Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 3703–3707, June 1988
Biochemistry

Genetic characterization of a brain-specific form of the type I regulatory subunit of cAMP-dependent protein kinase

(gene cloning/cDNA expression/testis/DEAE-cellulose chromatography)

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Communicated by Edmond H. Fischer, January 19, 1988

ABSTRACT **An isoform (*RIβ*) of the regulatory type I subunit gene of cAMP-dependent protein kinase (EC 2.7.1.37) has been characterized in mouse. The open reading frame of the RIβ cDNA is 72% identical in nucleotide sequence with the previously cloned *RI* gene, now referred to as *RIα*. Both genes code for a protein of 380 amino acids and their proteins are 82% identical in amino acid sequence. Sequence similarity is highest in the regions that form the pseudosubstrate-binding site of the catalytic subunit and the two cAMP binding domains. The amino-terminal portion shows the greatest dissimilarity, suggesting that the isoforms may differ in their dimerization properties or interaction with other proteins. In contrast to *RIα*, which is constitutively expressed in all tissues, *RIβ* is expressed in a highly tissue-specific manner. Brain and spinal cord contained significant levels of RIβ mRNA, testis RNA gave a detectable signal, and all other tissues tested were negative. Expression of a RIβ cDNA in NIH 3T3 cells resulted in the appearance of a RI subunit protein that migrated more slowly than RIα after $NaDodSO_4$/PAGE. The native form of RIβ in brain could also be distinguished from RIα by its abnormal migration on $NaDodSO_4$/PAGE. RIβ protein produced in 3T3 cells was shown to be functional by its ability to form a cAMP-dependent holoenzyme with the catalytic subunit.**

Protein sequence analysis suggests that the family of serine/threonine- and tyrosine-phosphorylating enzymes diverged from an ancestral catalytic protein and evolved a wide range of regulatory domains and substrate specificities (1). In many cases, these domains control the enzyme by inhibiting activity in the absence of specific inducers. cAMP-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37; referred to here as PKA) is unlike many other protein kinases in that the regulatory domain of this enzyme is derived from a separate gene. The PKA holoenzyme contains a regulatory subunit dimer and two catalytic subunit proteins. The enzyme is activated when the regulatory dimer binds four molecules of cAMP and the catalytic subunits are released (2).

Two forms of PKA regulatory subunit, RI and RII, have been purified to homogeneity (3, 4). These proteins are similar in the carboxyl half of the molecule, which interacts with cAMP, but quite dissimilar in the amino-terminal region, which controls R subunit dimerization. Both proteins possess low- and high-affinity binding sites for cyclic nucleotide (5), but they can be distinguished by their selective binding of cAMP analogues (6). RI and RII are also different in apparent molecular mass on $NaDodSO_4$/PAGE, with RI (49 kDa) being smaller than RII (54 kDa), and the two isoforms can be separated by ion-exchange chromatography (7). An additional difference between these subunits lies in the "hinge" region of the protein, which binds and inhibits the catalytic (C) subunit (8). Unlike RI, the pseudosubstrate binding site of RII can be phosphorylated, resulting in a change in affinity between RII and C. RI is also phosphorylated at sites near the amino terminus *in vivo*, but the importance of these covalent modifications is unknown (9).

The functional significance of having two types of PKA, often within the same cell, is poorly understood. This is complicated by the recent discovery that a second form of C subunit gene is expressed throughout mammalian tissues (10) and that an isoform of the *RII* gene is also expressed in specific tissues (11). Here we present evidence for a regulatory subunit gene that is expressed only in brain and testis. DNA sequence comparisons, as well as biochemical characteristics of the expressed protein, demonstrate that this regulatory subunit gene is a related form of *RI*. We have designated this gene *RIβ*.

MATERIALS AND METHODS

The techniques used in this work have been described in detail elsewhere (12, 13). For ion-exchange chromatography, cell pellets were homogenized in 50 mM Tris·HCl, pH 7.6/4 mM dithiothreitol/1 mM EDTA/0.1 mM phenylmethylsulfonylfluoride/0.25 mM isobutylmethylxanthine. The extract was sedimented at 100,000 × *g* for 1 hr at 4°C and the supernatant was filtered through a 0.2-μm-pore Acrodisc (Gelman). Soluble protein was loaded (<10 mg) onto a Bio-Gel DEAE-cellulose ion-exchange column and subjected to high-performance liquid chromatography.

