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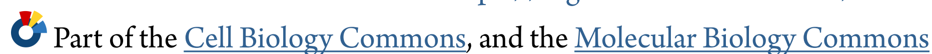
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Cγ- AND Cα-SUBUNITS PROVIDE THE SPECIFICITY FOR cAMP/ PKA FUNCTION

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A Dissertation Submitted to the Faculty of

Eastern Virginia Medical School & Old Dominion University

in Partial Fulfillment of the Requirement for the Degree of

DOCTOR OF PHILOSOPHY

Biomedical Sciences

May, 1997

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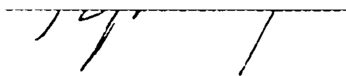

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ABSTRACT

Cγ-AND Cα-SUBUNITS PROVIDE THE SPECIFICITY FOR PKA/cAMP FUNCTION

Wei Qing Zhang

Eastern Virginia Medical School and Old Dominion University, 1997

Advisor: Dr. Stephen J. Beebe

The Cα and Cγ subunits of the cAMP-dependent protein kinase (PKA) are two highly homologous (83% amino acid identity), yet functionally distinct isozymes *in vitro* for substrate and pseudosubstrate specificity, and in intact cells for cell phenotypes (Beebe, 1992).

To determine the molecular mechanisms underlying the distinct functions in cell phenotypes, *in vitro* experiments were designed to make a detailed comparison of Cγ and Cα for substrate and pseudosubstrate specificity. To this end, Cγ and Cα were expressed in mammalian cells, bacteria and Sf9 insect cells using baculovirus. Abundant expression of active enzyme was cell system specific. Cγ and Cα expressed in Sf9 cells and bacteria, respectively, were purified to homogeneity. Kinetic analysis showed that Cγ and Cα shared primary substrate phosphorylation specificity. Cγ exhibited similar or lower Km values, but lower Vmax values in substrate phosphorylation than Cα. Different isoforms of PKI and R-subunit expressed in bacteria were purified to homogeneity. The kinetic comparison on these pseudosubstrate-inhibition of phosphotransferase activity showed Cγ was insensitive to PKI-inhibition and required the C-terminal residues in the pseudosubstrate site for RIα-inhibition. *In vitro* data suggested that Cγ had unique substrate and pseudosubstrate specificity. Intact cell experiments were designed to determine the distinct role of Cγ and Cα in the regulation of cAMP-responsive gene expression. To this end, Cγ and Cα were compared in the regulation of CRE(cAMP responsive elements)-reporter gene activity, and in the events which were associated with CRE-reporter gene expression using HEK293 cells and Y1/Kin8 clones permanently transfected with Cγ- and Cα-subunits. In response

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to cAMP stimulation, both Cγ and Cα migrated to the nucleus, phosphorylated CREB (cAMP responsive element binding protein) and increased the levels of immunoreactive CREB. Phosphorylated CREB in the nuclear extracts of Cγ and Cα clones bound to a CRE element in super gel mobility shift assay. Surprisingly, Cα, but not Cγ induced CRE-reporter gene activity and only Cα clone restored steroidogenesis to cAMP resistant Kin8 mutant. However, Cγ could both positively and negatively modulate Cα-mediated regulation of reporter gene activity. Results from Gal4-luc/Gal4-CREB reporter gene study showed that CRE and CREB dimerization were not necessary for Cγ to modulate the Cα-mediated regulation of CRE-reporter gene activity, suggesting that Cγ and Cα played different roles in the regulation of cAMP-responsive gene activity by an intra-CREB-molecular mechanism.

The presence of two kinetically and functionally distinct C-subunit isoforms provides the potential to fine-tune and /or to diversify cAMP signal transduction downstream of PKA activation, which is mediated by R-subunits and PKI, and/ or activation of C-isoforms with different specificity.

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THIS DISSERTATION IS DEDICATED TO

MY GRANDMOTHER, WHO LOVED ME THE MOST IN THIS WORLD,

AND MY SON (JACK ZHANG), WHO IS THE MOST IMPORTANT TO ME !

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ACKNOWLEDGMENT

First, I would like to express my sincere gratitude to the members of my research guidance committee: Dr.Gerald J. Pepe, Dr Howard D. White, Dr. Peter F. Blackmore, Dr. Mark S. Elliott, Dr. Christropher J. Osgood and Dr. Xi Jing for their kind help and important advice for my study and research.

Especially, my thanks go to my advisor, Dr. Stephen J. Beebe, for his kind and very patient help for enriching both my academic and cultural life, and for leading me into protein kinase research area. He has not only been an excellent academic mentor, but also a valued friend. Thank you, Stephen.

My appreciation is extended to the following people for them in a variety of ways to help me during my dissertation research.: Dr. Cathy Holroyd, Dr.Miker Uhler, Dr.Susan Didorm, Dr. Erwin Reimenn, Dr. Susan Taylor, Dr. Pat Quinn, Mrs. Kim Lattanzio, Dr. Briam Hemming, Dr. Stanley McKnight, Dr.Celest Smith, and Dr. Jackie Corbin. Thanks also go to my colleagues and friends: Burk, Bobby, Troy and Rick.

I would also like to thank my son, Jack Zhang, and my wife Lucy so much for their support throughout these years.

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CHAPTER I

INTRDUCTION

A. Background and Significance of Research

1.Protein kinase and phosphorylation coordinate cell function: Protein phosphorylation is one of the most highly conserved and successful mechanisms to evolve for the regulation of cell function in multicellular organisms (Hunter et al., 1995). Many critical proteins and enzymes are regulated by reversible covalent modification with the addition of a phosphate. The protein kinase family that catalyzes these reactions is becoming bigger in number and more diverse in functions. The functional regulation by phosphorylation in the eukaryotic cells is initiated by extracellular signals that generate intracellular second messengers (cAMP, cGMP, Ca^{2+}, phospholipids and others), which activate downstream protein kinases. There are two major classes of kinase: those showing specificity for serine/threonine, and those that transfer phosphate to tyrosine. Many mutant oncogenic proteins are protein kinases and have been demonstrated to disturb normal signaling pathways, which serves as a important molecular mechanism accounting for the origin of some tumors. Many multiple-substrate protein kinases play an integral role in signal transductions (Walsh et al., 1994). Of these, cAMP-dependent protein kinase is a prototypic model.

2.The cAMP-dependent protein kinase (PKA) is the prototype and best characterized kinase in eukaryotic cells: PKA is composed of a regulatory (R) subunit dimer and two monomeric catalytic subunits (Beebe et al., 1986, and Talor et al., 1989). The R-subunit family consists of four distinct gene products (RIα, RIβ, RIIα, and RIIβ) and the C-subunit family consists of 3 distinct gene products (Cα, Cβ, and Cγ) (Beebe, 1994). In the absence of cAMP, the C-subunit is bound as an inactive kinase to R-subunit, which functions in part as a pseudosubstrate, inhibitor and regulator of kinase activity. Each R-subunit of PKA contains the sequence Arg-Arg-Gly-Ala-Ile, which matches the consensus sequence for phosphorylation except for the presence of alanine in place of

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serine. In the R_2C_2 complex, this pseudosubstrate of R occupies the catalytic site of C, thereby preventing the entry of proteins substrates. In the presence of cAMP, which binds in two distinct sites on R-subunit, C-subunit is released as a free, active phosphotransferase, which phosphorylate protein substrates and mediates all of the well defined actions of cAMP (Figure 1). A PKA R-subunit model for cAMP binding was first derived by analogy to the crystal structure of the catabolic activator protein (CAP) from bacteria (Beebe et al., 1986) and more recently from the crystal structure of an RIα-subunit amino terminal deletion mutant (Su et al., 1995).

PKA, the second protein kinase to be purified and characterized, is one of the simplest members of kinase family owing to its well defined simple structure and mechanism of activation. The crystal structure of the catalytic subunits of PKA (Cα), complexed with ATP and 20-residue inhibitor (PKI_{5-24}), is reviewed and correlated with chemical and mutagenetic data (Taylor et al 1993). The crystal structure of the C-subunit serves as a prototype for all kinases, and shows a striking convergence of protein kinase structure with biochemistry and genetics (Knighton et al., 1991). The well-defined molecular mechanism for the catalysis by the PKA Cα-subunit serves as general template for the entire family of the enzymes.

3.The C-subunit serves as a model for all phosphotransferases and mediates all of the well characterized actions of cAMP PKA : All kinases share a highly conserved catalytic core, which is based on sequence comparisons and the C-subunit structure resolved from X-ray crystallography. The C-subunit is considered as a model for all eukaryotic protein kinases. The C-subunit is composed of two lobes, and catalysis occurs in the cleft between these lobes (Zheng et al., 1993). The smaller lobe (residues 40-125) functions to bind ATP/Mg^{2+} for phosphate transfer and the larger lobe (residues 140-280) functions to bind the substrate, to position the phosphate of ATP for transfer, and to promote catalysis. C-subunit catalyzes the transfer of γ-phosphate of ATP to substrate by an ordered bi-bi mechanism in which ATP binds first (Whitehouse et al., 1983).

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However, Kong's reporter (Kong and Cook, 1988) indicated that substrate phosphorylation by PKA followed the sequential random mechanism. No definitive conclusion has been made yet. The terminal anhydride bond is then proposed to undergo a conformational change, induced either as a consequence of ATP binding *per se* or by the subsequent binding of protein substrate. Catalysis then ensues, leading to the sequential release of phospho-substrate and ADP (Figure 2).

The resolved catalytic structure of C-subunit has broad significance because residues 40-280 constitute a conserved catalytic core that is shared by more than a hundred different protein kinases. Most of the highly conserved residues among protein kinases are clustered near the surfaces of the cleft between the small lobe and large lobe. These include all 11 residues that are conserved in the protein kinase family. Two extended chain structures outside of the loop domains are important for substrate, R-subunit and inhibitor recognition (Brushia et al., 1995; Knighton et al., 1991). These chains have been shown to mediate substrate recognition. Cγ and Cα differ in several regions of these chains, which may explain differences in observed isoform-specificity for substrates, R-subunits and inhibitors. The C-subunit of PKA regulates a wide range of functions including energy metabolism, gene expression, proliferation, and differentiation, as well as immune and reproductive functions in selected cell types (see Beebe, 1994 for review).

Two natural, highly homologous, but functionally distinct isozymes, Cγ and Cα, provide a focused structural map to correlate structural and functional differences (Beebe, 1992 and 1994). Chemical modification and site directed mutagenesis studies have generally supported C-subunit model derived from crystal studies, but additional studies about the molecular basis for the structural and functional differences between the Cγ and Cα is necessary. Comparison of these two natural variants will provide good models to further develop our understanding of protein kinase function in cAMP-mediated signal transductions.

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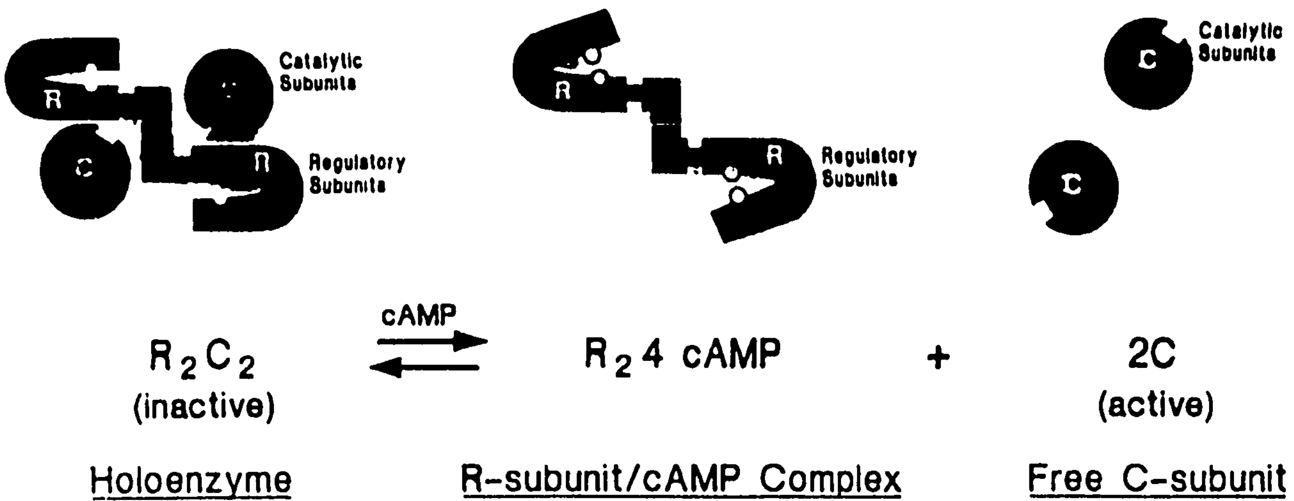

Figure 1 Diagram for the Structure and Activation of PKA by cAMP
The binding of four moleculaes of cAMP activates protein kinase A by dissociating the inhibited holoenzyme (R_2C_2) into a regulatory subunit (R_2) and two catalytical active subunits (2C).

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Figure 2. Diagram for the Catalytic Mechanism of Protein Phosphorylation by PKA
In Panel A, E: PKA; A: Mg^{2+}-ATP; B: Protein substrate. In Panel B, D-P: ATP; A: Protein substrate.

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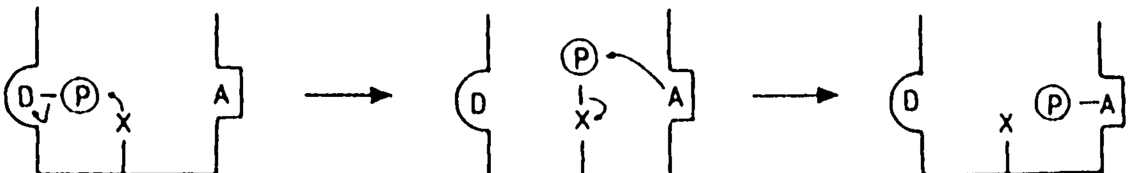
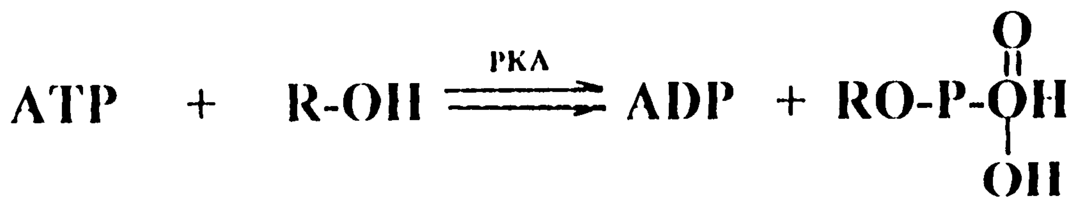
A. Sequential Ordered Mechanism

B. Covalent phosphoenzyme intermediate



C. Overall Reaction



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4. The Structural Distinction Of cAMP-Response Between the Prokaryote and Eukaryote: Cyclic AMP (cAMP) has been known as an important second message for the regulation of cell proliferation and differentiation in all living cells. However, cAMP uses different mediators to regulate cell function in prokaryotic and eukaryotic cells. The earliest defined role for cAMP was in bacteria for the regulation of gene expression controlling carbohydrate metabolism (Krebs et al., 1979; Beebe and Corbin, 1988 for a review). In bacteria, cAMP regulates gene expression by binding with a protein called the catabolic activator protein (CAP) (Crombrugghe et al., 1984). CAP has two kinds of binding sites, one for binding cAMP and the other for binding DNA. cAMP binding induces a conformation changes in CAP and makes CAP bind specific DNA regions which serve as promoter elements in a cAMP-responsive genes. So cAMP directly regulates transcription by binding CAP. However, in eukaryotes, there is not a single mediator protein like CAP for the regulation of cAMP-responsive gene expression. The function of CAP is shared by at least three proteins. These include that 1) PKA regulatory-subunit, which exhibits highly conserved cAMP binding sites for cAMP binding; 2) Nuclear trans-activating transcription factors, which belongs to CREB/ ATF-1 family (Gonzalez et al., 1989). CREB/ATF members have DNA binding domains that recognize and bind cAMP-responsive elements (CRE) as promoter for cAMP-responsive gene expression; 3) and PKA catalytic subunits, which phosphorylate and regulate regulatory enzymes and trans-activating transcription factors, but does not have a prokaryotic counterpart. Consequently, in eukaryotes, cAMP causes a conformational change in the R-subunit to dissociate R-subunit from C-subunit. The C-subunit does not have a DNA binding domain, but C-subunit can regulate gene expression by phosphorylating trans-activating transcription factors. These phosphorylated transcription factors via C-subunit phosphorylation can recruit other transcription cofactors to CRE site, then together activate CRE-containing gene expression. The advantages for the presence of multiple components in cAMP response in eukaryotic cells are considered as a way to provide more versatile and flexible control for the regulation of gene expression in response to

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the cAMP stimulation. Actually, this multiple-step control has evolved for the highly differentiated and sophisticated functions of the eukaryotic cell (Miller et al., 1993).

5.PKA is well defined intracellular mediator of cAMP action in regulation of eukaryotic gene expression: AMP is an ubiquitous intracellular second messenger whose functions in eukaryotes is mediated by the activation of cAMP-dependent protein kinase (PKA). Once activated by cAMP. PKA performs diversified functions in the cell by phosphorylating a wide variety of substrate proteins as a response to cAMP stimulation. C-subunits were first recognized for the phosphorylation of enzymes that regulate carbohydrate and lipid metabolism (Krebs and Beavo. 1979; Beebe and Corbin, 1986). We now know that Cα-subunits can translocate to the nucleus after the release of regulatory R-subunit, and activate trans-activating transcription factors (Nigg et al.,1985). One of the best characterized proteins which is involved in cAMP-responsive gene transcription and activated by C-subunit phosphorylation is the cAMP-responsive element binding protein (CREB) (Montminy et al., 1987, Hoffer et al.,1988) CREB is a 43 kDa monomeric protein that binds as a dimer to an consensus-palindromic 8-nucleotide sequence (5'-TGACGTCA-3') termed as cAMP-responsive element (CRE). The CRE element is found in the 5' promoter regions of cAMP-responsive genes (Comb et al., 1986, Montminy et al., 1986), and stimulates CRE-containing gene transcription. CREB is one member of a large family of transcription factors that exhibit basic region/leucine zipper motifs, which are involved in dimerization and binding-efficiency to CRE-elements in the promoter region of CRE-containing genes (Gonzalez et al., 1989). The functions of these trans-activating factors in ATF-1/CREB family are regulated by protein phosphorylation, which modulate their nuclear transport or DNA-binding affinity (Hunter et al., 1992). These trans-activating factors can form homodimers or interact with other transcription factor members to form heterodimers with their C-terminal zinc-finger motif after their phosphorylation (Waeber et al., 1991). Although dimers of transcription factors can efficiently bind CRE elements consensus sequences (Foulkes et al., 1991a), they still cannot

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activate downstream gene expression. Other proteins termed as co-transcription factors. are necessary to stimulate CRE-containing gene expression by interacting with phospho-CREB dimers So far, clearly defined co-factors include CREB binding protein (CBP) and p300 (adenoviral E1A-associated cellular protein) (Hoffler et al., 1988; Lundblad et al., 1995) Upon elevation of intracellular cAMP, CREB is phosphorylated by PKA at single phospho-acceptor site, Ser-133 This phosphorylation event is believed to bring about a conformational changes in CREB, but recent reports indicate that CREB phosphorylation by C-subunits does not induce the changes in global conformational and CRE-binding affinity in CREB (Richards et al., 1996) Although unphosphorylated-CREB still can bind to CRE sequence, only phospho-CRE can bind with phosopho-CBP (Chrivia et al., 1993). After the formation of phospho-trimer CREB-CBP, RNA polymerase II can be recruited to CRE promoter site to initiate CRE-containing gene expression (Lee et al., 1990; Brindle et al., 1993) Therefore, PKA phosphorylation and activation of CREB occurs by the production of its specific, complementary interactions with these co-factors (Figure 3) (Richards et al., 1996)

There are several levels to manipulate the regulation of CRE-containing gene transcription The ratio of heterodimers and homodimers among the same transcription factor family members will affect the transcription efficiency of CRE-containing genes The phospho-CREB homodimer enhances the CRE-containing gene expression (Gozalez et al., 1991), but the heterodimers of CREB and CREM, termed as cAMP responsive element modulator, and homodimer of CREM inhibit CRE-containing gene expression (Folkes et al., 1991) CREM protein is highly homologous to CREB in sequence and in the efficiency and specificity for CRE-binding, but in contrast to CREB, CREM acts as a down-regulator of cAMP-responsive gene expression (Fulkes et al., 1991) These trans-activating factors act as competitive antagonists by physically occupying CRE binding site, but not inducing transcription

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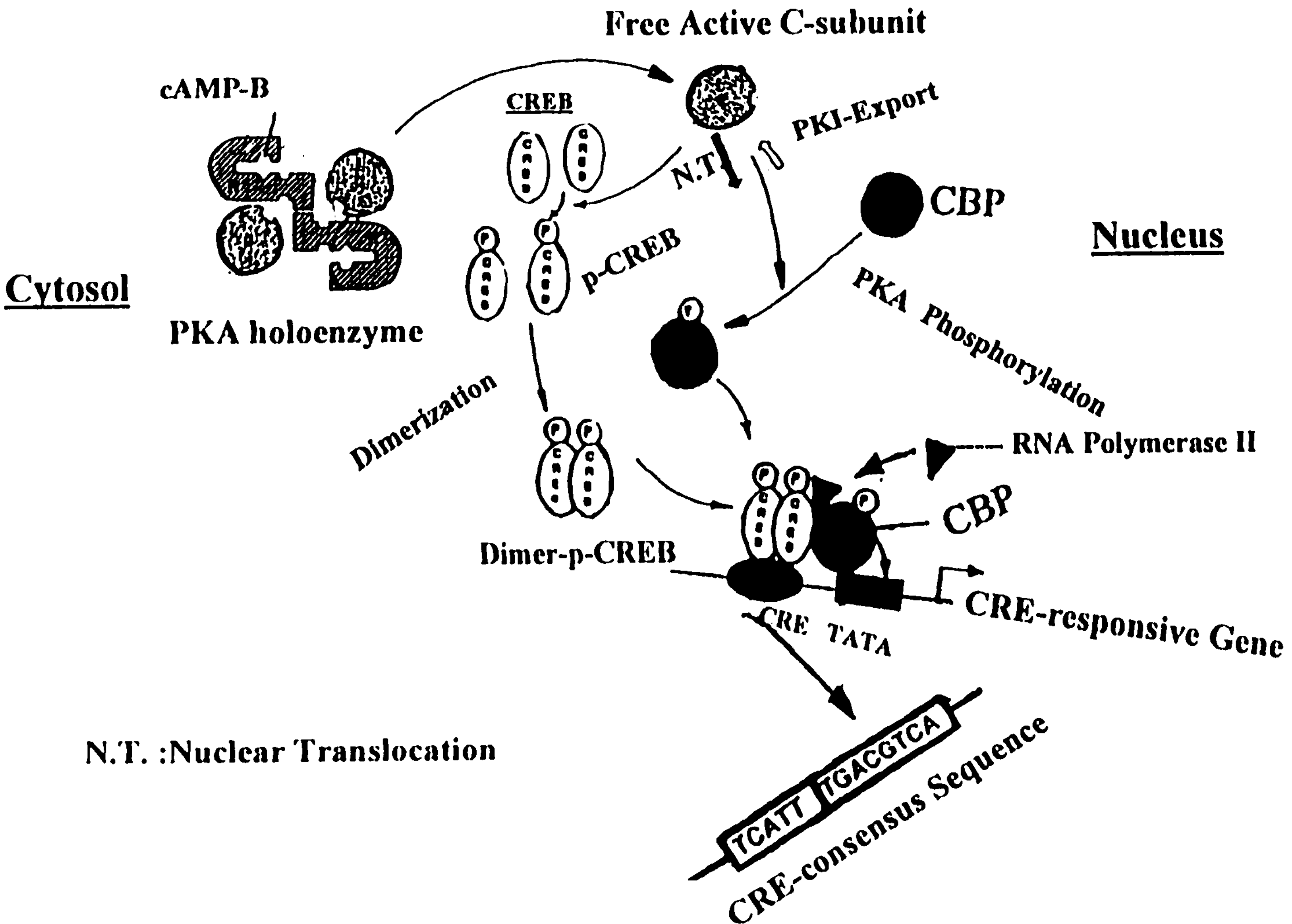

Figure 3 cAMP Regulates Eukaryotic Gene Expression by the Activation of PKA

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CRE-containing gene expression is also manipulated by variations in CRE sequence in different CRE-containing genes, which determines the presence of cell- or tissue-specific cAMP responses via distinct binding specificity to certain activated trans-activating factors. Consequently, the binding efficiency of these factors to respective CRE sequence is modulated by nucleotides flanking the CRE (Liu et al., 1991). There are some cAMP-responsive genes which do not use CRE as promoter. Here cAMP-responsive gene expression is regulated by cAMP-receptor protein in the cytosol. Most of these genes are involved in the generation of steroids hormones. The exact molecular mechanism for the regulation of these non-CRE cAMP-responsive gene expression is unclear.

