Novel Function of the Regulatory Subunit of Protein Kinase A: Regulation of Cytochrome *c* Oxidase Activity and Cytochrome *c* Release†

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ABSTRACT: There have been speculations that the regulatory (R) subunit of the cAMP-dependent protein kinase (PKA) may have other functions. A recent study has shown that the catalytic (C) subunit of PKA may be regulated in a cAMP- and R subunit-independent manner. However, evidence linking a function to the R subunit apart from inhibiting the C subunit has been elusive. In this report, interaction cloning experiments showed that the RIα subunit association with the cytochrome *c* oxidase subunit Vb (CoxVb) is cAMP-sensitive. Interaction was detected with a GST-RIα fusion protein as well as by coimmunoprecipitation. Transient treatment with cAMP-elevating agents inhibited cytochrome *c* oxidase in Chinese hamster ovary (CHO) cells with a concomitant decrease in cytochrome *c* levels in the mitochondria and an increase in its release into the cytosol. Furthermore, mutant cells harboring a defective RIα show increased cytochrome *c* oxidase activity and also constitutively lower levels of cytochrome *c* in comparison to either the wild-type cells or the C subunit mutant. These results suggest a novel mechanism of cAMP signaling through the interaction of RIα with CoxVb thereby regulating cytochrome *c* oxidase activity as well as the cytochrome *c* levels.

The cAMP signal transduction pathway can be elicited by various physiological ligands in cells and is critically involved in the regulation of metabolisms, cell growth and differentiation, and gene expression (*1*−*3*). The PKA[1] holoenzyme is composed of two genetically distinct subunits, R and C, forming a tetrameric holoenzyme R_2C_2 which dissociates into an $R_2(cAMP)_4$ dimer and two active C subunits in the presence of cAMP. There are two major R subunit isoforms which are further distinguished as RIα and RI*β*, and RIIα and RII*β*, and three isoforms of the C subunit, Cα, C*β*, and C*γ* (*1*−*3*).

For approximately forty years, the R subunit has been the only known receptor for cAMP in cells and cAMP binding to the holoenzyme is the accepted mechanism that regulates PKA activity. The only known function for the R subunit is its inhibition of the C subunit. However, this paradigm of cAMP signaling is challenged by a recent study that shows a novel mechanism of C subunit activation in a cAMP- and R subunit-independent manner (*4*). It was found that the C subunit of PKA can bind to the NF-*κ*B−I*κ*B complex. Inducers of NF-*κ*B cause degradation of I*κ*B and activation of the bound C subunit which can subsequently phosphorylate NF-*κ*B and increase its transcriptional activity (*4*). Therefore, activation of the C subunit kinase is not limited to cAMP and the R subunit.

There has been speculation that the R subunit has functions independent of its interaction with the C subunit. Consistent with this notion, the RII subunit was found to inhibit phosphorylase phosphatase and also the activity of a purified high molecular weight phosphoprotein phosphatase in a cAMP-dependent manner (*5*, *6*). Furthermore, on the basis of its homology to the bacterial catabolite activator protein (CAP) (*7*), sequence-selective binding of the RII subunit to double helical DNA was demonstrated (*8*), suggesting that the RII subunit may regulate gene transcription in eukaryotes.

RIα also interacts with the ligand-activated epidermal growth factor receptor complex by binding to the SH3 domains of the Grb2 adaptor protein (*9*). In addition, direct interaction between RIIα and the $p34^{cdc2}$ protein kinase cell cycle regulator has been demonstrated, suggesting that these two pathways may cross-talk in the regulation of cell division (*10*). Therefore, we speculate that protein−protein interactions of the R subunits may not be limited to the PKA holoenzyme complex alone but may include other important target proteins.

We have recently shown that the mouse adrenocortical carcinoma Y1 and the CHO cells harboring defective RIα subunits of PKA exhibit increased resistance to cisplatin as well as reduced P-glycoprotein expression (*11*, *12*). However, the C subunit mutants, also displaying repressed PKA activity, are equally sensitive to cisplatin and express

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[1] Abbreviations: R, regulatory subunit; PKA, cAMP-dependent protein kinase; CoxVb, cytochrome *c* oxidase subunit Vb; AKAP, A-kinase anchoring protein.

