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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 oncoprotein 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 downregulates thyroid-specific gene expression.

Ras is a small GTP binding protein that serves as a central molecular switch. Ras links activated receptor tyrosine kinases with downstream signaling systems 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 or 3T3-LI adipocytes) (3, 4) or deregulated growth and dedifferentiation (5, 6). Signaling proteins that couple Ras to receptor tyrosine kinases have been identified and characterized (for review see Ref. 7). Moreover, the formation of the complex between Ras-GTP and Raf-1 is essential for the subsequent activation of the downstream mitogen-activated protein kinase cascade (8). Recent work has demonstrated that Ras recruits Raf to the plasma membrane (9), where another tyrosine kinase-generated signal activates the membrane-bound Raf (10). cAMP blocks mitogenic signaling in fibroblasts by reducing the affinity of Raf-1 for Ras (11). The reduction in binding affinity is correlated with the phosphorylation of a consensus PKA substrate site in the N-terminal regulatory domain of Raf-1 (11, 12). These findings indicate an antagonistic relationship between the Ras and cAMP signals (13).

Signals carried by cyclic AMP are received, amplified, and transmitted by PKA.[1] In eukaryotic cells the multiple isoforms of the regulatory (R) and catalytic (C-PKA) subunits assemble to generate several distinct PKA holoenzymes. The characteristics of the PKA holoenzyme are largely determined by the structure and properties of their R subunits; the C-PKA subunits exhibit similar kinetic features and substrate specificities (14). The specific regulatory roles of PKA isoenzymes remain to be determined (14–16). Typically, PKAI is a soluble cytoplasmic enzyme (see Ref. 17). In contrast, PKAIIα and PKAIIβ are often associated with the cytoskeleton and the Golgi apparatus, centrosomes, and the perinuclear area (18–20). The specific localization of the PKAII isoenzymes is determined by the binding of the R subunit to compartimentalized cellular proteins (21–23, 59). In addition to their distinctive cellular locations, the R subunits differ in their regulation and biochemistry. R subunits are differentially expressed in neuronal and neuroendocrine cells (24). RI has a higher affinity for cAMP than RIIα and RIIβ (25, 26) and also turns over more rapidly (27). The RII subunits in the PKAII holoenzyme are phosphorylated by the catalytic subunit (25). Taken together, these observations suggest that PKAI and PKAII 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 Ats-aza). N cells grown at 33 °C ($N^{33^{\circ}}$) are dedifferentiated, whereas cells grown at 39 °C ($N^{39^{\circ}}$) 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 CREBs (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 translocation 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 dedifferentiation 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, which confers resistance to the neomycin analog G418 (33). RSV-NEO is a construct expressing 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-isoamyl alcohol extraction (34). 20 μg of each RNA sample were electrophoresed on a 1% agarose gel containing formaldehyde and transferred to nylon membranes (Amersham Corp.) using standard capillary blotting techniques. Blots were prehybridized for 1 h at 65 °C in O.5 M sodium phosphate buffer, pH 7.3, 7% SDS, 1 mM EDTA. Hybridation was carried out at 65 °C for 16 h in the same solution containing 1×10^6 cpm/ml of [^{32}P]dGTP-labeled probe. Membranes were washed three times for 2 min in 300 ml of prewarmed (65 °C) washing solution (40 mM Na_2HPO_4-NaH_2PO_4, 1% SDS) and once in 100 ml of the same solution on a shaking platform for 30 min in an incubator at 65 °C. Probes were ~0.35-kilobase PCR fragments corresponding to RIα, RIIα, RIIβ, and GAPDH labeled with ^{32}P as follows: 10 ng of the amplified DNA fragment (purified by electroelution) were labeled via 10 PCR cycles (1 min at 95 °C, 1 min at 60 °C, and 3 min at 72 °C) in a volume of 30 μl of PCR buffer (see below) containing 50 mCi of (3000 Ci/mmol) [^{32}P]dGTP, 2 nmol of dATP, dTTP, and dCTP, 10 pmol of each of the appropriate oligonucleotide primers, and 0.75 units of *Taq* DNA polymerase.

cDNA Synthesis and Reverse Transcriptase-PCR—Single strand cDNA synthesis was performed on 1 μg of total RNA in 20 μl of reaction buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 15 mM $MgCl_2$, 10 mM dithiothreitol, 500 μM dNTPs) containing 20 pmol of random primers and 200 units of Moloney murine leukemia virus RNase H Reverse Transcriptase (Superscript-Life Technologies, Inc.) at 42 °C for 2 h. After phenol-chloroform extraction and ethanol precipitation, the single strand cDNA was resuspended in 50 μl of H_2O. 2 μl of the cDNA was amplified in 100 μl of buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1, 5 mM $MgCl_2$, 0.001% gelatin, 200 μM dNTPs) containing 20 pmol of each oligonucleotide primer. One unit of *Taq* DNA polymerase was added after the first denaturation (5 min at 97 °C). Samples were then subjected to 16 cycles consisting of 1 min at 95 °C, 1 min at 60 °C, and 1 min at 72 °C. The last extension was carried out for 12 min.

Semiquantitative reverse transcriptase-PCR was performed coamplifying RIα, RIIα, RIIβ, and GADPH cDNAs (GADPH primers were added after the first five cycles). 20 μl of the PCR products were resolved on four different 1.5% agarose gels, blotted onto different nylon membranes (Amersham Corp.), and hybridized with specific probes (see above). Membranes were then exposed to a preflashed x-ray film (Beta-Max, 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).