RESULTS

RIβ mRNA Is Expressed Preferentially in Brain. While characterizing the 3′ end of the mouse *RI* subunit gene of PKA, a genomic fragment (*RIβ-11*) was isolated that was similar to the last exon of RIα (H. Knickerbocker and G.S.M., unpublished data) but showed significant changes in DNA sequence; 5 of the final 56 predicted amino acids in this exon were different, and almost half of the conserved residues (23/51) showed changes in codon usage. To determine whether this related form of *RI* DNA was an expressed gene, RNA was isolated from various mouse tissues, subjected to blot hybridization analysis, and probed at high stringency with *RIβ-11*-derived RNA (Fig. 1). A 2.8-kb transcript was readily detectable in brain, and lower amounts of the RIβ mRNA were present in testis RNA. This RIβ transcript is clearly distinguishable from the 3.2- and 1.7-kb mRNA derived from the ubiquitous *RI* gene (now referred to as *RIα*). We have quantitated the amount of RIβ mRNA present in these samples by a solution hybridization method using ^{32}P-labeled RNA (12) and determined that whole brain contains on average about 96 molecules of RIβ mRNA per

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Abbreviations: PKA, cAMP-dependent protein kinase; RI and RII, PKA regulatory subunit types I and II; C, PKA catalytic subunit.

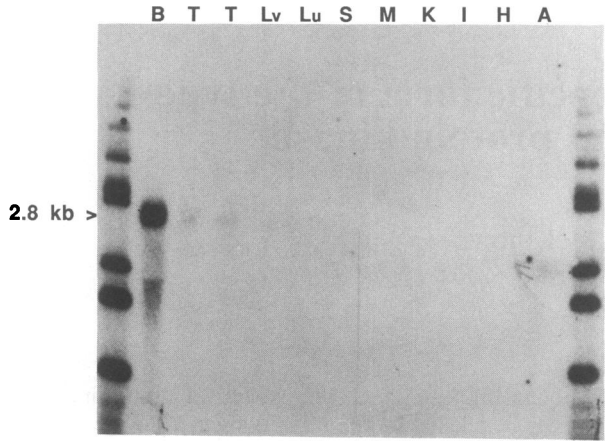


FIG. 1. Detection of RIβ mRNA in mouse tissue. Total RNA was isolated from various mouse tissues (13), electrophoresed under denaturing conditions through a 1% agarose gel, and blotted onto Nytran (Schleicher & Schuell). The filter was hybridized overnight with a ^{32}P-labeled RNA probe corresponding to 420 bases of *RIβ* genomic DNA (13) and then washed and exposed to film overnight. The sources of mouse RNA are indicated as follows: B, brain; T, testis; Lv, liver; Lu, lung; S, spleen; M, skeletal muscle; K, kidney; I, small intestine; H, heart; and A, adipose tissue. The size of the RIβ mRNA [2.8 kilobases (kb)] was determined by using denatured ^{32}P-labeled DNA markers.

cell and testis contains 2 molecules per cell, and in other tissues RIβ mRNA is undetectable (data not shown).

RIβ Amino Acid Sequence. By using the genomic fragment of RIβ as a hybridization probe, a λgt11 mouse brain cDNA library was screened and the largest cDNA (clone P4.4) was isolated, restriction mapped (Fig. 2*A*), and sequenced. The nucleotide sequence of clone P4.4 predicted an open reading frame of 1140 nucleotides, coding for a protein of 380 amino acids (Fig. 2*B*). The overall similarity of nucleotide sequence within the coding regions of *RIβ* and *RIα* is 72%. No similarity in sequence was observed in 90 base pairs (bp) of 5′ and 450 bp of 3′ flanking DNA.

The predicted protein sequence of the RIβ protein is 82% identical to RIα. The region of greatest dissimilarity occurs within the first 80 residues of the amino terminus, where 35 amino acid differences are found. This region of the protein controls, in part, regulatory subunit dimerization (14) and is the most variable among the four regulatory subunits characterized in mammals. The RIβ subunit contains a pseudosubstrate site (Agr-Arg-Gly-Gly) at the same position (95–98) as in RIα, where the sequence is Arg-Arg-Gly-Ala. The RII subunits contain an Arg-Arg-Xaa-Ser at this position, which is autophosphorylated (4, 11). Very few differences in amino acid sequence (90% identical) occur in the second half of the protein. This region is important for cAMP binding and contains a duplication that is conserved in all regulatory subunit proteins (13).

