Proliferation of Transformed Somatotroph Cells Related to Low orAbsent
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 (PRKARIA) 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 PRKARIA nor loss of heterozygosity was identified. By realtime 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 of the 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, adenocortical, 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 (PRKARIA) 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 thyrotropin (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.

PRKARIA 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 PRKARIA 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 (Gsa) analysis was performed in GH-omas, as described previously (12).
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 ΔΔCt 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-ΔΔCt, where ΔΔCt = [Ct (PKA) - Ct (ACTB)]sample - [Ct (PKA) - Ct (ACTB)]calibrator.

PKA R1A Degradation. To determine the degradation pathway of PKA R1A, we incubated GH3 cells and cells obtained by enzymatic digestion from 4 GH-secreting adenomas, and each determination was done in quintuple. We incubated GH3 cells and cells obtained by enzymatic digestion from 20,000 cells per well) in the presence of test substances (5, 10, 100 μmol/L cAMP or 500 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 4 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 (Novoceastra, 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

PKRARI A Sequencing Analysis. Analysis of the 12 exons and flanking regions of PRKARIA 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’t 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 Ct 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
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 Lyososomal 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 μM/L for 20 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 μM/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 μM/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 μM/L, 8-Cl cAMP caused a dose-dependent increase of cell proliferation (Fig. 5), the maximum effect being observed at 100 μM/L (362 ± 42% over basal levels). Coincubation with the PKA inhibitor PKI (5 μM/L) resulted in a significant reduction of this effect (Fig. 5). Conversely, a reproducible but nonsignificant increase (58 ± 22% over basal levels) of cell proliferation was induced by 8-Br cAMP only at 100 μM/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 ± 15% versus basal; Fig. 6), whereas a moderate response to
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 pigment-
transit from G0 to G1 phase and stimulate cell growth in others. The action of growth factors in some cell lines, or, conversely, promote the mitogenic action of G1 phase progression, in human 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 PRKAR1A 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 PRKAR1A 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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