The cDNA fragments corresponding to the RIα, RIIα, and RIIβ were ampified with primers: RIα-F, 5′-GGCGTTGAGGGAGGCAGACG-3′, 5′ end at position +1; RIα-R, 5′-GATTTGGGGGTGGAGGAGAG-3′, 5′ end at position 361 (35); RIIα-F, 5′-TGGAGTACTTCACAAGCCTG-3′, 5′ end at position 8; RIIα-R, 5′-AAGCTGTTCCTGATCCAGGT-3′, 5′ end at position 342 (36); RIIβ-F, 5′-ACCCCCAGTAAGGGTGTCAAC-3′, 5′ end at position 161; and RIIβ-R, 5′-GTTCCTCTGTCGATGACGTA-3′, 5′ end at position 544 (37).

Antibodies and Immunoprecipitation—Polyclonal antibodies against RIIβ and PKA catalytic subunits were prepared as described previously (22). Specific anti-RIIβ or anti RII or anti-C-PKA antibodies were generated by immunizing rabbits with a synthetic RIIβ peptide (peptide 31–57 from the AUG of the rat sequence) or RII (peptide containing the residues 53–73 from the start codon of the rat protein), respectively, cross-linked to soybean trypsin inhibitor. The total IgG was purified, and the specificity of each preparation was tested by immunoprecipitation, immunofluorescence, and immunoblot by preadsorbing the antibodies to the specific peptides or control peptides. Anti-α-mannosidase antibodies were a gift of K. Moreman (University of Georgia, Athens, GA).

Cell proteins were labeled with [^{35}S]methionine (1 Ci/mmol, 0.150 mCi/ml) for 4 h in methionine-free medium. Cells were collected and lysed in RIPA buffer (50 mM Tris-HCl, pH 8, 0.15 M NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 5 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml pepstatin, and phenylmethylsulfonyl fluoride 0.5 mM). Immunoprecipitation was performed 2 h with the specific antibody (1:200 anti-RIIβ). Samples were then incubated for 1 h with 40 μl (packed bed volume) of protein A-Sepharose CL-4B, followed by five washes with 1 ml of RIPA buffer. Beads were boiled in Laemmli buffer. SDS-PAGE was performed in 10% polyacrylamide gel. After drying, the gel was exposed to X-Omat S film at −80 °C.

Overlay-Far Western Analysis of RII Binding Proteins—Cells were washed twice and scraped in phosphate-buffered saline (PBS). The cell pellet was lysed in AT buffer (60 mM KCl, 15 mM NaCl, 14 mM β-mercaptoethanol, 2 mM EDTA, 15 mM Hepes, pH 7.9, 0.3 M sucrose, 5 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml pepstatin, and 0.5 mM phenylmethylsulfonyl fluoride) by passage through a 24-gauge tuberculin syringe (10 times). The cell lysate was fractionated into "particulate fraction" (pellet) and "cytosol" (supernatant) by centrifuging 30 min at 100,000 × *g*. The particulate and cytosolic fractions were resuspended in AT buffer containing 0.1% SDS, 1% Triton X-100. The samples (80 μg of protein) were subjected to electrophoresis in 10% SDS-PAGE. The resolved polypeptides were transferred to nitrocellulose filter (0.45 mm, Schleicher & Schuell). The probe (RIIα) was labeled with PKA catalytic subunit and processed as described (21).

Nuclear and Cytoplasmic PKA Assay—The cells were lysed in AT buffer containing 0.1% Triton X-100 by incubation for 5 min on ice. The lysate was layered on 1 volume of a sucrose cushion (AT buffer containing 1 M sucrose) and centrifuged at 10,000 × *g* for 5 min. The pellet represents purified nuclei; the upper phase represents cytoplasm. The nuclear fraction contained approximately 90% of the transcription factors: TTF1, CREB, and PAX8. In addition, each preparation was stained with propidium iodide to check purity. Assays (final volume, 25 μl) were performed at 30 °C for 10 min in a solution containing 100 μM ATP, [γ-^{32}P]ATP (Amersham Corp.) (125–150 cpm/pmol) at a final concentration of 10 μCi/100 μl of reaction mixture, 10 mM $MgCl_2$, 20 mM Hepes, pH 7.4, 100 μM kemptide (Sigma). When measuring PKA holoenzyme, 10–50 μM cAMP was added. PKA activity was fully inhibited by adding a specific PKA inhibitor peptide (PKI, 10 μM) containing a PKA pseudophosphorylation site (Sigma). Kemptide phosphorylation was monitored by spotting 20 μl of the incubation mixture on phosphocellulose filters (Whatman, P81) and washing with 75 mM phosphoric acid as described previously (30). The radioactivity retained on the filters was determined by scintillation counting in 4 ml of scintillation liquid (Ecolite, ICN). Holoenzyme activity was calculated by subtracting values obtained in the absence of cAMP and in the presence of PKI (+cAMP) from the values obtained in the presence of cAMP. Free C-PKA activity was evaluated by subtracting cpm obtained in the absence of cAMP from the values obtained in the presence of PKI. Data were expressed as picomoles of [^{32}P]phosphate transferred to the pep-

tide substrate during a 10-min incubation in the presence (PKA holoenzyme) 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 (Technogenetics) antibody for 60 min. Coverslips were observed by a Zeiss Axiomat microscope.

