Disruption of Protein Kinase A Regulation Causes Immortalization and Dysregulation of D-Type Cyclins

Kiran S. Nadella¹ and Lawrence S. Kirschner¹²

¹Human Cancer Genetics Program and ²Division of Endocrinology, Diabetes and Metabolism, Department of Internal Medicine, The Ohio State University, Columbus, Ohio

Abstract
Phosphorylation is a key event in cell cycle control, and dysregulation of this process is observed in many tumors, including those associated with specific inherited neoplasia syndromes. We have shown previously that patients with the autosomal dominant tumor predisposition Carney complex carry inactivating mutations in the PRKARIA gene, which encodes the type 1A regulatory subunit of protein kinase A (PKA), the cyclic AMP–dependent protein kinase. This defect was associated with dysregulation of PKA signaling, and genetic analysis has suggested that complete loss of the gene may be required for tumorigenesis. To determine the mechanism by which dysregulation of PKA causes tumor formation, we generated in vitro primary mouse cells lacking the Prkar1a protein. We report that this genetic disruption of PKA regulation causes constitutive PKA activation and immortalization of primary mouse embryonic fibroblasts (MEFs). At the molecular level, knockout of Prkar1a leads to up-regulation of D-type cyclins, and this increase occurs independently of other pathways known to increase cyclin D levels. Despite the immortalized phenotype, known mediators of cellular senescence (e.g., p53 and p19ARF) seem to remain intact in Prkar1a−/− MEFs. Mechanistically, cyclin D1 mRNA levels are not altered in the knockout cells, but protein half-life is markedly increased. Using this model, we provide the first direct genetic evidence that dysregulation of PKA promotes important steps in tumorigenesis, and that cyclin D1 is an essential target of PKA.

Introduction
Carney complex is an autosomal dominant inherited neoplasia syndrome characterized by spotty skin pigmentation, myxomas, endocrine tumors, and schwannomas (1). We and others have shown that this disease is caused by inactivating mutations in the PRKARIA gene, encoding the type 1A regulatory subunit of the cyclic AMP (cAMP)–dependent protein kinase, protein kinase A (PKA; refs. 2–4). Furthermore, we have shown that mutation of this ubiquitously expressed regulatory subunit causes increased PKA activity in tumors from patients with Carney complex (2). PRKARIA (R1A) mutations in Carney complex tumors induce nonsense-mediated mRNA decay resulting from premature termination codons (3). Partial or complete loss of heterozygosity of PRKAR1A is seen in Carney complex–associated tumors (2) as well as in sporadic tumors of the thyroid and adrenal glands (5, 6), indicating that it is a tissue-specific tumor suppressor gene.

Because patient samples are rare, we have generated a mouse model to study this condition. Mice homozygous for a null allele of Prkar1a exhibit embryonic lethality, whereas heterozygotes, like their human counterparts, are tumor prone (7–9). To determine the mechanism by which loss of Prkar1a promotes tumors, we have chosen to use primary cultures of mouse embryonic fibroblasts (MEFs), which lack secondary alterations commonly found in immortalized cell lines. Using Cre-lox technology, we generated Prkar1a−/− MEFs in vitro and compared them with otherwise genetically identical cells. We report that removal of Prkar1a from cells leads to immortalization in the absence of transformation. In probing the molecular basis for this observation, we find that there is strong up-regulation of cyclin D1, which is likely linked to the immortalized phenotype. This increase in cyclin D1 protein levels occurs independently of other known pathways, suggesting that PKA itself functions as a regulator of cyclin D1 protein levels.

Materials and Methods

Generation of Prkar1a−/− mouse embryonic fibroblasts. Mice carrying a conditional null allele of Prkar1a (8) were crossed, and primary MEFs were prepared from embryonic day 13.5 embryos. Each MEF line was derived from an individual embryo and PCR genotyped as described (8). Cells were cultured in DMEM with 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) containing 14% fetal bovine serum (HyClone, South Logan, UT). All the experiments presented here were done in two to three independent MEF lines prepared from different litters. Mice used for preparation of MEFs were maintained in accordance with the highest standards of animal care in accordance with Institutional Laboratory Animal Care and Use Committee guidelines.

