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Proliferation of Transformed Somatotroph Cells Related to Low or Absent Expression of Protein Kinase A Regulatory Subunit 1A Protein

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ABSTRACT

The two regulatory subunits (R1 and R2) of protein kinase A (PKA) are differentially expressed in cancer cell lines and exert diverse roles in growth control. Recently, mutations of the PKA regulatory subunit 1A gene (*PRKAR1A*) have been identified in patients with Carney complex. The aim of this study was to evaluate the expression of the PKA regulatory subunits R1A, R2A, and R2B in a series of 30 pituitary adenomas and the effects of subunit activation on cell proliferation. In these tumors, neither mutation of *PRKAR1A* nor loss of heterozygosity was identified. By real-time PCR, mRNA of the three subunits was detected in all of the tumors, R1A being the most represented in the majority of samples. By contrast, immunohistochemistry documented low or absent R1A levels in all tumors, whereas R2A and R2B were highly expressed, thus resulting in an unbalanced R1/R2 ratio. The low levels of R1A were, at least in part, due to proteasome-mediated degradation. The effect of the R1/R2 ratio on proliferation was assessed in GH3 cells, which showed a similar unbalanced pattern of R subunits expression, and in growth hormone-secreting adenomas. The R2-selective cAMP analog 8-Cl cAMP and R1A RNA silencing, stimulated cell proliferation and increased Cyclin D1 expression, respectively, in human and rat adenomatous somatotrophs. These data show that a low R1/R2 ratio promoted proliferation of transformed somatotrophs and are consistent with the Carney complex model in which R1A inactivating mutations further unbalance this ratio in favor of R2 subunits. These results suggest that low expression of R1A protein may favor cAMP-dependent proliferation of transformed somatotrophs.

INTRODUCTION

Cyclic AMP is implicated in the regulation of a variety of cell functions that are, at least in part, related to protein phosphorylation through the activation of protein kinase A (PKA). In addition to the control of differentiated functions, such as motility, secretion, metabolism, differentiation, synaptic transmission, and ion channel activities, cAMP inhibits or stimulates cell proliferation depending on the cell type. In recent years, mutations of genes involved in cAMP signaling and resulting in the constitutive activation of cAMP formation have been identified as a cause of endocrine neoplasia. In particular, activating mutations of the α subunit of the stimulatory G protein gene (the so-called *gsp* oncogene) have been found in ~30 to 40% of growth hormone-secreting pituitary adenomas and in subsets of thyroid, adrenocortical, ovarian, and testicular stromal Leydig cell tumors (1, 2).

More recently, genetic defects downstream of cAMP production and affecting PKA complex have been identified in endocrine disorders associated with benign and malignant neoplasia. In mammalian cells there are two types of PKA, PKA1 and PKA2, which share common catalytic subunits but possess different regulatory subunits, R1 and R2 (3). Through gene cloning, four genes coding for different R isoforms, R1A, R1B, R2A, and R2B, that differ in tissue distribution, subcellular localization and biological properties, have been identified (3). Dramatic changes in the proportion of R1 and R2 during embryonic development, differentiation processes, and neoplastic transformation indicate distinct roles for these isoenzymes in growth control (4, 5). In particular, previous studies supported the view that R1 was related to cell proliferation whereas R2 was primarily involved in tissue differentiation (4–6). Accordingly, in a variety of human cancer cell lines, transformation coincides with a sharp increase in R1, whereas R2 overexpression reverts the malignant phenotype into a nontransformed phenotype (7–9). However, the involvement of R1 in promoting cell proliferation and transformation has been challenged by the recent identification of R1A gene (*PRKAR1A*) mutations causing the loss of R1 expression and function in patients with Carney complex, a familial multiple neoplasia syndrome characterized by the association of skin pigmentation, cardiac myxomas and different endocrine tumors, including growth hormone (GH)-secreting pituitary tumors (10, 11). On the basis of this evidence, it has been suggested that the impact of unbalanced R1 and R2 expression on cell growth may depend on the cell type (4).

The aim of this study was to evaluate the relative expression of the different PKA regulatory subunits in pituitary tumors and to examine the effect of their selective activation on proliferation of somatotroph-lineage cells, the pituitary cell type in which cAMP promotes proliferation.

