The v-Ki-Ras Oncogene Alters cAMP Nuclear Signaling by Regulating the Location and the Expression of cAMP-dependent Protein Kinase IIβ*

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The v-Ki-Ras oncprotein dedifferentiates thyroid cells and inhibits nuclear accumulation of the catalytic subunit of cAMP-dependent protein kinase. After activation of v-Ras or protein kinase C, the regulatory subunit of type II protein kinase A, RIIβ, translocates from the membranes to the cytosol. RIIβ mRNA and protein were eventually depleted. These effects were mimicked by expressing AKAP45, a truncated version of the RII anchor protein, AKAP75. Because AKAP45 lacks membrane targeting domains, it induces the translocation of PKAII to the cytoplasm. Expression of AKAP45 markedly decreased thyroglobulin mRNA levels and inhibited accumulation of C-PKA in the nucleus. Our results suggest that: 1) The localization of PKAII influences cAMP signaling to the nucleus; 2) Ras alters the localization and the expression of PKAII; 3) Translocation of PKAII to the cytoplasm reduces nuclear C-PKA accumulation, resulting in decreased expression of cAMP-dependent genes, including RIIβ, TSH receptor, and thyroglobulin. The loss of RIIβ permanently down-regulates thyroid-specific gene expression.

Ras is a small GTP binding protein that serves as a central molecular switch. Ras linked receptor tyrosine kinases with downstream signaling pathways that include Ser/Thr and dual specificity protein kinases (1, 2). Constitutive expression of activated Ras bypasses the transient, ligand-regulated activation of transmembrane receptor tyrosine kinases and tonically stimulates signaling molecules that in turn affect cell growth, proliferation, and differentiation. Depending on the cell type, Ras activation elicits differentiation (PC12 neuroendocrine cells) (24). RI has a higher affinity for cAMP than RIIα and RIIβ (25, 26) and also turns on more rapidly (27). The RII subunits in the PKAII holoenzyme are phosphorylated by the catalytic subunit (25). Taken together, these observations suggest that PKAII and PKAIIβ decode cAMP signals that differ in their duration and intended target.

We are investigating molecular mechanisms that underlie Ras-induced dedifferentiation of thyroid cells. Thyroid growth and differentiation are dependent on cAMP, which is generated by thyrotropin activation of adenylyl cyclase. The early steps in Ras-induced dedifferentiation have been examined in a thyroid cell line transformed with a temperature-sensitive variant of

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1 The abbreviations used are: PKA, cyclic AMP-dependent protein kinase; C-PKA, catalytic subunit of PKA; R, regulatory; PKC, protein kinase C; RSV, Rous sarcoma virus; NEO, neomycin; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PKI, PKA inhibitor peptide; PMA, phorbol 12-myristate 13-acetate; MTOC, microtubule organizing center.
Ki-Ras p21 (N cells, referred to previously as Ata-aza). N cells grown at 33 °C (N(33)) are dedifferentiated, whereas cells grown at 39 °C (N(39)) express thyroid-specific markers (28). Activation of the v-Ras oncogene is rapidly followed by stimulation of PKC (29, 30) and by inhibition of the accumulation of nuclear C-PKA after exposure to forskolin or 8-Br-cAMP (30). Exclusion of C-PKA from the nucleus is correlated with the loss of the transcriptionally active forms of the thyroid transcription factor, TTF1, and CREB (30). Conversely, C-PKA rescues inactive TTF1 present in nuclear extracts of Ras-transformed cells (31). Similarly, PKC depletion reverses inhibition of thyroglobulin promoter activity by Ras (32). Taken together, these observations suggest that Ras may repress thyroid-specific genes by blocking the accumulation of C-PKA in the nucleus.

In this manuscript we ask how Ras inhibits the nuclear localization of C-PKA. We show that Ras alters the composition of PKA isoenzymes by blocking the expression of the specific RIIβ isoform. We also demonstrate that the intracellular location of RII subunits (and PKAII) profoundly affects the nuclear accumulation of C-PKA and, consequently, cAMP-regulated thyroglobulin mRNA levels. We propose that the Ras-induced delocalization and the ultimate loss of the PKAIIβ isoenzyme explains the delocalization of Ras-transformed thyroid cells.

**MATERIALS AND METHODS**

**Cell lines, DNA Plasmids, and Transfections**—The TL cell line is derived from the FRTL-5 thyroid cell line, which has been extensively characterized with respect to thyroglobulin expression. It is TSH-dependent for growth. The Ras-transformed TL derivative lines are KM, which was transformed with wild type Ki-Ras virus, and the N derivative, which was described in Refs. 28 and 30.

