Oncogene (1999) 18, 4999–5004

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SHORT REPORT

Protein kinase A-Iα subunit-directed antisense inhibition of ovarian cancer cell growth: crosstalk with tyrosine kinase signaling pathway

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Expression of the RIα subunit of cAMP-dependent protein kinase type I is increased in human cancers in which an autocrine pathway for epidermal growth factor-related growth factors is activated. We have investigated the effect of sequence-specific inhibition of RIα gene expression on ovarian cancer cell growth. We report that RIα antisense treatment results in a reduction in RIα expression and protein kinase A type I, and inhibition of cell growth. The growth inhibition was accompanied by changes in cell morphology and appearance of apoptotic nuclei. In addition, EGF receptor, c-*erb*B-2 and c-*erb*B-3 levels were reduced, and the basal and EGF-stimulated mitogen-activated protein kinase activities were reduced. Protein kinase A type I and EGF receptor levels were also reduced in cells overexpressing EGF receptor antisense cDNA. These results suggest that the antisense depletion of RIα leads to blockade of both the serine-threonine kinase and the tyrosine kinase signaling pathways resulting in arrest of ovarian cancer cell growth.

Keywords: protein kinase A; antisense; ovarian cancer; tyrosine kinase; EGF receptor

The ability to block expression of individual genes that are causally related to diseases provides a powerful means to explore the molecular basis of diseases as well as the opportunity for therapeutic intervention. There is growing evidence supporting that the sequence-specific inhibition of gene expression by the use of antisense oligonucleotides can be a promising therapeutic innovation toward treatment of cancer and other diseases (Cho-Chung, 1996; Dean *et al*., 1996; Monia *et al*., 1996).

Cyclic AMP (cAMP) regulates various cellular functions, such as metabolism, secretion, cell proliferation, differentiation and gene induction, through the activation of cAMP-dependent protein kinases (PKA) (Krebs and Beavo, 1979). There are two types of PKA, type I (PKA-I) and type II (PKA-II), which share a common catalytic (C) subunit but contain different regulatory (R) subunits, RI and RII, respectively (Beebe and Corbin, 1986). Four isoforms of the R subunits, RIα, RIβ, RIIα and RIIβ, have been identified (McKnight *et al*., 1988; Levy *et al*., 1988). Importantly, differential expression of PKA-I and II has been correlated with cell differentiation and neoplastic transformation. RI/PKA-I is preferentially expressed in transformed cells or during early stages of ontogenesis (Lohmann and Walter, 1984; Cho-Chung, 1990), whereas expression of RII/PKA-II is induced in cancer cells growth arrested following treatment with cAMP analogs or differentiating agents (Schwartz and Rubin, 1985; Cho-Chung *et al*., 1989). Thus, the dual signals toward cell growth, positive and negative, transduced by cAMP may depend on the availability of RI and RII subunits, respectively.

It has been hypothesized that the RIα is an ontogenic growth-inducing protein, and its constitutive expression disrupts normal ontogenic processes, resulting in a pathogenic outgrowth, such as malignancy (Cho-Chung, 1990). Subsequently, it has been shown, through antisense strategy, that RIα has a role in neoplastic cell growth *in vitro* (Tortora *et al*., 1991; Yokozaki *et al*., 1993; Srivastava *et al*., 1998) and *in vivo* (Nesterova and Cho-Chung, 1995; Cho-Chung *et al*., 1997).

The epidermal growth factor (EGF)-related growth factors play a role in human cancer growth through autocrine and paracrine mechanisms (Aaronson, 1991). EGF-like growth factors, such as transforming growth factor-α (TGFα), bind to the extracellular domain of the EGF receptor (EGF-R) and activate its intracellular tyrosine kinase domain. Enhanced expression of TGFα and/or EGF-R has been detected in a majority of human carcinomas examined (Aaronson, 1991).

Functional interactions between PKA and EGF-R signaling pathways have been suggested. *ras*- and TGFα-dependent transformation brought about an early induction of RIα/PKA, while selective down-regulation of RIα/PKA-I resulted in inhibition of TGFα- and p21 ras-signaling (Tortora *et al*., 1989; Ciardiello *et al*., 1990).