Another way to diversify the regulation for cAMP-responsive gene expression includes multiple phosphorylation of CREB/ATF transcription members and/or other co-factors. It is well known that phosphorylation of CREB at Ser133 by PKA is major requirement for cAMP-responsive gene transcription, but the PKA phosphorylation of CREB on unique Ser-133 is not sufficient to fully activate CREB (Lee et al., 1990). Additional phosphorylation in three other Serine sites are necessary to induce full stimulation of CRE-containing gene transcription (Foil et al., 1994). It is now recognized that the phosphorylation of the first site appears to provide a signal for the phosphorylation of the second one, and both are required for full activity (Roach, 1991). Our preliminary data suggests that the differences between Cγ and Cα-subunit for the regulation of CRE-reporter gene activity may be mediated by a distinct Cγ phosphorylation n CREB, CBP or other co-transcription factors. It is also possible that Cγ modifies the other kinases-mediated phosphorylation of these transcription factors. Therefore, the regulation of cAMP-responsive gene expression becomes much more complicated than originally thought. These multiple controls in the activation of cAMP-mediated gene expression provide a molecular basis for the cell to manipulate its response to cAMP-stimulation in particular conditions. As a major mediator for cAMP-

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regulated function in the cell. PKA plays important role in gene expression via phosphorylation of ATF/CREB trans-activating factor and their cofactors.

Based all information about cAMP-responsive gene transcription, it is clear that there are multiple steps to manipulate cAMP response in the cell, and PKA plays a key role in the regulation of this pathway. Previous results show that unlike Cα, stable Cγ-transfection could not restore the steroidogenesis which was mediated by cAMP-responsive gene expression in Kin8/Y1 cells (Beebe et al., 1992). This observation implies that different C-subunit isozymes may play different roles in the regulation of cAMP-responsive gene expression by their different substrate and/or pseudosubstrate specificity, or distinct cellular localization.

6.PKA isoforms provide specificity to cAMP function: AMP regulates various kinds of cell functions, but different cells respond to the cAMP-stimulation differently. This implies that cAMP function will be diversified in the cell under certain conditions. It has been known for years that PKA mediates cAMP-responsive function. An array of PKA isozymes are expressed in mammalian tissue: genes encoding three C-subunits and four R-subunit isoforms have been identified (Beebe, 1994). The initial classification of PKAs depended on differences in the R-subunits and most attention to PKA isoform specificity has focused on them (see Beebe, 1994 for a review). The presence of multiple isoforms of PKA-subunits provides another potential for functional diversity.

The RI- and RII- subunit isoforms, which determine type I and II PKA, differ in molecular weight, amino acid sequence, phosphorylation state as well as tissue and subcelluar distribution. The RIIα- and RIIβ-subunits were best characterized for their interaction with A kinase anchoring proteins (AKAPs), providing a mechanism for PKA subcellular localization and functional specificity (Scott et al., 1992). The RI-subunit isoforms were reported to be selectively localized in some cell types, but the mechanism for localization was different from the mechanisms for the RII-

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subunits and had not been not clearly defined (Coghlan et al., 1993) All these differences mediated PKA isoform-specific function

It was known that different hormones activate specific PKA subtypes. For example, type-I PKA was preferentially activated by glucagon in hepatocytes and by corticotrophin releasing factor, isoproterenol and forskolin in pituitary derived at T-20 cells (Byus et al., 1979) In contrast, type-II was activated by human gonadotrophic hormone in ovarian follicles Recently, type-I PKA, but not type II, was associated with T cell activation and inhibition (Laxminarayana, 1993, Skalhegg et al., 1992). Although these observations indicate the different R-subunit provide the specificity for PKA function, this also may be explained simply by differential localization of PKA and compartmentalization of cAMP elevation (Coghlan et al., 1993) PKA is a multifunctional enzyme with a broad substrate specificity. Subcelluar localization may provide an important mechanism to diversify PKA functions. This thesis predicates that the presence of multiple C-subunit isozymes also contributes to the specificity of cAMP-response by the preferential phosphorylation of substrate by kinases with different substrate specificity Recent accumulated evidence (Beebe et al.,1992 and 1994) demonstrates that the C-subunits provide the specificity for the function of PKA in cAMP-response just like R-subunit.

7.Cγ may have distinct specificity for substrate and pseudosubstrate in vitro, and different functions for Y1 Kin8 cell phenotypes Until molecular cloning became available, C-subunit was thought to be common to all holoenzymes (Krebs and Beavo, 1979, Beebe and Corbin, 1986) The human Cα and Cβ isoforms, which share 92% identity at the amino acid level, are functionally identical (Beebe et al., 1990), but Cγ exhibits functional differences (Beebe et al., 1992 and 1994) Cγ shares 82% and 79% identity with Cα and Cβ, respectively In spite of high degree of identity, the differences between Cγ and Cα have profound effects on C-subunit substrate and pseudosubstrate specificity *in vitro* and function in intact cells Cγ differs from Cα in its

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interactions with substrates, inhibitors, and R-subunits, and one or more of these differences is responsible for the Cγ- and Cα-specific phenotypes in Y1/Kin8 cells (Beebe et al., 1992). This suggests that the C-subunit is not necessarily common in all PKA holoenzymes and that the C-subunit may provide specificity for PKA function in cell physiology (Beebe, 1994).

Several observations suggest that Cγ and Cα exhibit different characters, or that Cγ has some unique features. Firstly, *in vitro* study showed that Cγ phosphorylated the histone protein substrate better than Kemptide which has been believed as a specific substrate for PKA (Krebs et al., 1972). Secondly, Cγ kinase activity is not sensitive to PKI-inhibition, which is one of the unique features of Cγ, because the PKI has served as PKA specific inhibitor for long time (Walsh, 1970). A third observation indicated that the expression of Cγ and Cα in the cAMP-resistant Y1/Kin8 mutant adrenal cells resulted in distinct cell phenotypes. The Y1 adrenal mutant cell, Kin8, was chosen as an in intact cell model to compare the functions of Cγ and Cα, because this Y1/Kin8 mutant cell contains an RI subunit mutant and is defective in cAMP-mediated responses (Rae et al., 1979). This provided an opportunity to transfect Cγ and Cα to bypass the mutant and compare Cγ- and Cα-expression on cell phenotype. The data (Table 1) was referred from Beebe (1992).
The table 1 shows that Cγ and Cα differentially restore cAMP-dependent phenotypes to the cells. Both isozymes restored cAMP-mediated morphology changes and growth inhibition, but in contrast to Cα, Cγ expression did not restore steroidogenesis, which is dependent on cAMP-responsive gene expression. This result suggested that Cγ had a different function in the regulation of steroid gene expression from that of Cα.

Previous results suggested that Cγ and Cα had different R-subunit specificity. Cγ, but not Cα, restored type II PKA holoenzyme in Kin8 clone. Cγ always was associated with R-subunits as holoenzyme in Cγ clone, but free Cα was present in Cα clone. A higher cAMP concentration was required to dissociate Cγ-Type-I holoenzyme than Cα-type I holoenzyme *in vitro*. These results

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Table 1 cAMP-PKA-Mediated Phenotypes in Y1/Kin8

Clone	Steroidogenesis	Rounding	Proliferation Rate
Y1	+ + + +	+ + + +	+ +
Kin8	-	-	+ + + +
Cγ 7	+	+ + + +	+ +
Cα 2	+ + + +	+ + + +	+ +

For brevity, phenotypes are presented as relative responses to stimulation of Y1/Kin8 clones with 300 μM cAMP for 18 hours. Total steroids were quantified (after methylene chloride extraction of cell culture media) by fluorescence at 530 nm using an excitation wavelength of 470 nm. 20 α-hydroxyprogesterone was used as standard. Rounding was scored by visual observation and proliferation determined by cell counts and /or ^{3}H-thymidine incorporation

suggested that C-subunit isozyme had distinct specificity in the interaction with R-subunit (Beebe et al., 1997). All these observations provide a motive and justification for this dissertation research to compare Cγ and Cα isozymes *in vitro* and in intact cell models

Structure-function studies of PKA have supplied critical understanding to the biochemistry and physiology of PKA, but there are still some unknown answers to very important questions 1) What structural feature at the primary phosphorylation site of substrate and pseudosubstrate determines Cγ and Cα specificities ? 2) Do Cγ and Cα differ kinetically for the phosphorylation of substrate ? 3) Is it possible to correlate Cγ and Cα specificity for R-subunit *in vitro* and in intact cells? 4) Does Cγ-mediated differential regulation of CRE-reporter gene activity account for the absence of steroidogenesis in Cγ/Kin8 clone (Table 1) 5) Do differences between Cγ and Cα from partially purified preparations coincide with those differences of Cγ and Cα from homogeneous

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preparations? 6) What are the full contents for the distinct function of C-subunit isozymes? These results will enhance our understanding of mechanisms for the specificity of multisubstrate kinase

B. Rationale of Research

Cγ has been shown to differ from Cα *in vitro* for substrate and pseudosubstrate specificity, and in intact cells for different phenotypes and regulation of cAMP-responsive gene expression. It has been well known that various protein kinases perform their distinct cell functions through unique substrate phosphorylation. The observed distinctions between Cγ and Cα are most likely mediated by differences in the substrate specificity and/or various pseudosubstrates. To determine the molecular mechanism that accounts for distinction of Cγ and Cα in cell phenotypes and probably in the regulation of gene expression, I took the following experimental approaches: 1) make a detailed comparison of homogeneous Cγ and Cα for their substrate and pseudosubstrate specificity *in vitro*; 2) create an intact cell model for direct comparison of Cγ and Cα function in the regulation of cAMP-responsive CRE-reporter gene activity; 3) characterize nuclear functions for Cγ and Cα in Kin8 cells that permanently express each C-subunit isozyme. In summary, major rationality to carry out this dissertation research was to search for molecular explanations for the distinction of Cγ and Cα in cell function.

C. Research Hypothesis

All previously accumulated findings clearly demonstrated that Cγ has distinct specificity on substrate and pseudosubstrate and is distinct in the restoration of cell phenotype and the regulation of gene expression (Table 1).

It was hypothesized that Cγ and Cα play distinct cell functions by unique substrate and/or pseudosubstrate specificity, and both R-subunit and C-subunit isozymes provide the specificity for

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the diversified function of cAMP/PKA. This is a completely new idea, which may serve as a new model to study how isozymes of one kinase provide diversified function for signal transductions

To support this hypothesis, it was first important to confirm the presence of substrate and pseudosubstrate specificity. To this end, homogeneous preparations of both C-subunit were utilized to make a fully detailed comparison of both isozymes *in vitro*. This serves as basic evidence to explain their distinct functions in cell phenotype.

It is also important to search for possible molecular mechanism (s) underlying the distinct role of Cγ and Cα in the regulation cAMP-responsive gene expression using an transient and permanent transfection in intact cell models. This provides evidence to show how differently Cγ and Cα regulate cAMP-responsive gene activity and nuclear events in intact cells

D. Specific Aims of Research

1.Provide pure recombinant enzymes for in vitro characterization for substrate and pseudosubstrate specificity: Cγ and Cα have been expressed in mammalian adrenal Y1 mutant cells (cAMP resistant Kin-8 cell) by permanent transfection with Cγ and Cα cDNA (Beebe et al., 1992). The characterization of these clones provided a valuable intact cell model, because the mouse methallothinein promoter (pMT) has the advantage that C-subunit expression can be regulated by Zinc. But it is not practical to obtain sufficient amounts of homogenous recombinant proteins from this mammalian expression system for *in vitro* characterization, because pMT does not contain a strong expressing promoter compared with T4 in bacterial pET or baculovirus *polh* (polyhedron) promoter. There is also an inherent declined-tolerance to the accumulation of recombinant proteins in mammalian cells due to feedback and compensatory mechanisms that maintain mammalian cell normal function. The experiments proposed in this specific aim will provide a way to create sufficient quantities of native recombinant PKA C-subunit isozymes for *in*

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vitro characterization by the expression of both isozymes in heterologous systems using bacteria and using Sf9 insect cell-baculovirus system.

2. Characterize native recombinant C-isozymes for substrate and pseudosubstrate specificity

The recombinant PKA C-subunit proteins from mammalian cell exhibited differences in substrate and pseudosubstrate specificities. However, these studies were not conducted with homogeneous enzyme preparations, and kinetic analyses on these specificities have not been carried out due to the absence of purified C-subunits. The studies in this specific aim will provide kinetic comparison on the substrates and pseudosubstrate specificity for purified Cγ and Cα for the first time. This information will serve as a fundamental evidence to confirm previous data and correlate the results *in vitro* studies with the phenomenon observed in intact cell studies.

3.Search for molecular mechanism(s) which underlay the distinct role of Cγ and Cα in the regulation of cAMP-responsive gene expression. Although the previous observation showed that unlike Cα, Cγ could not restore the cAMP-responsive gene expression for steroidogenesis, it is unclear whether this really implied that Cγ played different roles in the regulation of cAMP-responsive genes expression or whether this distinction is universal or particular to this cell line. What molecular mechanisms underlay this kind of distinction ? To this end, two different strategies were designed. The first one was to utilize transient transfection of CRE-reporter gene with C-subunit expression vector as a model to determine general existence of distinct roles for C-subunit isozymes in the regulation of cAMP-responsive gene expression. The second set of experiments were designed to search for the differences between Cγ and Cα in the nuclear events that determined the regulation of CRE-containing gene activity in both permanently C-subunit expressing Y1/Kin8 clones after stimulation with zinc/cAMP. These events include 1) The levels of immunoreactive and catalytic C-subunit proteins in the nucleus before and after stimulation 2) CREB phosphorylation by C-subunits 3) the levels of immuno-reactive CREB protein in the

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nucleus of respective clone after stimulation. 4) transcription factors which may be involved in gene transcription as determined by CRE-mobility shift. 5) the necessity of a CRE and dimerization of CREB for the cooperative co-regulation of CRE-reporter gene activity by Cγ and Cα-subunits. Preliminary data showed that Cα, but not Cγ, stimulated CRE-reporter gene activity, but Cγ modulated Cα-mediated CRE-reporter gene activity both positively and negatively. After exploring all these possibilities, the results may provide a final answer for the question, or at least provide a valuable evidence to rule out lots of possibilities, or provide important clues to approach to a final answer.

The results of this dissertation research are expected to provide important information about function of C-subunit isozymes in the cAMP signal transductions pathway. This information may help to generate a new concept in cAMP signal transductions, which is that PKA C-subunit diversifies cAMP signaling downstream of PKA activation by the localization and / or release of C-subunit isozymes with different substrate specificities and functions.

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CHAPTER II

METHODS AND EXPERIMENTAL PROCEDURES

A. General Methods

1.Protein concentration Assay: Protein concentration was determined using the Bradford protein assay with bovine serum albumin as standard (Bradford, 1976).

2.Protein kinase activity assay: In the standard kinase activity assay, the activity of C-subunit was determined by the transfer of γ-^{32}P-ATP (200 μM) to peptide (65 μM Kemptide, LRRASLG) or protein substrate (85μM histone IIIs, Sigma) in the presence or absence of 1 μM PKI_{5-24} , a heat-stable peptide as a C-subunit specific inhibitor (Walsh and Krebs, 1972; Whitehouse and Uhler, 1982). The reaction (50 μl) was carried out at 30° C for 10 min.. Then 35 μl of reaction mixture was transferred to P-81 phosphocellulose paper and washed in 75 mM phosphoric acid to stop reaction, dried and counted in scintillation counter (Beebe et al., 1990, 1992)

3.[^{3}H]cAMP binding assay: Recombinant R-subunit activity was determined by ammonium sulfate-nitrocellulose [^{3}H] cAMP-binding assay as described before (Doskarand and Ogreid, 1988)

4.Immunoblot analysis of C-subunits: Crude lysates for the immunoblot analysis of C-subunits were prepared as indicated in particular cells. The supernatants (0.5-25 μg of proteins) were subjected to 9% SDS-PAGE. After electrophoresis, the proteins on the gel were transferred to Immobilon-P membranes (Millipore). The membranes were blocked by 5% BSA in Buffer A (50 mM Tris-HCl containing 0.05% Tween 20, pH 7.5) overnight at 4 °C or 4 hours at 37 °C, incubated with PKA C-subunit specific antibody (1:1000) in Buffer A for 4 hours at room temperature, and washed with Buffer B (Buffer A with additional 0.05% Tween 40) (3 × 10 min.) The membranes were incubated with goat anti-rabbit IgG second antibody (1:1000) (Amersham) for 2 hours, at room temperature and washed (3 × 10 min) with buffer A. Finally, the membrane

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was developed using Chemiluminescence (ECL) and exposed to film according to manufacture's protocols (Amersham).

5. Preparation of nuclear extracts: Nuclear extracts are prepared by a modification of the method as described by Dignam (1983). Briefly, 1×10^6 HEK or Kin8 cells were washed twice with ice-cold PBS, harvested in 5 ml PBS and centrifuged at 400g for 5 min. at 4 °C The pellet was washed with 4 packed cell volume of Buffer C containing 10 mM Tris-HCl, pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl and left on ice for 10 min. The cells were then lysed by 10 strokes of a Dounce homognizer using a type B pestle. The presence of intact cell and nucleus were determined by cytospin analysis. Intact nuclei were sedimented d at 4500 × g for 5 min at 4 °C, resuspended in 2 packed cell volume of buffer D (420 mM KCl, 20 mM Tris-HCl, pH 7.8, 1.5 mM $MgCl_2$, 20% glycerol) and incubated at 4° C with gentle agitation for another one hour Immediately before use, Buffer C and D were supplemented with 0.5 mM DTT, 0.4 mM PMSF, 10 µg/ml microcystin and 2 µg/ml each leupeptin and pepstatin After complete lysis of nuclei, the nuclear extract was centrifuged at 10,000 ×g for 30 min at 4 °C and supernatant was dialyzed twice against 500 ml buffer E (20 mM Tris-HCl, pH 7.8, 100 mM KCl, 0.2 mM EDTA, 20% glycerol) for 4 hr at 4 °C, aliquoted, frozen in liquid N_2 and stored at -80 °C Protein concentration was determined using the Bradford protein assay (1976) with bovine serum albumin as standard

B. Expression of C-Subunits in Mammalian Cells

1 Stable transfection of C-subunits in Y1 Kin8 cells Cγ and Cα were expressed in mammalian cells by permanently transfecting cAMP-insensitive mouse Kin8 adrenal cell mutants with Cγ or Cα cDNAs expression vectors driven by metallothionein promoter as previously described (Beebe et al., 1992).

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2. Partial purification of C-subunits from Cγ- and Cα-clones: Recombinant mammalian Cγ and Cα were partially purified from cell extracts by gradient elution on DEAE-Sepharose as holoenzymes as previously described (Beebe et al., 1992).

3. Detection of native Cγ-subunit in monocyte-like U937 cells: 200×10⁶ U937 cells were stimulated or not for 72 hours in the presence and absence of 50 μM 8-CPT-cAMP [8-(-chlorophenylthio0-adeneosine 3':5'-cyclic monophosphate. The cells were harvested by centrifugation (5,000g ×10 min). The cell pellets were washed twice by cold phosphate buffer saline (PBS) and pelleted again by centrifugation. The pelleted cells were resuspended in 4 ml buffer F (10mM potassium phosphate, pH 6.9, 1mM EDTA) and homogenized by hand glass homogenized (4 ×10). Crude lysate was centrifuged at 100,000×g for 1 hour to obtain clean supernatant. 4 ml aliquots of supernatant were applied into 2ml equilibrated DEAE-Sepharose column by buffer F. After washing the column with 20 ml of the same buffer, the first elution was prepared by eluting the column with 8 ml buffer F containing 0.15 M NaCl. The second elution fraction was prepared by eluting the column by 20 ml buffer F containing 0.5 M NaCl. 1 ml aliquots of each elution fraction was collected and used for protein kinase assay and immunoblot analysis using C-subunit-specific antibody.

C. Heterologous Expression and Purification of C-Subunits for in Vitro Characterization

1. Vector construction and expression of Cγ and Cα in bacteria. Human Cγ and mouse Cα cDNAs were expressed in bacteria using the pET expression system (Novagen). Construction of pET-Cα and expression of soluble, active Cα in bacteria was carried out as previously described (Uhler et al., 1987). The Cγ and Cα constructions (made by Dr. Holroyd in EVMS) were used to

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transform bacteria (E. coli., BL21DE3pLysS), which included a pLysS construct for the production of lysogen. Expression of recombinant protein was performed as previously described for Cα (Uhler et al., 1987). In contrast to Cα, the Cγ proteins had very low levels of kinase activity. No increase in soluble, active Cγ could be induced by incubation of Cγ with catalytic amounts of Cα and ATP/Mg^{2+}. The co-expression of Cγ with RII-subunit and the incubation of Cγ-subunit expressing E. coli in different media (LB or M9ZB) (Molecular cloning 1989) at different temperatures (4, 18, 25, or 36 °C.) for different times (1, 4, 18, 24, or 36 hours), or with zero, 0.04 or 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside) as inducer still could create active recombinant Cγ in bacteria. Denaturalization/renaturalization experiments were unable to active recombinant Cγ expressed under different conditions as mentioned.

2. Purification of Cγ- and Cα-subunits expressed in bacteria Cγ was assayed in crude bacterial extract or after negative chromatography with CM-Sepharose. In contrast to all the PKA C-subunits described, the soluble Cγ did not bind to CM-Sepharose and the flow through fractions were assayed for Kemptide and histone kinase activities in the presence and absence of $PKI_{(5-24)}$ (2μM). Cα was purified from recombinant Cα-expressing bacteria by sequential chromatography on CM-Sepharose and Shacryl S-300 gel filtration as described by Baude et al. (1994).

3. Vector construction and expression of Cγ and Cα in Sf9 cells using baculovirus The 1.6 kb Cγ and the 1.2 kb Cα cDNAs were separately cloned into the BamHI site of baculovirus transfer vector pVL1393. The recombinant Cγ baculovirus was produced by cotransfection of Sf9 cells (1.5×10^6) with 2 μg baculovirus DNA (AcNPV DNA) and 3 μg of pVL1393 Cγ or pVL1393 Cα transfer plasmid for homologous recombination (4 hours at 27°C). The recombinant Cγ and Cα baculovirus were produced and used to infect Sf9 insect cells according to the manufacture's protocols (Invitrogen). Twenty of each purified recombinant C-subunit baculovirus isolates were

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selected and measured for kinase activity and C-subunits by immunoblot analysis using C-subunit-specific antibody in Sf9 cells. The best recombinant C-subunit baculovirus isolate based on kinase activity and immunoreactive proteins was chosen for further studies. Sf9 insect cells were cultured in Graces' media with 10% FBS and 100 μg/ml penicillin and streptomycin at 27° C with flow air (O'Reilly et al., 19392). The ratio of virus and insect cells was optimized at 80 : 1 for infection. Debris was removed from culture media by centrifugation (2500 × g 15 min.). Cell lysates were prepared by hand homogenization and centrifugation (14000 × g, 20 min). The crude lysate supernatants were assayed for PKA activity and subjected to immunoblot analysis.

