

# Growth Inhibition of Human Ovarian Cancer Cells by Differential Modulation of Protein Kinase A Isozymes

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Type II PKA  
Growth inhibition

We examined the effect of modulation of PKA isozymes on the growth of human ovarian cancer cells. Three ovarian cancer cell lines, 2774, SK-OV-3, and OVCAR-3, were examined in this study. The treatment of 5  $\mu$ M 8-Cl-cAMP, which has been known to down-regulate RI (or type I PKA) and up-regulate RII (or type II PKA), markedly inhibited the growth of all cell lines (50-80% at day 6). To test whether alteration in PKA regulatory subunits level can change the growth characteristics of ovarian cancer cells, we introduced R11 $\beta$ - expression construct and R1 $\alpha$  antisense-expression construct into 2774 cells. The overexpression of R11 $\beta$  down-regulated R1 $\alpha$  protein, and the antisense-expression of R1 $\alpha$  up-regulated R11 $\beta$  protein, showing that the intracellular levels of RI and RII are reciprocally regulated. In both cases, cell growth was reduced by 30% at day 2. These results indicate that the growth of ovarian cancer cells is controlled by the signals from PKA isozymes, and the modulation of PKA isozymes can be employed for the human ovarian cancer therapy.

Cyclic AMP (cAMP), initially discovered as an intracellular signal transducer or second messenger (Sutherland and Fall, 1957), has been considered to mediate growth-controlling signals. In mammalian cells, cAMP acts mainly by binding to protein kinase A (PKA). There are two types of PKA, type I and type II, which share common catalytic (C) subunits, but are distinguished by the association of their regulatory (R) subunits, RI and RII, respectively. Four genetically distinct R subunit isoforms (R1 $\alpha$ , R1 $\beta$ , R11 $\alpha$ , and R11 $\beta$ ) and three C subunit isoforms (C $\alpha$ , C $\beta$ , and C $\gamma$ ) have been identified (McKnight et al, 1988).

Relationship between the differential expression of two types of PKA and cell growth/differentiation has been studied in a variety of systems (Cho-Chung, 1990). When the cells undergo active proliferation or are in an early developmental stage, high type I PKA activity and RI/RII ratio are observed. However, terminally differentiated and non-growing cells show a high type II PKA activity and a low RI/RII ratio.

Previous reports suggested that the actions of PKA isozymes might also be involved in the differentiation of ovarian cells. It was demonstrated that PKA activity in rat ovary increases progressively during postnatal development (DeAngelo et al., 1975). PKA isozyme distribution also changes during the ovarian development. Ovarian cytosol of 6-day-old neonates contains

largely type I PKA, whereas in 34-day-old or older rats, type II PKA predominates (Hunzicker-Dunn, 1982). Thus, two PKA isozymes may play different roles in the early development of rat ovaries.

Since PKA-mediated events seem to be involved in the differentiation process of ovarian cells, we hypothesized that the differential modulation of PKA isozymes would affect the growth of ovarian cancer cells. In the present study, we employed three human ovarian cancer cell lines to test whether the cAMP analogue, which has been known to inhibit the growth of other cancer cells by differentially modulating PKA isozymes, could inhibit the growth of human ovarian cancer cells. We also examined the effect of up- or down-regulation of PKA regulatory subunits on the ovarian cancer cell growth.

## Materials and Methods

### Cell culture

SK-OV-3 cells were maintained in Dulbecco's modified Eagle's medium. 2774 and OVCAR-3 cells were grown in RPMI 1640. All the media were supplemented with 10% heat-inactivated fetal bovine serum (GIBCO-BRL), 100 units/ml penicillin G, 100  $\mu$ g/ml streptomycin. For OVCAR-3 cells medium was supplemented with 10  $\mu$ g/ml insulin. Cells were maintained in a humidified atmosphere of 5% CO<sub>2</sub> at 37  $^{\circ}$ C.