AKAP45 and AKAP75 plasmids contain the AKAP coding region under the control of the cytomegalovirus promoter and the aminoglycoside transferase gene under the control of the long terminal repeats of Rous sarcoma virus. DNA transfections were carried out by the calcium phosphate procedure.

**RNA Analysis**—Total RNA was purified by homogenization in guanidium isothiocyanate and phenol-chloroform-isooamyl alcohol extraction (34). 20 μg of each RNA sample was electrophoresed on a 1% agarose gel, transferred to nitrocellulose filter (0.45 mm, Schleicher & Schuell), and hybridized with specific probes (see MATERIALS AND METHODS).

**DNA polymerase** was added after the first five cycles. 20 μl of the PCR products were resolved on four different agarose gels, blotted onto different nylon membranes (Amersham Corp.), and hybridized with specific probes (see above). Membranes were then exposed to a preflushed x-ray film (BetaMax, Amersham Corp.) for 2–12 h at ~80 °C; the intensity of hybridization was quantitated by densitometric analysis. Under these conditions the hybridization signal was linearly dependent on initial cDNA concentration (data not shown).

**DNA fragments** corresponding to the RIA, RIIα, and RIIβ were amplified with primers: RIA-F, 5′-GGCCGGGAGAGAGAGCACGC-3′, 5′ end at position +1; RIA-R, 5′-GGTTTGGGGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA...
tide substrate during a 10-min incubation in the presence (PKA holoenzym) or the absence (free C-PKA) of 10 μM cAMP. At the concentrations used, PKI did not inhibit the binding of phosphorylated kemptide to phosphocellulose filters.

**Immunoblot Analysis—Nuclear, cytosolic, or membrane proteins were resolved by SDS-PAGE (see above), transferred to nitrocellulose, rinsed in TBST (10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% Tween 20), incubated with 10% nonfat dry milk in TBST, and incubated with anti-C-PKA antibodies (see above) in 5% nonfat dry milk in TBST for 1 h. After washing (three times in TBST for 15 min), the nitrocellulose membranes were incubated with alkaline phosphatase-conjugated anti-rabbit IgG in 5% nonfat dry milk in TBST for 1 h and then developed (30).

**Immunofluorescence—**Cells were treated as described (see above), rinsed with PBS, and fixed with 3.7% formaldehyde in PBS for 30 min. After permeabilization with 0.2% Triton X-100 in PBS, the cells were incubated with 0.2% porcine skin gelatin in PBS for 1 h at 22 °C. Anti-PKA catalytic or regulatory subunit immunoreactivity was detected using the specific polyclonal antibody (see above) in PBS containing 0.2% gelatin for 45 min at 22 °C. Antigen was visualized with fluorescein-tagged or rhodamine-tagged goat anti-rabbit IgG (Techno-bodies) for 90 min. Coverslips were observed by a Zeiss Axiomat microscope.

**Photoaffinity Labeling with 8-Azido-[32P]cAMP—**Labeling with 8-azido-[32P]cAMP was performed by incubating cellular extracts (50 μg) or purified R subunits (1 ng/μl) with 1 μM 8-azido-[32P]cAMP (50 Ci/mM, 20 μCi/ICN) in 50 mM Tris-HCl, pH 7.4 for 1 h at 4 °C in the dark. This labeled cAMP (C-PKA). Covalent incorporation was accomplished by exposure of the reactions at 20 °C to UV light (254 nm) at a distance of 5 cm for 15 min.

**RESULTS**

**v-Ras Inhibits Nuclear Accumulation of C-PKA—**Inactive cytoplasmic PKA holoenzyme is dissociated by cAMP into R_cAMP and the catalytically active subunit, C-PKA. A fraction of the C-PKA migrates into the nucleus where it catalyzes the phosphorylation of specific substrates (e.g. CREB) (38). In v-Ras transformed thyroid cells (N33°) the nuclear accumulation of C-PKA is inhibited (30). This might be due to inefficient dissociation of the holoenzyme, inhibition of C-PKA translocation to the nucleus, and/or lack of retention of C-PKA or increased export from the nucleus. To distinguish among these possibilities, we analyzed PKA dissociation and C-PKA accumulation in the nuclei of control cells and of cells expressing active Ras. N33° or N39° cells were stimulated with forskolin for 40 min at 37 °C to increase intracellular cAMP levels. The nuclei and cytoplasm were then isolated, and the levels of holoenzyme and dissociated C-PKA were determined for each compartment. Fig. 1A shows that v-Ras did not prevent the dissociation of cytoplasmic PKA holoenzyme in response to cAMP. The ratio of C-PKA to total PKA increased in both N33° and N39° in response to forskolin. At 30 μM forskolin, the dissociation of holoenzyme and the accumulation of C-PKA in the cytoplasm was nearly complete. In contrast, the nuclear accumulation of C-PKA was blocked in N33° (Fig. 1B). This was also shown by Western immunoblot of isolated nuclear proteins (Fig. 1C). Note that nuclear C-PKA represents approximately 5–10% of total C-PKA (0.75 versus 7–12 pmol/μg protein). Thus, a large change of C-PKA in the nucleus can occur with a little alteration in cytoplasmic C-PKA content. At higher concentrations of cAMP (80 μM forskolin) some C-PKA accumulated in the nucleus of N33° (Fig. 1B), indicating that excess C-PKA generated by the massive dissociation of cytoplasmic PKA can partly overcome the block induced by the oncogene. To define further the mechanism by which v-Ras blocks nuclear accumulation of C-PKA, we isolated nuclei from N33° or N39°.