4. *Purification of Cγ expressed in Sf9 cells using baculovirus*: Three-days post-infected Sf9 cells were harvested by centrifugation at 1500×g for 5 min. All subsequent procedures were carried out at 4°C. After washing twice with PBS, pelleted Sf9 cells were homogenized by ground glass homogenized (10× 4 strokes) in Buffer F containing 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM PSF, and 50 mM benzamidene. The homogenate was centrifuged at 15800 × g for 20 min and the supernatant was applied to a CM-Sepharose column (million cells/ml resin). The column was thoroughly washed with Buffer F, and C-subunit was eluted with a NaCl gradient (0-0.6 M) in the same buffer. One-ml elution fraction was collected and assayed for histone kinase activity in the presence 1μM PKI_{5-24} and protein concentration. The peak of PKI-insensitive histone kinase activity was pooled, concentrated by Centricon-10 and applied to Sephacryl S-300 (Pharmacia) gel filtration column (2.5×70 cm) equilibrated in Buffer F containing 0.5 M NaCl. Fractions (0.5ml) were collected, and assayed for histone kinase activity in the presence of 1 μM PKI. 2.5-5.0 μg of purified Cγ protein was subjected to SDS-PAGE, stained with silver or Coomassie blue, and identified as a single protein with an apparent molecular weight of 41 kDa.

5. *Comparison of purified C-subunits on the heat-stability*: Purified Cγ-subunits expressed in Sf9 cell using baculovirus and Cα-subunit expressed in bacteria as described above were preincubated

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at 37 °C for varying time intervals and kinase activities were determined by the standard phosphotransferase assay as mentioned above. Kemptide (65 μM) or histone (85 μM) were used as peptide and protein substrates, respectively. The themostability was represented as the percentage of kinase activity without preincubation with same substrate.

D. Expression and Purification of Other Recombinant Pseudosubstrate Proteins for Cγ and Cα Characterization *in Vitro*

1. Expression and purification of RIα -subunit in bacteria: The expression, induction and purification of recombinant RIα-subunits was performed as previously described (Ringheim et al., 1990). Briefly, one liter of LB broth containing 50 μg/ml ampicillin was inoculated with an overnight culture (1ml) of RIα expressing E. coli., (gift from Dr Susan Taylor, UCSD), grown at 37°C for 40-44 hours, harvested by centrifugation, and resuspended in 15 ml of lysis Buffer G (50mM MES., pH6.5, 150 mM NaCl, 15 mM β-mercaptoethanol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 50 mM benzamidine). Bacterial lysates were prepared by nitrogen cavitation. Soluble proteins were precipitated by $(NH_4)_2SO_4$ with 60% saturation at 4 °C for one hour. The precipitated RIα was harvested by centrifugation at 10000 ×g for 20 min at 4 °C. The RIα pellet was resuspended in 2 ml Buffer H (10mM MES., pH 6.5, 2mM EDTA, 2mM EGTA, 5mM β-mercaptoethnol) and dialyzed against three × 500 ml Buffer H at 4 °C for 4 hours. The dialyzed soluble protein was applied to 30 ml DEAE-Sepharose column equilibrated in Buffer H. The RIα-subunit was eluted from the column with a NaCl gradient (0-0.5M) in Buffer H. Fractions exhibiting [^{3}H]-cAMP binding activities were pooled, and concentrated by Centricon-10. Concentrated RIα was applied to Sephedex G-50 chromatography (1.5 × 75 cm) and eluted by Buffer H containing 0.5 M NaCl. To prepare cAMP-free RIα, solid urea was added into the

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pooled RIα fractions from DEAE-Sepharose column to 8 M as final concentration, and cAMP was removed by Sephedex G-25 chromatography (2× 50 cm) equilibrated with Buffer H containing 8M urea. Fractions containing RIα were pooled and dialyzed for 12 hours at 4 °C against Buffer F immediately to remove urea. 5 μg proteins of purified RIα bound with cAMP was loaded for 9% SDS-PAGE, and stained by Coomassie blue as single protein band with 48 kDa as apparent molecular weight.

2.Expression and purification of His_{10}–RIIα in bacteria: Expression and purification of recombinant His10-RIIα in bacteria were conducted as previously described with some modifications (Baude et al., 1994). Briefly, E. coli, BL21(DE3)/pLysS, harboring mouse His- R-IIα\pET16b construct were grown in LB broth containing 25 μg/ml chloramphenicol, and 50 μg ml ampicillin overnight at 37 °C. 500-ml LB was inoculated with 7 ml overnight culture and grown to 0.8 O.D. at 600 nm, then induced with IPTG (0.4 mM). The bacteria was incubated for additional 2 hours, pelleted, and resuspended in 15 ml of Buffer I (50 mM Tris-HCl, 500mM NaCl, pH 7.8) containing 1 mM PMSF, 1 μg/ml pepstatin , and 1 μg/ml leupeptin. The supernatant was loaded onto a 10 ml column of ProBond (Ni^{2+}-nitrilotriacetic acid resin, Invitrogen) equilibrated with Buffer I. The column was washed with the same buffer, eluted with an imididazole gradient (60-1000 mM) in Buffer I, and assayed for ^{3}H-cAMP binding activity (Doskeland and Ogreid 1988) fractions containing His10-RIIα were pooled, dialyzed against Buffer F containing 10 % glycerol, and concentrated by Centricon-10. Concentrated his10 RIIα was further purified by a second 5 ml fresh ProBond column eluted as described above. The purified protein (5 μg) was subjected to SDS-PAGE stained with silver or Coomassie blue, and identified as a single protein with an apparent molecular weight of 51 kDa.

3.Expression and purification of MBP-PKIα and MBP-PKIβ in bacteria MBP-PKIs fusion protein expression vectors were transformed into E coli , BL21(DE3)/pLysS The bacteria

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harboring vectors for MBP-PKIα and MBP-PKIβ were grown in LB with 0.2 mM sucrose, induced and lyzed as described as (Baude et al.,1994). The individual bacterial lysate containing MBP-PKIα and MBP-PKIβ was applied to a Amylose (BioLabs) column (10 mg protein/mg resin). After completely washing the column with Buffer J (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4), the column was eluted by 0.2 M maltose in Buffer J. The protein concentration of each fraction was assayed by Bradford (1976). The protein fractions were subjected to 9% SDS-PAGE and stained by Coomassie blue. Proteins were showed as about 43.5 kDa and 45 kDa for MBP-PKIα and MBP-PKIβ as apparent molecular weight, respectively. At least 90% homogeneous preparation of MBP-PKIs was used as pseudosubstrate inhibitor for C-subunit in this study.

E. Methods for Substrate Specificity

1. Steady-state kinetic analysis of C-subunits for the phosphorylation of histone, synthetic peptides and utilization of Mg^{2+}-ATP: 250 ^{32}pmol incorp/min/ml of purified Cγ and Cα were used to determine steady-state kinetic parameters (Km and Vmax) for substrates and ATP using a modified protein kinase assay. The reactions at 30 °C were initiated by the addition of C-subunits and aliquots (35 μl) were removed to terminate the reaction after 3, 6, and 9 min. For the determination of apparent Km and Vmax values for Kemptide, the substrate concentration set as follow (μM): 1.9, 2.8, 4.2, 6.3, 9.4, 14, 27, 54, 109, 218, and 435. To determine the apparent Km and Vmax values for histone, the substrate concentration set as follow (μM): 0.37, 0.75, 1.5, 2.9, 5.9, 11.8, 23.5, 47, 94, and 188. For the determination of apparent Km and Vmax values for ATP, Kemptide and histone concentrations were held at 95 μM and 210 μM, respectively. The ATP concentration was set as the following (μM): 2.6, 3.9, 5.8, 8.7, 13, 19, 28, 94, 94, 210, and 467. To determinate the Km and Vmax values for synthetic peptides, the concentration of these

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peptides set as follow (μM): 2.5, 7.5, 22.5, 66.7, 200, 500 and 1000. The average values of each triplicate assay was used in this experiments. The data were analyzed by Lineweaver-Burk plot and Eadie-Hofstee plots.

2.Autoradiography analysis of protein phosphorylation profiles in the cytosol and nuclear extracts of Kin8 clones by endogenous and exogenous C-subunits: The cytosol and nuclear extracts from three C-subunit expressing clones (Kin8, Cα2 and Cγ7) were prepared as described above.

To determine phospho-protein profiles in the cytosol and nuclear extracts by exogenous homogeneous C-subunit isozymes, the cytosol and nuclear extracts of Kin8 clone were boiled for 3 min. at 100 °C to denature endogenous kinases. After centrifugation (5000 rpm × 5 min.), each 25μg preheat-treated cytosol and nuclear extracts (10 μl) were used as substrates for normal kinase assay with 10 μl purified C-subunit isozymes (0.15 μ mol Cα and equal amount of Cγ). After 10 min incubation at 30 °C, the sample buffer was added to stop reaction.

To determine the phospho-protein profile in cytosol and nuclear extracts by endogenous C-subunits, 25μg proteins (10 μl) of cytosol or nuclear extracts prepared from Cα Kin8 and Cγ Kin8 clones stimulated by Zinc(85 μM)/ 8-CPT-cAMP(50μM) for 18 hours were added into phosphotransferas assay mixture (30 μl) in the absence of substrate to initiate reaction. After 10 min. incubation at 30 °C, 10 μl 4 × SDS sample buffer was added to stop reaction. PKI(5-24) (1 μM) was added to some reactions to inhibit endogenous Cα activity as indicated in the Figure 25. Equal amounts of protein samples (25 μg) were loaded for 9% SDS-PAGE. Duplicate gels were performed. One piece gel was dried on filter paper for autoradiography. The other piece gel was stained by Coomassie blue.

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F. Methods for Pseudosubstrate Specificity

1.Inhibition of recombinant C-subunit kinase activity by RIα and RIIα The inhibition of purified C-subunits kinase activity by purified recombinant RIα and His_{10}-RIIα was carried out as described by Baude (1994). Purified recombinant Cγ-subunits expressed in Sf9 cells using baculovirus and Cα-subunits expressed in bacteria were derived from homogeneous preparations described above. The homogenous R-subunits were prepared as described above. 75 pmol of purified Cα and equal amounts of purified Cγ based on Kemptide kinase activity were used in these experiments. Various amounts of RIα and His_{10}-RIIα-subunits based on [^{3}H]-cAMP binding activity were preincubated with C-subunits in Tris-HCl (50 mM, pH 7.0) containing 2% BSA and 10mM DTT at 30 °C for 20 min.. After preincubation, the reaction was started by adding Kemptide, ATP (300 cpm/pmol) and magnesium acetate to a final concentration of 65 μM, 200 μM and 10 mM, respectively. The final 85 μM histone was added to substitute for Kemptide if protein substrate was used. After 2 hours incubation, 35 μl reaction mixture was applied to p81 paper to terminate the reaction. The control activities for both purified C-subunits were 5.2 (μM/min./mg) for Cα and 0.22 (μM/min./mg) for Cγ using Kemptide as substrate.

2. Determination of PKI IC50 values for C-subunits The inhibition of purified C-subunits by synthetic PKI_{5-24} peptide (Penecilla), homogeneous MBP-PKIα and MBP-PKIβ were carried out as described by (Baude et al., 1994). The purified C-subunits were derived from the same preparation as used for R-subunit inhibition. Briefly, various concentration of individual PKI inhibitor based on determination of protein concentration was incubated with purified C-subunits (5 nmol of Cα, and an equal amount of kinase activity of Cγ) in phosphotransferase assay mixture (Beebe et al., 1984) without substrate and γ-^{32}p-ATP at 30 °C for 15 min. After preincubation, the substrate and γ-^{32}P-ATP (300 cpm/pmol) was added to start the reaction. The 85 μM histone was

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used as protein substrate or 65 μM Kemptide was used as peptide substrate in this experiment After another 10 minute incubation, 35 μl reaction mixture was spotted onto p81 paper to terminate the reaction. The three time points (3, 6, 9 min.) of C-subunit kinase activity were assayed to examine linear catalytic rate with respect to C-subunits concentration over the time periods assayed. The IC_{50} values are defined as the concentration of inhibitor which inhibits C-subunit kinase activity by 50%. The control activities for C subunits were Cα (5.2 μ mol/min. mg) and Cγ (0.22 μmol/min./mg) using Kemptide as the substrate.

G. Methods for CRE-Reporter Gene Study

1. Transient Transfections: The HEK293 and Kin8 cells in six-well plates (with 65-70 confluent) were maintained in modified Eagle's medium supplemented by 10 % fatal calf serum and transiently co-transfected by different expression vectors by calcium phosphate coprecipitation (Melloon et al., 1989).

For CRE-luc reporter gene study, 0.3 μg α-luc-168 (kind gift from Dr. McKnight, University of Washitone) (Mellon et al., 1989) was cotransfected with different amount of pMT pCMV Cα or pMT/pCMV Cγ expression vectors as indicated in the Figure 28. pMT C-subunit expression vectors with mouse methallothionein-1 promoter were constructed by subcloning 1.2 kb mouse Cα and human 1.6 kb Cγ cDNA, respectively, into BamH1 site of pMT vector (Beebe et al 1992 . Uhler and McKnight 1987). The pCMV C-subunit expressing vectors contained same Cα and Cγ cDNA as subcloning into pMT C-subunit expression vectors. In addition to the SV40 promoter-directed Tn5-neomycin phosphotransferase gene as a dominant selectable marker in a pUC13-based parental plasmid, this pCMV was under the control of the human CMV promoter (Uhler and Abou-chebl 1992). The empty pMT/pCMV vector was used to make up a total of 5-10 μg for each

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transfection. After 4 hours infection, the cells were changed with fresh media and cultured for another 18 hours.

For Gal4-luc and Gal4-CREB reporter gene study, the same procedure as for CRE-luc system was performed for the transient cotransfection. But, pGal4-luc and pGal4-CREB (kind gift from Dr. Qunin, University of Pennsylvania) (Qunin, 1994) expression vectors were used to substitute CRE-luc reporter gene as reporter gene to similar transient cotransfection with pCMV C-subunit expression vectors. Gal4-luc /Gal4 CREB reporter gene study were designed to determine if the CRE and dimerization of CREB were essential to show cooperative co-regulation of Cγ and Cα for the regulation of cAMP-mediated reporter gene activity.

2.Luciferase assay: After 18-hours post-transfection culture, the transfected cells were washed twice with cold PBS and were lyzed in 200 μl lysis buffer K (1% Triton-x100, 25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$,4 mM EGTA and 1 mM DTT). The supernatant of lysed cells was prepared by centrifugation by 14000 rpm × 2 min and used for luciferase activity assay. Luciferase activity was assayed on a Monolight luminometer by using the enhanced luciferase assay kit (Promega) according to the supplier's directions.

H. Methods for Nuclear Characterization

1.Immunoblot analysis of CREB and phospho-CREB: The nuclear extracts were prepared from Kin8, Cα and Cγ clones . These cells were stimulated by 18 hr Zinc (80 μM) for 18 hours, then treated with 50 μM cAMP for various time. 15 μg nuclear proteins were loaded each lane for 9% SDS-PAGE analysis. After transfer, the membrane was blocked with 3% powered milk (wt/vol) in PBS for 2 hr. at 25 °C. The blocked membrane was incubated with CREB (1μg/ml) or phospho-CREB (0.5 μg/ml) antibody overnight at 4 °C. The membrane was washed 3 times × 15 min. with solution B. The washed membrane was incubated with peroxidase labeled anti-rabbit

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IgG (1:2000) as second antibody for 1.5 hr at 25 ° C (Amersham). The membrane was washed (3 × 25 min.) with solution A. The substrates are added to the membrane and incubated exactly 1 min The film exposure and development were according to manufacture's recommendation (Amersham).

3.Gel mobility Shift Assay: Nuclear extracts were prepared as described above The CRE-probe was a double strand 27 bp CRE consensus sequence: 5'AAGGTT(Santa Cruze Biotechnology) The probe was labeled ^{32}P by T4 kinase (Promega) at 37 °C for 30 min. The ^{32}P-labled CRE-oligonuleiotide was separated from free [^{32}P]ATP by centrifugation through gel-filtration spin column (P-10) (Amasham). The binding reactions were performed in 20 µl reaction Buffer L containing 20 mM Tris. 1 mM MgCl. 12 % glycerol. 0.1 mM DTT. 10-15µg of nuclear protein. 1 µg poly[dI-dc] (Pharmacia) as unspecific competitor. 10 ng unlabelled CRE oligonucleotide as specific competitor. The specific antiserum for phospho-CREB (2 ng/10 µg proteins of nuclear extracts) was added into the binding reaction mixture at this point and incubated for one hour at 4 °C for super-gel shift assay. After 25 min. incubation at room temperature. 0.5-1 ng (20,000-40,000 cpm) ^{32}P-labled consensus CRE-oligonucleotide was added to each reaction and further incubated for 30 min. at 4 °C. The sample was run on 5% non-denatured polyacrylamide gel in 1 × or 0.5 ×TBE (Tris-Boric-EDTA) at constant 25 mA for 2 hr The dried gel was exposed to film for autoradiography.

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CHAPTER III

RESULTS

A. Expression and Purification of Recombinant Proteins for *In Vitro* Study

It is hypothesized that different substrate and pseudosubstrate specificity of Cγ and Cα account for their distinct functions in Y1/Kin8 cell phenotype and regulation of cyclic AMP-mediated gene expression. Previous studies showed that Cγ and Cα had distinct substrate and pseudosubstrate specificity (Beebe et al.,1992), but the kinetic analysis was not carried out because of the absence of pure C-subunit preparation. To test this hypothesis and extend the previous results, Cγ and Cα had to be compared more fully *in vitro*. To this end, the presence of abundant homogeneous C-subunits and relevant proteins such as different isoforms of R-subunit and PKI as pseudosubstrate for C-subunits are necessary.

Cγ-and Cα-subunits have been successfully expressed in the mammalian cells and utilized as an important intact cell model to compare the functions of both C-subunit isozymes (Beebe et al. 1992). However, recombinant Cγ and Cα had not been purified to homogeneity in sufficient amounts for *in vitro* kinetic characterization. This situation suggested that C-subunits have to be expressed in other heterologous expression systems in such high efficiency that both C-subunits could be purified to homogeneity in sufficient amount for *in vitro* kinetic analysis. In addition, the different isoforms of PKI and R-subunits, as pseudosubstrate of C-subunits, have to be expressed and purified to homogeneity to conduct *in vitro* studies for pseudosubstrate specificity of C-subunits.

To express recombinant C-subunits efficiently, the respective Cγ and Cα cDNA were expressed in bacteria using the IPTG-inducible bacteriaphage T4 promoter in the pET (Novogen) bacterial expression vector and in Sf9 cells using the baculovirus with polyhedron (polh) as promoter as described in Methods. In addition, homogeneous preparation of RIα and his[illegible]-RIIα were prepared

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from respective bacteria by chromatography. MBP-PKIα and MBP-PKIβ expression vectors were transformed into E.coli., BE21.DE3 pLySs bacteria. The fusion MBP-PKI proteins were prepared from respective bacteria, and purified to 95% homogeneity by Amylose affinity column as outlined in Methods.

1.Expression of C-subunits in bacteria: Bacterial expression systems are very often used and considered as the most efficient system to express recombinant proteins. To obtain sufficient amounts of recombinant C-subunits for *in vitro* analysis, human Cγ and mouse Cα cDNAs were expressed in bacteria using the IPTG-inducible bacteriaphage T4 promoter and the pET expression system (see Methods for detail)

Expression of Cα in bacteria provided an abundant source of recombinant Cα for characterization as previously described (Uhler, 1988). Cα was soluble, active, easily purified and stable at 4 °C for at least three weeks. But four different Cγ/pET constructs produced inactive Cγ proteins. The four Cγ constructs from the human testis Cγ cDNA differed by inclusion or exclusion of the first of two in-frame AUG sequences, which were separated by 27 bp (9 amino acids), and by inclusion or exclusion of 3' untranslated sequences and a poly-A tail (0.6 kb) in the original Cγ cDNA (Beebe et al., 1990). All of the Cγ constructs expressed immunoreactive Cγ as the major protein in bacterial extracts (not shown data), but only the one with two AUGs and no 3'-untranslated sequence produced significant amounts of soluble proteins (Figure 4). The other three constructs expressed insoluble Cγ in bacterial inclusion bodies. The insoluble Cγ proteins had little kinase activity indicating that they were largely inactive (Table 2.). No increase in soluble, active Cγ could be induced from any of the constructs by a variety of approaches. Immunoreactive, but catalytically inactive Cγ protein levels varied under different culture and induction conditions as indicated in Figure 5. (See Methods for detail). For example, the best condition for immunoreactive Cγ protein expression in bacteria appeared to use LB as media after induction with 0.04 mM IPTG

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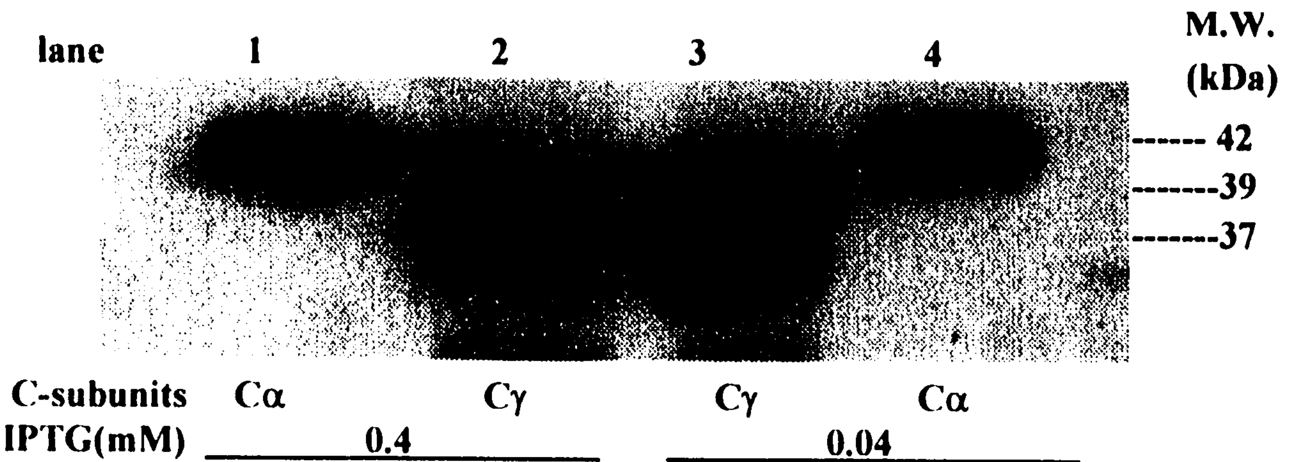

Figure 4 Immunoblot Analysis of C-Subunits Expressed in Bacteria
Bacteria expressing recombinant C-subunits were grown to 0.8 O.D. (600nm) in LB at 37 °C and induced by 0.04 or 0.4 mM IPTG for 4 hours at same temperature. 1.5 μg and 15 μg proteins from the lysates of bacteria expressing Cα and Cγ, respectively, were loaded for 9% SDS-PAGE, then probed for C-subunits by immunoblot analysis using C-subunit-specific antibody. Lane 1: Cα induced by 0.04 mM IPTG, Lane 2: Cγ induced by 0.4 mM IPTG, Lane 3: Cγ induced by 0.04 mM IPTG, and Lane 4: Cα induced by 0.04 mM IPTG

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Figure 5 Immunoblot Analysis of Cγ Expressed in Bacteria under Different Conditions Bacteria expressing recombinant C-subunits were grown to 0.8 O.D (600 nm) values in LB or M9ZB, then induced by 0.04 or 0.4 mM IPTG as inducer at 22 C or 37 C for different time as indicated in the figure Bacterial lysate was prepared as described in Methods 15 µg proteins from each bacterial lysate were loaded each lane of 9% SDS-PAGE and probed for C-subunit by immunoblot analysis using C-subunit-specific antibody as described in Methods

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as inducer for 4 hours induction at 37 °C.

