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# Point mutation of the autophosphorylation site or in the nuclear location signal causes protein kinase A $RII_\beta$ regulatory subunit to lose its ability to revert transformed fibroblasts

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**ABSTRACT** **The $RII_\beta$ regulatory subunit of cAMP-dependent protein kinase (PKA) contains an autophosphorylation site and a nuclear location signal, KKRK. We approached the structure–function analysis of $RII_\beta$ by using site-directed mutagenesis. $Ser^{114}$ (the autophosphorylation site) of human $RII_\beta$ was replaced with Ala ($RII_\beta$-P) or $Arg^{264}$ of KKRK was replaced with Met ($RII_\beta$-K). *ras*-transformed NIH 3T3 (DT) cells were transfected with expression vectors for $RII_\beta$, $RII_\beta$-P, and $RII_\beta$-K, and the effects on PKA isozyme distribution and transformation properties were analyzed. DT cells contained PKA-I and PKA-II isozymes in a 1:2 ratio. Overexpression of wild-type or mutant $RII_\beta$ resulted in an increase in PKA-II and the elimination of PKA-I. Only wild-type $RII_\beta$ cells demonstrated inhibition of both anchorage-dependent and -independent growth and phenotypic change. The growth inhibitory effect of $RII_\beta$ overexpression was not due to suppression of *ras* expression but was correlated with nuclear accumulation of $RII_\beta$. DT cells demonstrated growth inhibition and phenotypic change upon treatment with 8-Cl-cAMP. $RII_\beta$-P or $RII_\beta$-K cells failed to respond to 8-Cl-cAMP. These data suggest that autophosphorylation and nuclear location signal sequences are integral parts of the growth regulatory mechanism of $RII_\beta$.**

The structure of mammalian cAMP-dependent protein kinase (PKA) has been studied in considerable detail (1, 2). Two isoforms of PKA, termed PKA-I and PKA-II, are distinguished by their regulatory (R) subunits, RI and RII, respectively, that interact with an identical catalytic (C) subunit (3, 4). Through biochemical studies and gene cloning, four isoforms of the R subunits, $RI_\alpha$, $RI_\beta$, $RII_\alpha$, and $RII_\beta$, have been identified (5). Three isoforms of the C subunit, $C_\alpha$, $C_\beta$, and $C_\gamma$, have also been discovered (6, 7). However, preferential coexpression of any of these C subunits with the PKA-I or PKA-II R subunit has not been found (6, 7). RI and RII differ significantly in the N terminus at a proteolytically sensitive hinge region that occupies the catalytic domain of the C subunit in the holoenzyme complex (8). In this segment, RII contains the sequence Arg-Arg-Xaa-Ser (9), which can undergo autophosphorylation at Ser (10), whereas RI contains the sequence Arg-Arg-Xaa-Ala (11), which cannot be autophosphorylated but participates in the high-affinity binding of ATP in the PKA-I holoenzyme. Another distinction between RI and RII is that RII but not RI at C terminus contains four positively charged amino acids, positions 243–246, KKRK (12). Based on simian virus 40 large tumor antigen studies (13), the controlled exposure of this sequence might provide a signal for regulating the transport of the protein into the nucleus.

RI/PKA-I is preferentially expressed in transformed cells or during early stages of ontogenesis, whereas expression of RII/PKA-II is correlated with arrest of cell growth and differentiation (14–16). It has been shown (15, 17–21) that down-regulation of $RI_\alpha$ and the compensatory increase in $RII_\beta$ obtained by treatment with site-selective cAMP analogs such as 8-Cl-cAMP or with an antisense oligodeoxynucleotide targeted against the $RI_\alpha$ mRNA lead to arrest of cancer cell growth and induction of differentiation. Moreover, retroviral-vector-mediated overexpression of the $RII_\beta$ subunit induced differentiation in human leukemia cells and reverted the transformed phenotype of mouse fibroblasts (22).

In the present study, we approached the structure–function analysis of $RII_\beta$ by the use of site-directed mutagenesis. Two mutants, one at the autophosphorylation site ($RII_\beta$-P) and the other at KKRK nuclear location signal ($RII_\beta$-K), were prepared. We then introduced an expression vector containing the wild-type or mutant $RII_\beta$ cDNA under the transcriptional control of an inducible promoter into Ki-*ras*-transformed NIH 3T3 (DT) cells and examined the effects on cell growth and morphology of the transformed cells.

