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# Ras antagonizes cAMP stimulated glucagon gene transcription in pancreatic islet cell lines

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**Abstract** Ras, a GTP-binding protein, converts membrane tyrosine kinase signalling to changes in gene expression patterns. Utilising a rat glucagon promoter-CAT construct (p[−1.1]GLU-CAT) we demonstrate in transient transfection experiments that the oncogenic Ras inhibits cAMP-dependent activation of p[−1.1]GLU-CAT in both glucagonoma InR1-G9 and insulinoma β-TC1 cells. Conversely, the expression of a dominant negative mutant of Ras enhances the cAMP-induced activation of p[−1.1]GLU-CAT transcription in these cells. Our data suggests a functional interference of Ras with the cAMP-dependent transcription of the glucagon gene.

*Key words:* Ras; Glucagon gene; Gene transcription; Insulinoma; Glucagonoma; Protein kinase A

## 1. Introduction

Ras proteins belong to a large family of low molecular weight guanine nucleotides binding proteins which exert diverse functions in various systems as, for example, growth promotion of NIH-3T3 cells, maturation of *Xenopus laevis* oocytes, and inhibition of coupling between receptors and ion channels in neurones. A universal function for Ras has been established in controlling cell growth and differentiation (for reviews see [1–5]).

Ultimately, to promote these actions, Ras regulates the transcription of selected genes by altering the activity of specific nuclear transcription factors [6,7]. The linkages between extracellular stimuli, Ras, and the nucleus have been recently addressed. The intermediary role of a Grb2–mSos complex in tyrosine kinase receptor-triggered activation of Ras was described [8,9]. Furthermore, it was shown that Ras activation targets cRaf kinase to the plasma membrane which, in turn, switches on a series of kinases including MAP kinase and MAP kinases (MAPKs) [10–12]. MAPKs translocate to the nucleus upon activation; many transcription factors contain potential MAPK phosphorylation sites. Furthermore, MAPK clearly plays a role in the activation of some transcription factors [13].

The relationships between Ras and cAMP-dependent signal transduction pathways are complex and not fully elucidated. In yeast, Ras regulates adenylate cyclase [3,14]. The situation is not as clear in mammalian cells. Several data, although sometimes discordant, indicate that Ras interacts with the cAMP-regulated pathway of gene transcription. Kedar et al. [15] and Galien et al. [16] described that Ras stimulates gene transcription through the cAMP Responsive Element (CRE). Sassone Corsi et al. [17] and Fukumoto et al. [18] reported exactly the contrary. There is data in pituitary and thyroid cell lines showing that Ras and PKA signalling pathways are mutually antagonistic with respect to cell-specific transcriptional regulation of endocrine genes [19–21]. On the other hand, Kupperman et al. [22] reported that in thyroid cells, Ras and PKA cooperate in the regulation of gene transcription. Moreover, conflicting data have also been published on the relationships between Ras and PKC signalling pathways in regulating gene trancription. While Ras and PKC seem to cooperate in thyroid cells [20] they behave functionally antagonistic in $GH_4$ neuroendocrine cells [23].

Aim of our work was to elucidate the role of Ras in protein kinase A (PKA)- and protein kinase C (PKC)-stimulated glucagon gene transcription. The glucagon gene regulation has been recently focused on because of its significance in the hormonal control of fuel metabolism and particularly glucose homeostasis. In rodent islet cell lines, phorbol esters and analogs of diacylglycerol enhance glucagon biosynthesis [24] but a consensus DNA control element mediating phorbol ester effect on gene trancsription has not yet been identified in the glucagon gene 5′-flanking sequence. In contrast a perfect CRE was identified within 300 bp of the transcriptional start site of the rat glucagon gene [25]. To analyse in detail some of the properties of the glucagon gene transcriptional regulation with the specific aim of learning more about the role of Ras, PKA, and PKC, we expressed either activated V-12 Ras or N-17 Ras dominant negative mutant in insulinoma and glucagonoma cells utilising a glucagon promoter-CAT construct as reporter system, which is specifically inducible in these cells by PKA and PKC activators (R.G., H.-C.F., A. Volz and B.G., unpublished results).