Table 2 Specific Kinase Activity and PKI Sensitivity of t C-Subunits Expressed in Bacteria

C-subunit		Kinase activity (pmol/min/mg)
	PKI_{5-24} (1 μM)	
Cα	-	180428
	+	2579
Cγ	-	222
	+	169

* C-subunits were prepared as described in Methods. Kemptide (65 μM) and Histone-III (85 μM) were used as substrate for Cα and Cγ, respectively

These results suggested that some Eukaryote-specific factors might be necessary for the activation of Cγ by re-folding and/or posttranslational modification, which was absent in prokaryotic expression system. To express active Cγ-subunit, the baculovirus-Sf9 insect cell expression system was utilized to express both C-subunit isozymes.

2.Expression of C-subunits in Sf9 insect cells using baculovirus: The baculovirus-Sf9 insect system has become a popular vehicle to express recombinant proteins at levels as high 500 mg per liter in eukaryotic system (O'Reilly et al., 1992) Many of the pottranslational modification pathways present in mammalian systems are also utilized in baculovirus infected insect cells, allowing the production of recombinant protein which is antigenically, immunogenically, functionally similar to native protein. The baculovirus expression vector system offers significant advantages over prokaryotic and other eukaryotic systems for the production of many recombinant proteins (Summers et al., 1987; Miller et al., 1992, King et. al 1991) Because Cγ was expressed

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as inactive protein in bacteria, it lead us to express it in eukaryotic expression system, which may provide some crucial posttranslational modification to activate recombinant Cγ-subunit. To express both active C-subunit isozymes sufficiently, the Sf9-baculovirus (AcNPV) system was utilized. The same Cγ and Cα cDNAs that were used in bacteria were subcloned into baculovirus expression vectors (pVL 1393) as described in Methods. The purified recombinant Cγ and Cα baculovirus were used to infect monolayers of Sf9 insect cells for the expression of recombinant C-subunits.

To determine whether active Cγ was expressed efficiently in this system, Three-day post-infection crude lysates from mock-infected Sf9 cells (Sf9), wildtype baculovirus (wt) and Sf9 cells infected by purified recombinant Cγ isolates were compared for histone kinase activities in the presence or absence of 1 μM PKI inhibitor. The result showed that PKI-insensitive histone kinase activities in the lysates from the three-day post-infection Sf9 cells by recombinant Cγ-baculovirus were increased 20-50 fold above those from Sf9 cells with mock-infection or wild type virus infection, but PKI-sensitive histone kinase activity was not increased among those tested isolates as determined by kinase assay (Figure 6). To eliminate the possibility that other protein kinases contributed to this elevation of PKI-insensitive histone kinase activity in Sf9 cells due to baculovirus infection, immunoblot analysis using C-subunit specific antibody was utilized to detect immunoreactive C-subunits in those cell lysates as used for kinase assay. The result showed that PKI-insensitive kinase activity level correlated with the appearance of immunoreactive C-subunit protein levels (Figure 7), which suggested that elevated PKI-insensitive histone kinase activities in those Sf9 cells with Cγ-baculovirus infection was derived from the expression of recombinant Cγ-subunit. The results (Figure 6, and Figure 7) indicated that active Cγ has been expressed efficiently in Sf9 insect cells using baculovirus

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Figure 9. Comparison of Baculovirus Cα Isolates on the Kemptide Kinase Activity in Sf9 Cells

Sf9 cells (1.5×10^6) were seeded, grown in monolayer and infected by purified recombinant Cα-baculovirus. Infected Sf9 cells were cultured at 27 °C for three days. Sf9 cells with mock infection (Sf9), infected with wildtype baculovirus (wt) and infected by individual recombinant Cα baculovirus (isolates: 1-6) were harvested and lyzed as described in Methods. The Cα-subunits in these crude cell lysates were detected by standard kinase assay using 65 μM Kemptide as substrate in the presence or absence of 1 μM PKI_{5-24}. The data were derived from one representative experiments. Less than 10% variation of results were repeated by three independent experiments.

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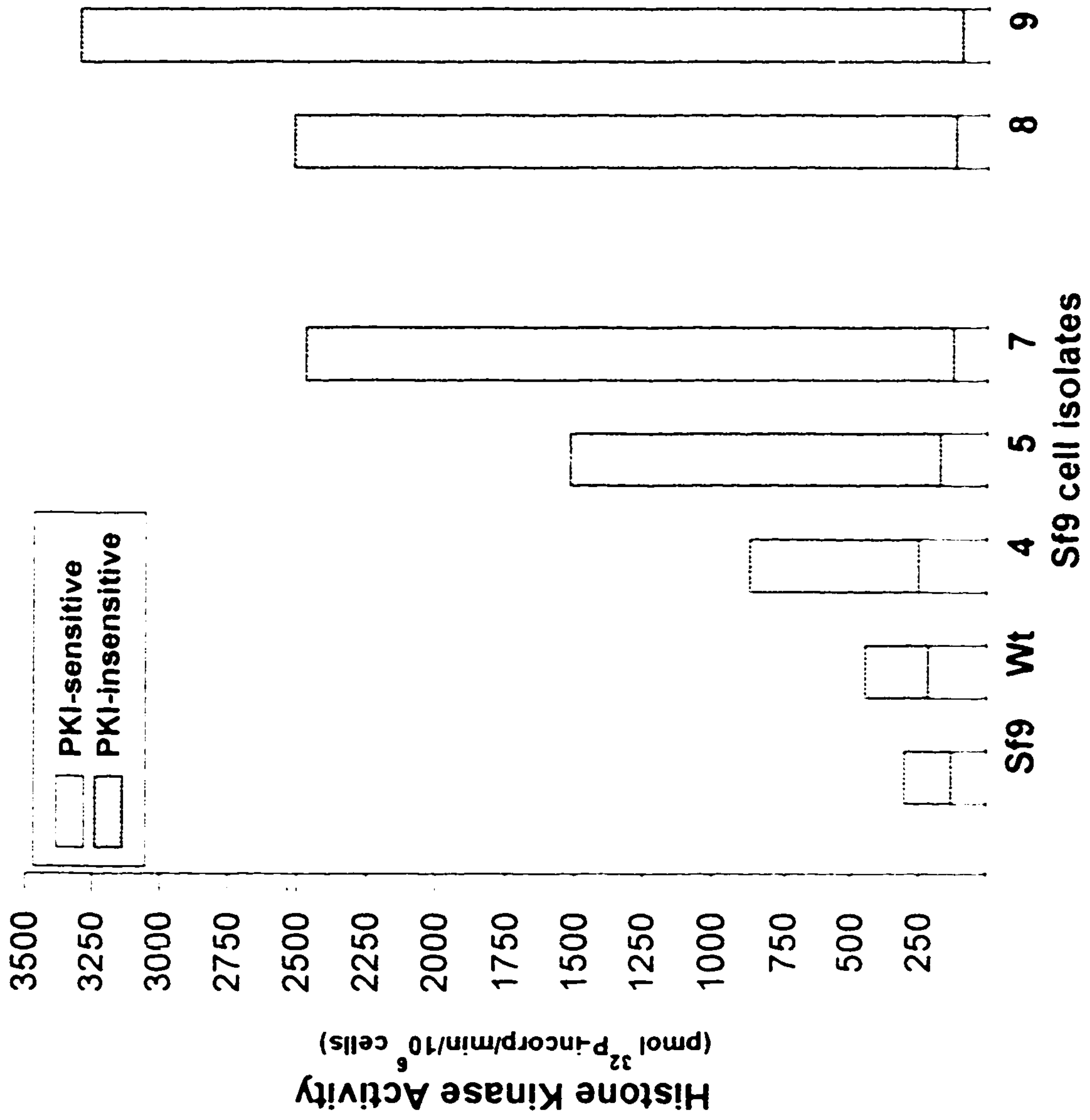

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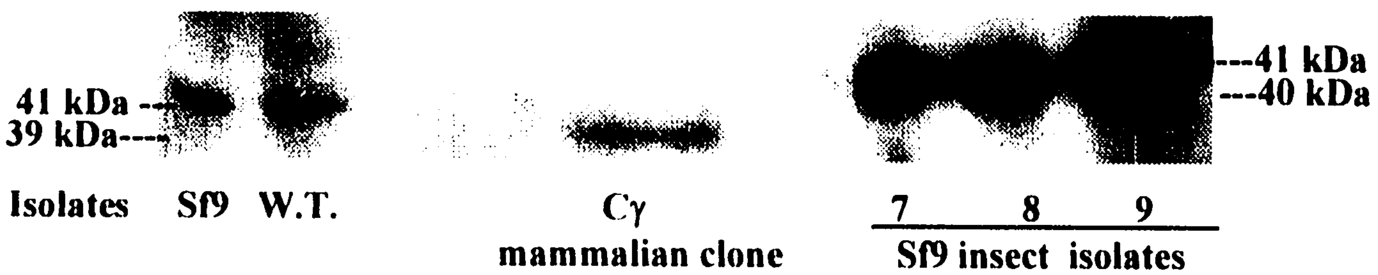

Figure 7. Immunoblot Analysis of Cγ-subunits Expressed in Sf9 Cells Using Baculovirus

Recombinant C-subunits in the same cell lysates as used for kinase assay in Figure 6 were detected by immunoblot analysis using C-subunit-specific antibody. Mammalian crude cell lysate was prepared from Kin8/C clone stimulated by Zinc(80 μM) and cAMP(50 μM) for 18 hours as described in Methods (Beebe et al., 1992). 10 μg proteins from those lysates of three-day post-infection Sf9 cells and mammalian Cγ/Kin8 (Cγ) clone were loaded for immunoblot analysis after 9% SDS-PAGE.

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To determine the expression curve of Cγ for future efficient purification, the kinetic profile for recombinant Cγ activity level expressed in Sf9 cells was observed. The result showed that optimal Cγ expression for purification from intact cells occurred 3 days post-infection, when 85% the recombinant Cγ was isolated from insect cells. Maximal expression occurred 7 days post-infection when 85-90% recombinant Cγ had been in the cell culture media (Figure 8). The monolayers of Sf9 cells infected by Cγ-baculovirus kept growing until complete lysis by baculovirus after seven-day post-infection. After 48 hr. infection by recombinant Cγ-baculovirus, the infected Sf9 insect cells were induced to generate long neturite-like structure, which was not observed in Sf9 cells infected by wildtype baculovirus (not shown data). The Cγ expressed in Sf9 cell using baculovirus was resolved as two immunoreactive protein bands with around 40–41 kDa as apparent molecular weight on the 9% SDS-PAGE (Figure 7), which was slightly larger than Cγ expressed in mammalian cells.

The Sf9 cells were infected with purified recombinant Cα baculovirus isolates. After three-days post-infection culture, infected Sf9 cells were compared with two control Sf9 cells with mock infection and infection by wildtype baculovirus on Kemptide kinase activities in the presence or the absence of PKI. Results showed that Cα was expressed as active protein based on only elevated PKI-sensitive Kemptide kinase activities (Figure 9) and immunoreactive proteins (Figure 10) in those Sf9 cells infected with Cα-baculovirus. PKI-insensitive kinase activity was elevated notably in one Sf9 cell infected by Cα-baculovirus isolate-4, but no immunoreactive C-subunit was detected (Figure 10), which suggested that this elevation of PKI-insensitive kinase activity was derived from other kinase. However, in contrast to Cγ expression in Sf9 cells, the progressive expression of Cα was terminated shortly after three day post-infection (Figure 11). The monolayers of Sf9 cells infected by recombinant Cα baculovirus stopped growing after 72 hr post-infection based on cell number. Infection of Cα-baculovirus still induced the Sf-9 cells to generate

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neturite-like structure after 48 hr post-infection. The total yield of recombinant Cα-subunit was lower than those found from Cγ expressed in the same system and much more lower than that found from Cα expressed in bacteria (Table 7) based on kinase assay.

The C-subunits have been expressed in three different systems, but the activity and expression level of each C-subunit varied remarkably in each system. The reason for this distinction is unclear. It is possible that the host cell somehow affected expression levels and processing of recombinant proteins. For example, Cγ expressed in Sf9 cells using baculovirus differed from those expressed in mammalian cells and bacteria in apparent molecular size on SDS-PAGE and in levels of active kinase activity. This phenomenon promoted us to ask this question. Do the different expression systems affect the substrate and pseudosubstrate specificity of these recombinant C-subunit isozymes? The answer to this question was imperative in determining if these recombinant C-subunit isozymes expressed in different expression systems could be fairly utilized for *in vitro* kinetic comparison on substrate and pseudosubstrate specificity.

3.Preliminary characterization of recombinant C-subunit isozymes expressed in three different systems: C-subunits have been successfully expressed in three expression systems. Figure 12 shows an immunoblot analysis of recombinant Cα-subunits (lanes 1,3, and 5) and Cγ-subunits (lanes 2,4,and 6) from crude extracts in three expression systems utilizing a C-subunit-specific antibody (provided by Dr. Bryan Hemming, Basal). Cα was expressed in Cα Kin8 clones (lane 1), in bacteria (lane 3) and in Sf9 cells(lane 5) as 41–42 kDa proteins. In all three expression systems, Cγ migrated as two bands. The C-subunits in the Cγ/Kin8 clone (lane 2) were 39 and 41 kDa, but the slow-migration band may be, at least in part, endogenous Cα. Two different Cγ/Kin8 clones produced essentially identical results (Beebe et al., 1992). Two Cγ bands were expressed in bacteria (lane 4) as 39–40 kDa and in Sf9 cells (lane 6) as 40–41 kDa.

It was necessary to determine whether the different expression systems affect the substrate.

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Figure 8. The Kinetics of Cγ-Subunit Expression in Sf9 Cells Using Baculovirus

Sf9 cells(1.5×10^6) were seeded, grown in monolayer and infected by purified recombinant Cγ-baculovirus (isolate 9) as described in Methods. The infected Sf9 cells were cultured for various times at 27 °C. Recombinant Cγ-subunit in the culture media and cell lysate prepared from these various days post-infection Sf9 cells were detected by kinase assay using 85 μM histone as substrate in the presence of 1μM PKI_{5-24}. The data were derived from one representative experiments. Less than 10% variation of results were repeated by three independent experiments.

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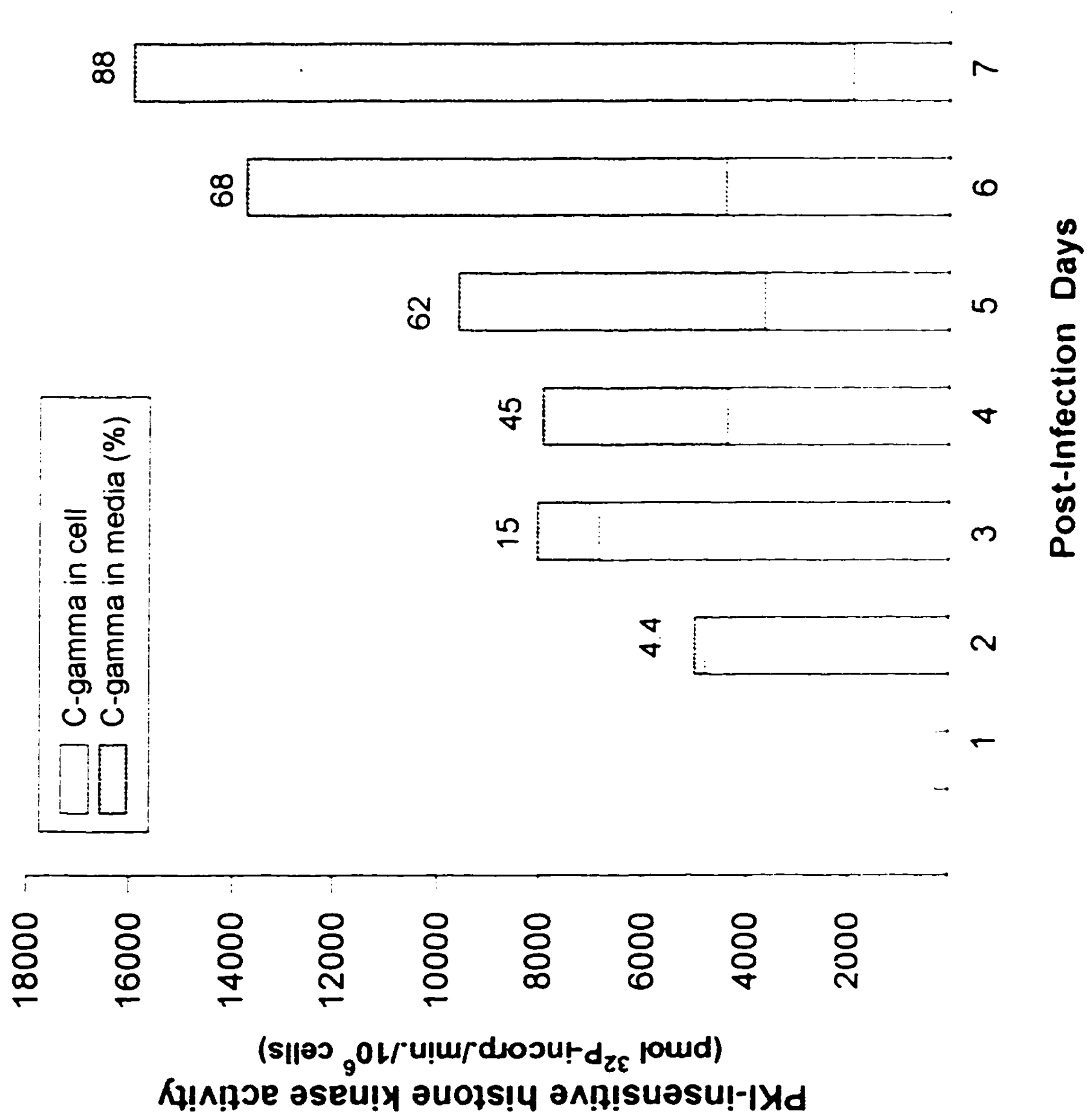

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Figure 6. Comparison of Recombinant Cγ-Baculovirus Isolates for Histone Kinase Activity in Sf9 Cells

Sf9 cells (1.5×10^6) were seeded, grown in monolayers and infected by purified recombinant Cγ-baculovirus isolates as described in Methods. Infected Sf9 cells were cultured at 27 °C for three days. Sf9 cells with mock infection (Sf9), infected with wildtype baculovirus (wt) and infected by individual recombinant Cγ baculovirus (isolates:4, 5, 7, 8 and 9) were harvested and lyzed as described in Methods. The Cγ-subunits in these crude cell lysates were detected by standard kinase assay using 85 μM histone as substrate in the presence or absence of 1 μM $PKI_{5\text{-}24}$. The data were derived from one representative experiments. Less than 10% variation of results were repeated by three independent experiments.

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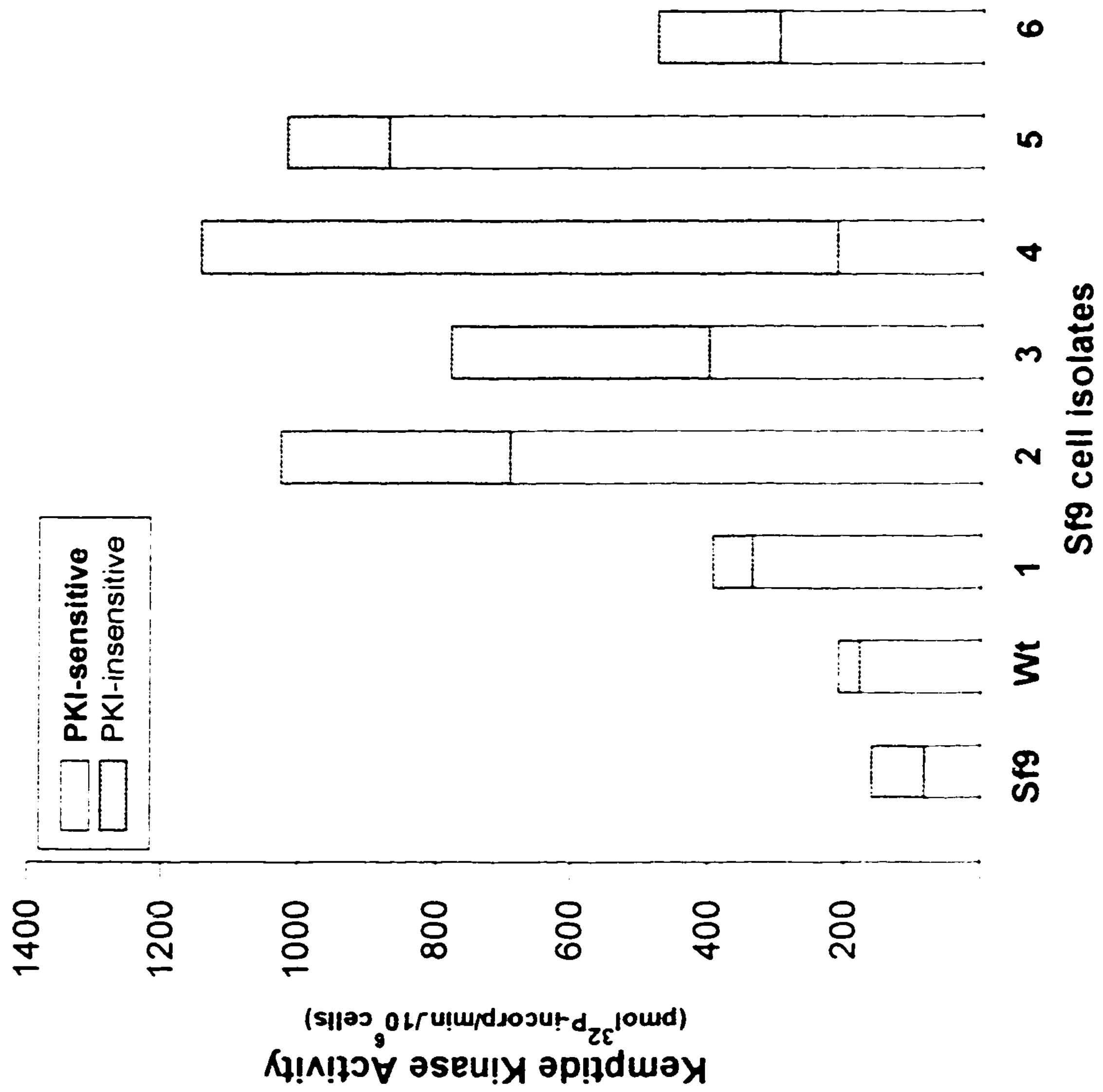

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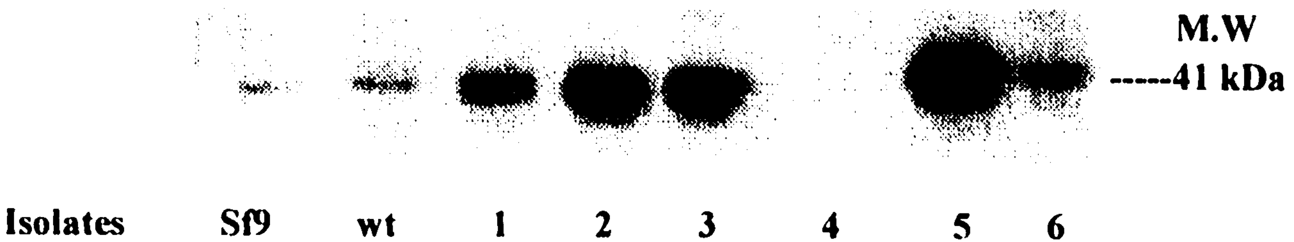

Figure 10. Immunobot Analysis of Cα-Subunits Expressed in Sf9 Cells Using Baculovirus

Recombinant Cα-subunits in the same cell lysates as used for kinase assay in Figure 9 were probed by immunoblot analysis using C-subunit-specific antibody. 10 μg proteins from the lysates of each three-day post-infection Sf9 cells were loaded for immunoblot analysis after 9% SDS-PAGE.