MATERIALS AND METHODS

Pituitary Tissue Samples and Cell Cultures. The study included 30 human pituitary adenomas, including 9 GH-secreting (GH-omas, 3 gsp+ and 6 gsp−), 12 nonfunctioning (NFPAs), 4 ACTH-secreting (ACTH-omas), 3 prolactin (PRL)-secreting (PRL-omas), and 2 thyreotropin (TSH)-secreting pituitary adenomas, surgically removed by the transsphenoidal route. Small adenoma fragments were fixed for immunohistochemistry, and the remaining tissues were quickly frozen for subsequent molecular analysis. Moreover, tissues from GH-omas as well as GH3 cells were placed in the appropriate sterile medium for cell culture, as described previously (12), to perform proliferation assays and to assess Cyclin D1 expression. Local ethics committee approval was obtained for all studies.

***PRKAR1A* Sequencing Analysis.** Genomic DNA was extracted with the phenol-chloroform method from adenomatous tissues (Nucleon-Amersham Life Science Europe, Milan, Italy). The 12 exons and flanking intronic sequences of the *PRKAR1A* gene (GenBank accession no. NM 002734) were amplified by polymerase chain reaction (PCR; primers and amplification conditions available on request). Direct sequencing of the amplified fragments was then performed with the AmpliTaq BigDye Terminator kit and 310 Genetic Analyzer (Perkin-Elmer Corp., Applied Biosystems, Foster City, CA). G protein stimulatory α subunit (Gsα) analysis was performed in GH-omas, as described previously (12).

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Note: A. Lania and G. Mantovani contributed equally to this work and should both be considered first authors.

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Real-time Reverse Transcription-PCR. Total RNA was isolated from tissue specimens with a commercial kit, Trizol (Invitrogen S.R.L., Milan, Italy) according to the manufacturer's instructions and 200 ng RNA was reverse transcribed (Applied Biosystems). PKA R1A, PKA R2A, and PKA R2B mRNA levels in pituitary adenomas were evaluated by real-time quantitative reverse transcription-PCR based on TaqMan methodology, with the ABI Prism 7700 Sequence Detection System (Applied Biosystems).

PKA R1A, PKA R2A and PKA R2B mRNA expression were determined applying the $\Delta\Delta C_t$ method, as described previously (13). We identified a calibrator sample that represents the unitary amount of the target of interest. The other samples express *n*-fold mRNA relative to the calibrator. As calibrator, we used one of the pituitary adenomas of the series under study. To normalize the amount of total RNA added to each reaction mixture, we quantified as internal RNA control the β-actin (ACTB) mRNA. Final amounts of target were determined as follows: target amount = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t = [C_t\ (PKAr) - C_t\ (ACTB)]_{sample} - [C_t\ (PKAr) - C_t\ (ACTB)]_{calibrator}$.

The primers and probe nucleotide sequence for β-actin mRNA were previously reported (13). For the quantification of PKA R1A, PKA R2A, and PKA R2B mRNA, we used a ready-to-use assay (identification numbers Hs00267597_m1, Hs00177760_m1, and Hs00176966_m1, respectively; Assay-on-Demand Gene Expression Products, Applied Biosystems), according to the manufacturer's instructions. All of the reactions were performed in duplicate. The thermal cycling conditions included 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. All of the reagents used for PCR were from Applied Biosystems.

Immunohistochemistry. Sections from paraffin-embedded tissues from 30 surgically removed pituitary adenomas were processed for immunohistochemistry, as reported previously (14). Specific monoclonal antibodies for PKA R1A, PKA R2A, and PKA R2B were used under the conditions specified by the manufacturer (BD Transduction Laboratories, Lexington, United Kingdom). Antigen-antibody detection was performed with the DAKO ChemMate En Vision detection kit (DAKO A/S, Glostrup, Denmark) according to the manufacturer's instructions. Sections were stained with 3,3′-diaminobenzidine substrate and counterstained with Meyer hematoxylin; slides were prepared for light microscopy examination, as reported previously (14). As positive controls, normal human adrenal tissues were used. Negative controls were obtained by occulting the primary antibody or by using an unrelated mouse monoclonal antibody. At least two blinded readers graded the specimens for all stainings. Briefly, PKA R1A, PKA R2A, and PKA R2B immunoreactivities were graded 0 to 3, with 0 = absence of immunoreactivity, 1 = $<$10%, 2 = 10 to 50%, and 3 = $>$50% in at least 400 cells in the main representative high power field.

***PKA R1A* Degradation.** To determine the degradation pathway of PKA R1A, we incubated GH3 cells and cells obtained by enzymatic digestion from 4 GH-omas with 10 μmol/L lactacystin or 100 μmol/L chloroquine (Sigma-Aldrich, Milan, Italy) for 20 hours at 37°C. Incubation was stopped by placing the cells on ice; the medium was removed, and cells were treated with 500 μL ice-cold lysis buffer in the presence of protease and phosphatase inhibitors. Western blot analysis of PKA R1A was performed with the same monoclonal antibody used for immunohistochemistry, detected by chemiluminescent method and the resulting bands evaluated by imaging densitometer, as previously reported (12). Experiments were repeated at least twice.