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P-glycoprotein levels comparable to those of wild-type cells. Thus, there is no apparent correlation between alterations of PKA activity, cisplatin resistance, and P-glycoprotein expression, therefore suggesting that the RIα subunit may have novel functions.

Despite the above studies, R subunit function that is independent of its interaction with the C subunit has not been established. To test this hypothesis, we showed in this study by interaction cloning experiments the association of RIα with the cytochrome *c* oxidase subunit Vb (CoxVb). The mammalian cytochrome *c* oxidase is a multisubunit enzyme complex of 13 different subunits and the terminal electron carrier of the respiratory chain. The interaction with CoxVb is cAMP-sensitive. The physiological significance of the RIα interaction with CoxVb is supported by the modulations of the cytochrome *c* oxidase activity and the mitochondrial cytochrome *c* levels in CHO cells with cAMP-elevating agents. These results demonstrate a novel cAMP-signaling pathway mediated by RIα, independent of the C subunit, and may have significant implications on the mechanism of cAMP in cell growth and apoptosis.

MATERIALS AND METHODS

Analyzing RIα Protein Interactions by the Yeast Two-Hybrid Cloning System. The yeast two-hybrid cloning experiments were conducted as described (*13*). The complete coding region of the mouse RIα cDNA was fused to the Gal4 DNA-binding domain in plasmid pAS2-1 (pAS2−RIα) and then transformed into *Saccharomyces cerevisiae* Y190 cells as a bait to screen a HeLa cell cDNA library fused to the Gal4 activation domain in the pGAD−GH vector. Positive clones were detected by β-galactosidase assay. Plasmids from positive clones were used to transform Y187 cells and were mated with the pAS2−RIα cells.

GST Fusion and Pull Down Assay. Expression of the glutathione S-transferase (GST)−RIα fusion protein and the partial purification with glutathione beads were performed as described (*14*). Wild-type RIα or mutant RIα(G200E) in pGEX−4T-1 was expressed in the *Escherichia coli* DH5a cells with IPTG. Cells were lysed by sonication, and the lysates were incubated with glutathione resin to immobilize the GST fusion proteins. GST−RIα or GST−RIα(G200E) beads were then incubated with yeast lysates overexpressing GAD−CoxVb either in the presence or in the absence of 100 μM 8-Br-cAMP. Proteins associated with the GST fusions were immunoblotted with anti-GAD antiserum or a monoclonal antibody against CoxVb.

Immunoprecipitation. CHO cells were harvested by washing in STE buffer (20 mM Tris-HCl, pH 7.2, 0.15 M NaCl, 1 mM EDTA, and 1% aprotinin), pelleted, and resuspended in RIPA buffer (20 mM Tris-HCl, pH 7.2, 0.15 M NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, and 1% aprotinin). The extracts were reacted with anti-RIα antisera, and the resulting complex was precipitated with protein A-Sepharose (Pharmacia Biotechnology Inc., Piscataway, NJ), resolved on SDS−PAGE and immunoblotted with anti-CoxVb monoclonal antibody.

Deletion Analysis of RIα Interaction with CoxVb. The amino terminal deletion mutant GST−RIα(Δ1−76) was constructed by subcloning a polymerase chain reaction (PCR) amplified product, using the RIα cDNA, into pGEX−4T-1. The carboxyl terminal deletion mutant GST−RIα(Δ77−380) was constructed by digesting GST−RIα with *Xho*I and *Not*I and then blunt-end ligated into pGEX−4T-1. Deletion mutants were expressed and bound to glutathione resin and then incubated with yeast lysates containing GAD−CoxVb, separated on SDS−PAGE and immunoblotted with anti-GAD antibody.

