8Cl-cAMP modifies the balance between PKAR1 and PKAR2 and modulates the cell cycle, growth and apoptosis in human adrenocortical H295R cells

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Abstract

Various types of protein kinase A (PKA) alterations have been observed in adrenocortical tumours and Carney complex (CNC). PKA is a heterotetramer of two regulatory and two catalytic subunits. The R1A and R2B proteins are the most abundant regulatory subunits in endocrine tissues. A decrease in R2B protein levels has been observed in adrenal adenoma, whereas tumours from patients with CNC display a decrease in R1A protein levels. Dysregulation of the balance between R1A and R2B may thus be involved in adrenal tumorigenesis. We investigated the impact of the differences in the balance of PKA subunits on cell growth using specific cAMP analogues. We assessed the effects of 8-chloroadenosine-cAMP (8Cl-cAMP), a site-selective activator of PKA R2B, in H295R adrenocortical cells. 8Cl-cAMP stimulated PKA activity, decreased R1A levels and increased R2B levels. It had no cytotoxic effects, initially stimulating DNA synthesis and then inducing apoptosis by disrupting G₂/M progression. We observed an initial accumulation of cells in the S phase, translocation of cyclin A to the nucleus, CDK2 activation, sustained DNA synthesis and proliferating cell nuclear antigen accumulation. Cell cycle arrest in the G₂ phase was parallel with the accumulation of cyclin B and the inactivation of CDC2 kinase. The 8CPT-cAMP, which activates the R2B subunit, had similar effects. R2B silencing reduced the apoptosis induced by tumour necrosis factor α and transforming growth factor β . Thus, R2B is a key regulator of proliferation/differentiation in H295R cell line along with the complex balance between the PKA subunits. Activation of PKA R2B and dysregulation of the R1A/R2B balance regulate cell cycle progression and apoptosis in adrenocortical cells by modulating cyclin production and cyclin-dependent kinase activities.

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Introduction

Aberrant cAMP signalling has been linked to adrenocortical and other tumours, mostly to endocrine tumours (Bertherat 2001, Bossis & Stratakis 2004). Based on the notion that proliferation, differentiation and steroid synthesis are principally dependent on ACTH-induced cAMP generation in the adrenal cortex, it has been suggested that changes in the cAMP cascade may result in the activation of cortisol synthesis and secretion and the development of adrenocortical neoplasia. Many defects downstream from cAMP production have been identified in adrenocortical tumours (Groussin *et al.* 2000, Rosenberg *et al.* 2002, Horvath *et al.* 2006, 2008, Libe *et al.* 2008), but their effects on proliferation and differentiation remain unclear. Among these defects, the *PRKARIA* alteration has a wide implication for the understanding of the cAMP signalling in adrenocortical tumours (Groussin *et al.* 2002a).

A high frequency of heterozygous inactivating germline mutations of the *PRKARIA* gene has been reported in patients with Carney complex (CNC; Kirschner *et al.* 2000, Veugeliers *et al.* 2004), which is characterised by spotty skin pigmentation (lentiginosis), endocrine hyperactivity with primary pigmented nodular adrenocortical disease (PPNAD) and cardiac myxoma (Carney *et al.* 1985, Groussin *et al.* 2002a,b, 2006). Somatic *PRKARIA* mutations have been demonstrated in sporadic secreting adrenocortical adenomas with clinical, hormonal and pathological characteristics similar to those of PPNAD (Bertherat *et al.* 2003). Moreover, *in vitro* studies have indicated that *PRKARIA* gene mutation (non-sense mRNA or truncated R1A) stimulates protein kinase A (PKA) activity (Groussin *et al.* 2002a,b, 2006). However, the relation of PKA type 1 and tumorigenesis in endocrine tumours is not exclusively due to *PRKARIA* gene mutation. The growth of several endocrine tumours is associated with the absence of R1A or very low concentrations of

this subunit (Griffin *et al.* 2004*a,b*). Indeed, tumoural pituitary cells are characterised by low or absent expression of the R1A subunit protein, and this is probably due to proteasome-dependent protein degradation (Yamasaki *et al.* 2003). In addition, the downregulation of *PRKARIA* expression by up to 70% in transgenic mouse tissues and embryonic fibroblasts results in concomitant increases in PKA kinase activity, cell proliferation and tumour formation (Griffin *et al.* 2004*a,b*, Veugelers *et al.* 2004). Moreover, we have shown recently that inactivating *PRKARIA* gene dysregulated cAMP pathway and reduced transforming growth factor β (TGF β or TGF β 1)-induced apoptosis by inhibiting SMAD3 expression in the human adrenocortical cell line (H295R; Ragazzon *et al.* 2009). These results suggest that the PKA type 1 may be considered as a tumour suppressor gene and as one of the key players in the development of adrenocortical tumours.

By contrast, the increase in PKA R1A levels has been associated with other types of human cancer tissues and cell lines, including retinoblastoma, breast cancers, malignant osteoblasts and serous ovarian tumours (Livesey *et al.* 1982, Miller *et al.* 1993, Stubbs *et al.* 1996, McDaid *et al.* 1999, Bossis & Stratakis 2004). Thus, based on the upregulation of R1A in several cancers, studies show that inhibition of R1A expression through antisense oligonucleotides (Nesterova *et al.* 2000, Cho-Chung 2004, Cho-Chung & Nesterova 2005) results in the growth arrest of several tumour cell lines. PKA R1A may be considered as an oncogene in these cancers (Nesterova & Cho-Chung 2000, Cheadle *et al.* 2008).

PRKARIA could act as a tumour suppressor gene and/or an oncogene, depending on the tumour and cell types. However, PKA holoenzyme is formed of two PKA regulatory subunits. The cAMP-dependent protein kinase type 1 (PKA 1) and cAMP-dependent protein kinase type 2 (PKA 2) have identical catalytic (C) subunits but different regulatory (R) subunits (R1 versus R2). The two subtypes are expressed in a balance of cell growth and differentiation (Cho *et al.* 2002, Bossis & Stratakis 2004, Neary *et al.* 2004). Tumours from patients with CNC display a decrease in R1A protein levels and a compensatory activation of PKA type 2 (Bossis & Stratakis 2004). Tumour lesions due to the downregulation of *PRKARIA* expression in transgenic mice are associated with an increase in total PKA type 2 activities and an increase in PKA R2B protein levels (Griffin *et al.* 2004*b*). In adrenocortical adenoma, Mantovani *et al.* (2008*a*) and Vincent-Dejean *et al.* (2008) have demonstrated a defect in R2B expression and synthesis in benign, cortisol-secreting tumours. Lastly, many studies have shown that PKA regulatory subunit switching elicits tumour reversion or enhanced differentiation. Downregulation of PKA type 1 and

overexpression of PKA type 2 in the PC3M prostate carcinoma cells lead to growth inhibition (Neary *et al.* 2004). By contrast, the imbalance in the PKA R1A/R2B ratio in favour of the R2B subunit induced by 8-chloroadenosine-cAMP (8Cl-cAMP) results in the cAMP-dependent proliferation of adenomatous somatotrophs (Lania *et al.* 2004). The overproduction of R2B in immortalised cell lines derived from a PPNAD bearing mutation inactivating *PRKARIA* increases cell proliferation (Nesterova *et al.* 2008). Moreover, disrupting the balance of PKA subunits in the H295R by inactivation of *PRKARIA* gene confers resistance to apoptosis (Ragazzon *et al.* 2009). However, no information is available on the PKA R2B involvement in this H295R cell line.