Photoaffinity Labeling with 8-Azido[^{32}P]cAMP—Labeling with 8-azido-[^{32}P]cAMP was performed by incubating cellular extracts (50 μg) or purified R subunits (1 ng/μl) with 1 μM 8-azido-[^{32}P]cAMP (50 Ci/mM, 20 μCi; ICN) in 50 mM Tris-HCl, pH 7.4 for 1 h at 4 °C in the dark in the presence or the absence of unlabeled cAMP. 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_2cAMP_4 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 ($N^{33°}$) 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. $N^{33°}$ or $N^{39°}$ 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. 1*A* 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 $N^{33°}$ and $N^{39°}$ 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 $N^{33°}$ (Fig. 1*B*). This was also shown by Western immunoblot of isolated nuclear proteins (Fig. 1*C*). 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 $N^{33°}$ (Fig. 1*B*), 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 $N^{33°}$ or $N^{39°}$. Confirming the experiments of Fig. 1, $N^{33°}$ cells treated with forskolin had little nuclear C-PKA compared with $N^{39°}$ 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 $N^{33°}$ and $N^{39°}$

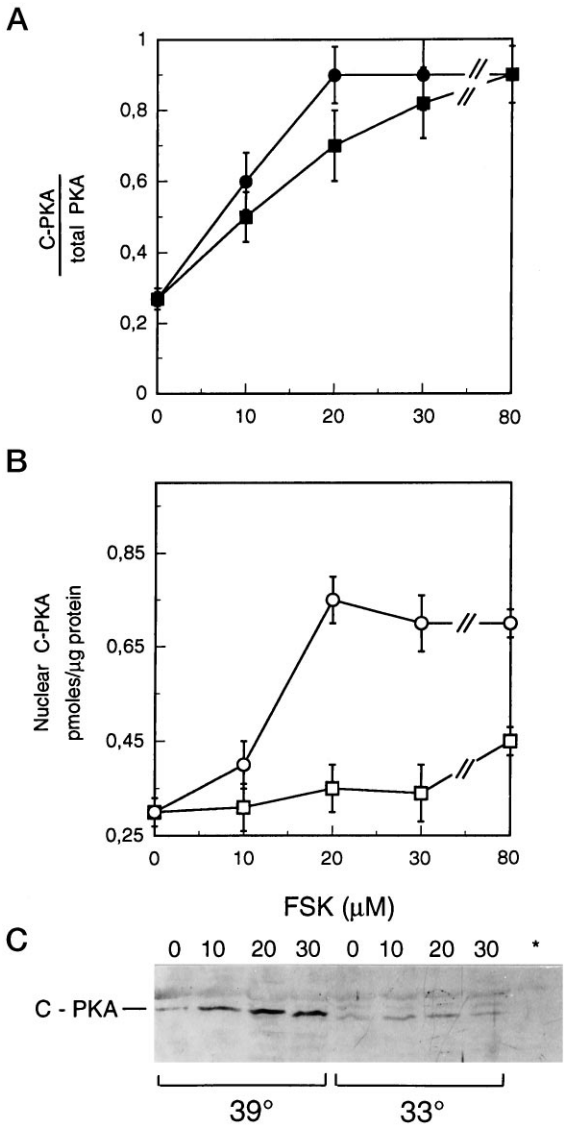

FIG. 1. **v-Ras inhibits nuclear accumulation of C-PKA subunit.** *A*, cytoplasmic PKA activation in $N^{33°}$ (■) and $N^{39°}$ (●) cells. Cytoplasmic fractions were prepared as described under "Materials and Methods." Cells were treated with the indicated concentrations of forskolin for 40′ at 37 °C. The activation of cytoplasmic PKA is represented as the ratio between C-PKA (−cAMP) and total PKA (+20 μM cAMP). PKA activity equals phosphotransferase activity inhibited by 10 μM PKI. The basal PKA activity (−cAMP, C-PKA) was 4 ± 0.3 and 5 ± 0.3 in $N^{33°}$ and $N^{39°}$, respectively. The basal PKA oloenzyme (+cAMP) was 16 ± 0.7 and 18 ± 0.8 pmol ^{32}P incorporated/10 min/μg of protein in $N^{39°}$ and $N^{33°}$, respectively. Immunoblots with anti-C-PKA antibodies indicated that the total amount of C-PKA was comparable in $N^{33°}$ and $N^{39°}$ cells. *B*, nuclear C-PKA accumulation in $N^{33°}$ (□) and $N^{39°}$ (○) cells. Nuclei were prepared by discontinuous sucrose gradient, and their purity was tested by histochemistry, as described under "Materials and Methods." C-PKA activity was measured as described under "Materials and Methods" and is reported as pmol of ^{32}P incorporated/min/μg of protein This experiment was repeated at least five times; variations in PKA values were less than 15%. *C*, Western immunoblot of nuclear PKA catalytic subunit in $N^{39°}$ and $N^{33°}$cells. Nuclear proteins (50 μg) were separated by SDS-polyacrylamide gel electrophoresis and probed with a specific anti-C-PKA antibody. The major band visible in the blot is C-PKA (39 kDa); in the lane marked by an *asterisk*, the antibody was preadsorbed with the specific C-PKA peptide used to immunize the rabbits (see "Materials and Methods"); *39* ° and *33* ° indicate nuclear proteins from cells expressing inactive or active Ras, respectively. The concentrations of forskolin are indicated (μM). Note that the difference in the nuclear content of C-PKA between $N^{39°}$ and $N^{33°}$ following forskolin stimulation is more evident by Western analysis than by enzymatic assay.

