Adenylate cyclase activity of NIH 3T3 cells morphologically transformed by *ras* genes

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The observed homology between G-proteins which regulate adenylate cyclase and *ras* proteins and the suggested role of *ras* in the regulation of adenylate cyclase in yeast prompted us to examine the regulation of adenylate cyclase in three cell lines: (i) NIH 3T3 cells, (ii) NIH 3T3 cells transformed by high levels of the normal ras^H gene product and (iii) NIH 3T3 cells transformed by a mutated ras^H gene product. We found that the regulation of adenylate cyclase by G-proteins is identical in the three cell lines, although the response of the transformed NIH 3T3 cells to agonists is strongly attenuated. Our data suggest that mammalian *ras* products do not interact directly with adenylate cyclase, although their increased expression may indirectly inhibit the interaction of adenylate cyclase stimulatory receptors with G-proteins.

Adenylate cyclase *Transformation* ras *gene*

1. INTRODUCTION

Genes of the *ras* family are present in all eukaryotes, including humans and yeast [1–4]. Activated versions of these genes have been found in a wide variety of spontaneous human tumors, in tumors experimentally induced in animals by chemical carcinogens or ionizing radiation, and in acute transforming retroviruses. These activated genes can induce cellular transformation of mammalian cell lines in vitro, and retroviruses carrying these genes are highly oncogenic in vivo.

Previous studies indicated that cells transformed by the *ras*-containing Kirsten murine sarcoma virus have alterations in cAMP metabolism [5,6]. Recent studies indicate significant similarities between mammalian and yeast *ras* genes. *ras* genes from these species can function in the heterologous system [7,8]. In addition, mammalian and yeast *ras* proteins share the capacity to bind guanine nucleotides and to hydrolyze GTP [9,10]. Activation of these genes via certain point mutations in their protein coding sequences is associated with decreased GTPase activity [11–13].

The cellular targets of *ras* genes have not been defined. Significant sequence homology between *ras* proteins and regulatory G-proteins of the mammalian adenylate cyclase system has been noted [14,15]. Recent observations in yeast suggest that yeast *ras* can positively regulate adenylate cyclase activity in yeast [16,17]. To determine whether the mammalian *ras* genes have analogous effects on the mammalian adenylate cyclase complex, we have undertaken a series of experiments to study the effect of *ras* gene products on the cyclase system in mammalian cells. Our results strongly suggest that *ras* proteins do not directly affect adenylate cyclase activity in these cells.

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2. MATERIALS AND METHODS

The v-RAS cell line (clone 13-3-B4) has been described [18]. It was obtained by transforming NIH 3T3 cells with the H-1 clone of Harvey murine sarcoma virus (Ha-MuSV), which encodes a mutated *ras* protein. This plasmid contains the circularly permuted full-length Ha-MuSV DNA [19]. The c-RAS line was a mass culture of NIH 3T3 cells transformed by a plasmid in which the normal rat c-Ha-*ras*-1 gene with intervening sequences was placed under the transcriptional control of a retroviral long terminal repeat (LTR) by placing the *ras* gene downstream from the Ha-MuSV STR. This 13 kb plasmid was constructed by Dr B.M. Willumsen (University Microbiology Institute, Copenhagen, Denmark) by ligating 3 different DNA fragments: (i) the 3.8 kb *Eco*RI/*Bam*HI fragment of pBR322, (ii) the 3.8 kb *Eco*RI/*Sst*II fragment of clone H-1 (this fragment of H-1 contains the viral LTR but lacks all coding sequences of the viral v-*ras* gene), and (iii) the 5.4 kb *Eco*RI/*Bam*HI fragment of the normal genomic rat c-Ha-*ras*-1 gene (clone pHLB [20]). Prior to ligation, the *Sst*II end of the H-1 fragment was made flush and converted to an *Eco*RI end via addition of an oligonucleotide linker. Following ligation of the 3 fragments, a clone (pBW192) in which the LTR was upstream from and in the same transcriptional orientation as the *ras* gene was identified and selected. Clone pBW192 induces foci of transformed NIH 3T3 cells with about 1/10 the efficiency of the H-1 clone (approx. 5 vs 5×10 foci per μg DNA). The foci induced by pBW192 usually appear 2–3 days later than do those induced by H-1.

