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ras oncogene-induced transformation of a rat seminal vesicle epithelial cell line produces a marked increase of adenylate cyclase and protein kinase C activities

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Cells transformed by Kirsten murine sarcoma virus (Ki-MSV) have basal adenylate cyclase activity (AC) higher than control cells and comparable level of forskolin-stimulated AC activity. Moreover, a higher protein kinase C (PKC) activity was found to be present in the transformed cells. The molecular mechanism underlying the increase of AC activity was investigated. Our findings strongly suggest that this biochemical event is due to a marked decrease of the α_i negative control of the enzyme, even though the α_i of transformed cells appears to possess fully functional domains interacting with both the effector enzyme and the agonist-activated receptor.

ras oncogene, Cell transformation; G-protein; Adenylate cyclase; Protein kinase C

1. INTRODUCTION

It is known that in cell transformation induced by *ras* oncogenes there is a marked modification of gene expression leading to significant changes of various cell differentiation characteristics. Most importantly, it has been suggested that the changes observed in the plasma membrane signaling metabolic pathways of *ras*-transformed cells could play a crucial role in the definition of the transformed cell phenotype [1].

The optimal activity of the cAMP signaling pathway occurring in the plasma membrane of eukaryotic cells, depends on the coordinate expression of three major protein components (receptor, transducer, amplifier). The expression of these specific proteins can be altered by cell transformation. An analysis of the relationship between the normal functions of this pathway and the process of gene expression could be of interest in elucidating the role played by the plasma membrane signaling pathways in differention of eukaryotic cells.

We investigated this problem by studying the cAMP signaling pathway in the rat seminal vesicle epithelial cell line SVC1 and in the corresponding Ki-MSV transformed cells (Ki-SVC1). The SVC1 clone is an androgen-dependent cell line derived from the Wistar-Fisher rat seminal vesicle secretory epithelium [2]. These cells, having a duplication time of about 24 h, retain in vitro differentiation markers such as the ability to synthetize and secrete in the medium a number of proteins, some of which are antigenically related to the protein SV-IV specific for this tissue. The infection of SVC1 with Ki-MSV, containing the v-*ras*-Ki oncogene, caused marked morphological changes, acquisition of typical neoplastic characteristics (growth in semisolid medium and induction of tumors after subcutaneous injection of transformed cells into singeneic rats) and a 25% decrease in their duplication time [3]. Transglutaminase (EC 2.3.2.13) activity was reduced to a half after cell transformation [4]. Dot-blot mRNA/cDNA hybridization experiments and immunological analyses of the proteins secreted from the normal and transformed SVC1 cells, indicate that the presence in the cells of oncogene v-*ras*-Ki decreases (20%) the expression of the gene coding for the SV-IV related antigens (unpublished data). Testosterone (TST) induced a mitogenic stimulus on both SVC1 and Ki-SVC1 cells. The Ki-SVC1 were less sensitive than SVC1 cells to mitogenic TST stimulus: 10-fold higher hormone concentrations were required to achieve a comparable proliferative response. The expression of specific TST receptors increased about three times in transformed cells (unpublished data). Furthermore, by using immunological techniques and the Fluorescence Activated Cell Sorter (FACS) it has been shown that in Ki-MSV infected cells the expression of genes coding for class I MHC antigens increases about 10 times [3].

In the present paper we report data showing that the *ras*-induced transformation of SVC1 cells produces a marked increase of both AC and PKC activities. The molecular mechanism underlying the increase in the activity of AC has been investigated and the results were discussed.

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2. MATERIALS AND METHODS

2.1. *Materials*

The following materials were obtained from the indicated sources: [α-^{32}P]ATP (29.6 TBq/nmol) and [α-^{32}P]NAD (29.6 TBq/mmol) from Du Pont-New England Nuclear; pertussis toxin (PT) from Sigma Chemical Co.

The sources of all other materials are described where required.

2.2. *Cell Culture*

SVC1 and Ki-SVC1 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomicin and 10 U/ml mycostatin. The cells were mantained at 37°C in an humidified atmosphere of 5% CO_2/95% air. The medium was changed every two days [2–4].