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Figure 11. The kinetics of Cα-Subunit Expression in Sf9 Cells Using Baculovirus

Sf9 cells(1.5×10^6) were seeded, grown in monolayer and infected by recombinant Cα-baculovirus (isolate 5). The infected Sf9 cells were cultured for various days (1 to 7 days) at 27 °C. Recombinant Cα-subunits in the culture media and cell lysates prepared from those various days post-infection Sf9 cells were detected by kinase assay using 65 μM Kemptide as substrate in the absence of PKI_{5-24}. The data were derived from one representative experiments. Less than 10% variation of results were repeated by three independent experiments.

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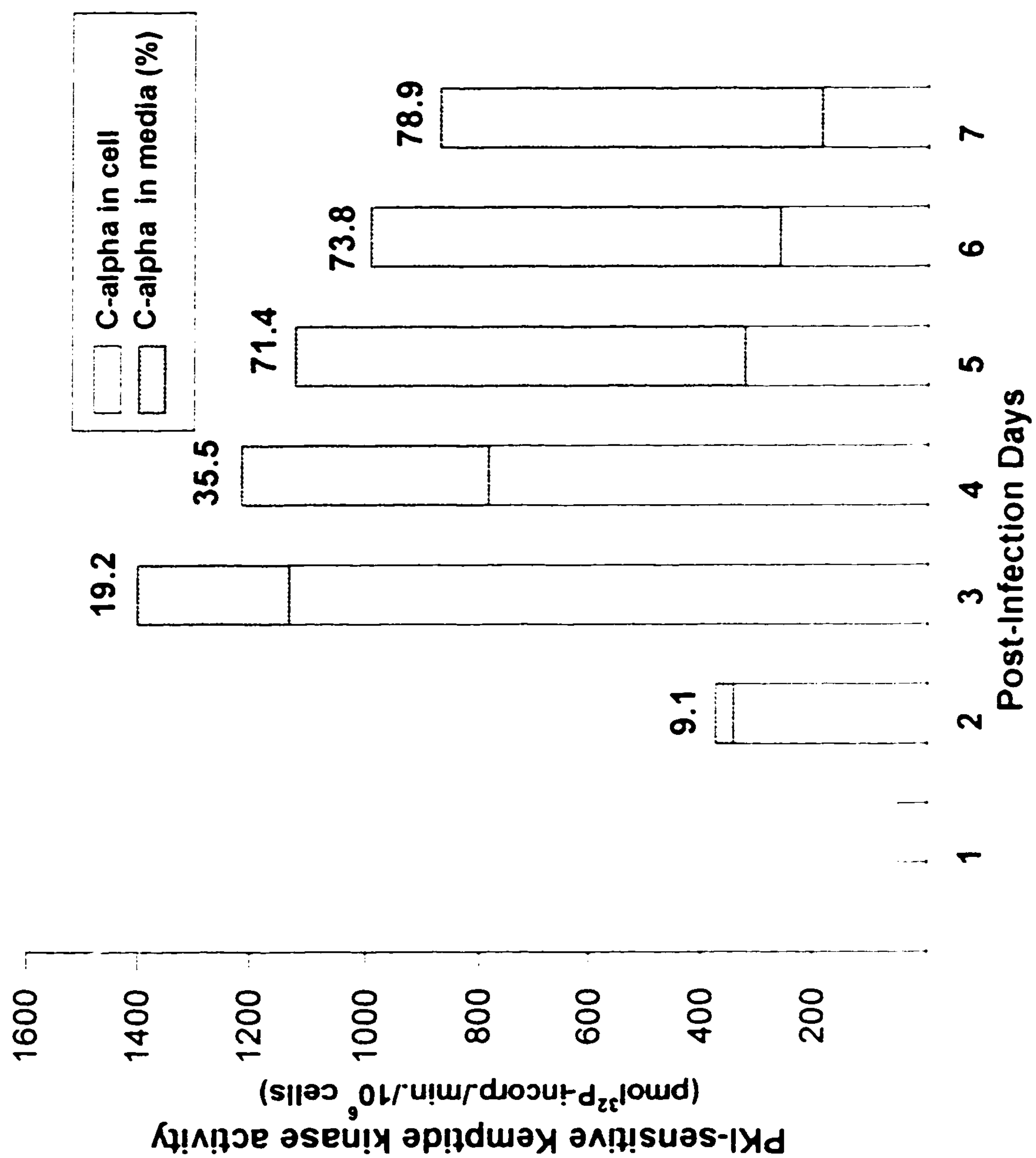

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pseudosubstrate specificity and activity of these recombinant C-subunit isozymes. In addition, the expression of Cα and Cγ subunits in Kin8 clones resulted in the expression of C-subunits with different sensitivities to the PKA-specific inhibitor proteins (PKIα and PKIβ) and a peptide, (PKI$_{5-24}$) (Beebe et al., 1992). Although Cγ mRNA and immunoreactive Cγ-subunit was increased in a zinc-dependent manner in Cγ/Kin8 clone (Beebe et al., 1992), the possibility that Cγ activated the PKI-insensitive activity of another kinase had not been ruled out. Furthermore, since there was some inhibitor sensitivity in Kemptide kinase activity from Cγ-expressing clones (Beebe et al 1992), it was not clear if inhibitor-sensitivity was protein substrate selective or if some Cα PKI-sensitive activity was present in Cγ preparation, this is possible because Kemptide is a better substrate than histone for Cα. Therefore, it is not only interesting, but also necessary to compare the recombinant C-subunits from all three expression systems for PKI-sensitivity and substrate specificity. To confirm the existence of unique isozyme-specificity for C-subunit isozymes, partially purified recombinant C-subunits were compared for specific kinase activity, substrate and inhibitor specificity as described in Methods.

Table 3 Specific Kinase Activity, Substrate and Inhibitor Specificity of Recombinant C-Subunits Expressed in Three Expression Systems (nM/min./mg)

Clones		Kemptide (KT)		Histone (HT)		KT/HT
		-PKI	+PKI	-PKI	+PKI	-PKI
Mammalian	Cα	1.15	0.04	0.21	0	5.41
Mammalian	Cγ	0.42	0.18	0.57	0.50	0.74
Bacterial	Cα	500.0	51.0	116.0	1.27	4.30
Bacterial	Cγ	0.13	0.11	0.22	0.17	0.59
Baculovirus	Cα	6.20	0.12	2.71	0.08	3.40
Baculovirus	Cγ	24.0	20.0	43.0	39.0	0.56

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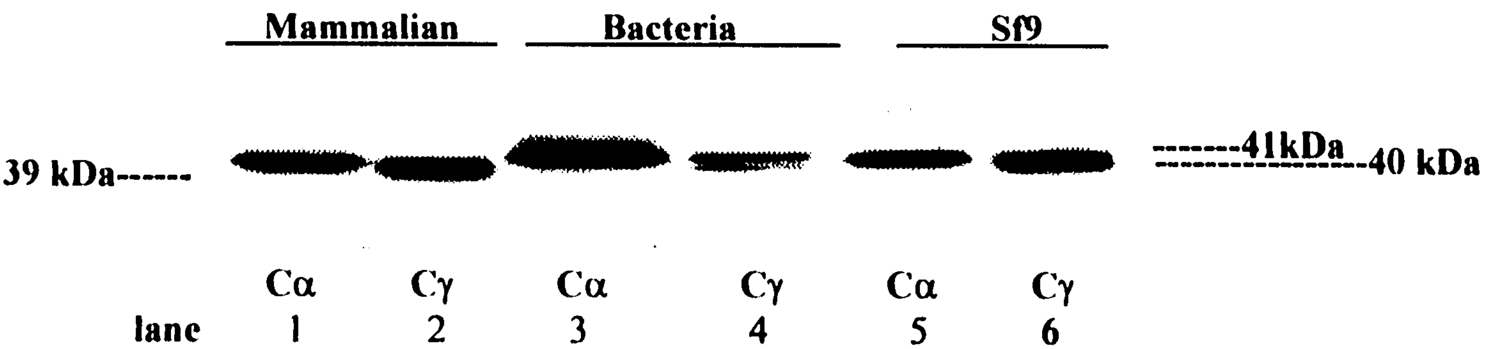

Figure 12 Immunoblot Analysis of C-Subunits Expressed in Three Expression Systems
Recombinant C-subunits were expressed in mammalian cells (Y1/Kin8) stimulated by zinc for 18 hours (lane:1-2) at 37 °C, in bacteria induced by 0.04 mM IPTG for 4 hours at 37 °C (lane 3-4), and in the monolayer of Sf 9 cells (lane:5-6) infected with recombinant C-subunit baculovirus for 72 hours at 27 °C. Cell lysate were prepared and detected for C-subunits by immonoblot analysis using C-subunit-specific antibody as described in Methods. 3 μg proteins of cell lysates from Kin8/ Cα2 (lane 1) and Kin8/Cγ7 (lane 2), 1.5μg proteins of cell lysates from bacteria expressing recombinant Cα (lane 3), 10μ g proteins of cell lysate from bacteria expressing recombinant Cγ (lane 4), 15μ g proteins of cell lysates from Sf9 cells infected with recombinant baculovirus Cα (isolate 5) (lane 5) and Cγ (isolate 9) (lane 6) were loaded immunoblot analysis after 9% SDS-PAGE

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Table 3 compares recombinant C-subunit activities from the indicated sources for PKI sensitivity and substrate phosphorylation by kinase activity assay. Characterization of Cα and Cγ expressed in all systems exhibited similar substrate and PKI inhibitor specificity. Results from Cγ in Sf9 cells using baculovirus and Cα from bacteria confirmed our original observations from the expression in Kin8 cells that Cγ was best defined as a PKI-insensitive histone kinase (HT) and Cα was best defined as a PKI-sensitive Kemptide kinase (KT) (Beebe et al., 1992). The KT/HT activity ratios for Cγ and Cα were 0.5-0.7 and 4.0-6.0, respectively. Unlike Cα, Cγ was insensitive to PKI_{5-24} with both substrates. Although optimal expression of these C-subunit isozymes is cell system dependent, Cα always exhibited PKI-sensitive and Cγ always exhibited PKI-insensitive activity. Furthermore, the KT/HT activity ratios for respective C-subunit are similar in all expression systems. However, the Cγ preparation from mammalian cell stable transfectants displayed some inhibition with Kemptide as substrate, which was best explained by contamination with endogenous Cα. It is therefore appropriate to use Cγ expressed in Sf9 cell using baculovirus and Cα expressed in bacteria for kinetic comparison based on presented results (Table 3). Considering expression level and kinase activity, Cγ and Cα expressed in Sf9 cells using baculovirus and bacteria, respectively, were chosen to be purified to homogeneity for kinetic comparison *in vitro*.

4. *Expression of native Cγ-subunits in monocyte like U937 cells.* Recombinant Cγ has been expressed in three cell systems and characterized as a PKI-insensitive histone kinase regardless of cell systems (Table 3). It is better to further confirm these results with native Cγ-subunit. Cγ subunit was reported to be expressed only in human testes (Beebe et al., 1990), which indicated that Cγ exhibited a limited and tissue-specific expression. Cγ gene has been cloned and shown to be intronless gene (Beebe at al., 1990). Many Cγ characteristics are consistent with other intronless gene products. For example, many intronless genes exhibit low-stability, low expression levels and

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limited distribution in tissues. The intronless gene products are often associated with development and/or cell differentiation, which is consistent with the expression of Cγ in testis, where sperm is differentiating after maturity. We hypothesized that Cγ might be present in other differentiating cells. Therefore, U937 cells, a undifferentiated monocyte-like cell which could be differentiated by agents such as cAMP, was utilized as cell line to determine if Cγ was present during differentiation. The U937 cells were induced to differentiate *in vitro* by 50 μM cAMP stimulation for 72 hours. The C-subunit was detected in undifferentiated and differentiated U937 cells by kinase assay and immunoblot analysis using C-subunit specific antibody. The results showed that after 72 hours of stimulation with cAMP, a Cγ-like protein was present based on several criteria, including the cAMP-dependent PKI-insensitive histone activity (Table 4) and immunoreactive Cγ-like proteins (Figure 13).

Previous data (L. K. Parvathenani's unpublished data at Easter Virginia Medical School) shown that after being treated by 50 μM cAMP, the U937 cells stopped proliferation, elevated the percentages of phagocytes, and reduced the generation of NBT, which are indicators of cell differentiation (Beebe and Corbin, 1986). The Table-4 showed when the U937 cells were induced to differentiate by cAMP-stimulation, the total PKA kinase was decreased about 40%.

Because the type-I kinase level was not changed during the differentiation of U937 cells, the decreased total kinase activity level was derived from the loss of type-II holoenzyme. The ratios of type-I / type-II was changed from 0.33 to 3.8. This result suggested that decreasing of type-II A kinase occurred during the differentiation of U937 cell differentiation. The previous data reported that type-II A-kinase mediated the cell differentiation (Beebe & Corbin, 1986), which suggested that this decreased type-II kinase activity might be involved in the differentiation of U937. Table-4 also showed that about 20% PKI-insensitive cAMP-dependent kinase activity was present in the crude lysates of both kinds of U937 cells. After separating type I and type II PKA holoenzymes in

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Table 4 Separation And Detection of Cγ Activity in Monocyte-Like U937 Cells[1]

Portion	PKI(1μM)	Total Kinase Activity		[2] %PKI-insen		[3] %cAMP-dependent	
		Differentiation [4]		Differentiation		Differentiation	
		-	+	-	+	-	+
Lysate	-	11,110	6,613	18	21		
	+	2001	1417			36	54
Elution I	-	972	1860	25	17		
	+	243	312			33	36
Elution II	-	2916	485	48	76		
	+	1399	368			43	66

[1]: 200× 10^6 U937 cells were used in this experiment. The two types of PKA holoenzymes were separated by two-step elution on 3DEAE-Sepharose chromatography as described in Methods [2] % PKI-insen.: kinase activity in the presence of 1 μM PKI_{5-24} / kinase activity in the absence of PKI × 100) Kemptide (65 μM) was used as substrate for the kinase assay in the presence of 5 μM cAMP [3] % cAMP-dependent means that kinase activity in the presence of 10 μM cAMP/(total kinase activity in the presence and absence of 10 μM cAMP) × 100; [4] -: undifferentiated cells; + differentiated cells

the cell crude lysate by DEAE-Sepharose chromatography eluted by two-step elution (0.15 M NaCl for type I and 0.5 NaCl for type II as described in Methods), most PKI-insensitive kinase activity was recovered from elution II (type-II holoenzyme) But, the PKI-insensitive cAMP-dependent kinase activity was only found in type-II holoenzyme of differentiated cells It was known that most of mammalian (Y1/Kin8 cells) PKI-insensitive cAMP-dependent kinase activity was detected in the type-II holoenzyme. Correspondingly, 39 kDa mammalian-specific Cγ protein

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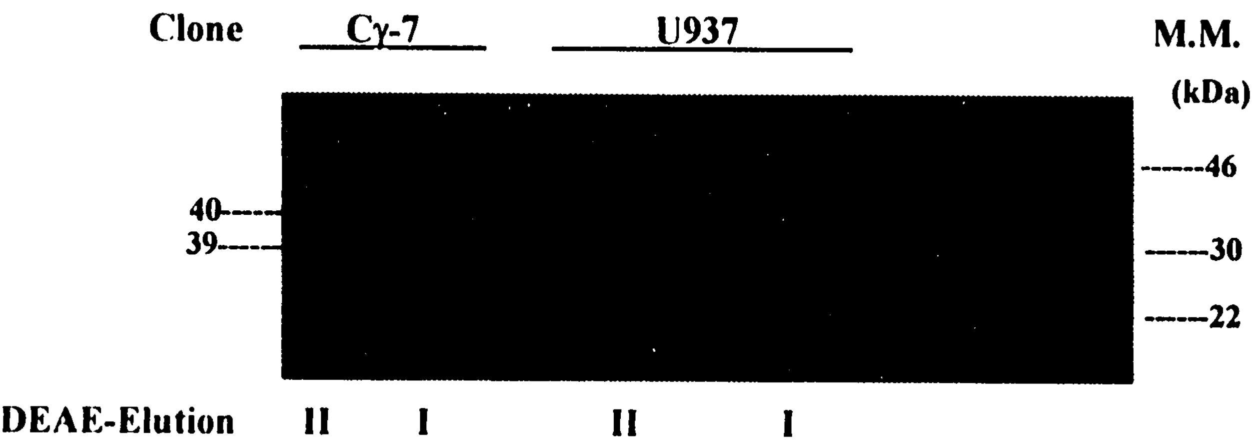

Figure 13. Immunoblot Analysis of Cγ-like Protein in cAMP-Treated Monocytes-Like U937 Cells
The crude lysates from U937 cells(200×10^{3}) stimulated by cAMP (50μ M) for 72 hours, and Kin8/Cγ cell stimulated by Zinc (85μM) for 18 hours, were separated into three portions on DEAE-Sepharose column by step-elution with 0.15 mM NaCl (elution-I) and 0.5 mM NaCl (elution-II) in 10 mM potassium-phosphate buffer (pH 6.8) as described in Methods. The C-subunits in individual elution from chromatography were assayed for PKI-sensitive and PKI-insensitive kinase activity with 85 μM histone or 65 μM Kemptide as substrate in the presence or absence of 1μ M PKI_{5-24}. 5μ g proteins from each elution fractions containing peak kinase activity were loaded for 9% SDS-PAGE, then probed for C-subunits by immunoblot analysis using C-subunit-specific antibody. Molecular weight marker (M.M.) is the same as described in Figure 17.

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band could be detected in type-II holoenzyme (Beebe et al., 1992). It was suggested that the increased PKI-insensitive cAMP-dependent kinase activity in type-II holoenzyme of differentiated U937 cells might be derived from Cγ-subunits. To test this, each elution fraction containing type-I and type-II holoenzymes were subjected to immunoblot analysis using C-subunit specific antibody. The parallel elution fraction from Cγ/Kin8 cells after Zinc/cAMP stimulation were used as positive control. Figure 10 shows that the 39-kDa Cγ-like immuno-reactive protein band only could be detected in the elution II portion (type-II) of differentiated U937 cells, which suggested that the elevation of PKI-insensitive cAMP-dependent kinase activity in type-II of differentiated U937 cells was probably derived from Cγ-subunits. The KT/HT ratio of this PKI-insensitive kinase was 0.75 (not shown data), which suggested that native Cγ-subunit seemed to be a PKI-insensitive histone kinase just like recombinant Cγ-subunits expressed in the heterologous system. Detection of Cγ-mRNA by northern-blot and RT-PCR in U937 is on the way to confirm the primary results. This cell line may become a model to study native Cγ-subunits as positive control.

5. Purification of recombinant Cγ-subunits expressed in Sf9 cells using baculovirus. Baculovirus recombinant Cγ was purified to homogeneity by sequential chromatography using CM-Sepharose and Sephacryl-SH300. This was a similar protocol described by Baude (1994) for the purification of bacterial Cα. Figure 14 shows the PKI-insensitive histone kinase activity profile in Cγ-baculovirus-infected cell lysate on CM-Sepharose chromatography eluted by gradient salt (0-0.6M NaCl). In this profile, two PKI-insensitive histone kinase could be revealed. The two PKI-insensitive kinase activity peaks were eluted by approximate 0.16-0.2M and 0.22-0.30 M NaCl, respectively. It was of interest to identify these two PKI-insensitive kinase peaks for their substrate specificities and components, which might show some information about different modified forms of recombinant Cγ due to the presence of two close Cγ bands in all three expression systems.

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The Table 5 showed that the kinase in the first peak was substrate-dependent PKI-sensitive kinase. When the Kemptide was used as substrate, this kinase was completely sensitive to PKI-

Table 5 Substrate and Inhibitor Specificity of Two Kinase Activity Peaks from CM-Sepharose Chromatography for Cγ Purification

PEAK	Kemptide(KT)		Histone(HT)		Ratio(KT/HT)	(% of total activity)
	-PKI	+PKI	-PKI	+PKI	-PKI	
I	7.75	0	5.84	3.54	1.32	36
II	7.76	8.94	31.93	42.58	0.24	64

* The kinase activity unit was nmol ^{32}P incorp./min/mg

inhibition. In contrast, around 50-60% kinase was insensitive to PKI-inhibition with histone as substrate. The kinase in the second peak was completely insensitive to PKI-inhibition regardless of substrate. The kinase activity ratio (KT/HT) for first and second peak were 1.32 and 0.24, respectively. Each fraction from both peaks were subjected to immunoblot analysis using the C-subunit-specific antibody. Figure 15 demonstrates that the first peak (fraction 6-20) contained one C-subunit band at approx. 41 kDa. The second peak (fraction 22-38) was resolved as two close bands, which were 41 kDa and 40 kDa. However, after gel-filtration on Sephacryl S-300, only one PKI-insensitive histone kinase peak existed (Figure 16), which contained one C-subunit band with 41kDa (Figure 17). Based known distinctions between Cγ and Cα on the substrate specificity (KT/HT), PKI-sensitivity and apparent molecular weight, the explanation for these two peaks derived from the elution profile by CM-Sepharose chromatography was that the first peak contained endogenous Cα-subunits and lesser amount of active recombinant Cγ-subunits or some

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other kinase, which accounted for PKI-insensitive histone kinase activity. The second peak contained two isoforms of recombinant Cγ-subunits.

After a two-step procedure, Cγ-subunits could be purified to homogeneity as judged by silver staining (Figure 17) and Coomassie blue staining (Figure 18). The final recovery of purified baculovirus recombinant Cγ was 21% and purification was 270-fold (Table 6). The two steps contributed equal efficiency for the purification of Cγ protein. Specific activity of purified baculovirus recombinant Cγ was 0.82 units (1μmol.^{32}P-incorp./min/mg=1units) with histone as substrate, and 0.31 units with Kemptide as substrate, which was relatively low if compared with bacterial Cα (5.8-6.96 units) (Baude et al., 1994) and much less than mammalian Cα-subunits (55 units) (Olsen & Uhler, 1989).