Retroviral infections. High titer retroviruses were produced by transient transfection of retroviral constructs using Phoenix-Eco packaging cells (10) by calcium phosphate precipitation (ProFection calcium phosphate kit, Promega, Madison, WI). Passage 2 MEFs were infected with virus and selected for 4 days in the presence of either puromycin (2.5 µg/mL) or hygromycin (400 µg/mL). The following previously described plasmids were used: pBABE-puro, pBABE-puro-Cre, pBABE-hygro (11), and pBABE-puro-cyclin D1 (12). pBABE-hygro-hemagglutinin (HA)-R1A was prepared by subcloning the HA-tagged human PRKAR1A cDNA from pREP4-HA-R1A (13) into the pBABE-hygro vector.

Cell proliferation and synchronization. The 3T3 assay was done essentially as described (14). To assess serum-free growth, 300,000 cells were plated in 100-mm dishes containing complete medium. After 24 hours, cells were trypsinized and counted. The medium in the remaining plates was replaced by serum-free DMEM, and cells were counted every 24 hours. For cell cycle studies, subconfluent MEFs were synchronized by incubation in serum-free DMEM for 72 hours and then stimulated to proliferate by addition of DMEM containing 14% serum. Cells were harvested at the indicated times for Western blotting.

Western analyses. MEFs were lysed in M-PER protein extraction reagent (Pierce, Rockford, IL) unless otherwise stated. Proteins were resolved in SDS-PAGE gels and transferred to nitrocellulose (Pall, East Hills, NY), and

Requests for reprints: Lawrence S. Kirschner, Human Cancer Genetics Program, Ohio State University, 544 TMRF, 420 West 12th Avenue, Columbus, OH 43210. Phone: 614-292-1190; Fax: 614-688-4006; E-mail: Lawrence.Kirschner@osumc.edu.

doi:10.1158/0008-5472.CAN-05-3183

the blots were developed with Western lightning reagents (Perkin-Elmer, Boston, MA). The antibodies used in this study were as follows: PKA-R1A, PKA-R2A, PKA-R2B, PKA-CA, and BB (554136) were from BD Biosciences (San Jose, CA). Cyclin D1 (SC-718), cyclin A (SC-396), cyclin E (SC-481), cyclin B1 (SC-245), proliferating cell nuclear antigen (SC-56), p16 (SC-1207), p19 (SC-1062), p21 (SC-471), cyclin-dependent kinase (CDK) 4 (SC-601), CDK2 (SC-163), and E2F1 (SC-251) were from Santa Cruz Biotechnology (Santa Cruz, CA). p21 (2552), phosphorylated extracellular signal-regulated kinase (ERK) p44/p42 (9102), ERK p44/p42 (9102), phosphorylated Creb (9196), phosphorylated glycogen synthase kinase 3β (GSK3β; 9363), phosphorylated p38 (9216), and p38 (9212) were from Cell Signaling Technologies (Beverly, MA). Monoclonal anti-HA (MMS-101P) was from Covance (Princeton, NJ). Actin (A5060) was from Sigma (St. Louis, MO). PTEN monoclonal antibody was a kind gift from Dr. C. Eng (The Ohio State University, Columbus, OH), and cyclin D3 monoclonal antibody was a gift from Dr. D. Guttridge (The Ohio State University, Columbus, OH).

Quantitative real-time PCR analyses. mRNA was isolated from Prkar1a+/- and Prkar1a-/- MEFs by Trizol reagent (Invitrogen) and reverse transcribed using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative real-time PCR (qRT-PCR) was done using an iCycler and iCycler SYBR Green reagents (Bio-Rad). Cyclin D1 primers were forward: ATGTGAGTCTATTCCAAACC and reverse: TTGACTCCAGAAGGGCTG.

