Protein kinase A and its role in human neoplasia: the Carney complex paradigm

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Abstract

The type 1 alpha regulatory subunit (R1α) of cAMP-dependent protein kinase A (PKA) (PRKAR1A) is an important regulator of the serine-threonine kinase activity catalyzed by the PKA holoenzyme. Carney complex (CNC) describes the association of ‘spotty skin pigmentation, myxomas, and endocrine overactivity’; CNC is in essence the latest form of multiple endocrine neoplasia to be described and affects the pituitary, thyroid, adrenal and gonadal glands. Primary pigmented nodular adrenocortical disease (PPNAD), a micronodular form of bilateral adrenal hyperplasia that causes a unique, inherited form of Cushing syndrome, is also the most common endocrine manifestation of CNC. CNC and PPNAD are genetically heterogeneous but one of the responsible genes is PRKAR1A, at least for those families that map to 17q22-24 (the chromosomal region that harbors PRKAR1A). CNC and/or PPNAD are the first human diseases to be caused by mutations in one of the subunits of the PKA holoenzyme. Despite the extensive literature on R1α and PKA, little is known about their potential involvement in cell cycle regulation, growth and/or proliferation. The presence of inactivating germline mutations and the loss of its wild-type allele in CNC lesions indicated that PRKAR1A could function as a tumor-suppressor gene in these tissues. However, there are conflicting data in the literature about PRKAR1A’s role in human neoplasms, cancer cell lines and animal models. In this report, we review briefly the genetics of CNC and focus on the involvement of PRKAR1A in human tumorigenesis in an effort to reconcile the often diametrically opposite reports on R1α.

Endocrine-Related Cancer (2004) 11 265–280

Introduction

The complex of ‘spotty skin pigmentation, myxomas, endocrine overactivity, and schwannomas’ or Carney complex (CNC) (Mendelian Inheritance in Man catalog number 160980) was described in 1985 (Carney & Young 1992). Isolated patients with characteristics similar to CNC had previously been described under the acronyms NAME (nevus, atrial myxomas and epitheliomas) and LAMB (lentigines, atrial myxomas and blue nevi). Today, it is accepted that most of these patients had CNC (Carney et al. 1985a, Carney 1995, Stratakis et al. 1998, Stratakis 2000).

CNC is a form of multiple endocrine neoplasia. Patients often have tumors of two or more endocrine glands, including primary pigmented nodular adrenocortical disease (PPNAD), growth hormone (GH)- and prolactin-producing pituitary adenoma, thyroid adenoma or carcinoma, testicular neoplasms (primarily large-cell calcifying Sertoli cell tumor (LCCSCT)), and ovarian cysts (Stratakis 2000). Tumors in the parathyroid glands or pancreas have not been observed. Non-endocrine tumors that occur frequently are myxomas and ear canal trichofolliculo-epitheliomas. Additional but rare manifestations include psammomatous melanotic schwannoma (PMS), breast ductal adenoma and osteochondromyxoma.

In most cases of CNC, the endocrine features are reminiscent of McCune–Albright syndrome (MAS) while skin abnormalities are similar to several lentiginoses and/or hamartomatoses syndromes, such as the Peutz–Jeghers, Cowden, Bannayan–Zonana (Bannayan–Myhre–Smith), Birt–Hogg–Dubé and neurofibromatosis (NF) syndromes (Stratakis 2000).

To date, approximately 400 patients with CNC are listed in the National Institutes of Health and Mayo Clinic registry (Stratakis et al. 2001). Although the sample is relatively small, the prevalence does not differ between
genders, various races or ethnic groups. Most of the patients belong to families in which the disease is inherited in an autosomal dominant fashion (Carney et al. 1985b). The remaining cases are classified as sporadic. Detailed family data have demonstrated significant variability in clinical manifestations between patients, including members of the same family. This clinical variability may be responsible for the apparent ‘skip’ of a generation in extended CNC pedigrees that renders designation of some cases as ‘sporadic’.

CNC symptoms and diagnosis

CNC is a developmental disorder. The median age at detection is 20 years. Abnormal skin pigmentation may be present at birth; however, the lentigines usually do not assume their characteristic morphology until around puberty. Unlike other pigmented skin lesions, lentigines associated with CNC tend to fade after the fourth decade of life. Other pigmented lesions, including blue and other nevi, café-au-lait spots, and depigmented lesions may also be present at birth and are referred to as ‘birthmarks’; more frequently, however, these lesions develop in the early childhood years (Carney 1995). The café-au-lait spots in CNC are usually smaller and less pigmented than those in MAS; they also tend to fade with time. Their shape is more reminiscent of the NF syndromes.

Heart or cutaneous myxomas and Cushing syndrome due to PPNAD are the clinical conditions with which most CNC patients are first diagnosed (Atherton et al. 1980, Rhodes et al. 1984, Stratakis et al. 1999). There seems to be a bimodal age distribution of PPNAD among CNC patients: a minority of patients present in the first 2–3 years, whereas the majority manifest in the second and third decade of life (Fig. 1). Among the endocrine tumors, PPNAD is the most frequent manifestation of the disease, occurring in about one-quarter of the patients. This estimate, however, is likely to be low; biochemical screening by a dexamethasone-stimulation test can now detect patients with PPNAD-associated subclinical, atypical or periodic Cushing syndrome. Histological evidence of PPNAD has been found at autopsy in almost every patient with the complex. PPNAD often presents a similar clinical outcome with other forms of micronodular adrenocortical disease (MiAD) and could be misdiagnosed (see below).

Heart myxomas in CNC, unlike their sporadic counterparts, are equally distributed among ages and genders. They usually also present multicentrically in one, or all cardiac chambers, without predilection for the left side of the heart.

Cutaneous myxomas in CNC can occur anywhere, but relatively frequent sites include the eyelid, external ear canal, breast and nipples. Occasionally, myxomas can be found in the oropharynx, the female genital tract and pelvis.