Fractionation of Cell Extracts and Cytochrome c Oxidase Assay. Fractionation of CHO cells was as described before (*15*). CHO cells were homogenized in a buffer containing 10 mM Hepes (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, 0.25 M sucrose, and protease inhibitors. Cell lysates were centrifuged, and the pellet was washed once and recentrifuged. The combined supernatants were centrifuged at 10000*g* yielding a crude mitochondria fraction. Supernatants of the 10000*g* spin were further centrifuged at 100000*g* to obtain a cytosolic protein fraction.

Cytochrome *c* oxidase activity was measured as described before (*16*). Approximately 1×10^5 CHO cells were plated in triplicate in 96-well microtiter plates and treated with either 10 μM forskolin or 50 μM dibutyryl cAMP for 30 min. Media were removed, and cells were permeabilized by the addition of 0.01% saponin and agitated, followed by the addition of substrate medium. Absorbances were then measured spectrophotometrically.

RESULTS

Despite various analyses, it has still not been possible to unambiguously link a function to the R subunit that is independent of its interaction with the C subunit (*5*, *6*, *8*−*12*). To test this hypothesis, we examined the protein−protein interaction of the RIα subunit using the yeast two-hybrid cloning system (*13*). We screened the human HeLa cell cDNAs for encoded proteins capable of binding to the mouse RIα cloned into the yeast expression vector pAS2-1. A total of 29 positive clones were identified, and of which 23 encode for the full-length cytochrome *c* oxidase subunit Vb (CoxVb), 5 for cytokeratin 18, and 1 for an anonymous expressed sequence tag (EST). Control experiments showed that CoxVb is an RIα binding protein (Figure 1A). Matings of yeast containing the CoxVb cDNA with those expressing the RIα−Gal4 DNA binding domain (Gal4DB) fusion bait indicated positive interaction, while those expressing either Gal4DB alone or an irrelevant protein, the snf1 protein kinase, were negative. As expected, the two-hybrid system also positively identified interactions between RIα and itself (dimerization) (Figure 1A).

To provide biochemical evidence for this interaction, we further studied the binding of CoxVb to RIα by expressing a GST−RIα fusion protein in *E. coli* and then partially purified it by binding to glutathione beads (*14*). GST−RIα has an apparent molecular weight of approximately 76 kDa (Figure 1B) and reacted with anti-RIα antiserum by immunoblot analysis (data not shown). The GST−RIα affinity beads were reacted with yeast lysates containing either GAL4 activation domain (GAD)−RIα or GAD−CoxVb fusion protein and then immunoblotted with anti-GAD antibody (Figure 1B). GST−RIα (lanes 3 and 6), but not GST (lanes 2 and 5), associated with GAD−RIα and GAD−CoxVb from the cell lysates, thus indicating the specific interactions between RIα and these proteins.