## MATERIALS AND METHODS

**Materials.** 8-Cl-cAMP, sodium salt, was provided by K. P. Flora, National Cancer Institute (NCI), National Institutes of Health (Bethesda). 8-$N_3$-[$^{32}$P]cAMP (60 Ci/mmol; 1 Ci = 37 GBq) was from ICN Pharmaceuticals. DT cells were a gift from R. Bassin (NCI). The polyclonal anti-$RII_\beta$ antibody was kindly provided by S. D. Park (Seoul National University, Seoul, Korea). Protein A-Sepharose CL4B was purchased from Pharmacia LKB. The Y13-259 antibody was kindly provided by D. Lowy (NCI).

**Cell Cultures.** DT cells were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO), supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, penicillin (50 units/ml), streptomycin (500 μg/ml), 20 mM Hepes (pH 7.4), and 4 mM glutamine (ICN Biomedicals), in a humidified atmosphere of 95% air/5% $CO_2$ at 37°C.

**Mutagenesis.** The $RII_\beta$-P mutant was prepared by replacing the autophosphorylation site $Ser^{114}$ of human $RII_\beta$ with Ala by using the altered sites *in vitro* mutagenesis system (catalog no. Q6210, Promega). The following primer was used (T340 → G mutation underlined): 5′-TCACAAGGCGTGCCGCAG-TATGTGCAGAAGCTTATAATCC-3′ (nt 326–365). The $RII_\beta$-K mutant was prepared by replacing $Arg^{264}$ at KKRK sequence of human $RII_\beta$ with a Met by the method of recombination PCR (23). The following primers were used (G791 → T mutation underlined; region of overlap in primers A and B in boldface type): A, 5′-**CAATGCCAAAAAGATA-AAAATGTATG**AAAGCTTTATTGAG-3′ (nt 777–816); B,

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Abbreviations: PKA, cAMP-dependent protein kinase; R, regulatory; C, catalytic.

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5′-ATACATTTTTATCTTTTTGGCATTGTTTTT-CACAATTATT-3′. DNA sequencing analysis verified that no new mutations were introduced by PCR in mutants $RII_\beta$-P or $RII_\beta$-K.

**Construction of Retroviral Vectors.** The full-length cDNA encoding the human $RII_\beta$ subunit (24) (provided by T. Jahnsen, Rikshopitalet, Oslo) was introduced in the sense orientation into the retroviral vector OT1521 (25) (provided by M. L. Cutler, NCI) as described (26). Mutant $RII_\beta$-P and $RII_\beta$-K cDNAs were also introduced into OT1521. The resulting retroviral vector plasmids contain a viral long terminal repeat-driven neomycin-resistance gene and the mouse metallothionein-1 promoter, inducible by heavy metals such as $CdCl_2$ or $ZnSO_4$ (25), which controls the expression of the $RII_\beta$ cDNAs.

**Transfections and Production of Stable Lines.** DT cells ($10^6$ cells per 100-mm dish) were transfected with 10 $\mu$g of retroviral vector plasmid containing wild-type or mutant $RII_\beta$ or control plasmid OT1521 by calcium phosphate precipitation (27). At 48 hr after the transfection, the neomycin analog G418 was added to the medium, and the resistant colonies were isolated after 2–3 weeks of selection. The colonies were then examined for their expression of the $RII_\beta$ gene. Those that overexpressed the gene were pooled and used for the experiments.

**Photoaffinity Labeling Followed by Immunoprecipitation of R Subunits.** The amount of R subunits of PKA in cell lysates was determined by photoaffinity labeling with 8-$N_3$-[$^{32}$P]-cAMP followed by immunoprecipitation with the R antibodies as described (28). Protein concentration (usually 1–5 mg/ml) was determined by the method of Lowry *et al.* (29) with bovine serum albumin as standard.