Immunofluorescence studies. MEFs were grown on glass coverslips, fixed in 4% paraformaldehyde, and permeabilized using 0.2% Triton X-100 analysis was representative of two independent experiments. A= with and without 5 μmol/L H-89 (Upstate) or 50 μmol/L forskolin (Sigma) for 12 hours. For half-life studies, MEFs were treated with cycloheximide for the indicated times and whole-cell lysates were made and then immunoblotted with cyclin D1 antibodies. For analysis of cyclin D1 degradation, asynchronous MEFs were treated with MG132 (30 μmol/L; Sigma) or calpain inhibitor 1 (100 μmol/L; Sigma) for the indicated times, and cells were harvested for preparation of whole-cell lysates. Western blots of the lysates were probed with the cyclin D1 antibody and quantitated as above.

Transient transfections. For transfections, 293T cells were seeded into 100-mm Petri dishes 1 day previously and transfected with either constitutively active PKA-C or dominant-negative PKA plasmids using Superfect (Qiagen) according to manufacturer’s recommendations. After 24 hours of transfection, cells were stimulated with 10 μmol/L forskolin (Sigma) for further 24 hours to induce the expression of R1A after which cells were harvested for protein preparation. All transfections were done a minimum of three times before calculating means and SDs.

Treatment with DNA-damaging agents. MEFs were expanded and plated onto 100-mm plates and grew them overnight to a confluency of 60% to 70%. The cells were washed with PBS and either exposed to UV-C radiation at 40 J/m² or treated with 3.5 μmol/L doxorubicin for 8 hours. MEFs were harvested after 8 hours of treatment for protein extraction.

Results

Generation and characterization of Prkar1a+/-, Prkar1a-/-, and Prkar1a-/- mouse embryonic fibroblasts. To explore the molecular basis for Prkar1a’s role in tumorigenesis, mice carrying a conditional null allele were bred and MEFs were prepared. Prkar1a-/-, Prkar1a-/-, and Prkar1a+/- cells were obtained in culture by infecting Prkar1aloxP/loxP or Prkar1a-/- cells with a retrovirus carrying Cre recombinase or a control virus lacking Cre. Genotyping and Western blotting confirmed the loss of Prkar1a coding sequences and protein (Fig. L4 and B). Although PCR was able to detect the intact conditional allele in Cre-treated cells at passage 5, there was no detectable Prkar1a protein by Western blotting. The cells exhibited a null genotype by passage 10. As expected, control virus-treated cells retained the intact conditional allele and the Prkar1a protein throughout the study, whereas Prkar1a-/- cells had intermediate Prkar1a protein levels. Interestingly, the PKA catalytic subunit was increased 2.5-fold in Prkar1a-/- cells, whereas the other regulatory subunits remained unchanged (Fig. L1B). To address the functional effects of these alterations, we measured total and free PKA activity in the cells using a novel nonradioactive PKA assay (see Materials and Methods). In agreement with the published data from Carney complex tumors (2), we observed an increased total and free PKA activity in Prkar1a-/- cells compared with heterozygote and the WT counterparts (Fig. 1C).

The effect of loss of Prkar1a on cell proliferation was assessed by 3T3 assay (Fig. 1D). Although WT MEFs underwent typical senescence after 10 to 12 generations, Prkar1a-/- cells proliferated unabated and continued to do so for >50 passages. Similar results were obtained in more than six independently replicated experiments. Furthermore, early-passage Prkar1a-/- MEFs were able to proliferate in serum deprived conditions, whereas control cells arrested within 24 hours of serum removal (Fig. 1E). Despite their cell cycle arrest, the cell cycle of the Prkar1a-/- cells was not transformed, as they retained contact inhibition, did not exhibit anchorage-independent growth, and did not form tumors in scid mice (data not shown).