Thyroid nodules (Stratakis et al. 1997), gonadal tumors (Premkumar et al. 1997, Stratakis et al. 2000) and schwannomas (Carney 1990, Carney & Stratakis 1998) and other lesions (Carney & Toorkey 1991, Carney & Stratakis 1996, Carney et al. 2001) may be present at the time of diagnosis but are rarely the reason for which most patients seek medical attention for the first time. LCCSCT (Premkumar et al. 1997, Stratakis et al. 2000) and thyroid nodules, appearing as microcalcifications and multiple, small, hypoechoic lesions upon testicular and thyroid ultrasonography respectively (Stratakis et al. 1997) appear often within the first 10 years of life. LCCSCT is usually multicentric, bilateral, but almost always benign. Testicular ultrasonography has also detected other tumors in CNC patients, including Leydig cell and (pigmented nodular) adrenocortical rest tumors. Thyroid cancer may develop in up to 10% of patients with CNC and thyroid nodules.

Clinically evident acromegaly is a relatively infrequent manifestation of CNC (Pack et al. 2000, Raff et al. 2000). However, asymptomatic GH and elevation of insulin-like growth factor type I levels and subtile hyperprolactinemia may be present in up to 75% of patients. Biochemical acromegaly is often unmasked by abnormal results of oral glucose tolerance tests or paradoxical responses to thyrotropin-releasing hormone administration. Somatotropin-mammotrop hyperplasia, a putative precursor of GH-producing adenoma, may explain the insidious and protracted period of establishment of clinical acromegaly in CNC patients.

PMS, a very rare tumor, has also been observed in CNC patients (Carney 1990, Carney & Stratakis 1998). PMS may occur anywhere in the central and peripheral nervous system but its most frequent site has been the gastrointestinal tract (esophagus, liver, stomach and rectum) and paraspinal sympathetic chain. CNC is the only genetic condition other than the NF syndromes and isolated familial schwannomatosis that includes schwannomas. The particular schwannoma in CNC is distinctive because of its heavy pigmentation (melanin), frequent calcification, and multicentricity. Imaging of the brain, spine, chest, abdomen (in particular the retroperitoneum), and the pelvis may be necessary for the detection of PMS, if there are suggestive symptoms.

The criteria used for the diagnosis of CNC are presented in Table 1 (Stratakis 2001); at least two of the major criteria need to be present for the establishment of the diagnosis. As has already been mentioned, spotty skin pigmentation is the most common clinical manifestation of CNC although it is not invariably present.
Genetics of MiAD, PPNAD and CNC

MiAD is a form of adrenocortical hyperplasia that was first recognized in the 1950s (De Moor et al. 1965). Like PPNAD, it leads to adrenocorticotropin (ACTH)-independent hypercortisolism that is caused by small nodules randomly arising within the cortex of both adrenal glands of affected individuals. ACTH is suppressed in both MiAD and PPNAD, and thus these conditions are readily differentiated from ACTH-dependent Cushing syndrome. Although for most of the 1980s and 1990s, several investigators, including our group, thought that all cases of MiAD were PPNAD, it is now becoming clear that non-pigmented forms of micronodular hyperplasia do not share the same genetic defects with the majority of PPNAD cases and probably constitute a different disorder, albeit with similar clinical outcome. Most cases of non-pigmented MiAD are sporadic and occur in very young children, but autosomal dominant inheritance cannot be excluded.

PPNAD leads to a pituitary-independent, primary adrenal form of hypercortisolism (Fig. 1). It is essentially a form of MiAD, perhaps the most frequent one, which is characterized by small, pigmented nodules that are surrounded by mostly atrophic cortex in an otherwise normal-sized gland (Shenoy et al. 1984). Most cases of PPNAD, inherited or sporadic, are associated with CNC (Stratakis et al. 2001).

Because of the similarities between CNC and MAS activating mutations of the GNAS1 gene (Stratakis 2001), or genes related to the cAMP-dependent PKA signaling pathway (Fig. 2) were long-considered possible candidates for CNC (De Marco et al. 1996). Genetic linkage analysis has identified two genetic loci harboring genes for CNC on 2p16 (CNC2 locus) and 17q22-24 (CNC1) (Stratakis et al. 1996, Casey et al. 1998). The PRKAR1A gene on 17q22-24 was positionally identified by our laboratory (Kirschner et al. 2000a), as the gene responsible for CNC in chromosome 17-mapping families. PRKAR1A mutations in patients with CNC were subsequently found by others, as well (Casey et al. 2000). Families that had recombinations with 17q22-24 but segregated with polymorphic markers from 2p16 are being used for the genetic mapping of the CNC region on 2p16 (see below).

Regulation of PKA activity by PRKAR1A

PRKAR1A encodes the regulatory subunit 1-alpha (R1α) of PKA, the main mediator of cAMP signaling in mammals (Scott 1991). The PKA holoenzyme is a tetramer consisting essentially of two dimers (for the most part, albeit not exclusively, homodimers (see below)): one composed of regulatory subunits and another of the two inactive catalytic subunits (Fig. 2). Co-operative binding of two cAMP molecules to each regulatory subunit results in dissociation and the consequent release (activation) of the two catalytic subunits. The latter phosphorylate, in turn, a wide variety of substrate proteins on serine or threonine residues (Tasken et al. 1993).

