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Ala^{99}ser mutation in RI$_\alpha$ Regulatory Subunit of protein kinase A causes reduced kinase activation by cAMP and arrest of hormone-dependent breast cancer cell growth

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Abstract

Expression of the RI$_\alpha$ regulatory subunit of protein kinase A type I is increased in human cancer cell lines, in primary tumors, in cells after transformation, and in cells upon stimulation of growth. Ala^{99} (the pseudophosphorylation site) of human RI$_\alpha$ was replaced with Ser (RI$_\alpha$-p) for the structure-function analysis of RI$_\alpha$. MCF-7 hormone- dependent breast cancer cells were transfected with an expression vector for the wild-type RI$_\alpha$ or mutant RI$_\alpha$-p. Overexpression of RI$_\alpha$-P resulted in suppression of protein kinase A type II, the isozyme of type I kinase, production of kinase exhibiting reduced cAMP activation, and inhibition of cell growth showing an increase in G_0/G_1 phase of the cell cycle and apoptosis. The wild-type RI$_\alpha$ overexpression had no effect on protein kinase A isozyme distribution or cell growth. Overexpression of protein kinase A type II regulatory subunit, RII$_\beta$, suppressed RI$_\alpha$ and protein kinase A type I and inhibited cell growth. These results show that the growth of hormone-dependent breast cancer cells is dependent on the functional protein kinase A type I. (Mol Cell Biochem **195**: 77–86, 1999)

Key words: protein kinase A, site-directed mutagenesis, breast cancer, growth arrest, CAMP response element

Abbreviations: PKA – cAMP-dependent protein kinase; C – catalytic subunit; R – regulatory subunit; PKA-1 and PKA-H – PKA type I and type II, respectively; RI, RH – R subunit of PKA-1 and PKA-H, respectively; RI$_\alpha$-p – autophosphorylation mutant of RI$_\alpha$; CRE – CAMP responsive element; CREB – CRE binding protein; CAT – chloramphenicol acetyltransferase

Introduction

cAMP-dependent protein kinase (PKA) is the major mediator of the CAMP signal transduction pathway in mammalian cells [1, 2]. This enzyme consists of two catalytic (C) subunits and a regulatory (R) subunit dimer. Activation occurs when two CAMP molecules bind to each R subunit of PKA, resulting in the release of the C subunits.

There are two types of PKA, type I (PKA-I) and type II (PKA-H), which share a common C subunit but contain distinct R subunits, RI and RII, respectively [2]. Through biochemical studies and gene cloning, four isoforms of the R subunits (RI$_\alpha$, RI$_\beta$, RII$_\alpha$, and RII$_\beta$) have been identified [3].

Varying the ratio of two isoforms of PKA has been linked to cell growth and differentiation [4, 5]. An enhanced expression of RI/PKA-I correlates with active cell growth and

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cell transformation, whereas a decrease in RI/PKA-I and an increase of RII/PKA-II are related to growth inhibition and differentiation-maturation [4, 5].

RI is the major, or sole, R subunit of PKA detected in a variety of types of human cancer cell lines [4]. The majority of primary human breast carcinomas examined show an enhanced expression of RI and a higher ratio of PKA-I/PKA-II as compared with normal counterparts [6–8]. Importantly, the relative overexpression of the RI_α subunit of PKA was associated with poor prognosis in patients with breast cancer [9]. Moreover, an antiestrogen, tamoxifen-induced regression of breast tumors correlated with a reduction in the tumor RI_α mRNA levels following the antiestrogen treatment [10].

In order to investigate the role of RI_α in the growth of hormone-dependent breast cancer cells, we approached the structure-function analysis of RI_α by the use of site-directed mutagenesis. We prepared a mutant of RI_α at its pseudo-phosphorylation site; Ala^{99} of human RI_α was replaced with Ser (mutant RI_α-p). We targeted this site for mutation because the RI and RII are significantly different in this proteolytically sensitive hinge region of amino terminus that occupies the peptide substrate binding site of the C subunit in the holoenzyme complex [11]. In this segment, RII contains the sequence (Arg-Arg-X-Ser), which can undergo autophosphorylation at Ser, whereas RI contains the sequence (Arg-Arg-X-Ala), which cannot be autophosphorylated but participates in the high-affinity binding of ATP in the type I holoenzyme.

We transfected MCF-7 human breast cancer cells with expression vector [12, 13] for the wildtype RI_α or mutant RI_α-p. MCF-7 cells were also transfected with the wild-type RII_β expression vector, which was previously shown to up-regulate RII_β/PKA-II and down-regulate RI_α/PKA-I [13, 14]. We then examined the distribution of PKA isozymes, the cAMP activation of intracellular PKA, and cell growth in these cells.

Materials and methods

Materials

Human breast cancer cell line MCF-7 was obtained from the Breast Cancer Task Force of the National Cancer Institute. Human RII_β [15], RII_α [16], and RI_α [17] cDNAs were kindly provided by Dr. Tore Jahnsen, Rikshopitalet, Oslo, Norway. 8-N_3-[^{32}P]$_C$AMP (60 Ci/mmol) and [γ-^{32}P]ATP (25 Ci/mmol) were from ICN Pharmaceuticals, Inc. (Irvine, CA, USA). [^{14}C]chloramphenicol (58.4 mCi/ml) was from DuPont-NEN (Boston, MA, USA). The 18-mer human RI_α antisense and sense oligodeoxynucleotide phosphorothioates [18] were synthesized at Midland Certified Reagent (Midland, TX, USA).