Loss of Prkar1a is associated with up-regulation of cyclin D1. Owing to the functional association of cyclins with cell proliferation and cell cycle regulation, we evaluated cyclin levels in...
asynchronous populations of cells. Unexpectedly, we observed a striking (3.5-fold) up-regulation of cyclin D1 in Prkar1a/C0 MEFs compared with the heterozygous and WT cells (Fig. 2A). This elevation in cyclin D1 was observed at early passages (passage <10), at a time when there was no significant difference in the growth rate between knockout and WT cells. Confocal microscopy confirmed the presence of increased cyclin D1 and showed that the protein has increased nuclear retention (Fig. 2B). Consistent with these observations, tumors obtained from Prkar1a+/C0 mice showed increased nuclear localization of cyclin D1 by immunohistochemistry (Fig. 2C). Other cyclins were not significantly altered in the knockout cells either at the mRNA level (data not shown) or at the protein level (Fig. 2A). In concert with the elevated cyclin D1 levels, an intact RB pathway was demonstrable by normal expression and phosphorylation of RB and normal S-phase reentry kinetics after serum starvation and release as shown by Western blotting (see below).

To explore the mechanism of up-regulation of cyclin D1 levels, we first examined cyclin D1 mRNA levels. Neither microarray hybridization nor qRT-PCR (Fig. 2D) showed a significant change in the levels of cyclin D1 mRNA, suggesting that post-transcriptional mechanisms might account for the change. Because cyclin D1 levels are tightly regulated by proteolysis, we sought to determine if there was an alteration in protein stability. Cycloheximide treatment (Fig. 2E) showed that cyclin D1 had a significantly longer half-life in the knockout cells (76 minutes) compared with WT cells (23 minutes). Treatment of cells with the proteasome inhibitor MG132 led to a modest increase in cyclin D1 in the cells, whereas treatment with a calpain inhibitor led to a marked increase in levels (Fig. 2F). These data indicate that calpain is primarily responsible for cyclin D1 degradation in these cells, as has been observed in other mouse fibroblasts (17), and that protein stabilization is primarily responsible for the observed increase in cyclin D1 protein levels.

Up-regulation of cyclin D1 in Prkar1a/C0 mouse embryonic fibroblasts is independent of other signaling pathways. Because GSK3β is known to phosphorylate cyclin D1 at the G1-S transition and target it to the cytoplasm for further degradation (18), we examined its activity in the WT and knockout cells. There was no detectable difference between knockout and WT cells, indicating that the alterations in stability and increased nuclear retention are not due to changes in GSK3β activity (Fig. 3A).

To assess the potential contributions of other (non-PKA-mediated) pathways to the elevated cyclin D1 levels, we measured activity of other pathways shown previously to regulate levels of this protein, including Ras/Raf/ERK, phosphatidylinositol 3-kinase/Akt, and nuclear factor-κB (NF-κB; refs. 19–22). GTP-loaded Ras was measured by a pull-down assay as a direct measurement of Ras activity (Fig. 3A), which showed that the active Ras levels were equivalent or even slightly decreased in Prkar1a/C0 cells when compared with their WT or heterozygous counterparts. We also

---


---

Figure 1. Targeted disruption of Prkar1a leads to up-regulation of PKA activity and an immortalized phenotype. A, Prkar1aloxP/loxP MEFs were infected with the control or Cre retrovirus at passage 1 and PCR genotyped at passage 5 (P5) or passage 10 (P10). B, Western blot of the same MEFs with the indicated antibodies. C, measurement of total and free PKA activity in Prkar1a-/-, Prkar1a+/-, and Prkar1a+/- MEFs at passage 8. D, 3T3 assay for measuring rate of proliferation in MEFs infected with control or a Cre retrovirus. E, assessment of serum-independent growth in Prkar1a-/- and Prkar1a+/- MEFs at passage 8.
measured the levels of the activated (phosphorylated) form of the ERKs p42 and p44. In agreement with the Ras data, no excess accumulation of phosphorylated ERK was detected. Furthermore, changes in phosphorylated p38 levels did not account for the changes in cyclin D1, excluding the possible involvement of mitogen-activated protein kinases (MAPK) in this process (Fig. 3B).

Similarly, immunoblotting with an antibody that recognizes all forms of phosphorylated Akt revealed that levels of total and phosphorylated Akt were unchanged in the knockout cells (Fig. 3B).