Protein Kinase A Activity. PKA activity was measured with a nonradioactive PKA kinase activity assay kit (Stressgen, Victoria, BC, Canada) in 2 μg of cell extracts from six GH-secreting tumors and from GH3 cells. The assay is based on a solid phase ELISA that uses a specific synthetic peptide as a substrate for PKA (kempeptide) and a polyclonal antibody that recognizes the phosphorylated form of the substrate. PKA activity reflects the enzymatic activity after stimulation with 5 μmol/L cAMP, free PKA activity represents basal activity, in the absence of cAMP stimulation, and total PKA activity is calculated as the difference between cAMP-stimulated PKA and the PKA inhibited by the protein kinase inhibitor PKI (5 μmol/L).

Cell Proliferation. Cell proliferation was assessed by colorimetric measurement of 5-bromo-2′-deoxyuridine (BrdUrd) incorporation during DNA synthesis in proliferating cells (Cell Proliferation Biotrak Elisa, Amersham, Piscataway, NJ). Briefly, cells were cultured in 96-well plate (20,000 cells per well) in the presence of test substances (5, 10, 100 μmol/L 8-Cl cAMP or 100 μmol/L 8-Br cAMP alone or in combination with 5 μmol/L PKA inhibitor PKI, Sigma-Aldrich, Milan, Italy) for 72 hours at 37°C and then with BrdUrd for 2 hours to allow BrdUrd incorporation in newly synthesized cellular DNA. Proliferation was expressed as relative fluorescence units (RFU). All of the experiments were repeated at least 3 times on 2 different GH3 clones and on 4 GH-secreting adenomas, and each determination was done in quintuple.

Cyclin D1 Expression. After 24 hours of serum starvation, cells obtained by enzymatic digestion from four GH-omas were incubated with different agents (100 μmol/L 8-Cl cAMP or 100 μmol/L 8-Br cAMP, alone or in combination with 5 μmol/L PKA inhibitor PKI) for 8 hours at 37°C. The determination of Cyclin D1 was performed after immunoprecipitation of cell lysates with a specific monoclonal antibody (Novocastra, Newcastle, United Kingdom) and Western blotting, as reported previously (12). The same experiment was repeated in GH3 cells transfected with siRNA. Experiments were repeated at least twice.

Synthesis and Transfection of Small Interfering RNA. Small interfering RNA (siRNA) for rat *R1A* gene was synthesized by Ambion, and GH3 cells were transfected with the double-stranded RNA with amine transfection reagent according to the manufacturer's instructions (Ambion, Austin, TX). Cells were exposed to double-stranded RNA and transfection reagent for 96 hours before performing Western blot analysis with the specific R1A antibody. Corresponding scrambled siRNA for the same regulatory subunit and siRNA for GAPDH were used as internal negative and positive controls, respectively.

Statistical Analysis. The results are expressed as the mean ± SD. A paired or unpaired two-tailed Student's *t* test was used to detect the significance between two series of data. $P < 0.05$ was accepted as statistically significant

RESULTS

***PRKAR1A* Sequencing Analysis.** Analysis of the 12 exons and flanking regions of *PRKAR1A* did not reveal mutations of the gene in any of the adenomas included in the study. Two known polymorphisms (15) in the noncoding sequence, *i.e.,* a T insertion in intron 3 (exon 4 IVS –5) and a base substitution (A to C) in the 5′-untranslated region of exon 1A, were found in 11 and 16 tumors, respectively. Twenty-four of 30 tumors were heterozygous for at least one polymorphism, thus excluding a loss of heterozygosity in these informative samples.

Real-time PCR. PKA R1A, R2A, and R2B mRNA expression levels were evaluated by real-time PCR in 18 of 30 pituitary adenomas included in the study. The mean C_t value of the internal control (ACTB) was 20.08 ± 0.79 (range 18.6–21.3) documenting the appropriate quality of RNA. PKA R1A, R2A, and R2B mRNA expression was detected in all of the samples examined. Although variable, PKA R1A mRNA levels were significantly higher than those of R2A and R2B mRNA (4.7 ± 2 *versus* 2.2 ± 1.2 and 2.3 ± 0.7, respectively; $P < 0.005$; Fig. 1). Accordingly, in almost all tumors analyzed, the R1A/R2B ratio was higher than 1 (2.2 ± 1.0). Finally, there was