In the present study, we have examined the effect of the sequence-specific inhibition of RIα gene expression on the growth of OVCAR-8 ovarian cancer cells. We also examined the effect of RIα antisense on the EGF-R signaling pathway and, conversely, the effect of EGF-R antisense gene overexpression on the RIα/PKA-I expression in OVCAR-8 cells. We used a mixed backbone RIα antisense, namely phosphorothioate oligodeoxynucleotide (PS-oligo DNA), containing segments composed of PS-2′-O-methyloligo RNA,

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Received 11 November 1998; revised 11 March 1999; accepted 11 March 1999

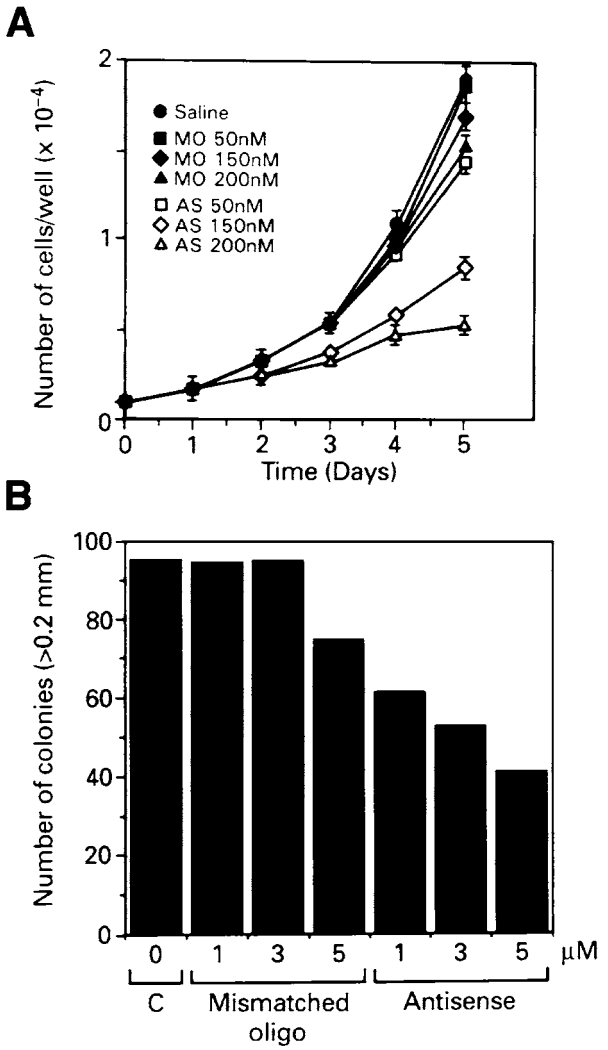

Figure 1 RIα antisense inhibits ovarian cancer cell growth in monolayer culture and soft agar. (**a**) Monolayer growth. OVCAR-8 human ovarian cancer cell line obtained from DCT-Tumor Repository (NCI-Frederick Cancer Research Facility) was cultured in IMEM medium supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS; Gibco BRL, Life Technologies Inc., Grand Island, NY, USA), 2 mM glutamine and penicillin-streptomycin in a humidified incubator (95% air and 5% CO_2) at 37°C. For cell growth experiments, cells (1×10^3) were seeded onto 24-well plates. At 3–5 h after seeding, antisense or control oligonucleotide at indicated concentrations were added. To increase the delivery of oligonucleotides into the cells, cationic lipid DOTAP was used according to the manufacturer's direction (Boehringer Mannheim). The oligonucleotides used in the present study include: HYB0165, 5′-[GCGU]GCCTCCTCAC[UGGC]-3′, RIα antisense (Nesterova and Cho-Chung, 1995) of phosphorothioate oligodeoxynucleotide containing segments composed of four 2′-O-methyl-phosphorothioate oligoribonucleotide (bracketed); HYB0295, 4-base mismatched (underlined) oligonucleotide, 5′-[GC*A*U]GC*T*TCC*A*CAC[*A*GGC]-3′, and HYB0674, random sequence oligodeoxynucleotide, 5′-[NNNN] NN NNN NN [NNNN]-3′. the bracketed segments represent 2′-0-methyl-phosphorothioate oligoribonucleotide, and N = A, T, C, G of random sequence. These oligonucleotides were kindly provided by Dr Sudhir Agrawal (Hybridon, Inc.). Control (saline) and antisense (AS) or mismatched oligonucleotide (MO)-treated cells were harvested at the time indicated and cell numbers were counted in triplicate by a Coulter counter. Data represent average values ± s.d. of six independent experiments (**b**) Soft agar growth. Cells (1×10^4) were seeded in 1 ml of 0.3% Difco Noble agar (Difco, Detroit, MI, USA) in culture medium. This suspension was layered over 1 ml of 0.6% agar-medium base layer in 6-well plates. The antisense and control oligonucleotides were added in the top and base layers. After 7 days of incubation, an additional 1 ml of medium containing the oligonucleotides was added to the plates. After 14 days, control (C) and oligonucleotide treated cells were stained with nitro blue tetrazolium (Sigma). Colonies larger than 0.2 mm were counted with an Artek 880 colony counter (Artek Systems, Inc., Farmingdale, NY, USA). The data represent average values of three independent experiments