**Preparation of Purified Nuclei.** All procedures were performed at 0–4°C. The cell pellets ($3 \times 10^7$ cells) after two washes with PBS were suspended in 2 ml of ice-cold isotonic lysis buffer [0.25 M sucrose/10 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$/0.5 mM EDTA/0.5 mM dithiothreitol/0.5 mM phenylmethylsulfonyl fluoride/1.0 mM benzamidine/leupeptin (30 $\mu$g/ml)/aprotinin (5.0 $\mu$g/ml)/pepstatin (5.0 $\mu$g/ml)] and equilibrated with $N_2$ at 600 psi in a Parr cell disruption chamber (Parr Instrument Co., Moline, IL) for 6 min. Rapid decompression produced a cell lysate, which was centrifuged at $900 \times g$ for 10 min. The pellets were gently suspended in lysis buffer and sedimented again as above. Crude nuclear fractions were resuspended in lysis buffer, layered on lysis buffer containing 2.0 M sucrose (30), and centrifuged at $120{,}000 \times g$ in a swing-out rotor for 1 hr. The resulting pellets of purified nuclei were washed twice in lysis buffer, suspended in the same buffer, and stored at −70°C.

**RNA Preparation and Northern Blot Analysis.** Total cellular RNA preparation, Northern blot analysis, and hybridization of RNA with the $^{32}$P-labeled human $RII_\beta$ (24) and $C_\alpha$ (31) probes were as described (22).

## RESULTS

**Overexpression of Wild-Type and Mutant $RII_\beta$ Subunits of PKA.** We identified the colonies that overexpressed the $RII_\beta$ gene by analyzing mRNA levels. Northern blot analysis showed expression in pooled colonies of DT-$RII_\beta$, DT-$RII_\beta$-P, or DT-$RII_\beta$-K cells, but not in DT cells, of the 4.4-kb $RII_\beta$ mRNA that hybridized with the human $RII_\beta$ cDNA (Fig. 1). Photoaffinity labeling with 8-$N_3$-[$^{32}$P]cAMP demonstrated an increased expression of RII protein with a decrease in RI protein in DT-$RII_\beta$ cells compared to the parental DT control cells (8-Cl-cAMP untreated) (Fig. 2). In mutants, $RII_\beta$-P- and $RII_\beta$-K-overexpressing cells, RII expression was much greater than in wild-type $RII_\beta$-overexpressing cells. DT cells after treatment with 8-Cl-cAMP exhibited an increased ratio of RII/RI as did the wild-type and mutant $RII_\beta$-overexpressing

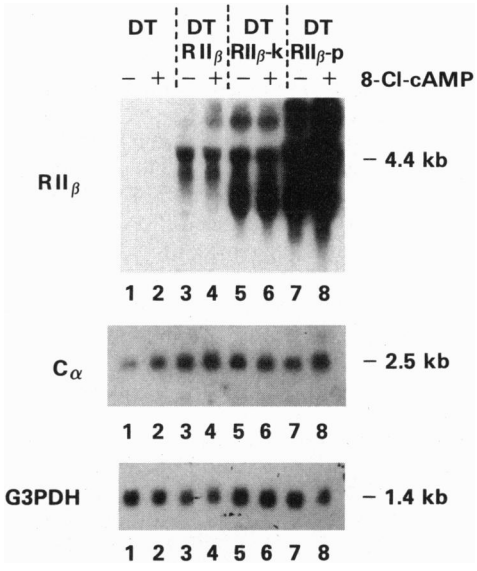


FIG. 1. $RII_\beta$ mRNA expression in wild-type and mutant $RII_\beta$-transfected Ki-*ras*-transformed NIH 3T3 cells (DT). Total RNAs isolated from untransfected control (DT) and wild-type or mutant $RII_\beta$ retroviral-vector-transfected (DT-$RII_\beta$, DT-$RII_\beta$-P, or DT-$RII_\beta$-K) cells treated with $ZnSO_4$ (60 $\mu$M) in the absence or presence of 8-Cl-cAMP (5 $\mu$M) for 48 hr were subjected to Northern blot analysis with $^{32}$P-labeled cDNA probes as indicated. Data represent one of three experiments that gave similar results.

DT cells, but the RII species that increased was the murine species of $RII_\beta$, which has a lower molecular mass (51–52 kDa) (32) than the human $RII_\beta$ (52–53 kDa) (33, 34). Photoaffinity labeling followed by immunoprecipitation with a polyclonal antibody raised against the human $RII_\beta$ that specifically cross-reacts with both human and mouse $RII_\beta$ identified expression of human $RII_\beta$ in DT-$RII_\beta$ cells, but not in DT cells (22). In DT cells, only mouse $RII_\beta$ was identified (22). Both human and mouse $RII_\beta$ elicited an enhanced expression upon treatment of these cells with 8-Cl-cAMP (22). At 60 $\mu$M $Zn^{2+}$, the expression of $RII_\beta$ protein reached its maximum in both wild-type and mutant $RII_\beta$ transfectants (data not shown). Thus, 60 $\mu$M $ZnSO_4$ was used throughout the experiments to stimulate $RII_\beta$ expression. $ZnSO_4$ at 60 $\mu$M was not toxic, but 75 $\mu$M $ZnSO_4$ was slightly toxic based on cell growth.