Cholera toxin and pertussis toxin were obtained from List (CA). All other chemicals were obtained from Sigma, except for forskolin which was obtained from Calbiochem, and R0-20-1724 which was obtained from Hoffman-LaRoche (NJ). The methods used for each biochemical assay are given in the appropriate figure legend.

3. RESULTS AND DISCUSSION

The *ras* genes can be activated to induce morphological transformation of mammalian cells by two mechanisms: elevated expression of a normal *ras* protein or amino acid substitution in the *ras* protein [3,4]. We studied various parameters of adenylate cyclase activity on 3 different cell lines: (i) NIH 3T3 cells (wild type), (ii) NIH 3T3 cells transformed by high levels of the normal rat cellular (c) ras^H gene (c-RAS cells), and (iii) NIH 3T3 cells transformed by Ha-MuSV, which expresses the mutant viral (v) ras^H gene (v-RAS cells). Compared with the protein encoded by the normal c-ras^H gene, v-ras^H encodes a protein that contains two activating amino acid substitutions.

Table 1

Effect of cholera toxin and of pertussis toxin on the adenylate cyclase

	% conversion of [^{3}H]ATP to [^{3}H]cAMP/h		
Toxin	3T3 (WT)	c-RAS	h-RAS
Cholera toxin	15.2 ±2.5	12.9 ±1.1	15.9 ±1.3
Pertussis toxin	1.52±0.03	1.63±0.22	1.3 ±0.17
Basal (IBMX)	0.31±0.07	0.36±0.08	0.32±0.10

Cells were planted 24 h prior to each experiment in 24-well Costar dishes at a density of 2×10^5 cells/well. They were grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal calf serum. Prior to labelling with [^{3}H]adenine (15–30 Ci/mmol, New England Nuclear) cells were washed twice with DMEM (2 ml/well) at 37°C and then incubated with 2 Ci/well [^{3}H]adenine for 2 h. Maximal incorporation of [^{3}H]-adenine into the ATP pool was achieved at 45 min (not shown). The wells were then washed 3 times with warm DMEM prior to the addition of the toxins. Then 2 μg/ml (final concentration) of either cholera toxin (Schwartz-Mann) or pertussis toxin (List) was added to the medium and the medium supplemented with 0.5 mM (final concentration) of the phosphodiesterase inhibitor R020-1724 in a final volume of 0.5 ml/well. At different time points 0.4 ml ice-cold 25% trichloroacetic acid was added immediately followed by 100 μl of a solution containing the following nucleotides (pH 7.4): adenine, adenosine, cAMP, AMP, ADP, and ATP at a final concentration of 0.5 mM. Then 3000–3500 cpm of [^{32}P]cAMP (ICN, CA) were added to each well and the sample analyzed for [^{3}H]cAMP according to Salomon [25]. The ^{32}P counts were used to calculate the extent of conversion of [^{3}H]ATP into [^{3}H]cAMP. The total incorporation of [^{3}H]adenine into the [^{3}H]ATP pool was determined in each experiment. Accumulation of [^{3}H]cAMP was linear up to over 2 h. Data shown are for a 1 h time point and are the average of 3 wells plus the standard error of the mean

The c-RAS cells contain about 10-times as much ras^H protein as do the v-RAS cells; both lines grow in agar, but the v-RAS cells form agar colonies faster than c-RAS (not shown).

These cell lines were used to study the possible interaction between *ras* proteins and the components of the hormonally regulated adenylate cyclase. The complex is composed of two classes of receptors: stimulatory (R_s) and inhibitory (R_i), which interact with the catalytic unit (C) through a stimulatory G-protein (G_s) [21] and an inhibitory protein (G_i) [22], respectively. We first examined the adenylate cyclase activity in response to agents which directly affect the G-protein system: cholera toxin, which activates C through ADP-ribosylation of the G_s [23] protein, and pertussis toxin, which activates C through ADP-ribosylation of the G_i protein [22,24]. Table 1 shows that the response of wild-type NIH3T3 and the two *ras* transformed cell lines to the bacterial toxins was almost identical. These results suggest that *ras* transformation does not directly affect the interactions between either G_i or G_s with the catalytic subunit of adenylate cyclase.