Table 6 Purification of Cγ-Subunit Expressed in Sf9 Insect Cells Using Baculovirus

Steps	Protein (mg/ml)	Specific Activity (μmole/min/mg)	Total Activity (μmole/min)	Purification (fold)	Yield (%)
Lysate*	4.25	0.003	0.74	1	100
CM-Sepharose	0.21	0.058	0.39	19	53
Sepharycal-SH300	0.008	0.818	0.15	273	21

The lysate was prepared from 400× 10^{6} Sf9 cells. 85(μM) histone was used as substrate for the kinase assay in the presence of 1 μM PKI$_{5-24}$

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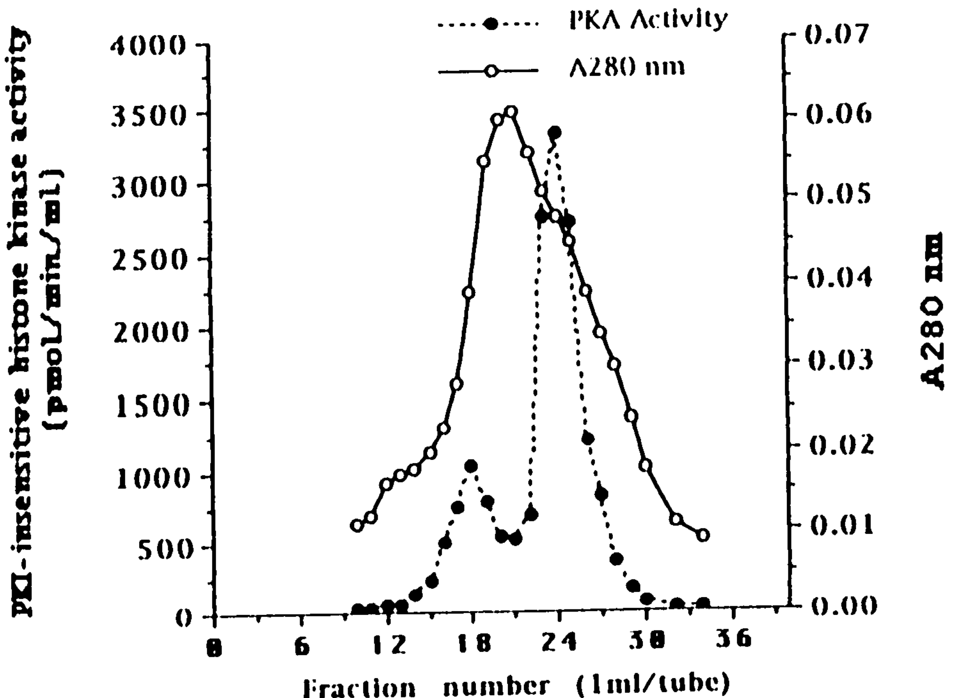

Figure 14 CM-Sepharose Chromatography of PKI-Iinsensitive Histone Kinase Activity in the Sf9 Cells Infected by Cγ-Baculovirus
400× 10^6 monolayer of Sf9 cells were seeded, grown to 65-75% confluent and infected by purified recombinant Cγ-baculovirus (isolate 9) The infected Sf 9 cells were cultured at 27 °C for 72 hours. The cell lysate from these three-day post-transfection Sf9 cells were prepared as described in Methods The Sf9 cell lysate (40 mg of proteins) was applied to 25 ml pre-equilibrated CM-Sepharose col9umn (1.5×15 cm), washed and eluted by 0-600 mM NaCl gradients (100 ml) with column buffer as described in Methods One-ml fractions was collected and assayed for kinase activity using 85 μM histone as substrate in the presence of 1 μM PKI_{5-24} and protein concentration as described in Methods The data was plotted by Sigma-plot software (1995)

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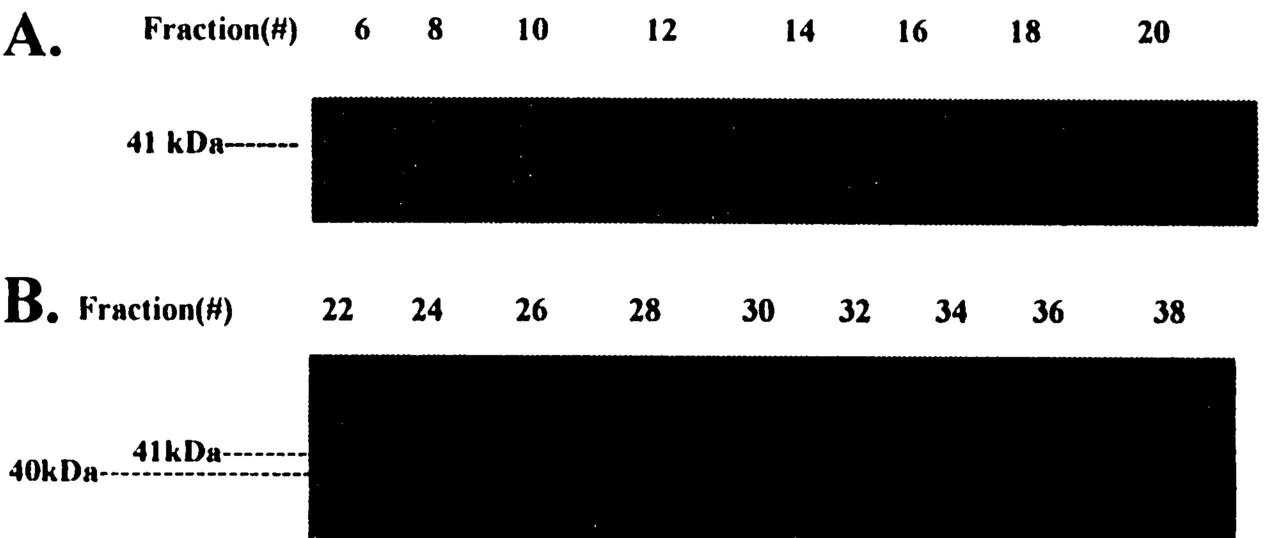

Figure 15. Immunoblot Analysis of Two C-Subunit Kinase Activity Peaks from Elution Profile of CM-Sepharose Chromatography

Various elution fraction containing histone kinase activities (Panel A. fraction 6--20, Panel B. fraction 22-38.) from CM-Sepharose NaCl gradient elution (0-0.6M) were probed for Cγ-subunits by immunoblot analysis using C-subunit-specific antibody. 25 μl of each fraction was loaded in each lane for 9% SDS-PAGE

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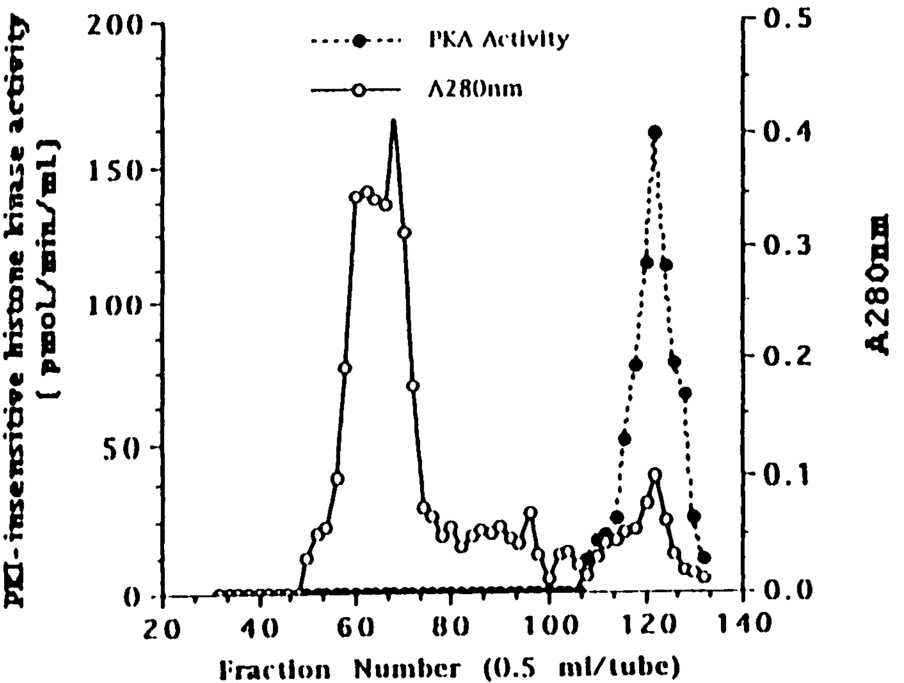

Figure 16 Sephacryl-S 300 Gel Filtration Chromatography of PKI-Insensitive Histone Kinase Activity in the Elution Fractions from CM-Sepharose Chromatography

Fractions 6-40 from the CM-Sepharose column were pooled and concentrated using Amicon-filtration. The concentrated sample (10 mg) was applied into 350 ml Sephacryl-S 300 gel filtration column (2.5 × 125 cm) which was pre-equilibrated in 10 mM potassium phosphate-5 μM EDTA buffer (pH 6.9) containing 0.5 M NaCl. The column was eluted with same buffer at a flow rate of 0.5 ml/min. Each elution fraction (0.5 ml) was collected and assayed for histone kinase activity in the presence of 1 μM of PKI_{5-24} and protein concentration as described in Methods

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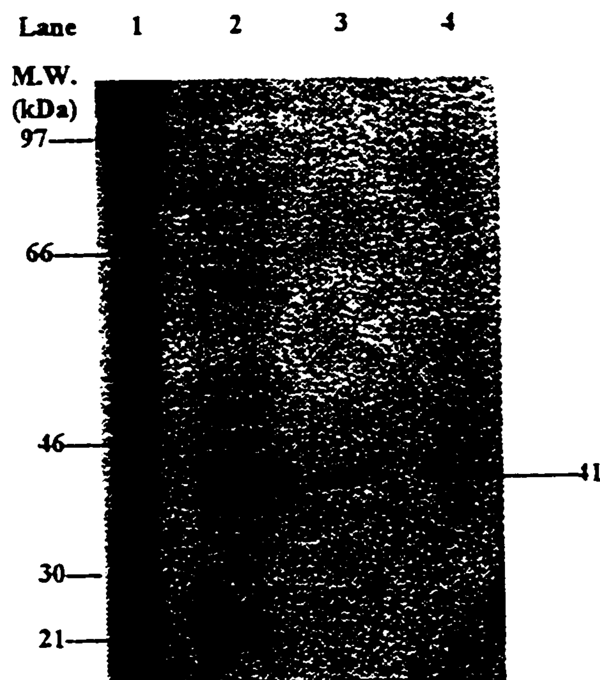

Figure 17. Analysis of Recombinant Cγ Purification by Silver-Stained-SDS-PAGE
Proteins from three stages of purification were analyzed by silver-stained SDS-PAGE as described in Methods. 15 μg Sf9 cell lysate (lane 1), 10 μg CM-Sepharose fractions (#26) containing PKI-insensitive kinase activity (lane 2), 2.5 μg (lane 3) and 5 μg (lane 4) concentrated Sephacryl S-300 fraction (#115-130) containing PKI-insensitive kinase activity were loaded for 9% SDS-PAGE. Molecular weight marker (M.M.) (Amersham) were myosin (220 kDa); phosphorylase b (97.4kDa); Bovine serum albumin (66kDa); Ovalbumin (46kDa); Carbonic anhydrase (30 kDa); Trypsin inhibitor(21.5 kDa).

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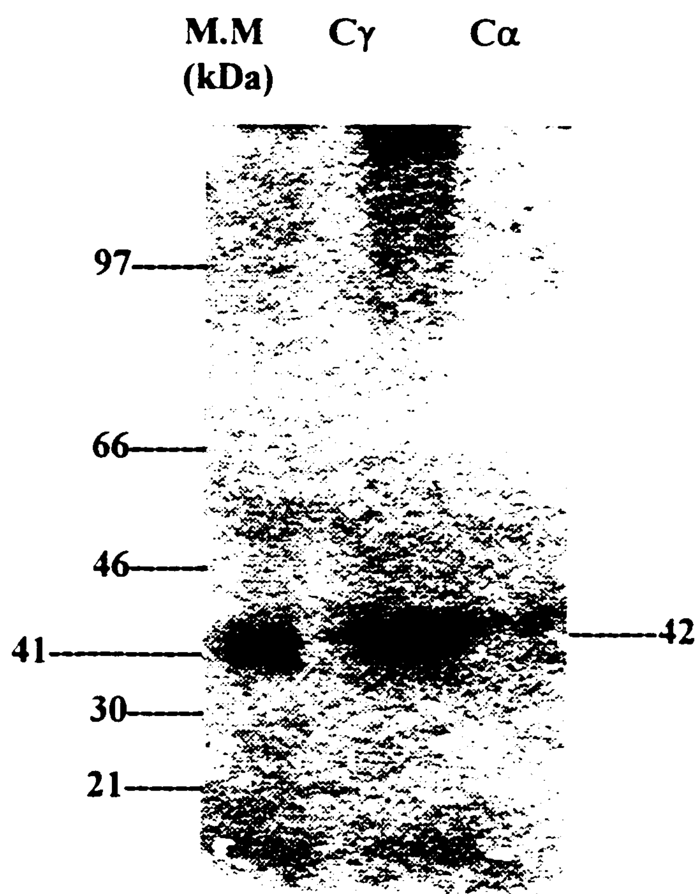

Figure 18. Analysis of Purified C-Subunits by Commassie Blue Stained-SDS-PAGE Recombinant Cγ expressed in Sf9 cells using baculovirus (recombinant Cγ-baculovirus isolate: 9) and recombinant Cα expressed in bacteria were purified by sequential chromatography on CM-Sepharose and Sephacryl S-300 as described in Methods 5μg purified Cγ and 5μg purified Cα were loaded for 9% SDS-PAGE The gel was stained by Coomassie blue. M.M.: molecular weight marker as described in Fig 17

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6 Purification of recombinant Cα-subunit expressed in bacteria

Table 7 Purification of Recombinant Cα-Subunits Expressed in Bacteria*

	Protein (mg/ml)	Specific Activity (μMole/min/mg)	Total Activity (μMole/min)	Purification (fold)	Yield (%)
Lysate**	3.81	0.093	14.174	1	100
CM-Sepharose	0.24	1.402	8363	15	59.2
3 Sepharycal-SH300	0.022	5.321	1923	62	23.0

*The lysate was prepared from 500 ml bacterial broth. The Kemptide (65μM) was used as substrate for kinase assay (Beebe et al.,1986).

As shown in Table 7, the same procedure as described by Baude (1994) for Cα purification was utilized to purify recombinant bacterial Cα-subunits. After two sequential chromatography steps (not shown), about 25% of total Cα-subunit was purified to homogeneity as judged by Coomassie blue stained-SDS-PAGE (Figure 18). Purified Cα 2.5 μg was stained better with Coomassie-blue than with sliver staining as (not shown data). Only one major kinase peak was revealed in PKI-sensitive kinase activity profile in CM-Sepharose column eluted by gradient salt (0-0.6 M NaCl) (not shown data). Because the Cα-subunit was expressed as the predominant protein in bacteria and was relatively stable at 4 °C, the final purification of recombinant Cα-subunit was only 65-fold. The recovery was 23%. In contrast to Cγ purification, the CM-Sepharose chromatography was major-step for the purification of bacterial Cα, which can be

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reflected by one major kinase peak in CM-Sepharose elution profile and less peaks in the elution-profile of Gel-filtration (not shown data). The specific activity of purified bacterial Cα was 5.3 μmol ^{32}P-incorp./min./mg protein, which was 10-fold lower than those found from recombinant *Aplysia* C-subunit (61 units) (Beley et al., 1992) and native mammalian Cα-subunits (55 units) (Olson & Uhler 1989). However, this specific activity was similar to that for recombinant bacterial Cα purified by Baude (5.8 units) (Baude et al., 1994).

7.*Stabilization of purified C-subunits:* It was imperative to have an efficient way to keep C-subunits stable during and after purification for kinetic analysis. The first experiment was conducted to compare the stability of both recombinant C-subunits in crude lysate stored at 4 °C. The result (Figure 19) showed that Cγ lost kinase activity faster than Cα, which indicated that Cγ was relatively in-stable at 4 °C. The lower stability of C-subunits may be an result of the presence of protease in the preparation. The stability of partially purified Cγ and Cγ after elution from CM-Sepharose showed that both C-subunit kinase activities decreased around 40% in the first two days. But, the Cγ lost almost 95% of its original kinase activity after 6 days storage at 4 °C and the partially purified Cγ only lost 60% of its kinase activity (Figure 20). This result indicated that purification stabilized the Cγ-subunits kinase activity, which might be resulted from separating Cγ-subunits from protease. The same phenomenon was observed in Cα (not shown data).

To further compare the stability for both C-subunits, both homogeneous recombinant C-subunits were compared for heat-stability by pretreatment at 37 °C and then assayed with histone and Kemptide as substrate. The result (Figure 21) showed that both C-subunits lost 15-35% total activity after first 6-minutes heat-treatment, but after six to 18 minutes, Cγ almost lost 90% of total activity, but Cα only lost 40% total activity. As predicted, recombinant Cα could tolerate the heat-treatment better than recombinant Cγ-subunits. The result also showed that substrate affected both C-subunits heat-stability. With histone as a substrate, 20% more of total kinase activity of

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both C-subunits sustained. Although exact molecular mechanism for this was unclear, this distinction appeared to be due to the difference between protein and peptide substrates rather than substrate-specific action on enzyme.

Glycerol is often used as a stabilizer to store protein or enzymes in frozen condition. In this study (Figure 22), 10-30% glycerol greatly stabilized Cα (Figure 22 A) and Cγ (Figure 22 B) stored in -70°C as determined by kinase assay. Even after five-month storage in 10-30% glycerol at -70 °C, 85% original kinase activity of both purified C-subunits were remained (not shown data). But, the kinase activity dropped rapidly if restored at 4 °C after thawing from -70°C (Figure 23). In addition, the freeze-thaw cycles destabilized the kinase activity for both enzymes (Figure 24).

From these stability studies, it was noted that Cγ was relatively unstable when compared to Cα, which may be considered as another unique feature for Cγ-subunit. But, it was noted that Cγ kinase activity was stable as Cα during 2-hour incubation at 30 C° for kinase assay in the presence of 2% BSA (not shown data). All results in this study indicated that purified C-subunit preparation were stable when stored in -70 °C with glycerol immediately after purification. Once thawed, the enzyme preparation could not be refrozen.

8. Expression and purification of recombinant RIα-subunits from bacteria. Regulatory subunit (R) of cAMP-dependent kinase (PKA) is the only protein which inhibits C-subunit kinase activity by forming holoenzyme. R-subunit can be divided into two types, RI and RII. Each of them can subdivided into two isoforms, suggesting the presence of four forms of R-subunits in the cell, including RIα, RIβ, RIIα and RIIβ. Previous data demonstrated that Cγ differed from Cα in the interaction with two types of R-subunit in the permanently Cγ/Cα transfected Y1 Kin8 mutant cell (Beebe et al., 1997). For example, only Cγ transfectants (Cγ/Kin8) could restore Cγ-RII holoenzyme (type II), not free Cγ-subunit was present in the Cγ clone, and higher cAMP

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Figure 19. Comparison of Cγ and Cα-Subunits in Crude Lysate on Stability Stored at 4 °C

Cγ-subunit in the lysate was prepared from the monolayler of Sf9 cells infected by recombinant baculovirus (isolate:9) for three days as described in Methods. Cα-subunit in the lysate was prepared from recombinant Cα expressing bacteria induced by 0.4 mM IPTG at 37 °C for four hours as described in Methods. Both C-subunits in crude cell lysate were stored in 4 °C. The kinase activities in these lysates were measured at various days after storage by standard kinase assay using 85 μM histone in the presence of 1 μM PKI_{5-24} for Cγ activity assay and 65 μM Kemptide as substrates in the absence of PKI_{5-24} for Cα activity assay.

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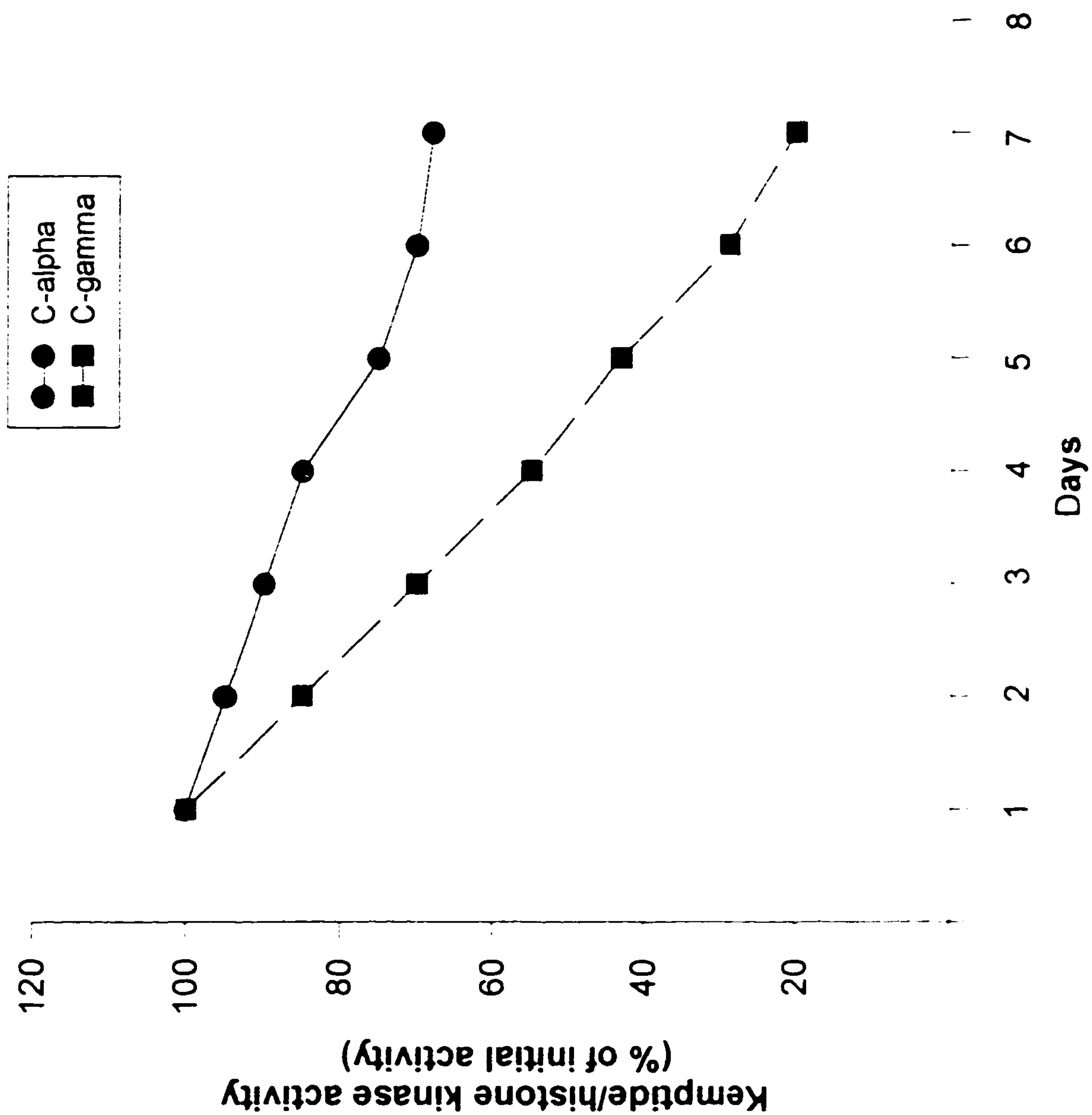

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Figure 20. The Effects of Purification on Cγ-Subunit Kinase Activity

The Cγ in crude lysate was prepared from the monolayer of Sf9 cells infected by recombinant Cγ-baculovirus (isolate:9) for three days as described in Methods. The partially purified Cγ was prepared from same crude lysate by one-step (0.5 NaCl) elution on CM-Sepharose chromatography as described in Methods. Both Cγ-preparations were compared on PKI-insensitive histone (85 μM) kinase activity by standard kinase assay in the presence of 1 μM PKI_{5-24} at various days after storage at 4 °C.