which combines the favorable antisense properties of PS-oligo DNA (RNase H activation) and of PS-2′-O-methyloligo RNA (increase in nuclease resistance and duplex stability). Such mixed backbone (hybrid) oligonucleotides have been shown to have improved antisense activity over PS-oligonucleotides (Metelev *et al*., 1994; Cho-Chung *et al*., 1997). As control, we used 4-base mismatched oligonucleotide and random sequence oligonucleotide.

We examined the effect of one time treatment (at 5 h after seeding) of RIα antisense oligonucleotide on the monolayer growth of OVCAR-8 cells. Cationic lipid, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP), was used in all cell culture experiments to facilitate intracellular uptake of oligonucleotides (Srivastava *et al*., 1998). The antisense treatment resulted in inhibition of cell proliferation in a time- and concentration-dependent manner exhibiting a 75% inhibition at 200 nM by day 5 (Figure 1a). The growth inhibition was not due to the nonspecific cytotoxicity since over 90% of the cells were viable at 5 days post antisense treatment. By comparison, 4-base mismatched (Figure 1a) and random sequence control (data not shown) oligonucleotides had little (<15%) or no growth inhibitory effect. We next examined the antisense effect on the anchorage-independent growth of OVCAR-8 cells. RIα antisense at 1, 3 and 5 μM concentrations in the absence of DOTAP produced inhibition of the colony formation of cells in soft agar in a dose-dependent manner (Figure 1b). Four-base mismatched oligonucleotide had no effect on colony formation at 1 and 3 μM concentrations, but had a minimum inhibitory effect at 5 μM concentration (Figure 1b).

We examined whether RIα antisense could specifically inhibit the RIα gene expression. RIα antisense treatment for 5 days at 150 nM concentration brought about a marked reduction in RIα mRNA level (Figure 2a left panel, lane 3). Mismatched oligonucleotide had no effect on RIα mRNA level (Figure 2a left panel, lane 2). Importantly, the mRNA levels of the RIIα and Cα subunits of PKA were not affected by the RIα antisense treatment (Figure 2a right panel).

We next examined whether the RIα antisense treatment could specifically decrease the amount of RIα protein in OVCAR-8 cells. Cells were analysed for the content of each of the R subunits of PKA at 5 days after one treatment with RIα antisense (150 nM). Cell extracts were photoaffinity-labeled with 8-N_3-[^{32}P]cAMP and then immunoprecipitated with the monospecific anti-RIα, -RIIα and -RIIβ antibodies, and the immunoprecipitated proteins were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The RIα levels in cells treated with RIα antisense were markedly decreased (Figure 2b, lane 7) as compared to 4-base mismatched control-oligo treated (Figure 2b, lane 4) or the untreated control cells (Figure 2b, lane 1). The antisense treatment had little or no effect on the RIIα protein level (Figure 2b,

lane 8) and RIIβ protein was not detected in both antisense-treated and untreated cells (Figure 2b). Quantification by densitometric tracings of autoradiographs (Figure 2b) showed that the RIα and RIIα protein levels in the antisense treated cells were 10 and 90%, respectively, of those in untreated control cells. The sequence-specific effect of RIα antisense was clearly demonstrated by the 4-base mismatched (Figure 2) and random sequence (data not shown) control oligonucleotides that could not mimic the antisense effect of RIα downregulation.