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 $N^{33°}$ *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*).

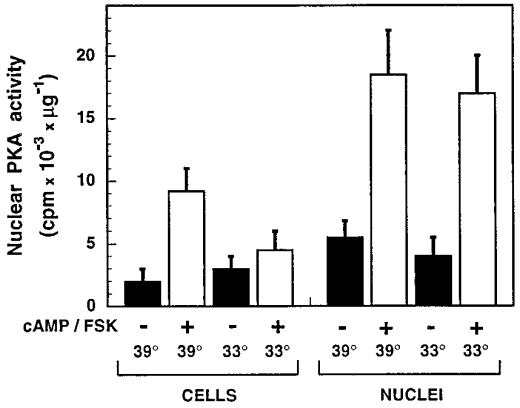

FIG. 2. **Isolated nuclei from Ras-transformed cells accumulate exogenous C-PKA.** Nuclear C-PKA activity in N cells grown at 33 and 39 °C and in isolated nuclei from the same cells. The cells were stimulated 40 min at 37 °C with 50 µM forskolin, as described in the legend to Fig. 1. Nuclei were isolated as described under "Materials and Methods." Nuclei (~10^6) were incubated 15 min with 0.5 µg of purified PKAIIα from rabbit muscle (specific activity, 10^5 cpm/µg/pmol kemptide in the presence of 10^{-4} 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 µM of PKI. By immunofluorescence analysis C-PKA was detected inside the nuclei.

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 $N^{33°}$ compared with $N^{39°}$ 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 RIIβ Subunit—Thyroid cells contain three PKA regulatory subunit isoforms: RIα, RIIα, and RIIβ. R subunit homodimers bind two C-PKA monomers to generate tetrameric holoenzymes that are named according to the cAMP binding subunits. PKAIα was separated from PKAII (a mixture of α and β) by chromatography on DEAE cellulose and the amounts of type I and type II enzymes were quantified by enzymatic assays. PKAI 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 PKAIIβ from PKAIIα, fluctuations in RII isoform expression are not detected by this method of analysis.

