Evidence for a Second Isoform of the Catalytic Subunit of cAMP-dependent Protein Kinase*

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We have used a previously characterized mouse cDNA clone for the catalytic (C) subunit of cAMP-dependent protein kinase (Uhler, M. D., Carmichael, D. F., Lee, D. C., Chrvia, J. C., Krebs, E. G., and McKnight, G. S. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 1300–1304), which we designate Ca, to isolate cDNA clones coding for a second isoform of the C subunit, CB. Ca cDNA clones hybridize to a 2.4-kilobase mRNA on Northern blots whereas CB cDNA clones detect a 4.3-kilobase mRNA. Nucleotide sequence comparison between Ca and CB cDNA clones shows that the CB cDNA codes for a protein which shows 91% identity with Ca. Determination of mRNA levels for CB in various tissues shows that it is most highly expressed in brain although it is detectable in all tissues examined. The presence of two genes coding for the C subunit of cAMP-dependent protein kinase may explain past reports of heterogeneity in C subunit protein preparations.

Many of the biological effects of cAMP are thought to involve activation of cAMP-dependent protein kinase via binding of cAMP to the regulatory (R)1 subunit and subsequent release of the catalytic (C) subunit from the holoenzyme (1). Two different types of cAMP-dependent protein kinase have been distinguished based on their order of elution from DEAE-cellulose and have been termed Types I and II (2). The R subunit of the Type I enzyme (RI) has a molecular weight of 49 kDa on SDS-polyacrylamide gels, and the amino acid sequence of bovine skeletal muscle RI has been reported (3). At least two forms of the Type II subunit (RII) have been identified: a 54-kDa RII found in most tissues (4) and a 51-kDa form found in brain, granulosa cells, and adrenal tissue (5, 6). In contrast, C subunit preparations isolated from the different types of cyclic AMP-dependent protein kinase and from different tissues have identical molecular weights of 40 kDa by SDS-polyacrylamide electrophoresis (7–10). In addition these different C subunit preparations also have similar biochemical and enzymatic properties (7–10). Nevertheless, at least three forms of this protein have been separated by isoelectric techniques (9).

We have previously described mouse cDNA clones for the Ca subunit which is 98% identical with the published bovine heart C subunit protein sequence (11). We have used the Ca cDNA under low-stringency hybridization conditions to isolate cDNA clones coding for proteins related to the C subunit. Here we report the isolation of cDNA clones coding for a protein (CB) which shows 91% amino acid identity with Ca. MATERIALS AND METHODS

Construction and Screening of cDNA Libraries—Phage λgt10 and λgt11 cDNA libraries from BALB/c mouse heart, brain, and S49 lymphoma cells were constructed as described (12). Phage plaques were transferred to nitrocellulose filters, denatured, and hybridized under low (25 °C) or high (42 °C) stringency in HYB buffer (60% formamide, 0.75 M NaCl, 0.075 M sodium citrate, 50 mM Na2HPO4, pH 7.5, 5 mM EDTA, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% SDS, 100 µg/ml of salmon sperm DNA). The nitrocellulose filters were baked at 20 °C (low stringency) or 65 °C (high stringency) in 0.9 M NaCl, 0.09 M sodium citrate, 50 mM Na2HPO4, pH 7.5, 0.1% SDS. A 700-base pair cDNA insert (11) was isolated from a S49 lymphoma cDNA library by low stringency hybridization using the 75-base pair Sau3AI-EcoRI Ca fragment employed previously to isolate the Ca cDNA clone MC8 (11). The Ca cDNA insert was then used to isolate MC31 and MC32 cDNA clones from a mouse heart library and to isolate MC33 from a mouse brain cDNA library.

Subcloning and Sequencing of cDNA Inserts—The EcoRI cDNA inserts of MC31, MC32, and MC33 were subcloned into the EcoRI site of pUC13 for restriction mapping. Restriction fragments of the MC31, MC32, and MC33 inserts were then subcloned into M13 phage vectors and both strands were sequenced using the dideoxy termination method as described (11). No differences in overlapping sequences were found between the three Cβ cDNA clones.

Northern Blot Analysis—Samples were denatured in 20 mM MOPS, pH 7.0, 1 mM EDTA, 5 mM sodium acetate, 2.2 M formaldehyde, and 50% formamide at 66 °C for 15 min. The samples were loaded on a 1% agarose gel and run in the same buffer without formamide. The gel was then blotted to nitrocellulose, baked at 80 °C for 2 h, and hybridized for 16–20 h.

Quantitation of Ca and Cβ mRNA Levels—Quantitation of Ca and Cβ mRNA levels was performed as described (11) except that the hybridizations were performed at 80 °C rather than 70 °C to increase the specificity of the hybridization. The SP6 transcript used to measure Cβ mRNA level corresponded to the 5′ EcoRI/SacI fragment of MC31.