Expression of RIβ Protein in NIH 3T3 Cells. To characterize the RIβ protein, a eukaryotic expression vector was constructed and transfected into mouse NIH 3T3 cells (Fig. 3*A*). This plasmid (RIβ-EV) was made by inserting the RIβ cDNA, by using *Bam*HI linkers, into the *Bgl* II restriction site of Zem 3, a pUC13-derived plasmid containing the zinc-inducible mouse metallothionein-1 promoter (MT-1) and the polyadenylylation signal sequence from the human growth hormone gene (17). After calcium phosphate-mediated cotransfection (18) with the neomycin resistance plasmid PKOneo (19) and selection in G418, several NIH 3T3 cell clones were isolated that expressed the RIβ cDNA. Fig. 3 *B* and *C* shows the zinc-inducible expression of RIβ mRNA and protein, respectively, in clone Rβ17. As indicated, incubation of cells in 80 μM zinc sulfate, a concentration that induced a significant accumulation of RIβ mRNA, stimulated the appearance of a regulatory subunit protein with an apparent molecular mass of 53 kDa. This was a surprising result, because both the *RIβ* and *RIα* genes code for proteins containing 380 amino acids, both with a molecular mass of approximately 49 kDa.

RIβ protein having been produced in cells, it was important to determine whether this regulatory subunit could associate with C subunit to form a functional holoenzyme. To test for this we incubated Rβ17 cells with zinc, to induce expression of RIβ protein, and then subjected cell extracts to DEAE-cellulose ion-exchange chromatography on a HPLC column. PKA binds to DEAE specifically through an interaction of the regulatory subunit with the anion-exchange resin. The different forms of the holoenzyme, as well as "free" regulatory subunit, are then separated by increasing NaCl concentration (8); type I holoenzyme elutes from DEAE at lower concentrations of NaCl, beginning at about 30 mM, whereas type II holoenzyme elutes above 100 mM NaCl. RI subunit not associated with C protein elutes just prior to the type II holoenzyme.

After fractionation of Rβ17 cell extracts we observed only type II holoenzyme (Fig. 4); type I holoenzyme was undetectable. To verify that cells synthesized the RIβ protein in response to zinc treatment, we probed the indicated fractions by using the immunoblot method. The bar in Fig. 4 indicates those fractions that contained RIα and RIβ protein. This result demonstrates that 3T3 cells contain a significant amount of free RI protein and that overproduction of additional RI has no effect on the type of holoenzyme that appears in cells.

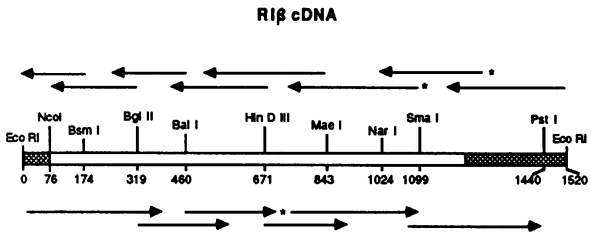
If the "free" RIβ protein overexpressed in 3T3 cells is functional, then extracts prepared from zinc-treated cells should inhibit kinase activity in a cAMP-dependent manner. To test for this, we incubated 6 ng of pure bovine C subunit (gift from J. Scott, University of Washington) with cell extracts derived from zinc-treated and non-zinc-treated cells under conditions that favor holoenzyme formation (20). Kinase activity was then measured (Fig. 5). As indicated, exposing C subunit to increasing amounts of extract isolated from zinc-treated Rβ17 cells resulted in a significant decrease in catalytic activity. Enzyme inhibition was not due to degradation, because equal amounts of kinase activity were recovered in both types of samples when assayed in the presence of cAMP. These results argue that the newly expressed protein is a functional regulatory subunit; RIβ associates with C subunit to block activity and RIβ binds cAMP, resulting in holoenzyme derepression.

Detection of RIβ Protein in Brain. The mobility difference between RIβ and RIα after $NaDodSO_4$/PAGE facilitates the direct assay for this protein in mouse tissues. The identification of RIβ in brain after DEAE-cellulose chromatography is shown in Fig. 6. The kinase activity profile demonstrates a peak of cAMP-independent activity (free C subunit) eluting near the start of the NaCl gradient, as well as the type II holoenzyme. In agreement with previous reports, brain has significant levels of only type II holoenzyme (21). RI immunoblot analysis demonstrates the elution in the free RI-subunit fractions of a regulatory subunit with the same mobility characteristics as RIβ. This result agrees with the suggestion that a second form of RI subunit is present in brain and argues that the apparent size difference of the protein made in 3T3 cells transfected with the RIβ cDNA is not artifactual. Interestingly, a third band of even higher apparent molecular mass was observed in the free RI fractions of brain, but not 3T3 cells (Fig. 4). The origin of this band is unknown, but it may represent another isoform of RI.

