Decreased adenylate cyclase responsiveness of transformed cells correlates with the presence of a viral transforming protein

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The adenylate cyclase responsiveness of transformed fibroblastic and epithelial cell lines to forskolin, fluoride, guanine nucleotides and cholera toxin was reduced compared to their parental counterparts. This phenomenon was observed in lines transformed by either RNA or DNA tumor viruses, and in the case of polyoma virus, coincided with the expression of middle T antigen. The data suggest that decreased responsiveness of adenylate cyclase to non-hormone activators is a general consequence of viral transformation and may be related to viral regulation of protein kinase activity.

Adenylate cyclase *Viral transformation* *Protein kinase*

1. INTRODUCTION

Numerous changes accompany transformation of various cell types with RNA tumor viruses, including alterations of membrane lipids [1], proteins [2], adhesive properties [3] and growth characteristics [4]. Morphological changes which accompany transformation of certain cell lines have been correlated with decreased intracellular cAMP levels: usually a consequence of decreased adenylate cyclase activity rather than increased phosphodiesterase activity (review [5]). Virtually all of the above studies were done with fibroblasts, transformed by RNA tumor viruses. Since current methodology permits correlation of cellular effects of transformation with specific viral gene products, it is possible to extend previous studies and to compare the effects of transformation with either RNA or DNA viruses on fibroblasts and differentiated epithelial cells.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Madin Darby canine kidney cells (MDCK) and a line of MDCK cells transformed with Harvey sarcoma virus (MDCK-HSV) were kindly provided by Dr Thomas Shih (NCI); normal rat kidney cells (NRK) and a line transformed with Kirsten sarcoma virus (NRK-KSV) or the Schmitt-Rupin strain of Rous sarcoma virus (NRK-RSV) were provided by Dr Ira Pastan (NCI); Chinese hamster lung cells (CHL) and a line of CHL transformed with simian virus 40 (CHL-SV 40) and a line of CHL transformed with simian virus 40 (CHL-SV 40) were from Dr George Martin (NIADDK); rat embryonic fibroblasts (RAT-1) as well as transformed lines were from Dr Yoshiki Ito (NIAID). Rat-1 lines transformed with specific polyoma virus gene products were as follows; PyT, wild-type polyoma virus, 1837, large T antigen, MT 2-6, middle T antigen, dl 23-14, wild-type polyoma virus lacking the specific substrate for phosphorylation. All lines were maintained in Dulbecco's modified minimal essential media with 5% fetal bovine serum (HYClone) in a humidified atmosphere in 5% CO_2.

2.2. *Membrane preparation*

Sucrose gradient purified membranes were prepared as in [6]. To prepare partially purified membranes cells were scraped, homogenized in hypotonic medium and debris removed by centrifugation at 500 × *g*. The supernatant was then

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centrifuged at 50000 × *g* for 20 min to pellet the partially purified membranes.

2.3. *Adenylate cyclase*

Adenylate cyclase activity was measured as in [7] with 20–30 μg purified membranes/100 μl in a final concentration of Tris-acetate (pH 7.5), 100 μM ATP, 10 mM $MgCl_2$, 100 μM cAMP, 5 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, 100 μM dithiothreitol, 10 μM GTP and 1 μCi [^{32}P]ATP, for 10 min at 30°C. Cyclic AMP was separated from ATP as in [7].

2.4. *ADP-ribosylation*

Purified membranes were incubated under adenylate cyclase conditions ([^{32}P]ATP was omitted) in the presence of 1 mM thymidine, 10 μM NAD^+ and 3 μCi [^{32}P]NAD^+ with 100 μg/ml activated cholera toxin [8] for 15 min at 30°C. ADP-ribosylated products were visualized by autoradiography of proteins separated as in [9].

3. RESULTS AND DISCUSSION

The hormone responsive adenylate cyclase system is composed of 3 major components [10]. The catalytic component (C) converts Mg^{2+}-ATP to cAMP. Its activity is regulated by guanine nucleotide regulatory proteins (N_s and N_i), which interact with stimulatory (R_s) or inhibitory (R_i) hormone receptors. Various compounds can activate adenylate cyclase by direct interaction with C or N_s. Cholera toxin catalyzes the ADP-ribosylation of N_s [11], resulting in the persistent activation of C. Gpp(NH)p, the non-hydrolyzable analogue of GTP, specifically binds to N_s, while fluoride, whose precise site of action is unknown, seems to require the presence of a functional N_s [12] to activate adenylate cyclase. Most adenylate cyclase systems also can be maximally activated by forskolin [13], which does not require functional N_s [14], although its site of action is not known [15].