Confirming the experiments of Fig. 1, N33° cells treated with forskolin had little nuclear C-PKA compared with N39° cells (Fig. 2). Incubation of the nuclei from untreated cells for 30 min with cAMP, PKA holoenzyme and ATP, or purified C-PKA (not shown), led to the accumulation of C-PKA in both N33° and N39°.
nuclei (Fig. 2; for details see “Materials and Methods”). These results imply that the nuclear retention of exogenous C-PKA is not altered in Ras-transformed cells. Instead, activated Ras appears to inhibit a cytoplasmic reaction that is essential for the translocation of C-PKA to the nucleus. The accumulation of C-PKA in the nuclei of N33° in vitro but not in vivo probably reflects the very high concentrations of the subunit in the in vitro experiments (approximately 10–20-fold higher than endogenous C-PKA levels in vivo).

The possibility that v-Ras limits the storage capacity of nuclei for C-PKA was tested by following the accumulation of nuclear C-PKA at very short periods after cAMP stimulation. Reduced nuclear C-PKA levels in N33° compared with N39° was seen as early as 2–10 min after forskolin stimulation (data not shown). These data also indicate that the import rather than the retention of C-PKA is inhibited in v-Ras transformed cells. However, our data does not exclude the possibility that C-PKA is rapidly exported from the nuclei of v-Ras transformed cells.

v-Ras Selectively Modulates the Expression of the PKA RII\(\beta\) Subunit—Thyroid cells contain three PKA regulatory subunit isoforms: RIIa, RII\(\alpha\), and RII\(\beta\). R subunit homodimers bind two C-PKA monomers to generate tetrameric holoenzymes that are named according to the cAMP binding subunits. PKAI\(\alpha\) was separated from PKAII (a mixture of \(\alpha\) and \(\beta\)) by chromatography on DEAE cellulose and the amounts of type I and type II enzymes were quantified by enzymatic assays. PKAI\(\alpha\) concentrations increased by 50% in v-Ras transformed cells, whereas total PKAII content was unchanged by oncogene expression (Ref. 26 and data not shown). Because ion-exchange chromatography does not resolve PKAII\(\beta\) from PKAII\(\alpha\), fluctuations in RII isoform expression are not detected by this method of analysis.

**Fig. 2.** Isolated nuclei from Ras-transformed cells accumulate exogenous C-PKA. Nuclear C-PKA activity in N cells grown at 33 and 39 °C and in isolated nuclei from the same cells. The cells were stimulated 40 min at 37 °C with 50 \(\mu M\) forskolin, as described in the legend to Fig. 1. Nuclei were isolated as described under “Materials and Methods.” Nuclei (\(\sim\)10\(^6\)) were incubated 15 min with 0.5 \(\mu g\) of purified PKAII\(\alpha\) from rabbit muscle (specific activity, 10\(^{5}\) cpm/\(\mu g\)/pmol kemptide in the presence of 10\(^{2}\) M dBt-cAMP) in a buffer containing 1 mM Mg-ATP (see “Materials and Methods”). At the end of incubation, nuclei were washed twice with the same buffer containing 0.1% Triton X-100 and purified by sedimentation through a sucrose cushion. The enzyme activity shown was specifically inhibited by 10 \(\mu M\) of PKI. By immunofluorescence analysis C-PKA was detected inside the nuclei.