Cell cultures

MCF-7 cells were grown in Improved Minimum Essential Medium (Biofluids, Inc., Rockville, MD, USA), supplemented with 10% heat-inactivated fetal bovine serum, penicillin (50 units/ml), streptomycin (500 µg/ml), in humidified atmosphere of 5% CO_2 at 37°C.

Mutagenesis

Site-directed mutagenesis was carried out by Kunkel method [19]. For the mutagenesis of RI_α-p, primer 5′ ^{213} AGG-CGA-CGA-GGT-ICT-ATC-AGC-GCT-G ^{307} 3′ was used (mutation ^{295}G $\rightarrow$ T, underlined).

Construction of retroviral vectors

The full-length cDNA encoding wild-type or mutant RI_α and wild-type RII_β was introduced into the retroviral vector OT1521 [20] at an *Eco*RI site as described [12]. Since the *Eco*RI fragments of RI_α contain an internal *Eco*RI site, a condition of partial digestion was employed to avoid digestion of internal *Eco*RI site. The vector OT1521 contains poly (A) sites at the long-terminal (LTR) U3 region and the distance between *Eco*RI and the poly (A) site is 1.3 kb [20]. The unique restriction site in each gene and the cloning site were used to verify the correct orientation of these inserted genes. The resulting retroviral vector plasmids contain a viral LTR-driven, neomycin-resistance gene and the mouse methallothionein-1 promoter, inducible by heavy metals such as CdCl2 or $ZnSO_4$ [20], which controls the expression of the protein kinase subunit cDNAs [12].

Transfection and selection of stable cell lines

MCF-7 cells (10^6 cells per 60-mm dish) were transfected with 10 µg of retroviral vector constructs containing wild-type or mutant RI_α or wild-type RII_β or control plasmid OT1521 by calcium phosphate precipitation [21]. Stably transfected cells were selected by growing cells in the presence of 200–400 µg/ml G418. The pools of transfectants were separated into individual clones by dilution culture.

RNA preparation and Northern blot analysis

Total cellular RNA preparation, Northern blot analysis, and hybridization of RNA with the ^{32}P-labeled human RI_α, RII_β, and C_α probes were as described [22].

Photoaffinity labeling and immunoprecipitation of R subunits

Exponentially growing cells were treated with 130 μM $ZnSO_4$ for 24 h. Cell extract preparation and photoaffinity labeling with 8-N_3-[^{32}P]cAMP and immunoprecipitation of R subunits were as described [23].

DEAE-cellulose chromatography and protein kinase assay

Exponentially growing cells were treated with 130 μM $ZnSO_4$ for 24 h. Cell extract preparation, DEAE-cellulose chromatography, and protein kinase assay were as described [24].

CRE-dependent gene expression

Transfection and CAT assay were carried out as described [12].

Monolayer growth

2.0×10^4 cells were seeded onto a 6-well plate at day minus 1. On day 0 medium was removed and new medium containing 130 μM $ZnSO_4$ was added. Cells were cultured for 5 days. At days 1, 3, and 5, cells were counted (in duplicate) by Coulter counter.

Soft agar growth

5.0×10^4 cells were seeded in 1 ml of 0.3% Difco Noble agar in culture medium containing 130 μM $ZnSO_4$ This suspension was layered over 1 ml of 0.8% agar-medium base layer in a 35-mm diameter dish, and after 7 days cells were stained with nitro blue tetrazolium overnight. Colonies larger than 0.1, 0.2, 0.35 mm were counted with an Artek 880 colony counter.

Preparation of antisera specific for RI_α, RII_α, and RII_β

To produce polyclonal antibodies specific for each R subunit, the amino terminal parts of cDNAs of RI_α, RII_α, and RII_β (bases 15–222 in RI_α [17], bases 36–397 in RII_α [16], and bases 33–355 in RII_β [15]), which are the most divergent regions in these genes, were inserted into pGEX-2T or pGEX-3X vector (Pharmacia Biotech, Piscataway, NJ, USA), and *E. coli* JM 109 cells were transformed with these plasmids. The glutathione-S-transferase fusion proteins overexpressed in *E. coli* were then resolved by SDS-polyacrylamide gel electrophoresis, and each fusion protein band was cut in the gel and electroeluted. Eluted proteins were injected into rabbits several times. After bleeding, bloods were clotted at 37°C for 1 h and centrifuged at 10,000 g for 10 min. Supernatants were used as antisera.

Cell cycle analysis

Cells (1×10^6) were seeded onto 10-cm diameter dishes and cultured until cells were confluent. Confluent cells were harvested and reseeded at 1×10^6 cells/plate. After cells attached to plate (0 h), 130 μM $ZnSO_4$ was added and cells were grown for 18 h, then harvested, and fixed with ice-cold 70% ethanol. Fixed cells were treated with RNase (1 mg/ml) at room temperature for 30 min and stained with 10 μg/ml of propidium iodide (Sigma, St. Louis, MO, USA) for flow cytometric analysis (FACScan, Becton Dickinson, San Jose, CA, USA). The data were analyzed with the Modifit software (Becton Dickinson) using the method of Dean [25]. More than 90% of confluent cells accumulated into G_0G_1 phase.