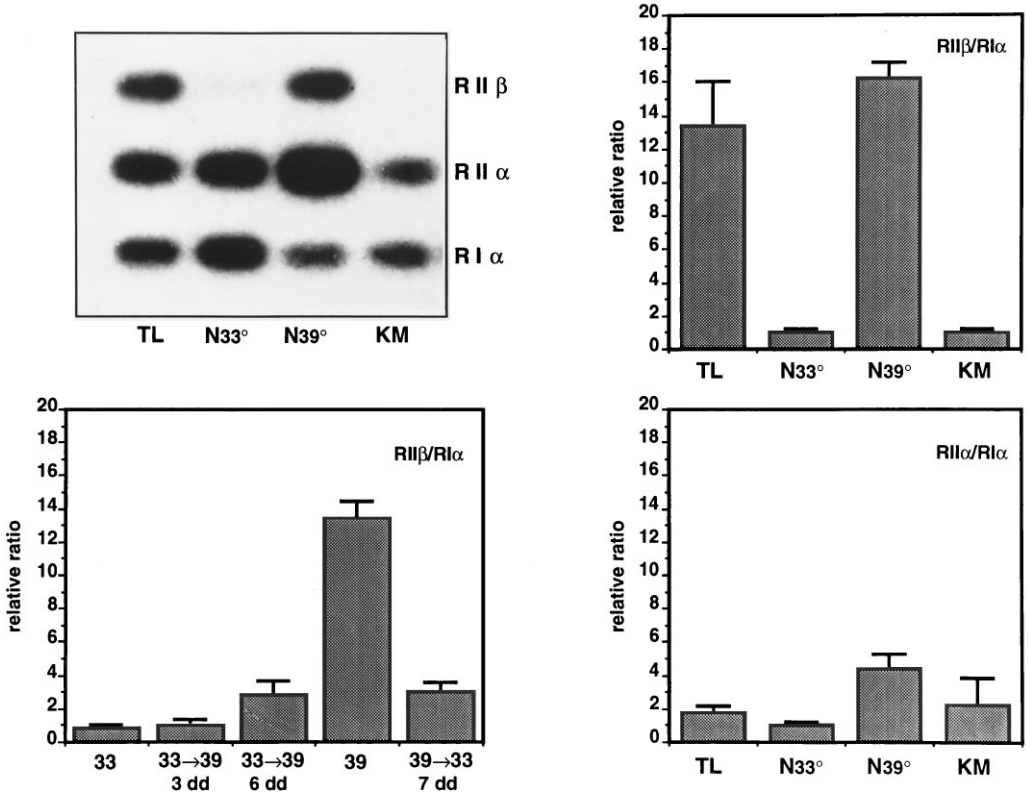

FIG. 3. **Relative abundance of RIIβ and RIIα mRNAs in differentiated and Ras-transformed cells.** Semiquantitative PCR of reverse-transcribed RNA isolated from the indicated cell lines was performed as described under "Materials and Methods." After PCR amplification, the mixture of products was electrophoresed in an agarose gel, blotted, and hybridized with specific probes. *Top left*, a representative autoradiogram of the hybridization signals corresponding to RIa, RIIα, and RIIβ. Comparable exposures were chosen and analyzed by densitometric scanning. The values obtained were normalized to RIα 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 RIα, RIIα, 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 RIα 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 RIα, 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 RIα, RIIα, and RIIβ subunits. Values are normalized to RIα mRNA content. The RIIβ/RIα ratio was 13-fold higher in differentiated (TL) cells than in the v-Ras transformed KM line and 16-fold higher in $N^{39°}$ than in $N^{33°}$. The RIIβ/RIα ratio decreased when $N^{39°}$ 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 RIIβ 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 RIα- and RIIα-specific probes. No significant changes in the levels of RIα 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 $N^{33°}$ and $N^{39°}$ (RIIβ and RIIα, *upper* and *lower panel*, respectively). In $N^{39°}$, RIIβ was located predominantly in the membrane fraction, as it was in differentiated TL cells. Upon shift of $N^{39°}$ to 33 °C for 1 week, RIIβ concentrations were reduced, and the protein was recovered mainly in the cytosolic fraction ($N^{39-33°}$). Three weeks at 33 °C induced a further depletion of RIIβ ($N^{33°}$). RIIα was present both in the cytosolic and membrane fractions in thyroid cells. Neither the partition nor the amount of RIIα changed with temperature shift (Fig. 4, *lower panel*). As a control for temperature effects, the amount and localization of RIIβ in differentiated cells (TL) grown at 39 °C was shown to be unaffected by a shift to 33 °C for 2 weeks (data not shown).

From these data we conclude that Ras activation induces the gradual translocation of RIIβ from the membrane fraction to the cytosol. Later, the RIIβ polypeptide concentration declines and cannot be detected in chronically Ras-transformed cells (KM or $N^{33°}$ 4 weeks). These observations are consistent with the specific decrease in RIIβ mRNA seen 1 week following Ras activation (Fig. 3).

Redistribution of RIIβ Is an Early Event Following Ras Activation—To understand how v-Ras elicits the loss of RIIβ protein, we monitored RIIβ distribution by immunofluorescence with anti RIIβ antibodies in N cells at early times following Ras activation. Fig. 5 shows the distribution of RIIβ in differentiated TL (Fig. 5*a*), transformed KM (Fig. 5*b*), or N cells grown at 39 or 33 °C for 2 weeks (Fig. 5, *c* and *d*, respectively). The fluorescence signal was concentrated in the Golgi-centrosome area in TL and $N^{39°}$. The signal was greatly reduced in KM or distributed diffusely in the cytosol in $N^{33°}$ (Fig. 5, *b* and *d*, respectively). $N^{39°}$ cells (Fig. 5*e*) that were treated for 1 h with PMA (220 ng/ml) (Fig. 5*h*) or shifted for 1 (Fig. 5*f*) or 4 (Fig. 5*g*) 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 increasingly 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-centrosome 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).

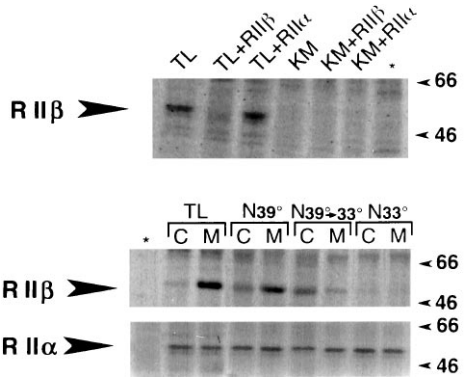

FIG. 4. **RIIβ polypeptide is depleted in Ras-transformed cells.** The cell lines indicated (TL, the differentiated thyroid cell line and KM, the same cell line transformed with the wild type Ki-Ras) were metabolically labeled with [^{35}S]methionine (6 h). Total proteins were immunoprecipitated with antibodies directed to the N-terminal peptide of rat RIIβ and analyzed by SDS-PAGE as described under "Materials and Methods" (*upper panel*). In the lanes indicated, the antibodies were preadsorbed before the immunoprecipitation with purified recombinant RIIβ (10 ng) (33) or purified RIIα (1 μg) (Sigma). The *lower panel* shows the immunoprecipitation with antibodies directed *versus* the N terminus peptides of RIIβ (*lower panel*, *upper segment*) or RIIα (*lower panel*, *lower segment*) of ^{35}S-labeled proteins derived from membranes (*M*) and 100,000 × *g* supernatant (*C*) (*upper panel*) of TL or N (see "Materials and Methods") cells grown at 39 °C, at 33 °C for 3 weeks, or shifted from 39 to 33 °C for 1 week. *Arrows* indicate molecular mass markers on the right side and the position of the bands corresponding to RIIβ or RIIα on the left. The *lanes* marked by an *asterisk* indicate immunoprecipitations performed with preimmune sera. Quantitative analysis of several experiments indicates that that the reduction of RIIβ in KM cells is ~15–20-fold relative to the differentiated controls (TL or $N^{39°}$ cells). In $N^{33°}$ cells the amount of RIIβ protein depends on the period of Ras activation: it is ~4–5-fold reduced after 1 week at 33 °C and becomes 7–8-fold less than the levels in $N^{39°}$ cells after 3 weeks at 33 °C.