## 2. Experimental

### 2.1. Materials, plasmids, and cells

Forskolin (FK) was from Sigma, phorbol-myristate, 12 acetate (PMA) was from Fluka. Restriction endonucleases and DNA modification enzymes were from Boehringer. p[−1.1]GLU-CAT (containing the reporter gene CAT directed by ~1.1 kb of the glucagon gene 5′-flanking region) was previously described [26] and obtained from Dr. J. Habener (Boston, MA), pRSV-CAT (in which CAT reporter gene transcription is directed by the Rous sarcoma virus LTR) was obtained from Dr. M. Beato (Marburg, Germany) [27]. c-Ha-*ras* and V-12-*ras* cDNAs were kindly provided from Dr. E. Santos (Bethesda, MD) and Dr. H. Land (London, UK), respectively. The expression vector pMAM-neo was

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obtained from Clontech. To construct the pMAM-V-12-*ras* expression vector, the V-12-ras cDNA was cloned into the multicloning site of pMAM-neo. To obtain the dominant negative N-17 c-Ha-*ras* mutant, the cDNA of c-Ha-*ras* was mutagenized by a PCR-mediated strategy [28]. A point mutation (G→A) in the second position of codon seventeen was introduced and checked by sequencing [28]. The mutated cDNA was introduced into the multicloning site of pMAM-neo vector (obtaining the pMAM-N17-*ras* construct). Consequently to the mutation, an Asp residue (instead of a Ser residue) was present in the Ras protein at position 17. Cos7, SV40-transformed African green monkey kidney, FL, human ovary carcinoma, NCI H-64, human small-cell lung carcinoma, A431, human vulval epidermoid carcinoma, and HIT-15, rat insulinoma cell lines were grown in DMEM supplemented with 10% Fetal Calf Serum (FCS) and 2 mM glutamine. InR1-G9, hamster glucagonoma cells were cultured in RPMI supplemented with 10% FCS and 2 mM glutamine. β-TC1 mouse insulinoma cells were cultured in DMEM supplemented with 12.5% horse serum, 2.5% FCS, and 2 mM glutamine and were used for experiments between passages 19 and 28.

*2.2. Transfection and chloramphenicol-acetyltransferase (CAT) assay*

Cell transient transfection in suspension (using the diethylaminoethyl-dextran method) and CAT assays were performed essentially as described by Gherzi et al. [29].

*2.3. RNA isolation and Northern blot analysis*

Total RNA was prepared from cultured cells by the guanidine/cesium chloride method [28]. Total RNA (30 μg per lane) was denatured, electrophoresed and blotted to Hybond N+ nylon membranes as reported [30]. Filters were hybridised using radiolabeled c-Ha-*ras* and G3PDH (Clontech) c-DNA probes and washed as previously described [30].

## 3. Results

PKA and PKC activators increase glucagon gene transcription in insulinoma and glucagonoma cells ([24,25]; R.G., H.-C.F., A. Volz and B.G., unpublished results). Forskolin (4 μM)~2.5- and ~12-fold stimulates the p[−1.1]GLU-CAT activity

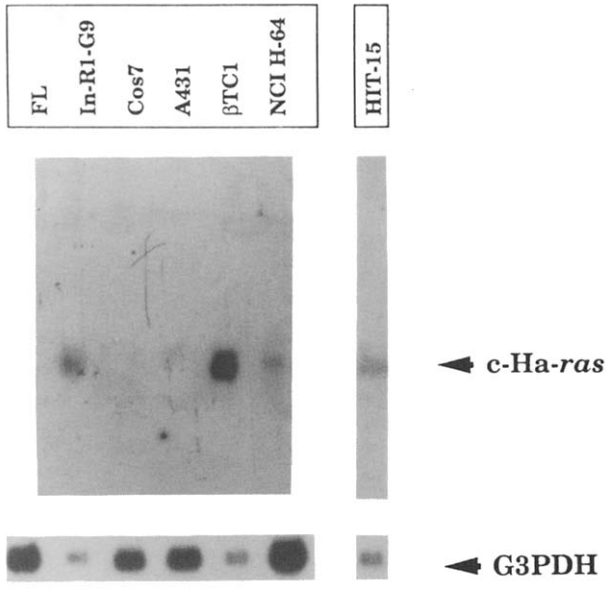


Fig. 1. Northern blot analysis of c-Ha-*ras* gene expression in cultured cell lines. Northern blots of total RNA prepared from the indicated cell lines were hybridized with c-Ha-*ras* (upper panels) and G3PDH (lower panels) cDNA probes, washed, and autoradiographed. The figure shows representative autoradiograms (exposed 72 h at −80°C for c-Ha-*ras* or 4 h at −80°C for G3PDH) of two performed. A single blot was subsequently hybridized with c-Ha-*ras* and G3PDH probes.