RESULTS

In order to isolate cDNA clones coding for proteins related to the Ca protein, a 75-base pair Sau3ΑI-EcoRI fragment of the Ca gene (11) was used to screen an S49 lymphoma cDNA library under low-stringency hybridization conditions. A cDNA clone that hybridized to this probe but did not have a restriction map consistent with that of the Ca cDNA clones was then used under conditions of high stringency to isolate cDNA clones containing the entire coding region of Cβ. The composite restriction map for clones MC31, MC32, and MC33 is shown in Fig. 1 and clearly distinguishes these cDNAs from the previously characterized Ca cDNAs (11).

Sequence analysis of these three clones showed that they contained a 1053-nucleotide open reading frame identical in size to that found for the Ca cDNA (Fig. 2). Within the coding regions, however, there is an 80% nucleotide homology between the mouse Ca and Cβ cDNAs. The Cβ nucleotide...
Fig. 1. Restriction map of the composite Cβ cDNA sequence. The topmost bar represents the 1272-base pair composite cDNA sequence obtained from the individual clones MCp1, MCp2, and MCp3. All restriction sites for BglII, PstI, SacI, and SalI restriction endonucleases are indicated above the bar. The solid black area within the bar indicates the protein coding region of the cDNA. The positions and sizes of the black bars below the composite restriction map indicate the relative size and location of individual Cβ cDNA clones. The MCp3 clone extends in the 3' direction another 400 base pairs past the SacI site, but this region was not sequenced. Synthetic EcoRI sites, but this region was not sequenced. Synthetic EcoRI

sequence predicts a protein of 351 amino acids that has 91% identity to mouse Ca and half of the amino acid differences are conservative substitutions. The homology between Ca and Cβ is weakest in the amino-terminal regions of the two proteins with only 73% identity over the first 70 amino acids. The two amino acid residues that have been implicated in ATP binding for the porcine C subunit, Lys73 and Cys194, are conserved in both mouse Ca and Cβ (13, 14). Comparison of the two protein sequences shows that Cβ has a charge of +4 at pH 7.0, while Ca would have a charge of +6. In spite of this charge difference, it is not clear that these two proteins could be resolved by isoelectric focusing techniques. The 192 nucleotides' of the initiation codon were 71% GC, similar to the 84% GC observed for the Ca cDNA (11).

In order to determine the presence of both Ca and Cβ mRNA in mouse cell lines and normal mouse tissues, the Ca

nucleotide sequence correspond to the position of the last amino acid displayed on that line within the Cβ protein sequence. Assuming a position of -1 for the initiator methionine.
was the same as that used previously (11). The SP6 probe for Cp was identical to untransfected cells when measured using the SP6 solution hybridization assay. Cross-hybridization between Ca and Cp SP6 probes was demonstrated using an SP6 RNA transcript complementary to either Ca and Cp. Arrows indicate a 2.4-kb RNA species hybridizing to the Ca probe and a 4.3-kb RNA hybridizing to the Cp probe.

**TABLE I**

Levels of Ca and Cβ mRNAs in mouse tissues

Levels of Ca and Cβ mRNA in mouse tissues were determined using an SP6 RNA transcript complementary to either Ca and Cβ mRNA in a solution hybridization assay as described (11) except that the hybridizations were carried out at 80°C. The SP6 probe for Ca was the same as that used previously (11). The SP6 probe for Cβ was generated using the EcoRI/ScaI fragment of MCp1. The absence of cross-hybridization between Ca and Cβ SP6 SP6 probes was demonstrated using total nucleic acid samples from cells transfected with a Ca expression vector that contained levels of Ca mRNA 10-fold higher than untransfected cells. Cβ mRNA levels in these cells were identical to untransfected cells when measured using the SP6 solution hybridization assay.

| Tissue         | Ca mRNA level | Cβ mRNA level |
|----------------|---------------|---------------|
|                | molecules/µg RNA | molecules/µg RNA |
| Brain          | 74            | 20            |
| Heart          | 81            | 11            |
| Intestine      | 9             | 4             |
| Kidney         | 44            | 10            |
| Liver          | 66            | 17            |
| Lung           | 8             | 9             |
| Muscle         | 72            | 12            |
| Pancreas       | 12            | 4             |
| Spleen         | 5             | 4             |
| Testis         | 105           | 5             |

Ca and Cβ cDNAs were used for Northern blot analysis of Ca and Cβ mRNA. Fig. 3 shows that, whereas the Ca mRNA is 2.4 kb in length (11), the Cβ mRNA is 4.3 kb in all tissues examined. In addition, the Northern blots show that the relative ratio of Ca to Cβ mRNA varies dramatically between different tissues. For example, there appear to be approximately equal amounts of Ca and Cβ mRNA in anterior pituitary AtT-20 cells, but in S49 lymphoma cells the Ca mRNA predominates. In normal mouse tissues, brain appears to contain the largest amount of Cβ mRNA although detectable levels appear in all tissues. Northern blot analysis under conditions of low stringency with either Ca or Cβ probes shows only two bands at 2.4 and 4.3 kb (data not shown); however, this does not exclude the possible existence of mRNAs coding for other isoforms of C since they might be expressed in tissues we have not examined or have less nuclear acid homology to Ca and Cβ than Ca and Cβ have to each other.