DISCUSSION

We have characterized a mouse regulatory subunit gene of PKA that is expressed primarily in brain. The coding region

A



5' CGCGTCCCGCGTCCTCGCGCAGCCTCCTCCGGCCTCGGGCACCGCGCATCTACAGGAGCATAGAAGACATTCTGACC

B

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Met Ala Ser Pro Ser Cys Phe His Ser Glu Asp Glu Asp Ser Leu Lys Gly Cys Glu Met Tyr Val Gln Lys His Gly Ile Gln Gln Val Leu Lys Glu Cys Ile Val   35
ATG GCC TCC CCA TCA TGC TTC CAC TCG GAG GAT GAG GAC TCT CTG AAA GGA TGC GAG ATG TAC GTG CAG AAA CAT GGC ATC CAG CAG GTG CTC AAA GAA TGC ATT GTG
      G   T GGC AGT ATG GCA AC  AGT       A     CGG AG    C CGG  A         C    T              G   C AAT         GCC C         G   C  C    C
            Gly     Met Ala Thr         Glu     Arg         Arg Glu         Leu                    Asn         Ala Leu         Asp Ser

His Leu Cys Val Ala Lys Pro Asp Arg Pro Leu Arg Phe Leu Arg Glu His Phe Glu Lys Leu Glu Lys Glu Glu Asn Arg Gln Ile Leu Ala Arg Gln Lys Ser Asn   71
CAC CTC TGT GTC GCC AAG CCG GAC CGG CCA CTG CGA TTC CTC CGG GAG CAC TTT GAG AAG TTG GAG AAG GAG GAA AAC AGG CAG ATC CTG GCT CGG CAG AAG TCA AAC
  G   G   C ACT A G CG    C   G A    C A   GC        T       A T          G                   G GC   A       T  A  TG   TA        A A C GG
Gln         Thr Thr Arg     Glu         Met Ala                 Tyr         Arg                   Ala           Gln Cys Leu         Thr Gly

Ser Gln Cys Asp Ser His Asp Glu Glu Ile Ser Pro Thr Pro Pro Asn Pro Val Val Lys Ala Arg Arg Arg Arg Gly Gly Val Ser Ala Glu Val Tyr Thr Glu Glu  107
TCC CAG TGT GAT TCC CAC GAT GAG GAG ATC TCC CCA ACA CCT CCA AAC CCC GTG GTC AAG GCG CGT CGC CGG CGG GGC GGT GTG AGT GCT GAA GTC TAC ACT GAA GAA
AT   GT AC    C   G AGG   G   C           T  T C    C   C   T   A       G     GC   A       C   A   T  C  A C                T           G  G
Ile Arg Thr         Arg Glu Asp                                             Gly                 Ala Ile

Asp Ala Val Ser Tyr Val Arg Lys Val Ile Pro Lys Asp Tyr Lys Thr Met Thr Ala Leu Ala Lys Ala Ile Ser Lys Asn Val Leu Phe Ser His Leu Asp Asp Asn  143
GAT GCT GTC TCC TAC GTG AGG AAG GTC ATT CCC AAG GAC TAT AAG ACC ATG ACC GCG CTG GCC AAG GCC ATT TCT AAG AAC GTG CTC TTT TCT CAC CTG GAC GAC AAC
        C           T  A       T       A  A  T           A    G T  T T A              C GAA       T       G       A       T  T  T
      Ala                                                  Ala                          Glu

Glu Arg Ser Asp Ile Phe Asp Ala Met Phe Pro Val Thr His Ile Gly Gly Glu Thr Val Ile Gln Gln Gly Asn Glu Gly Asp Asn Phe Tyr Val Ile Asp Gln Gly  179
GAG AGA AGT GAC ATA TTT GAC GCC ATG TTT CCT GTC ACT CAC ATC GGT GGG GAA ACA GTC ATA CAG CAA GGG AAT GAA GGA GAT AAT TTC TAT GTG ATT GAC CAA GGA
                T       T  T           A     T C TTT   T C    A  G  G  T  T           T G         G       C                   T
                                           Ser Phe     Ala                              Asp