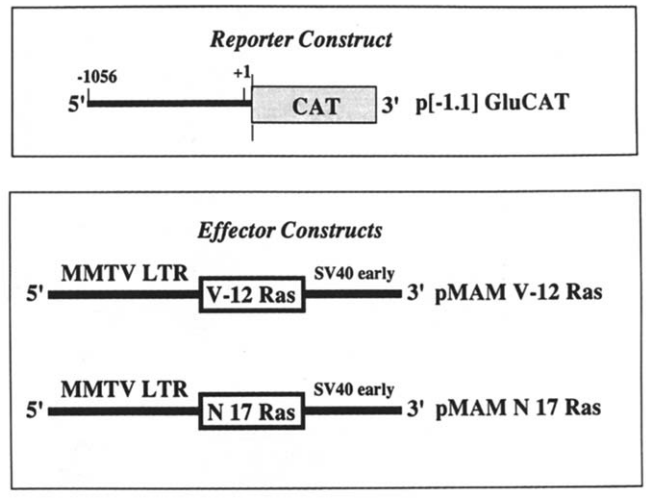


Fig. 2. Schematic representation of plasmid vectors used in transient transfection experiments. p[−1.1]Glu-CAT that comprises approximately 1.1 kb of the rat glucagon gene 5'-flanking region linked to the CAT gene was used as the reporter construct. pMAM-V-12-ras and pMAM-N17-ras were obtained as detailed in section 2 and used, together with the pMAM-neo vector, as the effector constructs in transient transfection experiments.

in glucagon-producing InR1-G9 cells and in insulin-producing β-TC1 cells, respectively. Furthermore, PMA (160 nM) enhances p[−1.1]GLU-CAT activity through a cAMP-independent pathway in both cell lines (~3-fold in InR1-G9, and ~6-fold in β-TC1) [RG]. Therefore, these cell lines are appropriate tools to investigate whether (and by which mechanism) Ras affects PKA- and PKC-induced gene transcription in endocrine cells.

First, the expression levels of the Ha-*ras* gene were investigated in InR1-G9 and β-TC1 cells using a full-length c-Ha-*ras* cDNA as probe for Northern blots. As shown in Fig. 1, β-TC1 and InR1-G9 express Ha-*ras*, the mRNA levels being ~6-fold higher in β-TC1 than in InR1-G9. This data is supported by a previous report that Ha-*ras* is expressed in rat pancreatic islets [31]. Out of five other cell lines analysed, only HIT-15 insulinoma cells and NCI H-64 small lung carcinoma cells express Ha-*ras* (Fig. 1). Thus, to determine whether the endogenous Ha-*ras* signalling pathway is involved in either basal or activated glucagon gene transcription, we performed transient co-transfection experiments using p[−1.1]GLU-CAT as the reporter construct and a vector capable to express a dominant negative mutant of Ras (pMAM-N17-*ras*) [32,33] as the effector (Fig. 2). The activity of our mutant was confirmed by evaluating its ability to inhibit the proliferation of transfected NIH-3T3 cells [32,33] (data not shown). Results presented in Fig. 3, demonstrate that transfection of pMAM-N17-*ras* strongly enhances either basal (non-stimulated) or FK- and TPA-stimulated p[−1.1]GLU-CAT activity in InR1-G9 cells. Similar results, although smaller in magnitude, were obtained using β-TC1 cells (data not shown). As a control, we co-transfected insulinoma cells with pMAM-N17-*ras* and pRSV-CAT. In both InR1-G9 and β-TC1, pMAM-N17-*ras* does not affect pRSV-CAT activity (data not shown). These observations indicate that Ha-*ras* is involved in the control of glucagon gene transcription in insulinoma cell lines. It is important to note that in all gene transfer experiments the total DNA transfected was fixed using 'non specific' pMAM-neo plasmid DNA.