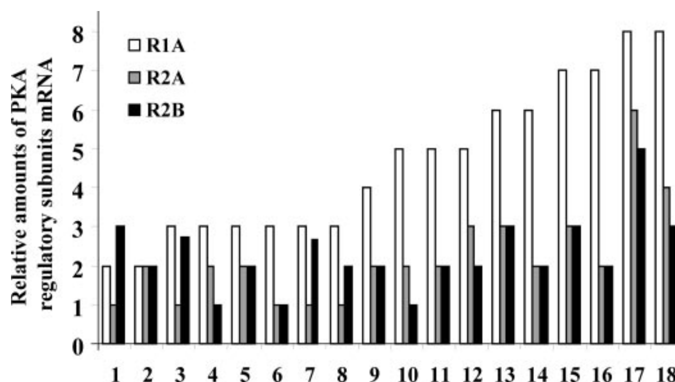
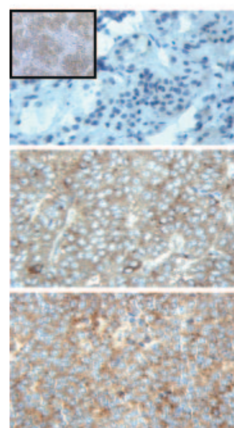


Fig. 1. PKA regulatory subunit (R1A, R2A, R2B) mRNA expression in pituitary tumors (GH-oma, $n = 7$; NFPA, $n = 7$; ACTH-oma, $n = 3$; PRL-oma, $n = 1$) by real-time PCR. PKA R1A, PKA R2A, and PKA R2B mRNA expression were determined applying the $\Delta\Delta C_t$ method with β-actin mRNA as internal RNA control. All reactions were performed in duplicate, and data are expressed as relative amounts of mRNA levels (*vertical axis*).

Fig. 2. Immunostaining for the three PKA regulatory subunits (R1A, R2A, and R2B) performed in one GH-secreting adenoma, as representative example (*left panel*). Similar data were obtained in 30 functioning and nonfunctioning pituitary tumors. Tissue sections were incubated with specific monoclonal antibodies. The immunoreactivity was visualized by diaminobenzidine, positive cells giving a *brown color* at the site of reaction. Tumors showed a differential pattern of subunit expression and graded with score 0–3: *0*, absence of immunoreactivity; *1*, <10%; *2*, 10–50%; *3*, >50% in at least 400 cells in the main representative high power field. *Inset*, immunostaining for R1A subunit performed in one normal adrenal used as positive control. All photomicrographs are shown at ×25. *, no. of pituitary adenomas.



Protein	*Staining pattern*	*Staining score* 0	1	2	3
R1A	**Diffuse cytoplasmatic**	**16***	**14***	**-**	**-**
R2A	**Diffuse cytoplasmatic** **Perinuclear dots**	**-**	**-**	**2***	**28***
R2B	**Diffuse cytoplasmatic**	**-**	**-**	**1***	**29***

no difference in the levels of expression of the three subunits between GH-omas expressing or not the *gsp* oncogene (data not shown). Similarly, no correlation with clinical parameters such as age of the patient, size of the tumor, aggressiveness, hormone secretion, and responsiveness to medical treatment was observed (data not shown).

Immunohistochemistry. No immunoreactivity for R1A subunit was found in 16 of 30 pituitary tumors, whereas in the remaining tumors, a low number of cells (<10%) showed a weak cytoplasmic staining (Fig. 2). Conversely, all tumors showed a strong positivity for both R2A and R2B subunits, which were detected in >50% of the total cell population in most cases. The R2B staining was exclusively cytoplasmic, whereas immunopositivity for R2A was also localized to perinuclear dots (Fig. 2). The same pattern of staining, showing barely detectable R1A and high levels of R2A and R2B, was observed in GH3 cells (data not shown).

Effect of Lysosomal and Proteasome Inhibitors on R1A Protein Levels. Cultured GH3 cells and primary cell cultures obtained from GH-omas were treated with a lysosomal (chloroquine) and a proteasome inhibitor (lactacystin) to evaluate R1A degradation processes. Proteasome blockade by lactacystin (10 μmol/L for 20 hours) hours induced a significant increase in R1A protein levels as assessed by immunoblotting analysis in human tumoral cells (Fig. 3) as well as in GH3 cells (data not shown). By contrast, no effect on R1A protein levels was induced by the lysosomal inhibitor chloroquine (100 μmol/L for 20 hours).

