fig. 1. Time course of adenylate cyclase activity and DNA synthesis in *ts* K-NRK cells. Adenylate cyclase was assayed in absence (open circles) and presence (solid circles) of 10 μM GTP. Cells were rendered quiescent by incubation in serum-deficient medium for 48 hr at 41 °C. Cells were harvested at the time points indicated, and adenylate cyclase activity was measured in crude membrane preparations as described in "Materials and Methods." The points are means $\pm$ SEM of at least 18 determinations. DNA synthesis was determined by autoradiography as described in "Materials and Methods."

2A). This increased sensitivity to GMPPNHP did not occur in normal NRK cells that had been shifted from 41 °C to 36°C for the same time period (Fig. 2B).

The viral *p21* protein might have stimulated the adenylate cyclase catalytic subunit. If this were the case, a potent catalytic subunit stimulator, such as forskolin [20], should override *p21* action. To test this, the stimulation of adenylate cyclase by 10 μM GMPPNHP and 10 μM forskolin was compared in membranes prepared from normal NRK cells and *ts* K-NRK cells held at 41 °C and 5 hr after shifting them to 36°C. The 41 °C to 36°C temperature shift did not alter the ability of either agent to stimulate the adenylate cyclase of normal NRK cells (Fig. 3). By contrast, the adenylate cyclase of *ts* K-NRK cells at 36°C was more sensitive to stimulation by both GMPPNHP and forskolin than was the enzyme of *ts* K-NRK cells that had been held at 41 °C (Fig. 3). In addition, forskolin did not override the effect of *p21* reactivation; hence, it is probable that the viral protein affected one of the adenylate cyclase's regulatory components.

Since adenylate cyclase activity is regulated by both stimulatory (Gs) and inhibitory (Gi) G proteins [21], *p21* could have increased the enzyme's activity either by activating the Gs protein or by inactivating the Gi protein. To determine which of the two regulatory proteins was affected by *p21*, we took advantage of the fact that the Gs and Gi proteins have different Mg^{2+} requirements [22]. The two activities cannot easily be separated in the presence of high Mg^{2+} concentrations (ie, the 10 mM Mg^{2+} used in the experiment and shown in Fig. 2). However, at lower Mg^{2+} levels (eg, 1 mM) that are suboptimal for Gs but optimal for Gi, it is possible to detect changes in Gi activity. Therefore, adenylate cyclase was assayed in the presence of 1 mM Mg^{2+} in membranes from quiescent *ts* K-NRK cells at 41 °C in serum-deficient medium and in the same cell 5 hours after a *p21*-activating shift to 36°C.

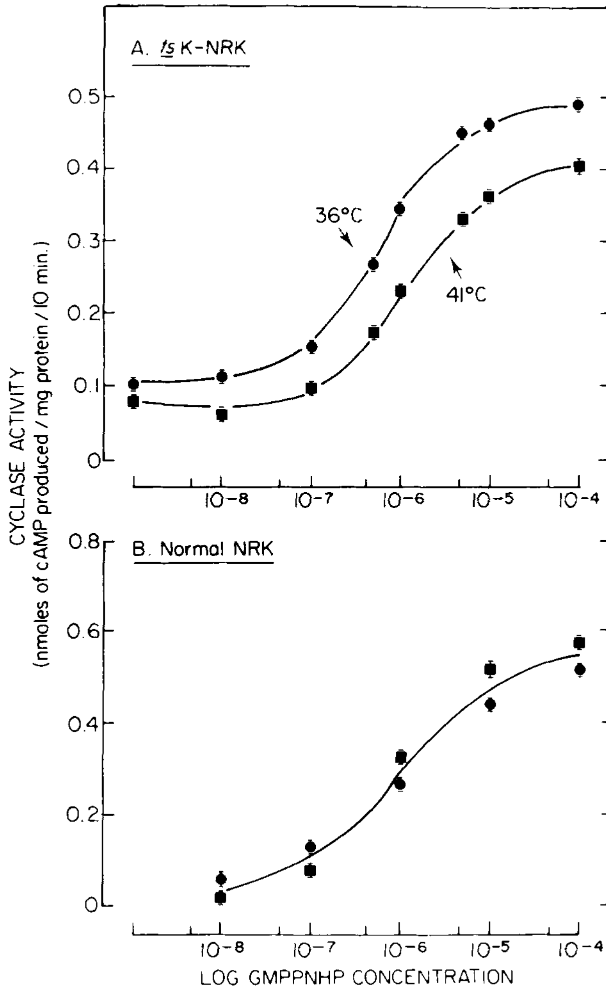

Fig. 2. Sensitivity to GMPPNHP of adenylate cyclase in membranes from *ts* K-NRK cells at 41°C and after 5 hours at 36°C. Adenylate cyclase activity was determined as described in "Materials and Methods." Values are means ± SEM of three determinations. Identical experiment, but using membranes from normal NRK cells that were shifted to 36° for the same time period.