2.3. *Membrane preparation*

The monolayer confluent cells were scraped, washed three times in PBS and the final pellet suspended in a sucrose saline buffer (0.33 M sucrose; 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 2 mM PMSF, 5 μg/ml leupeptin) at a final concentration of 4–6×10^{6}cells/ml. Three ml aliquots of cell suspension were homogenized with a loose-fitting glass–glass Dounce homogenizer (30 strokes). The membrane pellet, obtained by centrifugation of the homogenate at 27,000 × *g* for 20 min at 4°C, was resuspended in the specific buffer required in the different types of assay.

2.4. *Cell treatment with TPA*

Confluent cell cultures were incubated for 10 min in the presence of their culture medium supplemented with a final concentration of 1 μM 12-*O*-tetradecanoylphorbol 13-acetate (TPA) in 0.1% DMSO. Controls were obtained incubating the cells in the same culture medium supplemented with the corresponding volume of 0.1% DMSO used as TPA solvent.

2.5. α_i *ADP-ribosylation in vivo*

This assay was carried out according to Kopf and Woolkalis [5] by using confluent monolayer cells and 10 ng PT per ml of culture medium. After 18 h, the cells were used for membrane preparation to evaluate their AC and protein kinase activities.

2.6. *Adenylate cyclase assay*

The AC assay was performed according to a published procedure [6]. The reaction mixture contained, in a final volume of 100 μl, 50–100 μg of membrane proteins as enzyme source. Forskolin, NaF and mastoparan were added to the incubation mixture as AC modulators at the final concentration reported in Figures or Tables. The AC-specific activity was expressed as [^{32}P]cAMP formed per mg of membrane proteins. The c-AMP was recovered from reaction mixtures by chromatographic procedures [7].

2.7. *Protein kinase activities*

The incubation mixture used for evaluation of PK activity contained in a final volume of 250 μl: 25–50 μg of enzymic source, 20 mM Tris-HCl, pH 7.5, 0.75 mM $CaCl_2$, 10 mM magnesium acetate, 0.1 mM ATP, 0.2 mg/ml histone H1(Boehringer), 0.05 mg/ml leupeptin, [γ-^{32}P]ATP (500 Ci/mmol, NEN-Du Pont). The PKC activity was triggered by adding to the reaction mixture 1.6 μg of 1,2-diolein and 24 μg phosphatidyl-serine in 20 mM Tris-HCl pH 7.5. The reaction was carried out for 3 min at 30°C and then terminated by addition of 1ml of cold 25% TCA. Samples were filtered through Millipore filters (0.45 μm) and the radioactivity of dry filters was measured by a liquid scintillation counter [8]. The PK or PKC specific activity was expressed as nmoles of ^{32}P-phosphate incorporated/mg protein/min. The enzymic sources used were proteins of the cytosolic fraction (obtained as supernatant of 27,000 × *g*, 20 min centrifugation of cell homogenates) and membrane proteins partially purified by DEAE cellulose chromatography [9].

2.8. *Immunodetection of αG-proteins*

Proteins from membrane preparations were separated by SDS-PAGE on 10% polyacrylamide gels [10], and transferred onto nitrocellulose sheets (Schleicher & Schuell) with a Trans-Blot apparatus (Bio-Rad). The anti-α,G-proteins antibodies were purchased from NEN-Du Pont. Goat rabbit anti-IG antibodies, conjugated with alkaline phosphatase (BioRad) were used as detection system according to the manufacturer instructions.

2.9. *Protein concentration measure*

The protein concentration was determined according to Bradford [11].

3. RESULTS AND DISCUSSION

3.1. *Basal and F$^-$, but not forskolin-stimulated, AC activity is markedly increased in Ki-MSV-transformed cells*

The results reported in Table I show that *ras*-virus transformed cells have a markedly higher basal AC activity compared with the control cells (four times increase). The increase is also present when the enzymic activity is stimulated by F$^-$. In contrast, when AC is directly stimulated by forskolin, both control and *ras*-virus transformed cells show a comparable level of enzymic activity.