Protein Kinase A Activity in Tumoral Somatotrophs. We measured PKA activity, as well as the response to cAMP, in cell extracts from six GH-secreting pituitary tumors and from GH3 cells. All of the samples that were considered showed a similar free PKA activity; and after exposure to cAMP (5 μmol/L), all of the tumors responded with a significant increase in PKA activity (Fig. 4). This response was totally abrogated by the addition of the PKA inhibitor PKI (Fig. 4), resulting in a high total PKA activity in both GH-omas and GH3 cells.

Effect of cAMP Analogs on Cell Proliferation. We investigated the proliferation rate of GH3 cells induced by 8-Br cAMP and 8-Cl cAMP, two cAMP analogs with different selectivity for PKA regulatory subunits, the former activating all of the regulatory subunits and the latter able to selectively activate the R2 subunit. At concentrations higher than 5 μmol/L 8-Cl cAMP caused a dose-dependent increase of cell proliferation (Fig. 5), the maximum effect being observed at 100 μmol/L (362 $\pm$ 42% over basal levels). Coincubation with the PKA inhibitor PKI (5 μmol/L) resulted in a significant reduction of this effect (Fig. 5). Conversely, a reproducible but nonsignificant increase (58 $\pm$ 22% over basal levels) of cell proliferation was induced by 8-Br cAMP only at 100 μmol/L (Fig. 5). The same experiments performed on primary cultures from four GH-omas showed a slight increase of cell proliferation induced by 8-Cl cAMP, that did not reach the statistical significance, probably because of the low and variable proliferation rate that characterized these neoplasia (Fig. 5).

Effect of R1/R2 Ratio Modifications on Cyclin D1 Expression. The exposure to 8-Cl cAMP induced a significant increase of Cyclin D1 levels in the four GH-omas used for proliferation studies (+80 $\pm$ 15% *versus* basal; Fig. 6), whereas a moderate response to

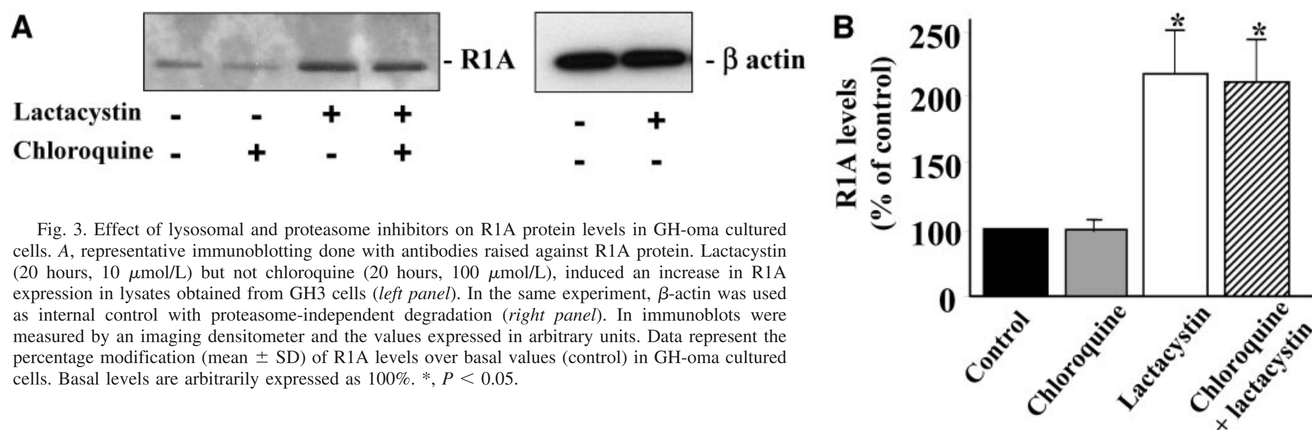


Fig. 3. Effect of lysosomal and proteasome inhibitors on R1A protein levels in GH-oma cultured cells. *A*, representative immunoblotting done with antibodies raised against R1A protein. Lactacystin (20 hours, 10 μmol/L) but not chloroquine (20 hours, 100 μmol/L), induced an increase in R1A expression in lysates obtained from GH3 cells (*left panel*). In the same experiment, β-actin was used as internal control with proteasome-independent degradation (*right panel*). In immunoblots were measured by an imaging densitometer and the values expressed in arbitrary units. Data represent the percentage modification (mean $\pm$ SD) of R1A levels over basal values (control) in GH-oma cultured cells. Basal levels are arbitrarily expressed as 100%. *, $P < 0.05$.

