Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 6687–6692, June 1998
Biochemistry

The RIIβ regulatory subunit of protein kinase A binds to cAMP response element: An alternative cAMP signaling pathway

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Communicated by Arthur B. Pardee, Dana–Farber Cancer Institute, Boston, MA, April 6, 1998 (received for review February 26, 1998)

ABSTRACT **cAMP, through the activation of cAMP-dependent protein kinase (PKA), is involved in transcriptional regulation. In eukaryotic cells, cAMP is not considered to alter the binding affinity of CREB/ATF to cAMP-responsive element (CRE) but to induce serine phosphorylation and consequent increase in transcriptional activity. In contrast, in prokaryotic cells, cAMP enhances the DNA binding of the catabolite repressor protein to regulate the transcription of several operons. The structural similarity of the cAMP binding sites in catabolite repressor protein and regulatory subunit of PKA type II (RII) suggested the possibility of a similar role for RII in eukaryotic gene regulation. Herein we report that RIIβ subunit of PKA is a transcription factor capable of interacting physically and functionally with a CRE. In contrast to CREB/ATF, the binding of RIIβ to a CRE was enhanced by cAMP, and in addition, RIIβ exhibited transcriptional activity as a Gal4-RIIβ fusion protein. These experiments identify RIIβ as a component of an alternative pathway for regulation of CRE-directed transcription in eukaryotic cells.**

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

There are two types of PKA, type I (PKA-I) and type II (PKA-II), that share a common C subunit but contain different R subunits (RI and RII, respectively) (2). Through biochemical studies and gene cloning, four isoforms of the R subunits (RIα, RIβ, RIIα, and RIIβ) have been identified (3). Varying the ratios of two isoforms of PKA has been linked to cell growth and differentiation (4, 5). An enhanced expression of RI/PKA-I correlates with active cell growth and cell transformation, whereas a decrease in RI/PKA-I and an increase of RII/PKA-II are related to growth inhibition and differentiation and/or maturation (4, 5).

Overexpression of the RIIβ subunit of PKA in several cancer cell lines results in a striking shift in PKA isozyme distribution, growth arrest, differentiation, and reverse transformation (4, 6, 7). The growth inhibition and reverse transformation correlated with nuclear translocation of RIIβ, because the mutant RIIβ that failed to translocate into the nucleus was incapable of inducing reverse transformation (6).

In this study, we examined whether RIIβ is a nuclear factor that can mediate cAMP responses in cAMP-responsive element (CRE)-containing genes in eukaryotic cells. Ki-ras-transformed NIH 3T3 (DT) cells and DT cells infected with a retroviral vector containing the human RIIβ gene (8) (DTRIIβ) or mutant RIIβ-P (6) (DTRIIβ-P) were used in the present study. The infectants were grown in the presence of 60 μM $ZnSO_4$ for 48 hr before experiments to maximally induce the infected genes (6). On the basis of cell growth, $ZnSO_4$ treatment at 60 μM for 5 days was not toxic to DT, DTRIIβ, or DTRIIβ-P cells (6).

MATERIALS AND METHODS

Materials. DT (NIH 3T3 fibroblasts transformed by v-Ki-*ras* oncogene) and DTRIIβ (DT cells infected with RIIβ retroviral vector) cells were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum, penicillin, and streptomycin (6). 8-Cl-cAMP was obtained from the National Cancer Institute, Drug Synthesis and Chemistry Branch (Bethesda, MD). 8-N_3-[^{32}P]cAMP (60.0 Ci/mmol; 1 Ci = 37 GBq) was obtained from ICN Pharmaceuticals. $\Delta-71$ chloramphenicol acetyltransferase (CAT) (9) and glutathione *S*-transferase (GST)-CREB (10) were provided by M. R. Montminy (Joslin Diabetes Center, Boston, MA).

The double-stranded oligonucleotide containing a single octamer of CRE (TGACGTCA, underlined) 5′-AGAGATTGCC<u>TGACGTCA</u>GAGAGCTAG-3′; Sp1, 5′-ATTCGATC<u>GGGGCGGGGCGAG</u>C-3′; Oct-1, 5′-TGTCGA<u>ATGCAAAT</u>CACTAGAA-3′; and AP-1, 5′-CGCTTGA<u>TGAGTCA</u>GCCGGAA-3′ were from Santa Cruz Biotechnology. The double-stranded oligonucleotide trioctamer of CRE 5′CCTGACGTCATGACGTCATGACGTCA-3′ was prepared as described (11).§ These oligonucleotides were labeled with ^{32}P at the 5′ end with T4 kinase (GIBCO/BRL).

Photoaffinity Labeling and Immunoprecipitation of R Subunits. The photoactivated incorporation of 8-N_3-[^{32}P]cAMP and immunoprecipitation of R subunits was performed as described (12). The polyclonal antibodies (13) specific for the RIα, RIIα, and RIIβ proteins were used in the immunoprecipitation.

