1992

The Cγ Subunit is a Unique Isozyme of the cAMP-Dependent Protein Kinase

Stephen J. Beebe
Old Dominion University

Paul Salomonsky

Tore Jahnsen

Yixin Li

Original Publication Citation

Beebe, S.J., Salomonsky, P., Jahnsen, T., & Li, Y. (1992). The Cγ subunit is a unique isozyme of the cAMP-dependent protein kinase. *Journal of Biological Chemistry*, *267*(35), 25505-25512.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 267, No. 35, Issue of December 15, pp. 25505-25512, 1992
Printed in U.S.A.

The Cγ Subunit Is a Unique Isozyme of the cAMP-dependent Protein Kinase*

(Received for publication, July 13, 1992)

Stephen J. Beebe‡§, Paul Salomonsky‡, Tore Jahnsen¶, and Yixin Li‡‖

From the ‡Departments of Pediatrics and Physiology, Pediatric Research Center, Eastern Virginia Medical School, Norfolk, Virginia 23501 and the ¶Department of Medical Biochemistry and Rikshospitalet, University of Oslo, Oslo N-0317, Norway

There are at least three isozymes (Cα, Cβ, and Cγ) of the mammalian catalytic (C) subunit of cAMP-dependent protein kinase (PKA) (Beebe, S., Oyen, O., Sandberg, M., Froysa, A., Hansson, V., and Jahnsen, T. (1990) *Mol. Endocrinol.* 4, 465–475). To compare the Cγ and Cα isozymes, the respective cDNAs were expressed in permanently transformed Kin-8 PKA-deficient Y1 adrenal cells using the mouse metallothionein promoter. The recombinant C subunits were characterized as immunoreactive, zinc-inducible, cAMP-dependent kinase activities. In contrast to Cα, histone was a better substrate than Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide) for Cγ. Furthermore, Cγ histone kinase activity was not inhibited by the protein kinase inhibitor peptide (5–24 amide), which has been widely used as a PKA-specific inhibitor. The major Cγ peak (type I) eluted from DEAE-Sepharose at a higher NaCl concentration (120 mM) than the Cα type I eluted (70 mM). Cγ and Cα type II eluted between 220 and 240 mM NaCl. Cγ required higher concentrations of cAMP than Cα did for dissociation from the mutant type I holoenzyme. These differences provided a basis for the separation of the mutant RI-associated isozymes on DEAE-Sepharose. Both Cα (41–42 kDa) and Cγ (39–40 kDa) were identified by a C subunit antibody after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis. Zinc induced the PKA-mediated rounding phenotype in Cγ and Cα clones, thereby restoring the cells to the parent Y1 adrenal cell phenotype. Collectively, these data indicate that Cγ is an active PKA C subunit but suggest that Cγ and Cα have different protein and peptide recognition determinants.

cAMP-dependent protein kinase (PKA)[1] is an integral component of the cAMP signal transduction pathway and mediates most of the actions of cAMP (for reviews see Taylor *et al.*, 1990; Beebe and Corbin, 1986). PKA has been implicated as a modulator within many signaling cascades such as those responsive to β agonists, ACTH, dopamine, and sex hormones, among others. These cascades have target responses in many cellular compartments including the cytoplasm, membrane, mitochondria, and nucleus. Unlike most other kinases, PKA consists of two different kinds of subunits, and each subunit consists of multiple isoforms, which are distinct gene products. The inactive PKA holoenzyme is a tetramer consisting of a dimeric, cAMP-binding regulatory (R) subunit and two monomeric catalytic (C) subunits. Cyclic AMP dissociates and activates the phosphotransferase activity of the C subunits. The two major types of PKA holoenzymes, type I and type II, are characterized by the R subunit isoforms (RIα, RIβ, RIIα, and RIIβ). The ratios of these forms vary among tissues and species and are expressed differently during development and oncogenesis, although distinctive roles for the isozymes have not been directly established. There are at least three mammalian isoforms of the C subunit, designated Cα, Cβ, and Cγ (Beebe *et al.*, 1990). Recently, a second Cβ cDNA, designated Cβ2, was identified in bovine as a possible alternatively spliced Cβ gene product (Wiemann *et al.*, 1991). In yeast, three distinct C subunit gene products have been described (Toda *et al.*, 1987) and in *Aplysia*, two alternately spliced C subunits have been identified (Beushausen *et al.*, 1988). Earlier reports described C subunits with different isoelectric points (Sugden *et al.*, 1976) with different interactions with the PKA inhibitor protein after nondenaturing gel electrophoresis (Van Patten *et al.*, 1991) and a mute C subunit (Reed *et al.*, 1983), but none of these has been specifically correlated with C subunit clones. The Cγ subunit appears to be testis-specific, and the Cγ gene has been located to chromosome 9 (Foss *et al.*, 1992). At the nucleotide level, Cα and Cγ share the most homology in the open reading frame (86%) and in 3′-untranslated sequences (81%), suggesting a common origin. Based on the deduced amino acid sequences, Cγ is the most distinct protein among the isozymes, but it has not been isolated or studied at the protein level (Beebe *et al.*, 1990).

The analysis of PKA has been facilitated by the availability of relatively specific peptide substrates (Kemp *et al.*, 1977) and specific heat-stable PKA inhibitors (Ashby and Walsh, 1973; Beale *et al.*, 1977) that bind competitively and with high affinity to the substrate binding site of the free C subunit (Demaille *et al.*, 1977; Whitehouse *et al.*, 1983). These inhibitors are expressed in a variety of tissues, including skeletal muscle, brain, and testis (Olson and Uhler, 1991). A unique testis inhibitor isoform has been cloned (Van Patten *et al.*, 1991) which is 41% identical to the skeletal muscle PKI but contains the conserved pseudosubstrate site in the amino-terminal domain. The amino-terminal 25 residues of the skeletal muscle PKI have been identified as the active fragment and have been used extensively as a PKA-specific in-

* This work was supported by the Jeffress Memorial Trust and by National Institutes of Health Grant GM 44777 (to S. J. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Dept. of Physiology, Eastern Virginia Medical School, P. O. Box 1980, Norfolk, VA 23507. Tel.: 804-446-5610; Fax: 804-624-2269.

‖ Present address: Dept. of Toxicology, Graduate School, University of Kentucky, Lexington, KY 40506.

[1] The abbreviations used are: PKA, protein kinase A; ACTH, adrenocorticotropic hormone; R, regulatory; C, catalytic; PKI, protein kinase inhibitor.

hibitor peptide (PKI-5–24-amide) (Scott *et al.*, 1985; Cheng *et al.*, 1986). This peptide inhibitor has been shown to block the kinase activity of Cα and Cβ, but until this time Cγ had not been tested.

The present study was designed to express the Cγ cDNA in mammalian cells and to determine if the Cγ protein is an active kinase that binds to the R subunits, is inhibited by PKI, and mediates the actions of cAMP in intact cells. The Y1 adrenal mutant cell, Kin-8 (Rae *et al.*, 1979), which contains an RI subunit mutant (Olson *et al.*, 1991) and is defective in cAMP-mediated responses, was chosen for this purpose. In this report, Cγ and Cα are shown to differ regarding interaction with a mutant Kin-8 RI subunit and sensitivity to a PKI peptide. However, both Cγ and Cα are identified by a C subunit-specific antibody and appear, at least in part, to transform the mutant Kin-8 adrenal cell to the native Y1 phenotype.

