Protein Kinase A RIα Antisense Inhibition of PC3M Prostate Cancer Cell Growth: *Bcl*-2 Hyperphosphorylation, Bax Up-Regulation, and Bad-Hypophosphorylation

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ABSTRACT

It has been shown that expression of the RIα subunit of cyclic AMP (cAMP)-dependent protein kinase is enhanced in human cancer cell lines, primary tumors, and cells after transformation. Using an antisense strategy, we have shown that RIα has a role in neoplastic cell growth *in vitro* and *in vivo*. In the present study, we have investigated the sequence- and target-specific effects of exogenous RIα antisense oligodeoxynucleotides (ODNs) and endogenous antisense gene on tumor growth, apoptosis, and cAMP signaling in androgen-insensitive prostate cancer cells, both *in vitro* and in nude mice. Here, we show that an RIα antisense, RNA/DNA mixed backbone ODN exerts a reduction in RIα expression at both the mRNA and protein levels, up-regulation of both the RIIβ subunit of cAMP-dependent protein kinase or protein kinase A and c-AMP-phosphodiesterase IV expression, and inhibition of cell growth. Growth inhibition was accompanied by changes in cell morphology and the appearance of apoptotic nuclei. In addition, *Bcl*-2 hyperphosphorylation; increase in the proapoptotic proteins Bax, Bak, and Bad; and Bad hypophosphorylation occurred in the antisense-treated cells. These effects of exogenously supplied antisense ODN mirrored those induced by endogenous antisense gene overexpression. The RIα antisense ODNs, which differed in sequence or chemical modification, promoted a sequence- and target-specific reduction in RIα protein levels and inhibited tumor growth in nude mice. These results demonstrate that in a sequence-specific manner, RIα antisense, via efficient depletion of the growth stimulatory molecule RIα, induces growth inhibition, apoptosis, and phenotypic (cell morphology) changes, providing an innovative approach to combat hormone-insensitive prostate cancer cell growth.

INTRODUCTION

The growth and differentiation of normal prostate cells depend on androgen stimulation. However, in response to androgen-deprivation therapies, prostate cancer cells soon become independent of androgen, and, thus, treatment of prostate tumors is somewhat difficult. The mechanism underlying this switch from androgen sensitivity to insensitivity may involve a CRE[2] present in the promoter region of the androgen receptor gene (1). The ability to regulate androgen receptor gene via cAMP is lost in androgen-insensitive prostate tumor cells (2). Thus, deregulation of the cAMP signaling may be an important mechanism of androgen insensitivity in prostate cancer.

The primary mediator of cAMP action in mammalian cells is PKA (3). Two types of PKA, type I (PKA-I) and type II (PKA-II), share a common catalytic subunit but contain distinct regulatory subunits, RI and RII (4). Expression of the RIα subunit of PKA increases in various human tumors and cell lines, including cancers of the breast (5–8), ovary (9, 10), lung (11), and colon (12–14). Overexpression of the RIα subunit of PKA correlates with poor prognosis and survival of cancer patients (6, 7, 9, 10, 15), and conversely, specific inhibition of RIα expression by an antisense ODN inhibits growth and modulates cAMP signaling in cancer cells (16–20).

In the present study, we examined the downstream biological effects of RIα antisense in the androgen-insensitive prostate cancer cells in the *in vitro* and *in vivo* tumor models.

MATERIALS AND METHODS

Cell culture medium, fetal bovine serum, MEM nonessential amino acids, antibiotic-antimycotic, and phosphocellulose discs were purchased from Life Technologies, Inc. (Rockville, MD). cAMP, Kemptide, and protein kinase inhibitor protein were from Sigma Chemical Co. (St. Louis, MO). [γ-^{32}P]ATP was purchased from ICN (Costa Mesa, CA). Antibodies against the Cα, RIα, RIIα, and RIIβ subunits of PKA and Bad were obtained from Transduction Laboratories (Lexington, KY). Anti-*Bcl*-2, Bax, Bak, and phospho-Bad antibodies were from Upstate Biotechnology (Lake Placid, NY). Antiactin anti-

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[2] The abbreviations used are: CRE, cyclic AMP responsive element; cAMP, cyclic AMP; PKA, cyclic AMP-dependent protein kinase; ODN, oligodeoxynucleotide; PS-ODN, phosphorothioate oligodeoxynucleotide; MBO, mixed backbone; PDE IV, phosphodiesterase IV; CREB, cyclic AMP response element binding protein.

body was obtained from Oncogene Research Products (Cambridge, MA).

Oligonucleotides. The oligonucleotides used in the present study include the human RIα antisense PS-ODN (16) targeted against codons 8–13 of human RIα. The second-generation RNA/DNA MBO PS-ODN contains four 2′-*O*-methyl ribonucleotides (RNA) at both the 5′ and 3′ ends (20) and the mouse RIα antisense PS-ODN targeted against codons 8–13 of mouse RIα (16). A four-base mismatched ODN and random sequence RNA/DNA MBO ODN (20) were used as control ODNs. These oligonucleotides were synthesized as described previously (18).