Glu Val Asp Val Tyr Val Asn Gly Glu Trp Val Thr Asn Ile Ser Glu Gly Gly Ser Phe Gly Glu Leu Ala Leu Ile Tyr Gly Thr Pro Arg Ala Ala Thr Val Lys  215
GAA GTA GAT GTA TAT GTG AAC GGG GAA TGG GTG ACC AAC ATC AGT GAG GGG GGA AGC TTC GGG GAG CTG GCT CTC ATC TAC GGC ACC CCC AGA GCG GCT ACC GTG AAG
  A G       C       C   T AAT         CA      GT G T G G   A  A   G       T  A              T G   T   T   A   A          A   C   T   C   A
    Met                   Asn         Ala     Ser Val Gly

Ala Lys Thr Asp Leu Lys Leu Trp Gly Ile Asp Arg Asp Ser Tyr Arg Arg Ile Leu Met Gly Ser Thr Leu Arg Lys Arg Lys Met Tyr Glu Glu Phe Leu Ser Lys  251
GCC AAG ACG GAC CTC AAG CTC TGG GGT ATC GAC CGT GAC AGC TAC AGG CGC ATC CTC ATG GGA AGC ACA CTG AGG AAA CGC AAG ATG TAT GAG GAG TTC CTC AGC AAA
  A       A A  G G   A  G       C           A           C A A A                       T    C A  G A G              A  A       T  T
            Asn Val

Val Ser Ile Leu Glu Ser Leu Glu Lys Trp Glu Arg Leu Thr Val Ala Asp Ala Leu Glu Pro Val Gln Phe Glu Asp Gly Glu Lys Ile Val Val Gln Gly Glu Pro  287
GTC TCC ATC CTA GAA TCC CTG GAG AAG TGG GAA CGC CTG ACT GTA GCT GAT GCC CTG GAG CCT GTG CAG TTT GAA GAT GGA GAG AAA ATT GTT GTG CAG GGG GAG CCT
  G   T  T T    G   T       C           G   T   C   A       C       A T           C                   C   G  C  G       A  A
                          Asp                                                                         Gln

Gly Asp Asp Phe Tyr Ile Ile Thr Glu Gly Thr Ala Ser Val Leu Gln Arg Arg Ser Pro Asn Glu Glu Tyr Val Glu Val Gly Arg Leu Gly Pro Ser Asp Tyr Phe  323
GGA GAT GAC TTC TAC ATC ATC ACA GAG GGC ACT GCT TCA GTC CTC CAG CGA CGA TCC CCC AAT GAG GAG TAC GTG GAA GTG GGG CGC CTT GGA CCC TCT GAC TAC TTT
  G       G     T       T TT            A     G T   G   G       T   G   A GAA   C   A   A  TT   T           A   A   G   G   T       T   T
        Glu     Phe       Leu             Ala                       Glu             Phe

Gly Glu Ile Ala Leu Leu Leu Asn Arg Pro Arg Ala Ala Thr Val Val Ala Arg Gly Pro Leu Lys Cys Val Lys Leu Asp Arg Pro Arg Phe Glu Arg Val Leu Gly  359
GGG GAG ATT GCC CTG CTG CTG AAT CGG CCC CGT GCA GCC ACT GTG GTG GCC CGG GGT CCC CTC AAG TGT GTG AAG TTA GAC CGG CCT CGT TTT GAG CGT GTC CTG GGC
  T   A                   A       T   T   G   T               T           C   T T G       C   T       G               G       A   C       T
                        Met

Pro Cys Ser Glu Ile Leu Lys Arg Asn Ile Gln Arg Tyr Asn Ser Phe Ile Ser Leu Thr Val                                                          380
CCC TGC TCT GAG ATC CTG AAG AGG AAC ATC CAG CGT TAC AAC AGC TTC ATC TCC CTA ACT GTC TGAGCTTGTGTTGCGCCTGCACCCCTGGGGGCC     3'
  G       A   C       C     C                 AG                G G       G T C
            Asp                               Gln               Val       Ser
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FIG. 2. Predicted sequence of the RIβ protein and its comparison to RIα. (*A*) Restriction sites for various endonucleases are indicated along the 1520-bp cDNA, P4.4. The open area of the bar indicates the protein-encoding region. The arrows identify fragments that were subcloned in pGEM-3 (Promega Biotec, Madison, WI) and sequenced by the dideoxy method. Asterisks indicate clones that were generated by exonuclease treatment. (*B*) The 1250 nucleotides of RIβ cDNA are shown together with the predicted amino acid sequence of the RIβ protein. Below this sequence are shown the nucleotide differences observed in the open reading frame of the *RIα* gene, followed by the corresponding changes in RIα amino acid sequence. The numbers to the right of the RIβ protein sequence indicate amino acid number for the last residue displayed on that line as counted from the alanine that follows the initiator methionine. The bar positioned over residues 95–98 identifies one region thought to interact with the C subunit, while the two solid bars in the second half of the protein identify a sequence duplication conserved in all cAMP-binding proteins (13).