Specific cAMP analogues can preferentially activate the different PKA subtypes (Dyson *et al.* 2009). The regulatory subunits of PKA each possess two cAMP-binding sites positioned in tandem at the carboxy-terminus of the protein. While highly conserved among all the regulatory subunits, these binding sites are not equivalent in their affinities for cAMP, and are distinguished as sites A and B within each regulatory subunit (Tasken & Aandahl 2004).

To evaluate the stimulatory effects of PKA type 2 on adrenocortical tumours, we used 8Cl-cAMP, which is the most potent cAMP analogue that induces inhibition of cell growth in a variety of cancer cell types (Cho *et al.* 2002). 8Cl-cAMP binds to A and B sites of PKA R1 with a higher affinity than cAMP, thereby activating PKA type 1, and decreasing the concentrations of R1A protein and mRNA as demonstrated in HL-60 leukaemia cells (Rohlf *et al.* 1993). 8Cl-cAMP also binds strongly to site B of PKA type 2, increasing R2B protein levels (Rohlf *et al.* 1993). We compared the 8Cl-cAMP effects with other selective cAMP analogues. 8CPT-cAMP is a cAMP analogue that is highly selective for site B of PKA type 2, and it does not bind to PKA type 1 (Knutsen *et al.* 1996). The Sp-diastereomer of AMP (SP-cAMP) is a potent, membrane-permeable activator of both cAMP-dependent PKA type 1 and type 2. NCI-H295R, a steroid-secreting adrenocortical cell line, is the main model of human adrenocortical tumour (Groussin *et al.* 2000, Liu *et al.* 2004*a*, Rainey *et al.* 2004, Jaroenporn *et al.* 2008). The cell line is generally used to study the effects of antitumour drugs or steroid synthesis.

Thus, we have investigated the effects of these cAMP analogues on the cell cycle and the regulation of cyclins and cyclin-dependent kinase activity in the H295R cells. We show that the stimulation of PKA activity with cAMP analogues, the dysregulation of the R1A/R2B balance and the increase in R2B have major impacts on the cell cycle, and allow to identify the precise role of R2B in this context.

Materials and methods

Materials

We used various cAMP analogues, which are cAMP site selective of PKA type 1 or 2, and act as phosphodiesterase (PDE)-resistant protein kinase activators. 8Cl-cAMP (Biolog, Life Science Institute) binds to A and B sites of PKA R1, thereby activating type 1 PKA and decreasing the concentrations of R1A protein and mRNA (Rohlf *et al.* 1993). 8Cl-cAMP also binds strongly to site B of PKA R2, activating type 2 PKA and increasing R2B protein levels (Rohlf *et al.* 1993). It inhibits the growth of several types of cancer cells. 8CPT-cAMP is a cAMP analogue that is highly selective for site B of PKA type 2 (8CPT-cAMP, Biolog, Life Science Institute; Knutsen *et al.* 1996). The SP-cAMP (Calbiochem by VWR International, Fontenay-sous-Bois cedex, France) is a potent, membrane-permeable activator of cAMP-dependent PKA 1 and PKA 2.

Cell culture

Human H295R adrenocortical carcinoma cells were grown in DMEM–Ham's F12 supplemented with 50 units/ml penicillin, 50 mg/ml streptomycin, 2% Ultrosor G2 (Biosepra, Fremont, CA, USA) and ITS (5 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml selenium; Sigma) at 37 °C under an atmosphere of 5% carbon dioxide/95% air (Groussin *et al.* 2000, Rizk-Rabin *et al.* 2008). Cells were cultured in 6-well plates (350×10^3 cells/well) for protein extraction and 5-bromo-2'-deoxyuridine (BrdU) incorporation; in 12-well plates (250×10^3 cells/well) for cell cycle profile determination, synchronisation, proliferating cell nuclear antigen (PCNA) staining and apoptosis assays, and in 96-well plates (10×10^3 cells/well) for MTT assays. Cells were allowed to attach for 48 h, and were then incubated with the cAMP analogues for 4 or 7 days. Medium and drugs were replenished on days 3 and 5.

Human embryonic kidney 293T (HEK293T) cells and MCF-7 cells were grown in DMEM supplemented with 10% FCS.

MTT cell survival and BrdU incorporation

The MTT assay is based on the conversion of the yellow tetrazolium salt MTT (Cell Titer 96 Non-Radioactive Cell Proliferation Assay, Promega) to purple formazan crystals by metabolically active cells. It provides an estimate of the number of viable cells (Rizk-Rabin *et al.* 2008). H295R cells were used to seed 96-well plates at a density of 10×10^3 cells, and were cultured for 48 h. They were then incubated with the cAMP analogues, at the concentrations indicated in the figures, for 24 h,

48 h, 4 or 7 days. The MTT reagents were used according to the manufacturer's recommendations. Optical absorbance was measured at 570 nm in a microplate reader. For BrdU incorporation, the cells were used to seed six-well plates, and were treated as described above. They were incubated with BrdU (10 µM; Sigma) for 6 h before each time point (days 2, 4, 6 and 7). Cells were then harvested, washed in PBS and fixed in 70% ethanol (500 µl). For BrdU analysis, cells were centrifuged, rinsed twice in PBS, incubated with 2 M HCl at room temperature for 20 min, collected by centrifugation, and washed twice in PBS and once in PBS+0.5% Tween+0.05% w/v BSA. They were then incubated with 20 µl of monoclonal anti-BrdU monoclonal FITC antibody for 20 min according to the manufacturer's instructions (BD Biosciences, Le Pont de Claix cedex, France). They were centrifuged, rinsed in PBS, suspended in 500 µl PBS supplemented with 50 µg/ml propidium iodide plus 100 µg/ml RNase A and analysed by flow cytometry using a FACscan (EPICS XL Coulter, Villepinte, France). Data were analysed with Multicycle software. All experiments were carried out three times. The histogram was constructed with at least 20 000 cells.