**PKA Isozyme Activity in Wild-Type and Mutant $RII_\beta$-Overexpressing Cells.** To study the specific effects of wild-type and mutant $RII_\beta$ overexpression on both PKA isozymes, we chromatographed cell extracts on DEAE ion-exchange columns and then material was eluted with a linear salt gradient

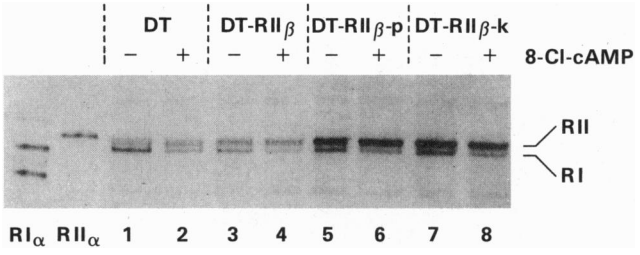


FIG. 2. $RII_\beta$ protein expression in wild-type and mutant-$RII_\beta$-transfected Ki-*ras*-transformed NIH 3T3 cells (DT). Cells, DT, untransfected control, and transfected with wild-type or mutant $RII_\beta$ retroviral vector, were grown in the presence of 60 $\mu$M $ZnSO_4$ and in the absence or presence of 5 $\mu$M 8-Cl-cAMP for 48 hr before harvesting. Cell extracts were photoaffinity labeled with 8-$N_3$-[$^{32}$P]cAMP. Lanes: $RI_\alpha$, 48-kDa RI from rabbit skeletal muscle (Sigma); $RII_\alpha$, 56-kDa RII (Sigma). The data represent one of three experiments that gave similar results.

(33) and assayed for PKA activity (33) and cAMP-binding activity (18). Chromatography separated two major peaks of PKA activity that coincided with peaks of cAMP-binding activity (data not shown). PKA-I was eluted between 40 and 80 mM NaCl, and PKA-II was eluted between 190 and 270 mM NaCl. In addition, two major species of cAMP-binding peaks with no PKA activity were eluted at 130 and 300 mM NaCl. Parental cells contained 2-fold more PKA-II than PKA-I. The $RII_\beta$ transfectants markedly reduced PKA-I levels to <5% of parental cell levels and increased PKA-II levels 3-fold. In $RII_\beta$-P or $RII_\beta$-K transfectants, PKA-I was completely eliminated, and PKA-II increased 3- to 4-fold over parental cell levels. Thus, $RII_\beta$, $RII_\beta$-P, or $RII_\beta$-K overexpression led to marked reduction or complete elimination of PKA-I and an increase of PKA-II, resulting in changing the ratio of PKA-I to PKA-II.

**Autophosphorylation of $RII_\beta$.** RII subunit of PKA is characterized by its autophosphorylation site at the RII–C subunit interaction site (9). Mutant $RII_\beta$-P has no autophosphorylation site due to the $Ser^{114} \rightarrow$ Ala substitution. As shown in Fig. 3, when cell lysates from $RII_\beta$-P-overexpressing cells were incubated in the presence of ATP at 4°C for 10 min followed by 8-$N_3$-[$^{32}$P]cAMP labeling and SDS/PAGE, no shift in the $RII_\beta$ band mobility was observed. Under the same conditions, wild-type $RII_\beta$ showed slower mobility after incubation with ATP.