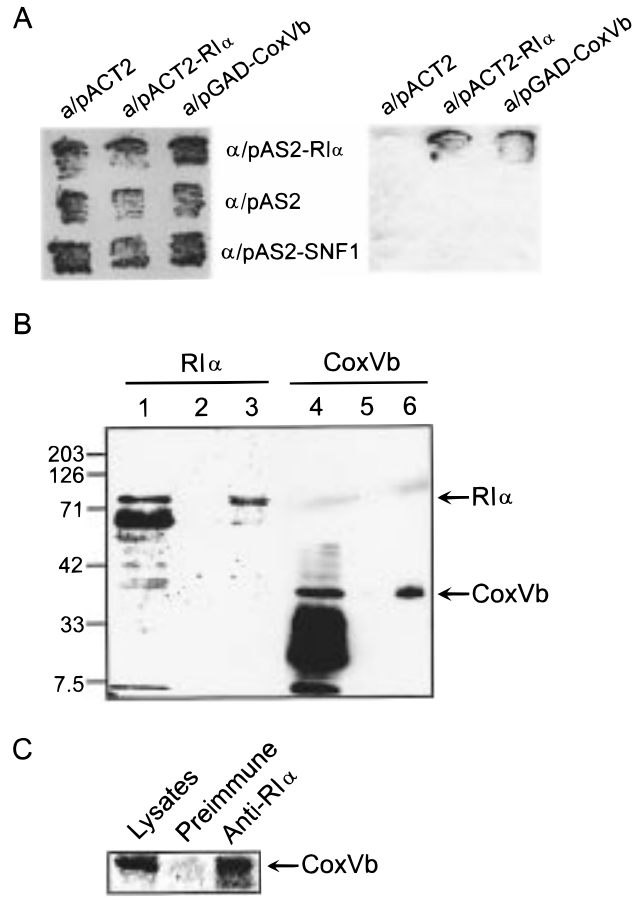

FIGURE 1: Interaction of CoxVb with RIα. (A) Mouse RIα was used as bait to screen a HeLa cDNA two-hybrid library. Test crosses are shown for yeast matings (*MAT***a** × *MAT***a**, left panel) in which association of expressed proteins resulted in the expression of the *β*-galactosidase reporter (indicated in black, right panel). Interaction of RIα with itself (dimerization) was a positive control, and the Snf1 protein kinase, SNF1, was a negative control. (B) GST, GST−RIα wild type, or mutant proteins were immobilized on glutathione resins and then eluted by boiling in gel-loading buffer, resolved on SDS−PAGE, and stained with Coomassie blue: lanes 1 and 4, yeast lysates containing either GAD−RIα or GAD−CoxVb fusion protein, respectively; lanes 2 and 5, GST; lanes 3 and 6, GST−RIα. (C) CHO cell lysates were immunoprecipitated with rabbit preimmune serum (lane 2) or anti-RIα antiserum (lane 3). The precipitated complexes were incubated with protein A-Sepharose, separated by SDS−PAGE, and immunoblotted with anti-CoxVb antibody: lane 1, 40 μg of total CHO cells lysates.

To further illustrate the specificity of the RIα−CoxVb interaction, lysates from CHO cells were subjected to immunoprecipitation with anti-RIα antiserum. The precipitated complex was immunoblotted with a monoclonal antibody against CoxVb, and our results confirmed that CoxVb can be coimmunoprecipitated with RIα in CHO cell lysates (Figure 1C), thus demonstrating unambiguously the interaction of RIα with CoxVb in mammalian cells. The control preimmune serum did not coprecipitate RIα and CoxVb (Figure 1C). These results showed that RIα interacts with CoxVb in a heterologous yeast two-hybrid system and in mammalian cells.

We next determined the domain in RIα that associates with CoxVb. Deletion constructs of RIα at the amino terminus containing the dimerization region as well as the A-kinase anchoring protein (AKAP) binding domain (*17*) and at the carboxyl terminus including the autoinhibitory region and the two tandem cAMP binding sites were created (Figure 2A). As shown in Figure 2B, deletion of the carboxyl terminus, GST−RIα(Δ77−380), did not significantly affect its association with CoxVb, whereas deletion of the amino terminus, GST−RIα(Δ1−76) virtually abolished its interaction with CoxVb. These results suggest that association of CoxVb with RIα at the amino terminus occurs either at the dimerization domain or at the site required for AKAP binding, which encompasses approximately the first thirty amino acid residues of RIα (*17*).

If the association of RIα with CoxVb represents a novel mechanism of cAMP signaling, then the interaction should be inducible by cAMP. Bacterially expressed GST−RIα and GST−RIα mutant proteins were incubated with GAD−CoxVb in the presence or the absence of 100 μM 8-Br-cAMP. The mutant RIα carries a mutation that converts Gly^{200} to a glutamic acid (G200E) in the site A cAMP binding domain which prevents the dissociation of C subunits (*18*). In the absence of cAMP, CoxVb bound to both GST−RIα and GST−RIα(G200E) (Figure 3, lanes 3 and 5). Addition of cAMP caused dissociation of CoxVb from GST−RIα (lane 4), reminiscent of the interaction of R and C in the holoenzyme complex. In contrast, addition of cAMP to GST−RIα(G200E) did not result in dissociation of CoxVb (lane 6). This is expected because of the lowered affinity of the mutant RIα for cAMP (*18*). These results demon-