Finally, to determine if increases in cyclin D1 could be attributed to NF-κB activation, we used a luciferase reporter gene driven by NF-κB response elements to assess the activity of this pathway. This experiment revealed that NF-κB activity was not increased in the knockout cells and was, in fact, somewhat reduced.4

At the functional level, we tested if elevated cyclin D1 levels were associated with increased CDK activity. CDK4 and CDK2 activities were measured in WT and knockout cells and found to be essentially unchanged (Fig. 3C). This observation prompted us to look at the amounts of CDK4 physically associated with cyclin D1 by coimmunoprecipitation experiments. This experiment, done either by immunoprecipitation with anti-CDK4 and immunoblotting with cyclin D1 or in the converse fashion, showed that the amount of CDK4 bound to cyclin D1 did not change with loss of Prkar1a (data not shown). Interestingly, there is a consistent up-regulation of p16INK4a levels, a known CDK4/CDK6 inhibitor (see below; ref. 23).

Regulation of cyclin D1 by protein kinase A. To confirm that the increases in cyclin D1 were directly due to increases in PKA activity, we treated WT and knockout cells with the PKA inhibitor H-89 or with forskolin, a stimulator of adenylate cyclase. In both WT and Prkar1a−/− cells, H-89 produced a small but consistent decrease in total cyclin D1 levels at doses where the compound predominantly inhibits PKA (24), whereas forskolin did not alter total cyclin D1 levels (Fig. 4A). Interestingly, we noted a shift in the relative abundance of the cyclin D1 bands, with a decrease in the upper band noted in response to H-89, and an obvious increase in the slower migrating form of cyclin D1 on forskolin treatment. To confirm the identity of the slower migrating form of the protein as a phosphorylated isoform, protein lysates were treated in vitro with phosphatase to induce nonspecific protein dephosphorylation (Fig. 4A, rightmost lanes). This treatment abolished the upper cyclin D1 band with only minimal effects on the lower (presumably unphosphorylated) form of the protein.

To further confirm the role of PKA in regulation of cyclin D1, we transfected 293T cells with the PKA catalytic subunit or with a dominant-negative Prkar1a isoform that does not bind cAMP (25). Western blot analysis showed that dominant-negative PKA consistently down-regulated endogenous cyclin D1 levels (Fig. 4B), although no significant changes were seen with overexpression of

---

4 K.S. Nadella and L.S. Kirschner, unpublished observations.
the active catalytic subunit. The induction of dominant-negative PKA was confirmed by increased expression of R1A, providing evidence that the observed changes in cyclin D1 were effects specific for PKA action.

**Immortalization of Prkar1a−/− mouse embryonic fibroblasts is independent of p53/p19ARF and other pathways.** To assess for possible non–cyclin D1–mediated effects and to further explore the mechanism of cellular immortalization, we sought to determine if senescence pathways remained intact. Surprisingly, measurement of p53 activity by a luciferase reporter showed that the p53 pathway did not differ significantly between WT and knockout cells (Fig. 5A). Serially passaged Prkar1a−/− and Prkar1a+/+ MEFs showed intact p53, p19ARF, and p16INK4a which showed evidence for loss of p53 and p19ARF (Fig. 5B). The levels of p16INK4a consistently remained up-regulated in knockout cells (Fig. 5A). The results of serial passage (passage 22) Prkar1a−/− MEFs, the p53 pathway remained intact as judged by p53 stabilization and growth arrest (shown by induction of p21Cip1) after exposure to UV and DNA-damaging agents, such as doxorubicin (Fig. 5C). Examination of the tumor suppressor genes involved in cellular senescence detected only moderate down-regulation of p21Cip1 and a marked up-regulation of p27Kip1 (Fig. 5D). Confocal microscopy of p27Kip1 confirmed the notable up-regulation of the protein and further showed that the protein remained localized in the nucleus (Fig. 5E). A similar analysis of p21Cip1 confirmed slight quantitative reductions as well as a redistribution from a predominantly nuclear localization to a mixed cytoplasmic-nuclear distribution (Fig. 5F).

**Figure 3.** Other mediators of cyclin D1 increases are not altered in Prkar1a−/− MEFs. A, measurement of GSK3β activity in MEFs by Western blotting using phosphorylated GSK3β antibody. The genotypes of the cells are shown on top of each set of lanes. B, measurement of GTP-loaded Ras, total and phosphorylated ERK, Akt, and p38MAPK in MEFs. C, measurement of CDK4 and CDK2 kinase activities in MEFs of the indicated genotypes.