MATERIALS AND METHODS

Construction of the Cγ Expression Vector and Transfection of Kin-8 Cells—The full-length 1.8-kilobase Cγ cDNA (Cγ4, Beebe *et al.*, 1990) was subcloned into the *Bgl*II site of pZEM (Uhler and McKnight, 1987) downstream of the mouse metallothionein promoter. The Cγ cDNA was removed from pSK+ (Bluescript) with *Eco*RI, the ends were filled with Klenow, and *Bam*HI linkers were ligated to the Cγ cDNA prior to subcloning into pZEM. To include a selection marker, a 2.2-kilobase neomycin resistance fragment was cloned into a unique *Bam*HI site of the Cγ pZEM construct. The PMT Cγ-neo expression vector construct was restriction mapped to determine orientation of the Cγ cDNA, purified by $CSCl_2$ centrifugation, and used for transfections. The pZEM expression plasmid, the 2.2-kilobase neomycin resistance sequence, and the PMT Cα-neo expression plasmid were gifts from Dr. Mike Uhler, University of Michigan. The Kin-8 cells were transfected with PMT Cγ-neo and PMT Cα-neo expression vectors using the calcium phosphate precipitation method (Wigler *et al.*, 1977) as described previously (Beebe *et al.*, 1992).

Kin-8 cells were a gift from Dr. Bernard Schimmer, University of Toronto. They were grown in 40–50% Ham's F-10 medium with glutamine, 10% fetal bovine serum (HyClone Laboratories), and neomycin (10 mg/ml, Sigma) prior to transfection. After transfection cells were grown in the presence of 500 μg/ml G418 (GIBCO). G418-resistant clones were isolated using glass cloning cylinders, expanded in culture, and analyzed for C subunit expression using PKA activity and C subunit immunoblot analysis. For induction of Cγ and Cα expression via the metallothionein promoter, cells were grown to 70–80% confluence and incubated for 18–20 h in the presence of 90 μM zinc. Preliminary experiments indicated that induction of PKA activity was near maximal between 12 and 22 h of zinc stimulation.

Assay of the cAMP-dependent Protein Kinase Activity—Tissue culture plates or flasks containing the parent Kin-8, Cγ, or Cα clones were washed three times with 10 ml of phosphate buffered saline (20 mM $NaPO^4$ (pH 7.6), 150 mM NaCl). When cells were prepared for ion-exchange chromatography, the last wash was with 10 mM potassium phosphate (pH 6.9), 1 mM EDTA, and 250 mM sucrose. Cells were scraped in a small volume of homogenizing buffer (0.5 ml/10^2-cm plate or 1 ml/162-cm^2 flask). Homogenizing buffer included 10 mM potassium phosphate (pH 6.9), 1 mM EDTA (KP buffer), 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, and 2 μg/ml pepstatin. The cells were homogenized in a Dounce homogenizer with pestle A using 4 × 10 up-down strokes. After homogenization approximately 85–95% of the cells included trypan blue. The homogenate was centrifuged at 14,000 × *g* for 20 min, and the supernatants were analyzed further.

The PKA activity assay and the determination of the PKA activity ratio were carried out as described previously (Beebe *et al.*, 1988) using the method of Roskowski (1983) with the following modifications. The extract was assayed for PKA activity using a series of dilutions including 50–200 μg/ml extract protein. When present, saturating concentrations of Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide; Sigma) (65 μM) and cAMP (100 μM) were used. Under these conditions the PKA activity of the Kin-8 clone was 90–100% of the maximal activity of the Y1 adrenal cell. When histone (Sigma) was substrate the standard PKA assay included subsaturating concentrations (1.8 mg/ml) to avoid a high background activity in the absence of enzyme and an apparent cAMP-independent, histone-mediated dissociation of the holoenzyme. These conditions were somewhat variable depending on the histone preparation. Maximal histone kinase activity required 7.2 mg/ml histone.

Immunoblot Analysis of C Subunit—Cell extracts were prepared as described for PKA assay and electrophoresed on 9% polyacrylamide gels according to Laemmli (1970). The proteins were transferred to Immobilon P (Millipore), and the filters were blocked overnight or for 1 h in 5% bovine serum albumin in Tris-buffered saline (100 mM Tris, 0.9% NaCl (pH 7.5)) containing 0.1% (v/v) Tween 20 (TTBS). The filters were incubated for 1 h with an affinity-purified polyclonal rabbit anti-bovine catalytic subunit (a gift from Dr. Brian Hemmings, Friedrich Miescher Institute, Basel, Switzerland), and washed with TTBS (three times, 15 min each). The filters were then transferred to a solution containing 5 μg/ml biotinylated second antibody (goat anti-rabbit IgG) (Vectastain), incubated for 30 min, washed as before, and then incubated for 30 min with Vectastain Reagent A (Avidin DH) and Reagent B (biotinylated horseradish peroxidase). Finally, the filters were incubated in phosphate-buffered saline containing the substrate diaminobenzidine (0.5 mg/ml), cobalt chloride (0.02%), and H_2O_2 (0.03%) until color developed (generally 10–60 s). The molecular weights were determined using the rainbow marker (Amersham Corp.) and/or biotinylated standards (Bio-Rad).

Determination of the PKA Activation Ratio Using CM-Sepharose Chromatography—The determination of the dissociation ratio is based on the separation of free C subunit, which binds to CM-Sepharose, from the holoenzyme, which does not. Extracts were prepared from each clone after an 18-h stimulation with 90 μM zinc. Generally six 162-cm^2 flasks for each experiment were used. To mimic the conditions of the PKA activity assay, 1.25-ml aliquots of the extract were incubated for 10 min at 30 °C in the absence or pressure of various concentrations of cAMP. For the Cα clone, which contained overexpressed free C subunit, the extract was prechromatographed on a CM-Sepharose column (1.5 × 5.6 cm), and the flow-through fraction, which contained holoenzyme, was used as the starting Cα preparation. The preparations were then applied to 1-ml CM-Sepharose columns that were equilibrated in KP buffer and various concentrations of cAMP. Each column was then washed with 2 ml of KP buffer to remove unbound protein. The column was then eluted with 2 ml of KP buffer containing 500 mM NaCl. Two fractions from each column were collected: the pooled flow-through and wash fractions represented the unbound fraction, and the elution fraction represented the bound fraction. Each fraction was assayed in the standard PKA assay in the presence of cAMP (100 μM) and in the absence and presence of PKI peptide (1 μM). The activity was measured as pmol of ^{32}P incorporated/min/fraction. The activation ratio was determined as the ratio of the activity in the bound fraction divided by the activity in the bound plus the unbound fractions (*i.e.* the total activity recovered from the column). Total recoveries (activity recovered in both fractions divided by activity applied to each column) were between 65 and 85%. Within each experiment, recoveries among the columns did not vary by more than 10%.

RESULTS

Transfection of Kin-8 Adrenal Cells with C Subunit Expression Plasmids and Analysis of Cγ and Cα Kin-8 Clones—The Cγ cDNA was cloned into the Zn^{2+}-inducible expression vector, PMT-neo, containing the metallothionein promoter, the human growth hormone polyadenylation site signal, and the neomycin phosphotransferase gene (Uhler and McKnight, 1987). The Cα expression vector, containing the same elements and the mouse Cα cDNA, was a gift from Dr. Mike Uhler, University of Michigan, and has been characterized previously (Uhler and McKnight, 1987). Each expression vector was transfected into the PKA mutant Y1 adrenal cell, Kin-8, using the calcium phosphate precipitation method and grown in the presence of the selection marker, G418. After transfection 12 putative Cγ and 20 putative Cα clones were identified as G418-resistant. Eleven Cγ and three Cα clones were isolated, expanded in culture, and further analyzed. For a positive identification of Cγ- and Cα-expressing clones, cell extracts from each clone were prepared and assayed for Zn^{2+}-inducible, cAMP-dependent protein kinase activity in the

presence or absence of PKI. This allowed a rapid analysis of a relatively large number of clones in a relatively short time period. Initial experiments utilized Kemptide as substrate. In the presence of Zn^{2+} stimulation, five Cγ clones and all three Cα clones expressed kinase activity above the Kin-8 control activity levels. The Cγ clones expressed a 25–100% increase in kinase activity, whereas the activity in Cα clones was increased 2–5-fold. Only two of the 11 G418 resistance Cγ clones expressed significantly elevated activity above the Kin-8 control to warrant further study. Both of these (see Fig. 1) were induced with zinc. The two Cα clones that were tested expressed similar amounts of elevated kinase activity, but only one of them (Cα2, see Fig. 1) was induced with zinc. Low basal "leaky" expression and good induction with zinc were important criteria for further study since the potential for kinase expression and reversion to the wild type Y1 phenotype may cause unstable cell lines because of the "killing" effect of cAMP and PKA activity. Two Cγ clones and two Cα clones were further tested using histone and Kemptide as substrate (Schimmer, 1985).