Gel Retardation Assay. Nuclear extracts were prepared by the method of Dignam *et al.* (14). The DNA binding assay was performed by a modification of the method of Fried and Crothers (15). Briefly, nuclear extracts (10 μg of protein) were preincubated with poly(dI-dC)·poly(dI-dC) (1 μg), DTT (0.3 mM), and binding buffer (12 mM Tris·HCl, pH 7.9/2 mM $MgCl_2$/60 mM KCl/0.12 mM EDTA/12% glycerol) for 30 min at 4°C. ^{32}P-labeled oligonucleotide was then added and the

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Abbreviations: PKA, cAMP-dependent protein kinase; C subunit, catalytic subunit; R subunit, regulatory subunit; RI and RII, R subunit of PKA-I and PKA-II, respectively; CRE, cAMP-responsive element; CAT, chloramphenicol acetyltransferase; GST, glutathione *S*-transferase.

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§The description of the trioctamer of the CRE sequence in ref. 11 contained a typographical error, 5′-TGA<u>G</u>GTCA-3′, in which the underlined G should have been C.

reaction mixtures were incubated for 10 min at 37°C. The reaction mixtures were then separated on a 4% polyacrylamide gel, and the gel was dried and autoradiographed.

Southwestern Blot Analysis. Southwestern blot analysis was performed as described by Silva *et al.* (16) with minor modifications. Briefly, nuclear extracts (50 μg of protein) or immunoprecipitates obtained from 50 μg of nuclear proteins were electrophoretically separated on SDS/8.5% polyacrylamide gels. The gels were incubated for 3 hr with renaturation buffer (50 mM NaCl/10 mM Tris·HCl, pH 7.4/2 mM EDTA/0.1 mM DTT/4 M urea). Renatured gels were electrically transferred onto nitrocellulose membrane. The blotted nitrocellulose membranes were preincubated for 2 hr at room temperature with preincubation buffer (10 mM Tris·HCl, pH 8.0/2 mM $MgCl_2$/1 mM 2-mercaptoethanol/50 mM NaCl/1% BSA/0.5% gelatin). ^{32}P-labeled oligonucleotide (0.1 μg, 2×10^6 cpm/ml) was then added and the nitrocellulose filters were incubated for 5 hr at room temperature with gentle agitation. The washed nitrocellulose membranes were subjected to autoradiography.

UV Cross-Linking Assay. UV cross-linking assay was performed by the method of Chodosh (17). Briefly, nuclear extracts (60 μg of protein) or the deoxycholate eluate (18) from CREB or RIIβ immunoprecipitates (obtained from 60 μg of nuclear protein) were incubated with a ^{32}P-labeled oligonucleotide, DTT, binding buffer, and poly (dI-dC)·poly (dI-dC) for 30 min at 4°C. The incubation mixtures were irradiated under a Fotodyne UV lamp (wavelength, 310 nm; intensity, 7,000 mW/cm^2), and then 1 μl of 0.5 M $CaCl_2$, 4 μg of DNase I, and 1 unit of micrococcal nuclease were added. The mixture was digested for 30 min at 37°C, an equal volume of 2× SDS/sample buffer was added, and the mixture was boiled for 5 min at 100°C. Samples were subjected to SDS/PAGE in 10% gels, and gels were dried and autoradiographed.

Preparation of R Subunit-GST Fusion Proteins. The N-terminal parts of human cDNAs of RIα, RIIα, and RIIβ (bases 15–222 in RIα, bases 36–397 in RIIα, and bases 33–355 in RIIβ) were inserted into pGEX-2T or pGEX-3X vector (Pharmacia Biotech) (13). For preparation of GST-RIIβ_L (GST-RIIβ whole molecule fusion protein), the human cDNA of RIIβ (bases 78–1,156) was inserted into pGEX-4T-1 vector (Pharmacia Biotech) and *Escherichia coli* JM 109/BL21 cells were transformed with these plasmids. The GST fusion proteins overexpressed in *E. coli* were then purified by using the bulk GST purification module (Pharmacia Biotech).

Transient Transcription Assays. Cells were transfected with 20 μg of somatostatin-CAT fusion gene (Δ−71 SS-CAT plasmid) (9) (provided by M. R. Montminy), by the calcium phosphate precipitation method (19). After 24 hr, fresh medium was added, and the cells were treated for the final 18 hr with $ZnSO_4$ (60 μM) with or without forskolin (10 μM) and then assayed for CAT activity as described (19). Cell lysates preparation and CAT assay were performed as described (19).

Gal4 Experiments. The plasmids used to generate the GalCREB-1 or GalRIIβ fusion proteins for the expression analysis contain the first 147 amino acids of Gal4 fused in-frame to the entire coding region of CREB-327 or the entire ORF of human RIIβ, by using the fusion junction described (20). The GalUAS reporter construct pG5E4CAT contains the CAT gene under the control of an adenoviral E4 TATA box with five copies of a GalUAS immediately upstream as described (20). COS cells were transfected with 5 μg of GalUAS