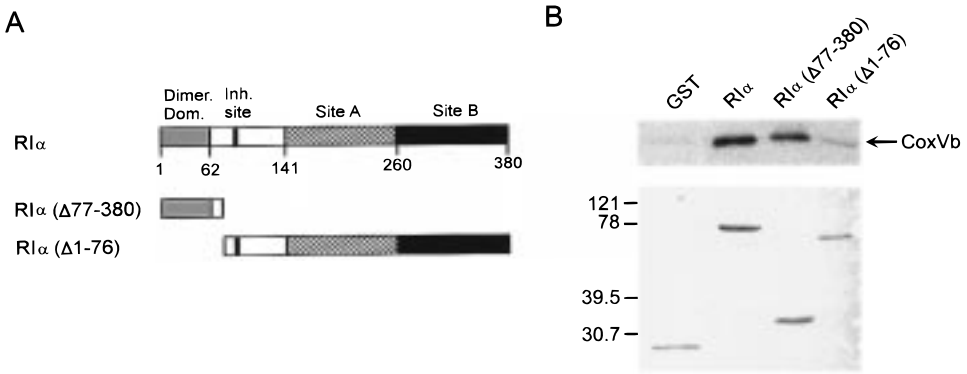

FIGURE 2: CoxVb interacts with the N-terminus of RIα. (A) Schematic diagram of RIα and the deletion constructs, RIα(Δ77−380), and RIα(Δ1−76): Dimer. Dom., N-terminal dimerization domain; Inh. Site, autoinhibitory site; Site A and Site B, tandem cAMP-binding domains. (B) GST, GST−RIα, GST−RIα(Δ77−380), and GST−RIα(Δ1−76) proteins were immobilized on glutathione resins and then incubated with yeast cell lysates containing GAD−CoxVb fusion. The complexes were eluted in gel-loading buffer, separated by SDS−PAGE, and immunoblotted with anti-GAD antibody. The lower panel is the Ponceau S stained-nitrocellulose membrane. The position of GAD−CoxVb is indicated.

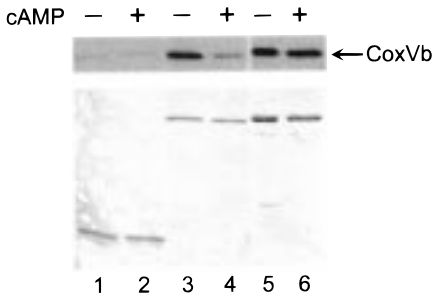

FIGURE 3: Interaction of RIα with CoxVb is regulated by cAMP. GST (lanes 1 and 2), GST−RIα (lanes 3 and 4), or GST−RIα-(G200E) (lanes 5 and 6) immobilized on glutathione resins was incubated with yeast cell lysates containing GAD−CoxVb. The complexes were then incubated at room temperature for 15 min in the presence or the absence of 100 μM 8-Br-cAMP. The associated proteins were eluted by boiling in gel-loading buffer, analyzed by SDS−PAGE, and immunoblotted with anti-GAD antibody. The lower panel is the Ponceau S stained-nitrocellulose membrane. The position of GAD−CoxVb is indicated.

strated that binding of CoxVb to RIα can be directly regulated by cAMP, thus showing for the first time that the cAMP-signaling pathway can regulate other target proteins apart from the inhibition of the C subunit kinase activity through the R subunit.