Fig. 1 shows the protein kinase activities in the presence of cAMP from the parent Kin-8 clone, two Cγ-transfected clones, and a Cα-transfected clone using histone and Kemptide as substrates in the pressure and absence of inhibitor peptide, PKI. The basal and Zn^{2+}-induced increases in cAMP-dependent activity were most evident in Cγ clones when histone was the substrate. The non-Zn^{2+}-induced histone kinase activities of Cγ7 and Cγ10 extracts were 2.5–3.0-fold higher than the activities in Kin-8 parent. Zinc stimulation increased the cAMP-dependent activity in these clones 3.5-5.0-fold over Kin-8 controls and 2–3-fold above the no-Zn^{2+} controls. Cγ7 had lower basal and greater Zn^{2+} induced histone kinase activity than Cγ10. When Kemptide was the substrate the evidence for Cγ expression was less clear. In contrast, Cα expression was readily evident and apparently greater than Cγ expression with either Kemptide or histone as substrate. Basal non-Zn^{2+}-stimulated activity in Cα2 was 2-fold greater than Kin-8 controls and Zn^{2+}-stimulated Cα2 kinase activity by 2-fold.

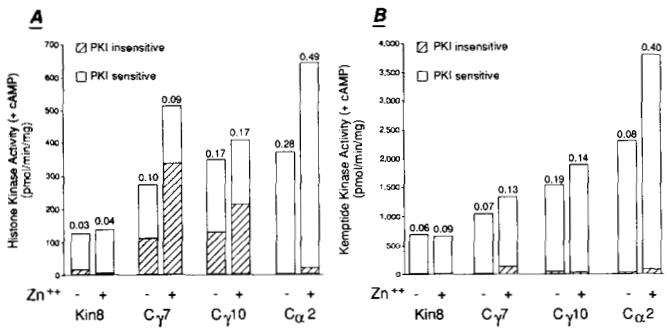

FIG. 1. **Comparison of zinc inducible, PKI-insensitive histone and Kemptide kinase activity in extracts from Kin-8 cells permanently transfected with expression vectors containing human Cγ and mouse Cα cDNAs.** Kin-8, Cγ, and Cα clones were grown to 70–80% confluence and were stimulated for 18 h in the presence or absence of 90 μM zinc prior to harvest. Cells from two 10-cm^2 plates were scraped in 1 ml of homogenizing buffer, homogenized, microcentrifuged, and the supernatant extracts were assayed for PKA activity using histone IIIS (*panel A*) and Kemptide (*panel B*) as substrates in the presence of 100 μM cAMP as described under "Materials and Methods." Activity for each substrate is expressed as that which is sensitive (*open bars*) and insensitive (*striped bars*) to the PKI peptide (1 μM). *Numbers* above each *bar* indicate the PKA activity ratio (activity measured in the absence divided by activity measured in the presence of cAMP) in the absence of PKI peptide.

The most notable difference between Cγ and Cα clones and best discriminator for Cγ expression was the absence of PKI inhibition in Cγ extracts (Fig. 1) and partially purified Cγ preparations (Figs. 2–6) when histone was used as substrate. The increase in PKI-insensitive, cAMP-dependent histone kinase activity was 50–100-fold and was Cγ clone-specific. In contrast the histone kinase activity in Kin-8 and Cα clones was inhibited 90–95% by PKI. Zinc increased activity in Cγ clones 1.5–3-fold above no-zinc controls. The cAMP-dependent, PKI-insensitive activity was about 65–70% of the total activity in the Cγ7 clone and somewhat lower in Cγ10. In the absence and the presence of zinc PKI-sensitive activities in Cγ7 and Cγ10 were similar to the PKI-sensitive activity in the Kin-8 parent. No changes in PKI-insensitive activity were observed in Kin-8 or Cα2 in the presence of zinc (Fig. 1) or 8-chlorophenylthio-cAMP (not shown). The ratios of activity with Kemptide and histone as substrates for the extracts of the clones were also different. In the presence of zinc the Kemptide to histone kinase activity ratio of Kin-8 and Cα 2 extracts was between 4.0 and 6.0. In contrast, the same ratio for Cγ7 extracts was between 2.0 and 3.0. As the PKI-insensitive activity was enriched (see below) the Cγ7 Kemptide to histone kinase activity ratio was as low as 0.40. Since histone appeared to be a better substrate than Kemptide for the kinase activity in Cγ clones, histone was used routinely to evaluate Cγ activity and to compare it with Kin-8 and Cα2 activity.

Fig. 1 also indicates the protein kinase activity ratios for each clone in the presence and absence of zinc stimulation. The basal activity ratio of Cα2 was relatively low, and activity was stimulated about 10-fold by cAMP when Kemptide was the substrate. When Cα2 and two other Cα clones were stimulated by zinc, the activity ratio increased to about 0.5. Chromatography confirmed the presence of an excess free C subunit above endogenous R subunit levels by identifying PKI-sensitive activity in the flow-through fractions of DEAE-Sepharose and in the 500 mM NaCl elution fraction of CM-Sepharose when Cα2 extracts were chromatographed. In contrast, extracts from Cγ clones had low activity ratios in the absence and presence of Zn^{2+} stimulation. Little or no PKI-insensitive activity appeared in the DEAE-Sepharose flow-through or CM-Sepharose high salt elution fractions when Cγ extracts were chromatographed.

Immunodetection of C Subunits in Cγ and Cα Expression Clones—Although the amino acid homology between Cγ and Cα was lower than homology between Cβ and Cα, it was likely that a polyclonal antibody made against Cα would recognize Cγ. Consequently, immunodetection of Cγ with a C subunit-specific antibody (provided by Dr. Brian Hemmings) represented a direct and rapid method to prove that the PKI-insensitive activity in Cγ7 was PKA. Fig. 2 shows a Western blot of purified bovine heart C subunit and equal amounts of extract protein from Cγ7, Cα2, and Kin-8 clones. Lanes containing the C subunit standard, Cα2, and Kin-8 each contained a single 42-kDa band. The Cγ7 lane contained a 41–42-kDa and a 39–40-kDa band. The histone kinase activities in the presence of 100 μM cAMP (pmol of ^{32}P incorporated/min/mg of extract protein) were 738, 3,289, and 5,187 for Kin-8, Cα2, and Cγ7, respectively. This represented a 4.5- and a 7.0-fold stimulation above Kin-8 for Cα2 and Cγ7, respectively, and was proportional to the intensity of the corresponding immunoreactive bands.