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*Construction of expression vectors and introduction into 2774 cells*

OT1521 retroviral vector (McGeady et al., 1989) was used to generate expression constructs. The constructs for the overexpression of RII $\beta$  (Tortora and Cho-Chung, 1990a) and antisense expression of RI $\alpha$  (Tortora et al., 1994) were described previously. Retroviral constructs were introduced into 2774 human ovarian cancer cells using replication-deficient retrovirus system and the selections of high expressor clonal lines were performed as described previously (Kim et al., 1996).

*Western blot analysis*

Cells were treated with 0 to 120  $\mu$ M ZnSO<sub>4</sub> for two d to induce the expression of various constructs before analysis. Cell extracts were prepared as described (Tortora et al., 1990b). Western blot analysis was performed as before (Kim et al., 1996) using ECL detection system (Amersham).

*Monolayer growth assay*

To examine the growth of ovarian cancer cells, we used microculture tetrazolium assay (Alley et al., 1988). Five thousand cells in 0.1 ml of culture medium were seeded on each well of flat-bottomed 96-well plate. After 8 h, 0.1 ml of culture medium containing additives was added to each well. To examine the effect of 8-Cl-cAMP, final 5  $\mu$ M 8-Cl-AMP was used, and for the induction of introduced genes, cells were treated with ZnSO<sub>4</sub> at a final concentration of 60  $\mu$ M. To determine the cell growth, 50  $\mu$ l of 2 mg/ml MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) was added to each well. After 4 h incubation, the medium was removed and the colored material was dissolved in 150  $\mu$ l of dimethyl sulfoxide. The absorbance at a wavelength of 570 nm was measured using a microplate reader (BIO-RAD).

**Results**

*Effects of 8-Cl-cAMP on the growth of ovarian cancer cells*

In an attempt to look at the effect of differential modulation of PKA isozymes on human ovarian cancer cell growth, we examined the changes in the growth rate of three lines of ovarian cancer cells, 2774, SK-OV-3, and OVCAR-3, after the treatment of a cAMP analogue. In the present study, we used 8-Cl-cAMP to modulate PKA isozymes, because it is known to inhibit growth of cancer cells by down-regulation of type I PKA activity and up-regulation of type II PKA activity (Rohlf et al., 1993). Thus, effect of 8-Cl-cAMP on the cell growth may well represent the relationship between the signals from PKA isozymes and the cell growth.

Treatment of 5  $\mu$ M 8-Cl-cAMP on ovarian cancer cells brought about 50-80% growth inhibition at day 6

