1992

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Original Publication Citation
The $\text{C}_\gamma$ Subunit Is a Unique Isozyme of the cAMP-dependent Protein Kinase*

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There are at least three isozymes ($\text{C}_\alpha$, $\text{C}_\beta$, and $\text{C}_\gamma$) of the mammalian catalytic ($\text{C}$) subunit of cAMP-dependent protein kinase (PKA) (Beebe, S., Oyen, O., Sandberg, M., Froyba, A., Hansson, V., and Jahnsen, T. (1990) Mol. Endocrinol. 4, 465-475). To compare the $\text{C}_\gamma$ and $\text{C}_\alpha$ 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 $\text{C}_\alpha$, histone was a better substrate than Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide) for $\text{C}_\gamma$. Furthermore, $\text{C}_\gamma$ 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 $\text{C}_\gamma$ peak (type I) eluted from DEAE-Sepharose at a higher NaCl concentration (120 mM) than the $\text{C}_\alpha$ type I eluted (70 mM). $\text{C}_\gamma$ and $\text{C}_\alpha$ type II eluted between 220 and 240 mM NaCl. $\text{C}_\gamma$ required higher concentrations of cAMP than $\text{C}_\alpha$ 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 $\text{C}_\alpha$ (41-42 kDa) and $\text{C}_\gamma$ (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 $\text{C}_\gamma$ and $\text{C}_\alpha$ clones, thereby restoring the cells to the parent Y1 adrenal cell phenotype. Collectively, these data indicate that $\text{C}_\gamma$ is an active PKA C subunit but suggest that $\text{C}_\gamma$ and $\text{C}_\alpha$ have different protein and peptide recognition determinants.

cAMP-dependent protein kinase (PKA) 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 (R1α, R1β, RIα, and RIβ). 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 $\text{C}_\alpha$, $\text{C}_\beta$, and $\text{C}_\gamma$ (Beebe et al., 1990). Recently, a second $\text{C}_\beta$ cDNA, designated $\text{C}_\beta_2$, was identified in bovine as a possible alternatively spliced $\text{C}_\beta$ gene product (Wiemann et al., 1991). In yeast, three distinct C subunit gene products have been described (Toda et al., 1987) and in Apysia, two alternately spliced C subunits have been identified (Besthuisen 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 $\text{C}_\gamma$ subunit appears to be testis-specific, and the $\text{C}_\gamma$ gene has been located to chromosome 9 (Foss et al., 1982). At the nucleotide level, $\text{C}_\alpha$ and $\text{C}_\gamma$ 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, $\text{C}_\gamma$ 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 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 Ca or 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 subunita, 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 Ca are shown to differ regarding interaction with a mutant Kin-8 RI subunit and sensitivity to a PKI peptide. However, both Cγ and Ca 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., 1992) was subcloned into the BamHI site of pZEM (Uhler and McKnight, 1987) downstream of the mouse metallothionein promoter. The Cγ cDNA was removed from pSK+ (BlueScript) with EcoRI, the ends were filled with Klenow, and BamHI 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 HindIII site in the Cγ gene to create the PMT Ca-neo expression vector. The PMT Ca-neo expression construct was restriction mapped to determine orientation of the Cγ cDNA, purified by CsCl centrifugation, and used for transformations. The pZEM expression plasmid, the 2.2-kilobase neomycin resistance sequence, and the PMT Ca neo expression plasmid were gifts from Dr. Mike Uhler, University of Michigan, and has been characterized 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 then further analyzed. For immunoblot analysis of C subunit expression, the Cγ cDNA was subcloned into the BglI site of pZEM (Uhler and McKnight, 1987) and this expression vector construct was restriction mapped to determine orientation of the Cγ cDNA, purified by CsCl centrifugation, and used for transformations. The pZEM expression plasmid, the 2.2-kilobase neomycin resistance sequence, and the PMT Ca neo expression plasmid were gifts from Dr. Mike Uhler, University of Michigan, and has been characterized previously (Beebe et al., 1992).