Western blotting

Whole-cell protein lysates were obtained by extraction with RIPA lysis buffer containing 50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.1% v/v Nonidet P-40 (NP40), 1 mM dithiothreitol (DTT), protease inhibitor cocktail (Roche), 1 mM phenylmethylsulphonyl fluoride (PMSF), and anti-phosphatase inhibitor, 1 mM sodium orthovanadate, and 1 mM okadaic acid (Sigma). Cell cytosols were extracted by incubation for 5 min with buffer A containing 20 mM HEPES, pH 7.9, 20 mM NaCl, 50 mM NaF and 0.5% v/v NP40. Cells were scraped off and collected by centrifugation at 13 000 g for 15 min. The resulting supernatant was collected, and the nuclear fraction was obtained from the pellet by incubation with buffer B, which contained 20 mM HEPES, pH 7.9, 50 mM NaF, 200 mM EDTA, 420 mM NaCl, 25% v/v glycerol and 1.5 mM MgCl₂. Both buffers A and B were supplemented with protease inhibitor cocktail, 1 mM PMSF and anti-phosphatase inhibitors as for the RIPA buffer. The nuclear pellet was incubated for 15 min and centrifuged, and the supernatant was collected. Equal amounts of protein, as determined with the Bio-Rad protein assay (Bio-Rad), were separated by electrophoresis in 10% SDS gels and transferred electrophoretically onto membranes. Western blotting was performed with primary mouse antibodies directed against R1A (1/250, Becton Dickinson Transduction Laboratories), R2A (1/250) and R2B (1/1000), and catalytic subunits

(1/1000; Santa Cruz Biotechnology, Tebu, France). The anti-cyclin E antibody was obtained from BD Biosciences. The anti-cyclin A and B antibodies (1/200) and antibodies against the cyclin-dependent kinases CDK2 and CDC2 (listed as CDK1 in the HUGO Database)/p34 were obtained from Santa Cruz Biotechnology. We used anti- β -actin antibody (1/2500; Sigma) as a standard for cell lysates and anti-lamin A/C antibody (1/1000; Cell Signaling) as a standard for nuclear samples. The antigen–antibody complexes were visualised with appropriate secondary antibodies (Santa Cruz) by chemiluminescence detection (ECL kit, Amersham). The signals were digitised with the Gene Tool analysis system.

Immunoprecipitation and histone kinase assay

Cells were incubated with RIPA buffer for 30 min as described above. Cells were scraped off, sonicated twice at 40 Hz for 20 s each, and centrifuged for 10 min at 13 000 g, and the supernatant was collected. An aliquot of each sample (equivalent of 200 μ g total protein) was incubated with 2 μ g of specific anti-CDK2 or anti-CDC2 antibody, with gentle shaking, for at least 4 h at 4 °C. Samples were then incubated with protein G plus agarose (20 μ l) or protein A/G plus agarose (20 μ l; Santa Cruz) in RIPA buffer for 2 h for immunocomplex formation. The beads were collected by centrifugation, and washed three times with RIPA buffer and twice with kinase reaction buffer (50 mM Tris–HCl, pH 7.5, 15 mM $MgCl_2$ and 1 mM DTT). Before *in vitro* kinase assays, the immunoprecipitated CDK2 and CDC2 were subjected to SDS-PAGE in 12.5% acrylamide gels and were electrophoretically transferred to membranes, which were incubated with antibodies against either CDK2 or CDC2. The antigen–antibody complexes were visualised as described above. For *in vitro* kinase assays, we measured CDK2- and CDC2-associated H1 kinase activities as described by Porter *et al.* (2001). The phosphorylated reaction products were resolved by SDS-PAGE in 12.5% acrylamide gels, and γ - ^{32}P incorporation was visualised by autoradiography.

Cell cycle distribution

We seeded 12-well plates with the cells, which were cultured and then harvested, rinsed with PBS and fixed in 500 μ l of 70% ethanol. The cells were centrifuged, rinsed in PBS and suspended in 500 μ l of 50 μ g/ml propidium iodide in PBS supplemented with 100 μ g/ml RNase A. They were analysed by flow cytometry using a FACscan machine (EPICS XL Coulter). Data were analysed with Multicycle software. All experiments were carried out three times. Each histogram was constructed with at least 10 000 cells (Rizk-Rabin *et al.* 2008).

PCNA immunostaining

Monoclonal phycoerythrin (PE)-conjugated antibody against PCNA was used as an essential marker for DNA synthesis. Cells fixed in 70% ethanol were centrifuged, washed in PBS and permeabilised by incubation for 5 min with Triton X-100 (0.25%). They were then incubated with 20 μ l FITC-conjugated antibody against PCNA overnight at 4 °C (BD Biosciences). PCNA staining was analysed by flow cytometry with a FACscan machine (EPICS XL Coulter; Beppu *et al.* 1994).

Cell cycle synchronisation

We used H295R cells to seed 12-well plates at a density of 150×10^3 cells/well. The cells were cultured for 24 h and then treated for 24 h with aphidicolin (4 μ g/ml), an inhibitor of DNA synthesis (Sigma), or for 48 h with nocodazole (10 μ M), an anti-neoplastic agent interfering with microtubule polymerisation (Sigma). The cells were then washed three times with PBS and incubated in a medium with or without 8Cl-cAMP or 8CPT-cAMP for 6, 12, 24 or 48 h. Propidium iodide was added, and the distribution of cells in the various phases of the cell cycle was analysed by flow cytometry (FACscan).

Annexin V–FITC staining of apoptotic cells

Apoptotic cells were quantified with the Annexin V–FITC Apoptosis detection kit according to the manufacturer's instructions (Roche). The cells were analysed with a Coulter Epics Altra flow cytometer (Koopman *et al.* 1994, Rizk-Rabin *et al.* 2008).

PKA assay

The PepTag non-radioactive protein kinase assay kit (Promega) was used to measure PKA activity according to the manufacturer's instructions. The reaction buffer (supplied with the PepTag kit) contained 1 μ M cAMP to ensure PKA activation. Bovine PKA (Promega) was used as a control for kinase activity, and PKA inhibitor (PKI; Sigma) was used as a specific PKI. The PepTag A1 peptide substrate was subjected to electrophoresis for 20 min in 1% (w/v) agarose gels, and the separated bands were photographed with a phosphorimager. The intensities of the bands were analysed with Gene Tool software (Rizk-Rabin *et al.* 2008).

Transfection and electroporation of the cells

H295R cells were electroporated with AMAXA Nucleofector II (program T20 for H295R and program T30 for ATC1; Amaxa Biosystems, Cologne, Germany).