Apoptosis assay

To assay nuclear morphology (apoptotic nuclei), cells were washed with PBS, fixed with 70% ethanol for 1 h, and stained with 1 mm Hoechst 33258 (Sigma) for 30 min [26]. The nuclear morphology of cells was visualized by a fluorescence microscope (Olympus BH2).

Results

Overexpression of R subunits of PKA

We transfected MCF-7 cells with the amphotrophic recombinant retrovirus containing human RI_α, RI_α-p, RII_β, or control plasmid (OT1521) [20]. The vector constructs contained the neomycin phosphotransferase gene, which allows selection of transfectants by G418 resistance, and the mouse metallothionein-1 promoter, which permits the inducible expression of the inserted gene upon treatment with $CdCl_2$ or $ZnSO_4$ [12].

The individual clones were isolated from the pool of G418-resistant cells. The clones were treated with different concentrations of $ZnSO_4$ (50–150 μM) for 5–7 days and screened for overexpression of transfected gene by Northern analysis. Treatment with 130 μM $ZnSO_4$ for 6 days induced maximally the transfected R subunit genes and was not toxic to cells. Three high expressor clones were selected and pooled for the transfectants of RI_α and RI_α-p, and a single high expressor clone was selected from RII_β transfectants.

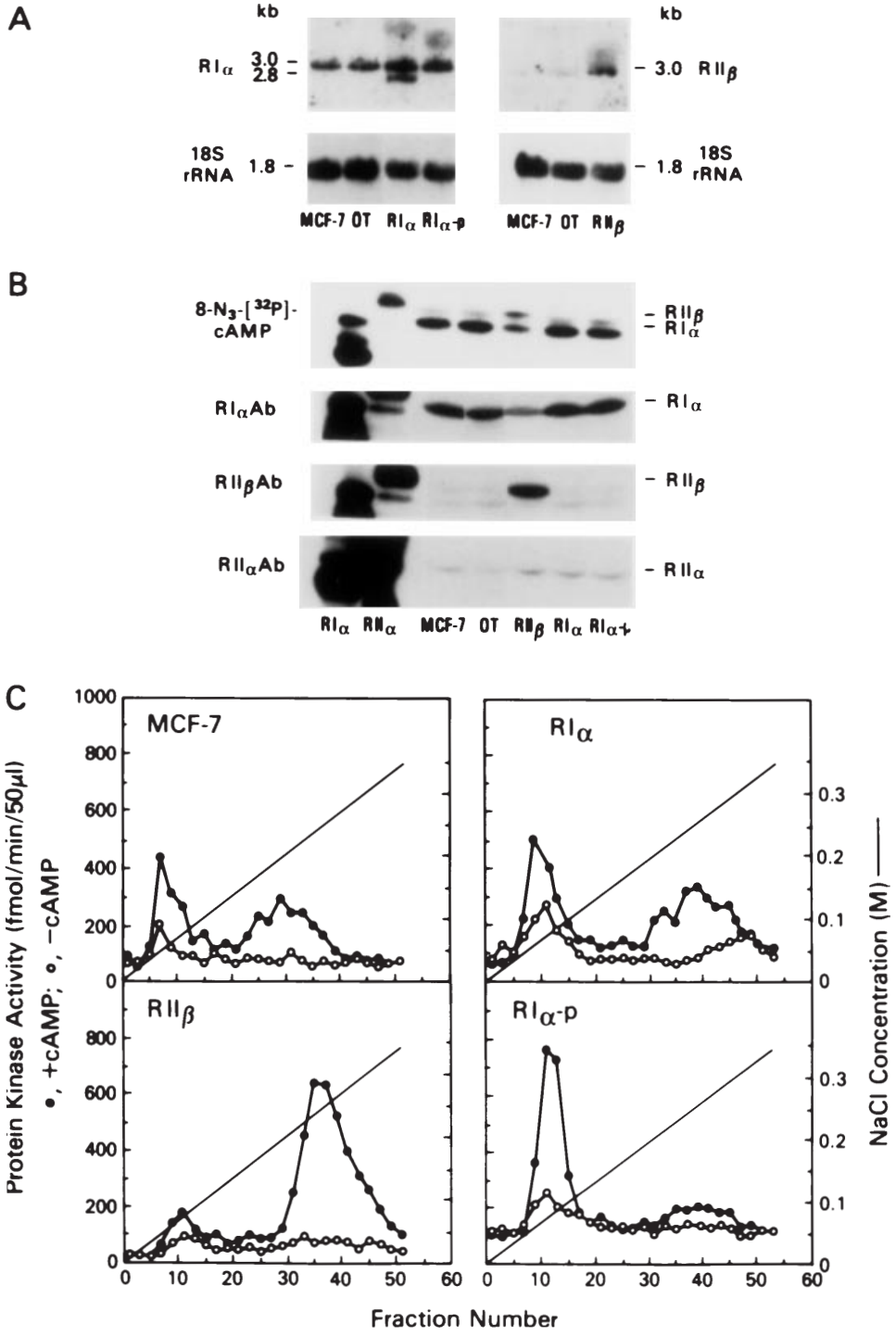


Figure 1. Overexpression of PKA subunits on protein kinase A isozyme distribution. (A) Northern analysis. RI_{α} (left panel) and RII_{β} (right panel) mRNA levels were measured by Northern blot analysis as described in 'Materials and Methods.' 18S rRNAwas used as an internal control. Lanes: MCF-7, parental cells; OT, RI_{α}, RI_{α}-p, and RII_{β} are transfectants of each vector construct. (B) Western analysis. Each R subunit protein level was measured by photoaffinity labeling with 8-N_3-[^{32}P]cAMP and immunoprecipitation followed by SDS-polyacrylamide gel electrophoresis as described in 'Materials and methods.' Lanes: RI_{α}, the M_r 48,000 RI from rabbit skeletal muscle (Sigma); RII_{α}, the M_r 56,000 RII from bovine heart (Sigma); other lane abbreviations are the same as (A). (C) DEAE-chromatography of PKA isozymes. DEAE-cellulose column chromatography and PKA assay were carried out as described in 'Materials and methods.' Kinase activities were determined in the absence (○) or presence (●) of 5 μM cAMP. Panel abbreviations are the same as (A). The data represent one of three independent experiments that showed similar results.

Figure 1A shows Northern blot analysis of the parental cells and transfectants. Densitomet tracing of autoradiographs showed that RI_α and RI_α-p transfectants (3 pooled clones) each exhibited a 2-fold increase in RI_α mRNA expression as compared with parental cells. RII_β transfectants (single clones) showed 5-fold increase in RII_β mRNA expression, as compared with parental cells. The mRNA levels of C_α did not change in any of the transfectants (data not shown).

