Published in final edited form as:

Horm Metab Res. 2014 November ; 46(12): 883–888. doi:10.1055/s-0034-1389951.

Comparison of the Effects of PRKAR1A and PRKAR2B Depletion on Signaling Pathways, Cell Growth, and Cell Cycle Control of Adrenocortical Cells

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Abstract

The cyclic AMP/protein kinase A signaling cascade is one of the main pathways involved in the pathogenesis of adrenocortical tumors. The PKA R1A and R2B proteins are the most abundant regulatory subunits in endocrine tissues. Inactivating mutations of *PRKARIA* are associated with Carney complex and a subset of sporadic tumors and the abundance of R2B protein is low in a subset of secreting adrenocortical adenomas. We previously showed that *PRKARIA* and *PRKAR2B* inactivation have anti-apoptotic effects on the adrenocortical carcinoma cell line H295R. The aim of this study was to compare the effects of *PRKARIA* and *PRKAR2B* depletion on cell proliferation, apoptosis, cell signaling pathways, and cell cycle regulation. We found that *PRKAR2B* depletion is compensated by an upregulation in the abundance of R1A protein, whereas *PRKARIA* depletion has no effect on the production of R2B. The depletion of either *PRKARIA* or *PRKAR2B* promotes the expression of Bcl-xL and resistance to apoptosis; and is associated with a high percentage of cells in S and G2 phase, activates PKA and MEK/ERK pathways, and impairs the expression of IκB leading to activate the NF-κB pathway. Nonetheless, we observed differences in the regulation of cyclins. The depletion of *PRKARIA* leads to the accumulation of cyclin D1 and p27kip, whereas the depletion of *PRKAR2B* promotes the accumulation of cyclin A, B, cdk1, cdc2, and p21Cip. In conclusion, although the depletion of *PRKARIA* and *PRKAR2B* in adrenocortical cells has similar effects on cell proliferation and apoptosis; loss of these PKA subunits differentially affects cyclin expression.

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Supporting Information for this article is available online at <http://www.thieme-connect.de/products>

Conflict of Interest

The authors declare that they have no conflict of interest in the authorship or publication of this contribution.

Keywords

PKA R1A; R2B; apoptosis; NF- κ B; cyclins; cdks

Introduction

The cAMP/PKA pathway is one of the major cell signaling pathways involved in the normal physiology and pathophysiology of the adrenal cortex [1]. PKA is a holoenzyme that is formed by 2 regulatory subunits, type I (R1A and R1B) and type II (R2A and R2B), and 4 catalytic subunits (C α , C β , C γ , and C δ). Signaling via cAMP/PKA can either inhibit or stimulate cell proliferation depending on the cell type [1, 2]. Most cells contain both PKAI and PKAII isozymes and their relative abundance varies among species and tissues. Alterations in cAMP-mediated signal transduction are associated with neoplastic transformation [1]. This is usually linked to changes in the ratio of PKA I to PKA II [3]. *PRKARIA* inactivating mutations are found in Carney complex (CNC) patients and are responsible for bilateral cortisol-secreting adrenocortical tumors, named primary pigmented nodular adrenocortical disease (PPNAD) [4]. Somatic *PRKARIA* mutations are also found in sporadic endocrine tumors [5]. PKA R1A is the most extensively studied PKA subunit because *PRKARIA* germ line mutations have been described in the CNC. However, subunits of PKA besides PKA R1A are also altered in endocrine tumorigenesis. Indeed, 2 studies described a new mechanism of cAMP pathway dysregulation in adrenocortical tumorigenesis involving the loss of PKA R2B protein in cortisol secreting adenoma due to a post-transcriptional mechanism [6, 7]. The loss of R2B was not associated with alteration of the other PKA subunits. However, in *Prkar2b* knockout mice, the production of R1A protein is upregulated in white adipose tissue to compensate for the loss of R2B protein, which is usually highly expressed in this tissue [8]. *Prkar2b* inactivation by siRNA in mice adrenocortical Y1 cells promotes cell proliferation [7].