HEK293 cells were transfected with the Lipofectamine 2000 protocol. We electroporated 5 million H295R cells (100 μ l of AMAXA electroporation buffer, kit R) with 4 μ l of 80 pmol/ μ l of each siRNA (program T20). The siRNA used for targeting *PRKAR1B* was AUGUGAUGGUGUUGGAAGAdTdT (siR2b), and the siRNA control was CAGUCGCGUUUGCGACUGGdTdT (siS; Dharmacon, Chicago, IL, USA). Cells were used to seed 12-well plates, in which they were cultured for 24 h. They were then cultured for 48 h in the presence or absence of 5 ng/ml tumour necrosis factor α (TNF α) or 25 ng/ml TGF β (Sigma).

Statistical analyses

Data were analysed by ANOVA and regression analysis with Fisher's projected least significant difference or Scheffe's *F* test for the comparison of means (StatView

5.0, SAS Institute, Cary, NC, USA). Data are expressed as means $\pm$ S.E.M. Values of $P < 0.05$ were considered significant. The number of experiments is defined in each figure legend.

Results

8Cl-cAMP regulates the level of PKA subunits and increases basal PKA activity

The PKA regulatory and catalytic subunits were affected differently by cAMP analogues (Fig. 1A). The results are expressed as fold inductions with respect to controls (the values for which were arbitrarily set to 1; Fig. 1B). Incubation with 8Cl-cAMP decreased the R1A/actin ratio ($P < 0.001$ on day 4 and $P = 0.01$ on day 7) and significantly increased the abundance of R2B on day 4 ($P = 0.004$) and day 7 ($P = 0.003$), as reported for other tumour cells (Kim *et al.* 2000, 2001). R2A levels were

Figure 1 Effects of 8Cl-cAMP on PKA regulatory subunits and PKA activity. (A) Western blots of the PKA subunits in cells treated with cAMP analogues (7 days). (B) The results are expressed as percentage relative band density/ β -actin of PKA subunits, normalised with respect to the corresponding controls (arbitrarily set to 1) in H295R treated cells. The results are the means $\pm$ S.E.M. of three duplicate independent experiments. Day 4 analogue-treated cells compared with control arbitrarily set to 1 (for 8-Cl-cAMP: R1A $**P = 0.01$, R2A $***P = 0.008$, R2B $***P = 0.004$, Ca $***P = 0.0005$; for 8CPT-cAMP: R2B $***P = 0.0001$, Ca $***P = 0.0005$; for SP-cAMP: R1A $**P = 0.02$, Ca $***P = 0.0009$). Day 7 analogue-treated cells compared with control (for 8Cl-cAMP: R1A $*P = 0.02$, R2A $**P = 0.01$, R2B $***P = 0.003$, Ca $***P < 0.0001$; for 8CPT-cAMP: R2B $*P = 0.05$, Ca $**P = 0.001$; for SP-cAMP: R1A $***P < 0.0001$, Ca $**P = 0.001$). (C) PKA type 1/type 2 ratio. The results are the means $\pm$ S.E.M. of three duplicate independent experiments, $*P < 0.05$ and $**P < 0.001$. (D) PKA activity. Cells cultured for 48 h were incubated with 100 μ 8Cl-cAMP, 8CPT-cAMP, SP-cAMP or vehicle for 15 min. Cell extracts were prepared with (not shown) or without cAMP. The free PKA/total PKA ratio showed that cells exposed to 8Cl-cAMP and 8 CPT-cAMP and extracted without cAMP had a higher PKA activity. The results are the means $\pm$ S.E.M. of three duplicate independent experiments, and $***P < 0.0001$.

increased by 8Cl-cAMP only on day 4 ($P=0.008$). Incubation with 8CPT-cAMP had no effect on R1A/actin and R2A/actin ratios, but it strongly increased the R2B/actin ratio ($P<0.0001$ on day 4 and $P<0.05\%$ on day 7). Similarly, incubation with SP-cAMP only increased R1A expression on day 7 ($P<0.0001$), but the R2B/actin and R2A/actin ratios remained unchanged. Significant accumulation of the Ca catalytic subunit was observed on day 7 with all cAMP treatments. The ratio of R1A PKA to R2B PKA subtypes was also significantly lower in 8Cl-cAMP-treated and 8CPT-cAMP-treated cells than in controls or SP-cAMP-treated cells (Fig. 1C).

Cells were incubated with the cAMP analogues for 15 min to determine whether the molecules had a direct effect on PKA activity. Basal PKA activity depended on the cAMP analogue. Cells incubated with 8Cl-cAMP had significantly higher levels of basal PKA activity corresponding to free PKA on total PKA ratio (measured in the absence of cAMP) than controls ($P<0.0001$; Fig. 1D). The PKA activity of 8Cl-cAMP-treated cells measured in the presence of cAMP (representing the total PKA activity) was significantly higher than basal levels of PKA activity ($P<0.05$; data not shown). Similarly, 8CPT-cAMP increased basal PKA activity in the absence of cAMP ($P<0.001$). SP-cAMP stimulation did not increase basal PKA activity to levels higher than those in control cell extracts. However, SP-cAMP-treated cells displayed higher levels of PKA activity than cAMP-treated control cells when assayed in the presence of cAMP ($P<0.05$; data not shown). PKA activity was completely abolished by the specific PKI (data not shown).

8Cl-cAMP regulates cell viability and DNA synthesis

The rate of cell viability was assessed by MTT assay in unsynchronised H295R cells. Population doubling times were long, and occurred at >96 h (4 days; data not shown) as also reported by Gazdar *et al.* (1990) and Rainey *et al.* (1994). Cell viability was assessed on days 1, 2, 4 and 7 (Fig. 2A and B). 8Cl-cAMP as the other cAMP analogues had no cytotoxic effects on H295R cells. Treatment with the different analogues did not affect cell viability on days 1 and 2 compared with untreated cells. On days 4 and 7, a slight but statistically significant increase in cell viability was observed in 8Cl-cAMP-, 8CPT-cAMP- and SP-cAMP-treated cells ($P<0.02$ and $P<0.05$ versus control; Fig. 2A). The cell viability increase represented 1.4- to 1.5-fold increase for 8Cl-cAMP respectively on days 4 and 7, and 1.3-fold induction for 8CPT-cAMP and SP-cAMP respectively on days 4 and 7 with respect to the corresponding control arbitrarily set to 1 (Fig. 2B). No significant differences in cell viability between treated groups were observed at any time point (Fig. 2A and B).

To assess whether 8Cl-cAMP modulates cell viability/proliferation, we evaluated DNA synthesis by H295R cells by monitoring BrdU incorporation for 6 h on days 2, 4, 6, and 7 after treatment with cAMP analogues (Fig. 2C). The study of BrdU incorporation along the kinetic time on days 2, 4, 6 and 7 showed that the level of replicating DNA (expressed as a % BrdU staining) in 8Cl-cAMP-treated cells was significantly higher than that of replicating DNA in untreated control cells on days 2, 4 and 6 ($P<0.003$ for days 2 and 4 and $P=0.05$ for day 6). Similar results were obtained for 8CPT-cAMP, while DNA synthesis in SP-cAMP-treated cells had the same level as the untreated cells (Fig. 2C). BrdU incorporation allows analysis of the pattern of the DNA synthesis along the time course in each group. DNA synthesis started to decrease on day 4 ($P=0.03$ versus day 2) and day 7 ($P=0.01$ versus day 6) in both untreated cells and SP-cAMP-treated cells. The decrease in DNA synthesis occurred later on day 7 in 8Cl-cAMP-treated cells as in 8CPT-cAMP-treated cells ($P=0.001$ and $P=0.012$ respectively versus day 6).