**Fig. 3.** Relative abundance of RII\(\beta\) and RII\(\alpha\) mRNAs in differentiated and Ras-transformed cells. Semiquantitative PCR of reverse-transcribed RNA isolated from the indicated cell lines was performed as described under “Materials and Methods.” After PCR amplification, the mixture of products was electrophoresed in an agarose gel, blotted, and hybridized with specific probes. Top left, a representative autoradiogram of the hybridization signals corresponding to RII\(\alpha\), RII\(\beta\), and RII\(\beta\). Comparable exposures were chosen and analyzed by densitometric scanning. The values obtained were normalized to RIIa content. Values reported on the top right, top left, and bottom left are the averages of at least three independent determinations. Cell lines are indicated for each column. No change in the relative ratios between mRNAs corresponding to the R subunits and reference genes was noted in total RNA extracted from TL or KM cells grown at 33 or 39 °C for 1 week (data not shown). Northern analysis of total RNA of the cell lines described was also performed with RII\(\alpha\), RII\(\beta\), and GADPH-specific probes. No significant changes were noted in the concentrations of these mRNAs (data not shown).
We therefore compared the mRNA levels of the R subunits in Ras-transformed and control cells. In endocrine tissues RIIα is the most abundant mRNA, followed by RIIα (10-fold less represented) and RIIβ mRNAs (5–10-fold less represented (39, 40). Because R subunit mRNA levels are of low abundance, we devised a sensitive method using reverse transcriptase PCR to detect changes in their relative ratios. Forward and reverse specific primers for RIIα, RIIα, RIIβ, and GADPH mRNAs were coamplified (10–15 cycles) with specific primers in the same test tube. The amplified bands were then separated by electrophoresis and hybridized with specific probes (see “Materials and Methods” for details). Under these conditions the hybridization signal was linearly dependent on initial cDNA concentration and the number of PCR cycles (data not shown). Fig. 3 shows the relative levels of mRNAs encoding RIIα, RIIα, and RIIβ subunits. Values are normalized to RIIα mRNA content. The RIIβ/RIIα ratio was 13-fold higher in differentiated (TL) cells than in the v-Ras transformed KM line and 16-fold higher in N39° than in N33°. The RIIβ/RIIα ratio decreased when N39° was shifted to 33 °C for 1 week. Inactivation of v-Ras increased the relative abundance of RIIβ mRNA. The changes in the mRNA ratios at 33 °C shown in Fig. 3 might reflect a decrease in RIIα mRNA, an increase in RI and RIIα mRNAs, or combinatorial effects. To discriminate among these possibilities, we performed a Northern analysis of total cellular RNA with RIIα- and RIIα-specific probes. No significant changes in the levels of RIIα and RIIα mRNAs were evident in transformed cells. In addition, the Northern analysis confirmed the PCR results (data not shown). Thus, Ras activation is associated with a substantial reduction in RIIβ mRNA content.

To determine if the reduction in RIIβ mRNA in Ras-transformed cells was associated with the loss of RIIβ protein, we measured RIIβ in total and fractionated cellular extracts by immunoprecipitation with anti-RIIβ-specific antibodies. RIIβ was present in differentiated TL cells but was undetectable in chronically Ras-transformed cells (Fig. 4, upper panel, compare TL and KM). Note that under the conditions used, the antibodies recognize RIIβ, but not RIIα (upper panel). RIIβ and RIIα proteins were measured in N39° and N33° (RIIβ and RIIα, upper and lower panel, respectively). In N39°, RIIβ was located predomina-

some area in TL and N39°. The signal was greatly reduced in KM or distributed diffusely in the cytosol in N33° (Fig. 5, b and d, respectively). N39° cells (Fig. 5e) that were treated for 1 h with PMA (220 ng/ml) (Fig. 5h) or shifted for 1 (Fig. 5f) or 4 (Fig. 5g) days to 33 °C showed an unusual RIIβ distribution. There was a dramatic change in the location of the RIIβ signal (cf. Fig. 5, e, f, and h), which moved from the Golgi-centrosome area to a narrow region around the nuclear envelope and later (after 3 days at 33 °C) diffused in the cytoplasm. With continued incubation at 33 °C, the cytoplasmic signal became increas-

ingly diffuse, and after 4 weeks the signal was reduced to the levels seen in KM cells (data not shown). When the cells were returned to 39 °C, RIIβ again accumulated in the Golgi-centro-

some region (Fig. 5, i and j). These effects were not due to the temperature shift per se, because TL cells did not redistribute the RIIβ-derived fluorescence signal under the same conditions (Fig. 5, k, l, and m). Activation of PKC mimicked the effects of v-Ras on RIIβ location, but the change was more rapid (1–3 h after treatment with PMA).

To characterize more precisely the subcellular compartment where RII accumulated, we performed indirect immunofluorescence of the Golgi apparatus and of the centrosome-located microtubule organizing center (MTOC), using anti-a-mannosidase and anti-a-tubulin antibodies, respectively. In most animal cells the Golgi complex and the MTOC are confined to the same centrosomal region near the nucleus.