Chromatographic Analysis of Cγ7 and Cα2 Extracts—The C subunit of PKA can be dissociated from the R subunit by cAMP. Since the C subunit is a relatively small protein kinase, it can be separated from many other kinases by gel filtration

chromatography. Fig. 3 *A* shows Sephacryl S-300 chromatography of the zinc-induced, histone kinase in the absence and presence of the PKI peptide from a Cγ7 extract that was concentrated with ammonium sulfate and preincubated with cAMP before chromatography. Identical experiments were carried out with extracts from the Kin-8 (not shown) and Cα2 clones (Fig. 3*B*). For Cγ7, two PKI-insensitive peaks of histone kinase activity were observed. The major peak eluted in fraction 43, near the ovalbumin standard and was inhibited 13% by the PKI peptide. The histone kinase activity of Cα2 (Fig. 3*B*) was inhibited 90–95% by PKI and eluted in the same fraction. Results similar to those shown for Cα2 were observed for the Kin-8 clone (not shown). A small peak, representing less than 5% of the recovered activity (total recovery in all experiments was 80–95%), eluted near the void volume and was present in all clones.

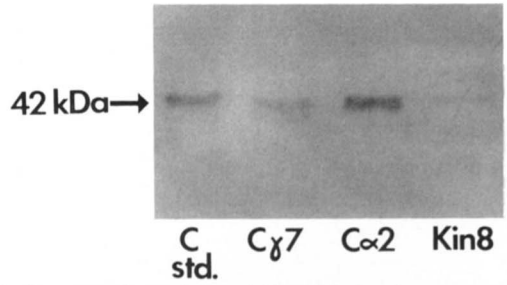

FIG. 2. **Immunodetection of C subunits in extracts from Cγ7, Cα2, and Kin-8 clones.** Cells were grown in 10-cm² plates to 70–80% confluence, stimulated for 18 h in the presence of 100 μM zinc, and extracts were prepared as described in the legend to Fig. 1 and under "Materials and Methods." Protein concentrations were determined by the method of Bradford (1976) and 2.9 μg of protein from each extract was subjected to 9% SDS-polyacrylamide gel electrophoresis. Proteins were blotted onto Immobilon filters (Millipore) and incubated with an affinity-purified anti-bovine C subunit antibody (see "Materials and Methods"). A pure bovine C subunit was used as a standard (M_r = 42,000), and apparent molecular weights were determined using the rainbow maker (Promega).

To characterize further the PKI-insensitive histone kinase activity in the Cγ7 clone and determine if it associated with RI or RII subunits, Cγ7 cell extracts were chromatographed on DEAE-Sepharose and eluted with NaCl gradients. Fig. 4*A* shows a composite DEAE-Sepharose elution profile for Cα2 (PKI-sensitive) and Cγ7 (PKI-insensitive and -sensitive). Similar amounts of extract proteins were loaded onto identical columns and eluted with 0–500 mM NaCl gradients. The column fractions were assayed for histone kinase activity in the presence and absence of PKI and the PKI-insensitive and PKI-sensitive activities were plotted against the NaCl concentration of each fraction. Essentially all of the activity in the Cα2 clone was sensitive to PKI (not shown), and 90% of it eluted at 70 mM NaCl. The Cγ7 was comprised of PKI-insensitive activity, which eluted at 135 mM NaCl, and PKI-sensitive, endogenous Kin-8 C subunit, which eluted as a relatively broad peak. When extracts from the Kin-8 clone were chromatographed peak I was 90–95% PKI-sensitive and eluted at 90 mM NaCl (not shown). All clones contained lower amounts of a second peak of activity, which eluted between 215 and 240 mM NaCl. In a total of three or four experiments for each clone, the Cγ (PKI-insensitive) peak I eluted at 126 ± 9 mM NaCl (mean ±S.E., n = 4); Cα peak I eluted at 67 ± 9 mM (n = 3) and Kin-8 peak I eluted at 90 ± 11 mM NaCl (n = 3). The elution positions of these peaks are generally typical for type I PKA. The kinase activity in peak II for each clone eluted from DEAE-Sepharose at greater than 200 mM NaCl, which is typical for type II PKA. The Cγ peak II was always larger and eluted earlier (218 ± 2 mM, n = 4) than the Kin-8 peak II (250 ± 8 mM, n = 3).

In three separate experiments with the zinc-stimulated Cγ7 clone, the ratio of PKI-insensitive to PKI-sensitive activity was greater than shown in Fig. 4*A* and indicated a decrease in PKI-sensitive histone kinase activity. Fig. 4*B* shows one of these experiments. The total amount of PKA histone kinase activity in both experiments was similar, but the PKI-insensitive activity accounted for 87% in experiment 4*B* and 55% in experiment 4*A* of the total activity. In the experiment

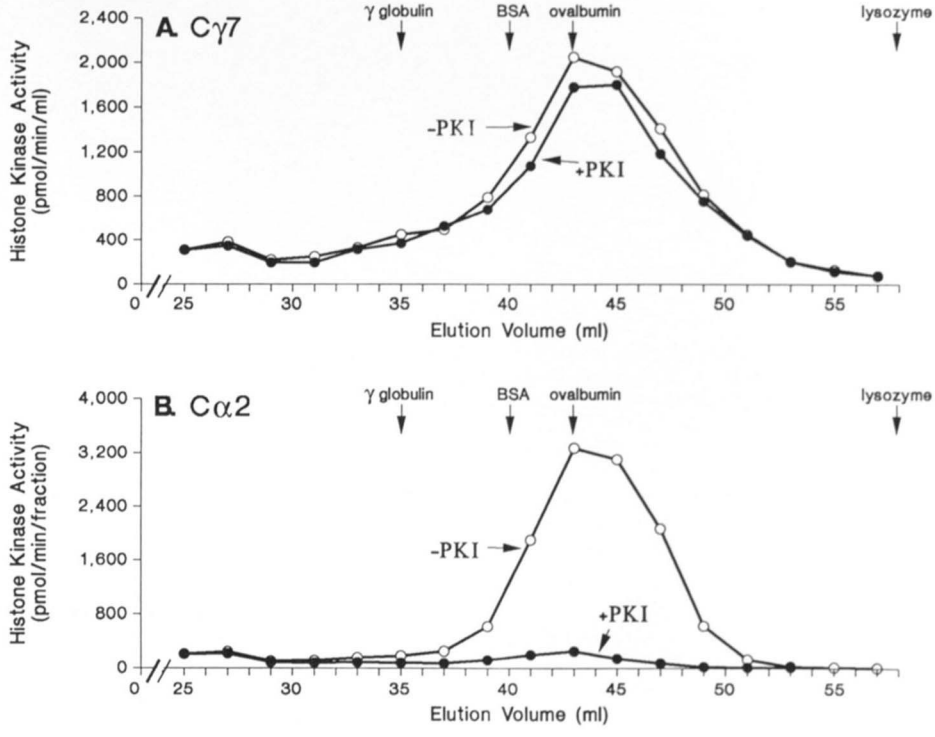

FIG. 3. **Sephacryl (S-300) gel filtration of the free C subunit from Cγ7 and Cα2 extracts.** Extracts from four 162-cm² flasks of Cγ7 (*panel A*) and Cα2 (*panel B*) cells (60–70% confluent) were prepared and made 100 mM phosphate (pH 6.9) and 70% ammonium sulfate. The precipitated protein was resuspended in 1 ml of 10 mM potassium phosphate (pH 6.9), 1 mM EDTA, 200 mM NaCl, and 1 mM cAMP. An 800-μl aliquot was applied to an S-300 column (1.5 × 20 cm) that was equilibrated in the same buffer. Fractions (0.5 ml) were collected and assayed for histone kinase in the absence and presence of PKI peptide as indicated. The protein standards were chromatographed individually under identical conditions, and the peaks were determined by absorbance at 280 nm. *BSA*, bovine serum albumin.