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-α-mannosidase and anti-α-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 MOTC, the Golgi apparatus, and RIIβ were also seen 3 h after

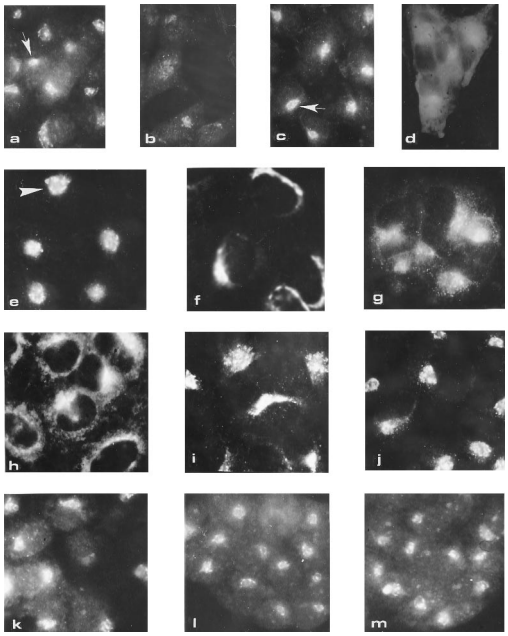

FIG. 5. **Early events following Ras activation.** RIIβ migrates to the perinuclear area and then diffuses in the cytoplasm. Localization by immunofluorescence of RIIβ in differentiated (TL, *a*; $N^{39°}$, *c* and *e*) and transformed cell lines (KM, *b*; $N^{33°}$, *d*). Cells were cultured to subconfluency on glass coverslips and stained with anti-RIIβ antibody as described under "Materials and Methods." RIIβ is principally associated with the centrosome-Golgi area close to the nucleus in *a* and *c* (*arrows*). In *d*, RIIβ shows a diffuse cytosolic distribution. In *b*, the RIIβ signal is significantly reduced and in some cells appears granular. From *e* to *j*, RIIβ staining is shown at higher magnification. $N^{39°}$ cells are shown in *e*. RIIβ forms a perinuclear ring in $N^{39°}$ cells shifted to 33 °C for 12 h (*f*). $N^{39°}$ cells shifted to 33 °C for 4 days show a diffuse staining of RIIβ in the cytoplasm (*g*). A similar redistribution of RIIβ is visible in $N^{39°}$ cells treated 3 h with PMA (220 ng/ml; *h*). When the cells in (*g*) were shifted back to 39 °C, RIIβ gradually relocated to its original perinuclear position. $N^{39°}$ cells shifted 4 days to 33 °C were returned to 39 °C for 3 (*i*) or 24 h (*j*). No RIIβ redistribution was observed in TL cells grown at 37 °C (*k*), shifted 4 days to 39 °C (*l*), or shifted to 33 °C for 24 h (not shown) or 4 days (*m*).

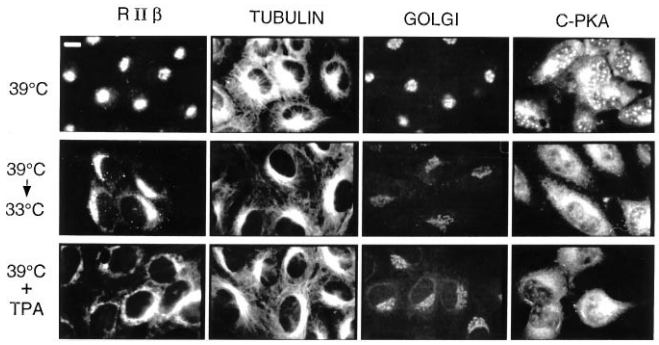

FIG. 6. **PKC stimulation or Ras activation induces redistribution of RIIβ, reorganization of the microtubules, and down-regulation of nuclear C-PKA.** Immunofluorescence staining with specific antibodies to RIIβ, α-tubulin, α-mannosidase (Golgi), and C-PKA of $N^{39°}$ cells (*top row*) shifted to 33 °C for 12 h (*middle row*) or treated with TPA (200 ng/ml) for 3 h (*bottom row*). The cells were treated with 0.250 mM 8-Br-cAMP for 15 min at 37 °C. In $N^{39°}$ cells both the anti-RIIβ and anti-α-mannosidase antibodies clearly stained a discrete iuxtanuclear region corresponding to the centrosome, as revealed by the MTOC staining with anti-α-tubulin antibodies. This pattern was evident in the large majority (90%) of $N^{39°}$ cells. In addition to its cytoplasmic localization, C-PKA accumulated as large dots in the nuclei. Shifting $N^{39°}$ cells to 33 °C (15 h) or stimulating cellular PKC with PMA induced a redistribution of RIIβ along the surface of the nucleus. A similar spread of the Golgi elements concomitant to a massive reorganization of the microtubules, with the appearance of a microtubule sheath in the form of a perinuclear ring, was observed in ~90% of the cells. Under these conditions the classical MOTC was not evident and nuclear staining of C-PKA was greatly reduced. Biochemical analysis revealed a consistent reduction of PKA activity in the nuclei of these cells. We noticed that in some cells (30%) the amplitude of the Golgi dispersal was greater than that observed for RIIβ. No change in the arrangement of microtubules, Golgi, or RIIβ staining was observed when control cells (TL) were shifted to 33° or 39 °C for 3 h or 1 day. cAMP stimulation did not alter the staining pattern of RIIβ, α-tubulin, and α-mannosidase (Fig. 5*e* and data not shown).

stimulation 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 PMA stimulation (12 h) or Ras activation (24–36 h) induced the movement of RIIβ to the cytoplasm (Figs. 4 and 5*d*). Eventually (after 4–6 weeks of exposure to active Ras), total RIIβ protein content decreased (Figs. 4 and 5*b*). 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 $N^{39°}$ or $N^{33°}$. Fig. 7*A* shows azido-cAMP binding activity and the immunoblot analysis of R subunits before or after forskolin stimulation. In Fig. 7*B*, 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. 7*B* 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 $N^{33°}$ cannot be accounted for by changes in RI. Instead we believe that a small fraction of anchored RIIβ-C_2 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 $N^{39°}$ and $N^{33°}$ (Fig. 7*C*).