The distribution of the RIIβ signal corresponded to the Golgi and the MTOC region (Fig. 6; 18, 19). 12 h of Ras activation induced a significant redistribution of RIIβ, Golgi, and microtubules, which assembled into bundles that ran in proximity to the cell nucleus. RIIβ formed a perinuclear ring largely coincident with the tubulin signal. Similar modifications of the MTOC, the Golgi apparatus, and RIIβ were also seen 3 h after...
stabilization of PKC with TPA. In these cells, the perinuclear microtubular array and RIIβ staining invariably overlapped. In about 30% of the cells, however, the staining of RIIβ and the Golgi apparatus did not overlap completely.

Redistribution of RIIβ to the perinuclear area was accompanied by a dramatic reduction in nuclear C-PKA (Fig. 6). As shown by staining with C-PKA-specific antibody, Ras activation or PKC stimulation induced a significant loss of nuclear C-PKA that was not reversed by cAMP treatment (Fig. 6; data not shown). Thus the early events following Ras activation are redistribution of RIIβ and a reduction in nuclear C-PKA, which as we show below is a result of this redistribution.

Longer periods of cAMP stimulation (12 h) or Ras activation (24–36 h) induced the movement of RIIβ to the cytoplasm (Figs. 4 and 5d). Eventually (after 4–6 weeks of exposure to active Ras), total RIIβ protein content decreased (Figs. 4 and 5b). This was accounted for by decreased synthesis, because the turnover of RIIβ protein was not affected by 1–3 days of Ras activation or by PKC stimulation (data not shown). Because RIIβ gene transcription is cAMP-dependent (41, 42), reduction of nuclear C-PKA probably accounts for the down-regulation of RIIβ mRNA and protein.

To determine whether v-Ras activation affected the cellular concentration of the other PKA regulatory subunits, we cross-linked labeled azido-cAMP with total proteins derived from N<sup>39°</sup> or N<sup>33°</sup>. Fig. 7A shows azido-cAMP binding activity and the immunoblot analysis of R subunits before or after forskolin stimulation. In Fig. 7B, extracts from unstimulated cells were cross-linked with increasing concentrations of azido-cAMP. To interpret this figure, recall that the RI isoform has a higher affinity for cAMP than RII. Treatment of cells with 40 μM forskolin increases the concentration of cAMP to levels sufficient to bind RI, RIIα, and RIIβ. Because RI when extracted from treated cells is bound to unlabeled cAMP, RIIα and RIIβ account for most of the bound azido-cAMP added to the extracts. The azido-cAMP titration in Fig. 7B confirms that RI has a higher affinity for cAMP than RII and indicates that RIIα has a higher affinity for cAMP than RIIβ.

Panels A and B in Fig. 7 indicate that although RIIβ is reduced, the concentration of the other subunits and their reactivity with cAMP are unaffected by exposure to v-Ras (see also Fig. 4). Thus, the down-regulation of nuclear C-PKA in N<sup>39°</sup> cannot be accounted for by changes in RI. Instead we believe that a small fraction of anchored RIIβ-C<sub>2</sub> pool is activated by cAMP to generate the C-PKA that translocates to the nucleus. Ligand blotting analysis with labeled RIIα further indicates that RIIα concentrations and partition do not change in N<sup>39°</sup> and N<sup>33°</sup> (Fig. 7C).

Centrosomal Localization of RIIβ Is Essential for Maintenance of cAMP-dependent Differentiation—Translocation of RIIβ to the cytosol thus appears to be temporally linked to the down-regulation of nuclear C-PKA in acutely Ras-transformed cells. To determine if these two events were causally linked, we performed experiments in which we manipulated the localization of RIIβ in normal differentiated cells. Thus, we could determine the consequences of PKAII delocalization on the thyroid phenotype in the absence of pleiotropic effects induced by the v-Ras oncogene or PKC activation. RIIβ is normally fixed to the cell membrane by anchor proteins. One of these
proteins (AKAP75) is abundant in brain and endocrine tissues and shows the same cellular distribution as RIIβ (22). We transected differentiated TL cells with expression vectors carrying a gene coding for a mutated version of an RIIβ anchor protein (AKAP75) lacking 180 N-terminal amino acids (AKAP45) or a control RSV-neomycin resistance gene. AKAP45 is a soluble protein that binds RIIβ efficiently and prevents membrane anchoring of PKAIIβ (33, 43). Our results show clearly that AKAP45 mimicks the effects of Ras transformation on nuclear C-PKA and thyroglobulin expression. Fig. 8 shows that the expression of AKAP45 induced the movement of RIIβ protein to the cytosol (compare A-45 with control cells; Fig. 8, C). Quantitative analysis of RIIβ immunoblots indicated that approximately 75% of RIIβ translocated to the cytosol; only a slight reduction in the total RIIβ content was evident (Fig. 8, lower panel). Fig. 9 shows that the localization α-mannosidase, which normally colocalizes with RIIβ, did not change in A-45 (Fig. 9, c and d). In the transfectants reported here (A-45), the cytosol/membrane partition and the total amount of RIIα were not affected (data not shown).