of this gene is closely related to the ubiquitous *RIα* subunit gene and predicts a protein that is 90% identical with RIα over 80% of its length (Fig. 2). This area of homology regulates the cAMP-dependent inhibition of enzyme activity. Sequences within the first 80 amino acids that are important for subunit dimerization, however, have diverged and are only 56% identical. This argues that dimerization may be subunit specific.

RIβ mRNA is at least 100 times more abundant in brain than in any other tissue analyzed in Fig. 1, with the exception of testis. Various regions of mouse brain such as cortex, hypothalamus, thalamus, pons/medulla, striatum, and hippocampus express equivalent amounts of RIβ mRNA (data not shown). Spinal cord also contains significant levels of RIβ mRNA. At present it is not known which cell types within nervous tissue express this gene. Recently, it has been discovered that RIβ expression in rat testis is derived exclusively from developing spermatocytes, beginning at the

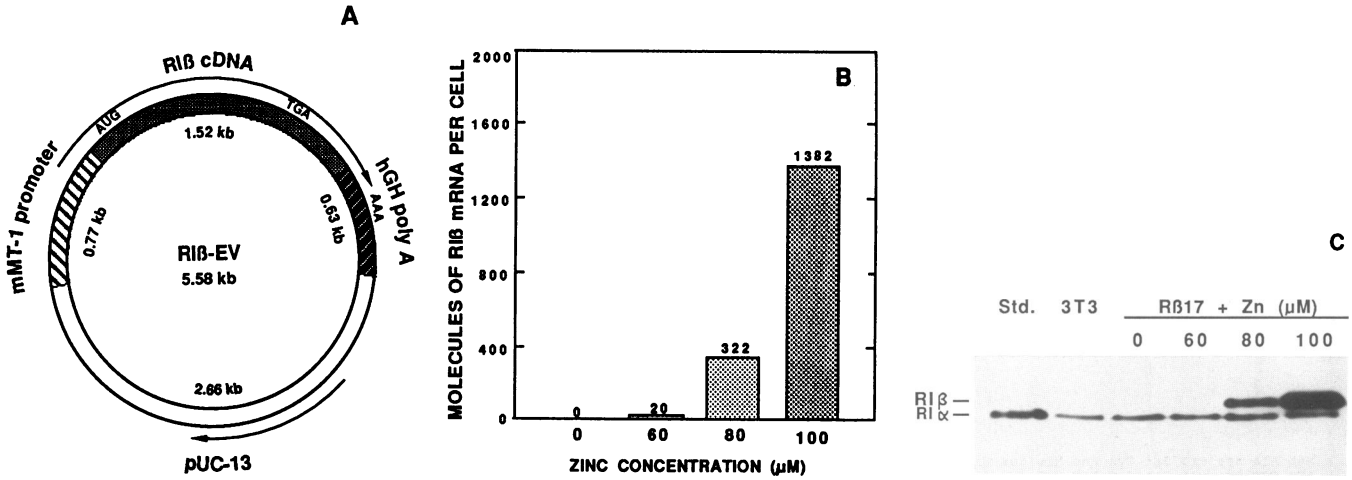

FIG. 3. RIβ expression in transfected 3T3 cells. (*A*) The RIβ cDNA expression vector, RIβ-EV, contains the RIβ cDNA flanked by the *Kpn* I/*Bam*HI fragment (approximately 700 bp) of the mouse metallothionein-1 promoter and 630 bp of the human growth hormone (hGH) 3′ untranslated and flanking sequences; the flanking sequence on the right provides a polyadenylylation signal sequence for the transcript. (*B*) RIβ mRNA accumulation. Subconfluent cultures of an RIβ-EV-transfected 3T3 clone (Rβ17) were incubated for 18 hr in various concentrations of zinc sulfate. Total nucleic acid was isolated and then assayed for RIβ mRNA by solution hybridization using a radiolabeled RNA probe specific for the hGH sequences present in the RIβ mRNA (13). (*C*) Total RI protein detected in zinc-treated control and Rβ17 cells. Cultures grown in parallel with those described above were harvested and frozen. Equivalent amounts of cell protein (300 μg) were subjected to $NaDodSO_4$/PAGE and assayed for RI by immunoblotting (15), using affinity-purified RI antibody (16). As a standard, lane 1 measures the immunoreactivity of 200 ng of pure bovine RI. The apparent molecular mass difference between RIα and RIβ protein is approximately 4 kDa, as determined from molecular mass standards.