There are four genes encoding the different regulatory subunits (R1α, R1β, R1Hα, R1ββ) and three encoding the catalytic subunits (Cα, Cβ, Cγ (Foss et al. 1994)). The four

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Diagnostic criteria for Carney complex. To make a diagnosis of Carney complex, a patient must either: (i) exhibit two of the manifestations of the disease listed below, or (ii) exhibit one of these manifestations and meet one of the supplemental criteria (an affected first-degree relative or an inactivating mutation of the PRKAR1A gene)</th>
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<tr>
<td><strong>Manifestations of disease</strong></td>
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<tr>
<td>1. Spotty skin pigmentation with a typical distribution (lips, conjunctiva and inner or outer canthi, vaginal and penile mucosa)</td>
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<td>2. Myxoma (cutaneous and mucosal)*</td>
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<td>3. Cardiac myxoma*</td>
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<td>4. Breast myxomatosis*</td>
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<td>5. PPNAD* or paradoxical positive response of urinary glucocorticosteroids to dexamethasone administration during Liddle's test</td>
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<td>6. Acromegaly due to GH-producing adenoma*</td>
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<td>7. LCCSCT* or characteristic calcification on testicular ultrasonography</td>
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<tr>
<td>8. Thyroid carcinoma (at any age)* or multiple, hypechoic nodules on thyroid ultrasonography in a prepubertal child</td>
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<tr>
<td>9. Psammomatous melanotic schwannoma*</td>
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<tr>
<td>10. Blue nevus, epithelioid blue nevus (multiple)*</td>
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<td>11. Breast ductal adenoma (multiple)*</td>
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<tr>
<td>12. Osteochondromyxoma*</td>
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<tr>
<td><strong>Supplemental criteria</strong></td>
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<tr>
<td>1. Affected first-degree relative</td>
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<tr>
<td>2. Inactivating mutation of the PRKAR1A gene</td>
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*With histological confirmation.
types of regulatory subunits have different expression patterns in mammals. While \( \text{RI}_a \) has ubiquitous distribution, \( \text{RI}_b \) is expressed primarily in brain, testis and B- and T-lymphocytes (Clegg \textit{et al.} 1988, Scott 1991). Similarly, \( \text{RII}_a \) has ubiquitous distribution, while \( \text{RII}_b \) is expressed in brain, adipose, and some endocrine tissues (Skalhegg & Tasken 1997). The regulatory subunits are responsible for the two main isoforms of PKA; type I PKA and type II PKA, which were identified and named after their order of elution in Sepharose gel denaturing elution anion exchange chromatography (McKnight \textit{et al.} 1988). Each isoform is composed of two regulatory subunits and two catalytic subunits (Fig. 2) (McKnight \textit{et al.} 1988, Scott 1991, Tasken \textit{et al.} 1997). Although in the majority of cases the tetramer consists of two homodimers (one composed of two identical regulatory subunits, and another composed of two identical catalytic subunits), heterodimers have been identified in some cell types (Tasken \textit{et al.} 1997).

Type I PKA contains either regulatory subunit \( \text{RI}_a \) or \( \text{RI}_b \) in its structure; type II PKA contains either regulatory subunits \( \text{RII}_a \) or \( \text{RII}_b \) (McKnight \textit{et al.} 1988, Scott 1991, Tasken \textit{et al.} 1997). Based on \textit{in vitro} studies, the catalytic subunits bind preferentially to regulatory subunits type II; however, upon PKA’s activation the catalytic subunits favor type I PKA (Gamm \textit{et al.} 1996). The different regulatory subunit isoforms play specific roles in response to certain agonists. \( \text{RI}_a \) is primarily involved in the control of cell proliferation and neoplastic transformation. It also plays an important role in the transition from the G1 to S phase of the cell cycle (Sewing & Muller 1994, Tortora \textit{et al.} 1997). The RIIs mainly participate in the control of differentiation, growth arrest and induction of apoptosis (Cho-Chung \textit{et al.} 1995). Overexpression of \( \text{RI}_a \) has been frequently detected in cancer cell lines (Ciardiello & Tortora 1998).

Within the cell environment, several cAMP–PKA pathways are operational at any given moment. Activated PKA can phosphorylate different targets in response to different stimuli. Signal specificity is mediated by tissue-specific expression of the R and C subunits, compartmentalization of the tetramer by A-kinase anchoring proteins (AKAPs) and other factors (Colledge & Scott 1999, Wang \textit{et al.} 2000, Griffioen & Thevelein 2002). Compartmentalization of the PKA holoenzymes is an important aspect of signal specificity: through it, cAMP messaging is targeted to specific subcellular locations, such as the cytoskeleton, plasma membrane, nucleus, Golgi apparatus, endoplasmic reticulum and other organelles. Compartmentalization is ensured via interactions of the R subunits with the AKAPs (Faux & Scott 1996, Dell’Acqua & Scott 1997). Initially, only type II PKA was observed to be targeted by AKAPs, but recent evidence also demonstrates the association of some AKAPs with type I PKA (Angelo & Rubin 1998, Kussel-Andermann \textit{et al.} 2000).

Figure 1 (A) Pediatric patient with Cushing syndrome caused by PPNAD (reported by Gomez-Mugurusa & Chrousos 1989). (B) The computed tomography scan shows adrenal glands of normal size but somewhat distorted shape due to the micronodular hyperplasia. (C) Macroscopically both glands are of normal size but pigmented small nodules are seen throughout the cortex.
PRKAR1A genetics in CNC

Linkage analysis and use of loss-of-heterozygosity (LOH) (by microsatellite markers) and allelic loss (by fluorescent in situ hybridization (FISH)) allowed us to identify PRKAR1A as the gene mutated in more than half of patients with CNC and/or PPNAD (Kirschner et al. 2000a,b). More than 70 kindreds have been registered in our collection of CNC patients at the National Institutes of Health and the Mayo Clinic; so far, complete information for their PRKAR1A mutational status is available in 53 (Sandrini & Stratakis 2003). Mutations have been found in 28 patients (Sandrini & Stratakis 2003). In almost all mutations the sequence change is predicted to lead to a premature stop codon; one mutation altered the initiator ATG codon (Kirschner et al. 2000b). The most frequent PRKAR1A mutation in CNC is a deletion that results in frameshift (c.578delTG in exon 4B of the gene) (Casey et al. 2000, Kirschner et al. 2000a); other frequent mutations are present in exons 2 and 6. Analysis of mRNA transcripts in patient lymphocytes treated with cycloheximide showed that mutant mRNAs containing a premature stop codon were unstable, due to nonsense-mediated mRNA decay—also, the predicted mPRKAR1A protein products were absent in these cells (Kirschner et al. 2000a,b). Tumors from CNC showed 17q22-24 LOH (Kirschner et al. 2000a) or allelic loss; the wild-type allele was lost in the lesions where this could be documented (Kirschner et al. 2000a).