The association of RIα with CoxVb raises questions about the functional significance of this interaction. Since CoxVb is one of the thirteen subunits of the mitochondrial cytochrome *c* oxidase enzyme complex (*19*), it is therefore conceivable that the oxidase activity may be regulated by cAMP through its interaction with RIα. To assess this possibility, we measured the cytochrome *c* oxidase activity in CHO cells following treatment with cAMP elevating agents. Exposure to either dibutyryl cAMP or forskolin for 30 min resulted in a markedly reduced rate of cytochrome *c* oxidase activity (Figure 4A), suggesting a physiological regulation of the oxidase activity by cAMP, presumably mediated through the interaction of RIα with CoxVb. In addition, it has also been shown that cisplatin-resistant human tumor cell lines have increased levels of cytochrome *c* oxidase activity compared with the parental cells (*20*). To show further that the regulation of the cytochrome *c* oxidase activity is due to RIα and not the C subunit, we then

Table 1: Cytochrome *c* Oxidase Activity of CHO Wild Type and PKA Mutant Cell Lines

cell line	mutation	cytochrome *c* oxidase activity (%)[a]
10001	wild type	100
10248	RI subunit	172.0 ± 9.4
10260	C subunit	96.6 ± 14.5

[a] Oxidase activity is a percent ratio of the mutant to the wild-type control.

measured the cytochrome *c* oxidase activity in the CHO cell lines 10248, which harbors a mutation in RIα subunit (*21*), and 10260, which has altered C subunits and possesses little type I and type II kinase activities (*22*). The 10248 and 10260 mutants were obtained through a combination of mutagenesis and selection for resistance to growth inhibition by cAMP from CHO cells. The mutants have impaired PKA activity resulting from mutations in either the R or the C subunit. The basal cytochrome *c* oxidase activity was found to be significantly higher in the 10248 RIα mutant than in the wild-type 10001 cells (Table 1). However, oxidase activity of the C subunit mutant 10260 cells was comparable to the wild-type cells (Table 1). Thus these results further support that RIα subunit may have functions independent of the C subunit and may regulate mitochondrial cytochrome *c* oxidase activity through its interaction with CoxVb.

In addition to its central role in aerobic energy production, mitochondria may also be involved in the triggering of apoptosis (*23*−*25*). Recent studies have shown that increased release of the mitochondrial peripheral membrane protein cytochrome *c* from the mitochondria into the cytosol may activate caspases and apoptosis (*15*, *26*, *27*). Since cytochrome *c* is a substrate for cytochrome *c* oxidase, therefore, it is possible that cAMP-regulated cytochrome *c* oxidase activity may influence the levels and the release of cytochrome *c* from the mitochondria. As shown in Figure 4B, in the presence of cAMP, the cytochrome *c* level in the mitochondria of 10001 cells was markedly reduced, accompanied by a concomitant increase in the cytosol. These results indicate that cAMP may either directly or indirectly modulate cytochrome *c* levels. Since the 10248 RIα mutant exhibits elevated cytochrome *c* oxidase activity, a subsequent

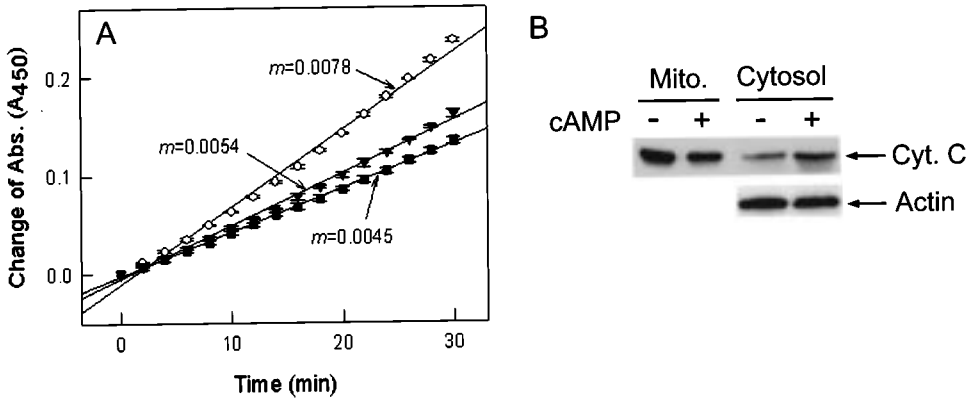

FIGURE 4: Regulation of cytochrome *c* oxidase activity by cAMP in CHO cells. (A) CHO cells were either untreated (○) or treated with 10 μM forskolin (■) or 50 μM dibutyryl cAMP (▼) for 30 min. Cells were permeabilized with saponin, and the cytochrome *c* oxidase activities were measured spectrophotometrically at 450 nm for 30 min. All the experiments were performed in triplicates for three times and values are means ± SD. The enzyme activity was determined by the slope of the curve. (B) Immunoblot of cytochrome *c* content in mitochondrial and cytosolic fractions in CHO 10001 cells, in the presence or the absence of 50 μM dibutyryl cAMP for 30 min. The position of cytochrome *c* is indicated.