3.2. *The level of G-protein α_i component is significantly lower in Ki MSV-transformed cells*

It is well established that adenylate cyclase enzyme is under control of two families of GTP-binding proteins (G_s and G_i proteins). These proteins connect the catalytic protein with receptors activated by stimulatory or inhibitory external signals. Both G-proteins are heterotrimers (α + β + γ), differing for the α component subunit (α_s or α_i) which binds GTP and swings, because of its intrinsic GTPase activity, between an active GTP-bound and an inactive GDP-bound structure [12]. In basal conditions, i.e. in the absence of known external signals, a continous inhibitory modulation is operated mainly by α_i. The finding that no difference was observed between the AC activity of SVC1 and Ki-SVC1 cells when the enzymic activity was directly stimulated with forskolin [13] bypassing the G-protein modulation, led us to suppose that an impaired α_i control could be

Table I

Adenylate cyclase activity SVC1 and Ki-SVC1 epithelial cells[a]

Addition	AC (pmol cAMP/mg protein/min)	
	SVC1	Ki-SVC1
None	23 ± 0.11	79 ± 1.5
Forskolin (10^{-5} M)	421 ± 19	400 ± 13
NaF (10^{-2} M)	137 ± 7.9	206 ± 9

[a] For experimental details, see Section 2. Each value represents the mean ± S.D. of at least three independent determinations performed in triplicate.

at the basis of the increased AC activity in the transformed cells. To verify this hypothesis, we measured by immunologic methods the levels of α_s/α_i proteins in control and transformed cells. The results shown in Fig. 1 indicate that the α_i amount is substantially lower in transformed than in control cells. By considering the continous inhibitory role of α_i on the AC enzyme, this finding strongly supports the above-mentioned hypothesis.

3.3. *Pertussis toxin-mediated α_i ADP-ribosylation is more effective in Ki-MSV-transformed cells*

To further investigate the role of α_i in the molecular mechanism underlying the increase of AC activity in transformed cells, the control and *ras*-virus transformed cells were treated in vivo with PT in order to obtain α_i-ADP-ribosylation and hence, its functional exclusion from AC modulation [14]. The results reported in Fig. 2 indicate that PT is much more effective in increasing AC activity in Ki-SVC1 than in SVC1 cells. Several mechanisms might be at the basis of this result: (i) the lower α_i amount in transformed cells could allow the PT to perform a maximal ADP-ribosylation; (ii) the toxin could find in transformed cells more favorable conditions for its activity [15]; (iii) both mechanisms could operate at same time.

3.4. *The 'cross talk' between PKC and AC is more intense in Ki-MSV transformed SV cells*

The AC signaling pathway has been shown to be connected and modulated by enzymic activities operating on other distinct signaling pathways [16]. As far as the Ca^{2+}, diacylglycerol, phosphatydylserine-dependent

Table II

Cytosolic and membrane-bound protein kinase activities in SVC1 and Ki-SVC1[a]

Enzymic source	nmol ^{32}P incorporated/mg protein/min		
	Addition		Calculated PKC (B–A)
	None (A)	Ca^{2+} + PL[b] (B)	
Crude cytosol			
SVC1	1.05 ± 0.02	1.19 ± 0.03	0.14
Ki-SVC1	1.86 ± 0.02	3.75 ± 0.04	1.89
M 100[c]			
SVC1	6.90 ± 0.17	8.69 ± 0.23	1.79
Ki-SVC1	0.39 ± 0.01	0.59 ± 0.03	0.2
M 350[c]			
SVC1	3.99 ± 0.10	2.50 ± 0.08	–
Ki-SVC1	1.75 ± 0.03	1.73 ± 0.05	–

[a] For experimental details, see Section 2. Each value represents the mean ± S.D. of at least three independent determinations performed in triplicate

[b] PL means: diacyl-glycerol + phosphatidyl-serine

[c] M 100 and M350 refer to solubilized membrane proteins, eluted from DE52 column by 100 mM and 350 mM NaCl, respectively.