Cell Culture and Oligonucleotide Treatment. PC3M cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mM MEM nonessential amino acids (pH 7.4), and antibiotic-antimycotic in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. For cell growth assays, PC3M cells were plated at 2×10^5 cell density in 60-mm culture dishes. After 12 h of seeding, cells were washed with fresh medium, and antisense and control ODNs were added to the culture medium at varying concentrations. To increase the uptake of ODN into the cells, the transfection reagent *N*-[1-(2, 3- Dioleoyloxy) propyll-*N*,*N*,*N*-trimethylammonium methyl sulfate was used according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN). The mismatched or random sequence ODNs were used as controls. Cells were harvested at the indicated times and counted using a ZI Coulter counter (Coulter Co., Miami, FL). Results are expressed as the mean cell number per dishes ±SD. Each assay was performed in triplicate.

Construction of Antisense RIα Vector and Production of Stable Transfectants. The 210-bp fragment of human PKA RIα cDNA was amplified by PCR using the upstream primer (5′-GCGC***GGATCC***ATGGAGTCTGGCA-3′, nucleotide positions 1–13) and downstream primer (5′-GCGCGAATTCTTTCTGCAGATTC-3′, nucleotide positions 198–210). The PCR product was cut with *Bam*HI/*Eco*RI and cloned into the *Eco*RI/*Bam*HI site of the OT1529 retroviral vector (21). OT1529 contains the mouse metallothionein (MT-1) promoter as the internal promoter and a gene encoding neomycin phosphotransferase, which confers G418 resistance and allows selection for stable transfectants. Cells were transfected with MT-antisense RIα vector using the lipofectin method (Life Technologies, Inc.). Stably transfected cells were selected by growing cells in the presence of G418 (400 μg/ml; Life Technologies, Inc.). To induce expression of the antisense RIα gene, cells were treated with 60 μM $ZnSO_4$ for 5 days.

RNA Preparation and Northern Blot Analysis. Total RNA was prepared using the Rneasy Midi kit (Qiagen), and 20 μg of each RNA sample were loaded onto an agarose/formaldehyde gel. PKA R- and C-specific probes were generated as described previously (21). Northern analyses were performed as described previously (21).

Tumor Growth and Antisense Treatment. PC3M human prostate carcinoma cells (2×10^6 cells) were inoculated s.c. into the left flank of nude mice. When tumors became palpable, antisense or control ODN (0.1 mg/0.1 ml saline/mouse, daily) or saline (0.1 ml/mouse) was injected i.p. into the mice. Tumor volumes were obtained from daily measurement and calculation as described in (16). At each indicated time, animals were sacrificed, and tumors, livers, and spleens were removed, weighed, immediately frozen in liquid N_2, and stored at −80°C until used.

Western Blot Analysis. PC3M cells were seeded at a density of 1×10^6 cells/100-mm plate and treated with antisense or control ODN (100 nM) for 2 days. Cells were washed twice with ice-cold PBS, lysed in Buffer 10 [Ref. 21; 20 mM Tris/HCl, 100 mM NaCl, 5 mM $MgCl_2$, 1% NP40, 0.5% sodium deoxycholate, 100 μM pepstatin, 100 μM antipain, 100 μM chymostatin, 10 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml trypsin inhibitor, and 1 mM benzamidine (pH 7.5)], and placed on ice for 15 min. Tumor and liver extracts were prepared as described in Ref. 16. Protein concentration was determined by the Bradford assay using the Bio-Rad Protein Assay Kit (Bio-Rad, Richmond, CA). Cell extracts (40 μg) were subjected to SDS-PAGE, and Western analysis was performed as described previously (22).

PKA Assay. Cells were lysed with Buffer 10 (see Western blotting method) on ice. PKA activity was measured by adding 10 μl of cell extract (10 μg of protein) to 40 μl of reaction buffer [50 mM Tris (pH 7.5), 20 μM Kemptide (a Ser peptide; Leu-Arg-Arg-Ala-Ser-Leu-Gly; Life Technologies, Inc.), 1.2 μM (γ-^{32}P) ATP (100–200 cpm/pmol), 10 mM $MgCl_2$, and 1 mM DTT] in the presence or absence of cAMP (5 μM) or protein kinase inhibitor protein (20 μM) and incubated for 5 min at 30°C. The reaction mixture (40 μl) was spotted onto phosphocellulose discs, then washed three times with 1.5% phosphoric acid. Filters were air dried, then counted using liquid scintillation counter (Beckman, Columbia, MD). One unit of PKA activity is defined as that amount of enzyme that transfers one pmol of ^{32}P from [γ-^{32}P] ATP to the recovered protein in 5 min at 30°C in the standard assay system.