pachytene stage (T. Jahnsen, O. Øyen, G.G.C., and G.S.M., unpublished data). This observation is intriguing and may help establish the function of the RIβ protein. The specificity of *RIβ* gene expression is striking when compared to the other subunit genes of PKA, in particular *RIα* (12). Characterization of the *RIβ* gene may identify DNA sequences important for brain- and spermatocyte-specific expression.

Expression of RIβ cDNA in 3T3 cells proved that this gene codes for a functional RI subunit protein. Induction of an expression vector containing the mouse metallothionein-1 promoter resulted in the accumulation of a protein detectable with affinity-purified RI antibody (Fig. 3). When analyzed by HPLC, RIβ protein eluted from DEAE at the same NaCl concentration as RIα (Fig. 4). RIβ is also a cAMP-binding protein, as indicated by its retention on cAMP affinity columns (data not shown). Full functional capacity, however, was demonstrated by the ability of RIβ to inhibit the activity of purified C subunit in a cAMP-dependent manner (Fig. 5).

The apparent molecular weight difference observed between RIβ and RIα protein in 3T3 cells by $NaDodSO_4$/PAGE might be due to post-translational modifications such as phosphorylation or to an unusual alteration in $NaDodSO_4$ binding due to differences in amino acid sequence. We believe that this latter explanation is correct, because RIβ protein synthesized in *Escherichia coli* migrates at the same position as the RIβ protein expressed in 3T3 cells (G.G.C., unpublished data). Moreover, the protein expressed in tissue

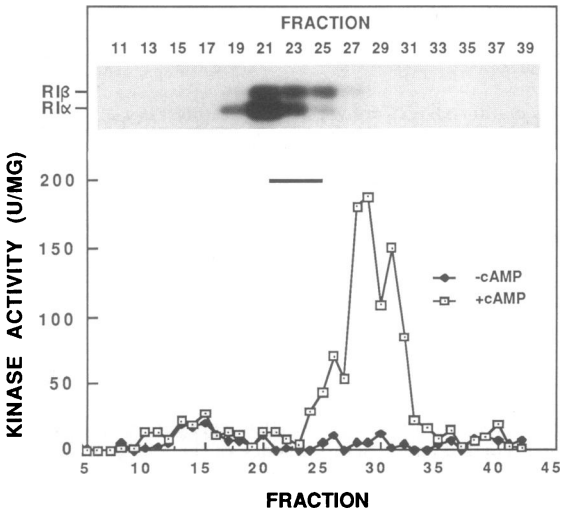

FIG. 4. DEAE-cellulose elution profile of RIβ protein. Extracts of zinc-treated Rβ17 cultures were loaded onto a DEAE column (HPLC), then eluted with increasing concentrations of NaCl. The subsequent fractions were assayed for protein kinase activity in the presence and absence of cAMP (13) and then precipitated with trichloroacetic acid for measuring RI protein by immunoblot analysis. The increase in NaCl concentration, measured by conductivity (data not shown), is first detected in fraction 10, and the concentration rises in a linear fashion to 300 mM in fraction 40. The major peak of PKA activity in 3T3 cells (type II) appears at about 150 mM NaCl. Fractions assayed by immunoblot analysis are indicated above the activity profile. The bar indicates those fractions in which RIα and RIβ protein was detected. PKA units (U) are defined in ref. 13.