**Nuclear Localization of $RII_\beta$.** Nuclear translocation of $RII_\beta$/PKA-II has been described as a mechanism for inducing the growth arrest of cancer cells treated with a site-selective cAMP analog, 8-Cl-cAMP (15, 19). Purified nuclei isolated from parental cells and transfectants were analyzed for the $RII_\beta$ content by photoaffinity labeling with 8-$N_3$-[$^{32}$P]cAMP and immunoprecipitation with monospecific anti-$RII_\beta$ antibody and SDS/PAGE. Fig. 4 shows that only in $RII_\beta$ cells was a substantial amount of $RII_\beta$ detected in the nuclei. Treatment of these cells with 8-Cl-cAMP (20 $\mu$M for 30 min) did not alter the amounts of $RII_\beta$ in the nuclei of parental cells or transfectants (Fig. 4). The large accumulation of $RII_\beta$ found in the nuclei of $RII_\beta$ cells is not likely due to the contamination by cytoplasmic or plasma membrane fractions. Specifically, the activities of lactic dehydrogenase and $Na^+$,$K^+$-ATPase were found to be at very low or undetectable levels of 0.05–0.08% and 2.0–3.9%, respectively, of the total homogenate activities (data not shown). The specificity of $RII_\beta$ accumulation in the nuclei of wild-type $RII_\beta$ cells was further supported by the findings that the mutant $RII_\beta$-P or $RII_\beta$-K expression was much greater than that of wild-type $RII_\beta$ as was shown in the total cell extracts (Fig. 2), but no accumulation of mutant $RII_\beta$ occurred in the nuclei (Fig. 4).

**Growth in Monolayer Culture.** We compared the growth properties of the wild-type and mutant $RII_\beta$ transfectants with

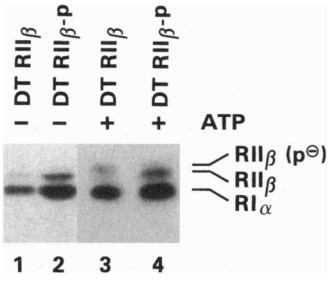


FIG. 3. Blockade of $RII_\beta$ autophosphorylation in $RII_\beta$-P-mutant-overexpressing cells. Autophosphorylation of $RII_\beta$ was performed in reaction mixtures containing 5 mM $MgCl_2$, 0.2 mM unlabeled ATP, 10 mM Tris·HCl (pH 7.1), and cell extracts that were photoaffinity labeled with 8-$N_3$-[$^{32}$P]cAMP. After incubation at 0°C for 10 min, the reaction was stopped and the R proteins were resolved by SDS/PAGE. $RII_\beta$ ($p^\ominus$), autophosphorylated $RII_\beta$.

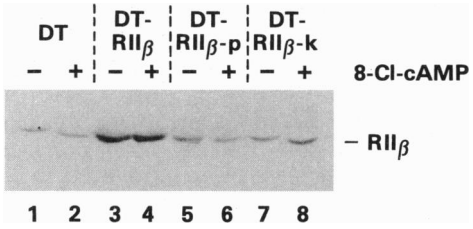


FIG. 4. Nuclear translocation of $RII_\beta$ protein in $RII_\beta$-overexpressing cells. Purified nuclei isolated from untransfected control (DT) and wild-type or mutant $RII_\beta$ retroviral-vector-transfected (DT-$RII_\beta$, DT-$RII_\beta$-P, or DT-$RII_\beta$-K) cells were treated with $ZnSO_4$ (60 $\mu$M) for 48 hr, untreated or treated for 30 min with 8-Cl-cAMP (20 $\mu$M), and subjected to photoaffinity labeling with 8-$N_3$-[$^{32}$P]cAMP, followed by immunoprecipitation with anti-$RII_\beta$ antibody and SDS/PAGE. The data represent one of three experiments that gave similar results.

nontransfectant parental cells. In monolayer culture of $RII_\beta$ transfectants growth was retarded 40% by day 6 compared with parental cells. $RII_\beta$-overexpressing cells responded to treatment with 8-Cl-cAMP (2.5 $\mu$M) by showing further growth retardation leading to growth arrest as did parental DT cells. In contrast, the $RII_\beta$ mutants, $RII_\beta$-P- and $RII_\beta$-K-overexpressing cells, grew at an even faster rate than parental control cells and exhibited an exponential growth rate in the presence of 8-Cl-cAMP.

**Soft Agar Growth.** Anchorage-independent growth has been widely used as one of the criteria to establish the transformed phenotype in cells. When we assayed the ability of these cells to grow in soft agar $RII_\beta$ cells showed >90% reduction in colony formation compared to the parental DT cells. These results indicate that in anchorage-independent conditions, the overexpression of the $RII_\beta$ subunit does not require further treatment with cAMP analog to cause arrest of cell growth. In contrast, $RII_\beta$-P- or $RII_\beta$-K-overexpressing cells and parental cells formed similar numbers of colonies. 8-Cl-cAMP (2.5 $\mu$M) treatment reduced the colony formation in $RII_\beta$ and parental DT cells but not in the mutant $RII_\beta$-overexpressing cells.