Morphological Determination of Apoptotic Nuclei. Cells were grown on ethanol-sterilized glass coverslips and treated with antisense or control ODN (100 nM) for 2 days. To examine whole cell morphology, cells were washed with PBS, fixed with 70% methanol for 5 min, and stained with Giemsa (Bio-Rad) for 30 min. Coverslips were rinsed with PBS, mounted on slides with 80% glycerol in PBS, and photographed using a Zeiss Axiovert 25 CFL inverted microscope. Morphological changes characteristic of apoptosis were determined by staining cell nuclei with Hoechst 33258 (Sigma Chemical Co.). After treatment, the coverslips were rinsed gently with PBS, fixed with 3.7% formaldehyde for 10 min, and stained with 1 μM Hoechst 33258 in PBS for 15 min. Coverslips were rinsed with PBS and mounted with SloFade antifade mounting medium (Molecular Probes, Eugene, OR). The slides were observed under a Zeiss Axiovert 25 CFL inverted microscope.

RESULTS

Inhibition of Cellular Proliferation. To evaluate the responsiveness of androgen-insensitive prostate carcinoma cells to RIα antisense ODN, we first analyzed the proliferation of PC3M cells in monolayer culture in response to increasing concentrations of antisense ODNs. To address the possibility of nonspecific cytotoxicity on exposure of cells to the ODN, a 4-base mismatched ODN or a scrambled sequence ODN were

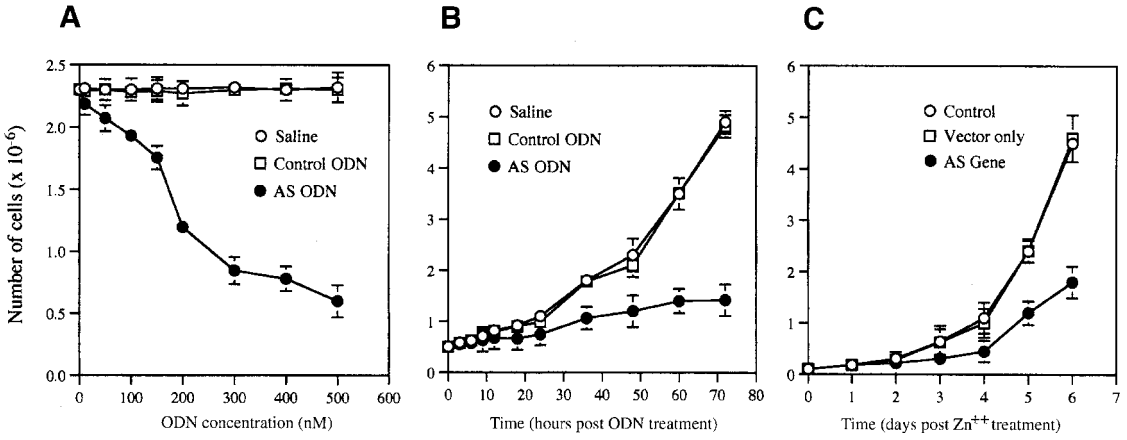

Fig. 1 Growth inhibition of PC3M cells in monolayer culture by RIα antisense ODN treatment or antisense gene overexpression. *A*, concentration dependence. Untreated (*Saline*) cells (○) or cells treated with RNA/DNA MBO antisense ODN (*AS ODN*; ●) or mismatched ODN (*Control ODN*; □) for 2 days at the indicated concentrations. *B*, time dependence. Cells were treated with ODNs as in *A*, at a concentration of 200 nM for the indicated times. In *C*, parental nontransfectants (○) and cells transfected with RIα antisense gene in OT1529 vector (●) or transfected with OT1529 vector only (□) were cultured in the presence of 60 μM $ZnSO_4$ for the indicated times. Cells were then harvested and counted by ZI Coulter Counter. The data represent average values ± SD of three independent experiments. Each cell count was performed in triplicate.

used as a control. Antisense ODN treatment inhibited growth of PC3M cells in a concentration-dependent (Fig. 1*A*) and time-dependent (Fig. 1*B*) manner. In contrast, the mismatched control ODN had no effect (Fig. 1, *A* and *B*). We next examined whether endogenous overexpression of an antisense gene could also inhibit growth in PC3M cells. As shown in Fig. 1*C*, the growth of cells transfected with the RIα antisense gene was inhibited, compared with parental nontransfectants. Cells transfected with the expression vector alone exhibited no growth retardation (Fig. 1*C*). Thus, both the exogenously supplied antisense ODN and the endogenously overexpressed antisense gene were capable of promoting growth inhibition in PC3M cells.

Down-Regulation of RIα, Up-Regulation of RIIβ, and Induction of cAMP-PDE IV. We next determined the effects of the antisense ODN or antisense gene on the levels of C and R subunits in PC3M cell extracts. Western blotting revealed a sharp down-regulation of RIα protein levels in both antisense ODN-treated cells and antisense gene transfectants, whereas the mismatched ODN had no effect (Fig. 2*A*). Moreover, RIα antisense, but not mismatched ODN, induced RIIβ protein expression (Fig. 2*A*), although the levels of RIIα and the Cα subunit did not change in RIα antisense ODN-treated cells or antisense gene transfectants (Fig. 2*A*).