Purified plasma membranes were prepared [6] 2–3 days after cells became confluent to eliminate density effects on adenylate cyclase activity. There was no detectable phosphodiesterase activity in such preparations. Membranes from MDCK-HSV cells exhibited activity in the presence of both Mg^{2+} and Mn^{2+} similar to parental cells, but the activation of adenylate cyclase by fluoride, Gpp(NH)p, cholera toxin and forskolin was reduced (fig.1). In contrast, with NRK-KSV and CHL-SV 40, basal as well as stimulated adenylate cyclase activity was reduced in the transformed lines (fig.1). The adenylate cyclase responsiveness of NRK-RSV was identical to that of the line transformed with KSV as compared to the parental line.

The decreased responsiveness of transformed cells to forskolin, usually the most potent activator of adenylate cyclase, was not the result of decreased potency of forskolin on transformed membranes. The K_{act} for forskolin activation of adenylate cyclase (5 μM) was similar in both parental and transformed MDCK and CHL membranes. Forskolin activation of all cell lines was maximal at 100 μM (not shown).

The catalytic (C) and regulatory (N) components of adenylate cyclase are thought to exist in the membrane as separate entities, which interact to stimulate ATP conversion to cAMP only after N is activated [16]. Cholera toxin covalently labels the M_r 45000 subunit of N_s with ADP-ribose [11], permitting quantitation of this component. However, the labeling pattern of MDCK-HSV and NRK-KSV was identical to that of their respective parental counterparts (not shown), suggesting that the quantity of N_s is not limiting for adenylate cyclase activation.

Given the current understanding of the adenylate cyclase system, the simplest explanations of the data would be that transformation:

(i) affects N_s domains relevant to its interaction with non-hormone activators;
(ii) affects N_i such that its activity is enhanced relative to N_s;
(iii) affects C such that its ability to be maximally stimulated is reduced; or
(iv) results in an as yet unidentified plasma membrane component which alters N_s or N_i regulation of C or C activity itself.

Studies are currently underway to explore these various possibilities.

To define specific viral gene products responsible for reduced adenylate cyclase activity, Rat-1 cells, a line of embryonic rat cells, and lines of Rat-1 cells transformed with plasmids containing single gene products of polyoma virus [17], were used. Polyoma virus, a DNA tumor virus, codes

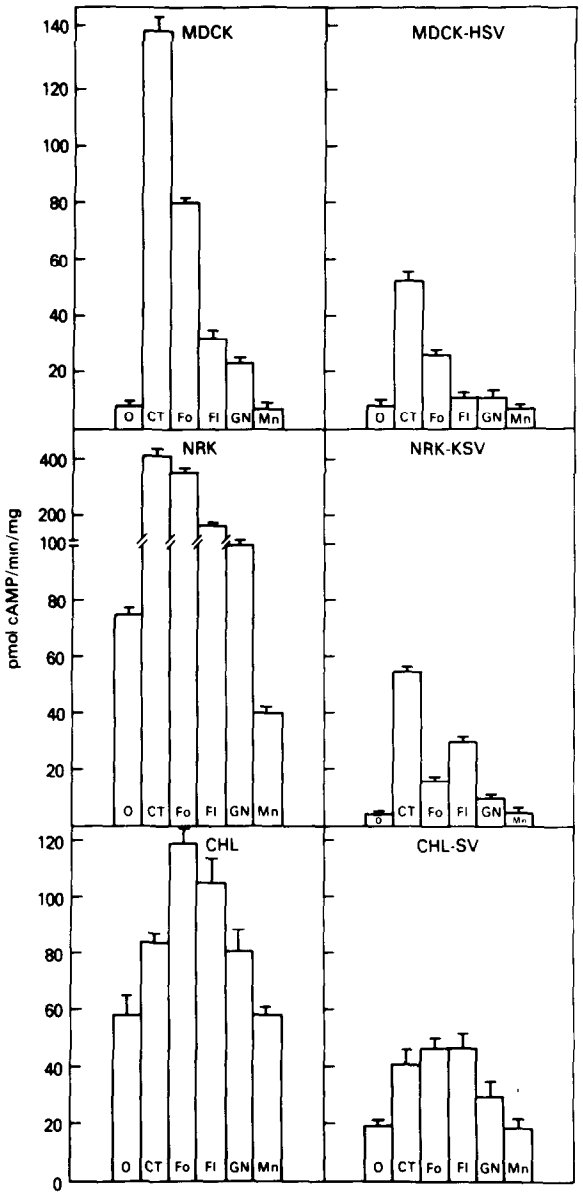

Fig. 1. Adenylate cyclase activity of normal and transformed MDCK, NRK and CHL cells. Adenylate cyclase activity of purified membranes was measured as described. The final concentrations of activators in the assay were all maximally stimulating: 2 mM Mn^{2+} replaced Mg^{2+}, 100 μg/ml cholera toxin (CT), 100 μM forskolin (Fo), 10 mM fluoride (Fl), 100 μM Gpp(NH)p (GN). Cholera toxin was activated as in [8], and 1 mM NAD^+ was present during the assay. There was no effect of NAD^+ on adenylate cyclase activity in the absence of cholera toxin.