The R subunit protein expression was determined by photoaffinity labeling with 8-N_3-[^{32}P]cAMP and immunoprecipitation with antibodies specific for each R subunit (Fig. 1B). Quantification by densitometric tracing of the photoaffinity labeled bands in the autoradiograms showed that the ratio of RI : RII in parental cells was 10 : 1.0. This RI : RII ratio did not change in cells transfected with control vector, OT1521 (vector without R subunit gene), RI_α, or RI_α-P transfectants. In RII_β transfectants, the RI : RII ratio was 1 : 1. Thus, overexpression of the RII_β gene markedly down-regulated RI_α protein expression. Parental MCF-7 cells contained low levels of RII_α, and the RII_α levels remained low in all transfectants.

Protein kinase A isozyme distribution in R subunit overexpressing cells

To examine the specific effects of R subunit overexpression on PKA isozyme distribution, we chromatographed cell extracts on DEAE-ion-exchange columns, and DEAE-bound materials were then eluted with a linear salt gradient [24] and assayed for PKA activity in the absence and presence of cAMP [24]. Chromatography separated two major peaks of PKA activity. PKA-I was eluted at 50–100 mM NACl and PKA-II was eluted at 150–250 mM NACl. Parental cells contained about equal amounts of PKA-I and PKA-II (Fig. 1C, MCF-7). The levels of PKA-I and PKA-II remained the same in OT1521 (vector without R subunit gene) transfectants (data not shown). Thus, the ratio of PKA-I : PKA-II (Fig. 1C) did not parallel the RI : RII protein ratio (10 : 1.0) (Fig. 1B). This indicates that the majority of RI_α did not form holoenzyme and that RII_α and RII_β had a stronger affinity for the C subunit than did RI_α in MCF-7 cells.

The RI_α transfectants increased both PKA-I and PKA-II levels 1.5-fold (Fig. 1C). Thus, the ratio of PKA-I : PKA-II in RI_α transfectants remained the same as that in parental cells. The RII_β transfectants increased PKA-II levels 3-fold and reduced PKA-I levels to 15% of parental cell levels (Fig. 1C). These data show that the wild-type RI_α overexpression did not suppress PKA-II holoenzyme formation, while the wild-type RII_β overexpression markedly suppressed PKA-I holoenzyme formation. These results indicate that RII_β had a stronger affinity for the C subunits than did RI_α, and are consistent with previous reports on ras-transformed NIH3T3 cells [27, 28] and LS-174T colon carcinoma cells [13] where overexpression of RII_α or RII_β eliminated PKA-I holoenzyme, while RI_α overexpression did not suppress PKA-II holoenzyme formation.

Importantly, in mutant RI_α-P transfectants, PKA-II levels were sharply reduced to 15% of parental cell levels and PKA-I levels increased 2-fold (Fig. 1C). Thus, unlike wild-type RI_α, the mutant RI_α-P overexpression led to marked down-regulation of PKA-II and an increase in PKA-I, resulting in changing the ratio of PKA-I to PKA-II. These data show that the introduction of the autophosphorylation site into RI_α increased its affinity for the C subunit above that of wild-type RI_α, RII_α, and RII_β.