Because RIα antisense treatment inhibited cell growth and decreased RIα protein levels, we examined whether antisense also influenced RIα mRNA levels (Fig. 2*B*). Both RIα antisense ODN treatment and antisense gene overexpression markedly reduced RIα mRNA levels (Fig. 2*B*). By comparison, the mismatched ODN had no effect (Fig. 2*B*). Both the antisense ODN and the antisense gene increased RIIβ mRNA levels without changing RIIα and Cα mRNA levels (Fig. 2*B*). Thus, the reduction in the levels of RIα protein and mRNA resulting from antisense treatment correlated with an increase in RIIβ mRNA and protein levels and a decrease in cell growth.

We have shown previously that the cAMP-inducible enzyme, PDE IV, increases in RIα antisense ODN-treated LS-174T colon cancer cells and in LNCaP prostate cancer cells (20). Fig. 2*B* shows that in cells exposed to RIα antisense, either through ODN treatment or gene overexpression, the PDE IV mRNA levels were markedly increased. Thus, RIα antisense induced PDE IV, a cAMP-inducible enzyme.

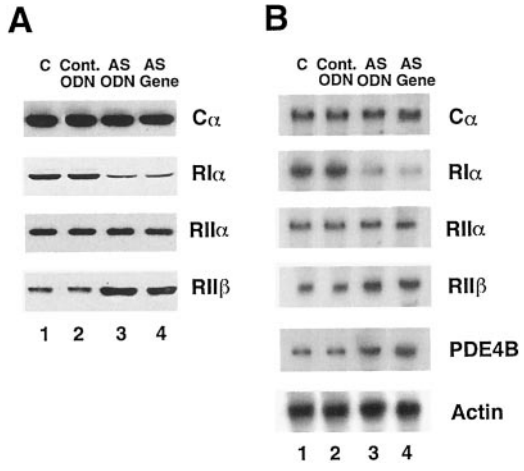

Fig. 2 Antisense suppression of RIα mRNA and protein expression and up-regulation of RIIβ and PDE IV. *A*, PKA R and C protein levels. Western blotting analysis was performed, as described in "Materials and Methods," in untreated control cells (*C*), in cells treated with mismatched control ODN (*Cont. ODN*), or antisense ODN (*AS ODN*) at 200 nM for 3 days and cells overexpressing the antisense gene and treated with 60 μM $ZnSO_4$ for 5 days (*AS Gene*). Data represent one of three independent experiments that gave similar results. *B*, mRNA levels for PKA R and C subunits and PDE IV. Northern blotting analysis was performed, as described in "Materials and Methods," for untreated control cells (*C*), cells treated with control ODN (*Cont. ODN*), or cells treated with antisense ODN (*AS ODN*) or overexpressing the antisense gene (*AS Gene*) that's described in *A*. Data represent one of three independent experiments that gave similar results.

Table 1 Increase in protein kinase A activity ratio in RIα antisense RNA/DNA ODN-treated PC3M cells.

Cells were treated with antisense ODN or mismatched control ODN (100 nM) for the indicated time intervals. Cell extracts were prepared, and PKA activity was measured as described in "Materials and Methods." PKA activity ratio was calculated as the ratio between free (basal) PKA and total PKA. Zero time indicates untreated control cells. Mismatched control ODN had no effect on both basal and total activity throughout the experimental time period. Data represent one of three independent experiments that gave similar results.

Antisense ODN treatment time (h)	Cell extract			
	Total PKA activity (units/mg protein)	Free PKA activity (units/mg protein)	PKA activity ratio −*cAMP*	
			+*cAMP*	%
0	4.2	0.46	0.11	100
3	4.2	0.47	0.11	100
6	4.2	0.47	0.11	100
9	4.1	0.47	0.11	100
12	4.1	0.49	0.12	109
18	4.0	0.50	0.13	118
24	3.8	0.50	0.13	118
48	3.6	0.50	0.14	127
60	3.5	0.57	0.16	145
72	3.4	0.59	0.17	155
96	3.4	0.60	0.18	164
120	3.4	0.60	0.18	164

Increase in PKA Activity Ratio. The induction of PDE IV, which contains a CRE enhancer in its promoter, suggests the activation of PKA. Therefore, we determined a PKA activity ratio, a measure of how much PKA is in its active form (free/total PKA), in antisense ODN-treated cells. As shown in Table 1, antisense ODN treatment substantially increased the PKA activity ratio in a time-dependent manner.