The above data showed that upon antisense treatment, the RIα expression at both mRNA and protein levels sharply decreased. In cells, RIα can exist either in its subunit form or in the form of the PKA-I holoenzyme. As the RIα subunit can act as a cAMP sink, RIα in the holoenzyme complex may be of functional importance. Therefore, we examined whether RIα antisense could influence the PKA isozyme distribution in OVCAR-8 cells. Cell extracts were subjected to diethyl-aminoethyldextran (DEAE) ion-exchange chromatography, and fractions were assayed for PKA activity in the absence and presence of cAMP. Saline-treated control cells showed two major peaks of PKA, PKA-I (Figure 3, peak I) and PKA-II (Figure 3, peak II), that were eluted at 40–80 mM and 220–300 mM NaCl, respectively, as was

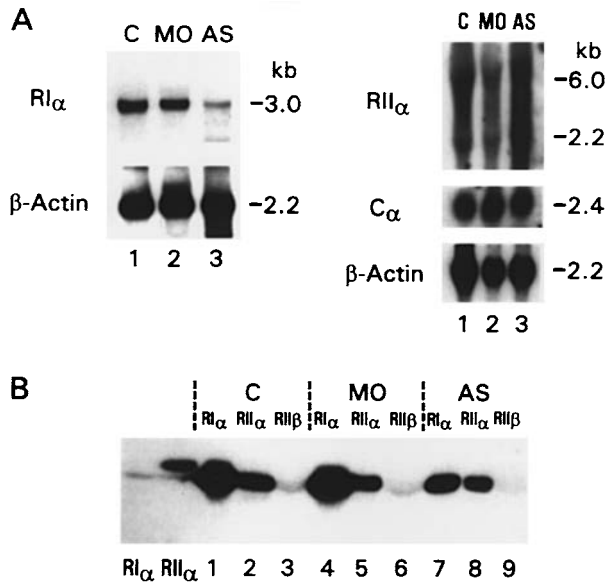

Figure 2 Sequence-specific inhibition of RIα gene expression. (**a**) RIα antisense effect on the mRNAs of PKA R and C subunit. The total RNAs isolated from untreated control (C) and antisense (AS) or control mismatched oligonucleotide (MO)-treated (for 5 days at 150 nM) cells were subjected to Northern blotting analysis using ^{32}P-labeled cDNA probes as indicated. Total cellular RNA preparation, Northern blot analysis and hybridization of RNA with ^{32}P-labeled DNA probes were as described earlier (Nesterova *et al*., 1996). DNA was labeled with α-^{32}P-dCTP according to a standard protocol for nick translation reactions using an Amersham nick translation kit. The specific radioactivity of labeled DNA equaled 3.7×10^6 c.p.m./μg DNA. Data represent one of three independent experiments that gave similar results. (**b**) Photoaffinity labeling-immunoprecipitation of PKA R subunits. Cell extracts from cells untreated (C) and treated with antisense (AS) or control mismatched oligonucleotide (MO) as in **a** above were photoaffinity-labeled with 8-N_3-^{32}[P]cAMP, immunoprecipitated with each R subunit antibodies, then subjected to SDS–PAGE. Cell extract preparation and the photoactivated incorporation of 8-N_3-[^{32}P]cAMP, immunoprecipitation using anti-RIα, anti-RIIα and anti-RIIβ polyclonal antibodies and SDS–PAGE of the dissolved immunoprecipitates were performed as described previously (Nesterova and Cho-Chung, 1995). The data represent one of three independent experiments that gave similar results