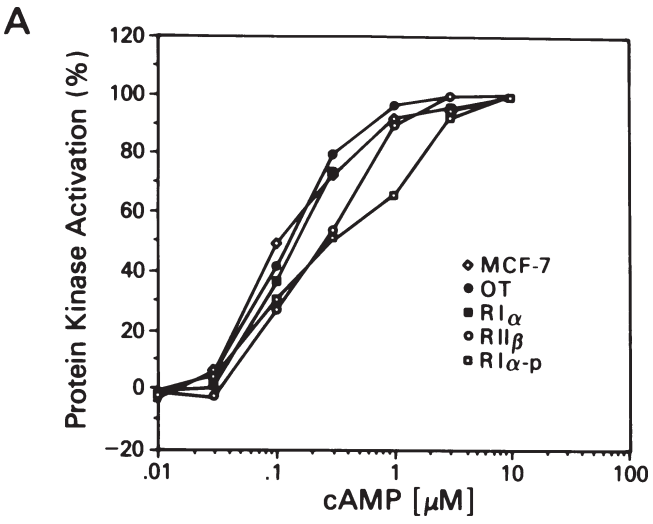
cAMP activation potency of protein kinase A in R subunit overexpressing cells

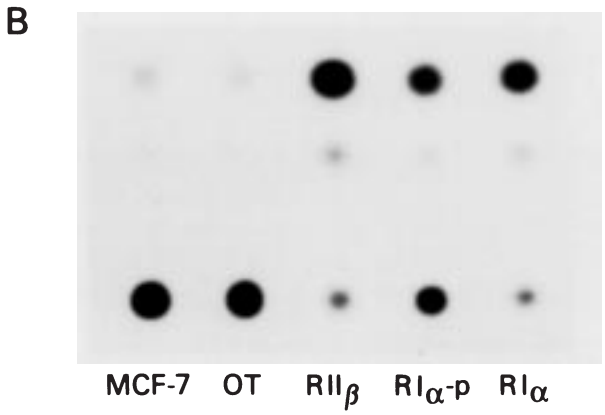
The above experiments demonstrated that the PKA R-subunit overexpression led to the changes in PKA isozyme patterns in the cell. We examined whether the changes in the isozyme distribution would bring changes in the cAMP activation potency of endogenous cellular PKA.

We measured the PKA activation in cell extracts with various concentrations of cAMP (Fig. 2A). In RI_α overexpressing cells, which contained PKA-I and PKA-II in an equal ratio (Fig. 1C) as in parental non-transfectants or OT (control vector) transfectants, the PKA activation constant for cAMP was very close to those parental or OT cells (K_a of cAMP: 0.102 µM for RI_α cells; 0.101 µM for OT cells; 0.095 µM for MCF-7 cells) (Fig. 2A). The PKA activation required a higher concentration of cAMP in RII_β overexpressing cells (K_α of cAMP: 0.230 µM) (Fig. 2A), in which PKA-II represents over 80% of total PKA (Fig. 1C). Most interestingly, the mutant RI_α-p overexpressing cells required the highest concentration of cAMP for PKA activation (K_a of cAMP: 0.290 µM) (Fig. 2A). The mutant RI_α-p, containing an autophosphorylation site, behaved like RII in its holoenzyme formation (Fig. 1C) and required the cAMP concentration even greater than RII for PKA activation.

These PKA activation experiments demonstrated that when cells contained a higher ratio of PKA-II to PKA-I, the PKA activation required higher concentrations of CAMP. However, experiments with cell extracts may contain artifacts of isozyme distributions, possibly due to dissociation and association of PKA holoenzymes during cell homogenization.

To avoid such a possibility we measured the PKA activation potency in intact cells by the use of transient transcription assays (Fig. 2B). The C subunit of PKA phosphorylates CRE binding protein (CREB), and the phosphorylated CREB enhances CRE-directed gene transcription (29). Measurements of CRE-directed gene transcription activity upon cAMP stimulation can be a measure of endogenous PKA





Forskolin

+	11	9	82	46	70
−	1.2	0.7	5.8	3.6	11

Percent Conversion

Figure 2. Mutant RI_{α}-p-overexpressing cells exhibit an increased affinity for the C subunit of PKA. (A) Cellular PKA activation by cAMP. PKA activation was measured by PKA assay (see 'Materials and methods') with various concentrations of cAMP using 30 μg proteins of cell extracts prepared from R subunit transfectants and 100 μM Kemptide. The activities stimulated by 10 μM cAMP minus the activities in the absence of cAMP were set as 100%. K_a of cAMP:MCF-7 cells – 0.095 μM; OT cells – 0.101 μM; RI_{α} cells – 0.102 μM; RII_{β} cells – 0.230 μM; RI_{α}-p cells – 0.290 μM. (B) Endogenous PKA activation measured by CRE-directed gene transcription. Upper panel is the CAT assay autoradiogram from forskolin-treated cells. Lower panel shows the percent conversion of [^{4}C]chloramphenicol into acetylated products in forskolin-treated or untreated cells. Abbreviations of symbols in (A) and lanes in (B) are the same as Fig. 1A. The data represent one of three independent experiments that showed similar results.

activation potency in intact cells. The R subunit transfectants and parental cells were transfected with the vector containing a fusion gene of CRE promoterchloramphenicol acetyl