CNC tumors showed an increased kinase activity in response to cAMP when compared with non-CNC tumors (Kirschner et al. 2000b). Our most recent data (unpublished observations) show that this is due to the imbalance between type I and type II PKA in CNC-affected cells. How these abnormalities tie with some of the known effects of cAMP and PKA on growth and proliferation remains to be seen. However, the fact that increased kinase activity in response to cAMP underlies tumorigenesis in CNC and/or PPNAD was also recently demonstrated by in vitro studies of a unique PRKAR1A mutation that leads to an expressed and translated product (Groussin et al. 2002a). Interestingly, in benign tumors from the proband there was no LOH, indicating that even in the presence of haplo-insufficiency tumors may form, perhaps due to the imbalance between type I and type II PKA in affected cells (Groussin et al. 2002a).

The CNC2 locus, possible other loci and heterogeneity in PPNAD

So far, some small families with CNC appear to collectively map to the CNC2 locus on 2p16 (Kirschner et al. 2000b) that was first identified (Stratakis et al. 1996). A comprehensive genetic and genomic map consisted of YACs, BACs and P1-clones and incorporating STS, EST, and polymorphic marker information from the publicly available databases has been built by our laboratory (Kirschner et al. 1999) and is continuously updated. BACs from this area were most recently used in FISH experiments to demonstrate that both allelic losses and amplifications occurred most frequently from a region of a length of approximately 100,000 bp in the center of the 2p16 cytogenetic band area and most proximal to marker D2S123. This region may overlap with a secondary amplicon that has been identified in thyroid tumors (Chen et al. 1998), although not the one containing the PKCε gene (Knauf et al. 1999), which is more telomeric to the CNC2 locus. It has also been shown that 2p16 abnormalities are found in tumors from patients with germline PRKAR1A mutations, indicating, perhaps, an interaction between the two loci at the somatic level.
(Matyakhina et al. 2003). The 2p16 locus is known to be involved in sporadic ACTs (Kjellman et al. 1999) and these data have been also confirmed by our group (unpublished observations).

In addition to families with CNC and/or PPNAD that map to 2p16 and do not have PRKAR1A mutations (Kirschner et al. 2000b), phenotype-genotype studies appear to identify a subgroup of pediatric patients consisting mainly of non-pigmented MiAD, who collectively do not have PRKAR1A mutations. We recently analyzed the data from 26 PPNAD/MiAD patients; 16 met the diagnostic criteria for CNC (Stratakis et al. 2001) and all had PPNAD, whereas ten did not meet these criteria and had isolated (i) PPNAD or iMiAD. This latter group as a whole presented earlier with Cushing syndrome (as the patient shown in Fig. 1) and there were significant gender differences: boys presented with the disease at a much younger age than girls. PRKAR1A mutations were present in only two patients who both had iPPNAD vs 10 of the 16 patients with CNC. Among pediatric patients with CNC (and PPNAD), patients with PRKAR1A mutations presented with the disease earlier than those without mutations in this gene. FISH with BAC probes from the CNC1 (17q22-24, B321G8) and CNC2 (2p16, B400) loci revealed CNC1 allelic losses more frequently in iPPNAD vs PPNAD associated with CNC, whereas CNC2 probe abnormalities were not different between the two groups (Sandrini et al. 2001).

Thus, genetic heterogeneity in micronuclear adrenocortical hyperplasias may be more extensive than previously thought. It is hard, however, to identify the genes for these disorders; they are exceedingly rare, and at least for MiAD, the vast majority of the cases are sporadic.

**PRKAR1A and cancer**

R1α, is the main regulator of cAMP-dependent PKA (Fig. 2), a pathway that when activated leads to inhibition of growth and/or proliferation in several cell lines (Cho-Chung et al. 1999). Dominant R1α mutations were identified in the 1970s and 1980s in CHO cells, as well as S49 mouse lymphoma cells following selection for growth in high concentrations of cAMP analogs that inhibited R1α-cAMP binding (Kx cells), lacked detectable C subunit (kin-8 cells) or had decreased levels of the holoenzyme (Vmax mutants) (Chin et al. 2002). ACTH acting through cAMP-dependent PKA, and thus through R1α, inhibited the proliferation of Y1 mouse adrenocortical cell line (Lotfi et al. 1997). Enhanced expression of R1α has also been shown in several human cancer tissues and cell lines, including retinoblastoma, renal and breast cancer, the transformed BT5C glioma cell line, malignant osteoblasts, in serous ovarian tumors vs mucinous, endometroid or clear cell lesions (Fossberg et al. 1978, Handschin & Epfenberger 1979, Livesey et al. 1982, Nakajima et al. 1984, Watson et al. 1987, Piroli et al. 1990, Bradbury et al. 1994, McDaid et al. 1999). Increased R1α expression has been shown to be associated with chemical and viral carcinogenesis and oncogene-induced cell transformation, such as N-methyl-N-nitrosourea-induced gastric tumors in rats, human adenovirus-12-transformed rat 3Y1 cells, MuSV-transformed NIH/3T3 clone 13-3B-4 mouse cells, transforming growth factor (TGF)-transformed or v-Ki-ras-transformed rat kidney cells or TGF-transformed rat kidney cells and the human mammary epithelial cell line MCF-10AHE mutated in c-H-ras or transformed by the c-erbB-2 oncogene (Ledinko & Chan 1984, Tagliaferri et al. 1985, Yasui & Tahara 1985, Clair et al. 1987, Tortora et al. 1989, Ciardiello et al. 1990, 1993). It is also known that under normal growth, the cAMP/PKA pathway is required for phosphorylation of the tyrosine kinase Src that, activating Rap1 and blocking the activation of Raf-1 by Ras, inhibits cell proliferation (Ciullo et al. 2001). PKA is also known to induce apoptosis in CD10+ B cell when induced by forskolin; activation of PKA decreased the expression of Mcl-1, an anti-apoptotic Bcl family member (Myklebust et al. 1999). Finally, recent experiments with sequence-specific inhibition of the PRKAR1A gene through antisense oligonucleotides resulted in differentiation of leukemia cells and growth arrest of mouse tumors and epithelial cancer cells (Tortora et al. 1991). In summary, these data pointed to the suggestions that ‘decreased type I/type II PKA ratios and or upregulation of type II PKA correlate with growth inhibition and cellular differentiation’ and that R1α-blockade ‘provides a single-gene targeting approach to treatment of cancer’ (Cho-Chung et al. 1999).