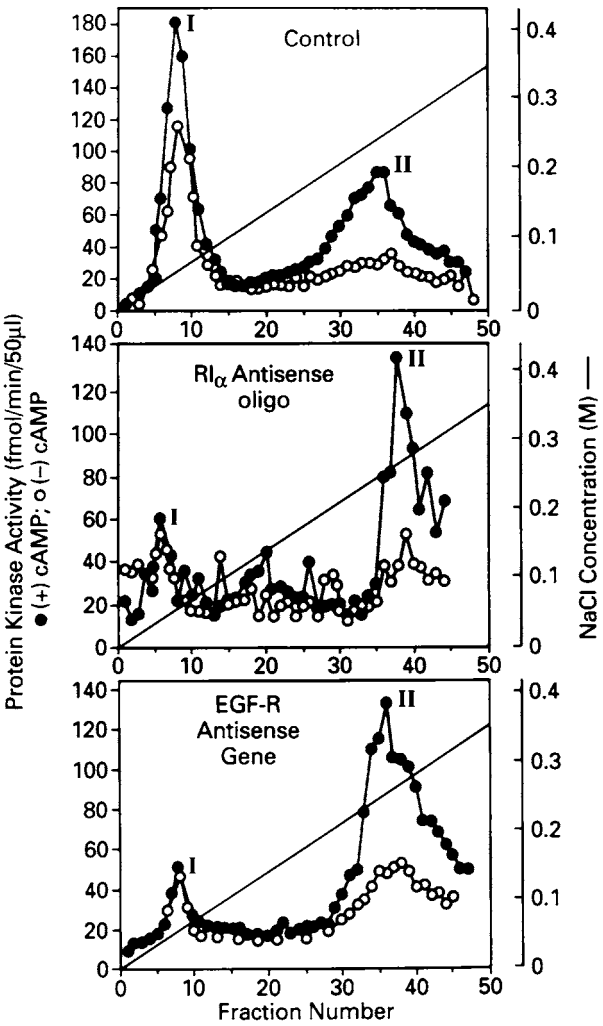

Figure 3 DEAE-chromatography of PKA isozymes in RIα antisense-treated cells and EGF-R antisense gene overexpressing cells. Construction and Transfection of EGF-R expression vectors: A 1.8 kb *Eco*RI fragment of pHER-64-1 (ATCC) coding for two cysteine-rich and transmembrane domains of EGF-R was blunted with T4 DNA polymerase and ligated to *Not*I linkers as described previously (Pegues and Stromberg, 1997). An expression vector pOPRSVI (Stratagene, La Jolla, CA, USA) was restricted with *Not*I, dephosphorylated with calf intestinal phosphatase and gel purified. The *Not*I modified 1.8 kb fragment was cloned into the *Not*I site of pOPRSVI in both sense and antisense orientation, which was determined by restriction analysis. OVCAR-8 ovarian cancer cells were stably transfected with EGF-R sense and antisense expression vectors, and the expanded clones were used in the experiment. Cell extracts (10 mg protein) of control (untreated) and RIα antisense-treated (150 nM for 5 days) or of EGF-R antisense gene overexpressing cells were subjected to DEAE chromatography (1×10 cm columns) and the column eluents were assayed for protein kinase A activity in the absence (○) and presence (●) of 5 μM cAMP by the method described previously (Nesterova and Cho-Chung, 1995). The experiments were repeated 2–3 times, and reproducible elution profiles were obtained. The salt concentrations were determined by the measurement of the conductivity. I, PKA-I; II, PKA II. The PKA-I and PKA-II activities were calculated by the area estimation of the peak I and II (i.e., activity in (+) cAMP minus activity in (–) cAMP

shown previously in other cells (Figure 3) (Nesterova *et al*., 1996). The antisense treatment (150 nM, for 5 days) selectively eliminated PKA-I, the RIα-containing holoenzyme without changing PKA-II activity (Figure 3). The antisense, however, brought about changes in the PKA-II profile of the cell. The control cells contained PKA-II with its peak eluted at 230 mM NaCl, whereas the antisense-treated cells contained the PKA-II with its peak eluted at 280 mM NaCl prominently (Figure 3). The PKA elution profile observed at a wide salt concentration range (200–300 mM NaCl) has been associated with the presence of multiple kinase species including either RII_2 C trimer or RII_2 C_2 tetramer with nonsaturating amounts of cAMP bound and cAMP unbound form (Cobb *et al*., 1987), the phosphorylated versus unphosphorylated (Nesterova *et al*., 1996; Budillon *et al*., 1995) and the RIIα versus RIIβ-containing PKA-II (Otten *et al*., 1991; Nesterova *et al*., 1996). The RIIα-containing PKA-II elutes at a higher salt concentration than the RIIβ-containing PKA-II, and PKA-II containing the mutant RIIβ lacking the autophosphorylation site elutes at a lower salt concentration than the wild-type RIIβ-containing PKA-II (Budillon *et al*., 1995). OVCAR-8 cells contained RIIα but not RIIβ (Figure 2). Thus, the PKA-II induced by RIα antisense treatment may represent the RIIα-containing PKA-II in which RIIα may be phosphorylated as its peak shifts toward a higher salt concentration (Figure 3). Four-base mismatched oligonucleotide treatment had no effect on the amount and profile of PKA-I and PKA-II in these cells (data not shown).