The nonhydrolysable GTP analogue GTPγS was used as the enzyme stimulator at concentrations ranging from 0.001 to 50 μM. There was significant inhibition of the adenylate cyclase from *ts* K-NRK cells held at 41°C by GTPγS concentrations higher than 5 μM, but there was no inhibition of the enzyme from *ts* K-NRK cells that had been shifted to 36°C (Fig. 4). Hence, it would appear that reactivating *p21* somehow interferes with the inhibitory action of Gi on the adenylate cyclase.

DISCUSSION

Reactivation of the temperature-sensitive, oncogenic viral Ki-RAS protein in quiescent serum-deficient *ts* K-NRK cells caused these cells to transit G_1 and replicate

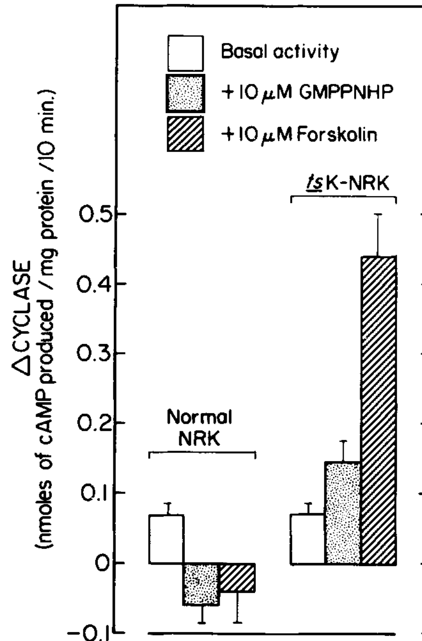

Fig. 3. Comparison of effect of 10 μM GMPPNHP and 10 μM forskolin on adenylate cyclase in normal NRK cells and *ts* K-NRK cells. Adenylate cyclase activity was determined as described in "Materials and Methods." Δ cyclase represents the *increase* in enzyme activity in membranes from cells 5 hr after a 41°C to 36°C temperature shift, relative to the activity in membranes from cells held at 41°C. Values are means ± SEM of three determinations.

DNA [16]. The reactivated RAS protein also stimulated adenylate cyclase activity. At first sight, it might seem that the present observations conflict with the finding that the mammalian Ha-RAS protein does not directly stimulate mammalian adenylate cyclase [8], although it does stimulate yeast adenylate cyclase [9]. However, the viral Ki-RAS protein does stimulate adenylate cyclase in *ts* K-NRK cells [14], but we do not know whether it does so directly or indirectly. The viral *p21* might have affected the action of the distantly related Gs or Gi proteins, or it may have mimicked the action of the Gs protein by directly stimulating the enzyme's catalytic component. A direct, Gs-like stimulation of the catalytic component is unlikely, since a powerful stimulator of the catalytic component, such as forskolin, was unable to override or mask the *p21*-induced activation. However, the fact that the cyclase's biphasic response to a nonhydrolysable guanyl nucleotide in the presence of a low Mg^{2+} concentration at 41°C was eliminated by reactivating *p21* at 36°C indicates that *p21*, either directly or indirectly, inactivated the Gi protein or dissociated it from adenylate cyclase.

The slowness of the stimulation of adenylate cyclase activity by *p21* reactivation suggests that its effect on the Gi protein is indirect. It appears that *p21* activation stimulates phosphatidylinositol degradation [23]. The fact that phosphatidylinositol degradation activates protein kinase C [24] suggests a possible mechanism of the *p21*-induced adenylate cyclase stimulation, since protein kinase C is known to increase adenylate cyclase activity in platelets [25] and pituitary cells [26] by inactivating the Gi protein.