The effects of PKA are associated with phosphorylation by the C subunit kinase of serine and threonine residues of target proteins (Fig. 2). Consequently, the R subunits are thought of mediating their action through inhibition of the C subunits (McKnight et al. 1988, Amieux & McKnight 2002, Chin et al. 2002). To the extent that C action mediates a mitogenic signal, when it does, R1α may be considered a ‘suppressor’ of this activity, as long as it restrains C subunits (Amieux & McKnight 2002). However in thyroid cells, cAMP may mediate mitogenic signals (although not only through PKA; Dremier et al. 2002). Studies in some human tumors tend to show lower type I PKA, in contrast to the investigations in cell lines, and mouse and rat cells cited above. In addition to data presented in this report from CNC and sporadic thyroid, adrenal and ovarian tumors (see below), R1α was found decreased in certain colon cancers (Carlson et al. 1999). Hepatoma cells also have
Table 2 Phenotypes of animal models of PKA regulatory subunit deficiency

<table>
<thead>
<tr>
<th>R subunit gene</th>
<th>Phenotype</th>
<th>Total PKA activity</th>
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<tbody>
<tr>
<td>Prkar1a --/-</td>
<td>Early embryonic lethality due to heart and other developmental defects</td>
<td>Increased</td>
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<tr>
<td>Prkar1b --/-</td>
<td>1. Defective hippocampal depotentiation, long-term-depression</td>
<td>Unchanged</td>
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<td></td>
<td>and other neuronal functional abnormalities</td>
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<tr>
<td></td>
<td>2. Reduced inflammation and response to pain</td>
<td></td>
</tr>
<tr>
<td>Prkar2a --/-</td>
<td>Normal: long-term follow-up (?)</td>
<td>Decreased</td>
</tr>
<tr>
<td>Prkar2b --/-</td>
<td>1. Lean phenotype, resistance to diet-induced obesity, increased</td>
<td>Decreased</td>
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<td></td>
<td>lipolysis, reduced plasma insulin and cholesterol</td>
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<td></td>
<td>2. Diminished motor learning</td>
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<td></td>
<td>3. Loss of haloperidol-induced catalepsy and gene expression</td>
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<td></td>
<td>4. Increased alcohol consumption and decreased alcohol-induced sedation</td>
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low R1α levels; PRKAR1A is the gene responsible for the tissue-specific extinguisher-1 phenotype (Jones et al. 1991). Overexpression of type I PKA interacts with activated epidermal growth factor receptor and stimulates growth and proliferation (Tortora et al. 1997). Furthermore, cisplatin resistance in cAMP-dependent PKA mutant cell lines indicates that the R subunits, R1α in particular, have non-PKA-mediated functions, including other protein–protein interactions and DNA binding (Liu et al. 1996, Cvijic & Chin 1997).

In support of the observations in CNC and sporadic endocrine tumors with regards to the function of R1α, and in contrast to cell line work listed above, is the observation that increased R1α levels in mouse in vivo are not associated with any tumors; they are rather associated with lean but otherwise healthy mice (Cummings et al. 1996, Amieux & McKnight 2002; see below, animal models) (Table 2). Indeed, the Prkar2b --/- mouse demonstrates compensatory increases of R1α protein expression in most tissues; this increase is associated with decreased cAMP-dependent total PKA activity which is, however, almost exclusively type I PKA (McKnight et al. 1998).

These data, albeit on the surface contradictory, point to the complex role of PKA and the R subunits in diverse functions, such as mediators of specific hormonal signaling (McKnight et al. 1988, Scott 1991, Amieux & McKnight 2002), G1/S phase progression (Van Oirschot et al. 2001), regulator of Ras/ERK signaling (Liebmann 2001, Pursiheimo et al. 2002) and fibroblast growth factor (FGF)-2 activated transcription (Pursiheimo et al. 2000), cytoskeleton formation and/or regulation (Han & Rubin 1996) and mitosis progression (Kerreyer et al. 1999), centrosome duplication or segregation (Imaizumi-Scherrer et al. 2001), and enhancer of transcriptional efficiency of target genes (Emanuel et al. 1998). These actions are further complicated by the tissue-specific expression of the various subunits that constitute the PKA tetramer, its cellular compartmentalization mediated by numerous anchoring proteins (Colledge & Scott 1999, Wang et al. 2001, Grifflon & Thevelein 2002), and its interactions with many of the major cellular signaling pathways, such as that of the mitogen-activated protein kinases (Schimmer 1995, Le & Schimmer 2001).

Is PRKAR1A involved in non-CNC tumors?