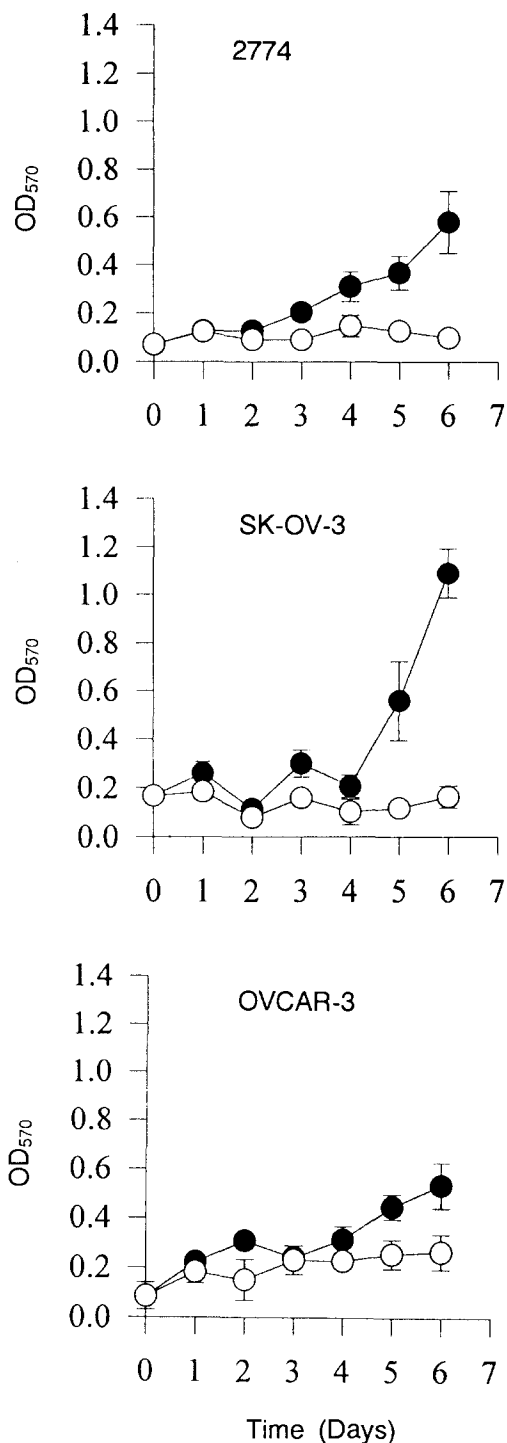


Fig. 1. Effects of 8-Cl-cAMP treatment on the cell growth of three ovarian cancer cell lines. Cells were cultured in the 96-well plate, and their growth rates were examined with a microculture tetrazolium assay. Absorbance at the wavelength of 570 nm was determined at the time of treatment (day 0) and every 24 h thereafter up to day 6. Closed circle (●) indicates untreated cells and open circle (○) indicates cells treated with 5  $\mu$ M 8-Cl-cAMP. Data indicate mean  $\pm$  S.D. of 6 separate determinations.

(Fig. 1). The level of growth inhibition was similar in 2774 and SK-OV-3 (about 80%) and was smaller in

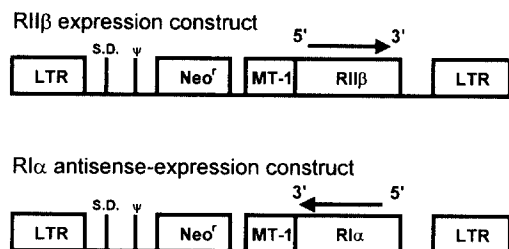


Fig. 2. Schematic diagram of the expression constructs for RII $\beta$  and RI $\alpha$  antisense. LTR, long terminal repeat from moloney murine sarcoma virus (MSV). S.D., splicing donor site.  $\psi$ , packaging site. Neo', neomycin phosphotransferase gene which confers G418 resistance. MT-1, mouse metallothionein-1 promoter. Arrows indicate the orientation of sense-strand.

OVCAR-3 (50%). These results suggest that the growth of ovarian cancer cells could be regulated by differential modulation of PKA isozymes with a cAMP analogue.

#### Introduction of RII $\beta$ - and RI $\alpha$ antisense-expression constructs into ovarian cancer cells

Since the growth of ovarian cancer cells was inhibited by 8-Cl-cAMP treatment, we hypothesized that the down-regulation of type I PKA and/or the up-regulation of type II PKA would induce growth inhibition of ovarian cancer cells. To test this hypothesis, we introduced the expression construct for RII $\beta$  and the antisense-expression construct for RI $\alpha$  into 2774 human ovarian cancer cells. Human RII $\beta$  (Levy et al., 1988) and RI $\alpha$  (Sandberg et al., 1987) cDNAs were inserted into the OT1521 (McGeady et al., 1989) retroviral vector in sense and antisense orientations, as shown in Fig. 2. OT1521 vector contains the neomycin phosphotransferase gene, which allows the selection of stable infectants by G418 selection, and the mouse metallothionein-1 promoter, which enables the inducible expression of the inserted gene upon treatment with CdCl<sub>2</sub> or ZnSO<sub>4</sub>. The expression constructs were introduced into the 2774 ovarian cancer cells and individual clones were isolated as described previously (Kim et al., 1996). The expressions of introduced genes were checked by Northern or Western analyses. Single high expressor clone that showed the highest level of RII $\beta$  mRNA in Northern analysis was selected as a 2774-RII $\beta$ . For RI $\alpha$  antisense-expression, RI $\alpha$  antisense-infected cells were screened for low expression of RI $\alpha$  in Western analysis. A clone of the lowest expression of RI $\alpha$  protein was selected as a 2774-RI $\alpha$ -r (Data not shown).