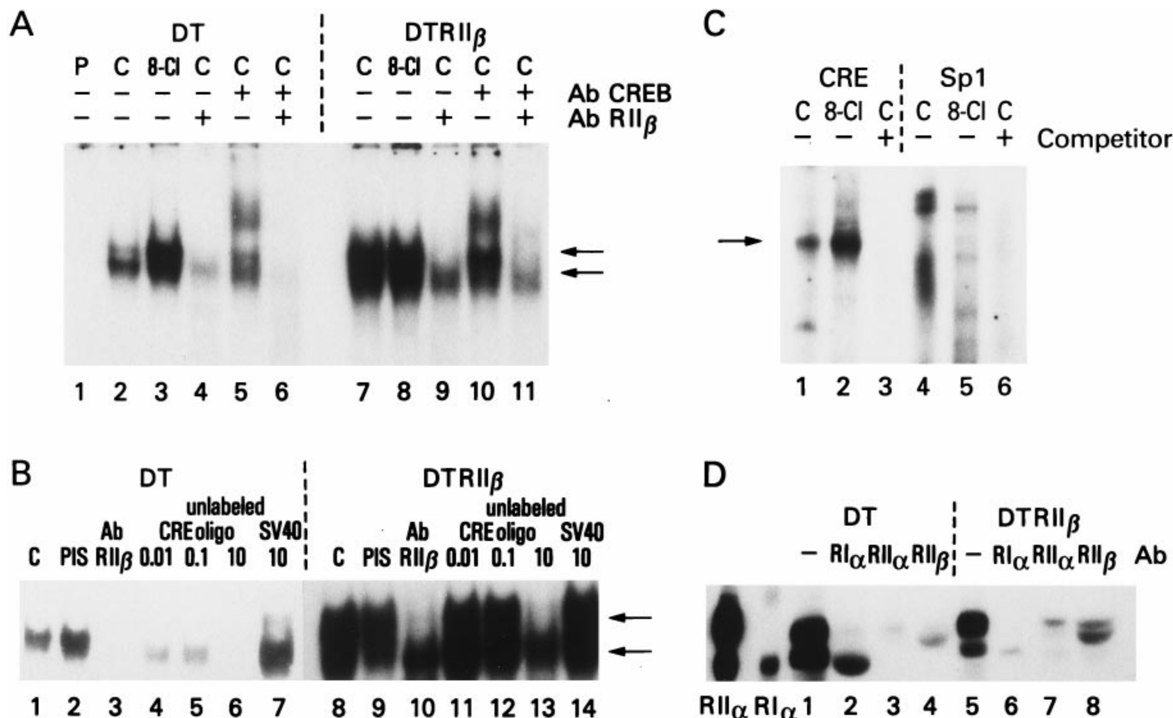

FIG. 1. Increased complex formation between CRE oligonucleotide and nuclear factor(s) in RIIβ-overexpressing cells. (*A*) Gel retardation assay with a trioctamer-CRE (11) oligonucleotide. Nuclear extracts from parental DT (lanes 2–6) and DTRIIβ (lanes 7–11) cells untreated (lanes C) or treated with 8-Cl-cAMP (5 μM, 18 hr) (lanes 8-Cl) were incubated with or without anti-RIIβ antiserum (13) or anti-CREB antibody (Santa Cruz Biotechnology). Lane 1 shows probe only. Arrows indicate DNA–protein complexes. (*B*) Competition experiment. Gel retardation assays were performed as described in *A*. Unlabeled CRE oligonucleotide (0.01–10 ng) or unlabeled simian virus 40 (SV40) oligonucleotide (10 ng) was used as indicated. PIS, preimmune serum. (*C*) Gel retardation assay with a single octamer-CRE oligonucleotide. Nuclear extracts from DT cells, untreated control (lanes C) and treated with 8-Cl-cAMP (5 μM, 18 hr; lanes 8-Cl) were incubated in the absence and presence of unlabeled competitor (lanes 1–3). Lanes 4–6 show Sp1 binding assayed in the same nuclear extracts. (*D*) Photoaffinity labeling and immunoprecipitation of R subunits. Lanes: 1 and 5, photoaffinity labeling only; 2–4 and 6–8, photoaffinity labeling followed by immunoprecipitation with anti-RIα, RIIα, or RIIβ antiserum (13), as indicated. RIα, 48-kDa RI (Sigma); RIIα, 56-kDa RII (Sigma). The data represent one of three to five experiments that gave similar results.

reporter pG5E4CAT and 5 μg of pJATLACZ along with 7.5 μg of pGalCREB-1 or 7.5 μg of pGalRIIβ. Transfections were performed by using Lipofectin. Cell extract preparation and CAT and β-galactosidase assays were performed as described (19).

RESULTS

Increase in CRE–Nuclear Protein Binding in RIIβ-Overexpressing Cells. The first series of experiments was conducted to determine whether RIIβ binds to DNA containing CRE consensus sequence (TGACGTCA) (21). In gel retardation assays, DTRIIβ cells (Fig. 1*A*, lane 7) exhibited increased CRE–nuclear protein binding compared with DT cells (Fig. 1*A*, lane 2). The binding of nuclear protein(s) to CRE was also increased when cells were treated with 8-Cl-cAMP (Fig. 1*A*, lane 3). 8-Cl-cAMP induces growth inhibition and differentiation in a broad spectrum of cancer cell lines (4, 22) and induces reverse transformation in DT cells (6). These effects of 8-Cl-cAMP correlate with down-regulation of RIα and nuclear translocation and up-regulation of the RIIβ subunit of PKA (4, 6, 22).