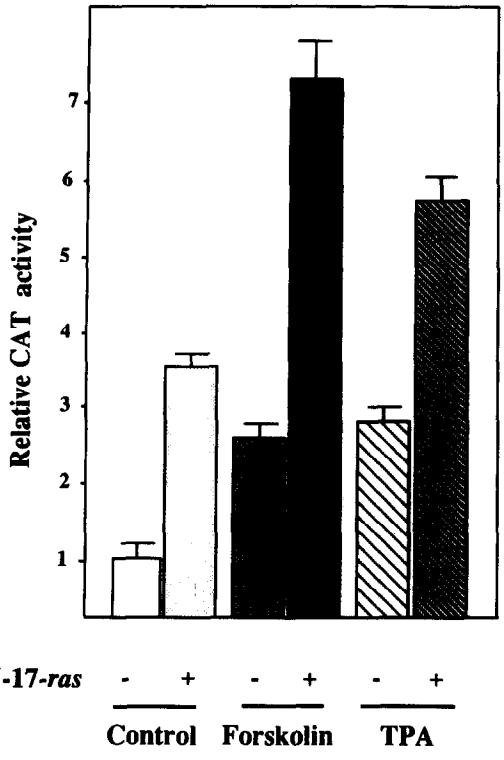


Fig. 3. A Ras dominant negative mutant enhances p[−1.1]Glu-CAT activity in transfected InR1-G9 cells. Subconfluent cultures of InR1-G9 cells were transiently co-transfected with 12 μg of p[−1.1]Glu-CAT (reporter vector) and 12 μg of pMAM-neo (indicated as −) or of pMAM-N17-*ras* (indicated as +), respectively. 32 hours later, cells were changed to serum-free medium in the absence or in the presence of 4 μM forskolin or 160 nM TPA. After further 16 h, cells were harvested and CAT activity in cell lysates was measured. The average (± S.E.M.) of four independent experiments performed in duplicate is presented.

To further investigate the role of p21-Ras in the regulation of glucagon gene transcription, we transfected InR1-G9 and β-TC1 cells with an expression vector coding for V-12-Ras (pMAM-V-12-*ras*) (Fig. 2) and measured the activity of the co-transfected p[−1.1]GLU-CAT. As depicted in Fig. 4, the basal activity of p[−1.1]GLU-CAT is not affected by the expression of V-12-Ras. On the contrary, oncogenic Ras strongly reduces FK-stimulated CAT activity in both InR1-G9 and β-TC1 cells (Fig. 4). Also the induction of p[−1.1]GLU-CAT activity by PMA is reduced in V-12-*ras* transfected cells, although in this case the effect of the activated oncogene is less pronounced (Fig. 4). The cotransfection of pMAM-V-12-*ras* does not affect the activity of pRSV-CAT in neither InR1-G9 nor β-TC1 cells (data not shown).

## 4. Discussion

Glucagon gene expression is enhanced, at the transcriptional level, by PKA and PKC activators in a strictly cell-specific manner ([24,25]; R.G.). Despite the huge amount of investigations published during the last few years, the mechanisms responsible for the transduction of different extracellular stimuli to the nucleus are far from being completely elucidated. However, the signal transduction pathways involving PKA, PKC, and Ras activation are supposed to play a crucial role in modulating the expression and/or the activity of nuclear transcription factors responsible for gene transcription regulation [6,7].

Recent experimental observations indicate that the stimulation of cAMP production and the activation of PKA inhibit proliferative signal transmission from Ras by preventing Ras-dependent activation of Raf-1 [34]. However, the relationship between PKA and Ras signal transduction pathways in regulating cell-specific gene transcription is still under discussion [15–22]. Our observations, the first in pancreatic endocrine cell lines, agree with data previously reported in pituitary cell lines [19–21]. Our data indicates that the Ras and the PKA signalling pathways are functionally antagonistic in regulating glucagon gene transcription in both glucagonoma and insulinoma cell lines.

Ras responsive elements (RRE) have been identified in the 5′-regulatory regions of different genes [35]. The sequences of RREs are very close to the sequence of either cAMP responsive