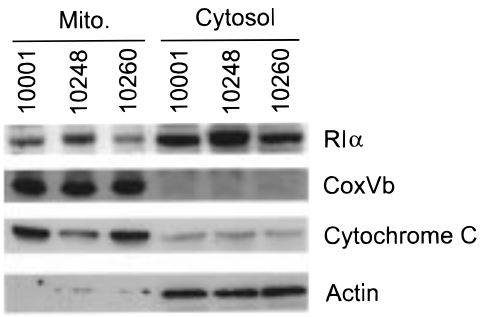

FIGURE 5: Cytochrome *c* levels are regulated by RIα. Mitochondrial and cytosolic fractions of 10001, 10248, and 10260 cells were prepared as described under Materials and Methods Approximately 15 μg of cytosolic protein or 5 μg of mitochondrial fraction was separated by SDS−PAGE and immunoblotted with antibodies for either RIα, CoxVb, cytochrome *c*, or actin.

survey of basal mitochondrial cytochrome *c* levels in 10001, 10248, and 10260 cells revealed that it was significantly reduced in the RIα mutant in comparison to the wild-type 10001 cells (Figure 5). The C subunit mutant 10260 showed cytochrome *c* levels comparable to those of the wild-type cells. No significant changes were observed in either the cytosolic cytochrome *c* or the CoxVb levels in all three cell types examined. Furthermore, a modest increase in RIα subunit was found in the 10248 mutant (Figure 5), consistent with previous reports of the stabilization of the mutant RI subunits (*28*). These results demonstrate that cAMP can regulate cytochrome *c* oxidase activity and cytochrome *c* levels, presumably through the interaction of RIα with CoxVb.

DISCUSSION

Previous studies with PKA genetic mutants of CHO and the mouse adrenocortical carcinoma Y1 cells implicate the RIα subunit of PKA in regulating cisplatin resistance, a function independent of the C subunit (*11*). Further investigation shows that P-glycoprotein expression in these mutants is also regulated in an RIα-dependent manner (*12*), leading us to speculate that the R subunit may have other functions. Our results in this report revealed a novel mechanism of cAMP signaling through RIα subunit interaction. One of the targets that interacts with RIα is CoxVb (Figure 1). We demonstrated the physical association of RIα with CoxVb in vitro as well as in CHO cell lysates by coimmunoprecipitation (Figure 1). The interaction of RIα with CoxVb occurs at the amino terminus of RIα and is regulated by cAMP (Figures 2 and 3). Consistent with the regulation of RIα interaction with CoxVb, the cytochrome *c* oxidase activity is inhibited by cAMP in CHO cells (Figure 4A). The reduced cytochrome *c* oxidase activity is accompanied by a decrease in cytochrome *c* levels in the mitochondria and a concomitant increase release of cytochrome *c* into the cytosol (Figure 4B). In addition, the cytochrome *c* level is also markedly decreased in the 10248 RIα mutant, but not in the C subunit mutant, compared to the wild-type cells (Figure 5). No significant changes in CoxVb levels were observed (Figure 5). These results demonstrate a novel mechanism of cAMP signaling whereby the R subunit may interact with other target proteins and modulate their activities in a cAMP-dependent manner. Whether the R subunit and CoxVb exist in a dimerized state, similar to its association with the C subunit, or in a complex including the C subunit remains to be determined.