More quantitative estimates of Ca and Cβ mRNA levels were obtained by using SP6 RNA probes in a solution hybridization assay under conditions where cross-hybridization of Ca and Cβ does not occur (Table I). Since the DNA content of the tissue nucleic acid samples varies from one tissue to another, the results are given both in mRNA molecules/cell and in µg of mRNA/µg of total RNA for all the tissues examined. These results generally agree with those seen in Northern blot analysis and show that the level of Cβ mRNA varies from 4 molecules/cell in intestine, lung, pancreas, and spleen to 20 molecules/cell in brain. The SP6 solution hybridization results confirm that the ratio of Ca to Cβ mRNA varies widely from tissue to tissue with testis having 20-fold more Ca than Cβ mRNA and spleen having approximately the same amounts of the two mRNAs. No consistent correlation between the Ca/Cβ mRNA ratios (Table I) and the relative ratio of Type I/Type II kinase is seen for mouse brain (1:9), heart (1:1), liver (1:1), and lung (6:1) (15). However, it should be noted that relative levels of Ca and Cβ protein in a particular tissue may differ substantially from that shown for...
the mRNAs depending on the translational efficiency and the rates of protein degradation.

**DISCUSSION**

Although many authors have assumed that C subunits from different tissues and holoenzymes types are identical (1, 2, 16), heterogeneity in C subunit preparations has been reported previously. Analysis of bovine heart Type II C subunit preparations using isoelectric focusing has shown three distinct isoelectric forms with pl values of 7.01, 7.48, and 7.78 (9). Since the C subunit is known to be both phosphorylated at Thr207 and Ser238 and myristylated at its amino terminus (1, 2), multiple isoelectric forms are not unexpected. However, Peters et al. (9) examined all three isoelectric forms and demonstrated similar phosphate content and amino-terminal blocking groups. Although the different isoelectric forms also had slightly different amino acid compositions, we found no clear correlation with the compositions of Co and Cβ presented here. A second report has compared the tryptic peptides generated from R and C subunits purified from either the Type I or Type II kinase from porcine skeletal muscle (10). Whereas the R subunits from either type of kinase had very different tryptic peptides, the C subunit preparations had very similar peptide maps with only minor differences. Finally, three forms of bovine heart C subunit complexed with the cyclic AMP-dependent protein kinase inhibitor protein were identified upon nondenaturing electrophoresis (17). The formation of one of the complexes required ATP while another showed no such dependence on ATP. The multiple forms of the inhibitor protein-C subunit complexes were suggested to represent multiple isoelectric forms of the C subunit interacting with the inhibitor protein. Unfortunately it is difficult to strictly correlate any of these earlier demonstrations of C subunit heterogeneity with the Co and Cβ sequences shown here due to species differences and uncertainty regarding the contribution of post-translational phosphorylation and myristylation events to the observed heterogeneity.

The existence of two distinct C subunits of cAMP-dependent protein kinase raises several interesting questions about their functional significance. The high degree of amino acid sequence homology, despite considerable difference in nucleic acid sequence, suggests that after an early gene duplication event, Co and Cβ subunits acquired distinct and essential roles as part of the cAMP-dependent protein kinase system. This conclusion is supported by the demonstration that bovine tissues also express a Cβ subunit that shows more homology to mouse Cβ (97% amino acid identity) than mouse Cβ shows to mouse Co.

It is possible that the amino acid sequence differences between Co and Cβ could lead to differences in substrate specificity between the two types of C subunit. The observation that substrate sequences on the amino-terminal side of phosphorylated serine residues generally fall into one of two classes, Arg-Arg-X-Ser or Lys-Arg-X-Ser, could be consistent with the existence of multiple forms of the C subunit (18). Since the regulatory subunits inhibit catalytic activity by binding to the same binding site as substrate proteins (2), one might also expect differences between Co and Cβ in their interactions with the various regulatory subunits. We suggest that Co and Cβ subunits do not represent Type I and Type II specific C subunits but that Co interacts with both RI and RII while Cβ may preferentially interact with a distinct R subunit isoform that has yet to be characterized. The crucial questions regarding the interaction of Cβ with different types of R subunits and the possible differences in substrate specificities between Co and Cβ can be addressed now that cDNAs for Co and Cβ have been isolated.

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