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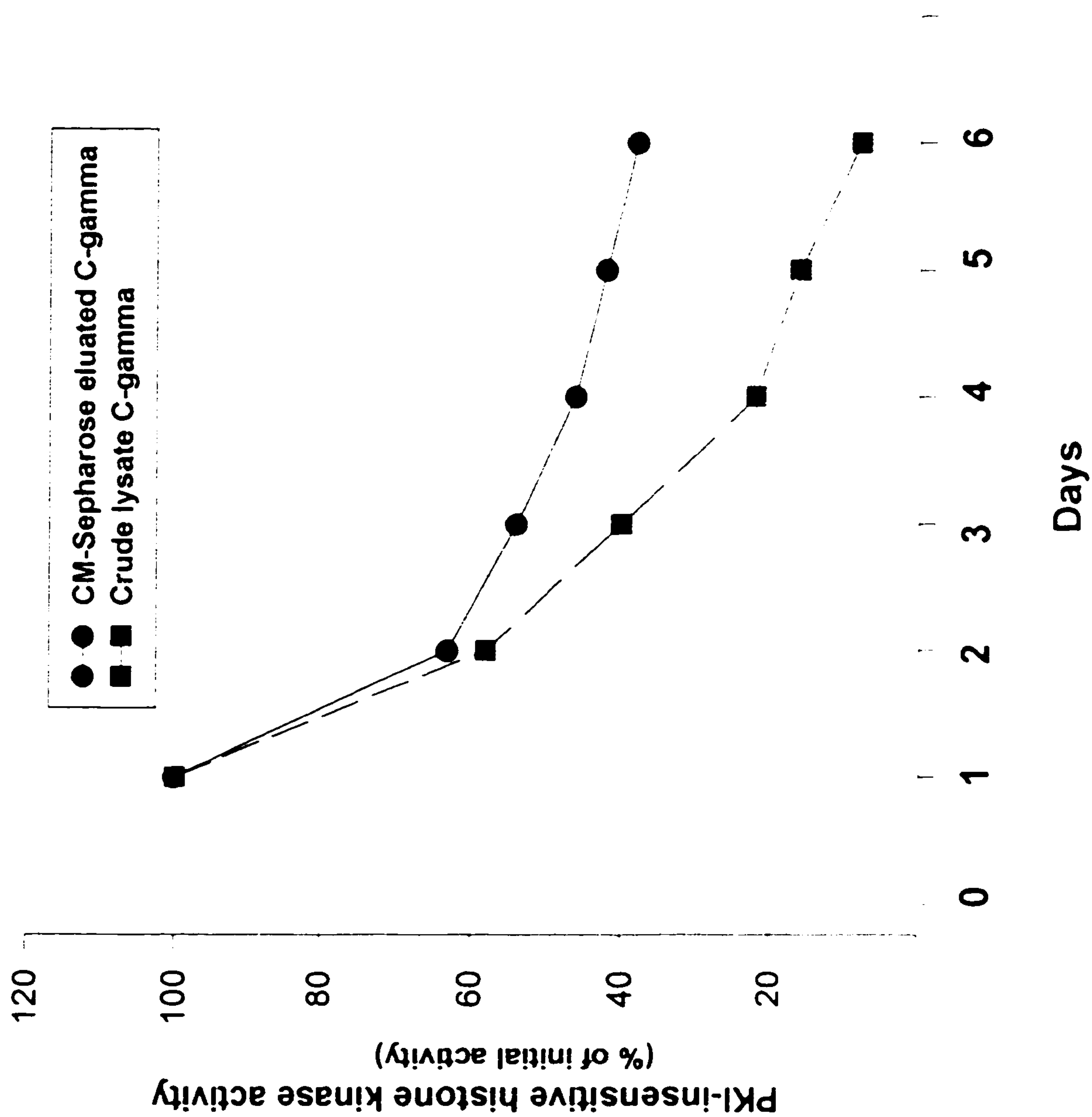

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Figure 21. Comparison of Purified Cγ- and Cα-Subunits on Heat-Stability

Homogeneous Cγ and Cα-subunits prepared as described in Methods were pre-incubated at 37 °C for various times in the presence of 1 mg/ml BSA and 15 % glycerol. The heat-treated C-subunits were assayed for remaining kinase in the presence of 1 μM PKI_{5-24} for Cγ and in the absence of PKI_{5-24} for Cα with 85 μM histone or 65 μM Kemptide as substrate.

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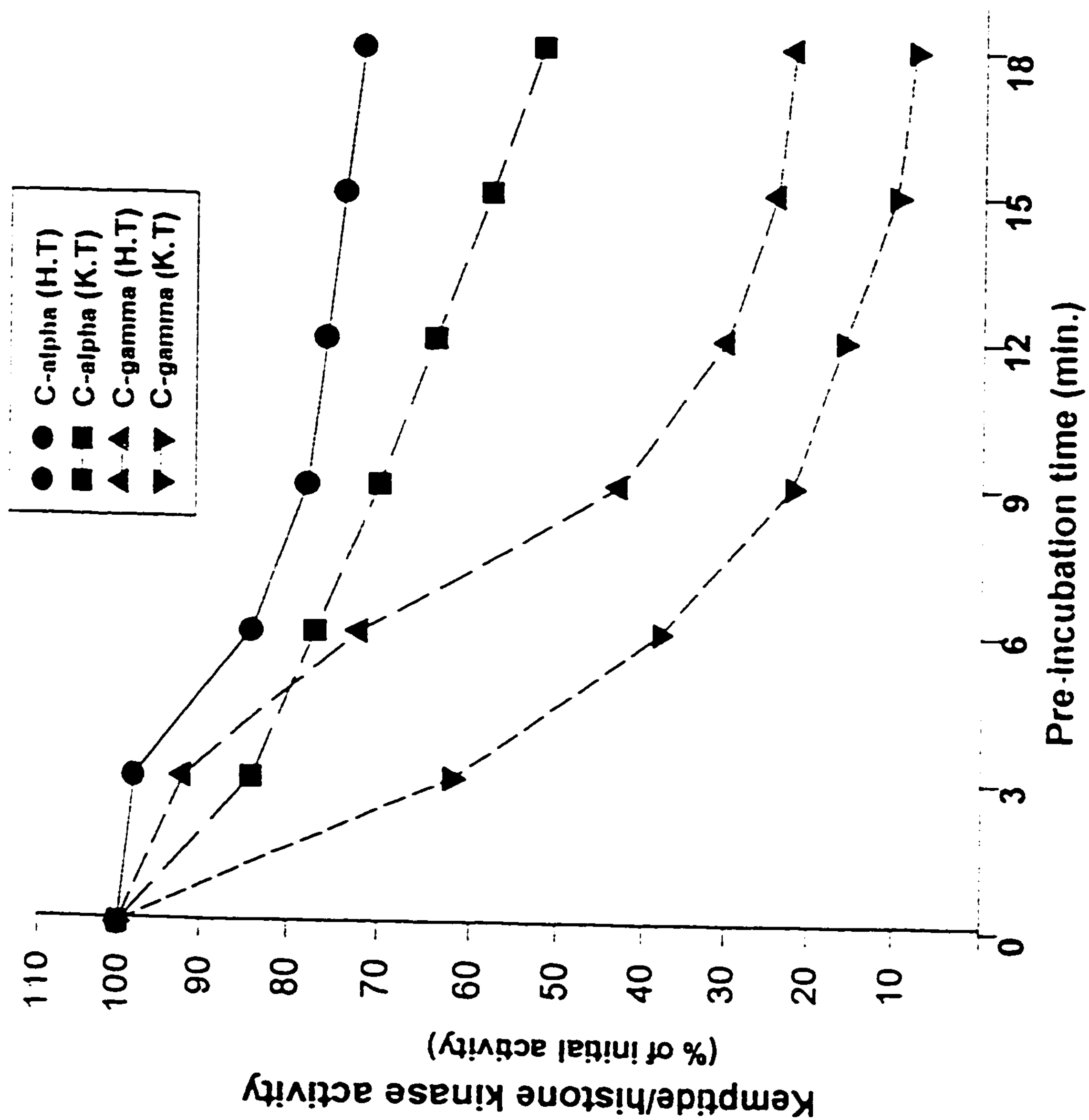

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Figure 22. Stabilization of C-Subunit Kinase Activity by Glycerol

Homogeneous C-subunit preparation as described in Methods were supplemented with 15% glycerol, 1 mg/ml DTT, 1 mg/ml benzemedian, 10 μg/ml PMSF, and aliquoted. The aliquots were stored at -70 °C. After various days of storage, aliquots were thaw at 4 °C and assayed for PKI-sensitive kinase activity for Cα (panel A) in the absence of PKI_{5-24} and PKI-insensitive kinase activity for Cγ (panel B) in the presence of 1 μM PKI_{5-24} with 65 μM Kemptide as substrates.

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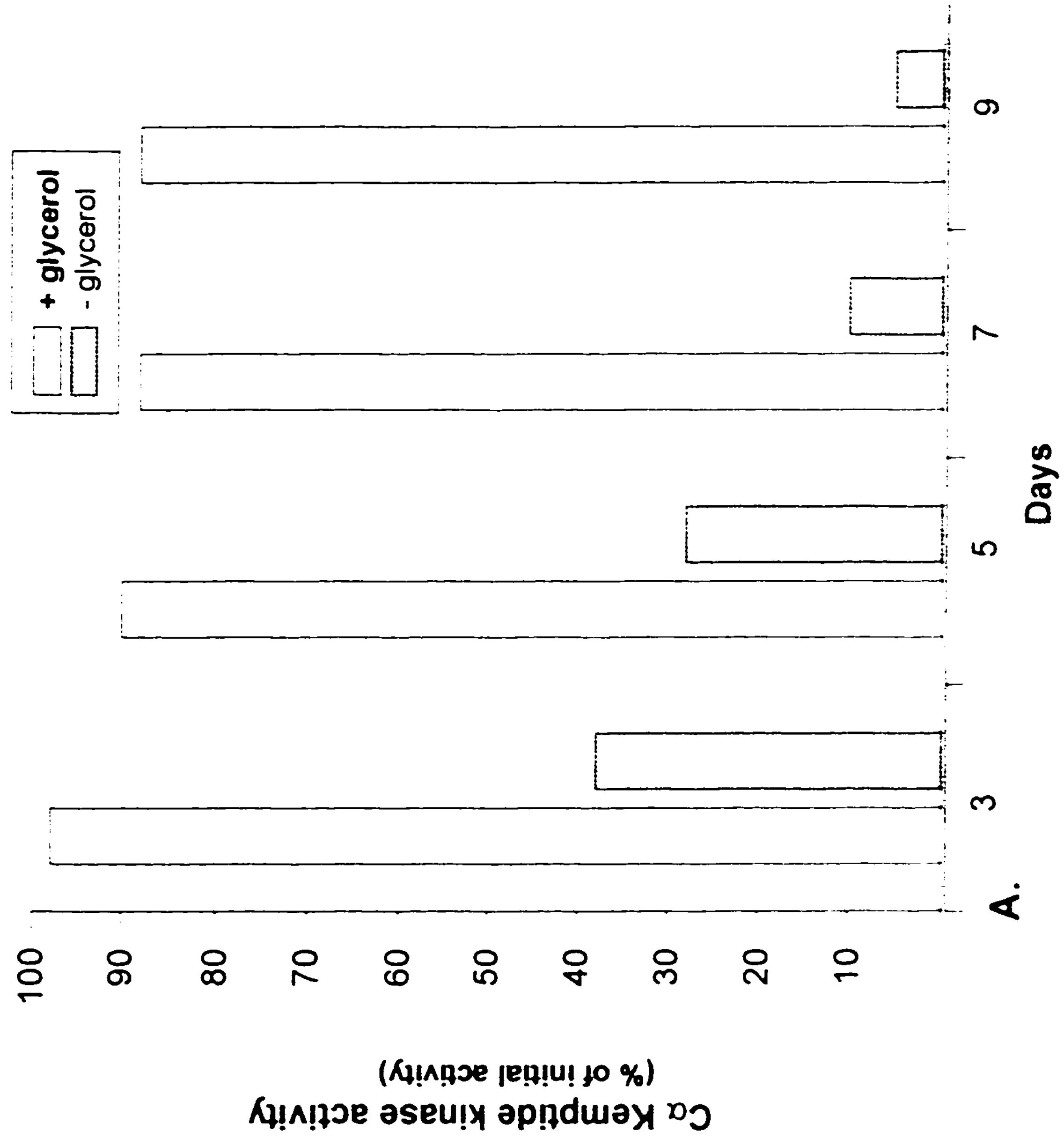

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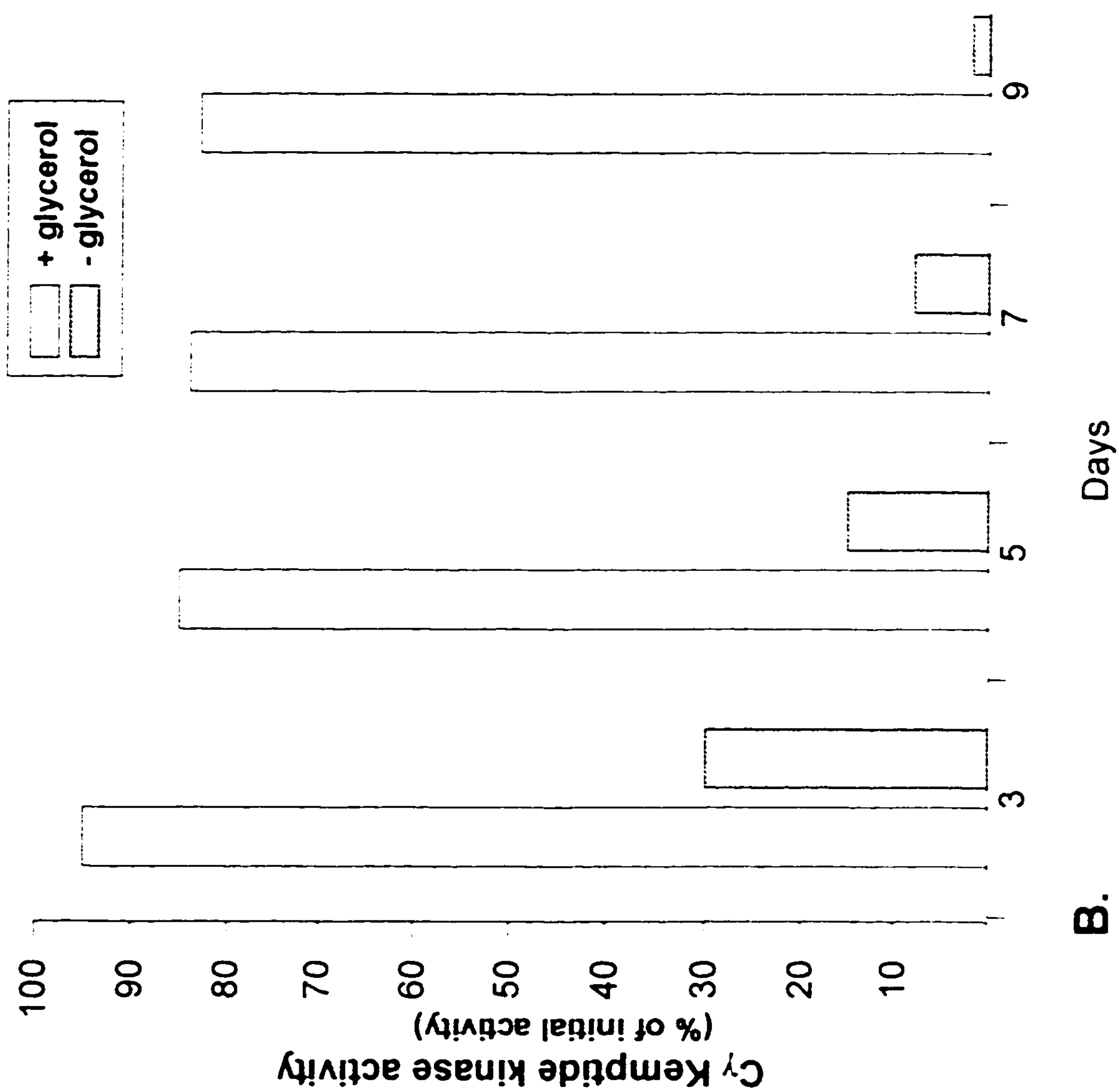

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Figure 23. Stability of C-Subunit Activity Stored at 4° C after Thaw from -70 °C

Homogeneous C-subunits were purified as described in Methods, aliquoted and stored in -70 °C. The aliquots were thaw and stored at 4 °C. The kinase activity of C-subunits 7in the thawed aliquots were assayed after various days of storage at 4 °C in the presence of 1 μM PKI_{5-24} with histone (85 μM) as substrate for Cγ and in the absence of PKI with Kemptide (65μM) for Cα.

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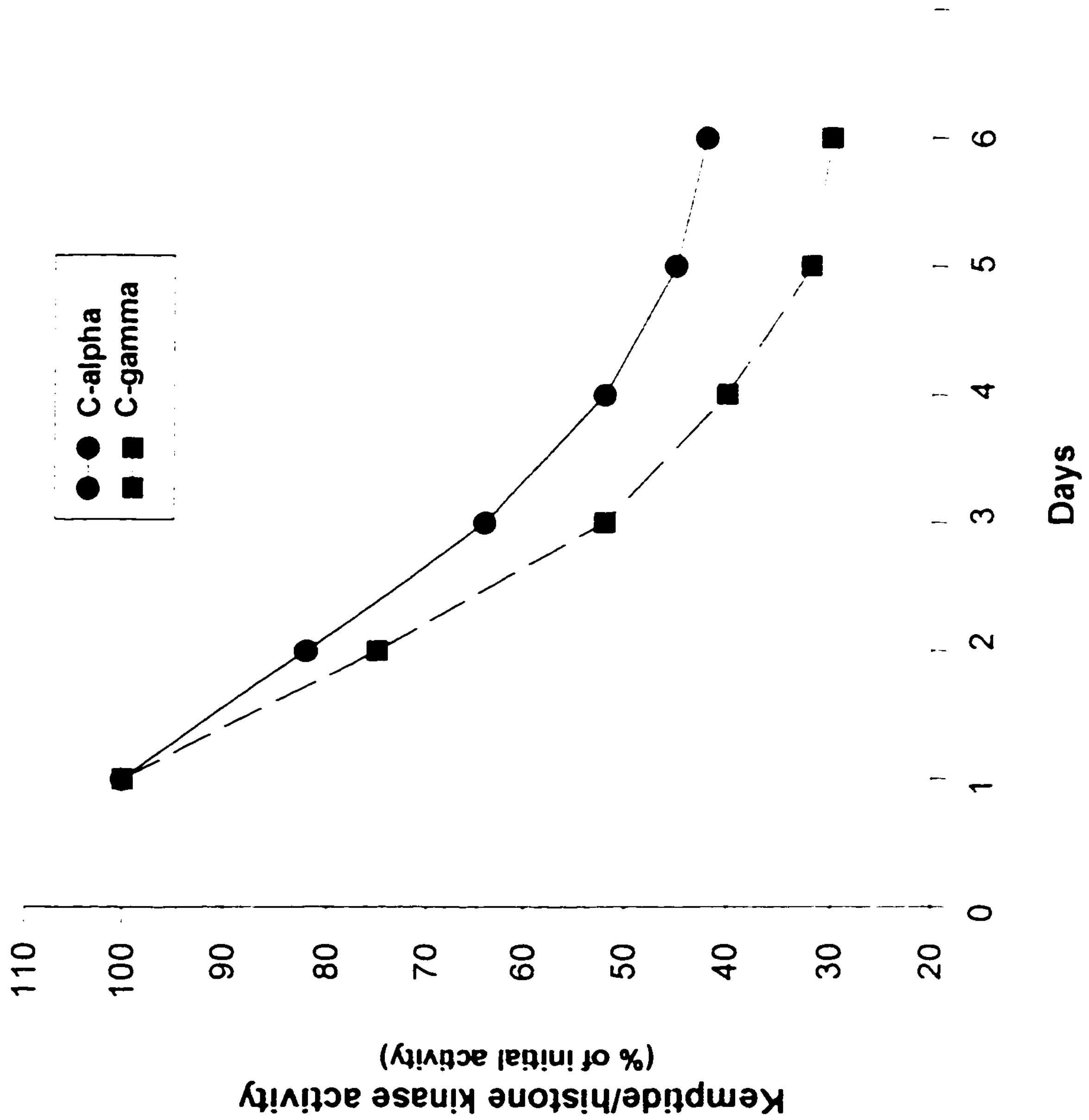

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Figure 24. The Effects of Frozen-Thaw Cycle on the Stability C-Subunits Kinase Activity
Homogeneous C-subunits were prepared, aliquoted and stored in -70 °C as described in Methods. The aliquotes were thawed at 4 °C and refrozen at -70 °C. The C-subunits after various number of freeze-thaw cycles were assayed for kinase activity in the presence of 1 μM PKI_{5-24} with histone (85 μM) as substrate for Cγ and in the absence of PKI with Kemptide (65μM) for Cα.

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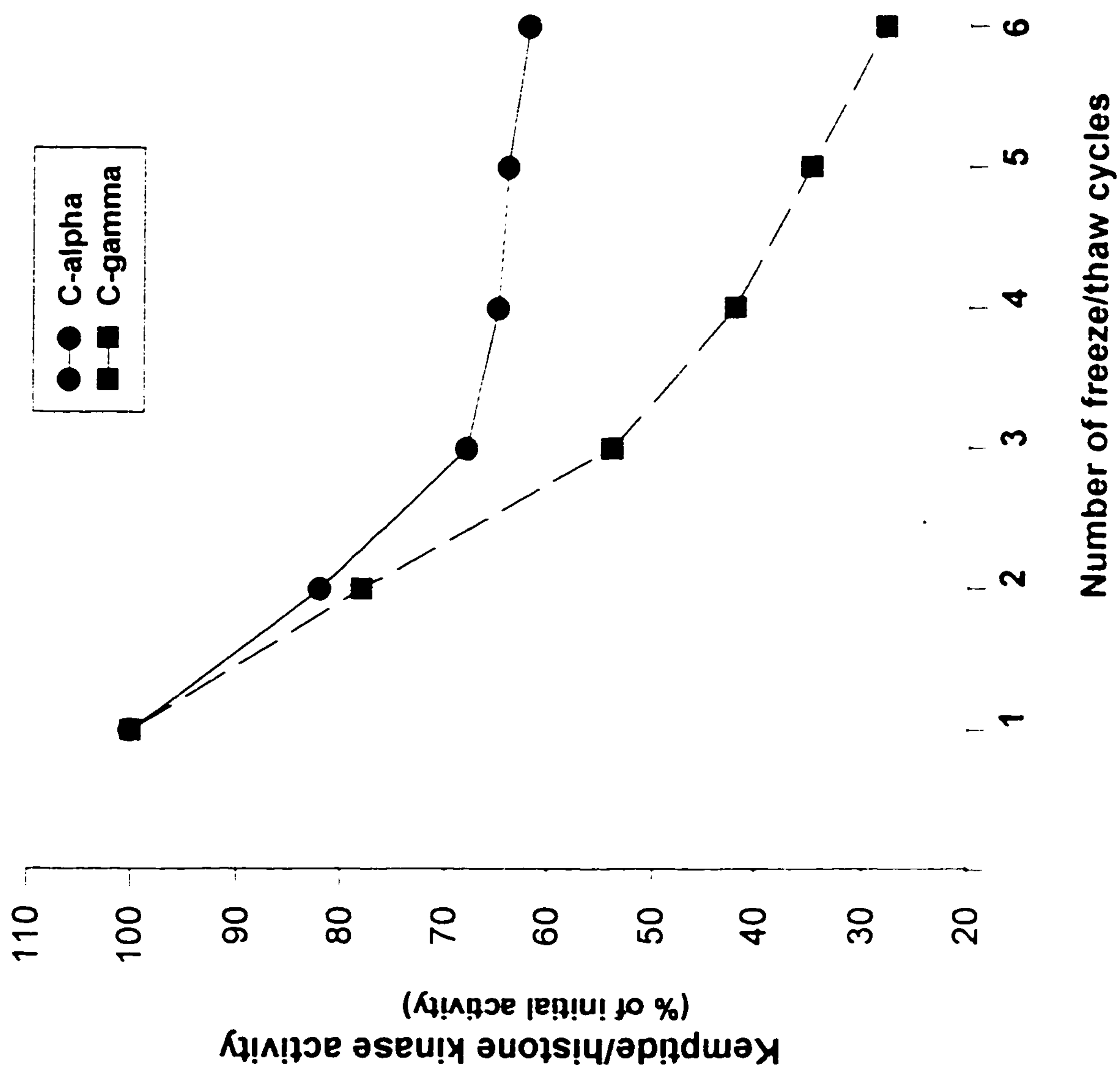

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concentration was required to dissociate Cγ-RI holoenzyme (type I) than Cα-RI holoenzyme in vitro. In order to analyze specific interaction between C-subunit and R-subunit more accurately, the inhibition of phosphotransferase activity of pure C-subunit isoforms by pure R-subunit isoforms *in vitro* was designed. The distinct affinity or interaction between the C-subunit and R-subunit was evaluated based on the IC50 values (the R-subunit concentration required to inhibit 50% of total C-subunit kinase activity). To this end, the two isoforms of R-subunit, recombinant RIα and his_{10}-RIIα were expressed in bacteria and purified into homogeneity

Recombinant RIα-expressing bacteria were grown, induced and lysed as described in Methods cAMP-bound RIα expressed in bacteria was purified to homogeneity, as judged by Coomassie blue stained-SDS-PAGE (Figure 25), by three steps (Table 8) as described in Methods The precipitation of RIα by $(NH_4)_2SO_4$ greatly improved the purity of RIα, but this step caused the loss of 40% total RIα-subunit. DEAE-Sepharose and Sephedex-Gel-filtration chromatography resulted in five-fold purification. The final recovery of purified recombinant bacterial cAMP-bound RIα was 18%. The pooled cAMP-bound RIα fraction from elution fractions of DEAE-Sepharose was denatured by adding solid urea to a concentration of 8 M After gel-filtration to remove cAMP and instant dialysis to remove urea, only 5.4% of total cAMP-bound RIα activity [^{3}H-cAMP-binding] was recovered as cAMP-free RIα, which suggested that some portion of denatured cAMP-bound RIα might be not renatured to native conformation, which resulted in lost normal cAMP-binding activity. This problem caused the IC50 values of cAMP-free RIα-inhibition for both C-subunits (shown later) to be relatively high The expression level of RIα-subunits in bacteria was only 5% of his_{10}-RIIα expression in bacteria.