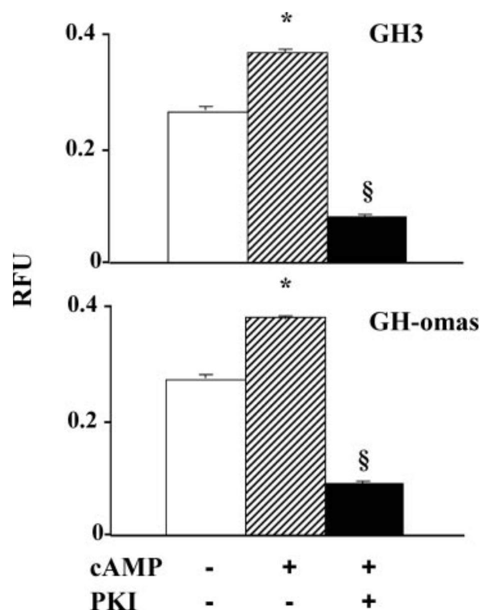


Fig. 4. PKA activity measured (*RFU*, relative fluorescence units) in cell extracts obtained from GH3 cells and six GH-omas. PKA activity was measured in basal conditions (free PKA activity) as well as after cAMP stimulation (PKA activity) in the absence or presence of PKA inhibitor PKI (5 μmol/L). *, $P < 0.05$ *versus* free PKA activity; §, $P < 0.05$ *versus* PKA activity.

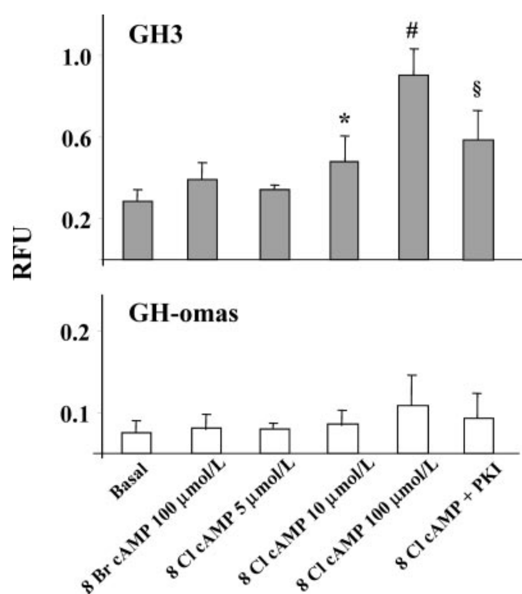


Fig. 5. Effect of different cAMP analogs on the proliferation of GH3 cells and cultured cells from four GH-omas. In all samples, 8-Br cAMP was ineffective, whereas 8-Cl cAMP, at concentrations >5 μmol/L, caused a dose-dependent increase of GH3 proliferation, this effect being PKA dependent (*top panel*). Conversely, a slight but not significant increase in cell proliferation was observed after 8-Cl cAMP incubation in GH-oma cultured cells (*bottom panel*). Data, expressed as relative fluorescence units (*RFU*), are the mean ± SD of cell number determinations carried out in triplicate for each clone tested. *, $P < 0.05$ *versus* basal; #, $P < 0.01$ *versus* basal; §, $P < 0.05$ *versus* 8 Cl cAMP.

8-Br cAMP was observed in one (30% *versus* basal). The elevation of Cyclin D1 levels induced by 8-Cl cAMP was significantly reduced by coincubation with PKA inhibitor PKI (Fig. 6). Similar results were observed in GH3 cells (data not shown).

The effect of changes in R1A/R2B ratio on cell cycle progression was further investigated in GH3 cells by silencing R1A RNA expression by siRNA transfection. In particular, the near total abrogation of R1A protein levels obtained by this manipulation was associated with an increase both in R2B (+85 ± 10% *versus* not transfected) and in Cyclin D1 expression (+200 ± 45% *versus* not transfected), consistent with the results obtained after 8-Cl cAMP treatment (Fig. 7).

DISCUSSION

The present study demonstrates that deficiency of the PKA regulatory subunit 1A (PKA R1A) is a common event in pituitary tumors and provides evidence for a proliferative role of the unbalanced expression of PKA isoenzymes in tumoral somatotrophs. In recent years, loss-of-function mutations of the gene encoding PKA R1A leading to uncontrolled activation of PKA catalytic subunits have been identified in patients with Carney complex, a familial multiple neoplasia syndrome characterized by the association of skin pigmen-