Change in Cell Morphology and Induction of Apoptosis. The morphology of PC3M cells exposed to antisense ODN or cells overexpressing the antisense gene was examined by staining with Giemsa (Fig. 3*A*). When cells were evaluated for morphology, only RIα antisense ODN-exposed cells or cells overexpressing the antisense gene displayed a unique morphology that is different from untreated control (saline treated; Fig. 3) and mismatched ODN-treated cells (data not shown). The control and mismatched ODN-treated cells exhibited an elongated shaped morphology with a higher ratio of nucleus-to-cytoplasm intensity (Fig. 3*A*). Cells exposed to endogenous or exogenous RIα antisense, however, exhibited a flat phenotype and an increased cytoplasm:nucleus ratio, and they grew sparingly (Fig. 3*A*).

Because the above data show that treatment with the RIα antisense ODN or overexpression of the antisense gene inhibited growth, it was of interest to examine if apoptosis was involved in this process. Treatment of PC3M cells for 3 days with RIα antisense ODN (0.2 μM) induced apoptosis, as evident from fragmented nuclei or condensed chromatin (Fig. 3*B*). The antisense treatment induced apoptosis in a concentration- and time-dependent manner, whereas mismatched ODN could not mimic the antisense effect (Fig. 3*C*). Under the experimental conditions, the saline-treated control cells exhibited no appreciable changes in nuclear morphology (Fig. 3*C*). The fragmented nuclear morphology and chromatin condensation observed in the antisense ODN-treated cells were mirrored in cells overexpressing the antisense gene (Fig. 3*B*).

Hyperphosphorylation of *Bcl*-2 and Up-Regulation of Bax. Some proteins within the *Bcl*-2 family, including *Bcl*-2 and *Bcl*-X_l, inhibit apoptosis, whereas others, such as Bax, Bak, and Bad, promote apoptosis. It has been shown that the microtubule-damaging drugs induce *Bcl*-2 hyperphosphorylation and apoptosis in cancer cells and that *Bcl*-2 hyperphosphorylation is mediated by activated protein kinase A (23). Because the above data show that treatment of cells with RIα antisense resulted in the activation of PKA and the induction of apoptosis, we examined the effect of RIα antisense on the expression of *Bcl*-2 family proteins. PC3M cells either treated with RIα antisense ODN or overexpressing the antisense gene exhibited a marked increase in the proapoptic proteins Bax, Bak, and Bad (Fig. 4). In addition, RIα antisense induced hyperphosphorylation of the antiapoptic *Bcl*-2 protein and a decrease in the phosphorylated form of Bad (Fig. 4), which is also antiapoptotic (24). p53 protein was undetected, and the $p21^{waf1/cip1}$ protein level was unchanged in the antisense-treated cells (Fig. 4). In comparison, the mismatched ODN could not mimic these effects (Fig. 4). These data confirm the above findings that RIα antisense induces apoptosis in PC3M cells.

Inhibition of *in Vivo* Tumor Growth. The effects of RIα antisense ODN on human prostate tumor growth *in vivo* was next examined using s.c. implanted PC3M cells in nude mice. To verify the sequence specificity of the antisense effects on *in vivo* tumor growth, we used three antisense ODNs that differed in sequence or in chemical modification: a PS-ODN, directed against codons 8–13 of human RIα (16); the immunosuppressive (25), less cytotoxic (26), and second-generation RNA/DNA MBO PS-ODN (20); and a nonimmunostimulatory 5′-CCG-containing (27) PS-ODN antisense, targeted to codons 8–13 of mouse RIα, which can cross-hybridize with human RIα (16). As shown in Fig. 5, all three antisense ODNs markedly inhibited growth. By day 9, tumor growth was inhibited by 60–90%, compared with untreated control tumors, and the antisense RNA/DNA MBO ODN exhibited the most potency (Fig. 5*B*). By comparison, the scrambled sequence control ODN produced no effect. Compared with untreated (saline-injected) control tumors, the weights of treated tumors after 4 days of treatment were 42, 63, 56, and 98% for antisense RNA/DNA MBO ODN, PS-ODN, mouse PS-ODN, and control ODN, respectively (Fig. 5*A*). There was no sign of systematic toxicity in ODN-treated animals, and the sizes of the liver and spleen remained unchanged.

Down-Regulation of RIα and Up-Regulation of RIIβ in Tumors *in Vivo*. We next determined whether antisense ODN-induced inhibition of *in vivo* tumor growth could be correlated with down-regulated RIα expression. Western blotting demonstrated sharp down-regulation of RIα protein levels in antisense-treated tumors, whereas random sequence ODN had no effect on RIα levels (Fig. 6*A*). Moreover, RIα antisense ODN, but not the control, increased RIIβ protein levels (Fig. 6*A*). The host livers contained a low level of RIα and high levels of RIIβ, and RIα antisense had no appreciable effect on any of the R and C subunits of PKA (Fig. 6*B*).