The responsiveness of the A-45 cells to cAMP was followed by measuring the accumulation of nuclear C-PKA at increasing cAMP concentrations. At 250 μM cAMP, A-45 had markedly reduced nuclear C-PKA concentrations compared with controls. To reach control levels, the A-45 cells required 750 μM cAMP (Fig. 10, top panel). The absence of nuclear C-PKA was not due to inhibition of cytoplasmic PKA holoenzyme dissociation, which was unaffected by the expression of AKAP45 (data not shown). AKAP45 also inhibited the activation of the cAMP-dependent CREB nuclear transacting factor. As shown in Fig. 10 (middle panels), the concentration of phosphorylated CREB (PCREB) was significantly (approximately 60%) reduced in A-45. The total CREB content remained at control levels (compare PCREB with CREB in Fig. 10). The thyroglobulin mRNA content in A-45 cells was also greatly reduced, reflecting the decrease of nuclear C-PKA (Fig. 10, lower panel).

We conclude that the induced translocation of PKAIIβ from juxtanuclear structures to the cytoplasm of thyroid cells significantly impairs C-PKA accumulation in nuclei and blocks the induction of thyroglobulin mRNA. This reproduces an effect of v-Ras on thyroid differentiation and indicates a specific role for PKAIIβ in the transmission of cAMP signals into the nucleus.

**DISCUSSION**

Ras and cAMP Signals in the Thyroid Cell—Thyroid cells exposed to v-Ras dedifferentiate. The down-regulation of thyroid-specific gene expression is associated with the inactivation of thyroid-specific transcription factors (TTF1 and PAX8) and with a more general down-regulation of cAMP-dependent promoters (31, 32, Fig. 3). Antagonism between oncogenic Ras and cAMP signaling is not confined to thyroid cells; oncogenic Ras likewise down-regulates the PKA-dependent rat prolactin promoter (44).

We followed the early biochemical events associated with the activation of the v-Ras oncogene by using cells transformed with a reversibly temperature-sensitive v-Ras variant (28). Decreased accumulation of nuclear C-PKA in cells treated with
cAMP was among the first responses to Ras activation (Fig. 6). cAMP, even at high concentrations, failed to induce thyroglobulin transcription in v-Ras-transformed cells. Nuclear C-PKA accumulation was also inhibited by stimulation with PKC for 3 h (30). The simultaneous inhibition of PKC and stimulation by cAMP restored differentiation in v-Ras transformed thyroid cells (32).

We present evidence in this manuscript that v-Ras significantly alters the location and the expression of the protein kinase A isofrom, PKAIIβ, in thyroid cells. PKAIIβ is expressed in thyroid cells and is localized on the membranes. The translocation of PKAIIβ occurs shortly after the activation of the oncogene and is reversible upon Ras denaturation. Down-regulation of RIIβ expression is a late response to the oncogene and leads to a permanent alteration in the composition of PKA holoenzymes in chronically Ras-transformed cells.

Within 3–24 h after exposure to v-Ras or active PKC, RIIβ translocated from the Golgi-centrosome region to the perinuclear area and to the cytosol. This movement was accompanied by a decrease in nuclear C-PKA concentrations. The levels of RIIβ protein and mRNA declined 4–7 days after exposure to v-Ras and were nearly undetectable in chronically transformed cells. The translocation and loss of RIIβ were associated with inactivation of the thyroid-specific transacting factor TTF1, diminished phosphorylation of PKA-regulated CREB transcription factor, and inhibition of thyroid-specific gene transcription (30, 31).

The translocation of RIIβ to the cytosol is directly related to the inhibition of cAMP signal transduction to the nucleus. We used a mutant PKAII anchoring protein, AKAP45, to mimic the effects of v-Ras. Expression of AKAP45 induced the cytosolic translocation of RIIβ in differentiated thyroid cells. These cells displayed down-regulation of thyroglobulin expression and reduced CREB phosphorylation (Fig. 10). Although cells transformed with Ras and cells expressing AKAP45 are qualitatively similar, they cannot be compared quantitatively. The translocation of RIIβ to the cytosol and subsequent loss of the protein is complete in Ras-transformed cells but only partial in cells expressing AKAP45.