**Cell Morphology.** When cells were evaluated morphologically, only $RII_\beta$ cells (Fig. 5*C*) displayed a morphology different from that of parental DT cells (Fig. 5*A*). These cells with or without 8-Cl-cAMP treatment exhibited an enlarged cytoplasm and a flat phenotype resembling that of the formerly described flat revertants (Fig. 5 *C* and *D*) (35). After treatment with 8-Cl-cAMP (2.5 $\mu$M) for 5 days, parental DT cells exhibited a flat phenotype resembling $RII_\beta$ transfectants (Fig. 5*B*). In contrast, cells overexpressing $RII_\beta$-P (Fig. 5 *E* and *F*) or $RII_\beta$-K (Fig. 5 *G* and *H*) displayed the transformed parental cell phenotype regardless of 8-Cl-cAMP treatment.

***ras* Gene Expression.** We examined whether overexpression of $RII_\beta$ has a direct effect on the viral *ras* gene expression. As shown in Fig. 6, $RII_\beta$ cells contained ras mRNA levels that were 15-fold higher than that of parental cells. In contrast, ras mRNA levels in the mutant $RII_\beta$ ($RII_\beta$-P and $RII_\beta$-K)-overexpressing cells were similar to the parental cell levels. Western blot analysis with anti-p21 antibody Y13-259 (36) demonstrated that the viral p21 expression was markedly enhanced in the wild-type but not mutant $RII_\beta$ cells compared to parental cells (data not shown).

## DISCUSSION

We have transfected v-Ki-*ras*-transformed NIH 3T3 clone DT cells with a retroviral vector coding for the wild-type or mutant human $RII_\beta$ subunit of PKA. The mRNA expression determined by Northern blot analysis demonstrated an enhanced expression of human $RII_\beta$ gene in both the wild-type and the mutant $RII_\beta$ transfectants. Photoaffinity labeling with 8-$N_3$-

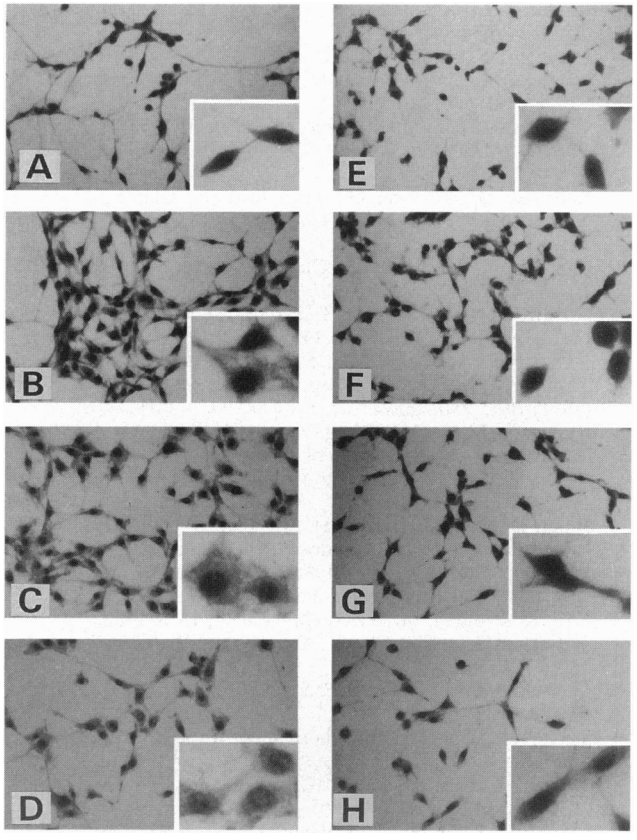


FIG. 5. Effect of wild-type and mutant $RII_\beta$ overexpression on the morphology of Ki-*ras*-transformed NIH 3T3 (DT) cells. Cells, untransfected control (DT) or transfected with wild-type $RII_\beta$, mutant $RII_\beta$-P, or $RII_\beta$-K retroviral vectors, were seeded at $1 \times 10^4$ cells per ml onto chamber tissue culture slides and were grown in the presence of 60 $\mu$M $ZnSO_4$ and in the absence or presence of 8-Cl-cAMP (5 $\mu$M) for 72 hr. Cells were fixed and stained with Wright's stain. (*A* and *B*) DT cells. (*C* and *D*) DT-$RII_\beta$ cells. (*E* and *F*) DT-$RII_\beta$-P cells. (*G* and *H*) DT-$RII_\beta$-K cells. (*B, D, F,* and *H*) +8-Cl-cAMP. (×50; *Insets*, ×150.)