8Cl-cAMP treatment leads to the accumulation of cells in the S and G₂ phases and enhances PCNA staining

As the high level of DNA synthesis by 8Cl-cAMP-treated cells was not reflected by the high proliferative rate in H295R cells, we analysed whether this mirrored a disturbance in cell cycle progression (Fig. 3A). Cells incubated with 8Cl-cAMP for 4 days were significantly fewer in the G₀/G₁ phase than controls (57.7 vs 66.7%, $P<0.001$) and more in the S phase (24.5 vs 15.5%, $P<0.001$; Fig. 3A and B). The accumulation of cells in the S and G₂ phases continued until day 7 (24 vs 19%, $P<0.05$ for the G₂ phase; Fig. 3B). The same decrease in the number of G₀/G₁ cells was observed 7 days after 8CPT-cAMP treatment (51.84 vs 63.1%, $P<0.001$), with an accumulation mostly in the S and G₂ phases being observed on day 7 (24.8 vs 19%, $P<0.05$ for the G₂ phase; Fig. 3A and B). About 66.7% of untreated H295R cells (controls) were in the G₀/G₁ phase, 15.5% were in the S phase and 18.3% were in the G₂/M phase (Fig. 3A and B). The SP-cAMP-treated cells displayed a similar distribution between the phases of the cell cycle as control cells.

We used a PCNA-PE antibody to assess the accumulation of cells in the S phase by flow cytometry (Beppu *et al.* 1994; Fig. 3C). The 8Cl-cAMP-treated cells displayed significantly higher levels of PCNA-PE staining (23.05%, $P<0.001$) than controls after day 7. The observed levels of PCNA staining were also higher in 8CPT-cAMP-treated cells (16.3%, $P<0.05$). The percentage of SP-cAMP-treated cells in the S phase was 1.24%.

Figure 2 Effects of 8CI-cAMP on cell survival and DNA synthesis. (A) Cell survival curve of H295R cells, as assessed by the MTT assay in the absence of treatment (control) (—◆—) and after incubation with 8CI-cAMP (—■—), 8CPT-cAMP (—▲—) and SP-cAMP (—◆—) for 1, 2, 4 or 7 days. Cells were plated 48 h before treatment. (B) Cell survival, and fold induction on control set to 1. Each MTT assay was performed in quintuplicate. In (A), the results are the means $\pm$ S.E.M. of one MTT experiment among three MTT independent experiments: b,c * P <0.05 for 8CPT-cAMP and SP-cAMP respectively; a ** P <0.02 for 8CI-cAMP versus untreated control. In (B), the results of fold induction with respect to the corresponding control set to 1 are the means $\pm$ S.E.M. of three MTT independent experiments: * P =0.05, ** P =0.02 and *** P =0.005 versus control (untreated cells). (C) BrdU incorporation reflects the newly replicated DNA (S phase) during the time of BrdU incorporations. Cells were labelled with BrdU for 6 h at the end of each incubation period (2, 4, 6 and 7 days). BrdU incorporation was analysed after incubation with BrdU FITC antibody and propidium iodide. The upper square shows the percentage BrdU incorporation during the S phase. The results are the means $\pm$ S.E.M. of three duplicate independent experiments. * P <0.05 and ** P =0.003 versus untreated cells for the corresponding day.

Figure 3 Effects of 8Cl-cAMP on cell cycle distribution and PCNA. (A) The distribution of cells in the various phases of the cell cycle was analysed by propidium iodide incorporation after stimulation for 4 and 7 days with 8Cl-cAMP, 8CPT-cAMP or SP-cAMP (100 μ M). (B) The cell cycle distribution is plotted as a histogram. 8Cl-cAMP induced the accumulation of cells in the S and G₂ phases, and decreased the percentage of cells in the G₀/G₁ phase. (C) The accumulation of cells in the S phase was assessed by PCNA staining and FACS flow cytometry. The results presented are an overlay of the PCNA staining of treated and untreated cells (controls). Observations depicted in (A) and (C) are from the same experiment. The percentage indicated is the mean $\pm$ S.E.M. of three duplicate independent experiments. * $P < 0.05$ and ** $P < 0.001$ versus control.

8Cl-cAMP causes the accumulation of cyclin B in the cytosol and the nucleus and modulates CDK2 and CDC2 kinase activities

The accumulation of cells in the S and G₂ phases may result from the deregulation of cell cycle regulatory proteins, disturbing the checkpoint transition. We analysed the concentrations of cyclin E, cyclin A and cyclin B in the cell cytosol and nuclear fractions by western blotting on day 4 (Fig. 4). All cAMP analogues decreased cyclin E concentration in the cytosolic extracts and increased cyclin E concentration in the nuclear extracts, reflecting the transition from the G₁ phase to the S phase. Similarly, cyclin A concentration was higher in the nucleus. The nuclear translocation of cyclin A is an indicator of the S phase. By contrast, only cells incubated with 8Cl-cAMP displayed a significant increase ($P < 0.001$) in cyclin B levels in cytosolic and nuclear extracts (Fig. 4A and B), reflecting abnormal cell cycle progression through mitosis. Cells incubated with 8CPT-cAMP also accumulated cyclin B, but only in

the nuclear fraction, again reflecting abnormal G₂/M cell cycle progression.