Antisense EGF-R cDNA transfection has been shown to impair cell proliferation in cancer cells (Ciardiello *et al*., 1998). To investigate the crosstalk between PKA and tyrosine kinase pathways, OVCAR-8 cells were transfected with the plasmid containing EGF-R cDNA in the antisense orientation. The clones that show retarded growth, cell morphology changes and the low levels of EGF-R protein (Alper *et al*., 1998) were examined for their PKA isozyme profile. Strikingly, the PKA profile of the antisense EGF-R cDNA transfectants was almost identical to that of the parental cells treated with the RIα antisense (Figure 3). The sense EGF-R cDNA transfectants exhibited the same PKA profile as that of nontransfectant parental cells (data not shown).

Our data that the selective downregulation of PKA-I without changing the level of PKA-II in EGF-R-antisense gene transfectants (Figure 3) suggest the possible crosstalk between PKA and tyrosine kinase signaling pathways. We therefore examined the effect of RIα antisense treatment on EGF-R, c-*erb*B-2, c-*erb*B-3 and c-*erb*B-4 protein levels in OVCAR-8 cells. Treatment of cells with RIα antisense (150 nM, 5 days) markedly reduced EGF-R, c-*erb*B-2 and c-*erb*B-3 levels (Figure 4). Quantification by densitometric tracings of the bands showed that these receptor levels in the antisense treated cells were $<10\%$ of that in the untreated control cells. The mismatched oligonucleotide could not mimic the effect of RIα antisense (Figure 4). However, c-*erb*B-4 expression was not downregulated but was upregulated ($\sim$twofold) non-sequence specifically by the both antisense and control antisense oligonucleotide treatment (Figure 4).

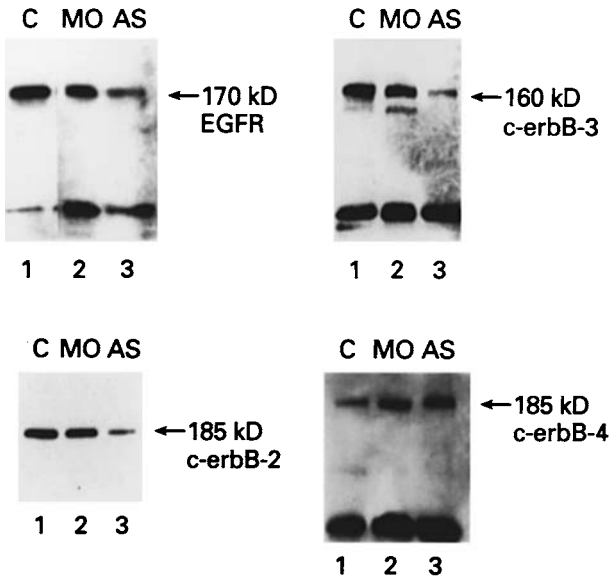

Figure 4 RIα antisense downregulates EGF-receptor and c-*erb*B proteins. Cell extracts from untreated control (C) and RIα antisense (AS) or control mismatched oligonucleotide (MO)-treated cells (150 nM for 5 days) were subjected to immunoprecipitation followed by Western blotting analysis for EGF-R and c-*erb*B proteins. Western blot analysis: Cells (1×10^6) were washed with PBS and lysed in 'Buffer 10' (Nesterova *et al*., 1996). Cell lysates were immunoprecipitated with anti-EGF-R antibody (LA-1) (Upstate Biotechnology, Lake Placid, NY, USA), anti-c-*erb*B-2 antibody (Ab-3) (Oncogene Research Products, Cambridge, MA, USA), anti-c-*erb*B-3 antibody (c-17) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti-c-*erb*B-4 antibody (Ab-1) (Neomarkers, Fremont, CA, USA) at 4°C overnight. The immunoprecipitates were incubated with Protein A sepharose at 4°C for 2 h, proteins bound to protein A-sepharose were recovered by centrifugation, released by heating, resolved by 4–12% SDS–PAGE and transferred to nitrocellulose sheets. The blots were first incubated with 4% non-fat dry milk for 1 h at room temperature and then probed with antibodies specific to EGF-R (1005) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), c-*erb*B-2 (Ab-3), c-*erb*B-3 (c-17) and c-*erb*B-4 (Ab-2) (Neomarkers, Fremont, CA, USA). Horseradish peroxidase was used as secondary antibody. Immunodection was performed using enhanced chemiluminescence method recommended by the manufacturer (Amersham Pharmacia Biotech). The data represent one of three independent experiments that gave similar results