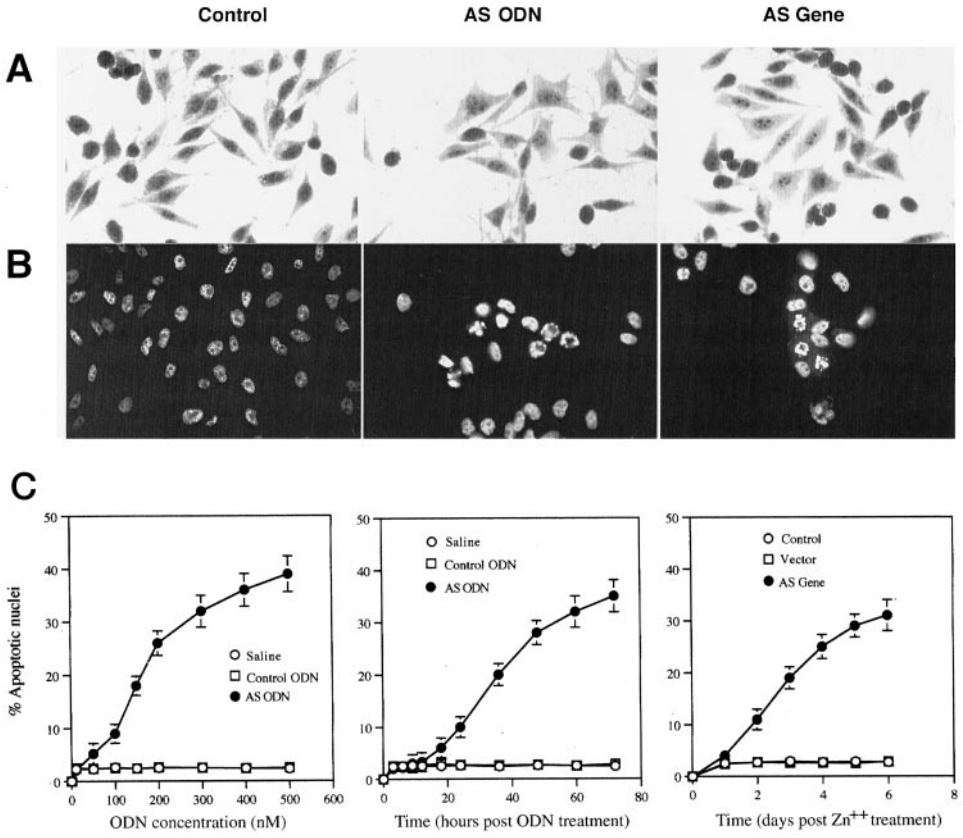

Fig. 3 RIα antisense induces changes in cell morphology and apoptosis. *A*, cell morphology; *B*, apoptotic nuclei; *C*, apoptotic nuclei counts. Untreated control cells (*Saline*), cells treated with 200 nM antisense ODN (*AS ODN*) for 3 days, or cells overexpressing antisense gene (*AS Gene*) were examined for cell morphology and apoptosis (see "Materials and Methods"). Whole cell and nuclear morphology: ×180. Data represent one of three separate experiments that gave similar results. Cells treated with mismatched control ODN exhibited the same morphology and nuclear appearance as that of saline-treated control cells.

DISCUSSION

We have shown in the present study that the growth of androgen-insensitive prostate cancer cells (PC3M) is arrested by an antisense ODN targeted against the RIα regulatory subunit of protein kinase A. This antisense inhibition of cell growth correlated with the down-regulation of RIα and the up-regulation of RIIβ, at both the mRNA and protein levels, without affecting the RIIα or Cα subunits of protein kinase A. Thus, the increased expression of the differentiation-inducible RIIβ (28, 29), which was not detected in the untreated control cells, compensated for the loss of RIα resulting from antisense treatment as was shown previously in other cancer cells (16, 17, 20, 30). Despite the often compensatory up-regulation of RIIβ, the PKA activity ratio, which measures the degree of PKA activation, increased in RIα antisense-treated cells (Table 1). This might have occurred because the rate of RIα loss exceeded that of RIIβ increase and because the C subunit was rescued from rapid proteolysis by the continued up-regulation of RIIβ in the cell. In fact, in such cells as LS-174T colon carcinoma and LNCaP prostate cancer cells, in which both PKA-I and PKA-II were expressed, the compensatory up-regulation of RIIβ occurred on RIα antisense treatment, and the Cα subunit was stable (20). However, in HCT-15 multidrug-resistant colon cancer cells, in which PKA-I is primarily expressed, antisense-directed loss of RIα was not balanced by an increase in RIIβ, and Cα subunit degradation increased because of a decreased half-life (20). Thus, the RIα down-regulation, the up-regulation of RIIβ, and the PKA activation show strong target specificity of RIα antisense and the biochemical adaptation of the cell to the loss of RIα.