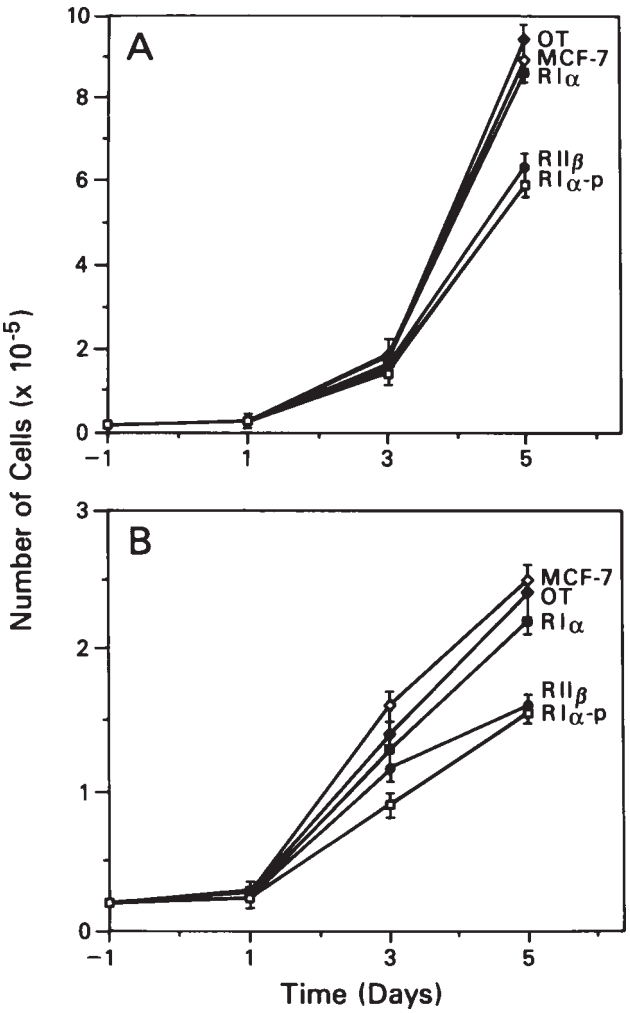


Figure 3. Mutant RI_{α}-p overexpressing cells exhibit retarded growth in monolayer culture. Cell growth was measured as described in 'Materials and methods' in medium containing regular serum (A) or phenol red-free medium containing dextran-coated charcoal-treated serum (B). Symbol abbreviations are the same as Fig. 1A. The data represent average values ± S.D. (n = 6) of three independent experiments.

transferase (CAT) and were treated with $ZnSO_4$ and forskolin. The basal CAT activity in the absence of forskolin was very low in both parental cells and transfectants (Fig. 2B). When these cells were treated with forskolin, the CAT activity increased dramatically. In the wild-type RI_{α} and RII_{β} cells, the CAT activity was 7- and 8-fold, respectively, that of parental cells in the presence of forskolin (Fig. 2B). In mutant RI_{α}-P transfectant, the CAT activity in the presence of forskolin was markedly reduced as compared to that in wild-type RI_{α} and RII_{β} transfectants. These results suggest that RI_{α}-p does not dissociate from its holoenzymes as readily as wild-type RI_{α} and RII_{β} in response to the intracellular cAMP stimulus.

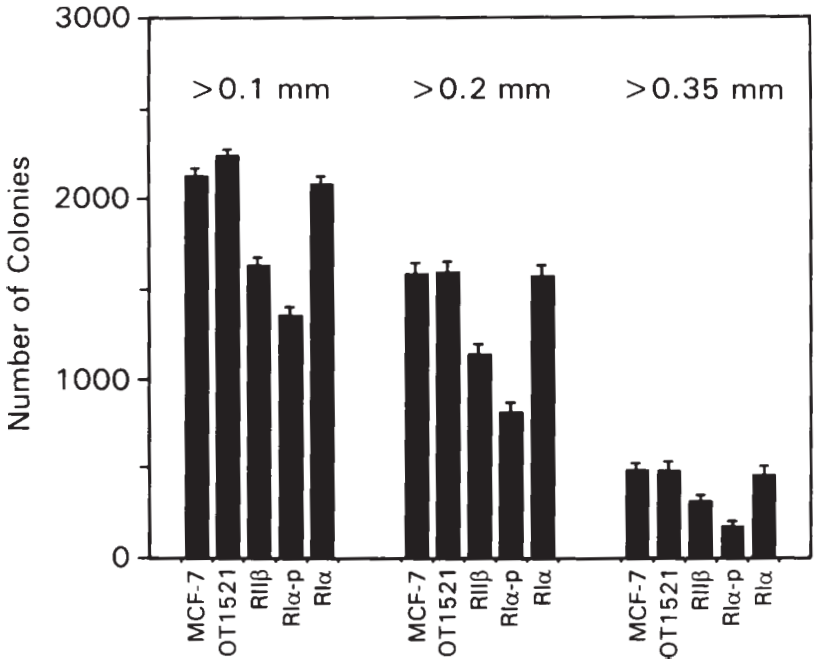


Figure 4. Mutant RI_α-p overexpression inhibits anchorage-independent growth. The soft agar growth (anchorage-independent growth) was measured in parental and R-subunit overexpressing cells as described in 'Materials and methods.' The data represent average values ± S.D. (n = 6) of three independent experiments.

Cell growth and PKA isozymes

We compared the growth properties of the PKA-R subunit transfectants with nontransfectant parental cells in monolayer culture containing the regular medium (Fig. 3A) or phenol red-free medium containing dextran-coated charcoal-treated serum (Fig. 3B). The RII_β cells, which showed 85% down-regulation of PKA-I, demonstrated a retarded growth of 40% on day 5 (Fig. 3). The RI_α-p cells, which produced the mutant RI_α containing the autophosphorylation site, showed a 45% growth inhibition on day 5 (Fig. 3). RI_α overexpression had no effect on cell growth (Fig. 3). Cell growth in soft agar produced similar results as that in monolayer growth (Fig. 4). These results show that either the reduction in wild type RI_α/PKA-I or the overexpression of the autophosphorylation mutant RI_α-p inhibited cell growth.