Assay of the CAMP-dependent Protein Kinase Activity—Tissue culture plates or flasks containing the parent Kin-8, Cγ, or Ca clones were washed three times with 10 ml of phosphate buffered saline (20 mM NaPO₄ (pH 7.5), 150 mM NaCl). When cells were prepared for ion-exchange chromatography, the last wash was with 10 ml potassium phosphate (pH 6.9), 1 ml EDTA, and 250 ml sucrose. Cells were scraped in a small volume of homogenizing buffer (0.5 ml/m³ cm plate or 1 ml/162-cm² 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 trypsin blue. The homogenate was centrifuged at 14,000g 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 used as a 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—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, 0.3% NaCl (pH 7.6)) 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 H₂O₂ (0.03%) until color developed (generally 10–60 s). The molecular weight data were determined using the rainbow protein 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 1.5 h stimulation with 50 μM zinc. Generally six 162-cm² 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 presence of various concentrations of cAMP. For the Ca clone, which contained overexpressed free C subunit, the extract was prechromatographed on a CM-Sepharose column (1.5 x 5.6 cm), and the flow-through fraction, which contained holoenzyme, was used as the starting Ca 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 [³²P] 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 from both fractions) after stimulation with 50 μM zinc 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 Ca Kin-8 Clones—The Cγ cDNA was cloned into the Zn²⁺-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 Cγ expression vector, containing the same elements and the mouse Ca 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 2 putative Ca clones were isolated, expanded in culture, and further analyzed. For a positive identification of Cγ- and Ca-expressing clones, extracts from each clone were prepared and assayed for Zn²⁺-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 Cy clones and all three Ca clones expressed kinase activity above the Kin-8 control activity levels. The Cy clones expressed a 25–100% increase in kinase activity, whereas the activity in Ca clones was increased 2–5-fold. Only two of the 11 G418 resistance Cy 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 Ca clones that were tested expressed similar amounts of elevated kinase activity, but only one of them (Ca2, 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 Cy clones and two Ca 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 Cy-transfected clones, and a Ca-transfected clone using histone and Kemptide as substrates in the presence and absence of inhibitor peptide, PKI. The basal and Zn\(^{2+}\)-induced increases in cAMP-dependent activity were most evident in Cy clones when histone was the substrate. The non-Zn\(^{2+}\)-induced histone kinase activities of Cy7 and Cy10 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. Cy7 had lower basal and greater Zn\(^{2+}\) induced histone kinase activity than Cy10. When Kemptide was the substrate the evidence for Cy expression was less clear. In contrast, Ca expression was readily evident and apparently greater than Cy expression with either Kemptide or histone as substrate. Basal non-Zn\(^{2+}\)-stimulated activity in Ca2 was 2-fold greater than Kin-8 controls and Zn\(^{2+}\)-stimulated Ca2 kinase activity by 2-fold.

The most notable difference between Cy and Ca clones and best discriminator for Cy expression was the absence of PKI inhibition in Cy extracts (Fig. 1) and partially purified Cy 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 Cy clone-specific. In contrast the histone kinase activity in Kin-8 and Ca clones was inhibited 90–95% by PKI. Zinc increased activity in Cy 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 Cy7 clone and somewhat lower in Cy10. In the absence and the presence of zinc PKI-sensitive activities in Cy7 and Cy10 were similar to the PKI-sensitive activity in the Kin-8 parent. No changes in PKI-insensitive activity were observed in Kin-8 or Ca2 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 Ca 2 extracts was between 4.8 and 6.0. In contrast, the same ratio for Cy7 extracts was between 2.0 and 3.0. As the PKI-insensitive activity was enriched (see below) the Cy7 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 Cy7 clones, histone was used routinely to evaluate Cy activity and to compare it with Kin-8 and Ca2 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 Ca2 was relatively low, and activity was stimulated about 10-fold by cAMP when Kemptide was the substrate. When Co2 and two other Ca 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 Co2 extracts were chromatographed. In contrast, extracts from Cy 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 Cy extracts were chromatographed.

Immunodetection of C Subunits in Cy and Ca Expression Clones—Although the amino acid homology between Cy and Ca was lower than homology between Ca and Co, it was likely that a polyclonal antibody made against Ca would recognize Cy. Consequently, immunodetection of Cy 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 Cy7 was PKA. Fig. 2 shows a Western blot of purified bovine heart C subunit and equal amounts of extract protein from Cy7, Ca2, and Kin-8 clones. Lanes containing the C subunit standard, Ca2, and Kin-8 each contained a single 42-kDa band. The Cy7 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, Co2, and Cy7, respectively. This represented a 4.5- and a 7.0-fold stimulation above Kin-8 for Co2 and Cy7, respectively, and was proportional to the intensity of the corresponding immunoreactive bands.