#### Overexpression of RII $\beta$ and the effect on the RI $\alpha$ level

To find out the optimum induction condition for the expression of introduced genes, we examined the toxicity of ZnSO<sub>4</sub> at various concentrations. Up to 120  $\mu$ M, ZnSO<sub>4</sub> did not exert any effect on the growth of 2774 cells (data not shown). Thus, 0 to 120  $\mu$ M ZnSO<sub>4</sub> was used to induce the introduced genes in further experiments.

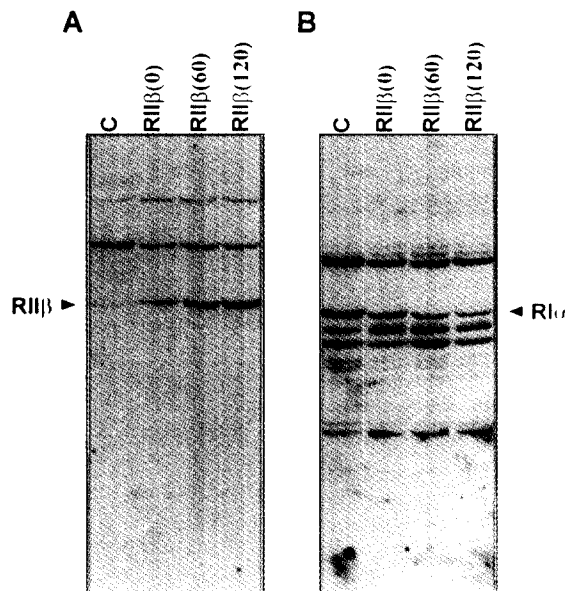


Fig. 3. Protein levels of RII $\beta$  and RI $\alpha$  in 2774-RII $\beta$ . Total cell extracts from 2774-RII $\beta$  were analyzed with Western blot analysis as described in "Materials and Methods". RII $\beta$  (panel A) and RI $\alpha$  (panel B) proteins were detected with polyclonal antiserum generated against recombinant RII $\beta$  and RI $\alpha$  proteins produced in bacteria, respectively. RII $\beta$  and RI $\alpha$  bands were indicated with arrowheads. C, parental cells; RII $\beta$  (0) 2774-RII $\beta$  cells not treated with ZnSO<sub>4</sub>; RII $\beta$  (60), and 2774-RII $\beta$  cells treated with 60  $\mu$ M ZnSO<sub>4</sub>; RII $\beta$  (120) and 2774-RII $\beta$  cells treated with 120  $\mu$ M ZnSO<sub>4</sub>.

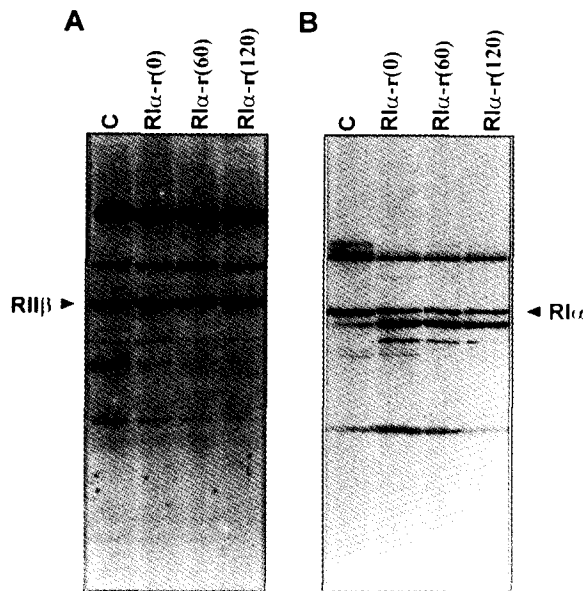
The expressions of RII $\beta$  in the 2774-RII $\beta$  at several concentrations of ZnSO<sub>4</sub> were compared with that of parental 2774 cells (Fig. 3A). The 2774 cells exhibited very low, but detectable level of RII $\beta$ , as reported previously (Seo et al., 1995). In 2774-RII $\beta$  cells, 7-, 12-, and 20-fold increases in RII $\beta$  level were observed at the induction conditions of 0, 60, and 120  $\mu$ M ZnSO<sub>4</sub>, respectively, as determined by Western analysis and densitometric scanning. The increase of RII $\beta$  protein brought about the decrease of RI $\alpha$  (Fig. 3B). When RII $\beta$  expression was induced with 120  $\mu$ M ZnSO<sub>4</sub>, RI $\alpha$  protein was decreased up to 30% of the parental cells. Even without the induction, RI $\alpha$  decreased to 50% of the control cells showing that increase in RII $\beta$  down-regulated RI $\alpha$ .