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 transfected 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).

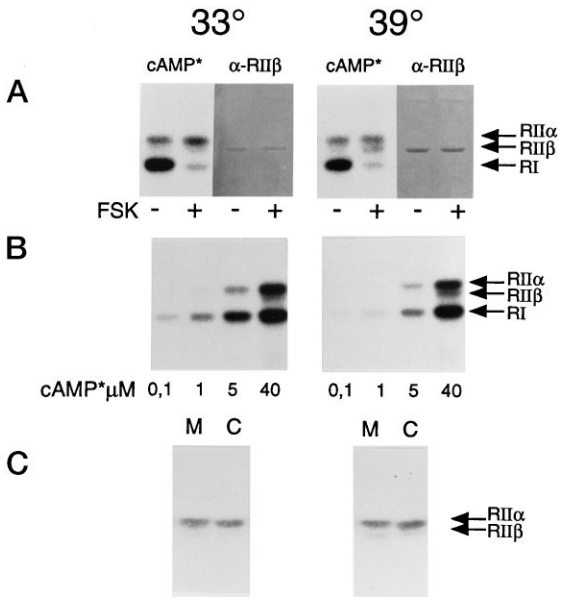

FIG. 7. **cAMP binding of RI, RIIα, and RIIβ in $N^{39°}$ and $N^{33°}$ cells.** 8-N_3[^{32}P]cAMP was cross-linked to total cell proteins (50 μg) as described under "Materials and Methods." *A*, extracts were cross-linked with labeled cAMP (*cAMP**) before (−) and after (+) stimulation of $N^{33°}$ and $N^{39°}$ cells with forskolin (40 μM, 40 min). Also shown (*α-RIIβ*) the immunoblot of the same gel with anti-RIIβ-specific antibodies. *B*, isolated cytoplasmic extracts derived from untreated $N^{33°}$ or $N^{39°}$ cells were cross-linked with increasing concentrations of labeled cAMP (*cAMP*μM*; indicated beneath the autoradiogram). *C*, the binding of labeled RIIα by the overlay method to membrane or cytoplasmic fractions derived from untreated $N^{33°}$ and $N^{39°}$ cells (see "Materials and Methods"). The faint band visible in the membrane fraction of $N^{39°}$ cells is RIIβ cross-reacting with RIIα probe. The specific regulatory subunits were identified as follows: RIIα, RIIβ by immunoblot with specific antibodies, and RI by cross-linking to 8-N_3[^{32}P]-cAMP.

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

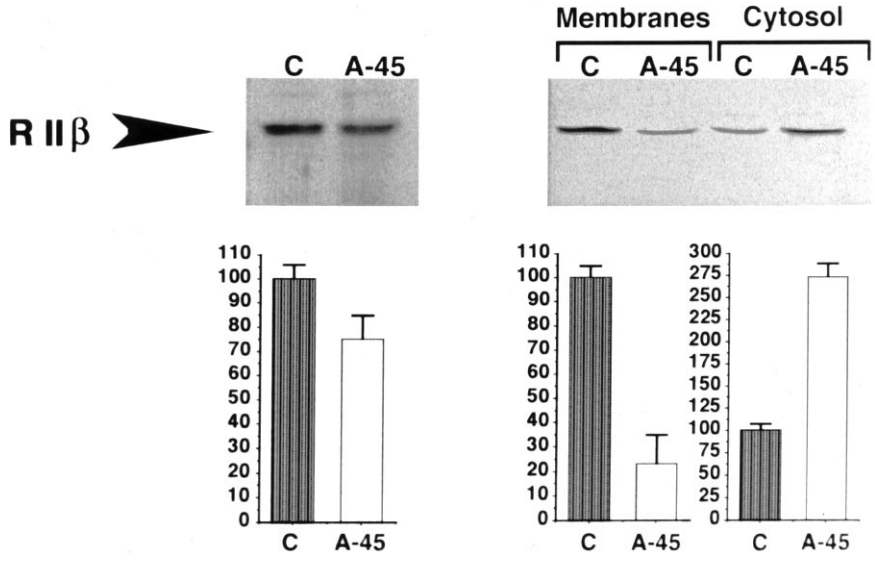

FIG. 8. **Overexpression of AKAP45 in differentiated thyroid cells delocalizes RIIβ to the cytosol.** TL cells were stably transfected with expression vectors encoding either RSV-NEO (*C*) or AKAP45 (*A-45*), an N-terminal deletion mutant of AKAP75. AKAP45 is a cytoplasmic high affinity RII binding protein (33). A pool of ~100 independent clones grown in the presence of TSH (see "Materials and Methods") was analyzed by immunoblot with specific anti-RIIβ antibodies. The *upper panels* show RIIβ (indicated by the *arrow*) in the total (*left*) or fractionated cell extracts (*right*). The *lower panel* shows the RIIβ content determined by densitometric scanning in two independent experiments. The *numbers* shown on the *ordinates* represent arbitrary values.