Somatic activating mutations in the PKA catalytic subunit alpha gene (*PRKACA*) were recently reported in cortisol-secreting adrenocortical adenoma. These mutations alter the interaction between C α and R2B proteins [9]. The effect of alterations to PKA signaling during endocrine tumorigenesis is likely to be complex, because the defective expression of PKA Type I and Type II enhances PKA activity but causes different types of tumors. Here, we used siRNA to inactivate either *PRKARIA* or *PRKAR2B* in human adrenocortical carcinoma H295R cells and studied the resulting effects on cell proliferation/apoptosis, signaling pathways, and cell cycle control. We show that the inactivation of *PRKARIA* or *PRKAR2B* had a common effect on the resistance of cells to apoptosis; however, this effect was mediated through distinct targets and at different components of cell cycle control. These findings indicate that PKA subunits, despite their similar properties, may regulate distinct stages of the cell cycle.

Materials and Methods

Cell culture and cell cycle, cell proliferation, and apoptosis analyses

Human H295R adrenocortical carcinoma cells were grown as previously described [2]. Cell cycle, cell proliferation and apoptosis were analyzed by flow cytometry as previously reported [2].

Analysis of RNA and protein

The abundance of total RNA and protein was assessed by Western blots (antibodies Table 1S) and real-time-PCR (primers Table 2S) as previously reported [2, 10].

PKA and NF- κ B pathway studies

The PepTag nonradioactive protein kinase assay kit (Promega) was used to measure PKA activity as reported [2]. DEAE column chromatography of PKA-I (Peak I) and PKA-II (Peak II) was performed in the absence or presence of 5 μ M cAMP as previously described [11]. Electrophoretic mobility shift assay (EMSA) to analyze the activation of NF- κ B was carried out with total homogenates and nuclear fractions and was analyzed by a radioactive labeled oligonucleotide probe containing the specific recognition sequence for NF- κ B as previously described [12]. For supershift assays, total cell extracts were incubated with specific antibodies for 30 min on ice before incubation with the labeled probe.

Transfection

Cells were transfected with siRNAs and the different luciferase reporter gene driven by the cyclin promoters described in Supporting Information as previously described [2, 10]. For the analysis of transcription, siRNA-treated cells were incubated with actinomycin D (5 μ g/ml) 48 h after transfection, and RNA was measured every 2 h (see, Supporting Information).

Statistical analyses

All statistical analyses were carried out with data from at least 3 duplicate experiments. Control conditions were set as 100 % and data were analyzed with Fisher's exact test. Significance was set at $p < 0.05$; $p < 0.01$, and $p < 0.001$.

Results

Effect of depletion of PRKAR1A and PRKAR2B on the abundance of PKA regulatory subunits

Knockout of R1A in H295R cells did not affect the abundance of the other PKA subunits in siR1A cells; however, in siR2B cells, the abundance of R1A protein was higher than in control (siCtl) cells without changes to the abundance of R1A mRNA (Fig. 1a). We assayed PKA activity, and found no difference in basal activity in the absence of forskolin (FSK) between siR1A or siR2B and siCtl cell lysates (Fig. 1b). However, FSK enhanced PKA activity after 5 min in siR2B cells, whereas it took 10 min in siR1A cells. In basal condition of PKA DEAE chromatography, PKA type I was the major activated fraction in H295R

control cells, whereas the addition of cAMP to the assay enhanced equally the activity of both type I and type II fractions (Fig. 1b). Knockdown of R1A impaired the activity of the type I fraction in the absence of cAMP; however, the addition of cAMP enhanced the activity of both PKA type I and type II (although the activity of PKA type I was lower than in the control). Depletion of R2B stimulated PKA type I activity. In these cells, the addition of cAMP enhanced PKA type I activity as in the control, but did not affect PKA type II activity (Fig. 1b).

Effect on apoptosis and cell proliferation

Annexin V staining showed no differences in the percentage of apoptotic cells between siR1A, siR2B, and siCtl cells in an unstimulated state (Fig. 1c). However, following the induction of apoptosis by either TNF α (Fig. 1c) or TGF β (Fig. 1SA), the percentage of apoptotic cells was significantly lower in both siR1A and siR2B cells than in control cells. The abundance of the pro-apoptotic Bax protein showed no significant variation between siR1A, siR2B, and siCtl cells, whereas the abundance of anti-apoptotic Bcl-xL protein was higher in both siR1A and siR2B cells than in control cells (Fig. 1c). The percentage of BrdU-positive cells corresponding to cells in S phase of the cell cycle was also higher in both siR1A and siR2B cells than in siCtl cells (Fig. 1c). The propidium iodide staining showed that the percentage of cells in the G1 phase was lower in siR1A and siR2B cells than in control cells, whereas the percentage of cells in the G2 phase was higher (Fig. 1SB). This suggests that siR1A and siR2B cells progress more quickly through the cell cycle than control cells.