Our data indicate that the cellular location of RIIβ in thyroid cells and therefore PKAIIβ determines whether cAMP signals

![Image](https://example.com/image1.png)

**Fig. 9.** Immunofluorescence analysis of RII and α-mannosidase in AKAP45 expressing cells. AKAP45 expressing cells (A-45) and control cells (C) were stained with specific antibodies to RIIβ and α-mannosidase, a specific Golgi marker. α and β are the cells stained with anti-RIIβ antibody; c and d represent the cells stained with anti-α-mannosidase antibody. In A-45 cells RIIβ was not concentrated only in the juxtanuclear centrosomal-Golgi region but was diffuse in the cytoplasm. Note that in some cells the Golgi signal was evident in a diffuse background. We have noticed that the heterogeneity of the immunofluorescent signal was dependent on the amount of AKAP-45 expressed. The Golgi apparatus did not show any significant change and α-mannosidase staining appeared similar in control (c) and A-45 cells (d).

![Image](https://example.com/image2.png)

**Fig. 10.** cAMP nuclear response is downregulated in AKAP-45 expressing cells. The upper panel shows nuclear accumulation of C-PKA subunit following acute stimulation with 8-Br-cAMP. RSV-NEO (■) and AKAP45 (●) transfected cells were TSH-starved for 2 days and then stimulated with TSH for 12 h. The cells were then treated with 250 or 750 μM 8-Br-cAMP for 40 min at 37°C. At the end of this period, the cells were collected, and the nuclei were prepared as described under "Materials and Methods." Catalytic activity was assayed in the presence and the absence of PKI. The PKA activity is reported in pmol of 32P incorporated into Kemptide/μg of nuclear protein. This experiment was repeated several times with equivalent results. Cells overexpressing AKAP75 were identical to RSV-NEO control cells with respect to nuclear C-PKA accumulation induced by cAMP (data not shown). Phosphorylation of CREB is reduced in cells expressing AKAP45. TL cells carrying RSV-NEO (C) or AKAP45 plasmids (A-45) were labeled for 4 h with 0.5 μCi/ml [32P]orthophosphate or 0.150 μCi/ml [35S]methionine for 12 h. During the final 45 min of labeling, cells were treated (+) or not (−) with 8-Br-cAMP. Nuclear proteins were prepared as described (30) and immunoprecipitated with antibody specific for phosphorylated CREB (54) or with a specific anti-CREB antibody (anti-KID domain, UBI). The immunoprecipitates were separated by SDSPAGE and visualized by autoradiography. CREB represents the CREB protein detected in [35S]methionine-labeled proteins. The lanes marked with an asterisk indicate the immunoprecipitation of control cell extracts with nonimmune serum. Densitometric scanning of several autoradiograms derived from three independent experiments indicated that CREB-P in AKAP45 expressing cells was reduced by ~60% ± 10. Reduction of thyroglobulin mRNA in cells expressing AKAP45. The lower inset shows a Northern analysis of 20 μg total RNA derived from AKAP-45 (A-45) or control (C) cells hybridized with a specific rat thyroglobulin (Tg) or GAPDH cDNA probes. The specific mRNA bands are indicated by the arrows.
Transformation and cAMP Signaling in Thyroid Cells

are efficiently transduced to the nucleus and suggest that the concentration of nuclear C-PKA is critical for the maintenance of thyroid cell differentiation.

It has recently been shown that cAMP antagonizes Ras-mediated signal transmission initiated by plasma membrane tyrosine kinase receptors (11, 13, 45, 46). Ras binds to and activates Raf-1, which initiates the mitogen-activated protein kinase cascade. This pathway is inhibited by PKA, which phosphorylates Raf-1 and reduces its affinity for Ras (11, 12). Recent evidence from our and other laboratories indicates that PKA also inhibits Raf in differentiated thyroid cells (47). The antagonism between v-Ras and cAMP signaling in thyroid cells occurs at two additional steps. Upstream to PKA, v-Ras reduces cAMP levels by inhibiting adenyl cyclase (48) and by reducing TSH receptor expression (49). And, as we show above, v-Ras antagonizes cAMP signaling to the nucleus by changing the location of PKAIIβ. This reduces nuclear C-PKA concentrations, down-regulating the expression of RIβ. The RIβ protein disappears in chronically transformed cells.