The anti-RIIβ antiserum added to the gel shift reactions brought about no supershift but resulted in a marked reduction in the CRE–protein complex formation (Fig. 1*A*, lanes 4 and 9). The reduction in the CRE–protein complex formation by RIIβ antiserum was the specific effect of RIIβ antibody because anti-RIα antiserum (data not shown) and preimmune serum (Fig. 1*B*, lanes 2 and 9) had no effect on complex formation. CREB (10) antibody caused a supershift (Fig. 1*A*, lanes 5 and 10). Interestingly, addition of both anti-CREB and anti-RIIβ antibodies almost totally abolished the CRE–nuclear protein binding (Fig. 1*A*, lanes 6 and 11), suggesting a possible role of RIIβ as a positive regulator of CRE binding for other nuclear factors, such as CREB. The specific nature of the CRE–nuclear protein binding was demonstrated by the ability of an unlabeled CRE oligonucleotide to compete for these complexes, whereas simian virus 40 oligonucleotide did not compete (Fig. 1*B*).

The CRE–nuclear protein binding was further assessed by the use of oligonucleotide containing a single CRE [instead of triplet-CRE (11) that was used in Fig. 1 *A* and *B*] and a non-CRE sequence, Sp1 oligonucleotide. Fig. 1*C* shows that the nuclear extracts from DT cells had a major band of CRE binding complex (Fig. 1*C*, lane 1), which was competed by the unlabeled CRE oligonucleotide (Fig. 1*C*, lane 3) and exhibited different mobility from that of Sp1 binding complex (Fig. 1*C*, lane 4). Importantly, 8-Cl-cAMP treatment markedly increased CRE binding (Fig. 1*C*, lane 2), whereas it inhibited Sp1 binding (Fig. 1*C*, lane 5).

The photoaffinity labeling with 8-N_3-[^{32}P]cAMP showed the DTRIIβ cells had an increased ratio of RII/RI proteins as compared with the parental DT cells (Fig. 1*D*, lanes 1 and 5). Immunoprecipitation with the anti-RIIβ antibody (13) demonstrated a high level of 52- to 53-kDa human RIIβ (23) in DTRIIβ nuclear extracts (Fig. 1*D*, lane 8). DT nuclear extracts contained a low level of 50- to 51-kDa mouse RIIβ (3) (Fig. 1*D*, lane 4). Low levels of RIIα were detected in DT and DTRIIβ cells (Fig. 1*D*, lanes 3 and 7). A high level of RIα was detected in DT nuclear extracts (Fig. 1*D*, lane 2) but not in DTRIIβ nuclear extracts (Fig. 1*D*, lane 6). Thus, the increase in nuclear content of RIIβ was correlated with an increased formation of CRE–nuclear protein complex in DTRIIβ cells as compared with DT cells.

Southwestern Blot and UV Cross-Linking Analysis of CRE–RIIβ Binding. Southwestern blot analyses (16) and UV cross-linking (17) allow for the identification of specific protein–DNA interactions and determination of the molecular size of the DNA binding proteins. Southwestern blot analysis revealed the presence of several species of CRE binding proteins, including the 50- to 53-kDa and 43-kDa proteins (Fig. 2*A*, lanes 2 and 4) in DT and DTRIIβ cells. By using the anti-CREB and anti-RIIβ antibody immunoprecipitates, the 43-kDa protein was identified as CREB (10) (Fig. 2*A*, lane 1) and the 50- to 53-kDa protein was identified as RIIβ protein (Fig. 2*A*, lanes 3 and 5). These results were confirmed with UV cross-linking assays. Nuclear extracts from DT, DTRIIβ, and mutant DTRIIβ-P [RIIβ lacks the autophosphorylation site; Ser^{114} of human RIIβ was replaced with Ala (6)] cells were immunoprecipitated with anti-RIIβ or anti-CREB antiserum. The immunoprecipitates were eluted with deoxycholate (18). The results showed that anti-RIIβ immunoprecipitates contained a