Chromatographic Analysis of Cy7 and Co2 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.
Expression of PKAγ in Mammalian Cells

To characterize further the PKI-insensitive histone kinase activity in the Cy7 clone and determine if it associated with RI or RII subunits, Cy7 cell extracts were chromatographed on DEAE-Sepharose and eluted with NaCl gradients. Fig. 4A shows a composite DEAE-Sepharose elution profile for Co2 (PKI-sensitive) and Cy7 (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 Co2 clone was sensitive to PKI (not shown), and 90% of it eluted at 70 mM NaCl. The Cy7 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 Cy (PKI-insensitive) peak I eluted at 126 ± 9 mM NaCl (mean ± S.E., n = 4); Co 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 Cy 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 Cy7 clone, the ratio of PKI-insensitive to PKI-sensitive activity was greater than shown in Fig. 4A and indicated a decrease in PKI-sensitive histone kinase activity. Fig. 4B 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 4B and 55% in experiment 4A of the total activity. In the experiment

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 Cy7 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 Co2 clones (Fig. 3B). For Cy7, 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 Co2 (Fig. 3B) was inhibited 90–95% by PKI and eluted in the same fraction. Results similar to those shown for Co2 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 Cy7, Co2, 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, = 42,000), and apparent molecular weights were determined using the rainbow maker (Promega).

Fig. 3. Sephacryl (S-300) gel filtration of the free C subunit from Cy7 and Co2 extracts. Extracts from four 102-cm² flasks of Cy7 (panel A) and Co2 (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.

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PKI insensitive, and the Ca2 peaks contained 90-95% PKI-clones. When analyzed by the Hill equation as reported previously for PKAs (Beebe and Corbin, 1984), the values for Cy7 and Ca2 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 A0.5 values. In contrast, the type II Cy7 and Ca2 enzymes had A0.5 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 Cy7, Ca2, and Kin-8 Clones—Initial experiments to purify free Cy 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 Cy bound more tightly than Ca 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 presence (holoenzyme plus free C subunit) of cAMP.

Fig. 5 shows the effect of cAMP on PKA dissociation in Cy7, Kin-8, and Ca2 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, Cy7 activity was determined in the presence of PKI, and Kin-8...
and Co2 activities were determined in the absence of PKI. The PKI-insensitive activity in the Cy7 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 Cy7 and Kin-8 extracts, which did not contain free C subunit, Co2 extracts were prechromatographed on a CM-Sepharose column to remove free C subunit prior to the cAMP treatment. A similar treatment of Cy7 and Kin-8 extracts made no difference in the results of the experiments. When Cy7 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 Co2 to higher levels as indicated by activation ratios as high as 0.6. Kin-8 extracts, and the PKI-sensitive activity in Cy7 extracts, representing endogenous Kin-8 C subunit (not shown), showed activation characteristics similar to those in Co2, although Co2 was dissociated slightly more for any given concentration of cAMP.

Separation of PKI-insensitive and PKI-sensitive Histone Kinase Activity in the Cy7 Clone—Fig. 6 shows the results of a protocol (see figure legend) that separated Cy 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 predominately PKI-sensitive, endogenous Kin-8 C subunit at 41-42 kDa in fraction 34 and a totally PKI-insensitive Cy 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 reversal of the Kin-8 mutant to the Y1 phenotype, the well characterized CAMP-induced morphological rounding effect (Schimmer, 1985) was analyzed when Kin-8, Co2, and Cy7 clones were treated with zinc to induce the expression of Cα and Cγ in transfected clones (Fig. 7). The untreated Kin-8, Co2, and Cy7 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 Co2 and Cy7 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 Co2 and Cy7 but not for Kin-8 (not shown). Fig. 7 shows the maximal morphological response of the Co2 and Cy7 clones to zinc (90 μM). Kin-8 did not show the rounding response except at high concentrations of zinc (≥ 120 μM). Rounding of Co2 occurred at 50-60 μM zinc, but the rounding of Cy7 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.

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, Cy is distinctly different from Ca regarding PKI peptide sensitivity. The PKI insensitivity of Cy is not dependent on the presence of the mutant RI or normal RII subunit, since Cy histone kinase activity was not significantly inhibited when tested as a free Cy 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 Cy 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 Cy activity. The presence of a unique PKA Cy subunit and a unique PKA inhibitor in the testis (Beale et al., 1977; Van Patten et al., 1991) suggests the possibility for distinct Cy PKA-mediated roles in reproductive function.

Other Cy properties also distinguish it from Ca. Cy bound more tightly than Ca did to the Kin-8 mutant R subunit, and full activation of Cy required different conditions. Cy 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 Cy holoenzyme. This is, in part, responsible for the relatively poor phosphorylation of Kemptide by Cy. It is possible that the tight binding of Cy to mutant Kin-8 RI subunit is primarily responsible for the successful expression of Cy since Cy expression in mouse L-cells and COS cells was of limited success. Unlike Ca, Cy 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 Cy subunit in DEAE-Sepharose flow-through and CM-Sepharose elution fractions. Furthermore, only two out of 11 Cy clones expressed sufficient Cy activity to warrant further study, but all three Ca clones tested overexpressed free Ca subunit in the presence of zinc. Taken together these data suggest that free Cy subunit may be unstable or rapidly turned over in the intact cell.