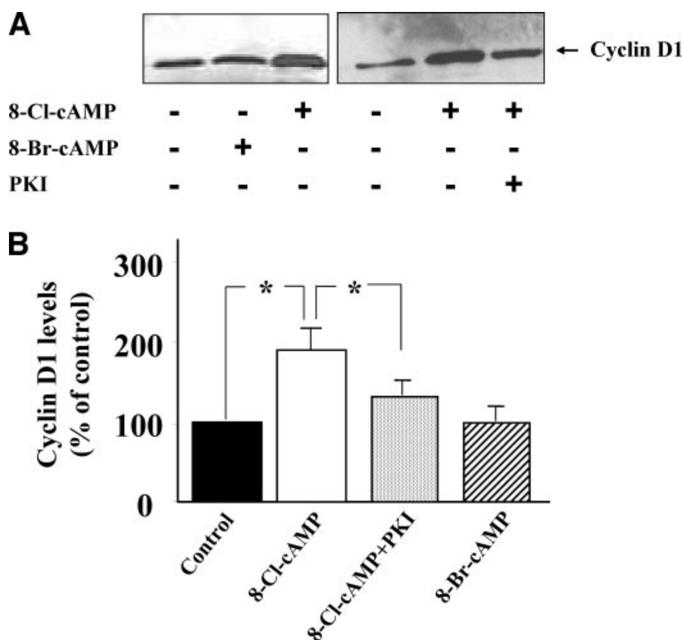


Fig. 6. Effect of cAMP analogs on Cyclin D1 expression in pituitary adenomas. *A*, representative immunoblotting performed with antibodies raised against Cyclin D1. Each lane was loaded with 20 μg of tumoral tissue homogenates. 8-Cl cAMP (100 nmol/L for 8 hours) induced a significant increase in Cyclin D1 levels in the four GH-omas studied. In *B*, immunoblots were measured by an imaging densitometer, and the values were expressed in arbitrary units. Data represent the percentage modification (mean ± SD) of Cyclin D1 levels over basal values (control) in four GH-omas. Basal levels are arbitrarily expressed as 100%. *, $P < 0.05$.

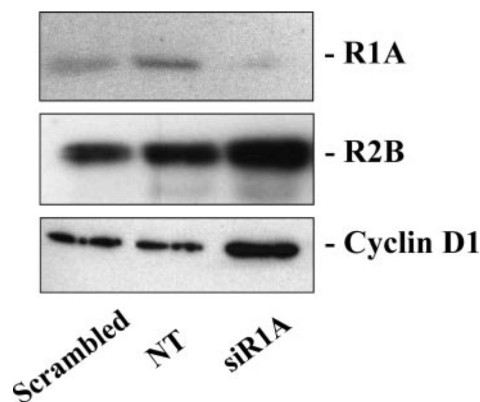


Fig. 7. Effect of R1A RNA silencing experiments on R1A (*top panel*), R2B (*middle panel*), and Cyclin D1 (*bottom panel*) protein levels in GH3 cells. Western blot analysis with the specific R1A, R2B, and Cyclin D1 antibodies were carried out after 96 hours' exposure to siRNA for rat R1A gene. The near total abrogation of R1A protein levels was associated with a significant increase both in R2B and in Cyclin D1 expression. The corresponding scrambled siRNA for R1A was used as internal negative control. *NT*, not transfected cells.

tation, cardiac mixomas, and different endocrine tumors, particularly GH-secreting pituitary tumors (10, 11). Consistent with the hypothesis that *PRKAR1A* may act as a tumor suppressor gene, in this syndrome, the loss of one allele due to germ-line mutations is generally associated with the loss of heterozygosity at the *PRKA1A* locus in the tumor tissue. Subsequently, the same genetic defects have been detected in some sporadic endocrine tumors, such as adrenal and thyroid adenomas and carcinomas (16–18). Conversely, a role of PKA R1A in the pathogenesis of pituitary tumors has been ruled out based on the absence of both gene mutations and loss of heterozygosity reported by previous studies and confirmed in the present series (15, 19, 20).

This study showed that, consistent with the absence of genetic defects, mRNA of the three subunits was detected in all of the pituitary tumors, R1A being the most represented regulatory subunit in the majority of pituitary samples, independently from the tumor type. R1A, R2A, and R2B mRNA levels were not affected by the presence of the *gsp* oncogene in GH-omas, suggesting that contraregulatory mechanisms associated with these mutations, such as cAMP degradation due to phosphodiesterase overactivity, prevented the up-regulation of PKA subunits, particularly R2, expression (21).