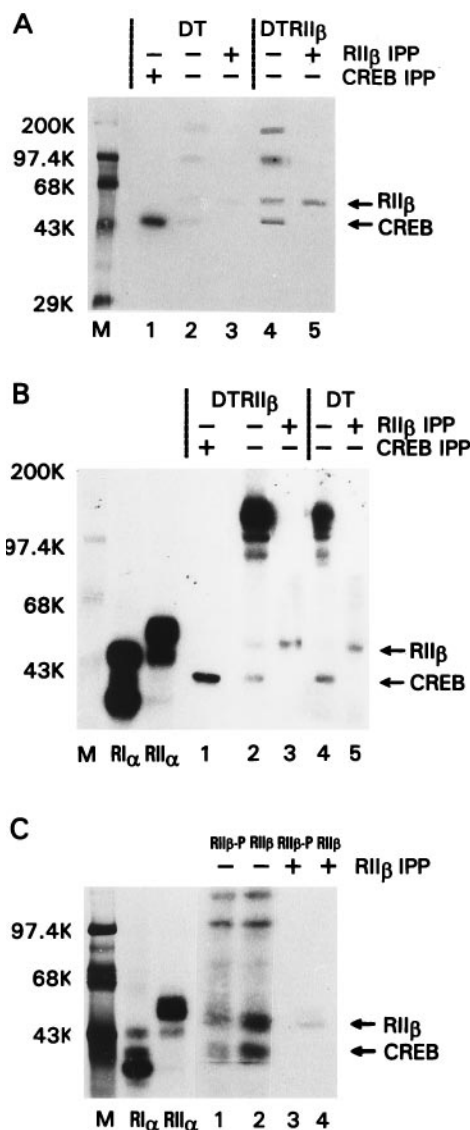

FIG. 2. Southwestern blot analysis and UV cross-linking assay demonstrate RIIβ binding to CRE oligonucleotide. (*A*) Southwestern blot analysis. Lanes: 2 and 4, DT and DTRIIβ cell nuclear extracts; 3 and 5, immunoprecipitated RIIβ from DT and DTRIIβ nuclear extracts; 1, immunoprecipitated CREB from DT nuclear extract; M, ^{14}C-labeled marker proteins. (*B*) UV cross-linking assay of DT and DTRIIβ cell nuclear extracts. Lanes: 2 and 4, DT and DTRIIβ cell nuclear extracts; 3 and 5, immunoprecipitated RIIβ from DT and DTRIIβ nuclear extracts; 1, immunoprecipitated CREB from DTRIIβ nuclear-extract; RIα, photoaffinity-labeled 48-kDa RI; RIIα, photoaffinity-labeled 56-kDa RII (Sigma). (*C*) UV cross-linking assay of DTRIIβ and DTRIIβ-P cell nuclear extracts. Lanes: 1 and 2, DT-RIIβ-P and DTRIIβ nuclear extracts; 3 and 4, immunoprecipitated RIIβ from DTRIIβ-P and DTRIIβ nuclear extracts. The data represent one of three to five experiments that gave similar results.