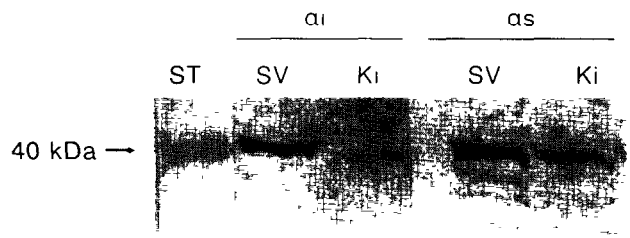

Fig. 1. Immunological detection of α_s and α_i in the protein extract of SVC1 or Ki-SVC1 cell membranes. Western blot of SVC1 or Ki-SVC1 membrane proteins. Fifty μg of crude cell membranes were fractionated on 10% SDS gels, transferred to nitrocellulose and probed with antisera raised against α_s or α_i. For experimental details, see Section 2.

protein kinases (PKC), these enzymes phosphorylate and functionally modulate either one of the three molecular components (receptor, α-G-protein subunit and cyclase enzyme) of the AC signaling pathway, depending on the investigated system [17–19]. We have found that in another epithelial cell line derived from rat thyroid the *ras* virus transformation produced increased AC and PKC activities together with an increased level of intracellular cAMP [6,8]. On the basis of these findings and considerations we have investigated the protein kinase activities in control and transformed cells.

The data reported in Table II show that the protein kinase activity distribution between cytoplasm and membrane in control cells is different from that occurring in Ki-MSV transformed cells. In the latter the PKC activities are higher in cytoplasm than in membranes. It has been shown that PKC phosphorylates the α subunit of the Gi proteins [16] and that the resulting covalent modification produces α_i functional inhibition and an enhanced AC activity. By considering the different PKC cellular distribution in control and *ras*-virus transformed cells, it seemed of interest to investigate whether an in vivo PKC activation by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) could have differentially affected the AC in the two cellular types. TPA is a well known tumor promoting agent, which acts on PKC activation and translocation from cytosol to membrane because of a structural similarity with the physiological activator diacyl-glycerol [20].

The results reported in the Table III indicate that that 1 μM TPA, is able to markedly increase, already after 10 min, the membrane-bound AC activity in virus transformed cells, while it does not have any effect on the AC of control cells. The results suggest that TPA in transformed cells can activate and translocate to plasma membrane an amount of PKC sufficient to phosphorylate and functionally exclude α_i so that the AC activity was enhanced.

The experimental results, so far described, converge on indicating that the AC basal activity is stronger in transformed than in control cells, because of a decreased α_i inhibitory modulation on AC activity. The

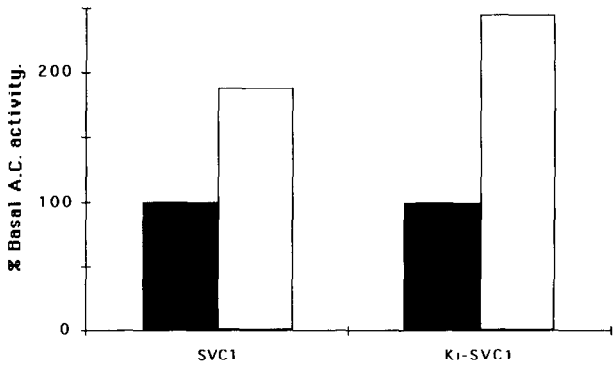

Fig. 2. The effect of PT treatment on AC activity of SVC1 or Ki-MSV-transformed (Ki-SVC1) epithelial cells. Black bars refer to untreated cells; white bars refer to PT-treated cells. Specific AC activity is respectively 23.3 ± 0.9 and 79.4 ± 2.1 in SVC1 and Ki-SVC1. For experimental details, see Section 2.