Demonstration of such specificity is usually considered to be the most important criterion for determining the true antisense mechanism underlying the biological hallmarks, such as effects on cell growth, cell death, and cell differentiation (16, 31, 32). Importantly, our results show that the target specificity of RIα antisense extended beyond that of direct target down-regulation, leading to the effect on the regulation of the cAMP signaling cascade. The antisense-induced activation of PKA (Table 1) led to an induction of PDE IV (Fig. 2*B*), a cAMP-responsive enzyme that contains CRE enhancer (33). Thus, the loss of RIα resulted in activation of cAMP signaling through activation of PKA, bypassing adenylate cyclase/cAMP. This was shown previously in other cancer cells (20). We have interpreted these results in the context of CRE-directed transcription. PKA is known to activate the transactivation activity of CREB (34) by phosphorylating Ser 133 (35). On the other hand, it has been shown that the phosphorylation of CREB at Ser 133 is also critical for growth factor induction of c-*fos* transcription (36, 37). Thus, in transformed cells, the growth factor-mediated phosphorylation of CREB may supersede that mediated by PKA and stimulate cell growth. On RIα antisense treatment, the activated PKA (free C subunit) may cause a switch in the mechanism of CREB phosphorylation from one mediated by growth factors to one mediated by PKA. This

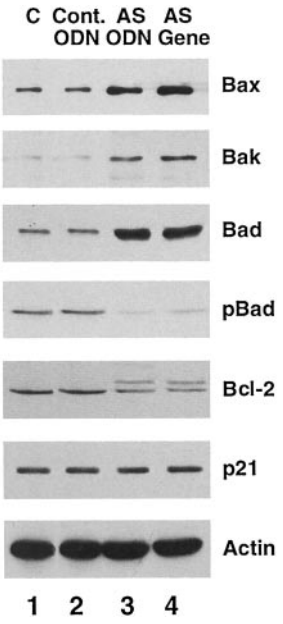

Fig. 4 RIα antisense induction of *Bcl*-2 hyperphosphorylation; up-regulation of Bax, Bak, and Bad proteins; and Bad hypophosphorylation. *Bcl*-2 family proteins were measured by Western analysis (see "Materials and Methods") in untreated control cells (*C*), mismatched control ODN (*Cont. ODN*), or antisense ODN (*AS ODN*)-treated cells (200 nM, 3 days) and cells overexpressing the antisense gene (*AS Gene*; 60 μM $ZnSO_4$, for 5 days). Data represent one of three separate experiments that gave similar results.

would inhibit the growth factor signals that stimulate cell proliferation and, thus, promote growth inhibition, consistent with a possible mechanism for the RIα antisense inhibition of tumor cell growth.

Antisense RIα not only blocked cell proliferation but also promoted apoptosis in PC3M cells. It has been shown that inactivation of the antiapoptotic function of *Bcl*-2 by phosphorylation occurs after treatment with the microtubule-damaging anticancer drug, paclitaxel, or the phosphatase inhibitor okadaic acid (23). It has also been shown that PKA is involved in *Bcl*-2 hyperphosphorylation and apoptosis induced by microtubule-damaging drugs (23). Our present study shows that an antisense ODN targeted against the RIα subunit of PKA induces *Bcl*-2 hyperphosphorylation and apoptosis (Fig. 3, *B* and *C*). These results suggest that the activated PKA (free C subunit) arising from the antisense-mediated depletion of RIα may be responsible for the *Bcl*-2 hyperphosphorylation observed in the antisense-treated cells.

It has been shown previously that *Bcl*-2 is phosphorylated on serine residues, and of the 17 serine residues present in *Bcl*-2, several could be the potential sites of phosphorylation by different kinases (38, 39). Recently, it has been shown that angiotensin type 2 receptor binding dephosphorylates *Bcl*-2 by activating the mitogen-activated protein kinase phosphatase 1 and induces apoptosis in PC12 cells (40). Furthermore, interleukin-3, erythropoietin, or the protein kinase C activator byrostatin-1 hyperphosphorylates *Bcl*-2 and suppress apoptosis in murine interleukin-3-dependent National Science Foundation/N1.H7 cells (41, 42). These studies show that *Bcl*-2 phosphorylation involves multiple kinases and that specific sites of phosphorylation may determine whether *Bcl*-2 loses or gains its antiapoptotic function. *Bcl*-2 phosphorylation by mitogen-activated protein kinase and protein kinase C may promote antiapoptotic activity of *Bcl*-2, leading to suppression of apoptosis, whereas *Bcl*-2 phosphorylation by PKA may suppress *Bcl*-2 function, leading to promotion of apoptosis.