Since the identification of the PRKAR1A gene in CNC, seven research studies (Fogt et al. 2002, Groussin et al. 2002b, Kaltsas et al. 2002a, 2002b, Bertherat et al. 2003, Yamasaki et al. 2003) of which three from our laboratory (Sandrini et al. 2002a, 2002b, Bertherat et al. 2003) have addressed the issue of whether PRKAR1A is mutated in other endocrine (Groussin et al. 2002b, Kaltsas et al. 2002, Sandrini et al. 2002a, 2002b, Bertherat et al. 2003, Yamasaki et al. 2003) and non-endocrine (e.g. cardiac myxomas; Fogt et al. 2002) tumors. Patients with sporadic PPNAD appear to frequently have PRKAR1A mutations (Groussin et al. 2002b), but this is not surprising given the data that we presented in the previous section.

Although none of the sporadic non-CNC-related cardiac myxomas (Fogt et al. 2002) and pituitary tumors (Kaltsas et al. 2002, Sandrini et al. 2002a, Yamasaki et al. 2003) investigated so far has been found to have PRKAR1A mutations, R1α transcript changes, or 17q22-24 allelic losses, we identified one PRKAR1A inactivating mutation (Q167X), which has not previously been seen in PPNAD or CNC, in an undifferentiated thyroid carcinoma (Sandrini et al. 2002b). This mutation was somatic, i.e. it was not present in the patient’s peripheral DNA (Sandrini et al. 2002b). Thyroid carcinomas, unlike follicular adenomas, showed both 17q22-24 LOH and PKA activity alterations; immunohistochemistry supported these molecular and biochemical findings (Sandrini et al. 2002b).
More recently we examined the hypothesis that somatic alterations of 17q and/or the \textit{PRKAR1A} gene were associated with the development of 44 sporadic adrenal tumors, in 29 patients with single adenomas, and 15 patients with cancer, including 23 patients with Cushing syndrome (Bertherat \textit{et al.} 2003). A BAC probe containing the \textit{PRKAR1A} gene and ten linked microsatellite markers in a 10 cM area flanked by D17S942 and D17S1295 were used for studying 17q allelic losses. The \textit{PRKAR1A} gene was then sequenced in all samples. Allelic losses by FISH were seen in six of ten tumors, for which either DNA from normal tissue was not available or marker analysis was uninformative. LOH was detected in 4 of 20 adenomas (25\%) and 8 of 15 cancers (53\%) for which paired blood and tumor DNA samples were available. Three previously undescribed \textit{PRKAR1A} inactivating mutations were identified; these were not present in the leukocytes of these patients or in samples from 200 normal controls. The mutations predicted premature termination of the \textit{PRKAR1A} translated product. Accordingly, Western blotting showed decreased RIIz protein content. PKA functional studies demonstrated PKA activity changes that correlated with the allelic studies; among tumors without \textit{PRKAR1A} mutations, those with LOH for 17q22-24 had more total PKA and free PKA activity than those without LOH. The three adenomas bearing \textit{PRKAR1A}-inactivating mutations were associated with cyclical CS or ‘paradoxical’ increases of cortisol secretion in response to dexamethasone administration. Other adenomas with allelic losses of the 17q22-24 \textit{PRKAR1A} region were associated more frequently with atypical forms of Cushing syndrome (Bertherat \textit{et al.} 2003).

Studies of the \textit{PRKAR1A} gene and 17q22-24 locus on sporadic ovarian, colon, and basal cell skin carcinomas, melanomas and benign nevi are ongoing in our laboratory. Among these investigations, the most complete is that of ovarian tumors. Although mutations were not identified, both 17q22-24 LOH and PKA functional changes have been identified in ovarian cancer, but not in ovarian cysts (Stergiopoulos S & C A Stratakis, unpublished observations).

\textbf{PRKAR1A and chromosome stability}

It has been well documented that R1z and R1Hz of PKA may be associated with the cytoskeleton in both interphase and mitotic nuclei (Vallee \textit{et al.} 1981, Imaizumi-Scherrer \textit{et al.} 2001); in particular, R1Hz and R1Hz\_beta subunits may be associated with pericentriolar matrix of the centrosome during interphase (Keryer \textit{et al.} 1999), and the catalytic subunits with microtubules or the mitotic spindle (Imaizumi-Scherrer \textit{et al.} 2001). Association with these structures, which are major components of the mitotic apparatus, suggests that \textit{PRKAR1A} may play a very important role in different phases of chromosomal replication, segregation and/or cell division.

The centrosome plays a vital role during cell cycle. Its main role is to organize the mitotic spindle. At the onset of mitosis, two centrosomes initiate microtubule assembly and form the spindle where chromosomes are captured, aligned and then separated by kinetochore-microtubule interaction (Balczon 1996, Pihan & Doxsey 1999, Ou & Rattner 2000, Doxsey 2001). Thus, alterations in centrosome function or regulation may result in failure of cell division and/or missegregation of chromosomes (Len-gauer \textit{et al.} 1998, Doxsey 2001). This idea has been supported by several studies showing a correlation between changes in the centrosomes and the presence of chromosomal instability in cancer (Lengauer \textit{et al.} 1998, Pihan \textit{et al.} 1998, Ghadimi \textit{et al.} 2000, Gustafson \textit{et al.} 2000, Sato \textit{et al.} 2001).