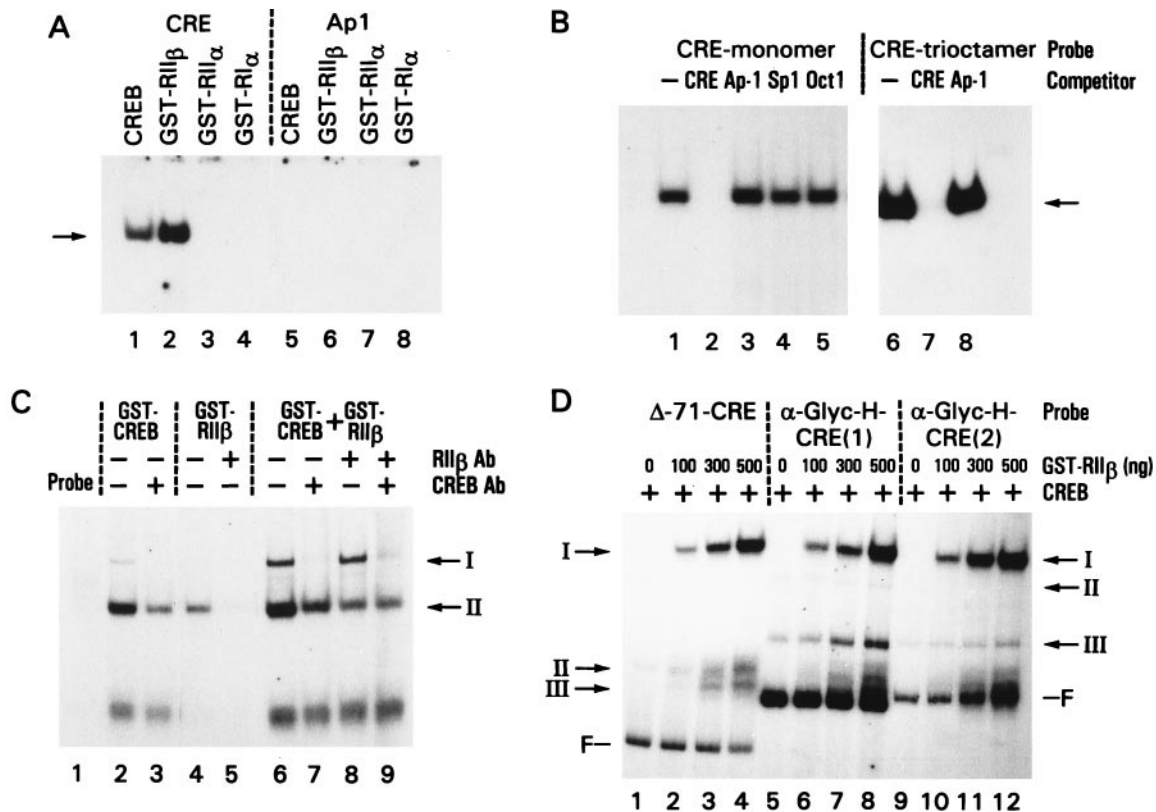

FIG. 3. GST-RIIβ fusion protein binding to the CRE. (*A*) Gel retardation assay of R subunits (N-terminal parts)-GST proteins. The purified RIIβ-, RIIα-, and RIα-GST proteins were used in the assays. Lanes: 1–4, ^{32}P-labeled CRE probe; 5–8, ^{32}P-labeled AP-1 probe; 1 and 5, 0.5 μg of CREB (CREB-1, bZIP, Santa Cruz Biotechnology); 2 and 6, 3 μg of GST-RIIβ; 3 and 7, 3 μg of GST-RIIα; 4 and 8, 3 μg of GST-RIα. (*B*) Gel retardation assay of GST-RIIβ_L (RIIβ whole molecule) protein. Lanes: 1–5, GST-RIIβ_L (500 ng) CRE-monomer-^{32}P probe in the absence (lane 1) and presence (lanes 2–5) of competitor as indicated; lanes 6–8 contained thrombin-digested GST-RIIβ_L (500 ng), CRE-trioctamer ^{32}P-probe in the absence (lane 6) and presence (lanes 7 and 8) of competitor as indicated. (*C*) Gel retardation assay of GST-RIIβ_L in the presence of GST-CREB. ^{32}P-labeled CRE-monomer was used. Lanes: 2 and 3, GST-CREB (10 ng) in the absence and presence, respectively, of anti-CREB antibody (Santa Cruz Biotechnology); 4 and 5, GST-RIIβ_L (100 ng) in the absence and presence, respectively, of anti-RIIβ antibody (Transduction Laboratories, Lexington, KY); 6–9, GST-CREB (10 ng) plus GST-RIIβ_L (100 ng) in the absence and presence of anti-CREB antibody or anti-RIIβ antibody as indicated. (*D*) Gel retardation assay of GST-RIIβ_L in the presence of CREB. ^{32}P-labeled probes are as follows. Lanes: 1–4, $\Delta-71$-CRE; 5–8, α-Gly promoter with one CRE [CRE(1)]; 9–12, α-Gly promoter with CRE dimer [CRE(2)]. All lanes contained 10 ng of CREB (CREB-1, bZIP, Santa Cruz Biotechnology) and the indicated amounts of GST-RIIβ protein; lanes 1, 5, and 9 contained CREB only. Bands I-III, GST-RIIβ- or CREB-CRE complexes; F, free probe. The data represent one of three to five experiments that gave similar results.

single species of CRE binding protein, RIIβ (Fig. 2 *B*, lanes 3 and 5, and *C*, lane 4). Both Southwestern blot analysis and UV cross-linking demonstrated that, apart from RIIβ and CREB, other higher molecular weight species of CRE binding proteins/complexes were also present in the nuclear extracts of DT, DTRIIβ, and DTRIIβ-P cells (Fig. 2). Interestingly, in mutant DTRIIβ-P cells, the CRE binding activities of both RIIβ and CREB were much reduced as compared with the wild-type DTRIIβ cells (Fig. 2*C*). However, the CRE binding activities of the high molecular weight nuclear proteins were the same in the wild-type and mutant RIIβ infectants (Fig. 2*C*).

Binding of GST-RIIβ Protein to CRE. We examined the CRE binding of GST fusion proteins of RIα, RIIα, and RIIβ (N-terminal regions of the R subunits). In gel retardation assays, only GST-RIIβ protein showed complex formation with CRE (Fig. 3*A*, lane 2); GST-RIIα and GST-RIα proteins showed no complex formation (Fig. 3*A*, lanes 3 and 4). The GST-RIIβ did not form complex with Ap-1 oligonucleotide (Fig. 3*A*, lane 6). Thus, the N-terminal region (107 amino acids) of RIIβ was capable of binding to CRE as much as CREB in a sequence-specific manner (Fig. 3*A*).

The GST-RIIβ_L (whole molecule RIIβ-GST fusion protein) also showed the sequence-specific binding to both CRE-monomer and -trioctamer oligonucleotides (Fig. 3*B*). We compared the ability of GST-RIIβ_L to form a complex with a CRE with that of GST-CREB (Fig. 3*C*). Both GST-CREB and GST-RIIβ form complexes with a CRE probe (Fig. 3*C*, lanes 2 and 4, respectively). The formation of these complexes are disrupted by the anti-CREB antibody (Fig. 3*C*, lane 3) and anti-RIIβ antibody, respectively (Fig. 3*C*, lane 5). The addition of both RIIβ and CREB to the CRE binding reaction led to the formation of a band with lower mobility (Fig. 3*C*, lane 6, complex I). This band was disrupted by the addition of the anti CREB antibody (Fig. 3*C*, lane 7) but not the addition of the anti-RIIβ antibody (Fig. 3*C*, lane 8). Complex one is therefore likely to be a higher-order ternary complex of CREB, RIIβ, and the CRE probe. However, the inability of the RIIβ antibody to disrupt this complex means that the presence of RIIβ in complex I, although likely, is not proven.