Effect on MAPK and NF- κ B pathways

As the activation of PKA and its crosstalk with MAP kinases are implicated in apoptosis and proliferation [13, 14] we analyzed the MEK/ERK pathway. Depletion of *PRKARIA* and *PRKAR2B* led to the MEK/ERK pathway activation. This pathway was more strongly activated in siR2B cells than in siR1A cells (Fig. 1SC).

NF- κ B (p50/p65 heterodimer) is one of the most important cell survival factors; it is sequestered by the I κ B α inhibitor in the cytoplasm and is activated upon its release and translocation to the nucleus, where it promotes gene transcription. Immunoblotting with I κ B α -specific antibodies showed that the amount of I κ B α protein was significantly lower in both siR1A and siR2B cells than in control cells (Fig. 1d). The knockdown of R1A or R2B promoted the movement of NF- κ B p50 protein from the cytosol to the nucleus. In contrast, only the knockdown of R2B promoted the translocation of NF- κ B p65(RelA) to the nucleus (Fig. 1d). We carried out gel shift experiments to investigate NF- κ B DNA binding activity and the composition of the complex. NF- κ B DNA binding activity was already high in H295R control cells. EMSA analysis of whole cell lysates (data not shown) and the nuclear fraction (Fig. 1SD) of H295R control cells revealed the presence of a major NF- κ B–DNA complex 1 and 2 minor complexes, 2 and 3, with higher motility. Knockdown of R1A or R2B stimulated NF- κ B DNA binding (Fig. 1SD). The NF- κ B complex 1 was supershifted with a p65(RelA) or p50 antibody, indicating the presence of both p65(RelA) and p50 in complex 1. Complex 2 was supershifted with the p50 antibody only and therefore may

comprise p50 but not p65(RelA). Complex 3 corresponded to a p50/p50 homodimer, because it disappeared with the p50 antibody (Fig. 1SD).

Differential regulation of cyclins and cyclin-dependent kinases

We studied the expression of cyclins and their distribution between the cytosol and the nucleus because knockdown of *PRKAR1A* or *PRKAR2B* was associated with a high percentage of cells in G2 phase (Fig. 1SB). Knockdown of *PRKAR1A* in H295R cells stimulated the transcriptional activity of the cyclin D1 promoter (Fig. 2SA) and led to the accumulation of cyclin D1 mRNA and protein (Fig. 2a). Cyclin D1 protein was accumulated in the cytosol, and its abundance was low in the nucleus. There were no differences in cyclins A, B, and E between siR1A cells and control cells (Fig. 2a).

Knockdown of *PRKAR2B* in H295R cells had a major effect on cyclin A and B. Indeed, the transcriptional activity of the cyclin A and B promoters (Fig. 2SA) and the abundance of cyclin A and B mRNA and protein (Fig. 2a) were all affected. Cyclin A accumulated in both the cytosol and the nuclear fractions, whereas cyclin B accumulated only in the nucleus. Cyclin D1 and cyclin E were not affected in siR2B cells (Fig. 2a). These effects are essentially transcriptional as shown by the treatment of siR1A and siR2B cells with actinomycin D for 2 h, which prevented the accumulation of cyclins mRNA (Fig. 2SB). Cyclin-dependent kinases (cdks) form complexes with cyclins: cdk4/cdk6/cyclin D, cdk6/cyclin E, cdk2/cyclin A, and cdc2/cyclin A and B, and their localization is a marker of cell cycle progression. Although siR1A cells expressed a high amount of cyclin D, the abundance of cdk4 and cdk6 protein was similar to control cells (Fig. 2b). The abundance of cdk2 and cdc2, but not other cdks, was higher in siR2B cells than in control cells and they accumulated in both the cytosol and nuclear fractions (Fig. 2b), concomitant with a high amount of cyclin A and B. We also examined the expression of cdk inhibitors, which regulate cyclin/cdk complexes. The abundance of p21Cip was significantly higher in siR2B cells than in control cells, and p27Kip accumulated in siR1A cells (Fig. 2c).