Centrosomal localization of PKA regulatory and catalytic subunits indicates that these proteins may be involved in C-subunit-mediated phosphorylation of other molecules important for cell cycle progression, or that they mediate other, direct effects in this process. PKA is involved in phosphorylation of centrin, a component of the centrioles and the pericentriolar matrix; phosphorylation of centrin is necessary for normal centrosome division and for formation of the mitotic spindle during the G2/M phase of the cell cycle (Lutz \textit{et al.} 2001). Compartmentalization of PKA subunits to the centrosome is secured by AKAP350/450/CG-NAP and pericentrin (the latter is also an AKAP; Schmidt \textit{et al.} 1999, Takahashi \textit{et al.} 1999, Witzak \textit{et al.} 1999, Diviani \textit{et al.} 2000). It is interesting that both these AKAPs target PKA to the same subcellular site, but probably spatially segregate independent signaling events within it (Diviani & Scott 2001). Pericentrin, interacts with \gamma-tubulin and the motor protein dynein and is involved in the organization of the mitotic spindle (Doxsey \textit{et al.} 1994, Dictenberg \textit{et al.} 1998, Purohit \textit{et al.} 1999). PKA anchoring at the centrosome through pericentrin is important for normal centrosomal function. Disruption of PKA anchoring results in spindle abnormalities, apparently due to deficient dynein function (Diviani \textit{et al.} 2000, Diviani & Scott 2001). In addition to PKA, several kinases involved in regulation of cell cycle progression are localized at the centrosome and mitotic spindle poles. These include Cdk2, Cdk4/6, polo-like and aurora kinases, pEg2 and others (Balczon 1996, Whitehead & Salisbury 1999, Nigg 2001). Mutations of polo-like kinase-1 result in centrosome abnormalities and chromosomal instability in rodent cells (Smith \textit{et al.}...
This observation suggests that changes in localization or activity of PKA subunits may induce chromosomal instability through deficient centrosomal structure and/or function. In addition, stathmin (a protein necessary for microtubule stability) is phosphorylated by PKA (Larsson et al. 1997); over-expression of stathmin mutants that cannot be phosphorylated by PKA prevents the assembly of the mitotic spindle in vitro (Gradin et al. 1998, Howell et al. 1999). Similarly, transfection of mouse hepatoma cells with wild-type or mutant RIα subunits resulted in aberrant mitosis with multipolar spindles and mono- or multinucleated giant cells (Imaizumi-Scherrer et al. 2001).

Microtubules, a main component of the cytoskeleton, are polar polymers assembled from tubulin subunits that act as tracks for motor proteins. Their primary roles are vesicle and organelle movement, spindle assembly and, as mentioned above, chromosome segregation (in mitosis and meiosis) (Desai & Mitchison 1997, Andersen 1999, Cassimeris 1999). Association of RIα and meiosis) (Desai & Mitchison 1997, Andersen 1999, Cassimeris 1999). Association of RIα and RIβ as well as the catalytic subunits of PKA with microtubules suggests that PKA could also be involved in vesicle and organelle formation and/or movement. In a study quoted in the previous paragraph, asymmetric distribution of both RIα and C subunits on microtubules was observed suggesting that PKA could be also associated with the motor proteins regulating cytokinesis during cellular division (Imaizumi-Scherrer et al. 2001).

One potential target for PKA regulation is the metaphase–anaphase transition. Errors that occur during this transition lead to numerical chromosomal aberrations as a result of asymmetric distribution of chromosomes. Many epithelial tumors exhibit chromosomal instability caused by abnormalities during this transition (Steibeck 1998). The primary regulatory protein complex allowing for the initiation of anaphase is the anaphase-promoting complex, otherwise known as the cyclosome (APC/C). The APC/C, a highly regulated ubiquitin ligase, promotes the transition from metaphase to anaphase by targeting key mitotic proteins for proteolysis. If chromosomes are properly attached and aligned, the APC/C is activated by Cdc20. APC/C\(^{C_{dc20}}\) activation is inhibited by the PKA pathway in both fission yeast and mammalian cell models (Kotani et al. 1998, Yanagida et al. 1999). APC/C control over mitosis progression is modulated by the phosphorylation of both PKA and Cdc2-cyclinB-activated polo-like kinase. These proteins are involved in the correct timing of substrate-specific ubiquitination through binding of activators and cell cycle-dependent phosphorylation (Yanagida et al. 1999, Zachariae 1999). Finally, sister chromatid separation is the main event in metaphase–anaphase transition; it is also regulated by APC/C and, thus, indirectly by PKA and other kinases (Glover et al. 1998, Kotani et al. 1999). Mutations in PKA could affect chromatid separation and, thus, cause chromosomal instability by yet another mechanism.

Is PRKAR1A a tumor-suppressor gene?

Although LOH and allelic losses of the 17q22-24 PRKAR1A locus have been seen in CNC tumors (Kirshner et al. 2000a) and, as discussed above, they are frequently detected in some sporadic tumors (Groussin et al. 2002b, Sandrini et al. 2002b, Bertherat et al. 2003), it remains unclear whether PRKAR1A’s multiple functions would classify this gene as a ‘classic’ tumor-suppressor.

LOH is a key pointer to the function of a gene as a tumor-suppressor, according to Knudson’s hypothesis (Mendelsohn & Liotta 1995). Tumor-suppressors, unlike oncogenes, generally operate in a recessive manner, requiring loss of both copies for tumorigenesis; haploinsufficiency leads to simple predisposition to tumor development. Furthermore, a gene with tumor-suppression function, when both its copies are lost, generally should exert inhibitory effects on the cell cycle, halt cellular proliferation or abate neoplastic transformation initiated by other molecular events, or activate senescence in the presence of unfavorable growth conditions (Mendelsohn & Liotta 1995). Examples of ‘classic’ tumor-suppressor genes, that meet all or part of these criteria are the retinoblastoma (RB1) and the TP53 genes. However, as we learn more about the functions of these and other genes, we find roles that defy the stereotypes. The TP53 gene provides a very instructive example of a tumor-suppressor gene that under certain conditions can act as a dominant oncogene. Transfection studies in NIH 3T3 cells showed that TP53 could function as an oncogene to immortalize and transform cells in conjunction with a mutant ras gene. And more recently, Lkb1, a serine-threonine kinase that functions as a tumor-suppressor, and is the mouse homolog of the LKB1 gene mutated in a disease that is related to CNC Peutz-Jeghers Syndrome, actually enhanced resistance to neoplastic transformation of mouse cells in vitro (Bardeesy et al. 2002).

PRKAR1A, it almost safely may be said, could not be a ‘classic’ tumor-suppressor gene because of its multiple interactions with major signaling pathways and often opposite effects on important cellular functions that we have reviewed in this report.