For all clones tested Ca expression appeared to be greater than Cy expression based on total protein kinase activity. However, full Cy 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 Co2 clone than in any of the Cy clones tested. This suggests that Cy may bind more tightly to the mutant RI subunit in the intact cell. However, it is possible that some of the differences in Cy and Ca expression are caused by the expression vector constructs rather than differences between the Cy and Ca proteins. The Cy expression vector contained more 3'-untranslated sequences than the Ca construct, but we did not directly study Cy mRNA expression. Nevertheless, it is clear that differences do exist between the Cy and Ca proteins, and some of these may account for some of the differences in the expression in the Cy7 and Co2 clones. If Cy does have a distinct substrate and protein/peptide binding specificity, as suggested by differences in Cy and Ca PKI sensitivity, the timely phosphorylation of some functionally relevant substrate by a rapidly turned-over cAMP-dependent, Cy-mediated mechanism could provide tight control of the phosphorylation of some temporally important phosphoprotein. In addition, tight binding of Cy 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 Cy expression more fully in tissues, cells, and tumors to determine Cy substrate specificity and to determine if Cy binds more tightly than Ca does to wild type R subunits.

The Cy type I holoenzyme eluted at higher salt concentrations from DEAE-Sepharose than Ca type I holoenzyme did. This property and the tight binding of Cy to mutant Kin-8 RI subunit provided a means to separate Cy 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 Ca2 and parent Kin-8 extracts, and two bands at 41–42 kDa and 39–40 kDa in Cy7 extracts. The smaller band was identified as Cy 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 Cy 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 Cy isozyme. The Cy expression vector construct contained a 27-base pair extension at the 5' end of the Cy cDNA, which potentially codes for 9 additional amino-terminal residues (Beebe et al., 1990).

Therefore, Cy could be expected to be larger than Ca. However, the translation start site for Cy has not been identified specifically, and it is not known if these residues are part of the recombinant Cy 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 Cy band could result from a dephosphorylated enzyme, but the presence or absence of Cy autophosphorylation has not been studied, and the effect of autophosphorylation on C subunit migration after SDS-polyacrylamide gel electrophoresis is not clear presently.

Cy 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 Cy activity was associated with type I, but in all experiments more type II holoenzyme was found in the Cy7 clone than in either the Kin-8 parent or the Ca2 clone. In contrast to Cy expression in Kin-8 cells, expression of Ca 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 Cy with mutant Kin-8 RI subunit, Cy may more readily associate with RII subunit than Ca does. Further studies are in progress to confirm these findings and to determine if Cy induces and/or binds to RIIα and/or RIIβ.

The expression of the Cy isozyme of PKA, as well as Ca (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 Cy 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 Ca expression, Cy 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 Ca clones, Cy clones contained higher levels of total PKA activity, which could shift the cAMP activation equilibrium to the right, and

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2 S. J. Beebe, P. Salomonsky, T. Johnsen, 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 express the enzyme expression 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 Cy7 clone appeared to express higher levels of the type II holoenzyme than Kin-8 did.

It is most likely that Cy subunit is responsible for the rounding phenotype. First zinc induced the rounding phenotype in the Cy7 clone in a concentration-dependent manner. PKI-insensitive activity generally accounted for 65–85% of the PKA activity in Cy7, and in some experiments Cy7 essentially replaced or diminished endogenous Kin-8 C subunits. Since type II PKA activity was primarily PKI-insensitive, there was a low cAMP activation constant, and was elevated in the Cy7 clone compared with Kin-8, it is quite possible that the zinc-cAMP-induced rounding response in the Cy7 clone was caused by Cy activation from type II PKA. Nevertheless, since Cy 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 Cy7 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 muta-RI subunit. In fact, expression of mutant RI subunit has not been reported previously. The PKI has been a valuable diagnostic. Although Cy 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 Cy subunit functions as a PKA phosphatase 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 Cy in cAMP signal transduction requires further study.

Acknowledgments—We express special thanks to Dr. Michael Uhlcr for providing the Cy expression vector and pZEM-neo; to Drs. Joëlle Huggenick and Michael Uhler for assistance in preliminary Cy expression studies; to Dr. Brian Hennings 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. Ervin 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