We then proceeded to examine the response of the adenylate cyclase in these cell lines to two stimulatory hormones: prostaglandin E_1 (PGE_1), and (−)-isoproterenol, which activate adenylate cyclase through prostaglandin and β-adrenergic receptors, respectively. In parallel we also analyzed the response of adenylate cyclase to the diterpene forskolin, which activates the catalytic unit of adenylate cyclase [26,27] but also synergizes with stimulatory hormones [28,29]. Examination of the response of the 3 cell lines to stimulatory hormones and forskolin revealed some differences. Fig.1 shows that wild-type 3T3 and c-RAS 3T3 responded well to PGE_1 whereas v-RAS 3T3 cells did not. The characteristic synergism between the stimulatory agent and low forskolin concentrations, which is clearly seen in wild-type 3T3 and c-RAS 3T3, is absent from v-RAS 3T3. The blunted hormonal response in v-RAS cells probably reflects a lack of PGE_1 receptors in these cells [30]. Diminished

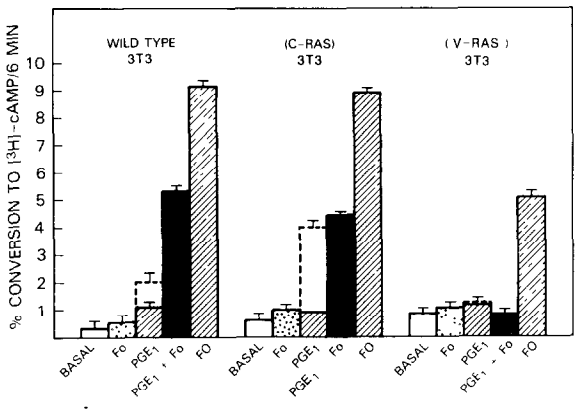

Fig.1. Response of 3T3 cells to PGE and forskolin. Assays were performed as described in table 1. Forskolin (Calbiochem) and PGE_1 (Sigma) when used were made as 0.1 M stock solutions in DMSO. Basal activity, R0-20-1724 (0.5 mM) only; Fo, 0.1 μM forskolin; PGE_1 (solid box), 1 μM, PGE_1 (dashed lined box), 100 μM; Fo + PGE_1, 0.1 μM forskolin + 1.0 μM PGE_1; F0, 100 μM forskolin. Note that *c-ras* 3T3 cells respond better to PGE_1 than wild-type 3T3 cells. This is due to the fact that these cells desensitize much more slowly to PGE_1 than do wild-type 3T3 cells (not shown); therefore, the longer the incubation times the higher the discrepancy in favor of C-RAS 3T3 cells. There is no clue as yet to the origin of this phenomenon.

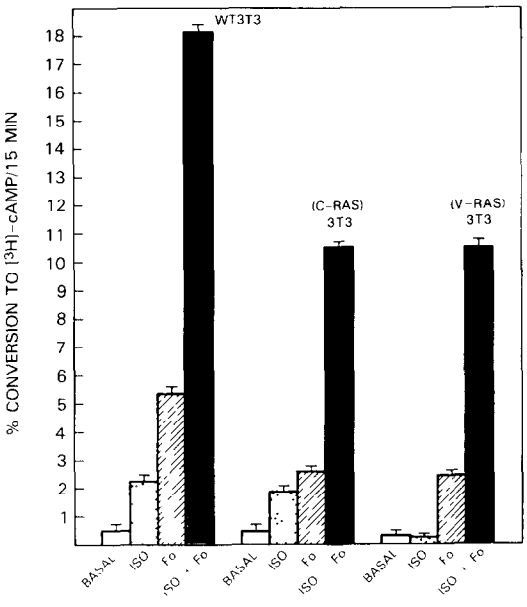

Fig.2. Response of 3T3 cells to (−)-isoproterenol and forskolin. Assays were conducted as described in table 1. Isoproterenol had a maximal effect on the intact cells at 1.0 μM and half-maximal effect at 30 ± 6 nM. Isoproterenol was used at 1.0 μM throughout. Fo, 0.1 μM forskolin; ISO + Fo, 1.0 M (−)-isoproterenol + 0.1 μM forskolin. Both wild-type 3T3 and C-RAS 3T3 cells undergo desensitization to isoproterenol.