In contrast to the pattern of R1A mRNA expression, pituitary tumors were characterized by a dramatic reduction or complete loss of R1A at the protein level, which was clearly detectable in normal adrenal tissue used as positive control. Therefore, because R2A and R2B were expressed at high levels, almost all of the tumors included in the study displayed a strong prevalence of R2 over R1 protein. The discrepancy between R1A mRNA and protein expression suggested the existence of a high rate of protein degradation in pituitary tumors. Indeed, the incubation of primary cell cultures as well as GH3 cells with the potent proteasome inhibitor lactacystin induced a striking elevation of R1A protein. This finding is in accordance with the notion that the physiologic degradation pathway of PKA R1A is proteasome-mediated (22). Moreover, although R1A is particularly susceptible to degradation when dissociated from the catalytic subunit, a persistent R1A dissociation as cause of low R1A expression seems unlikely, when the cAMP-inducible PKA activity recorded in all pituitary tumors examined is taken into account. However, in this study, it was not possible to ascertain whether the low expression of R1A protein was a tumor-related event. In fact, the precise assessment of R1A expression and degradation as well as PKA activity was unfeasible in the normal autoptic pituitary gland, because this subunit is a soluble cytoplasmic enzyme with a rapid turnover.

In this study, we asked what was the impact of unbalanced R1 and R2 expression on the proliferation of cells of the somatotroph lineage. It is well known that cAMP, through the activation of PKA, may exert different actions on cell proliferation. According to the cell type, it may have no influence, induce cell arrest, or even suppress mitogenic action of growth factors in some cell lines, or, conversely, promote the transition from G_0 to G_1 phase and stimulate cell growth in others (21). Evidence from *in vitro* studies and naturally occurring human diseases (gsp+ GH-secreting adenomas, and carney complex) indicate that somatotrophs belong to the set of cells that recognizes cAMP as a growth factor (21, 23). However, no information on the relative expression of PKA R1 and R2 in normal and tumoral somatotrophs is thus far available, although it is known that these isoenzymes are differentially expressed in a variety of human cancers and exert distinct roles in differentiation processes and neoplastic transformation (4, 6, 7). In particular, in contrast to the recent observation that loss of *PKRAR1A* was associated with multiple neoplasia, previous studies carried out on human cancers and cell lines, such as breast, colon and lung carcinomas, fibrosarcomas, gliomas, and leukemias, indicated that R1 was the major or the sole R subunit detectable and was primarily involved in cell proliferation (6, 7, 24–26). Conversely, R2 would be related to tissue differentiation and the induction of R2 subunits by 8-Cl-cAMP treatment in malignant cell lines was associated with growth arrest and reversion of the transformed phenotype (5, 8, 9, 27).

These considerations prompted us to examine the proliferative role of the two different PKA regulatory subunits in transformed somatotrophs, by using two cell models that displayed a strong prevalence of R2 over R1 protein, *i.e.,* the somatomammotroph GH3 cell line and human GH-omas. Increasing concentrations of 8-Cl cAMP, a cAMP analog able to selectively activate R2 subunits, induced a strong proliferation of GH3 and increased the expression of Cyclin D1, a key regulator of G_1 phase progression, in GH-omas (28), both parameters being marginally modified by the activator of all subunits. The effect of changes in the R1/R2 ratio on GH3 cells proliferation was further investigated by silencing R1A RNA expression. In particular, the decrease in the R1/R2 ratio obtained by this manipulation induced a dramatic increase in Cyclin D1 expression, thus confirming the R2-mediated stimulatory effect of 8-Cl cAMP on cell proliferation. Taken together, these data are consistent with previous observations indicating that cAMP-dependent pathway may activate proliferative signals in somatotrophs and provide evidence for a crucial role of the prevalent expression of R2 over R1 protein in this process (21). The observation that a low R1/R2 ratio promoted cell proliferation in the tumoral pituitary is consistent with the Carney complex model in which inactivating mutations of the R1A subunit further unbalance this ratio in favor of R2 subunits (10, 11).

Finally, the unbalanced R1/R2 expression was associated with well-differentiated pituitary neoplasia. This is consistent with the presence of *PRKA1A* mutations in adrenocortical adenomas and their absence in adrenocortical cancers (16), but it is the opposite of what is observed in thyroid tumors in which *PRKA1A* mutations are associated with the malignant phenotype (17). This suggests that with regard to the role of PKA, diverse tumorigenic processes exist in endocrine cells of different origin.

In conclusion, the results presented here demonstrate that tumoral pituitary cells are characterized by low or absent expression of the PKA R1A subunit protein. Defective expression of R1A at the protein level was not due to reduced transcription but was associated with proteasome-dependent protein degradation. Analysis of the proliferative response to cAMP analogs suggests that, in analogy with the proliferative phenotype resulting from loss-of-function *PRKAR1A* mutations in the Carney complex, unbalanced expression of the R1 and R2 PKA subunits underlies cAMP-dependent proliferation of somatotroph cells. How R1A or R2 excess insufficiency lead to tumorigenesis, however, remains unclear.

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