#### Reduction of RI $\alpha$ in 2774-RI $\alpha$ -r cells and the effect on the RII $\beta$ level

The expressions of RI $\alpha$  and RII $\beta$  were examined in 2774-RI $\alpha$ -r cells by Western blot analysis. At all induction conditions (0, 60, and 120  $\mu$ M ZnSO<sub>4</sub>), 2774-RI $\alpha$ -r showed 30% decreased RI $\alpha$  level compared to that of parental cells (Fig. 4B). RII $\beta$  protein was increased slightly (10-30%) at all induction conditions (Fig. 4A).

#### Monolayer growth

To examine the effect of the modulation of RI and RII levels on the ovarian cancer cell growth, we compared the growth rates of 2774-RII $\beta$  and 2774-RI $\alpha$ -r with that



**Fig. 4.** Protein levels of RII $\beta$  and RI $\alpha$  in 2774-RI $\alpha$ -r. Total cell extracts of 2774-RI $\alpha$ -r were analyzed with Western blot analysis as described in "Materials and Methods". RII $\beta$  (panel A) and RI $\alpha$  proteins (panel B) were detected with the same antibody as described in Fig. 3. RII $\beta$  and RI $\alpha$  bands were indicated with arrowheads. C, parental cells. RI $\alpha$ -r (0) 2774-RI $\alpha$ -r cells not treated with ZnSO<sub>4</sub>; RI $\alpha$ -r (60) and 2774-RI $\alpha$ -r cells treated with 60  $\mu$ M ZnSO<sub>4</sub>; RI $\alpha$ -r (120) and 2774-RI $\alpha$ -r cells treated with 120  $\mu$ M ZnSO<sub>4</sub>.

of parental cells. The growth of 2774-RII $\beta$  and 2774-RI $\alpha$ -r was reduced by 30% at day 2 as compared with parental cells. These data show that the up-regulation of RII $\beta$  and/or down-regulation of RI $\alpha$  can induce growth inhibition in human ovarian cancer cells.

### Discussion

In the present study, we have shown that 1) 8-Cl-cAMP induces growth inhibition in three lines of ovarian cancer cells, 2) overexpression of RII $\beta$  down-regulates RI $\alpha$  in a quantity-dependent manner, 3) down-regulation of RI $\alpha$  increases the level of RII $\beta$ , and 4) up-regulation of RII $\beta$  and/or down-regulation of RI $\alpha$  causes growth inhibition of ovarian cancer cells.

The close relationship between the activities of PKA isozymes and the cell growth has been well established in other systems (Cho-Chung, 1990). It has been reported that down-regulation of type I PKA (or RI $\alpha$ ) and up-regulation of type II PKA (or RII $\beta$ ) by cAMP analogues such as 8-Cl-cAMP brings about growth inhibition and differentiation in a variety of cancer cell lines (Katsaros et al., 1987; Tortora et al., 1989a). Suppression of type I PKA or RI $\alpha$  by the treatment of RI $\alpha$  antisense oligodeoxynucleotide also induced an increase of RII $\beta$  and type II PKA activity, and inhibited the growth of cancer cells (Tortora et al., 1991; Yokozaki et al., 1993). However, the effects of the modulation of PKA isozymes on the ovarian cancer cell growth have not been well examined.