Cyclins are active in complexes with cyclin-dependent kinases, and the consecutive activation of cyclin-cdk complexes is required for progression through critical checkpoints within the cell cycle. We assessed the kinase activities associated with immunoprecipitated CDK2 (responsible for S phase entry) and CDC2 (responsible for entry into and exit from mitosis) in cells incubated with the various cAMP analogues (Fig. 4C). The CDK2 and CDC2 immunocomplexes were labelled with the antibodies against CDK2 and CDC2 respectively (Fig. 4C Wb). The apparent molecular weight of the immunoprecipitated CDK2 and CDC2 proteins (33 kDa) on Coomassie blue-stained SDS-PAGE gels (Fig. 4C) was as expected. The amounts of immunoprecipitated CDK2 and CDC2 were similar in all treated and control cells (Fig. 4C Coomassie blue), but differences were observed in the corresponding kinase activities (Fig. 4C histone kinase). Levels of CDK2 kinase activity were higher in cells treated with 8Cl-cAMP, which had lower levels of

Figure 4 Effects of 8Cl-cAMP on the cyclin contents of cytosolic and nuclear extracts and on the cyclin-dependent kinases, CDK2 and CDC2. (A) Western immunoblot analysis of cyclin E (G₁), cyclin A (S) and cyclin B (G₂) in the cytosolic and nuclear extracts after 4 days of treatment. (B) Relative band density (cyclin/β-actin) of the cyclins in cytosolic extracts, and relative band density (cyclin/lamin A/C) in nuclear extract. The results are the mean ± S.E.M. of three duplicate independent experiments, **P* < 0.05 and ***P* < 0.001. (C) Western blot (Wb) of immunoprecipitated CDK2 or CDC2 with anti-CDK2 and anti-CDC2 respectively. Immunoprecipitation (Coomassie blue) of CDK2 and CDC2 proteins from cells incubated with cAMP analogues for 4 days. Autoradiography of CDK2- and CDC2-associated histone kinase activities assessed in immunoprecipitated CDK2 or CDC2 using histone H1 as a substrate. This is one of the representations. The relative activity of CDC2 and CDK2 blots is based on the measurement of the density of kinase band normalised to the density of the corresponding band of the loading immunoprecipitated protein complex visualised by Coomassie blue. The ratio of CDK2 activity for each treatment compared with control (set to 1) is 8Cl 3.16 ± 0.16, *P* < 0.0001; 8CPT 1.53 ± 0.048, *P* = 0.004; SP-cAMP 1.64 ± 0.17, *P* = 0.001; and for CDC2 kinase activity: 8Cl 0.732 ± 0.01, *P* < 0.0001; 8CPT 0.865 ± 0.012, *P* = 0.002; SP-cAMP 1.845 ± 0.05, *P* < 0.0001.

CDC2 kinase activity. Cells incubated with 8CPT-cAMP displayed levels of CDK2 kinase activity higher than those displayed by the control cells, but lower than those displayed by the cells incubated with 8Cl-cAMP. Levels of CDC2 kinase activity were lower in 8CPT-cAMP cells than in control cells. By contrast, cells incubated with SP-cAMP displayed higher levels of CDK2 and CDC2 kinase activities than control cells. However, CDK2 kinase activity levels were lower in SP-cAMP-treated cells than in cells incubated with 8Cl-cAMP (Fig. 4C), whereas SP-cAMP-treated cells had higher levels of CDC2 kinase activity than cells incubated with 8Cl-cAMP or 8CPT-cAMP (Fig. 4C).

8Cl-cAMP affects cell cycle progression

Two molecules were used to synchronise the cells – aphidicolin for the S phase and nocodazole for the G₂/M phase – to identify the checkpoint transition affected by 8Cl-cAMP (Uzbekov 2004). Cells were incubated with aphidicolin for 24 h, or with nocodazole for 48 h. They were then placed in a medium without aphidicolin or nocodazole, and incubated with the cAMP analogue or medium alone. The distribution of cells in the various phases of the cell cycle was analysed at 6, 12, 24 and 48 h. Incubation with aphidicolin for 24 h caused cells to accumulate in the late G₁/S phase,

at the G₁/S border (72% in the late G₁ phase; Fig. 5A). Most (64%) of the cells that were returned to medium alone were in the S phase at 6 h, progressed to the G₂ phase by 12 h, and divided and showed a normal cell cycle distribution 24 h after release (Fig. 5B). At 12 h, entry into the G₂ phase was delayed for cells incubated with 8Cl-cAMP. More number of these cells (65%) were

still in the S phase compared with those placed in the normal medium (47%, $P<0.001$; Fig. 5B). However, fewer 8Cl-cAMP-treated cells (7%) were present in the G₂ phase than control cells (30%, $P<0.002$). The 8Cl-cAMP-treated cells then progressed through the cell cycle at 24 and 48 h, as did those placed in the normal medium. However, a significantly smaller

Figure 5 Effects of 8Cl-cAMP on cell cycle progression in cells treated with aphidicolin or nocodazole. (A) Cell cycle distribution after incubation with aphidicolin for 24 h. (B) Cell cycle distribution of cells incubated in the normal medium, 8Cl-cAMP and 8CPT-cAMP for 6, 12, 24 and 48 h after the removal of aphidicolin. (C) Cell cycle distribution after incubation with nocodazole for 48 h. (D) Cell cycle distribution of cells incubated in the normal medium, 8Cl-cAMP and 8CPT-cAMP for 6, 12, 24 and 48 h after the removal of nocodazole. The representative cell cycle profile was obtained in one experiment and the percentages of cells in cell cycle phases are the means $\pm$ S.E.M. of three duplicate independent experiments. * $P<0.05$, ** $P<0.001$ and *** $P<0.0001$ in comparison with the control at the indicated phase.

percentage of the treated cells were in the G₀/G₁ phase at 48 h ($P<0.0001$ versus normal medium; Fig. 5B). As for 8Cl-cAMP, treatment with 8CPT-cAMP also delayed entry into the G₂ phase. At 12 h, the percentage of cells in the S phase was 65.6 vs 47% of those placed in the medium alone ($P=0.005$), and that of cells in the G₂ phase was 8.8 vs 31% of those placed in the medium alone (Fig. 5B).

Incubation with nocodazole for 18 h is effective in many types of cells, resulting in an accumulation of cells in the G₂/M phase (Uzbekov 2004), but H295R cells required incubation for 48 h to establish a partial G₂/M blockade, with fewer cells in the G₀/G₁ phase (48%; Fig. 5C) than non-synchronised control cells (66.7%; Fig. 3A), and in the accumulation of cells in the S phase (28%) and the G₂ phase (24%; Fig. 5C). Cells treated with 8Cl-cAMP for 48 h (after nocodazole) did not divide or enter the normal cell cycle (Fig. 5D). Cells remained blocked in the S (32%) and G₂ (49%) phases, with percentages of cells in these phases being well above those for controls placed in the medium alone (20% in the S phase, $P<0.001$, and 17% in the G₂ phase, $P<0.0001$; Fig. 5D). This resulted in a proportion of cells in the G₁ phase (25%) much smaller than the 59.5% in this phase in controls ($P<0.0001$). Cells incubated with 8CPT-cAMP

accumulated in the G₂ phase 12 h after release (53.6 vs 32.15% of those placed in the normal medium, $P=0.002$), and were blocked in the G₂ phase at 48 h (56% in the G₂ phase, 17% in the S phase and 26% in the G₁ phase versus 17, 20 and 59.42% respectively of those placed in the medium alone; Fig. 5D). The control cells gradually entered the normal cell cycle (Fig. 5D). Cells arrested in the G₂ phase showed a small sub-G₀ peak, indicating the appearance of apoptotic cells after incubation with 8Cl-cAMP and 8CPT-cAMP for 48 h (Fig. 5D).