Discussion

In this study, we showed that the depletion of the PKA subunits R1A and R2B in H295R cells have similar effects response regarding the resistance of cells to apoptosis and the stimulation of cell proliferation, although their loss differentially affects components of cell cycle control and signaling pathways. The depletion of either *PRKAR1A* or *PRKAR2B* promoted signaling via the PKA and MEK/ERK pathways. However, there were substantial differences between siR1A and siR2B cells in terms of the abundance and/or distribution of the molecular players involved in the activation of NF- κ B signaling and cell cycle control (cyclins/cdks). Inactivation of the regulatory subunit R1A, either by genetic mutation in humans or by targeted deletion in the mouse, promotes PKA activity, which has been attributed to the development of neoplasms [1]. The high PKA activity in cells depleted of R1A or R2B cells may be explained by a compensatory mechanism involving the upregulation of other PKA subunits. Here, the depletion of *PRKAR1A* by siRNA in our H295R cell model was not compensated by an accumulation of R2B protein or mRNA (Fig. 1a). This finding is consistent with a study involving HEK293 cells, in which the

introduction of the *PRKARIA* deletion (R1del6) led to aberrant cellular morphology and high PKA activity without affecting the abundance of type II PKA subunits [15]. Thus, a switch to type II PKA is not required to achieve high PKA kinase activity in H295R and HEK293 cells depleted of R1A. By contrast, the abundance of R1A protein, but not R1A mRNA, was higher in siR2B H295R cells than in control cells (Fig. 1a). This may explain the higher and faster stimulation of PKA activity by FSK in siR2B cells (5 min) than in siR1A cells (10 min). A similar accumulation of R1A protein was also reported in mouse adrenocortical Y1 cells after the inactivation of *Prkar2b* [7]. PKA activity is also higher in adenoma producing low amounts of R2B protein than in control tissue [6, 7].

The PKA pathway participates in crosstalk with other signaling pathways involved in cell growth. We found that the PKA depletion of R1A or R2B in H295R cells promoted the phosphorylation of both MEK and ERK; however, MEK and ERK were more strongly phosphorylated in siR2B cells than in siR1A cells. Activation of PKA and MAP kinase in adrenocortical cells may promote cell proliferation and survival [13, 14]. We found that the depletion of *PRKARIA* or *PRKAR2B* confers resistance to apoptosis in cells stimulated with TGF β or TNF α . Liu et al. [16] showed that the activation of PKA by dibutyl-cAMP stimulates TNF α -induced apoptosis in H295R cells. In our study, the resistance of H295R cells apoptosis induced by TGF β or TNF α was probably an indirect effect of the depletion of PKA subunits. We previously reported that high PKA activity in cells lacking *PRKARIA* inhibits the expression of SMAD3, which protects H295R cells from the apoptotic effect of TGF β [10]. We did not observe the downregulation of SMAD3 expression after the depletion of *PRKAR2B* in H295R cells (data not shown). However, the strong induction of the MEK/ERK pathway, and the high abundance of the antiapoptotic protein BclL may also counteract apoptosis in *PRKAR2B*-depleted cells. Moreover, we previously reported that the activation of PKA R2B by specific cAMP analogues triggers apoptosis in H295R cells [2].

The NF- κ B pathway plays an important role in protection against apoptosis in a variety of cell types. NF- κ B is also a target of PKA: NF- κ B p65(RelA) interacts with the PKA catalytic subunit, which phosphorylates it at Ser267 [17]. In addition, an A-kinase-interacting Protein 1 (AKIP1) promotes the interaction of NF- κ B p65(RelA) and PKA C α in breast cancer cell lines [18], in addition to their translocation to the nucleus [17]. We found that the depletion of either PKA R1A or R2B in H295R cells impaired the expression of I κ B α , as evidenced by its low abundance and the nuclear translocation of NF- κ B transcription factors. We found higher NF- κ B DNA-binding activity in both siR1A and siR2B cells than in control cells. We also revealed the presence of the same NF- κ B protein complexes: p65(RelA)/p50, p50/unknown compound, and p50/p50, with a pronounced shift of complex 1 and 2 with p50 antibody in siR1A cells than in siR2B. The distribution and abundance of NF- κ B proteins were different between siR1A and siR2B cells. Moreover, the abundance of NF- κ B protein and its translocation to the nucleus were not predictive of NF- κ B complexes. The depletion of either PKA subunit stimulated proliferation, as shown by the large percentage of cells in S phase (BrdU incorporation) and G2 Phase. However, the depletion of the PKA subunits affected different cyclins of G2 phase. Depletion of *PRKARIA* promoted the accumulation of cyclin D1 without affecting the abundance of cdk4