It has already been shown previously that PKA is present in the mitochondria of various rat tissues (*29*). Since binding of CoxVb to the amino terminus of RIα may overlap with the AKAP binding domain (Figure 2), it raises the possibility that CoxVb may be an AKAP which targets the subcellular localization of PKA to the mitochondria. However, the distinct properties of the cAMP-regulated RIα interaction with CoxVb are not previously observed with AKAP and PKA. Furthermore, the regulation of cytochrome *c* oxidase activity by cAMP (Figure 4A) adds fuel to the notion that RIα may modulate cytochrome *c* oxidase function through its association with CoxVb, thus arguing against CoxVb playing the role of the anchoring protein. Moreover, it has also been shown in some instances that AKAPs themselves can also be substrates for PKA on top of targeting the localization of PKA (*16*). Therefore, it is conceivable that CoxVb can be an AKAP and also be regulated by RIα.

What is the physiological significance of the interaction of RIα with CoxVb? Cytochrome *c* oxidase has been suggested to play key roles in the regulation of energy production, dependent on the level of respiration and oxidative phosphorylation in response to short-term and long-term cellular energy requirements (*19*). The role of CoxVb in the cytochrome *c* oxidase complex is unknown but may be involved in catalysis, regulation of catalysis, or the folding or stability of the catalytic subunits (*19*). We speculate that cytochrome *c* oxidase activity may be regulated by cAMP in response to cell growth. In the absence of cAMP, association of RIα with CoxVb is essential for the maintenance of basal cytochrome *c* oxidase activity. Upon exposure to cAMP, dissociation of RIα from CoxVb occurs, leading to an inhibition of the oxidase activity (Figures 3 and 4A). These observations are further supported by the results with the 10248 cells which have a defective RIα subunit with altered cAMP binding, thus preventing holoenzyme dissociation (*21*). Hence the mutation in RIα confers tight association with CoxVb (Figure 3), resulting in an apparent increase in the oxidase activity (Table 1). These observations are consistent with the growth inhibitory effects of cAMP in CHO cells (*30*) whereby the inhibition of cytochrome *c* oxidase activity by cAMP correlates with a decreased energy demand during growth inhibition (Figure 4A).

In addition to its growth inhibitory effects, cAMP is also known to induce cell death in various cell types (*31*). However, the mechanism of cAMP-induced cell death is not completely understood. Recent studies have demonstrated that increased release of the mitochondrial peripheral membrane protein cytochrome *c* from the mitochondria may activate caspases and apoptosis (*15*, *26*, *27*). We speculate that regulation of cytochrome *c* oxidase activity by cAMP, presumably mediated through RIα interaction with CoxVb, may also influence the levels of cytochrome *c*. Our results showed that inhibition of cytochrome *c* oxidase activity by cAMP is accompanied by a decrease in mitochondrial cytochrome *c* and a concomitant increase in its release into the cytosol (Figure 4). It has been shown that cytochrome *c* mRNA levels may be increased transcriptionally by cAMP (*32*). However, in our studies, transient exposure to cAMP causes a decrease in the mitochondrial cytochrome *c* levels

within 30 min. These results exclude the possibility of transcriptional regulation because we observe a decrease instead of an increase in cytochrome *c* levels in the presence of cAMP. Also the 60 min elapse for the induction of cytochrome *c* mRNA levels (*32*) differs significantly from the transient cAMP exposure in our experiments. In addition, although both the 10248 RIα mutant (*21*) and the 10260 C subunit mutant (*22*, *33*, *34*) have reduced PKA activity, only the RIα mutant shows altered cytochrome *c* levels (Figure 5), thus further supporting that the R subunit but not the C subunit may regulate cytochrome *c* levels.

In summary, our study provides evidence that the RIα subunit of PKA interacts with CoxVb and is cAMP-sensitive, resulting in the regulation of cytochrome *c* oxidase activity and the cytochrome *c* levels in the mitochondria. These results demonstrate a novel signaling mechanism of cAMP mediated by the interaction of RIα with other target proteins in addition to the C subunit. The effects of RIα on cytochrome *c* may have significant implications on the mechanism of cAMP-induced apoptosis in eukaryotic cells.

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