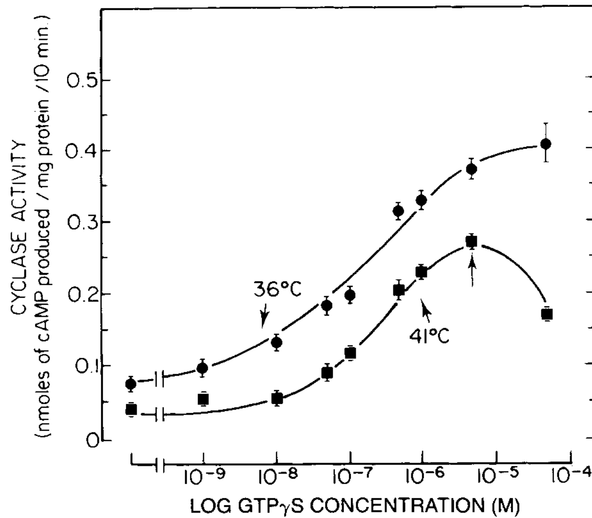

Fig. 4. Sensitivity to GTPγS of adenylate cyclase in membranes from *ts* K-NRK cells at 41°C and after 5 hours at 36°. Adenylate cyclase activity was determined as described in "Materials and Methods," but the enzyme was assayed at 1 mM Mg^{2+} instead of 10 mM Mg^{2+}. The arrow indicates the GTPγS concentration at which inhibition of adenylate cyclase occurs. Values are means ± SEM of three determinations.

Since the viral Ki-RAS protein does cause a burst of adenylate cyclase activity, similar to that which is required for G_1 transit of normal mammalian cells, and since a burst of cellular Ki-*ras* gene transcription coincides with the late G_1 burst of adenylate cyclase activity in regenerating rat liver cells and mouse 3T3 cells [11,12], it is tempting to speculate that it is the cellular Ki-RAS protein that causes this burst of adenylate cyclase activity. It is also tempting to speculate that the mitogenic, and ultimately the oncogenic, capability of the viral Ki-RAS protein is due at least in part to a stimulation of adenylate cyclase.

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REFERENCES

1. Kataoka T, Powers S, McGill C, Fasano O, Strathern J, Broach J, Wigler M: Cell 37:437–445, 1984.
2. Tatchell K, Chalell D, Defeo-Jones D, Scolnick E: Nature 309:523–527, 1984.
3. Matsumoto K, Uno I, Oshima Y, Ishikawa T: Proc Nat Acad Sci USA 79:2355–2359, 1982.
4. Matsumoto K, Uno I, Ishikawa T: Cell 32:417–423, 1983.
5. Uno I, Matsumoto K, Ishikawa T: J Biol Chem 257:14110–14115, 1982.
6. Toda T, Uno I, Ishikawa T, Powers S, Kataoka T, Broek D, Cameron S, Broach J, Matsumoto K, Wigler M: Cell 40:27–36, 1985.
7. Boynton AL, Whitfield JF: Adv Cyclic Nucleotide Res 15:193–294, 1983.

8. Beckner SK, Hattori S, Shih TY: Nature 317:71-72 1985.
9. Broek D, Samiy N, Fasano O, Fujiyama A, Tamanoi F, Northrup J, Wigler M: Cell 41:763-769, 1985.
10. Shih TY, and Weeks MO: Cancer Invest 2:109-123, 1984.
11. Goyette M, Petropoulos CJ, Shank PE, Fausto N: Mol Cell Biol 4:1493-1498, 1984.
12. Campisi J, Gray HE, Pardee AB, Dean M, Sonnenshein G: Cell 36:241-247, 1984.
13. Saltarelli D, Fischer S, Gacon G: Biochem Biophys Res Comm 127:318-325, 1985.
14. Franks DJ, Whitfield JF, Durkin JP: Biochem Biophys Res Comm 127:318-325, 1985.
15. Scolnick EM, Goldberg, RJ, Parks WP: Cold Spring Harbor Symp Quant Biol 39:885-895, 1974.
16. Durkin JP, Whitfield JF: Mol Cell Biol 6:1368-1392, 1986.
17. Franks DJ, Plamondon J, Hamet P: J Cell Physiol 119:41-45, 1984.
18. Bradford M: Anal Biochem 72:248-254, 1979.
19. Durkin JP, Whitfield JF: J Cell Physiol 120:135-145, 1984.
20. Seamon KB, Daly JW: J Cyclic Nucleotide Protein Phosphor Res 7:201-204, 1981.
21. Rodbell M: Nature 284:17-22, 1980.
22. Jakobs KH, Aktories K, Minuth M, Schultz G: Adv Cyclic Nucleotide Protein Phosphorylation Res 19:137-150, 1985.
23. Fleishman LF, Chahwala SB, Cantley L: Science 231:407-410, 1986.
24. Takai YK, Kikkawa U, Kaibuchi K, Nishizuka Y: Adv Cyclic Nucleotide Protein Phosphorylation Res 18:119-158, 1984.
25. Katada T, Gilman AG, Watanabe Y, Bauer S, Jacobs KH: Eur J Biochem 151:431-437, 1985.
26. Summers ST, Cronin MJ: Biochem Biophys Res Comm 135:276-281, 1986.