[$^{32}$P]cAMP demonstrated an increase in $RII_\beta$ protein levels in these transfectants reflecting their enhanced expression of $RII_\beta$ mRNA.

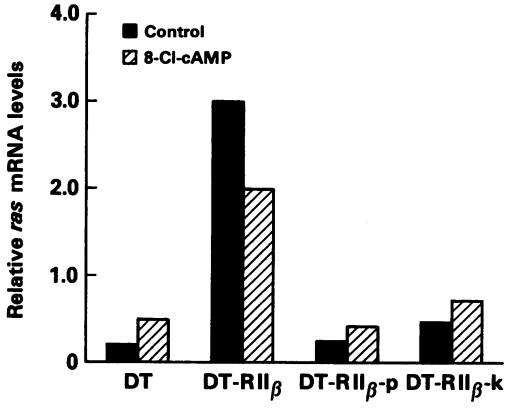


FIG. 6. Effect of wild-type or mutant $RII_\beta$ overexpression on ras mRNA levels. Total RNA isolated from untransfected control (DT) and wild-type or mutant $RII_\beta$-retroviral-vector-transfected (DT-$RII_\beta$, DT-$RII_\beta$-P, or DT-$RII_\beta$-K) cells treated with $ZnSO_4$ (60 $\mu$M) in the absence or presence of 8-Cl-cAMP (5 $\mu$M) for 48 hr was subjected to Northern blot analysis with a $^{32}$P-labeled cDNA probe of v-Ki-*ras* (0.62-kb fragment) (Clontech). The data represent quantification by densitometric tracings of autoradiographs and are average values of two experiments that gave similar results.

PKA-I isozyme is present in transformed but not untransformed NIH 3T3 cells (37, 38). In DT cells, PKA-I and PKA-II were present in a 1:2 ratio despite the fact that $RI_\alpha$ protein was the overwhelming R subunit species. This suggests that the C subunits are in equilibrium between PKA-I and PKA-II and that PKA-II formation is highly favored in DT cells. This preferential formation of PKA-II over PKA-I has also been observed in previous studies of PKA R subunit overexpression in R3T3 (*ras*-transformed NIH 3T3) cells (39) and AtT20 pituitary cells (39).

Overexpression of the wild-type and mutant $RII_\beta$ subunit resulted in a striking shift in PKA isozyme distribution. In wild-type and mutant $RII_\beta$-overexpressing cells, PKA-I was almost completely eliminated, and PKA-II was increased. Since we have introduced the human $RII_\beta$ gene into DT cells, the increase in PKA-II may represent PKA-$II_\beta$ ($RII_\beta$-containing PKA-II).

In the presence of low salt, PKA-II reassociates faster than PKA-I, and high salt favors the dissociation of PKA-I over PKA-II (40). MgATP prevents the dissociation of PKA-I but not of PKA-II (41). In addition, autophosphorylation of PKA-II favors its dissociation (42). In fact, it has been shown that an $Ala^{97} \rightarrow Ser$ replacement in the pseudophosphorylation site of bovine $RI_\alpha$ significantly reduces MgATP binding affinity for PKA-I holoenzyme (43). Conversely, replacement of the autophosphorylation site $Ser^{145}$ in the R subunit of yeast PKA with Ala significantly enhances the affinity of the R subunit for the C subunit (44).

To study the functional significance of the autophosphorylation site in $RII_\beta$, we constructed the mutant $RII_\beta$-P by a $Ser^{114} \rightarrow Ala$ replacement in the autophosphorylation site of human $RII_\beta$. We observed that in $RII_\beta$-P-overexpressing cells, the PKA-II peak was eluted at slightly higher salt concentration compared to wild-type $RII_\beta$-overexpressing cells. In addition, the $RII_\beta$-P transfectants were incapable of autophosphorylation *in vitro*. These effects of $RII_\beta$-P may be due to its ability to form highly stable holoenzyme, probably forming the ternary complex cAMP–$R_2C_2$–MgATP, and its high affinity for C subunit due to loss of autophosphorylation site.