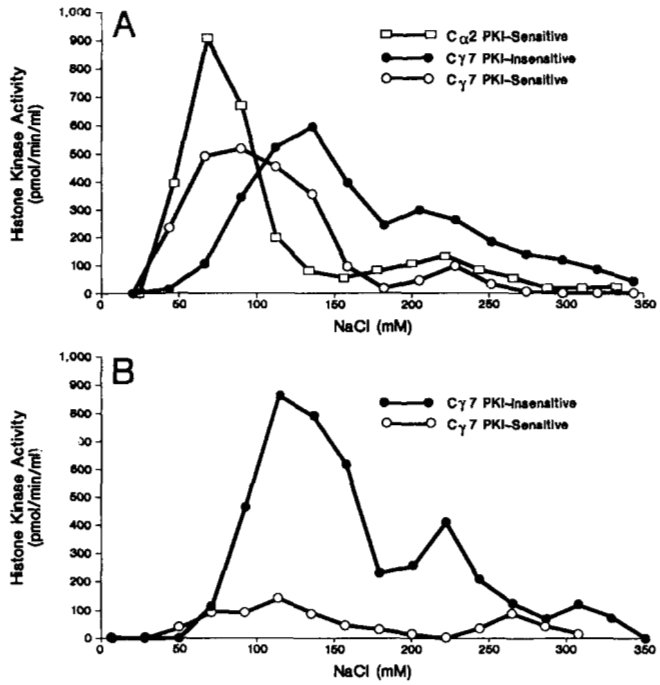

FIG. 4. **DEAE-Sepharose chromatography of histone kinase activities in Cγ7 and Cα2 cell clones.** Cells from six 162-cm^2 flasks (70–80% confluent) were stimulated with 90 μM zinc for 18 h, and cell extracts were prepared. The Cα2 extract (6.2 mg of protein) (*panel A*) was prechromatographed on CM-Sepharose to remove free C subunit. The CM-Sepharose flow-through fractions were pooled according to protein concentration prior to DEAE-Sepharose (1.0 × 13 cm) chromatography. Cγ7 extracts (*panels A* and *B*) (6.9 and 5.8 mg of protein, respectively) which did not contain free C subunits were chromatographed directly on separate but identical DEAE-Sepharose columns. All columns were equilibrated in homogenizing buffer, extracts were applied, and the columns were washed with column buffer. The DEAE-Sepharose columns were eluted with 0–500 mM NaCl gradients (50 ml). One-ml fractions were collected and assayed for sodium concentration and histone kinase activity in the presence of 100 μM cAMP and the absence and presence of 1 μM PKI peptide. *Panel A* represents a typical Cα2 activity profile that was sensitive to PKI peptide (*open squares*) (representing 95% of the total activity) and a Cγ7 profile of PKI-insensitive (*closed circles*) and PKI-sensitive (*open circles*) histone kinase activity. *Panel B* represents a second type of Cγ7 profile that was observed in three separate experiments. PKI-insensitive activity is considerably greater than PKI-sensitive activity (see also Fig. 3*A*).

illustrated in Fig. 3*A* the PKI-insensitive activity (87%) was also relatively high. In general, the zinc-induced PKI-insensitive activity in the Cγ7 clone varied between 50 and 90%. The conditions that account for these differences are under investigation.

The relatively poor phosphorylation of Kemptide and the PKI insensitivity of Cγ histone kinase activity removed two valuable PKA discriminators from the Cγ analysis. To substantiate further that Cγ was a PKA, the cAMP dependence of the PKI-insensitive activity from Cγ7 and PKI-sensitive activity from Cα2 was analyzed. In two separate experiments for each Cγ7 and Cα2 clone, peaks I and II were pooled, concentrated with 70% ammonium sulfate, and dialyzed extensively to remove salt. Each peak from both clones was then analyzed for cAMP activation in the presence of PKI for Cγ7 and the absence of PKI for Cα2. Peak I and II fractions from the column of the Cγ7 clone were 85–90% PKI-insensitive, and the Cα2 peaks contained 90–95% PKI-sensitive activity. As expected the concentration of cAMP required to activate peak I half-maximally was high for both clones. When analyzed by the Hill equation as reported previously for PKAs (Beebe and Corbin, 1984), the $A^{0.5}$ values for Cγ7 and Cα2 were 10–20 μM, and the Hill coefficients were 0.2–0.4, indicating negatively cooperative activation or a mixture of PKAs with different cAMP $A^{0.5}$ values. In contrast, the type II Cγ7 and Cα2 enzymes had $A^{0.5}$ values for cAMP activation between 150 and 200 nM and Hill coefficients of 1.2–1.3.

cAMP-induced Dissociation of C Subunit from Holoenzyme in Cγ7, Cα2, and Kin-8 Clones—Initial experiments to purify free Cγ subunit utilized the cAMP-specific elution of the C subunit from DEAE-Sepharose-immobilized holoenzyme. However, the 3 mM cAMP eluate was 90–95% PKI-sensitive, and PKI-insensitive activity remained on the column and was eluted with high salt. These results suggested that Cγ bound more tightly than Cα did to the R subunit. To analyze the effects of cAMP without the complication of possible histone-induced dissociation, experiments were designed to analyze the effects of cAMP on PKA dissociation in the absence of a substrate. In these studies CM-Sepharose chromatography was used to separate the free C subunit, which binds to the column and elutes with high salt, and the holoenzyme complex, which does not bind and appears in the flow-through fraction. This is analogous to the PKA activity ratio in the PKA assay, when activity is measured in the absence (free C subunit) and pressure (holoenzyme plus free C subunit) of cAMP.

Fig. 5 shows the effect of cAMP on PKA dissociation in Cγ7, Kin-8, and Cα2 extracts. Extracts were incubated for 10 min at 30 °C to mimic PKA assay conditions and then chromatographed on CM-Sepharose. Two fractions were collected from each column: the nonbound holoenzyme and the bound C subunit. The activation ratio (see "Materials and Methods" and Fig. 5), which determines the extent of PKA dissociation, was measured. The total recoveries of activity among different experiments were between 65 and 85%, and recoveries among different treatments within individual experiments did not vary by more than 10%. With histone as substrate, Cγ7 activity was determined in the presence of PKI, and Kin-8

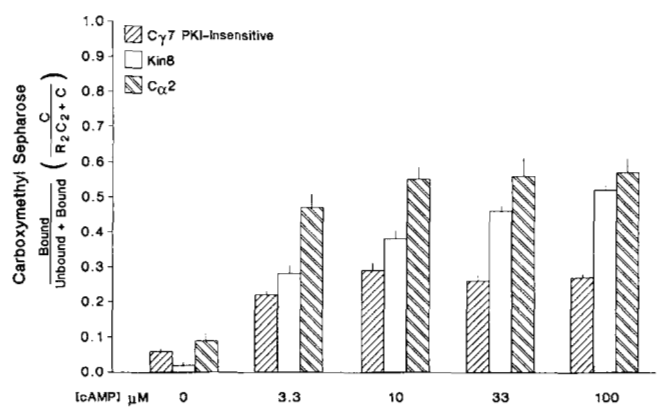

FIG. 5. **Separation of holoenzyme and C subunit from Cγ7, Cα2, and Kin-8 clones on CM-Sepharose after dissociation of holoenzymes with cAMP.** Cells from six 162-cm^2 flasks for each clone (70–80% confluent) were stimulated with 90 μM zinc for 18 h, and extracts were prepared. Holoenzyme preparations were incubated for 10 min at 30 °C, chromatographed on a 1-ml CM-Sepharose column, washed, and eluted with 500 mM NaCl as described under "Materials and Methods." The CM-Sepharose flow-through and elution fractions were assayed for histone kinase activity in the absence and presence of PKI peptide for Cγ7 and the absence of PKI peptide for Cα2 and Kin-8. The activation ratios were calculated as indicated on the *y* axis of the figure and as described under "Materials and Methods." The experiment represents the mean ± S.E. from three separate experiments for each clone.