**Figure 4.** Elevations of cyclin D1 are a direct effect of elevated PKA activity. A, representative immunoblot showing cyclin D1 and CDK4 levels in H-89 or forskolin (FSK)–treated MEFs. Right, Prkar1a−/− cell lysates treated with and without a phosphatase and probed for cyclin D1; bottom, quantitation of the bands for total cyclin D1 normalized against CDK4 loading control (left) as well as the ratio between upper and lower cyclin D1–specific bands (right). B, representative Western blot of 293T cell lysates transiently transfected with constitutively active (CA) and dominant-negative (DN) PKA plasmids. Bottom, quantitation of the cyclin D1 and Prkar1a levels, normalized to actin. *, P = 0.01, compared with untransfected cells.

**Cell cycle–dependent regulation of cyclin D1 in Prkar1a−/− and Prkar1a+/+ mouse embryonic fibroblasts.** To better understand the regulation of cyclin D1, cells synchronized for 72 hours by serum deprivation were stimulated to proliferate by addition of serum. As expected, cyclin D1 accumulated after 9 hours in WT cells, whereas in Prkar1a-null MEFs, levels did not vary significantly over the experimental time course. Examination of the pattern of band mobility also showed differences: a doublet of cyclin D1 was observed only from 0 to 6 hours in the WT cells, but two bands were seen at all times in the Prkar1a−/− MEFs (Fig. 6A and B). This observation implies a cell cycle–dependent regulation of cyclin D1 synthesis, which is abolished in the presence of elevated PKA activity.

Cyclin D1 levels rose during G1 coincident with the decrease of p27Kip1 levels in control cells, whereas the oscillations of cyclin D1, p27Kip1, and CDK4 roughly paralleled each other in Prkar1a−/− MEFs.
MEFs, suggesting that a similar mechanism (i.e., phosphorylation) may also be involved in their up-regulation. We observed similar deregulation of cyclin D3 levels in R1A-null MEFs (Fig. 6A and B), whereas cyclin D2 levels were essentially undetectable in the cells (data not shown), which is in agreement with the data reported in MEFs (26). Timely synthesis of different cell cycle markers was observed in WT cells, suggesting a stringent regulation of cell cycle. In contrast to the dysregulation of D-type cyclins, the expression of other cell cycle markers was not significantly different between knockout and WT MEFs.

To confirm that loss of Prkar1a was the primary factor responsible for the observed changes, we reintroduced HA-tagged PRKAR1A (13) into null and WT MEFs to see if the defect could be rescued. Prkar1a<sup>−/−</sup> cells into which HA-R1A had been reintroduced were synchronized by serum deprivation for 72 hours and harvested at different time points after addition of serum-containing medium. Although not fully reverted to normal, the kinetics of cyclin D1, p27<sup>Kip1</sup>, and CDK4 in HA-R1A cells were closer to those observed in WT cells (Fig. 6C). Specifically, cyclin D1 levels increased starting from 6 hours, coincident with a decrease of p27<sup>Kip1</sup> levels; in addition, the cyclin D1 doublet was detected only at early times after release into the cell cycle, confirming a partial restoration of normal cyclin D1 dynamics.

We ascribe the observation that we were only able to observe partial restoration of normal cell cycle regulation to the fact that HA-tagged R1A expression was generally quite poor in the knockout compared with robust expression in WT cells (data not shown).

**Discussion**

In this article, we report the initial phases of our work to understand the molecular basis by which patients with the Carney complex develop tumors. Compared with the study of human or mouse tumors in vivo, the in vitro system used in this article has the advantages that the cells are uniform and that they can be analyzed immediately after the relevant genetic event has occurred. In this way, the changes responsible for molecular alterations in the early phases of tumor initiation can be more easily studied.