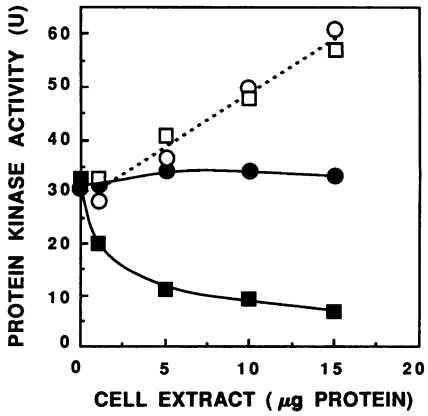

FIG. 5. cAMP-dependent inhibition of kinase activity by RIβ protein. Purified bovine C subunit (6 ng) was incubated at 30°C in standard kinase reaction buffer (minus cAMP, ^{32}P-labeled ATP, and the artificial substrate Kemptide) with increasing amounts of cell extract prepared from zinc-treated (□, ■) or non-zinc-treated (○, ●) cultures. Ten minutes later samples were assayed for protein kinase activity in the absence (■, ●) or presence (□, ○) of 5 μM cAMP.

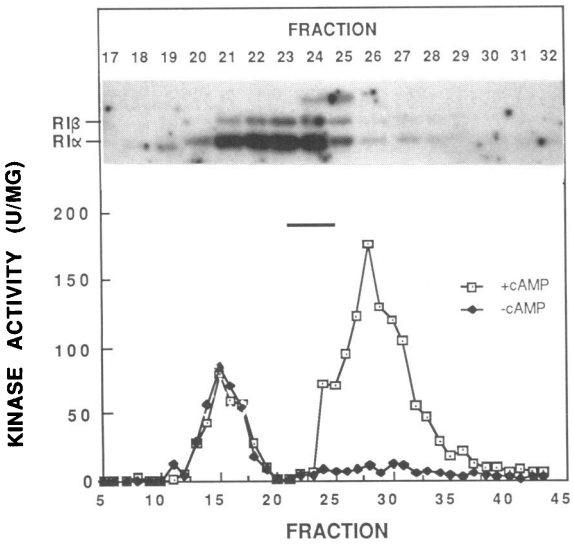

FIG. 6. Ion-exchange elution profile of mouse brain. Extracts of soluble protein prepared from whole mouse brain were eluted from DEAE-cellulose with increasing concentrations of NaCl. The subsequent fractions were assayed for protein kinase activity in the presence and absence of cAMP and then precipitated with trichloroacetic acid for measuring RI protein by immunoblot analysis. The increase in NaCl concentration, measured by conductivity (data not shown), is first detected in fraction 10, and the concentration rises in a linear fashion to 300 mM in fraction 40. Elution of free C subunit occurs near the start of the salt gradient, and the major peak of PKA (type II) appears at about 150 mM NaCl. Fractions assayed by immunoblot analysis are indicated above the activity profile. The bar indicates those fractions in which RIα and RIβ protein was detected.

culture cells mimics the migration characteristics of a RI protein in brain (Fig. 6). Proof that this latter protein is RIβ, however, awaits availability of subunit-specific antibodies. These probes will also be useful in determining the homology of RIα and RIβ to the third and highest molecular mass form of RI observed in brain.

A fundamental question in the PKA field concerns the types of holoenzyme that are formed in cells. Many tissues produce both RI and RII protein and contain different ratios of type I and type II holoenzyme (8). In this regard, overexpression of RI protein failed to change the exclusive formation of type II enzyme in NIH 3T3 cells (Fig. 4). This demonstrates that the relative amount of RI protein is not the rate-limiting factor for RI–C association. One mechanism that does control type I formation in cells, however, is the relative amount of C subunit. When either the Cα or the Cβ subunit protein is overproduced in 3T3 cells by gene transfection, an immediate increase in holoenzyme is observed (22). When analyzed by ion-exchange chromatography this new holoenzyme is entirely type I (unpublished observation).

cAMP is the second messenger for numerous hormones and neurotransmitters, and it influences ion channel function and synaptic transmission (23). Not surprisingly, the brain is a rich source of PKA (24). Having at least two kinds of C subunit and four different R subunits, the brain may possess eight distinct forms of PKA, each of which could differ in affinity for cAMP, intracellular localization, or modification by other kinases. These multiple forms of enzyme would permit changes in PKA function and generate greater diversity in cAMP-regulated responses. For instance, it has been postulated that long-term changes in transmitter release in *Aplysia* may result from the induction of a new type of regulatory subunit that has greater sensitivity to cAMP and locates near the presynaptic membrane (25). Further functional studies should determine whether RIβ might be capable of performing in this or similar roles within the central nervous system.

We thank Dr. Michael Uhler for construction of the mouse brain cDNA library, Dr. John Scott for purified C subunit protein, Dr. Erwin Reimann for critical review of the manuscript, and Thong To for expert technical assistance. This work was funded by National Institutes of Health Grant GM 32875.

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