In accordance with this, our present data show that the antisense-induced *Bcl*-2 hyperphosphorylation occurs along with the up-regulation of the proapoptotic proteins Bax, Bak, and Bad and with the hypophosphorylation of Bad (Fig. 4). Although the mode by which *Bcl*-2 affects the process of cell death is not fully understood, recent studies indicate that the *Bcl*-2 protein binds to other proteins with which it has amino acid sequence homology, including Bax, *Bcl*-X_l, *Bcl*-Xs, Mc1–1, Bak, Bik, and Bad (43–45). The functional significance of many of these *Bcl*-2 family protein-protein interactions remains unclear. However, the heterodimerization of *Bcl*-2 with Bax appears crucial in preventing Bax-mediated apoptosis (44); hyperphosphorylated *Bcl*-2 is less able to form heterodimers with Bax (23, 46). On the other hand, the phosphorylated Bad releases *Bcl*-2 from mitochondria to the cytoplasm leading to a *Bcl*-2-promoted cell survival pathway (24). Thus, dephosphorylated Bad remains bound with *Bcl*-2 in the mitochondria, preventing the *Bcl*-2 promotion of cell survival. Our present data about RIα antisense promotion of *Bcl*-2 hyperphosphorylation, along with up-regulation of Bax and hypophosphorylation of Bad, clearly favor apoptosis over cell survival.

The results observed for three RIα antisense ODNs that differ in sequence or chemical modification show highly sequence-specific antisense effects on the inhibition of *in vivo* tumor growth. The RNA/DNA MBO antisense was the most potent, and the human RIα antisense PS-ODN exhibited the least potency in growth inhibition. RIα antisense inhibition of tumor growth accompanied the target specificity of the antisense illustrated by RIα down-regulation and the compensatory up-regulation of RIIβ. However, in host livers, which express very low levels of RIα and high levels of RIIβ currently beyond the experimental limits of detection, antisense treatment brought about no appreciable changes in these protein levels. One possible explanation for these differential effects between tumor and liver is the different basal levels of RIα and RIIβ in these tissues. A second explanation would be the difference in the half-lives of RIα and RIIβ, both at the mRNA and protein level. Finally, the tissue specificity between tumor and liver, with regard to ODN uptake, pharmacokinetics, stability, and tissue retention time, may be another factor. Nevertheless, the differential effects of antisense RIα observed between tumors and host livers are striking and may have important implications for the clinical development of this antisense.

Our present study, which demonstrates target-specific and sequence-specific growth inhibitory effect by antisense RIα, clearly supports a use for this antisense ODN in combating the androgen-independent growth of prostate cancer and other cancers in which expression of the protein kinase A RIα is increased.

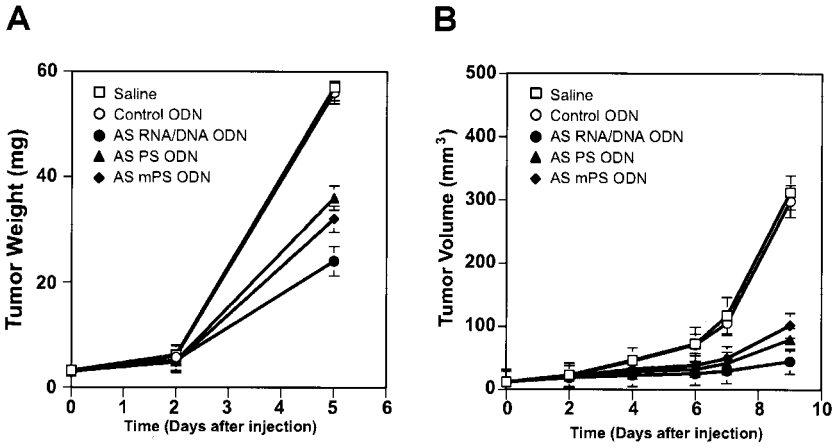

Fig. 5 Sequence-specific, oligonucleotide-dependent inhibition of *in vivo* tumor growth. PC3M cells were inoculated s.c. into nude mice (see "Materials and Methods"). □, saline; ○, control ODN (scrambled sequence); RIα antisense ODN (*AS ODN*) of (●) RNA/DNA MBO ODN; ▲, PS-ODN, at (◆) mouse PS-ODN was injected daily into mice (see "Materials and Methods"). *A*, tumor weights determined on day 5 and tumor volumes measured daily ≤day 9 (*B*; see "Materials and Methods"). Data represent average values ± SD of five tumors per group.

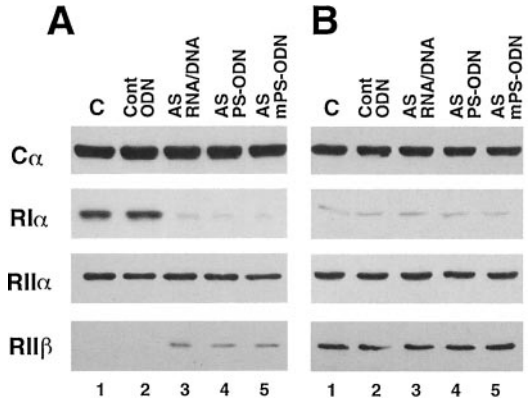

Fig. 6 Antisense down-regulation of PKA RIα subunit and up-regulation of RIIβ subunit in tumors but not in host livers. Tumors and host livers obtained in Fig. 5*A* were analyzed for PKA R and C subunit levels by Western blotting (see "Materials and Methods"). *A*, tumor; *B*, liver. Data represent one of two separate experiments that gave similar results.

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