and cdk6. A reduction in the abundance of cyclin D1 is required for DNA synthesis during S phase, and cells subsequently produce a high amount of cyclin D1 when they enter G2 phase. Thus, cyclin D1 has been proposed to serve as an active switch to regulate cell cycle progression [19]. In MEF depleted of *Prkar1a* and in PPNAD cells [20], cyclin D is preferentially present in the cytoplasm and not the nucleus; however, this does not prevent cell cycle progression. Cyclin D1 is a proto-oncogene and its sequestration is observed in the cytoplasm of mammalian cancer MCF-7 cells [21]. The strong stimulation of NF- κ B activity in siR1A and siR2B cells did not explain the accumulation of cyclin D in siR1A cells, which is a target gene of the NF- κ B pathway, and not in siR2B cells. In contrast with our results, the accumulation of cyclin D1 in MEF depleted of *Prkar1a* is not linked with the activation of NF- κ B [20]. The depletion of *PRKAR2B* in H295R cells affected the abundance and distribution of cyclins A and B that are involved in the progression of the cell cycle during S and G2 phases. Indeed, these cyclins accumulated in both the cytoplasm and nuclear fractions of siR2B cells, similar to cdk2 and cdc2. Thus, the depletion of *PRKARIA* and *PRKAR2B* has different effects on the H295R cell line. Silencing of *PRKARIA* may lead to the immortalization of the cells as shown by Nadella et al. [20], whereas the depletion of *PRKAR2B* enhances cell cycle progression. The loss of either subunit inhibits apoptosis. Further studies are needed to explain the differential regulation of cyclins. Altogether these findings underline the complexity of the cellular effects of alterations to PKA subunits that occur in adrenocortical tumors.

Supplementary Material

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Acknowledgments

We thank the members of the FACS Core Facility of Institute Cochin for their cooperation. This study was supported by grants from INSERM the Conny-Maeva Charitable, and the funding of the Seventh Framework Program (FP7/2007–2013) under grant agreement 259735.

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Fig. 1.

Effect of *PRKARIA* or *PRKAR2B* depletion on the abundance of other PKA subunits, cell signaling pathways, apoptosis and cell proliferation. **a** Histogram showing the abundance of PKA R1A, R2B and Caα mRNA and protein. **b** PKA activity of cells incubated with FSK for 5 or 10 min, 48 h after siRNA transfection. DEAE Chromatography indicates the activity of Type I and Type II PKA in the absence or presence of cAMP in siR1A and siR2B cells. **c** Apoptosis was assessed by annexin V-FITC/IP staining in transfected cells with or without TNFα. Results are expressed as the percentage of viable cells (hatched bars) and cells undergoing apoptosis (annexin +/IP, plus annexin +/IP +, black bars). Western blot of Bcl-xL shows the accumulation of the protein in both siR1A and siR2B cells. No differences were observed in the abundance of Bax. BrdU incorporation reveals that the proportion of cells in S phase is higher in both siR1A and siR2B cells than in control cells. **d** NF-κB cell signaling; the abundance of IκBα expression is low in siR1A and siR2B cells. Analysis of NF-κB p50 and p65 in cytosolic and nuclear fractions reveal different pattern of NF-κB proteins in siR1A and siR2B cells.

Fig. 2.

Effects of *PRKAR1A* or *PRKAR2B* depletion on cell cycle regulators. **a** The abundance of cyclin mRNA and the distribution of cyclin proteins between the cytosol and the nucleus. Cyclin D accumulates in siR1A cells whereas cyclins A and B accumulate in siR2B cells. **b** The distribution of cdk between the cytosol and nucleus is consistent with the distribution of their partner cyclins. **c** The abundance of the cdk inhibitors p21Cip and p27 Kip is affected by the depletion of PKA subunits.