and Cα2 activities were determined in the absence of PKI. The PKI-sensitive activity in the Cγ7 clone accounted for 20–35% of the total activity and showed the same cAMP activation effects as the Kin-8 clone (not shown). For comparison with Cγ7 and Kin-8 extracts, which did not contain free C subunit, Cα2 extracts were prechromatographed on a CM-Sepharose column to remove free C subunit prior to the cAMP treatment. A similar treatment of Cγ7 and Kin-8 extracts made no difference in the results of the experiments. When Cγ7 extracts were incubated with 3–100 μM cAMP, the activation ratio increased to 0.2–0.35 but not higher (Fig. 5). In contrast, the same concentrations of cAMP activated Cα2 to higher levels as indicated by activation ratios as high as 0.6. Kin-8 extracts, and the PKI-sensitive activity in Cγ7 extracts, representing endogenous Kin-8 C subunit (not shown), showed activation characteristics similar to those in Cα2, although Cα2 was dissociated slightly more for any given concentration of cAMP.

Separation of PKI-insensitive and PKI-sensitive Histone Kinase Activity in the Cγ7 Clone—Fig. 6 shows the results of a protocol (see figure legend) that separated Cγ from endogenous Kin-8 C subunit by utilizing differential responses of each isoform to cAMP activation (Fig. 5) and elution from DEAE-Sepharose (Fig. 4). As shown in Fig. 6, histone kinase activity and immunoblot analysis (*inset*) identified a predominantly PKI-sensitive, endogenous Kin-8 C subunit at 41–42 kDa in fraction 34 and a totally PKI-insensitive Cγ at 39–40 kDa in fraction 40. As expected from studies in crude extracts, the ratios of activity with Kemptide and histone as substrates for fractions 34 and 40 were different. In data not shown, the Kemptide to histone activity ratios for fractions 34 and 40 were 5.81 and 0.43, respectively. Furthermore, the activity of neither fraction was stimulated by cGMP with either substrate (not shown).

Expression of Cγ and Cα and PKA-mediated Morphological Changes in Kin-8—To determine if Cγ functioned as a PKA in intact cells and promoted the reversion of the Kin-8 mutant to the Y1 phenotype, the well characterized cAMP-induced morphological rounding effect (Schimmer, 1985) was analyzed when Kin-8, Cα2, and Cγ7 clones were treated with zinc to induce the expression of Cα and Cγ in transfected clones (Fig. 7). The untreated Kin-8, Cα2, and Cγ7 cells grew as flat and broad monolayers that tightly adhered to the culture dishes. When each clone was treated with 90 μM zinc for 18 h, the Cα2 and Cγ7 cells, but not the Kin-8 cells, assumed a retracted, rounded morphology that was more refractile to light. A concentration-dependent rounding response was observed for Cα2 and Cγ7 but not for Kin-8 (not shown). Fig. 7 shows the maximal morphological response of the Cα2 and Cγ7 clones to zinc (90 μM). Kin-8 did not show the rounding response except at high concentrations of zinc (≥ 120 μM). Rounding of Cα2 occurred at 50–60 μM zinc, but the rounding of Cγ7 required 80–90 μM zinc. These intact cell responses are consistent with the *in vitro* data demonstrating that Cα is more easily dissociated than Cγ is from the Kin-8 R subunit.

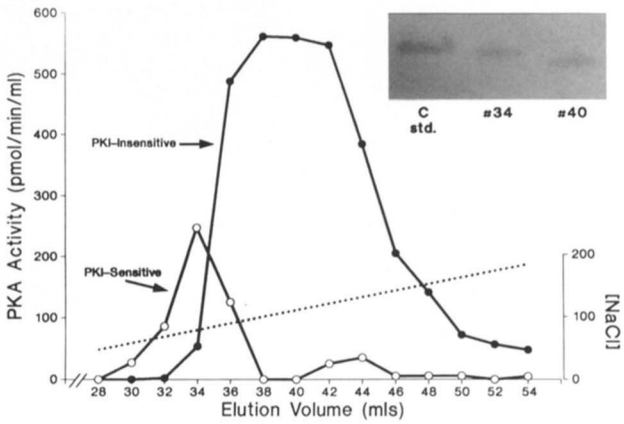

FIG. 6. **Separation of PKI-insensitive Cγ and PKI-sensitive endogenous C subunit from the Cγ7 clone by DEAE-Sepharose chromatography.** Cells (60% confluent) from eight 162-cm² flasks were stimulated with 90 μM zinc for 21 h, and extracts were prepared. The extract was made 1 mM cAMP, incubated on ice for 30 min, and loaded onto a CM-Sepharose column (1.5 × 7 cm) that was equilibrated in KP buffer with 1 mM cAMP. The CM-Sepharose flow-through fractions were pooled and loaded onto a DEAE-Sepharose column (1.0 × 16.5 cm) equilibrated with the same buffer. The column was eluted with a 0–300 mM NaCl gradient (60 ml), 1-ml fractions were collected and assayed for sodium concentration and histone kinase activity in the presence of 100 μM cAMP and in the presence and absence of 1 M PKI peptide. *Inset*, a 100-μl aliquot from fractions 34 and 40 was precipitated with 10% tricholoroacetic acid, subjected to 9% SDS-polyacrylamide gel electrophoresis, blotted onto Immobilon P filters (Millipore), and probed with the C subunit antibody (see "Materials and Methods"). The C subunit standard (42 kDa) was from bovine heart.

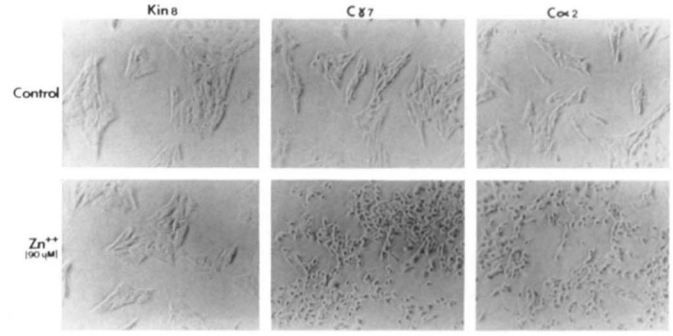

FIG. 7. **Zinc stimulation of Cγ and Cα expression induces the rounding phenotype in Cγ7 and Cα2 clones but not in the Kin-8 parent cell.** Clones were grown in 10-cm² plates until nearly confluent. Each clone was stimulated for 18 h in the absence (*control*) and presence of 90 μM zinc. Cells were photographed using an Nikon inverted phase contrast microscope at a magnification of 200 ×.