The higher-order complex also forms in the presence of GST-RIIβ_L and recombinant CREB without the GST tag on natural CREs (Fig. 3*D*). The amount of complex I increased with increasing amounts of RIIβ when the amount of recombinant CREB in the binding reactions was held constant. This was observed with a somatostatin CRE (Fig. 3*D*, lanes 1–4), and the α subunit of the glycoprotein hormone promoter (α-Glyc-H-CRE) in the presence of one (Fig. 3*D*, lanes 5–8) and two (Fig. 3*D*, lanes 9–12) of the two CREs naturally present in this promoter. The exact nature of the faster migrating complexes, II and III, was not investigated, but because they form more readily in the presence of increasing

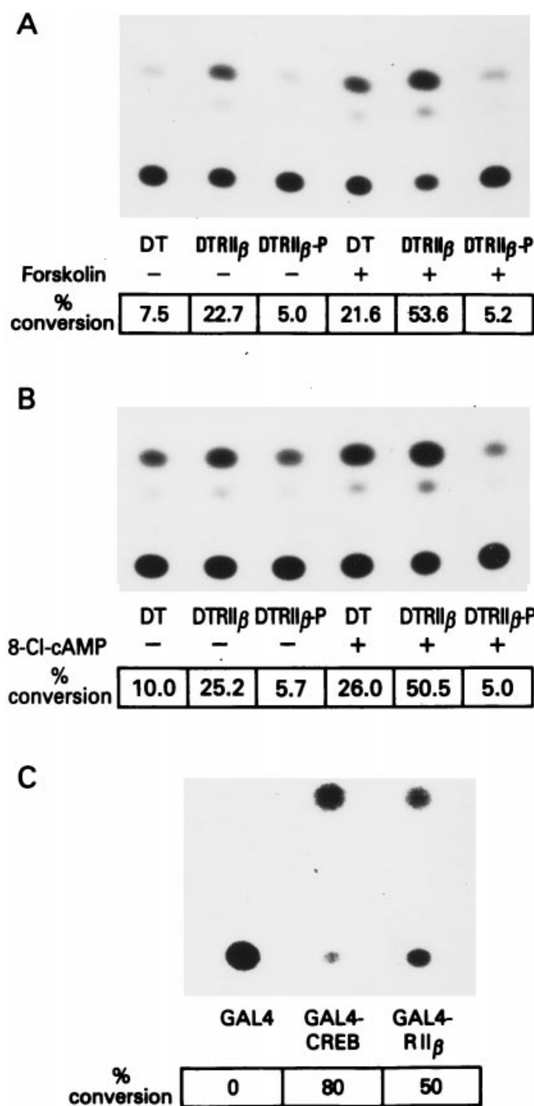

FIG. 4. RIIβ induction of CRE-directed and Gal4-directed gene transcription. (*A*) Transient somatostatin-CAT assay. DT, DTRIIβ, and DTRIIβ-P were transfected with $\Delta-71$ SS-CAT plasmid (20 μg), treated or untreated with forskolin (18 hr, 10 μM), and assayed for CAT activity and β-galactosidase activity. (*B*) Transient somatostatin-CAT assay of cells treated or untreated with 8-Cl-cAMP. DT, DT-RIIβ, and DT-RIIβ-P cells transfected with $\Delta-71$ SS-CAT plasmid, and treated or untreated with 8-Cl-cAMP, and assayed for CAT activity as described in *A*. (*C*) Gal4 experiment. COS cells were transfected with GalUAS reporter construct, pG5E4CAT, pJATLACZ, and pGalCREB-1 or pGalRIIβ and assayed for CAT activity as described for *A*. CAT activities normalized to β-galactosidase activities are shown as mean values of percent conversion derived from three or four experiments.

GST-RIIβ, they are likely to contain RIIβ as a major component.

Transactivation Activity of RIIβ. We examined whether the RIIβ-overexpressing cells are capable of enhancing the CRE-directed gene transcription (Fig. 4*A*). In the absence of cAMP agonist, the RIIβ-overexpressing DTRIIβ cells exhibited 3-fold greater transcription activity of somatostatin-CAT gene as compared with parental DT cells. The CAT activity in OT1521 control vector transfectants was the same as that in DT cells. When DT cells were treated with forskolin (10 μM), the CAT activity increased to the levels of DTRIIβ cells in the absence of forskolin treatment. The CAT activity in DTRIIβ cells increased further upon forskolin treatment (Fig. 4*A*). Most interestingly, in the mutant RIIβ-P-overexpressing cells (DTRIIβ-P), the CAT activity was much reduced below that of parental DT cells, and the activity remained low on stimulation with forskolin (Fig. 4*A*). Fig. 4*B* shows that treatment with 8-Cl-cAMP increased the CAT activity in DT and DT-RIIβ cells but not in the mutant RIIβ-P-overexpressing cells, supporting the data of forskolin effects on the CAT activities of these cells (Fig. 4*A*). Moreover, an increase in CAT activity shown in DT cells upon 8-Cl-cAMP treatment coincides with the increase of CRE binding demonstrated in these cells on 8-Cl-cAMP treatment (Fig. 1 *A* and *C*). Thus, RIIβ displayed positive effects on the CRE-directed gene transcription in the absence of cAMP agonist, and 8-Cl-cAMP and forskolin further increased such transcription.

To test whether RIIβ protein alone can induce gene transcription, we constructed a plasmid that directs the synthesis of a GalRIIβ fusion protein and examined the consequences of expression on a GalUAS reporter construct. We compared the behavior of a GalRIIβ and GalCREB-1 (20) fusion protein in COS cells. As shown in Fig. 4*C*, GalRIIβ was capable of inducing CAT activity, although the CAT activity by GalRIIβ was lower than that induced by GalCREB-1. These data suggest that RIIβ protein has the ability to stimulate transcription.