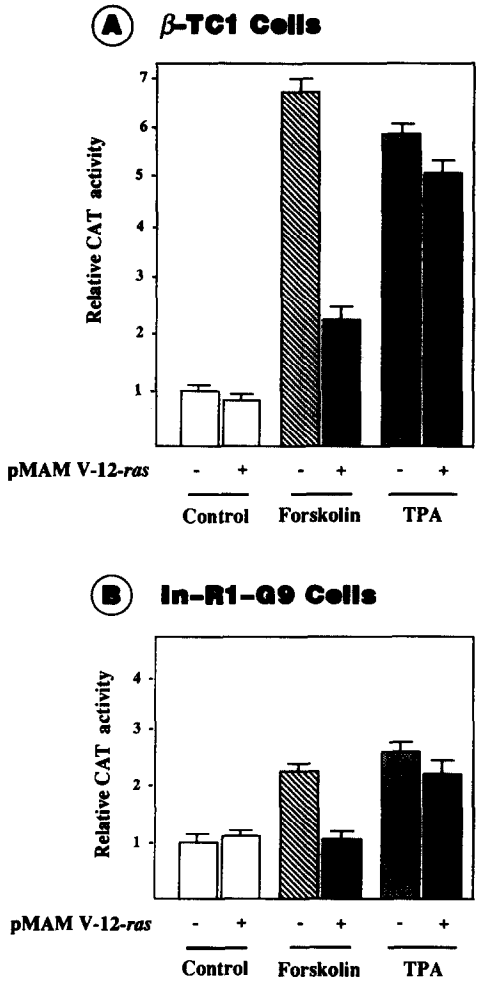


Fig. 4. The activated V12-ras reduces p[−1.1]Glu-CAT activity in transfected β-TC1 and InR1-G9 cells. Subconfluent cultures of β-TC1 (panel A) and InR1-G9 (panel B) cells were transiently co-transfected with 12 μg of p[−1.1]Glu-CAT (reporter vector) and 12 μg of pMAM-neo (indicated as −) or of pMAM-V12-*ras* (indicated as +), respectively. 32 hours later, cells were changed to serum-free medium in the absence or in the presence of 4 μM Forskolin or 160 nM TPA. After further 16 h, cells were harvested and CAT activity in cell lysates was measured. The average (± S.E.M.) of four independent experiments performed in duplicate is presented.

element (CRE) or TPA responsive element (TRE) [36]. Nevertheless, the role of AP-1 and CREB transcription factors in mediating the transcriptional effects of Ras has not been yet fully elucidated [16]. A sequence analysis of the ~1.1 kb 5'-flanking region of the rat glucagon gene (performed using the DNA Strider 1.2 software [37]) revealed the presence of a palindrome which comprises a canonical CRE at position −295/−288 [36]. This sequence differs for two bases from the sequence supposed to be the RRE in the β-polymerase promoter [15]. Moreover, at position −252/−245 we identified the sequence 5'-CTGACTGA-3' which corresponds to the RRE present in the interleukin 2 promoter [35]. Ras was recently shown to be activated after insulin stimulation of various cells and it was concluded that Ras is possibly involved in insulin's signal transduction pathway [38,39]. Recently, we characterised insulin receptors in InR1-G9 cells and found that insulin inhibits glucagon gene transcription in these cells [40]. Philippe suggested that this insulin effect is mediated via an 'insulin-response element' localised in the A domain of the glucagon gene promoter G3 element [41]. This DNA region contains the RRE present in the interleukin 2 promoter. Furthermore, Philippe found two proteins that bind the 'insulin-response element' in a sequence-specific manner [41]. These two DNA-binding proteins are not A cell-specific since they are present also in nuclear extracts from JEG-3 chorioncarcinoma and HeLa cells [41]. Based on these and our present data it is possible to hypothesise that Ras mediates insulin-induced negative regulation on glucagon gene transcription.

Activated Ras has been reported to act via both PKC-dependent and PKC-independent pathways [20,23]. Recently, Gutierrez-Hartmann and co-workers [23] have demonstrated that Ras and PKC signalling pathways are separate and mutually antagonistic in regulating rat prolactin promoter activity in $GH_4$ neuroendocrine cells. Our evidence that, in endocrine pancreatic cell lines, a Ras dominant negative mutant strongly enhances glucagon gene promoter activity suggests that in endocrine pancreatic cell lines Ras and PKC are also not cooperative. The relatively small effect of V-12-Ras expression on PKC-stimulated glucagon gene transcription further supports the assumption that Ras and PKC are not synergistic in regulating p[−1.1]GLU-CAT activity.

In conclusion, in neuroendocrine cells, the transcriptional regulation of specific genes seems to be controlled antagonistically by PKA and Ras, respectively. This agrees with results provided by cell proliferation studies [34]. The relationships between PKC and Ras in specifically regulating neuroendocrine genes transcription is complex as underscored by our results.

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