The mediation of FSH-induced granulosa cell differentiation by cAMP-signal pathway has been demonstrated (Knecht et al., 1981). FSH also increases RII protein (Darbon et al., 1984) and mRNA (Ratoosh et al., 1987) levels in rat granulosa cells. These previous reports suggest that the malfunction of the cAMP signal pathway can disable the differentiation of hormone-dependent ovarian cells and that the growth of the ovarian cancer cells may be controlled by the modulation of PKA signal pathway.

The growth of ovarian cancer cells was markedly inhibited (50-80%) by 8-Cl-cAMP treatment (Fig. 1). The effects of 8-Cl-cAMP treatment have been examined in a variety of cancer cells or transformed cells: The treatment of 8-Cl-cAMP 1) induces the nuclear translocation of RII into the nucleus (Clair et al., 1987; Ally et al., 1988; Ally et al., 1989), 2) down-regulates RI and up-regulates RII in mRNA and protein levels (Ally et al., 1988; Ally et al., 1989; Tortora et al., 1989a, b; Rohlf et al., 1993), 3) down-regulates type I PKA activity and up-regulates type II PKA activity (Rohlf et al., 1993), 4) increases cAMP-responsive element binding activity of nuclear extracts (Mednieks et al., 1989), and 5) reduces the expression of oncogenes such as *c-myc* (Ally et al., 1989; Tortora et al., 1989a), and *N-ras* (Ally et al., 1989) and oncogene products, transforming growth factor  $\alpha$  and p21<sup>ras</sup> (Tortora et al., 1989b). Also, the differentiation of HL60 cells by 8-Cl-cAMP was abrogated by the treatment of RII $\beta$  antisense oligodeoxynucleotide (Tortora et al., 1990b), showing that the RII $\beta$  protein or type II PKA takes an important part in the differentiation induced by 8-Cl-cAMP. These previous reports suggest that the growth inhibition brought about by 8-Cl-cAMP treatment in the ovarian cancer cells could be mimicked by the up-regulation of type II PKA and down-regulation of type I PKA.

Growth inhibition of cancer cells or transformed cells by the overexpression of RII $\beta$  has been reported previously (Budillon et al., 1995; Kim et al., 1996; Nesterova et al., 1996). However, the overexpression of RII $\beta$  has not been tried in the hormone-dependent ovarian cells. The results obtained in the present study showed that overexpression of RII $\beta$  also induces growth inhibition in ovarian cancer cells (Fig. 5). Effects of RI $\alpha$  antisense-expression were also of our interest because down-regulation of type I PKA by RI $\alpha$  antisense-expression in SK-N-SH human neuroblastoma cells could not induce growth inhibition (Kim et al., 1997). In 2774 ovarian cancer cells, RI $\alpha$ -antisense expression as well as RII $\beta$  overexpression resulted in marked growth inhibition (Fig. 5). Although we are not aware of the causative physiological difference between the neuroblastoma cells and the ovarian cancer cells, we suspect that the endogenous PKA isozyme status may be responsible in part for the disparity. In the case of neuroblastoma cells, there was no detectable endogenous RII $\beta$  mRNA or protein, and RII $\beta$  was not induced by the down-regulation of RI $\alpha$ . However, the