**The role of increased protein kinase A activity in tumor formation.** The most striking observation made in this report is that removal of Prkar1a from primary cells leads to immortalization without transformation. This finding is in contrast to prior studies indicating that down-regulation of PRKAR1A suppressed cell proliferation in a variety of cell types, including prostate and pancreatic cancer cells (27, 28). Similarly, as has been observed in

![Figure 5](cancerres.aacrjournals.org)
Carney complex tumors (2), we found increased basal and total PKA activity in the Prkara knockout cells (Fig. 1C). In the literature, compounds that stimulate PKA in vitro have generated conflicting reports regarding proliferation, with some investigators reporting growth-inhibitory effects, whereas others describe growth promotion (29). The explanation for these apparent discrepancies may lie in the fact that the prior studies were done in immortalized cell lines in tissue culture, whereas we have used primary cells. Observations on cells that have undergone multiple mutations to generate a continuous cell line may not accurately reflect the situation in tumor initiation in vivo.

The fact that we are able to recapitulate one important aspect of tumorigenesis (i.e., immortalization) in this primary cell culture model bespeaks the importance of the proper model system for understanding biological observations. Consistent with the tumor suppressor function in vivo, Prkara deficiency prevented culture-induced senescence. In addition to the loss of replicative senescence, knockout of Prkara also allows the cells a gain of growth factor independence, a second key feature in the development of cancer (30). However, in order for Carney complex patients to develop aggressive tumors (e.g., thyroid cancer), secondary and/or tertiary mutations must occur in the immortalized cells. This is also modeled nicely in vitro by the fact that introduction of activated Ras into the Prkara-null MEFs leads to a transformed phenotype.5

The increase in PKA activity caused by loss of Prkara may also provide a model with which to understand benign tumorigenesis induced by activation of PKA caused by mutations in G-protein-coupled receptors (e.g., the thyrotrphin receptor in thyroid adenomas) or in G proteins themselves (e.g., McCune-Albright syndrome or somatotroph pituitary adenomas; ref. 31). In each of these cases, the establishment of autonomous hyperproliferative cells may be due to the immortalizing effect of increased PKA signaling, although, as above, there is little impetus toward malignant transformation in the absence of additional mutations. In fact, down-regulation of Prkara has recently been described in sporadic growth hormone–producing adenomas (32), although mutations in PRKAR1A are rarely, if ever, observed in these tumors (33, 34).

Effects on modulators of cell senescence. Cellular immortalization is an essential step toward malignant transformation of normal cells. In primary MEFs, cellular senescence is mediated via regulators of cell cycle progression, most prominently p53 and p19ARF (35, 36). In Prkara knockout cells, p53/p19ARF and RB activity remained intact. We found that p16INK4a levels were up-regulated in the knockout MEFs. p16INK4a can compete with cyclin D1 for binding to CDK4, which may account for the lack of change of CDK4 activity in spite of the increased cyclin D1 levels (37). Together, these observations suggest that loss of Prkara immortalizes MEFs without directly impairing the function of p53/p19ARF or the RB pathway. Interestingly, we have observed a consistent up-regulation of p27Kip1, which may also occur at the post-transcriptional level (data not shown). As levels of p27 and cyclin D1 have been shown to correlate with each other (38), this finding is somewhat unsurprising. This observation is likely based on the known association of these proteins, leading to p27Kip1 inhibition of the activity of cyclin D-CDK4 and cyclin E-CDK2 complexes (39, 40). Furthermore, it has also been shown (26) that p27Kip1 stabilizes cyclin D1 and results in the formation of inactive stable complexes with CDK4; thus, up-regulation of p27Kip1 in Prkara−/− MEFs may partially explain the increased stability of cyclin D1 in these cells. It is somewhat unusual, however, that p27 retains its nuclear localization, as high levels of nuclear p27 have generally been noted to be growth inhibitory. The relationship between elevation in cyclin D1 and p27 are currently under further investigation.

Relationship between protein kinase A and D-type cyclins in neoplastic growth. D-type cyclins are the major common targets by which cells proliferate in response to environmental signals. Intriguingly, MEF cells that carry a knockout of the Men1 gene also exhibit increased levels of cyclin D1 (41), although if this was a transcriptional or post-transcriptional event is currently unknown. This common pathway provides a molecular link to the overlapping spectrum of tumors in patients with MEN1 and Carney complex, which includes pituitary and adrenal lesions, as well as skin abnormalities (42).