In contrast to $RII_\beta$-P transfectants are the $RII_\beta$-K transfectants that contain a mutation at the KKRK nuclear location signal site. In $RII_\beta$-K-overexpressing cells, the PKA-II peak was eluted at slightly lower salt concentration compared to wild-type $RII_\beta$-overexpressing cells. These results suggest that the mutation may have caused a reduction in cAMP-binding ability of $RII_\beta$, thus forming a greater amount of the $RII_\beta$-containing PKA-II holoenzyme in the cAMP-unbound than in the cAMP-bound state. Differential elution profiles of DEAE chromatography of cAMP-unbound vs. cAMP-bound PKA-II have been obtained (45).

We compared growth and transformed properties of the wild-type and mutant $RII_\beta$ gene transfectants with parental control cells by using the following criteria: (*i*) growth in monolayer culture, (*ii*) anchorage-independent growth, and (*iii*) cell morphology. We found that by all criteria, the mutant $RII_\beta$-P- or $RII_\beta$-K-overexpressing cells and parental control cells behaved as transformed cells. In contrast, the $RII_\beta$ cells exhibited restrained growth in monolayer culture, limited capacity to form colonies in soft agar, and change in morphology compared with parental cells. Thus, $RII_\beta$ cells had the properties of untransformed rather than transformed cells. The parental cells responded to 8-Cl-cAMP treatment by exhibiting restrained growth and changes in morphology as did $RII_\beta$-overexpressing cells. In contrast, $RII_\beta$-mutant-overexpressing cells did not respond to 8-Cl-cAMP treatment, suggesting that the mutant $RII_\beta$ gene was dominant with respect to the endogenous mouse $RII_\beta$ gene in these cells.

We measured the *ras* expression to determine whether suppression of *ras* gene is involved in $RII_\beta$-induced reverse transformation, since DT cells are transformed by the *ras* gene.

The *ras* expression was markedly enhanced, rather than suppressed, in $RII_\beta$ cells. In contrast, little or no change in *ras* expression was found in mutant $RII_\beta$-overexpressing cells. Thus, an enhancement of *ras* expression was associated with $RII_\beta$ overexpression. Enhanced *ras* expression has been associated with cell differentiation (46). Whether the enhanced *ras* expression in $RII_\beta$ cells is causally related to or the result of the reverse transformation induced in $RII_\beta$ cells is not clear.

$RII_\beta$ cells markedly reduced $RI_\alpha$ and PKA-I isozyme levels; however, elimination of $RI_\alpha$/PKA-I alone may not be the mechanism that elicits untransformed properties in $RII_\beta$ cells, since both $RII_\beta$-P- and $RII_\beta$-K-overexpressing cells sharply down-regulated $RI_\alpha$/PKA-I but failed to show untransformed properties.

This laboratory has shown (26) that PKA-II is necessary for the transcription of the CRE-containing somatostatin gene and for the cAMP-induced phenotypic change in the A126 cell line, a mutant of PC12 pheochromocytoma that is deficient in PKA-II but contains the wild-type level of PKA-I. We hypothesize that the lack of nuclear catalytic activity of PKA could be ascribed to a lack of PKA-II. As $RI_\alpha$, $RII_\alpha$, and $RII_\beta$ subunits compete for a limited pool of the C subunit and $RII_\alpha$ remains constant by forming the most favored complex (47), the ratio of PKA-I/PKA-II would be regulated mainly by the changes in the amounts of $RI_\alpha$ and $RII_\beta$.

Strikingly, $RII_\beta$ overexpression accompanied nuclear accumulation of $RII_\beta$, supporting its functional significance as the mutated $RII_\beta$ molecules ($RII_\beta$-P or $RII_\beta$-K) were incapable of translocating into the nucleus. The mutant $RII_\beta$-P that lacks the autophosphorylation site but contains an intact nuclear location signal KKRK was incapable of nuclear translocation. This may suggest that either the phosphorylated form of $RII_\beta$ is necessary or the KKRK sequence alone is insufficient and requires additional sequences at the autophosphorylation site for the nuclear translocation.

Thus we have shown the functional importance of both the autophosphorylation site and nuclear location signal KKRK in $RII_\beta$, as mutants at either of these sites failed to mimic the effects of $RII_\beta$. The precise mechanism and the site of action of the $RII_\beta$ subunit in growth control and reverse transformation remain to be elucidated.


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