DISCUSSION

The stable transfection of a mouse Kin-8 mutant of the Y1 adrenal cell with an expression plasmid that contained the human Cγ subunit cDNA and a mouse metallothionein promoter resulted in the zinc-inducible expression of a unique Cγ subunit of PKA that is cAMP-dependent but not inhibited by the PKI peptide, 5–24 amide. This inhibitory peptide has a high affinity pseudosubstrate recognition domain of the PKI protein that is specific for Cα (Scott *et al.*, 1985; Cheng *et al.*, 1986) and Cβ (Olsen and Uhler, 1989) but apparently not for Cγ. The crystal structure of the Cα subunit complexed with the PKI (5–24 amide) has been resolved (Knighton *et al.*, 1991). This model proposes that the peptide occupies a cleft between two lobes of the subunit, interacting with amino acids that are not conserved among kinases. Interestingly, according to crystal structure analysis, the amino acids of the Cα subunit that bind to the PKI peptide are common among all three mammalian C subunit isozymes. Therefore, it is not readily apparent why Cγ activity is not inhibited by PKI. Since C subunit residues that interact with the PKI peptide are widely separated in the Cα sequence (Knighton *et al.*, 1991), it is possible that other amino acid substitutions modify the secondary structure of Cγ and alter sensitivity to the inhibitor. It is conceivable that in contrast to PKI inhibition of Cα, inhibition of Cγ requires additional sequences in the PKI protein. Alternatively, it is possible that Cγ is more similar to protein kinase G and does not recognize amino-

terminal sequences of PKI (Glass *et al.*, 1986). Nevertheless, Cγ is distinctly different from Cα regarding PKI peptide sensitivity. The PKI insensitivity of Cγ is not dependent on the presence of the mutant RI or normal RII subunit, since Cγ histone kinase activity was not significantly inhibited when tested as a free Cγ subunit after S-300 gel filtration nor as a cAMP-dependent Kin-8 mutant type I holoenzyme or a Kin-8 type II holoenzyme after DEAE-Sepharose chromatography. It is of interest to determine if recombinant Cγ produced in other cells or in bacteria is also insensitive to PKI and to determine if the PKI proteins from skeletal muscle and testis inhibit Cγ activity. The presence of a unique PKA Cγ subunit and a unique PKA inhibitor in the testis (Beale *et al.*, 1977; Van Patten *et al.*, 1991) suggests the possibility for distinct Cγ PKA-mediated roles in reproductive function.

Other Cγ properties also distinguish it from Cα. Cγ bound more tightly than Cα did to the Kin-8 mutant R subunit, and full activation of Cγ required different conditions. Cγ required micromolar concentrations of cAMP for activation in the presence of histone and millimolar cAMP concentrations in the absence of histone. Preliminary data indicate that histone, but not Kemptide, promotes the dissociation of Cγ holoenzyme.[2] This is, in part, responsible for the relatively poor phosphorylation of Kemptide by Cγ. It is possible that the tight binding of Cγ to mutant Kin-8 RI subunit is primarily responsible for the successful expression of Cγ since Cγ expression in mouse L-cells and COS cells was of limited success.[3] Unlike Cα, Cγ did not accumulate above the levels of endogenous R subunits in the Kin-8 cell as indicated by low cAMP activity ratios in crude extracts and the absence of free Cγ subunit in DEAE-Sepharose flow-through and CM-Sepharose elution fractions. Furthermore, only two out of 11 Cγ clones expressed sufficient Cγ activity to warrant further study, but all three Cα clones tested overexpressed free Cα subunit in the presence of zinc. Taken together these data suggest that free Cγ subunit may be unstable or rapidly turned over in the intact cell.

For all clones tested Cα expression appeared to be greater than Cγ expression based on total protein kinase activity. However, full Cγ expression may not occur under present assay conditions because of the tight binding to the mutant RI subunit. Nevertheless, the induction of immunoreactive C subunit and the return of the mutant Kin-8 cell to the rounding Y1 cell phenotype (wild type) were more readily elicited in the Cα2 clone than in any of the Cγ clones tested. This suggests that Cγ may bind more tightly to the mutant RI subunit in the intact cell. However, it is possible that some of the differences in Cγ and Cα expression are caused by the expression vector constructs rather than differences between the Cγ and Cα proteins. The Cγ expression vector contained more 3′-untranslated sequences than the Cα construct, but we did not directly study Cγ mRNA expression. Nevertheless, it is clear that differences do exist between the Cγ and Cα proteins, and some of these may account for some of the differences in the expression in the Cγ7 and Cα2 clones. If Cγ does have a distinct substrate and protein/peptide binding specificity, as suggested by differences in Cγ and Cα PKI sensitivity, the timely phosphorylation of some functionally relevant substrate by a rapidly turned-over cAMP-dependent, Cγ-mediated mechanism could provide tight control of the phosphorylation of some temporally important phosphoprotein. In addition, tight binding of Cγ to R subunits could also serve to target some temporally important phosphorylation event(s) that require relatively high cAMP levels. It will be important to characterize Cγ expression more fully in tissues, cells, and tumors to determine Cγ substrate specificity and to determine if Cγ binds more tightly than Cα does to wild type R subunits.

The Cγ type I holoenzyme eluted at higher salt concentrations from DEAE-Sepharose than Cα type I holoenzyme did. This property and the tight binding of Cγ to mutant Kin-8 RI subunit provided a means to separate Cγ from endogenous Kin-8 C subunits completely. After Western blotting an anti-C subunit antibody specifically identified C subunit bands at 42 kDa for purified bovine heart, 41–42 kDa in Cα2 and parent Kin-8 extracts, and two bands at 41–42 kDa and 39–40 kDa in Cγ7 extracts. The smaller band was identified as Cγ because when the isozymes were separated the smaller band, but not the larger band, was observed in the cAMP-dependent, PKI-insensitive fraction. Although the smaller immunoreactive Cγ band was identified in crude extracts as well as partially purified preparations, it has not been ruled out that it may result from proteolysis rather than from a difference in the migration of the intact Cγ isozyme. The Cγ expression vector construct contained a 27-base pair extension at the 5′ end of the Cγ cDNA, which potentially codes for 9 additional amino-terminal residues (Beebe *et al.*, 1990). Therefore, Cγ could be expected to be larger than Cα. However, the translation start site for Cγ has not been identified specifically, and it is not known if these residues are part of the recombinant Cγ protein. Phosphorylation is known to slow the migration of some proteins, including the RII subunit (Robinson-Steiner *et al.*, 1984), so the 39–40-kDa Cγ band could result from a dephosphorylated enzyme, but the presence or absence of Cγ autophosphorylation has not been studied, and the effect of autophosphorylation on C subunit migration after SDS-polyacrylamide gel electrophoresis is not clear presently.

Cγ binds to both the Kin-8 mutant RI subunit and the apparently normal RII subunit since two peaks of cAMP-dependent, PKI-insensitive histone kinase activity were separated by DEAE-Sepharose chromatography. Most of the Cγ activity was associated with type I, but in all experiments more type II holoenzyme was found in the Cγ7 clone than in either the Kin-8 parent or the Cα2 clone. In contrast to Cγ expression in Kin-8 cells, expression of Cα and Cβ in 3T3 fibroblasts and AtT-20 cells resulted in elevated levels of RI subunit but not RII subunit (Uhler and McKnight, 1987). These data suggest that in spite of the tight association of Cγ with mutant Kin-8 RI subunit, Cγ may more readily associate with RII subunit than Cα does. Further studies are in progress to confirm these findings and to determine if Cγ induces and/or binds to RIIα and/or RIIβ.

The expression of the Cγ isozyme of PKA, as well as Cα (Clegg *et al.*, 1989), in the Y1 adrenal Kin-8 mutant restores the normal, cAMP-induced rounding phenotype to the mutant cell and results in the morphology changes that are caused by cAMP and PKA. This suggests that Cγ is an active phosphotransferase in the intact cell as predicted by the deduced amino acid sequence, which contains highly conserved motifs that are common to serine/threonine kinases (Hanks and Quinn, 1991). This was not necessarily expected since, unlike Cα expression, Cγ expression results in tight binding to mutant RI and does not appear to produce an excess of free C subunit. However, if the rounding response is like many other cAMP effects, very low levels of PKA activation are sufficient to induce the response. Like the Cα clones, Cγ clones contained higher levels of total PKA activity, which could shift the cAMP activation equilibrium to the right, and

[2] S. J. Beebe, P. Salomonsky, T. Jahnsen, and Y. Li, unpublished data.