8Cl-cAMP induces apoptosis

We studied the early apoptotic pathway by annexin V staining to confirm that treatment with 8Cl-cAMP and the accumulation of cells in the S/G₂/M phase enhanced apoptosis (Koopman *et al.* 1994). A higher proportion of cells treated with 8Cl-cAMP than of control cells entered apoptosis on day 4 ($*P<0.05$, data not shown) and day 7 ($**P<0.001$). The proportion of cells treated with TGF β (25 ng/ml) entering apoptosis was also higher ($**P<0.001$; Fig. 6A and B). Significantly more number of the 8Cl-cAMP-treated cells than of the control cells were apoptotic (early, late

Figure 6 Effects of 8Cl-cAMP on apoptosis. Apoptosis was assessed by annexin V-FITC/IP staining, and measured by FACS flow cytometry in cells incubated with 8Cl-cAMP, 8CPT-cAMP, SP-cAMP or TGF β (25 ng/ml) for 4 or 7 days. (A) A representative dot plot. Points are the means $\pm$ S.E.M. of three duplicate experiments, expressed as percentage apoptosis. (B) Results are expressed as the percentage of cells undergoing apoptosis (early annexin⁺/IP⁻, late apoptosis annexin⁺/IP⁺ and necrosis annexin⁻/IP⁺). Points are the means $\pm$ S.E.M. of three triplicate independent experiments. $*P<0.02$ and $**P<0.001$.

apoptosis and necrotic cells; $**P < 0.001$; Fig. 6B). Similar results were obtained when cells were treated with 8CPT-cAMP ($*P < 0.02$; Fig. 6A and B).

R2B invalidation enhances cell survival and reduces apoptosis

We invalidated the *R2B* gene to determine whether R2B was a key regulator of cell proliferation or apoptosis in H295R cells. Electroporation with an siRNA against R2B was carried out, and its efficacy was confirmed by the decrease in *R2B*/actin ratio observed on western blots of H295R and HEK293 cells (Fig. 7A). As transient transfection methods allow only limit periods of time for studying H295R cell proliferation, we studied the effect of *R2B* invalidation on the apoptosis induced by TGF β and TNF α (Mikhaylova *et al.* 2007) over a maximum of 48 h. Flow cytometry with annexin staining showed that siR2B-transformed cells were more in the annexin-IP cluster (viable cells) in basal conditions (79.5 vs 72% control, $P < 0.05$; Fig. 7B and C). The invalidation of *R2B* significantly decreased the percentage of apoptotic cells after 48 h of stimulation with TNF α and TGF β , as shown by comparison with treated siS control (Fig. 7B and C). This effect on cell survival was cell specific. Treatment with TGF β and TNF α induced apoptosis in siS control HEK293 cells (TGF β 42.47%, TNF α 38.6 vs 21.12% siS control, $*P = 0.05$ and $**P = 0.02$ respectively) and in MCF-7 cells ($***P < 0.0001$; Stoika *et al.* 2008; Fig. 7C). Invalidation of *R2B* in HEK293T and MCF-7 cells had no effect on apoptosis induced by TGF β and TNF α in siS as shown by comparison with treated siS control cells.

Discussion

Here, we have demonstrated that altering the balance between PKA R1A and PKA R2B influences cell growth in the human adrenocortical carcinoma cell line H295R. The main finding of the study is that activation of R2B and enhancement of its expression using two selective cAMP analogue activators for R2B dysregulate cell cycle progression and induce apoptosis. R2B plays an important role in H295R apoptosis, which is confirmed by silencing *R2B* gene, since inactivation of R2B confers resistance to apoptosis.

8Cl-cAMP, a site-selective analogue for the type 2 PKA, may affect the growth of several types of cells through its extracellular conversion into a cytotoxic metabolite, 8Cl-adenosine (Lange-Carter *et al.* 1993, Robinson-White *et al.* 2008), or by influencing the ratio of PKA isozymes in the cell, thereby modifying PKA activity (Rohlf *et al.* 1993, Kim *et al.* 2001). In our study,

the effects of 8Cl-cAMP on cell viability, DNA synthesis and apoptosis in H295R cells are not linked to its conversion to 8Cl-adenosine, because 8CPT-cAMP, a site-selective cAMP analogue specific for the type 2 PKA, which is not converted to 8Cl-adenosine, has similar effects. Moreover, using a synthetic Ultrosor G serum rather than using foetal bovine serum may rule out the presence of enzymes catalysing the conversion to 8Cl-adenosine.

We found that the inhibition of H295R cell growth is a late event, occurring after the onset of apoptosis. 8Cl-cAMP has no cytotoxic effects on cells, and initially increases DNA-replicating cells and maintains the level high for over 6 days and then decreases DNA synthesis on day 7. However, the increase in DNA-replicating cells analysed by BrdU incorporation is not in concordance with the limited increase of 1.5-fold in cell viability on days 4 and 7. While the same fold increase in cell viability (1.3-fold) is observed in SP-cAMP-treated cells, no sustained DNA synthesis occurs. Viability of 8Cl-cAMP- and 8CPT-cAMP-treated cells decreases after 10 days of incubation (data not shown). The discordance between DNA synthesis and the limited increase in cell viability is explained by the fact that not all DNA-replicating cells divide. This is shown by the analysis of DNA content after propidium iodide incorporation where the S and G₂ phases increase and the G₁ phase decreases. An accumulation of DNA content in the S phase is observed on days 4 and 7. The S phase accumulation on day 7, which is not observed using BrdU, is due to the incorporation of propidium iodide in all the DNA-replicating cells, which is not limited to the newly replicating cells in a range of 6 h as for BrdU incorporation. The high PCNA staining also reflects the accumulation of the cells in the S phase. The accumulation in the S phase without induction of proliferation is also reported in primary mouse epidermal keratinocytes (Dransfield *et al.* 2001). 8Cl-cAMP-treated keratinocytes arrested in the G₂/M phase can still incorporate ³H thymidine, but cannot divide. 8Cl-cAMP as 8CPT-cAMP initially causes a transient stimulation of the cell cycle in H295R cells, which is followed by apoptosis and the inhibition of growth due to perturbation of the cell cycle.