To further confirm these results, we treated MCF-7 cells with RI_α antisense oligodeoxynueleotide [18] and measured cell growth. As shown in Fig. 5, 10 µM RI_α antisense inhibited cell growth 40% at day 5, while control sense oligodeoxynucleotide had no effect, further supporting the role of RI_α in MCF-7 cell growth.

To obtain experimental evidence that the activation of PKA-I but not PKA-II is involved in MCF-7 cell growth, we used a cAMP analog, 8-Cl-cAMP, which selectively activates PKA-I isozyme [4, 24, 30]. DEAE-column chromatography experiments showed a marked down-regulation of PKA-I without an effect on PKA-II in MCF-7 cells treated with 5 µM 8-Cl-cAMP for 48 h (data not shown). Over 80% growth inhibition was observed in these cells. We examined whether growth inhibition observed in the above experiments accompanied changes in cell cycle phase distribution and apoptosis (programmed cell death).

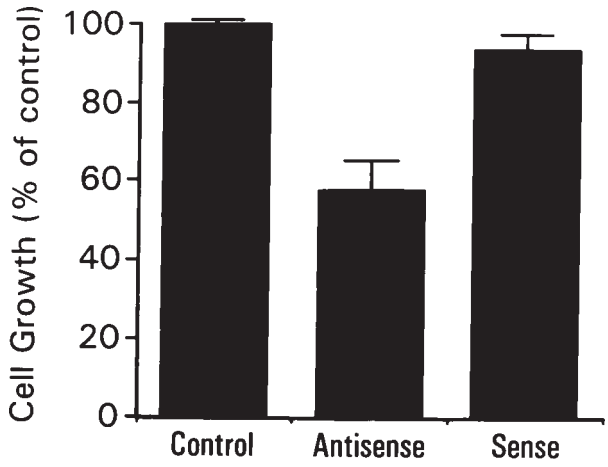


Figure 5. Growth inhibition by RI_α antisense oligonucleotide. Cells were treated with RI_α antisense [18] or control sense oligonucleotide (10 µM for 5 days) and cell growth was measured (see 'Materials and methods'). The data are expressed as percentage of control cells (cells treated with saline) and represent average values ± S.D. (n = 6) of three independent experiments.

Table 1. Cell cycle kinetics analysis of RI_a and RI_a-p transfectants and parental MCF-7 cells

	cell cycle(%)		
Cells	G_0/G_1	S	G_2/M
MCF-7	44.27 ± 6.07	42.06 ± 2.88	13.68 ± 6.65
MCF-7 OT	44.27 ± 6.64	40.39 ± 3.51	15.35 ± 5.87
MCF-7 RI_α	39.29 ± 4.37	39.64 ± 7.21	21.07 ± 3.37
MCF-7 RI_α-p	51.30 ± 8.29[a]	34.70 ± 4.87	14.00 ± 3.48

Cells were grown for 18 h in the presence of 130 µM $ZnSO_4$, harvested, treated with RNase and stained with 10 µg/ml of propidium iodine for flow cytometric analysis. Data represent mean ± S.D. obtained from four separate experiments. [a]P < 0.0 1 vs. MCF-7, MCF-7 OT, and MCF-7 RI_α.

Table 1 shows that the RI_α-p transfectants exhibited an increase in the percentage of cells in G_0G_1 phase of the cell cycle as compared to the wild-type RI_α transfectants or the nontransfectant parental cells. Although accumulation of RI_α-p cells in the G_0G_1 phase was small, it was significant in comparison to control cells ($p < 0.01$).

The RI_α-p transfectants also exhibited apoptosis as evident from fragmented nuclei or condensed chromatin (Fig. 6). Apoptotic nuclei were also observed in RII_β overexpressing cells, whereas wild-type RI_α overexpressing cells or parental non-transfectants exhibited no apopotosis (Fig. 6).

Discussion

We have shown in the present study that the growth of hormone-dependent breast cancer cells is dependent on the presence of one isoform of cAMP-dependent protein kinase, the type I protein kinase (PKA-I). The cell growth was experimentally inhibited by (i) depletion of PKA-I by overexpression of the other isoform of PKA, PKA-II, through overexpressing its regulatory subunit, RII_β, and (ii) overexpression of a functionally abnormal PKA-I containing the mutant RI_α-p, which behaved similarly to PKA-II in its activation by cAMP and holoenzyme formation, thus functionally abrogating the PKA-I.

To study the functional significance of RI_α on the growth of MCF-7 cells, we constructed a mutant RI_α, RI_α-p, by replacing the pseudophosphorylation site, Ala^{99} of human RI_α, with Ser. Our results show that RI_α-p overexpressed in the cell had a greater affinity for the C subunit of PKA than the wild-type RI_α resulting in increased formation of holoenzyme and exhibited a reduced activation by cAMP (Fig. 2). The RI_α-p overexpressing cells required a higher concentration of cAMP for activation of the endogenous PKA than did cells overexpressing the wild-type RI_α or RII_β (Fig. 2). Furthermore, we showed that the mutant RI_α-p overexpressing cells almost totally down-regulated PKA-II, whereas wild-type RI_α overexpression did not down-regulate PKA-II. As both RI_α- and RI_α-p-overexpressing cells up-regulated RI_α/PKA-I to a similar degree, the ability of RI_α-p overexpression to down-regulate PKA-II was not due to its greater overexpression (Fig. 1).