[3] S. J. Beebe, J. Huggenvick, and M. Uhler, unpublished data.

the enzyme may be more readily activated. Alternatively, higher kinase levels could induce the expression or formation of wild type I or type II holoenzymes, which are activated more readily than the mutant type I holoenzyme. This is not unreasonable since the Cγ7 clone appeared to express higher levels of the type II holoenzyme than Kin-8 did.

It is most likely that Cγ subunit is responsible for the rounding phenotype. First zinc induced the rounding phenotype in the Cγ clone in a concentration-dependent manner. PKI-insensitive activity generally accounted for 65–85% of the PKA activity in Cγ7, and in some experiments Cγ7 essentially replaced or diminished endogenous Kin-8 C subunits. Since type II PKA activity was primarily PKI-insensitive, had a low cAMP activation constant, and was elevated in the Cγ7 clone compared with Kin-8, it is quite possible that the zinc-cAMP-induced rounding response in the Cγ7 clone was caused by Cγ activation from type II PKA. Nevertheless, since Cγ binds more tightly to mutant RI subunit, at present it cannot be ruled out totally that endogenous C subunits are displaced to wild type RI subunit and they mediate the rounding response in the Cγ7 clone. However, no data are available to indicate that wild type RI subunit or wild type I holoenzyme is elevated, especially in the presence of mutant RI subunit. In fact, expression of mutant RI subunit has been shown to reduce and replace endogenous R subunits in several cell types (Clegg *et al.*, 1987).

In summary, transfection of Kin-8 adrenal cells with a Cγ expression vector results in the expression of a unique Cγ subunit of PKA that is cAMP-dependent when associated with a mutant RI or normal RII subunit and appears to bypass the RI subunit mutation in the Kin-8 cell restoring the cell to the wild phenotype, at least in part. However, in contrast to Cα and Cβ, Cγ it not inhibited by the PKI peptide. The presence of a PKA that is insensitive to this inhibitor has not been reported previously. The PKI has been a valuable tool for PKA analysis, but the data presented here indicate that some cautions may be required when utilizing PKI as a PKA diagnostic. Although Cγ mRNA has been detected only in human testis, a thorough analysis of human ovary, human brain, and tissues from other species has not been conducted. The Cγ subunit functions as a PKA phosphotransferase *in vitro* and in intact cells but may have a unique substrate specificity and different recognition determinants than Cα or Cβ. The potential for a unique role for Cγ in cAMP signal transduction requires further study.

Acknowledgments—We express special thanks to Dr. Michael Uhler for providing the Cα expression vector and pZEM-neo; to Drs. Jodie Huggenvick and Michael Uhler for assistance in preliminary Cγ expression studies; to Dr. Brian Hemmings for providing the C subunit antibody; to Dr. Bernard Schimmer for providing the Kin-8 cells and for helpful discussions during the course of these experiments; and to Dr. Erwin Reimann for providing purified catalytic subunit. We thank Dr. Gerry Pepe for a critique of the manuscript and Mary Beth Thompson for the typescript.

REFERENCES

Ashby, C., and Walsh, D. (1973) *J. Biol. Chem.* **248,** 1255–1261
Beale, E., Dedman, J., and Means, A. (1977) *J. Biol. Chem.* **252,** 6322–6327
Beebe, S., and Corbin, J. (1984) *Mol. Cell. Endocrinol.* **36,** 67–78
Beebe, S., and Corbin J. (1986) *Enzymes* **17,** 43–111
Beebe, S., Blackmore, P., Chrisman, T., and Corbin, J. (1988) *Methods Enzymol* **159,** 118–139
Beebe, S., Oyen, O., Sandberg, M., Froysa A., Hansson, V., and Jahnsen, T. (1990) *Mol. Endocrinol.* **4,** 465–475
Beebe, S., and Leyton, L., Burks, D., Ishikawa, M., Fuerst, T., Dean, J., and Saling, P. (1992) *Dev. Biol.* **151,** 48–54
Beushausen, S., Bergold, P., Sturner, S., Elste, A., Roytenberg, V., Schwartz, J., and Bayley, H. (1988) *Neuron* **1,** 853–864
Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
Cheng, H., Kemp, B., Pearson, R., Smith, A., Misconi, L., Van Patten S., and Walsh, D. (1986) *J. Biol. Chem.* **261,** 989–992
Clegg, C., Correll, L., Cadd, G., and McKnight, S. (1987) *J. Biol. Chem.* **262,** 13111–13119
Clegg, C., Ran, W., Uhler, M., and McKnight, S. (1989) *J. Biol. Chem.* **264,** 20140–20146
Demaille, J., Peters, K., and Fischer, E., (1977) *Biochemistry* **16,** 3080–3086
Foss, K., Simard, J., Brub, D., Beebe, S., Sandberg, M., Grzeschik, K., Gagn, R., Hansson, V., and Jahnsen, T., (1992) *Cytogenet. Cell Genet.* **60,** 22–25
Glass, D., Cheng, H., Kemp, B., and Walsh, D. (1986) *J. Biol. Chem.* **261,** 12166–12171
Hanks, S., and Quinn, A., (1991) *Methods Enzymol.* **200,** 38–62
Kemp, B., Graves, D., Benjamini, E., and Krebs, E. (1977) *J. Biol. Chem.* **252,** 4888–4894
Knighton, D., Zheng, J., Ten Eyck, L., Ashford, V., Xuong, N., Taylor, S., and Sowadski, J. (1991) *Science* **253,** 407–414
Laemmli, U. K. (1970) *Nature* **227,** 680–685
Olsen, S., and Uhler, M. (1989) *J. Biol. Chem.* **264,** 18662–18666
Olsen, S., and Uhler, M. (1991) *J. Biol. Chem.* **266,** 11158–11162
Olson, M., Krolczyk, A., Gorman, K., Steinberg, R., and Schimmer, B. (1991) *Endocr. Soc. Abstr.* **622,** 186
Rae, P., Gutmann, N., Tsao, J., and Schimmer, B. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 1896–1900
Reed, J., Gagelmann, M., and Kinzel, V. (1983) *Arch. Biochem. Biophys.* **222,** 276–284
Robinson-Steiner, A., Beebe, S., Rannels, S., and Corbin, J., (1984) *J. Biol. Chem.* **250,** 10596–10605
Roskowski, R., Jr. (1983) *Methods Enzymol.* **99,** 3–6
Schimmer, B. (1985) *Methods Enzymol.* **109,** 350–356
Scott, J., Fischer, E., Demaille, J., and Krebs, E. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 4379–4383
Sugden, P., Holladay, L., Reimann, E., and Corbin, J. (1976) *Biochem. J.* **159,** 409–422
Taylor, S., Buechler, J., and Yonemote W. (1990) *Annu. Rev. Biochem.* **59,** 971–1005
Toda, T., Cameron, S., Sass, P., Zoller, M., and Wigler, M. (1987) *Cell* **50,** 277–287
Uhler, M., and McKnight, S. (1987) *J. Biol. Chem.* **262,** 15202–15207
Van Patten, S., Ng, D., Th'ng, J., Angelos, K., Smith, A., and Walsh, D. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 5383–5387
Whitehouse, S., Feramisco, J., Casnellie, J., Krebs, E., and Walsh, D. (1983) *J. Biol. Chem.* **258,** 3693–3701
Wiemann, S., Kinzel, V., and Pyerin, W. (1991) *J. Biol. Chem.* **266,** 5140–5146
Wigler, M., Silverstein, S., Lee, L., Pellicer, A., Cheng, Y., and Axel, R. (1977) *Cell* **11,** 223–232