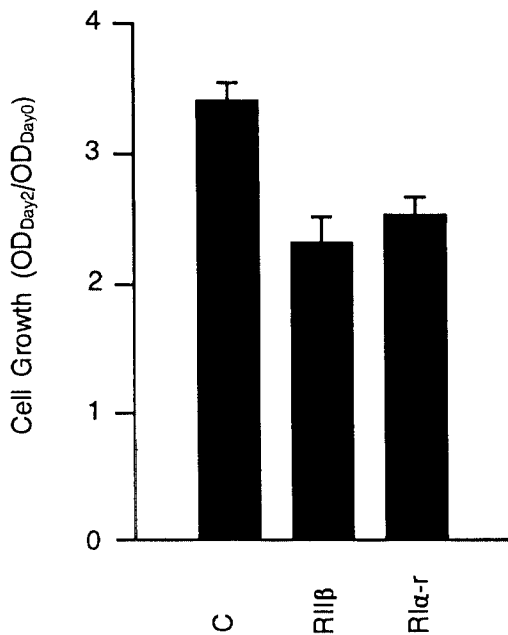


Fig. 5. Effects of RII $\beta$  overexpression and RII $\alpha$  antisense expression on the growth of 2774 cells. The monolayer growth rates of parental cells (C), 2774-RII $\beta$  (RII $\beta$ ), and 2774-RII $\alpha$ -r (RII $\alpha$ -r) were examined as described in "Materials and Methods". Cell growth was determined with a microculture tetrazolium assay and shown as OD<sub>570</sub> at day 2 divided by OD<sub>570</sub> at day 0. Data express mean  $\pm$  S.D. of 6 separate determinations.

basal level of RII $\beta$  could be observed in ovarian cancer cells, and RII $\beta$  was increased by the down-regulation of RII $\alpha$ . Thus, the basal level and/or increase of RII $\beta$  might be necessary for the growth inhibition by the down-regulation of RII $\alpha$ .

Overexpression of RII $\beta$  in 2774 cells resulted in the decrease of RII $\alpha$  (Fig. 3). The reciprocal regulation between RI and RII has been studied by the overexpression of PKA subunits. Overexpression of RII $\alpha$  in the ras-transformed NIH3T3 cells eliminated type I PKA, whereas the overexpression of RI did not alter PKA isozymes distribution (Otten and McKnight, 1989), suggesting that RII has higher affinity toward C subunit than that of RI. Overexpression of RII with high affinity leaves RI as free subunits, which are subject to degradation (Steinberg and Agard, 1981).

The increase of RII by the decrease of RII $\alpha$  has also been reported. In the studies using antisense oligodeoxynucleotides, the decrease of RII $\alpha$  was accompanied by the marked increase of RII *in vitro* (Tortora et al., 1991; Yokozaki et al., 1993) and type II PKA activity in nude mice injected with cancer cells (Nesterova and Cho-Chung, 1995). However, in neuroblastoma cells, the down-regulation of RII $\alpha$  and type I PKA by antisense expression of RII $\alpha$  could not induce an increase of RII or type II PKA activity (Kim et al., 1997). In the present study, we observed only slight increase of RII $\beta$  in RII $\alpha$  antisense-expressing cells. The difference in the changes of RII $\beta$  levels seems to be due to the differences in

the endogenous level of RII $\beta$  and in the level of RII $\alpha$  decrease. As shown in the study with neuroblastoma cells, without basal level of RII $\beta$ , decrease of RII $\alpha$  could not induce RII $\beta$  expression (Kim et al., 1997). However, in ovarian cancer cells, only 30% decrease in RII $\alpha$  resulted in the increase of RII $\beta$ . Thus, the endogenous PKA isozyme status may be important in the induction of RII $\beta$ . In the studies using RII $\alpha$  antisense oligodeoxynucleotide, RII $\alpha$  mRNA and protein were eliminated (Tortora et al., 1991; Yokozaki et al., 1993), but in the case of antisense expression, RII $\alpha$  protein still remained (Fig. 4). Thus, it may be reasoned that the antisense expression of RII $\alpha$  in this study was not so effective as RII $\alpha$  antisense oligodeoxynucleotide.

We have shown in the present study that the modulation of PKA isozymes, i.e., up-regulation of RII $\beta$  and down-regulation of RII $\alpha$ , can induce growth inhibition in ovarian cancer cells. Interestingly, decrease of RII $\alpha$  by antisense expression caused an increase of RII $\beta$  and growth inhibition, suggesting that the intracellular level of RII $\alpha$  can affect that of RII $\beta$  in ovarian cells. These results indicate that the modulation of PKA isozymes using cAMP analogues, antisense oligonucleotides, or gene transfer, could be applied to control the growth of ovarian cancer cells.

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