for 3 well defined tumor antigens, designated as small, middle and large T antigens [17]. Middle T antigen, which seems to be localized on the plasma membrane [18], is required for the maintenance of the oncogenic state [19]. Large T antigen, a DNA binding protein, is required for viral DNA replication and is responsible for the expression of altered growth characteristics which accompany transformation [20], but alone is not a transforming protein. The function of the small T antigen is less well characterized [21]. Like MDCK cells, membranes of PyT cells, a line of Rat-1 cells transformed with wild-type polyoma virus, were significantly less responsive to stimulators of adenylate cyclase (fig. 2), although basal activity was similar to the parental line. Membranes of 1837 cells (only large T antigen) exhibited responsiveness very similar to the parental line but membranes from MT 2-6 cells (only middle T antigen), exhibited almost total lack of all adenylate cyclase activity. A specific tyrosine residue (Tyr 315) of middle T antigen is

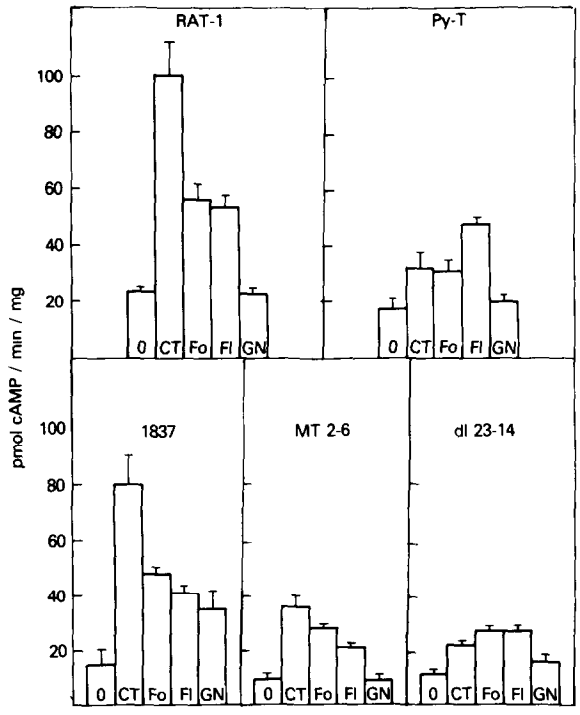

Fig. 2. Adenylate cyclase responsiveness of Rat-1 cells transformed with polyoma virus. Conditions were identical to those of fig.1.

phosphorylated in vitro [22]. The adenylate cyclase activity of a Rat-1 line (dl 23-14), transformed with virus lacking the nonapeptide around this tyrosine residue [23] was very similar to that of MT 2-6 (fig.2). While the transformation frequency of this deletion mutant is not significantly altered, decreased expression of the transformed phenotype is observed [24]. Therefore, although middle T is required for transformation, phosphorylation of middle T is not. It has also been demonstrated that phosphorylation of the transforming protein of RSV is not required for transformation [25]. The present studies (fig.2) suggest that like transformation, the effect on adenylate cyclase requires the viral transforming protein, but that phosphorylation of middle T is not relevant to either process.

It is surprizing that depressed adenylate cyclase activity is observed in epithelial cells (MDCK and CHL) or fibroblasts (NRK and Rat-1) transformed with RNA (HSV, RSV, KSV) or DNA (SV 40 and polyoma) tumor viruses. Cell lines transformed with KSV and HSV express the viral *ras* gene product (p 21) associated with the plasma membrane [26], a GTP binding protein capable of autophosphorylation [27]. Lines transformed with RSV express the viral *src* [28] gene product (pp 60) also associated with the plasma membrane [29], which exhibits tyrosine-specific protein kinase activity [30]. SV 40 transformation is accompanied by numerous changes in protein phosphorylation, several of which are also observed following transformation with RSV [31]. The nature of these substrates and their relationship to transformation are unknown. The middle T antigen of polyoma virus [32] is also associated with the plasma membrane [33]. A tyrosine kinase activity is detectable in middle T immunoprecipitates [22]. Such immunoprecipitates also contain proteins which correlate with the cellular homolog of *src* (pp60) [34] and epidermal growth factor receptor kinase [35], suggesting that middle T may associate with and regulate the activity of these kinases. While it is tempting to propose that viral-dependent phosphorylation regulates adenylate cyclase activity, this premise seems premature since the relationship between transformation and phosphorylation is not well defined at this point.

Although an explanation for the depressed adenylate cyclase activity following viral transformation is not readily apparent, it does seem significant that viral transformation with a variety of different viruses in a number of different cell lines has profound effects on the adenylate cyclase system. It is possible that these alterations in adenylate cyclase activity are related to the production of transforming factors by cells transformed with RNA and DNA viruses [36]. Whether these changes confer some advantage on the transformed cells is not known. However, further study of the consequences of virally regulated protein kinase activity on adenylate cyclase and other cellular regulatory systems may provide insight into critical factors involved in and contributing to malignant transformation.

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