DISCUSSION

We have demonstrated in the present study that the regulatory subunit RIIβ of PKA can interact with a CRE both physically and functionally. Our results showing that the RIIβ is a CRE transcription factor raises the possibility for an entirely different mechanism for cAMP regulation of gene expression. An established mechanism of cAMP/CRE-regulated transcription involves the CREB/ATF family of transcription factors. However, the CREB/ATF transcription factor family is unlikely to mediate all transcriptional responses to cAMP during development, differentiation, and endocrine homeostasis. The recent demonstration of CREB/ATF-independent cAMP gene regulation through orphan nuclear hormone receptor confirms the presence of multiple mechanisms for cAMP-regulated transcription (24, 25).

The function of R subunit in PKA has been confined to its inhibition of the C subunit. Recently, however, binding of R subunit to proteins other than the C subunit of PKA has been explored with the yeast two-hybrid system (26). Moreover, another mechanism of C subunit activation in a cAMP- and R subunit-independent manner has also been shown (27).

A function for the R subunit apart from kinase inhibition is also suggested by the finding that the cAMP binding domain of RII shares extensive homology with the bacterial catabolite activator protein (CAP) (28). CAP is not associated with a kinase but has the ability to regulate cAMP-mediated gene expression by binding to DNA. The evolutionary conservation between RII and CAP suggests that RII may have retained the DNA binding function of bacterial CAP, as well as its ability to bind cAMP.

The discovery of nuclear translocation of both the R and C subunits of PKA was made by Jungmann (29) in cells under gonadotropin stimulation. Previous studies from this laboratory have correlated the nuclear translocation of PKA RII subunit with tumor regression and reverse transformation. It has been shown by indirect immunofluorescence method that the RII but not RI subunit of PKA accumulates into nucleus in MCF-7 human breast tumors undergoing regression after hormone withdrawal of the host animals (30). Also, a rapid nuclear translocation of RII (within 30 min of cAMP stimulus) was detected by indirect immunofluorescence in the cAMP-analog-induced reverse transformation of Harvey murine sarcoma virus-transformed NIH 3T3 fibroblasts (31).

By several different approaches, we demonstrated in the present study that although it may not be sufficient, the RIIβ

regulatory subunit can participate in CRE-regulated transcription. Importantly, our results suggest that the transcriptional activity of RIIβ may trigger cAMP-induced growth inhibition and phenotypic reversion of the ras-transformed fibroblasts.

In further support of the role of RIIβ in CRE-directed transcription, expression of RIIβ-P, which lacks the autophosphorylation site, inhibits the cAMP response in cells expressing endogenous wild-type RIIβ (ras-transformed NIH 3T3, DT cells) (6). The dominant negative activity of RIIβ-P may be due to an ability to trap wild-type RIIβ in inactive dimers. Mutant RIIβ-P, in such inactive dimers, exhibits a reduced ability to bind to a CRE and shows weak CRE transcription activity when compared with wild-type RIIβ.

Importantly, we directly demonstrated RIIβ–CRE binding by using a GST-N-terminal part, as well as a whole molecule of RIIβ protein, demonstrating RIIβ has CREB/ATF-independent binding activity. We used the N-terminal part of the R subunits to construct the GST-R proteins because the N terminus contains the most divergent primary structures (amino acid sequence) among the RIα, RIIα, and RIIβ. In contrast to GST-RIIβ, the GST-RIIα protein did not show CRE binding. This may reflect the large primary structure difference between these two proteins. The homology of the N-terminal amino acid sequence included in these constructs is only 25%. Whether the inability of the GST-RIIα protein to bind CRE is due to the amino acid sequence difference awaits further studies. By using RIIβ whole molecule-GST fusion protein (GST-RIIβ_L), we further demonstrated that RIIβ is capable of binding the CRE-monomer and -trioctamer oligonucleotides, as well as $\Delta-71$ somatostatin and α-glycoprotein hormone promoters. Interestingly, RIIβ in the presence of CREB forms a higher-order complex with natural CREs.

The role of RIIβ in CRE-directed transcription is further supported by the previous reports that showed that transcriptional regulation of the somatostatin gene by cAMP requires PKA-II. The cAMP-regulated expression of a somatostatin fusion gene was greatly reduced in mutant PC12 line A126–1B2, which is deficient in PKA-II (9). Overexpression of RIIβ in A126–1B2 cells restored the somatostatin fusion gene transcription and cAMP-responsiveness, and RIIβ antisense specifically inhibited the RIIβ-induced CRE transcription (8).

We conclude that RIIβ, a cAMP receptor protein, is a nuclear factor that can participate in cAMP responses in CRE-containing genes in eukaryotic cells, perhaps in a manner similar to that in bacterial CAP. RIIβ may function by interacting with CREB and other CRE transcription factors or may act independently of them. Further studies on the RIIβ function may ultimately delineate the PKA isozyme-dependent function of cAMP (4), namely, the specificity of cAMP in eukaryotic gene regulation underlying cell growth, development, and differentiation.

We thank P. D. Drew for critical reading of the manuscript, K. Becker for his technical advice, and M. R. Montminy for providing us with $\Delta-71$ CAT plasmid and GST-CREB protein.

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