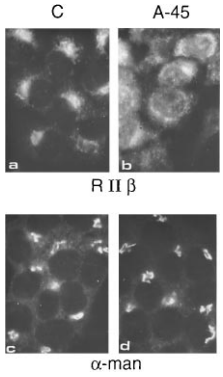

FIG. 9. **Immunofluorescence analysis of RII and α-mannosidase in AKAP45 expressing cells.** AKAP45 expressing cells (*A-45*) and control cells (*C*) were stained with specific antibodies to RIIβ and α-mannosidase, a specific Golgi marker. *a* and *b* are the cells stained with anti-RIIβ antibody; *c* 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*).

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 isoform, 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 mimick 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

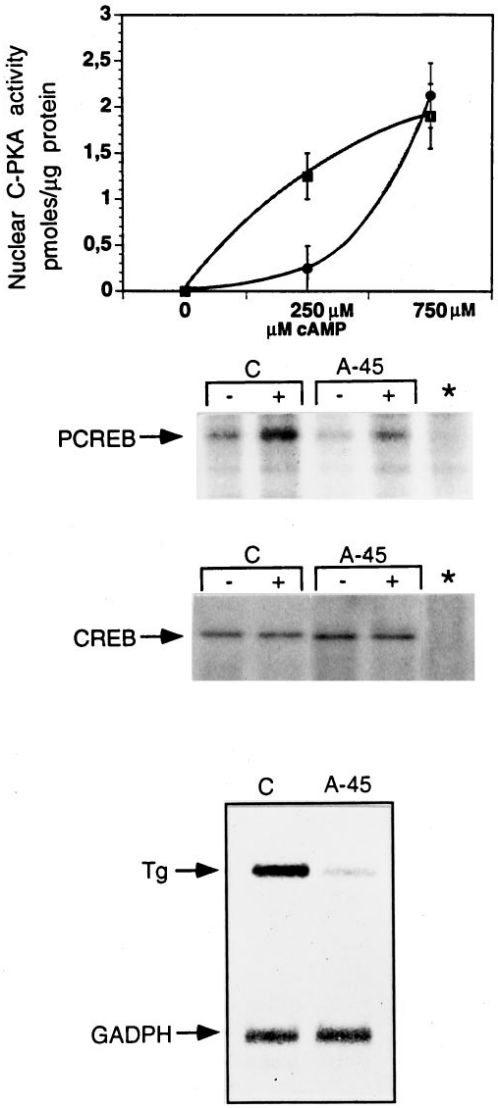

FIG. 10. **cAMP nuclear response is downregulated in AKAP-45 expressing cells.** The *upper panel* shows nuclear accumulation of C-PKA subunit following acute stimulation with 8-Br-cAMP. RSV-NEO (■) and AKAP45 (●) transfected cells were TSH-starved for 2 days and then stimulated with TSH for 12 h. The 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 ^{32}P 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 mCi/ml [^{32}P]orthophosphate or 0.150 mCi/ml [^{35}S]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 SDS-PAGE and visualized by autoradiography. PCREB indicates the phosphorylated CREB protein (*upper panel*); CREB represents the CREB protein detected in [^{35}S]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 GADPH cDNA probes. The specific mRNA bands are indicated by the *arrows*.

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 tha 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 adenylyl cyclase (48)[2] 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 RIIβ. The RIIβ 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 facilitate transmission of Ras signals, even in the absence of maximal stimulation of tyrosine receptors (50).

Localization of PKA and cAMP Nuclear Signaling—Nuclear responses to cAMP are mediated by the activation of transcription factors by C-PKA. After dissociation, C-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. 1*B*). 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 cAMP 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 RIIβ are initially unchanged (this work; 30), and expression of AKAP45 induces the cytosolic translocation of RIIβ and inhibits nuclear C-PKA accumulation without reducing the cellular content of the regulatory subunit (Fig. 8).

The Function of RIIβ—RIIβ is expressed predominantly in endocrine, brain, and reproductive tissues (22) and at low levels in fibroblasts and epithelial cells.[3] We have studied the transcriptional regulation of the different regulatory subunits. Transcription of the RIIβ gene, as determined by reverse transcriptase-PCR, is induced by persistent cAMP stimulation (24–36 h). In contrast, transcription of RIα and RIIα responds more rapidly to cAMP, requiring 6–12 and 12–24 h stimulation, respectively (data not shown). Delayed induction of RIIβ has also been reported in rat Sertoli cells treated with cAMP (41, 42). Prolonged cAMP stimulation maintains RIIβ expression in thyroid cells. These data suggest an autoregulatory loop between RIIβ and cAMP. PKAIIβ adapts 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 adenylyl 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 RIIβ 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 PKAIα, PKAIIα, 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, RIIβ might draw C-PKA from the nucleus at low cAMP concentrations. In this role, RIIβ could function as a repressor, reducing basal cAMP-induced transcription (55). At intermediate cAMP levels, RIIβ efficiently facilitates C-PKA import to the nucleus.

[2] P. Giuliano, unpublished observations.

[3] A. Feliciello, unpublished data.

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