The morphology of OVCAR-8 cells treated with RIα antisense was examined by staining with Giemsa (data not shown). Control cells exhibited a small, round-shaped morphology and grew in piling up fashion over other cells exhibiting pseudopodic behavior. RIα antisense treatment (150 nM, 5 days) brought about an elongated cell shape, increased cytoplasm to nucleus ratio, flat phenotype and sparse growth. The 4-base mismatched oligonucleotide failed to produce any morphological change. Because the antisense treatment brought about cell growth inhibition (Figure 1), we examined whether apoptosis (programmed cell death) was involved in the growth inhibition. Treatment of cells with RIα antisense (150 nM, 5 days) caused induction of apoptosis as evident from fragmented nuclei and/or condensed chromatin (data not shown). In contrast, mismatched oligo had no effect on apoptosis.

The mitogen-activated protein kinases (MAP kinases), $p^{42\ mapk}$ and $p^{44\ mapk}$, also called ERK-1 and ERK-2, are rapidly activated in cells stimulated with

various extracellular signals. They are thought to play a pivotal role in integrating and transmitting transmembrane signals required for growth and differentiation (Sturgill *et al*., 1988; Davis, 1993). Because both RIα antisense treatment and EGF-R antisense gene overexpression resulted in inhibition of cell growth and changes in cell morphology, we examined whether alteration in MAP kinase activity is involved in the growth inhibitory effects. The untreated control cells exhibited the maximum activation of MAP kinase after 4 min of EGF stimulation (data not shown). In RIα antisense-treated cells, the basal and EGF-stimulated MAP kinase activities were greatly reduced as compared to the control cells. In contrast, in EGF-R antisense gene overexpressing cells, the MAP kinase was constitutively activated to a high level and no longer stimulated with EGF. The control oligonucleotide treatment or EGF-R sense gene overexpression had no effect on MAP kinase activity.

In the present study we have shown that the RIα antisense treatment results in a reduction of RIα expression at both the mRNA and protein levels, inhibition of cell growth, changes in cell morphology and induction of apoptosis in OVCAR-8 ovarian cancer cells. The growth inhibition may have been due to the effect other than the blockade of RIα expression as nonspecific binding of oligonucleotides or its degradation products to biological targets has been shown (Stein, 1995). As discussed below, however, our data show that the antisense inhibition of RIα expression and modulation of protein kinase A isozymes are clearly related to the inhibition of OVCAR-8 cell growth. Thus, at most, nonspecific binding of oligonucleotide probably played a minimal role, if any, in the observed growth inhibition. The sequence-specific effect of RIα antisense was supported by our results showing: (1) the selective downregulation of RIα mRNA level without affecting the mRNAs of other subunit of PKA, RIIα or Cα, suggesting RNase-H activation as the underlying antisense mechanism of action; (2) the selective downregulation of PKA-I (the RIα-containing PKA holoenzyme) without changing the level of PKA-II (the RIIα-containing PKA holoenzyme); and (3) inability of 4-base mismatched or random sequence control oligonucleotides to downregulate RIα or inhibit cell growth.

In addition, the RIα antisense, in a sequence-specific manner, brought about downregulation of EGF-R, c-*erb*B-2 and c-*erb*B-3 protein levels. Importantly, like the RIα antisense-treated cells, the EGF-R antisense gene overexpressing cells also downregulated RIα/PKA-I levels without altering PKA-II level. The RIα antisense downregulation of the EGF-R supergene family and EGF-R antisense gene downregulation of RIα/PKA-I clearly demonstrate crosstalk between the serine/threonine kinase and tyrosine kinase signaling pathways.