First, PRKAR1A appears to have different effects under different conditions (cellular stage, differentiation, signaling mode). Otherwise, how can one reconcile the known inhibitory effects of cAMP on cell line proliferation, the data from targeted disruption of Prkar1a in mice (which showed increased PKA activity, Table 2), and the fact that PRKAR1A loss in human CNC tissues, not only
leads to increased PKA activity (as expected from the mouse model) but it is also associated with tumor formation, and in at least some cases cancers? PKA activity appears to interact differently in nascent mesoderm vs adult endocrine cells. In Sertoli, adrenocortical and Schwann cells, thyrocytes, somatotrophs, and melanocytes, cAMP/PKA ligands (follicle-stimulating hormone, ACTH, neuregulin, thyrotropin, GH-releasing hormone and melanocyte-stimulating hormone) can stimulate mitogenesis (Amieux et al. 2002).

Secondly, for some of its tumorigenic effects, LOH may not be necessary; haploinsufficiency may be sufficient, as we have recently suggested (Groussin et al. 2002a). So far, we have also not found significant 17q22-24 allelic losses in CNC-associated cardiac and skin myxomas (C A Stratakis, unpublished observations). The development of these tumors in CNC suggests a yet-uncharacterized mechanism for PKA-dependent proliferation of pluripotent primitive mesenchymal cells, despite the evidence that PKA can inhibit growth factor/receptor tyrosine kinase-mediated cell proliferation (Amieux & McKnight 2002). It is conceivable that in the sensitive balance that maintains normalcy in these cells, R1z haploinsufficiency is enough to lead to uncontrolled proliferation. This could be mediated through steadily increased PKA activity (the known effects of PKA on mesenchymal cells are derived from studies that looked only at acute increases of PKA-mediated signaling (Amieux & McKnight 2002), or through non-PKA-mediated R1z effects on DNA repair and elsewhere (Chin et al. 2002).

Our most recent data from studying transformed cell lines from CNC patients who carried inactivating PRKAR1A mutations in heterozygosity, indicated that R1z haploinsufficiency is sufficient for the appearance of increased PKA activity levels (Robinson-White et al. 2003). Increased cAMP-stimulated PKA activity in these cells was also associated with increased proliferation rates and ERK1/2 phosphorylation, suggesting that, unlike a ‘classic’ tumor-suppressor gene, PRKAR1A-haploinsufficient cells are not normal.

Furthermore, we recently reported that microdissected adrenal cortex from PPANAD specimens showed not only retention of heterozygosity, but also evidence of genomic amplification of the 17q22-24 locus, which was associated with increased immunoreactivity for R1z (Sandrini et al. 2002c).

Taken together, these data point to a non-conventional role of R1z in tumorigenesis. The potential interactions between PRKAR1A and other PPANAD or MiAD-causing genes (as suggested by abnormalities of the 2p16 chromosomal region in tumors of CNC or PPANAD patients with germline R1z defects) further enhanced our interest in trying to define this gene’s function.

**PKA and PRKAR1A in animal models**

A summary of what we know about the mouse animal models of the PKA regulatory subunits (McKnight et al. 1998, Cho-Chung et al. 1999, Amieux & McKnight 2002, Amieux et al. 2002), including Prkar1a−/− is presented in Table 2. In brief, these studies in mice and in other in vivo models (e.g. fruit fly) have suggested that PKA negatively regulates sonic hedgehog signaling (Jiang & Struhl 1995, Lepage et al. 1995, Li et al. 1995, Pan & Rubin 1995, Strutt et al. 1995). This action is mediated mainly by Prkar1a through its actions on the catalytic subunits (Cz and Cβ); the end-result of this interaction is inhibitory control of PKA activity (Amieux & McKnight 2002, Amieux et al. 2002). This is also supported by the fact that crossing Prkar1a−/− with PrkarCa−/− mice rescues the Prkar1a−/−-associated phenotype (although the mice still die later of other defects that have not been well characterized).

Several mice have been reported with early embryonic lethality caused by defects similar to those induced by Prkar1a deficiency, such as the FGF-4/FGF-8, FGF receptor-1, fibronectin, focal adhesion kinase, Src and Fyn mouse models, suggesting molecular pathways that may be interacting with PRKAR1A at least during early development (Yamaguchi et al. 1994, Furuta et al. 1995, Ilic et al. 1995, Georges-Labouesse et al. 1996, Ciruna et al. 1997, Klinghoffer et al. 1999, Liu et al. 1999, Sun et al. 1999, Ciruna & Rossant 2001). It is no surprise that the complete loss of all PKA activity (Cz−/− Cβ−/− mice) results in early embryonic lethality, too. However, crosses of the catalytic subunit knock-outs have various developmental defects. There is no information on tumorigenesis of any of these mice or the respective heterozygotes. Careful long-term follow-up data, however, are lacking.

**Concluding remarks**

Over the last decade, several lines of evidence have suggested that the PKA holoenzyme is involved in several processes including signal transduction, cell cycle regulation, chromosomal stability and cellular homeostasis. It remains to be seen whether the regulatory subunit PRKAR1A behaves as a tumor-suppressor, an oncogene or merely acts by modulating the levels of PKA activity. Germline mutations and subsequent LOH at the PRKAR1A locus in tumors of CNC patients are consistent with the Knudson ‘two hit’ theory; these data support a role for PRKAR1A as a tumor-suppressor gene, although similar observations have also been made.
recently for oncogenes (RET). Haplo-insufficiency of \textit{PRKAR1A} leads to an increase in kinase activity; the latter appears to be associated with the multiple tumor formation and tendency for carcinogenesis in CNC. It may well be that dysregulation of the PKA tetramer, rather than levels of a particular subunit, is the mediator of tumorigenesis; studies on cell lines and animal models are ongoing to define the tumor-suppression and/or oncogenic activity of \textit{PRKAR1A}.

\section*{Acknowledgements}

We thank Ms Caroline Sandrini (from Santa Catarina, Brazil) for the art and graphics included in Fig. 2.

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