Inhibition of the PKA pathway by Ras is a potential feedback mechanism that could dampen negative regulation of Ras-Raf signal transmission by cAMP. A decline in cAMP levels could contribute efficiently to nuclear C-PKA in thyroid cells. It is possible that C-PKA liberated by soluble PKAs is bound by cytosolic PKA substrates, thereby reducing the efficiency of nuclear accumulation. Alternatively, C-PKA at physiological concentrations may indeed enter the nucleus not by diffusion but via direct import through anchored PKA.

Because of its low affinity for cAMP and its membrane localization, RIβ might draw C-PKA from the nucleus at low cAMP concentrations. In this role, RIβ could function as a repressor, reducing basal cAMP-induced transcription (55). At intermediate cAMP levels, RIβ efficiently facilitates C-PKA import to the nucleus.

Localization of PKA and cAMP Nuclear Signaling—Nuclear responses to cAMP are mediated by the activation of transcription factors by PKA. After dissociation, PKA accumulates in the nucleus (18), where it phosphorylates transacting factors on specific threonine and serine residues. The modified transcription factors bind to DNA and/or other nuclear factors and stimulate transcription (30, 51, 52).

Nuclear translocation of C-PKA is the rate-limiting step in the coupling of hormonal stimulation and the transcription of CREB-dependent genes (53). The molecular mechanism responsible in vivo for the reversible movement of C-PKA between the cytoplasm and the nucleus is not known. Microinjection experiments with exogenous C-PKA suggest that nuclear entry of C-PKA can be explained by diffusion and is solely dependent on cAMP concentrations (54, 55). Note, however, that the levels of C-PKA in these experiments are significantly above physiological levels. In v-Ras transformed cells as well, some nuclear accumulation of C-PKA can be induced at high cAMP concentration (Fig. 1B). The diffusion hypothesis fails to explain why C-PKA exits the nucleus in the presence of high levels of cAMP or why there is a lengthy lag period between an increase in intracellular C-PKA and the nuclear accumulation of C-PKA (56). Furthermore, the export of C-PKA from the nucleus appears to be regulated. PKI and R subunits facilitate the exit of C-PKA from the nucleus (57, 58).

Our experiments indicate that the localization, rather than the concentration, of PKAIIβ, influences the nuclear concentration of C-PKA. Thus Ras or PKC activation leads to the rapid loss of nuclear C-PKA, even though the total levels of RIβ are initially unchanged (this work; 30), and expression of AKAP45 induces the cytosolic translocation of RIβ and inhibits nuclear C-PKA accumulation without reducing the cellular content of the regulatory subunit (Fig. 8).

The Function of RIβ—RIβ is expressed predominantly in endocrine, brain, and reproductive tissues (22) and at low levels in fibroblasts and epithelial cells. We have studied the transcriptional regulation of the different regulatory subunits. Transcription of the RIβ gene, as determined by reverse transcriptase-PCR, is induced by persistent cAMP stimulation (24–36 h). In contrast, transcription of RIα and RIα responds more rapidly to cAMP, requiring 6–12 and 12–24 h stimulation, respectively (data not shown). Delayed induction of RIβ has also been reported in rat Sertoli cells treated with cAMP (41, 42). Prolonged cAMP stimulation maintains RIβ expression in thyroid cells. These data suggest an autoregulatory loop between RIβ and C-PKA. PKAIIβ adapt the cell (mainly neurons and endocrine cells) to persistent and high concentrations of cAMP. Recall that these cell types express specific receptors that efficiently stimulate adenyl cyclase. We suggest that PKAIIβ responds to high and persistent cAMP levels, whereas PKAI is transiently activated by weak cAMP signals. The selective loss of RIβ should in principle result in significant changes of cAMP sensitivity.

Cytosolic translocation of PKAIIβ induced either by v-Ras, PKC, or overexpression of a mutant anchor protein, reduced nuclear C-PKA and thyroglobulin mRNA levels. It thus appears that PKAIIα, PKAIα, as well as cytosolic PKAIIβ, do not contribute efficiently to nuclear C-PKA in thyroid cells. It is possible that C-PKA liberated by soluble PKAs is bound by cytosolic PKA substrates, thereby reducing the efficiency of nuclear accumulation. Alternatively, C-PKA at physiological concentrations may indeed enter the nucleus not by diffusion but via direct import through anchored PKA.

Because of its low affinity for cAMP and its membrane localization, RIβ might draw C-PKA from the nucleus at low cAMP concentrations. In this role, RIβ could function as a repressor, reducing basal cAMP-induced transcription (55). At intermediate cAMP levels, RIβ efficiently facilitates C-PKA import to the nucleus.

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Transformation and cAMP Signaling in Thyroid Cells

25359

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