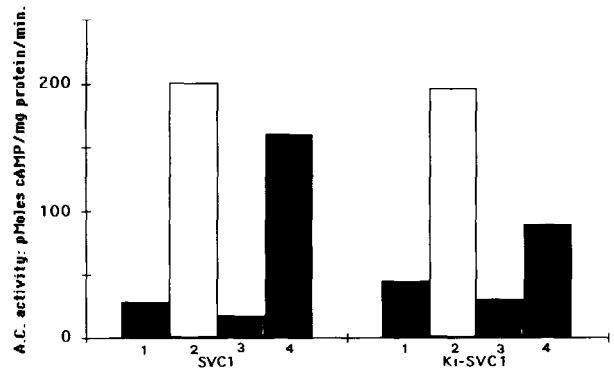

Fig. 3. The mastoparan effect on the basal or forskolin-stimulated AC activity of SVC1 and Ki-SVC1 cell membranes. (1) basal; (2) + forskolin; (3) + mastoparan; (4) + forskolin and mastoparan. For other experimental details, see Section 2.

decreased modulation, in turn, could be due to a diminished concentration of α_i associated with its higher susceptibility to covalent modification by either PT or PKC. This consideration suggests that in transformed cells the increase in the AC activity could result from a tighter connection with a PKC-mediated signal transducing pathway.

3.5. *The α_i interaction with the agonist-activated receptors and AC is equally effective in both control and transformed cells*

The above reported results do not exclude that α_i of transformed cell might be mutated either in the domain interacting with agonist-activated receptors or in the domain interacting with AC, or in both of them. We have functionally approached the problem by studying the AC activity of control and transformed cell membranes in presence or in absence of 0.3 μM mastoparan. Mastoparan is a fourteen residues amphiphylic peptide present in wasp venoms, which inserts it self in the membrane phospholipid bilayer and activates α_i by mimicking the domain of the agonist-activated receptor interacting with the G_i-protein [21]. The results shown in Fig. 3 indicate that mastoparan is effective in decreasing AC activity in transformed cell membranes and suggest that the α_i present in these membranes possesses domains interacting with agonist-activated receptors and AC as efficiently as in controls.

Table III

The effects of TPA on AC activity in SVC1 or Ki-SVC1 celsl[a]

Cells	AC activity (pmol cAMP/mg protein/min)	
	− TPA (vehicle)[b]	+ TPA
SVC1	69.1 ± 5.5	65.3 ± 10.7
Ki-SVC1	151.8 ± 6.3	273.4 ± 12.8

[a] For experimental details, see Section 2. Each value represents the mean ± S.D. of at least three independent determinations performed in triplicate.

[b] 0.1% dimethyl sulfoxide (DMSO) solution is used to dissolve TPA and it is able to increase, by itself, the AC activity both in control and in transformed cells. We have previously shown that the DMSO effect on AC is aspecific because of its attitute to interact with membrane lipids and its ability to decrease the membrane microviscosity.

4. CONCLUDING REMARKS

We have shown that Ki-*ras*-induced transformation is accompanied with an increase in the activity of both AC and PKC-associated signaling pathways. From the data obtained it appears that the molecular mechanism underlying this biochemical event could be a decrease of α_i concentration combined with a tighter PKC-mediated control of α_i. Activation of $p21^{ras}$, as measured by the transition from its inactive GDP-bound state to the active GTP-bound conformation, is considered a final and essential step in driving quiescent cells in to DNA replication process. Growing evidence suggests that distinct mitogen signaling pathways triggered either by ligands of receptor tyrosine kinases, such as insulin or epidermal growth factor (EGF), or by ligands not directly activating tyrosine kinases, such as thrombin and LPA (lipid lysophosphatidic acid), converge to a $p21^{ras}$ activation process as to a metabolic cross-point leading to DNA replication [22]. It is generally accepted that one of the first events in the process of *ras*-induced transformation is represented by an intracellular increase of the $p21^{ras}$ concentration. The component of this protein family expressed by the viral genome possesses a deficient intrinsic GTPase activity as a consequence of a point mutation of its gene and remains, therefore, for a longer time in the active GTP-bound state. The role played by activated $p21^{ras}$ in the triggering and regulation of DNA replication is well estab-

lished, although still unknown in its molecular details. Therefore, significant changes in the biochemical phenotype of transformed cells are to be expected. The challenge is to identify in the transformed cells the main metabolic steps leading from an altered membrane signaling pathway, such as the c-AMP pathway, to various changes in the normal program of gene expression and regulation of DNA replication.

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