The observation that PKA can affect protein levels adds a new level of complexity to understanding cyclin-mediated cell cycle

---

5 L.S. Kirschner, unpublished observations.
control. In vitro studies of cyclin D1 have indicated that the protein can be phosphorylated by PKA and that there are three potential PKA phosphorylation sites in the protein, including one in the cyclin box (43). Our data suggest that PKA activation leads to phosphorylation of cyclin D1, although whether this is a direct effect or the result of PKA activation of a secondary kinase is unknown at present. Our interpretation of the data presented here is that the majority of cyclin D1 runs as a singlet at M, 36,000, whereas the PKA-mediated phosphoisoform of the protein runs at a slightly reduced mobility. The exact nature of this isoform and its immunologic characteristics are under active investigation.

Although cyclin D1 levels have been shown to be elevated in breast cancers, there has been, in general, poor association between levels of elevation and tumor progression and/or prognosis. With the finding that cyclin D1 may be subject to post-translational modification affecting its activity, this question may need to be reconsidered. Interaction between cyclin D1 and estrogen receptor (ER) has been studied in the past (44, 45). However, phosphorylation certainly has the possibility to affect this interaction, and studies have already suggested that phosphorylation of the ER can affect its estrogen responsiveness (46). The implication of these observations for clinical cancers has yet to be addressed experimentally.

Cyclin D1 and cellular immortalization. Overexpression of cyclin D1 is associated with human cancer and is the most common genetic alteration seen in many types of cancers, including breast, head and neck squamous cell carcinomas, and mantle cell lymphomas (47, 48). Although cyclin D1 is overexpressed in oral esophageal squamous cell carcinomas, inactivating mutations of pRB were not observed. Furthermore, cyclin D1 overexpression alone has extended the replicative life span of primary human keratinocytes up to 80 PDs (49). In early studies of immortalized fibroblast lines, cyclin D1 overexpression was reported to enhance cell cycle transit times, although, as seen here, no effect on transformation was observed (50). Intriguingly, in that study, overexpression of cyclin D1 was sufficient to induce partial growth factor independence, similar to what we have observed for the Pkria1 knockout cells. The fact that increased cyclin D1 levels were seen in the early passages (passages 6-8) suggests that up-regulation of cyclin D1 could be an early event in immortalization, although further experiments are needed to determine if this finding is a cyclin D1 effect or is a direct result from PKA dysregulation.

Summary. The data presented in this article provide a good in vitro model for tumor initiation as observed in patients with the Carney complex. The observations presented here indicate an unexpected role for PKA in modulating cell cycle progression through regulation of cyclin D1 and p27kip1 protein levels. Dereulation of these key cell cycle proteins represents a previously unexplored PKA dependent mechanism for regulation of cell cycle at G0/G1 phase. The findings presented here also describe a novel relationship between PKA and D-type cyclins, with implications for both normal and tumor cell biology.

Acknowledgments

Received 9/5/2005; accepted 9/7/2005.

Grant support: Grants HD01323 and CA112268-02 (L.S. Kirschner) and National Cancer Institute grant CA6568 (Ohio State University Comprehensive Cancer Center).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank William H. Towns for expert technical assistance, Drs. G. Leone and D. Guttridge for sharing reagents and guidance, Drs. L. Wu and C. Timmers for technical advice, Dr. J. Boss for 6× His-Creb, Dr. M-D. Tsai for GST-RB, Dr. J. Bertherat for pREP4-HA-R1A, Drs. Stanley Mc Knight and Constantine Stratakis for the dominant-negative and constitutively active PKA plasmids, and Drs S. Jiang and M. Ringel for critical review of the article.

References

25. Meegh CJ, Correll GA, Cadd GG, McKnight GS. Inhibition of intracellular CAMP-dependent protein


Disruption of Protein Kinase A Regulation Causes Immortalization and Dysregulation of D-Type Cyclins

Kiran S. Nadella and Lawrence S. Kirschner