response to PGE has also been observed in other types of transformation [30–32], suggesting that this impaired response is not specific for *ras* transformed cells.

Interestingly, the response of the v-RAS 3T3 cells to (−)-isoproterenol was somewhat different from their response to PGE_1. Although v-RAS 3T3 cells, unlike c-RAS 3T3 and wild-type 3T3, did not respond to (−)-isoproterenol, they did respond to isoproterenol plus forskolin (fig.2). This finding may indicate a deficient ability of G_s to couple to the β-adrenergic receptor in the v-RAS 3T3 cells whose response to this ligand has been restored by forskolin. We do not know what accounts for the lesion in the β-receptor to G_s coupling in v-RAS 3T3 cells. Examination of the total number of β-adrenergic receptors in the 3 cell types revealed that v-RAS 3T3 cells possess almost as many receptors as c-RAS cells, although both transformed cell lines displayed many fewer receptors than the untransformed NIH 3T3 cells (table 2).

Table 2

Maximal ^{125}I-cyanopindolol binding sites in NIH 3T3, c-RAS/NIH 3T3 and h-RAS/NIH 3T3

Cell type	Maximal number of receptors	
	37°C	22°C
Wild-type NIH 3T3	8100 ± 650	35000 ± 1500
c-RAS/NIH 3T3	1400 ± 110	7800 ± 600
h-RAS/NIH 3T3	1000 ± 80	2650 ± 200

Cells were planted in 24-well dishes 24 h prior to the binding experiment as described in table 1. The serum-containing medium was removed and the cells washed twice with DMEM medium. Then triplicate wells were incubated with 0.5 ml DMEM containing ^{125}I-cyanopindolol (Amersham, 1950 Ci/mmol) in the concentration range 10–300 pM in the absence and presence of 1.0 M l-propranolol (ICI, England). Incubation was performed at 22 or 37°C for 2 h. The medium was subsequently removed, the cells washed 3 times with 2.0 ml ice-cold Dulbecco's PBS with Ca^{2+} and Mg^{2+}, then 0.5 ml of 0.5 N NaOH containing 0.1% Triton X-100 was added to each well. The well was scraped and the total volume removed for counting in an LKB gamma counter. Non-specific binding (5–15%) was subtracted and the data analyzed according to Scatchard using a computer program as described [33]. Binding curves were normal (non-cooperative) and the dissociation constant for ^{125}I-cyanopindolol was found to be 33 ± 4 pM at 23°C and 30 ± 3 pM at 37°C

None of the 3 cell lines responded to α_2-adrenergic agonists or adenosine A1 agonists which induce adenylate cyclase inhibition (not shown). However, G_i is probably present in these cells, since treatment with islet-activating protein (IAP, pertussis toxin) induced a 4–5-fold activation of the basal adenylate cyclase activity in all 3 lines tested (table 1).

In summary, our results are consistent with previous studies suggesting a decreased stimulation of adenylate cyclase activity in cells transformed by a variety of genes [30–32]. Our analysis has examined this system in detail for cells transformed by a *ras* proto-oncogene as well as for cells transformed by a mutated version of this gene. In contrast to results obtained with the yeast adenylate cyclase system, *ras* proteins have not been found to be positive regulators of mammalian adenylate cyclase. Recent in vitro reconstitution experiments between mammalian adenylate cyclase and mammalian *ras* proteins [34], which were published after we obtained the in vivo results reported here, also suggest that there is no direct interaction between *ras* and mammalian adenylate cyclase.

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