The ability of mutant RI_α-p to down-regulate PKA-II in intact cells was most striking because such ability cannot be observed with wild-type RI_α (Fig. 1C) as was shown previously [13, 27, 28]. This was also an unexpected finding in consideration of the *in vitro* behavior of the same RI_α mutant previously reported by Durgerian and Taylor [31]. These investigators showed by the use of purified preparations that

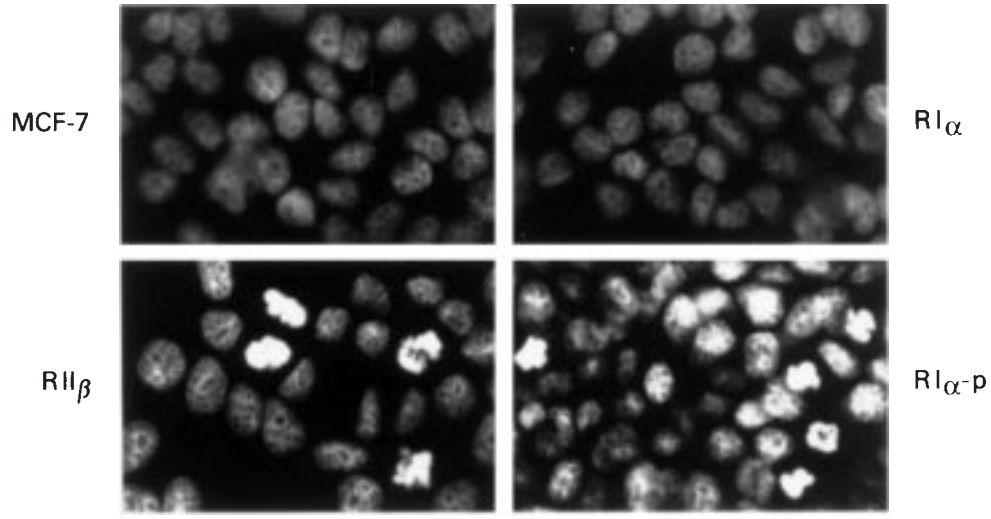


Figure 6. Apoptosis in PKA R subunit transfectants. Parental nontransfectants and R subunit transfectants were grown in the presence of 130 µM $ZnSO_4$ for 5 days as described in 'Materials and methods,' then apoptotic nuclei were assayed as described in 'Materials and methods.' Nuclear morphology (x2000).

introduction of the autophosphorylation site into RI_α results in much slower holoenzyme formation than in the wild-type RI_α in the presence of MGATP *in vitro.* However, these investigators did not examine the effect of overexpression of the mutant RI_α in holoenzyme formation of its own (PKA-I) or of the isozyme, PKA-II, in intact cells.

The mutant RI_α-p when overexpressed in intact cells may have interfered or competed with RII for PKA-II holoenzyme formation, probably due to its stronger affinity for the C subunit than RII (Fig. 2), resulting in down-regulation of PKA-II.

The RI_α-p overexpressing cells exhibited reduced cell growth. The functional abnormality of the mutant RI_α-p was clearly demonstrated in the cell cycle kinetics analysis. The RI_α-p transfectants exhibited an increase in the percentage of cells in G_0G_1 phase of the cell cycle as compared to the wild-type RI_α transfectants or the nontransfectant parental cells (Table 1). This increase in G_0G_1 phase in the cell cycle distribution in RI_α-p transfectants may be attributed to increased affinity of RI_α-p to the C subunit, resulting in an increased formation of holoenzyme and decreased activation by cellular cAMP as compared to the wild-type RI_α. These results suggest that RI_α may regulate the entry of cells from G_0G_1 phase to the S phase of the cell cycle in MCF-7 breast cancer cells. The role of RI_α in cell cycle progression has been shown previously in normal human mammary epithelial cell line, MCF-10A [32]. The retroviral vector-driven overexpression of RI_α but not of RII_β or C_α subunit of PKA enabled MCF-10A cells to grow in serum-free medium, and RI_α antisense treatment led to growth arrest of proliferating MCF-10A cells accompanying G_1 arrest of the cell cycle [32]. A role for RI_α in MCF-7 cell growth is further supported by RI_α antisense experiments (present study; [33]). RI_α antisense, which specifically depleted RI_α expression, caused growth inhibition, changes in cell morphology and induction of apoptosis in MCF-7 cells.

Our present finding that both RII_β-overexpressing cells (RI/PKA-I down-regulation) and RI_α-p-overexpressing cells (abnormal PKA-I expression) induce apoptosis suggests a role for RI_α in the survival of MCF-7 cells. Importantly, expression of the mutant RI_α-p, which acquired an autophosphorylation site and thus behaved like RII, inhibits cell growth and induces apoptosis in cells expressing endogenous wild-type RI_α. This dominant activity of RI_α-p may be due to an ability to trap wild-type RI_α in inactive dimers.

We conclude that RI_α subunit of PKA is essential for the optimal growth and survival of MCF-7 hormone-dependent breast cancer cells as depletion of the wild-type RI_α or overexpression of the functionally abrogated mutant RI_α led to retarded cell growth/apoptosis. Elucidation of the exact mechanism by which RI_α regulates the growth of hormone-dependent breast cancer cells awaits further studies.

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