Several previous reports support this communication. In NRK rat fibroblasts (Tortora *et al*., 1989) and Nog-8 mouse mammary epithelial cells (Ciardiello *et al*., 1990), TGFα and *ras*-induced transformation correlated with an increased expression of PKA-I along with downregulation of PKA-II. Conversely, the selective inhibition of PKA-I with a site-selective cAMP analog, 8-Cl-cAMP, resulted in downregulation of TGFα and $p21^{ras}$, upregulation of PKA-II, growth arrest and phenotypic reversion (Cho-Chung, 1990). More recently, an actual physical association between PKA-I and EGF-R has been shown by demonstrating the binding of RIα to the Grb2 protein (Tortora *et al*., 1997).

It has been shown that PKA directly regulates the *ras* signaling pathway by phosphorylating raf-1 kinase (Wu *et al*., 1993; Cook and McCormick, 1993) and that the activated α-subunit of the heterotrimeric guanine nucleotide binding protein inhibits proliferative signals from *ras* through cAMP and PKA (Chen and Iyengar, 1994). In these mechanisms of action, an increase in intracellular cAMP levels is an essential prerequisite. However, it has been shown that cellular cAMP levels do not strictly correlate with the transformation or reverse transformation processes, suggesting that cellular effectors other than endogenous cAMP levels, such as PKA, are critically involved in transformation and/or reverse transformation (Cho-Chung, 1990).

Our results show that the antisense depletion of RIα resulted in the downregulation of both basal and EGF-stimulated MAP kinase activities, whereas in EGF-R antisense gene overexpressing cells, the MAP kinase activity was constitutively activated to a high level and no longer stimulated with EGF. Because both the RIα antisense and EGF-R antisense gene overexpression led to cell growth inhibition, we speculate the following mechanism of action. In RIα antisense-treated cells, the EGF pathway was downregulated but was not totally blocked and, therefore, the EGF stimulation of MAP kinase was still functional even though the activity was low. The reduced MAP kinase activity may not be due to the PKA-phosphorylation of raf-1 kinase. The PKA activity ratio, the ratio of activity in the absence of added cAMP to that in the presence of cAMP, which measures the degree of the free C subunit release, was the same during the course of the antisense treatment for 5 days (Alper and Cho-Chung, unpublished). Thus, the RIα antisense inhibition of OVCAR-8 cell growth might involve blocking of PKA-I function in cell cycle progression as was previously shown in other cancer cells treated with RIα antisense. In HL-60 cells, RIα antisense treatment induced differentiation and resulted in an increase in the population of cells in S phase of the cell cycle, indicating inappropriate entry of cells into S phase (Cho-Chung *et al*., 1997). Assay of cyclin E demonstrated the depletion of RIα by the antisense caused deregulation of the cell cycle at two critical points, one at the entry into S phase and the other at G_2M phase, resulting in accumulation of cells in S phase (Cho-Chung *et al*., 1997), suggesting the critical role of PKA-I/RIα in the cell cycle regulation. In contrast, in EGF-R antisense gene overexpressing cells, the EGF-R pathway may be largely inhibited, if not totally blocked, and consequently cells find an alternative survival pathway resulting in constitutive activation of MAP kinase. The constitutive activation of MAP kinase has been implicated as a cellular survival factor during tumor promotion (Guyton *et al*., 1996).

Our results show that both cAMP and EGF signaling pathways are involved in OVCAR-8 cancer cell growth. Although these two signaling pathways crosstalk probably through PKA-I, blocking only one of these two pathways may result in activation of an

alternative pathway giving rise to some advantage to cell growth. Therefore, blocking both serine/threonine kinase and tyrosine kinase pathways would be a more effective therapeutic approach to the treatment of ovarian cancer.

Acknowledgements
We thank Dr Sudhir Agrawal (Hybridon, Inc., Milford, MA, USA) for providing the oligonucleotides and Dr Joyce Pegues (FDA, Bethesda, MD, USA) for her kind help in the construction of EGF-receptor expression vectors. This research was supported in part by a grant from the Gynaecological Cancer Fund of the Royal Hospital Foundation.

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