Two phases of the cell cycle are mainly affected by the R2B site-selective cAMP analogues: first, the transition of the cells from the S phase to the G₂ phase as demonstrated by the increase in the percentage of the S phase cells, the intense PCNA staining, sustained DNA synthesis (BrdU incorporation) and the delayed entry into the G₂ phase as shown by the aphidicolin synchronisation. Secondly, the transition from the G₂ phase to mitosis; the cells accumulated in the G₂ phase do not go on to divide. The prolonged arrest in the G₂ phase, as shown by nocodazole synchronisation, leads to a decrease in the proportion of cells in the G₀/G₁

Figure 7 Effects of PKA R2B invalidation on cell survival and the apoptosis induced by TGF β and TNF α in H295R, HEK293T and MCF-7 cell lines. Apoptosis was assessed by annexin V-FITC/IP staining, and measured by FACS flow cytometry in cells invalidated for PKA R2B by electroporation. Cells electroporated with scrambled siRNA (siS) or an siRNA against R2B (siR2B) were incubated for 48 h in the presence or absence of TGF β (25 ng/ml) and TNF α (5 ng/ml). (A) Validation of R2B silencing by western blotting and comparison with siS cells in H295R, HEK293 and MCF-7 cells. (B) A representative dot plot for annexin analysis in H295R cells. Points are the means $\pm$ S.E.M. of three duplicate independent experiments expressed as percentage apoptosis. (C) Results are expressed as the percentage of cells surviving (annexin $^{-}$ /IP $^{-}$ cells) and apoptotic cells (early annexin $^{+}$ /IP $^{-}$, late apoptosis annexin $^{+}$ /IP $^{+}$ and necrosis annexin $^{+}$ /IP $^{+}$). R2B invalidation resulted in a larger number of viable cells and a smaller number of apoptotic cells after treatment with TGF β and TNF α than for the treated control (siS) H295R cells. Points are the means $\pm$ S.E.M. of three duplicate experiments. * $P=0.05$, ** $P=0.01$ and *** $P<0.005$ for H295R cells; * $P=0.05$ and ** $P=0.02$ for HEK293 cells; *** $P<0.001$ for MCF-7 cells. Comparisons were performed between siS-treated cells versus siS-untreated control cells, and siR2B-treated or siR2B-untreated cells versus siS control cells of each treatment.

phase. 8Cl-cAMP targeting cell cycle phase is cell specific. 8Cl-cAMP acts on MCF-10A HE- and TE-transformed cells, causing these cells to accumulate in the G₀/G₁ phase and decreasing the proportion of cells in the S phase (Ciardiello *et al.* 1993). 8Cl-cAMP may also affect two phases of the cell cycle, G₀/G₁ and G₂/M, in HL-60 leukaemic cells (Pepe *et al.* 1991) and primary mouse epidermal keratinocytes (Dransfield *et al.* 2001).

The effect of 8Cl-cAMP and 8CPT-cAMP on the S and G₂/M phases in H295R cells is parallel to changes in cyclin levels and the activities of cyclin-dependent kinases. The increase in the percentage of H295R cells in the S phase and the early G₂ phase is linked to the translocation of cyclins E and A from the cytosol to the nucleus and the activation of total CDK2 as described previously (Pines & Hunter 1991, Bailly & Bornens 1992). 8Cl-cAMP and 8CPT-cAMP allow cells to enter the G₂ phase, as shown by the accumulation of cyclin B in the cytosol. They probably block the H295R cells in the G₂ phase by inducing cyclin B accumulation in the nucleus and decreasing total CDC2 kinase activity. The resulting interruption of mitosis then triggers apoptosis. A direct link has been found between PKA and the molecular mechanisms maintaining cell cycle arrest in the G₂ phase, with the CDC2–cyclinB complex being kept inactive by the PKA-dependent activation of Wee1 and inactivation of CDC25 in *Xenopus* oocytes (Duckworth *et al.* 2002, Shibuya 2003).

The two selective R2B cAMP analogues trigger apoptosis in H295R cells. This may be a link to their common effect on targeting R2B, decreasing the type 1/type 2 PKA ratio, increasing R2B synthesis and activating PKA, regardless of the effect of 8Cl-cAMP on activating R1A and decreasing its synthesis. The inactivation of R2B by siRNA decreases the basal levels of apoptotic H295R cells and confers resistance to the apoptosis induced by TNF α and TGF β . These findings are consistent with a role for R2B in apoptosis and the data obtained from the transformed mouse adrenal Y-1 cell line, in which R2B silencing increases proliferation (Mantovani *et al.* 2008b).

However, PKA type 1 and type 2 in adrenocortical human H295R cell line do not share the same feature as those in the endocrine tumours, where PKA subunits have compensatory effects and distinct roles (Bossis & Stratakis 2004, Griffin *et al.* 2004a,b). The effects of R2B and R1A are not antagonist when inactivated in H295R cells. We have recently shown that inactivation of R1A confers resistance to apoptosis induced by TGF β as does that of R2B (Ragazzon *et al.* 2009). PKA type 1 and type 2 may have complementary effects on H295R cells. Inactivation of both R1A and R2B confers resistance to apoptosis induced by TNF α in H295R cells. However, they delineate distinct regulation in the expression of pro-apoptotic and apoptotic factors Bax and Bcl2

proteins. Our preliminary results show that the expression of Bax is decreased and that of Bcl2 is increased under TNF α stimulation in R2B-inactivated cells only (data not shown). Activation of the PKA type 1 or type 2 may have the same cellular responses, but it modulates different molecular mechanisms and gene regulations depending on the sensitivity to cAMP concentration and the location of the PKA holoenzyme (Liu *et al.* 2004b, Tasken & Aandahl 2004, Dyson *et al.* 2009). It has been shown by injection of antisense oligonucleotides into the sensory neuron cell body that the two types of kinases have distinct but complementary functions in the production of facilitation at synapses of an identified neuron (Liu *et al.* 2004b). It has been suggested that PKA type 1 operates in the nucleus to maintain cAMP response element-binding protein-dependent gene expression, and PKA type 2 acts at sensory neuron synapses by phosphorylating proteins to enhance the release of a neurotransmitter. Such a distinct function is also reported in steroidogenic tissues for *Star* gene regulation. Using different selective cAMP analogues, Dyson *et al.* (2009) have shown that in MA-10 mouse Leydig tumour cells, *Star* gene expression is more dependent upon PKA type 1, while the post-transcriptional regulation of *Star* appears to be controlled by PKA type 2. These experiments delineate the discrete effects that PKA type 1 and type 2 exert on STAR-mediated steroidogenesis, and suggest complementary roles for each subtype in coordinating steroidogenesis.

In conclusion, we have shown that PKA R2B is involved in H295R cell growth. Targeting of PKA R2B, disturbing the balance between PKA isozymes by increasing their expression, influences cell cycle progression by targeting specific cyclins and G₂ cell cycle kinases, and induces apoptosis. The silencing of the *R2B* gene confers resistance to apoptosis. Our data associated to those on R1A silencing (Ragazzon *et al.* 2009) suggest complementary roles of both PKA R1A and PKA R2B in cell apoptosis. Comparative studies of molecular mechanisms involved in apoptosis induced by TGF β and TNF α are underway to delineate the effects that PKA type 1 and type 2 exert on the cAMP–PKA signalling pathways involved in adrenocortical tumours.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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