9. Expression and purification of recombinant his_{10}-RIIα-subunit from bacteria Recombinant bacteria harboring His_{10}-RIIα fusion protein expression vector was grown, induced and lysed as

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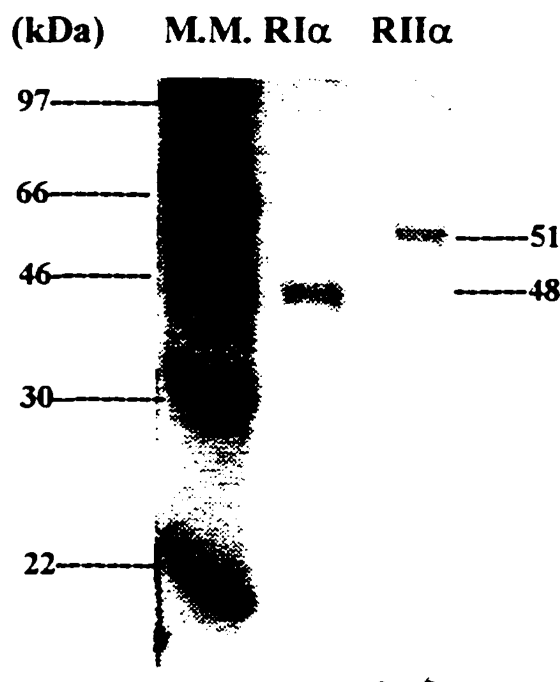

Figure 25. Analysis of RIα and His10-RIIα by Coomassie Blue-stained SDS-PAGE Bacteria expressing Recombinant RIα and his10-RIIα-subunit were grown and induced as described in Methods. Harvested recombinant R-subunit expressing bacteria were lysed by nitrogen cavitation. Recombinant RIα was purified by 65% ammonia sulfate precipitation and two sequential chromatography on DEAE-Sepharose and Sephedex G-50 as described in Methods. His10-RIIα was purified by twice of Ni^{2+}-chelated Pro-bond affinity column eluted by gradient imidizole (60-1000 mM) as described in Methods. The 5 μg proteins of each RI α and RII α preparation were loaded for 9% SDS-PAGE and stained by Coomassie blue staining. Molecular weight marker (M.M.) is the same as described in Figure 17.

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described in Methods. Being a his-tag fusion protein, his_{10}-RIIα-subunits could be purified to homogeneity by Ni^{2+}-chealated affinity column (Table 9). After the first-round of affinity column chromatography, two protein bands were revealed by silver stained-SDS-PAGE (not shown data). One protein had 62 kDa as apparent molecular weight, the other one appeared to be 51 kDa, which corresponded to reported apparent molecular weight of His_{10}-RIIα fusion protein (Baude et al., 1994). After second-round affinity column chromatography, only the 51 kDa band was revealed as judged by Coomassie blue stained SDS-PAGE (Figure 25) and silver staining (not shown data). The recovery of his_{10}-RIIα was near 50%. The cAMP binding ability of his_{10}-RIIα fusion protein was stable for several weeks at 4 °C.

Table 8 Purification of Recombinant RIα-Subunit Expressed in Bacteria*

Steps	Protein (mg/ml)	Specific Activity** (nmol[^{3}H]cAMP bound/mg)	Total Activity (nmol[^{3}H]cAMP bound)	Purification (fold)	Yield (%)
Lysate	4.53	1.63	147	1	100
$(NH_4)_2SO_4$	3.20	43.7	86	27	59
DEAE-Sepharose	0.61	169	58	104	40
1) Sephadex-G50	0.21	845	27	518	18
2)Sephadex-G25 (After 8 M urea treatment)	0.12	236	8	145	5.4

*The lysate was prepared from 1000 ml bacterial broth. ** Activity of R-subunit was determined by [^{3}H] cAMP bound binding assay (Doskarand & Ogreid, 1988)

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Table 9 Purification of Recombinant His_{10}-RIIα-Subunits Expressed in Bacteria

	Protein (mg/ml)	Specific Activity* (nmol[^{3}H]cAMP bound/mg)	Total Activity (nmol[^{3}H]cAMP bound)	Purification (fold)	Yield (%)
Lysate	4.57	46	6286	1	100
1° Pro-Bond	0.15	670	4768	15	75.6
2° Pro-Bond	0.059	1642	3099	36	49.3

* Activity of R-subunit was derived from the [^{3}H] cAMP binding assay (Comb & Corbin, 1986)

10.*Expression and purification of recombinant MBP-PKIα and MBP-PKIβ from bacteria*: PKI is small (80 amino acid residue), heat-stable, and potent PKA-specific inhibitor. PKI has two major isoforms, PKIα and PKIβ. The PKI_{5-24} and PKI_{14-24} are two PKI peptides which are synthesized based on PKI inhibitory sequence and exhibit nearly full inhibitory activity observed with the full length of PKI proteins. PKI may be important regulator for the function of PKA in the cell, but its functions have only recently been elucidated. PKI not only inhibits C-subunit kinase activity in the cell, but also down-regulate cAMP-mediates stimulation of gene expression by exporting C-subunit from nucleus, though the exact physiological function of PKI is not clear. The striking difference between Cγ-subunit and the other two isoforms of C-subunit is that Cγ kinase activity is insensitive to the inhibition by PKI_{5-24} and PKI_{14-24} (Beebe et al., 1992). But, little was known about the sensitivity of Cγ-subunit kinase activity to full length PKI protein. In addition, Cα and Cβ1 differ in the sensitivity to different PKI isoforms (Gamm et al.,1996). It is possible that Cγ has different sensitivity to full PKI protein from PKI peptide. To explore this possibility,

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homogeneous Cγ and Cα-subunits were compared on the sensitivity for PKIα and PKIβ. In this experiment, MBP-PKIα and MBP-PKIβ were utilized as two isoforms of PKI proteins.

MBP-PKI fusion protein expression vectors that express MBP-PKIα and MBP-PKIβ were transformed into E. coli. BE21 DE-3 pLySs bacteria. Recombinant bacteria containing respective fusion expression vectors were grown, induced and lyzed as described in Methods. Maltose binding fusion protein, MBP-PKIα and MBP-PKIβ were purified to at least 90% homogeneity by Amalose-affinity chromatography eluted with 0.1 mM maltose and judged by Coomassie blue stained-SDS-PAGE (Figure 26) as described in Methods. The two isoforms of PKI shared 59% amino acid identity (Baude et al., 1994), but both PKI fusion proteins had very close apparent molecular weight on the 9% SDS-PAGE. The MBP-PKIα appeared as 43.5 kDa protein band, which was slightly smaller than MBP-PKIβ (45kDa protein band). It was reported that MBP did not affect the PKI inhibition of C-subunit kinase activity (Gamm et al., 1995), full length purified PKI fusion proteins were used as potential inhibitor of C-subunit activity.

After the above series of experiments, the two recombinant C-subunit isozymes (Cγ and Cα) and four recombinant pseudosubstrate proteins (RIα, RIIα, PKIα and PKIβ) were successfully expressed and purified to homogeneity in sufficient amounts for *in vitro* kinetic analysis for substrate and pseudosubstrate specificity of both C-subunit isozymes.

B. Kinetic Analysis of C-Subunits for Substrate Specificity

As a prototype for all phosphortransferases, C-subunit of PKA is considered to be an important enzyme for cell function. Recent research derived from Cα knockout mice has provided evidence to show that C-subunit is vital to life (Qi et al.,1996). The C-subunits regulate cell physiological functions though phosphorylation of a wide variety of protein substrates. Some substrates are regulatory enzymes which are important in the regulation of cellular metabolism (Krebs et al.,

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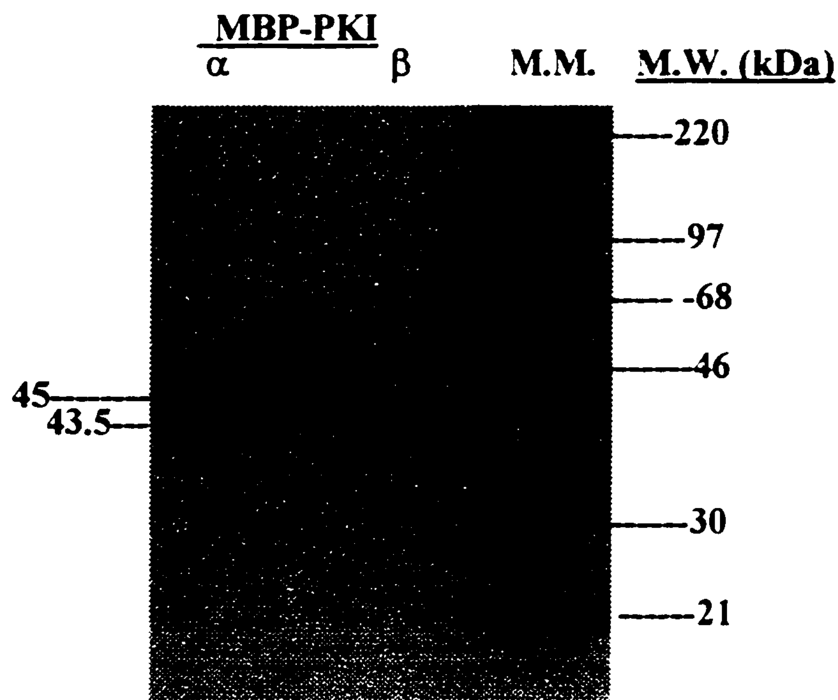

Figure 26 Analysis of Purified MBP-PKIα and MBP-PKI β by Coomassie Blue-Stained SDS-PAGE
Bacteria expressing recombinant MBP-PKIα and MBP-PKIβ fusion proteins were grown in LB with 0.25 mM sucrose at 37 °C to 0.6 as O D value (600nm), then induced by 0.4 mM IPTG for another 2 hours at same temperature. Harvested cells were lysed as described in Methods. MBP-PKI fusion proteins were individually purified by Amylose-affinity chromatography eluted by 0.1 mM maltose as described in Methods. 15 g of each purified fusion PKI proteins was loaded for 9% SDS-PAGE, and gel was stained by Coomassie blue. Molecular weight marker (M.M.) is the same as described in Figure 17

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1979, Beebe & Corbin, 1986, Levitan et al., 1994). Other substrates are trans-activating transcription factors that regulate cAMP-responsive gene expression (Montminy et al., 1990). Three different forms of C-subunits have been identified, but little is known about their substrate specificities. Generally, the various C-subunit isozymes were considered to have common substrate specificity. Since recombinant Cγ-subunit expressed in Y1/Kin8 cells was shown to phosphorylate histone better than Kemptide (Beebe et al., 1992), which has been considered as the best substrate for A-kinase (Kemp et al., 1977), and it suggested that substrate specificity for different C-subunit isoforms was not same (Uhler et al., 1986; Showers et al., 1986). Little is known about specific functions of Cγ. However, the unique substrate specificity predicts that Cγ has the potential to play a different role from other C-subunit isoforms in the regulation of cell function, and this is supported by the different phenotypes expressed in permanently Cγ/Cα transfected Y1/Kin8 mutants (Table 1). The different phenotypes could be due to the existence of substrate and/or pseudosubstrate specificity between Cγ- and Cα-subunits. To extend substrate specificity studies and to define unique catalytic features for Cγ, the kinetic comparisons of Cγ and Cα for substrate specificity becomes necessary. Availability of homogeneous recombinant C-subunit isozymes makes it possible to compare the kinetic parameters for both C-subunits. Identification of C-subunit isozyme-specificity for substrate phosphorylation and recognition may provide primary evidence to demonstrate the contribution of the C-subunit isozymes to the diversified function of PKA in the cAMP signal transduction pathway, or at least provide some information to explain how permanent Cγ- and Cα-transfection can induce distinct Y1/Kin8 cell phenotypes.

To make quantitative and qualitative comparisons of Cγ and Cα-subunits for substrate specificity, two experimental strategies were exploited. First the kinetic constants (Km and Vmax) were determined for the phosphorylation of histone and synthetic peptides as well as for utilization of Mg^{2+}-ATP. Second, the phosphorylation of native cellular proteins in cytosol and nuclear

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extracts of Kin8 clone by exogenous homogeneous C-subunits and by recombinant C-subunit expressed in Cγ and Cα clones were identified by autoradiography

1.*Kinetic parameters of C-subunits for the phosphorylation of Kemptide, histone and utilization of ATP-Mg²⁺*: Initial studies with partially purified Cγ and Cα expressed in Kin8 cells indicated that Cγ- and Cα-subunits had different substrate specificity based on their distinct KT/HT phosphorylation ratio (Beebe et al., 1992). Further studies with recombinant C-subunit isozymes expressed in different expression systems confirmed previous results from mammalian cells (Table 4). However, all these *in vitro* comparisons were based on partially purified enzyme preparation, which only provided a brief evaluation of the substrate specificity for both C-subunits. Since both Cγ and Cα-subunits have been purified to homogeneity (Figure 18), substrate specificity of C-subunit isozymes were further compared by kinetic analysis. Table 10 shows the results generated from kinetic comparison of both C-subunits on Kemptide and histone phosphorylation, and utilization of ATP-Mg^{2+} as phosphate donor. These kinetic parameters in Table 10 were derived from the Double-reciprocal plot of data as shown in Figure 27.

Compared with Cα, the Km values for Cγ were similar or lower for the substrates tested, the Km value with histone as substrate was nearly 7-fold lower for Cγ than for Cα. The Km value with Kemptide as substrate for Cγ were 4-fold lower than that for Cα. Compared with Cα, the Km values for ATP-Mg^{2+} was 2.5-fold lower with Cγ, regardless of the phospho-acceptor substrate used. Because crystal structure showed that the ATP-Mg^{2+} and substrate bind to different regions of C-subunit, there was no detectable effects of substrate on the Km values for both C-subunits.

A comparison of the Vmax values indicated that for the tested substrates, Cγ had a lower Vmax than Cα, regardless of the substrate, but the differences between the two isozymes were always smaller when histone was a substrate. Compared with Cα, the Vmax values for Kemptide and

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Figure 27. A Linearweave-Burk Plot of kinetic parameters for C-subunit substrate phosphorylation

Linear rates of substrate phosphorylation by homogeneous C-subunits were determined by standard kinase assay as described in Methods. Recipreoal initial substrate phosphorylation rates (1/V) were plotted against reciprocal substrates concentration (1/[S]). Panel A showed the douple-reciprocal plot for ATP utilization with Kemptide (A-1) or histone (A-2) as phosphoacceptors. The Panel B showed the douple-reciprocal plot for Kemptide (B-1) and histone (B-2) phosphorylation. The protein concentrations of Cγ and Cα for kinetic analysis were 0.83 and 0.23 μg/ml, respectively.

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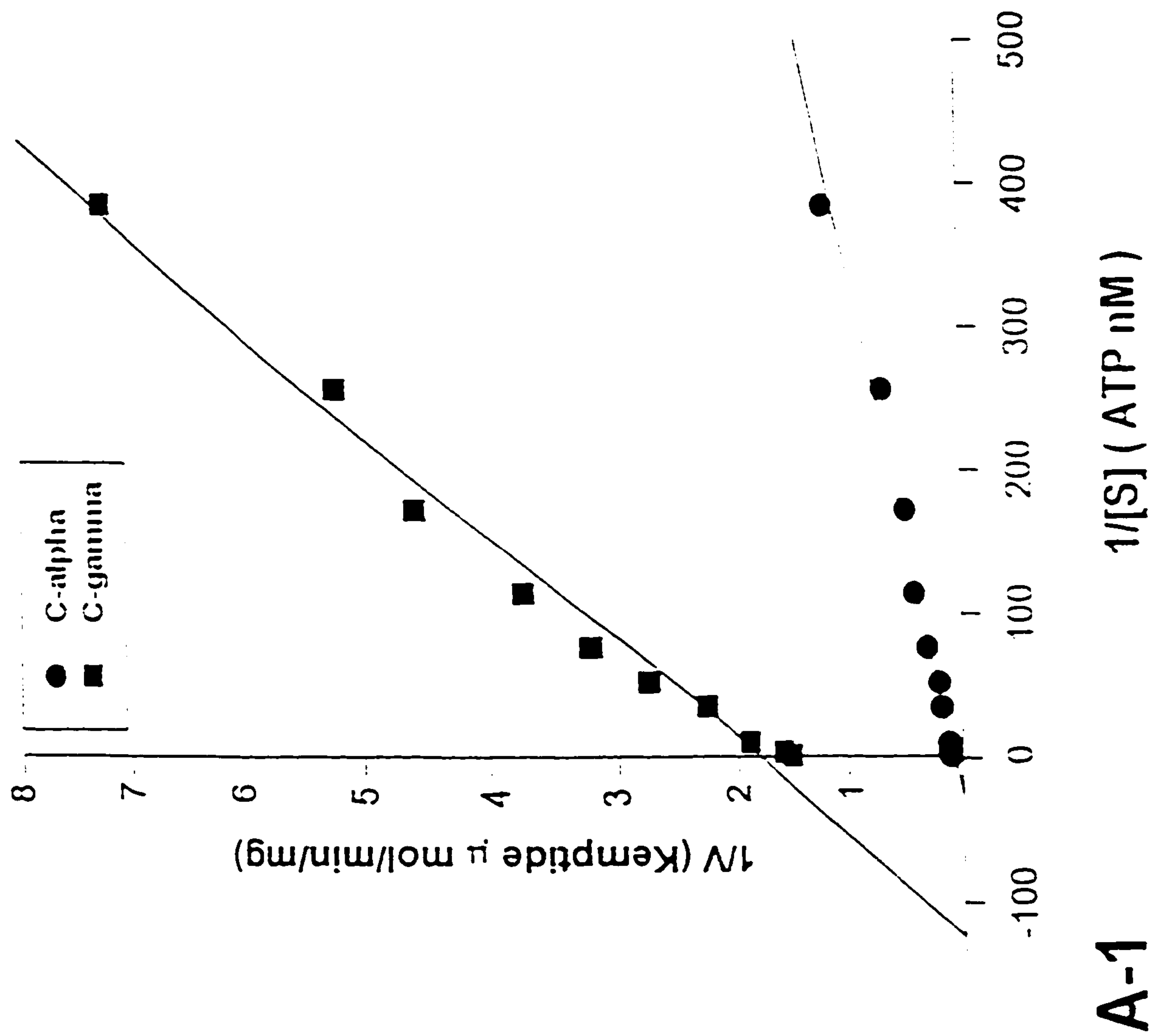

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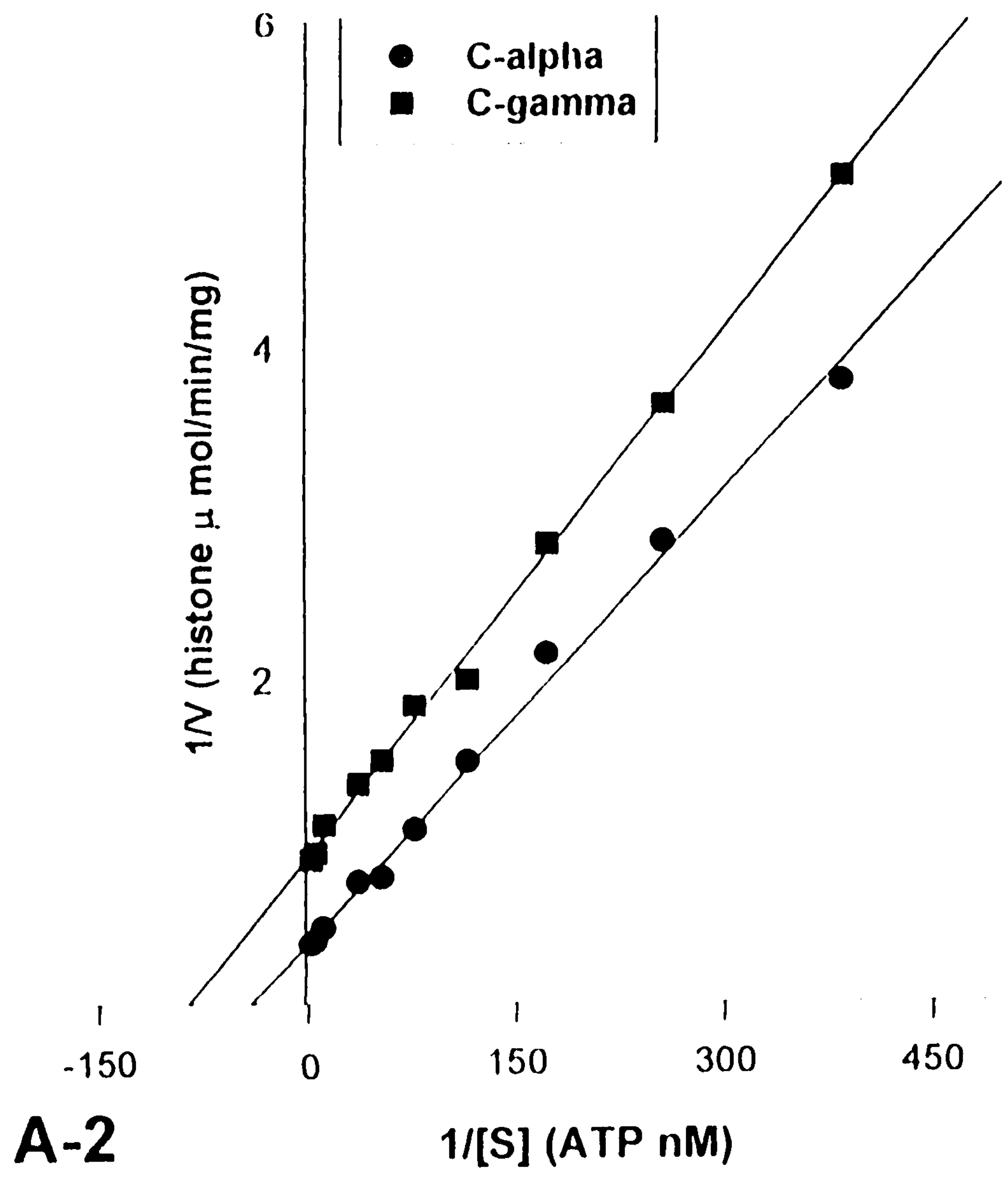

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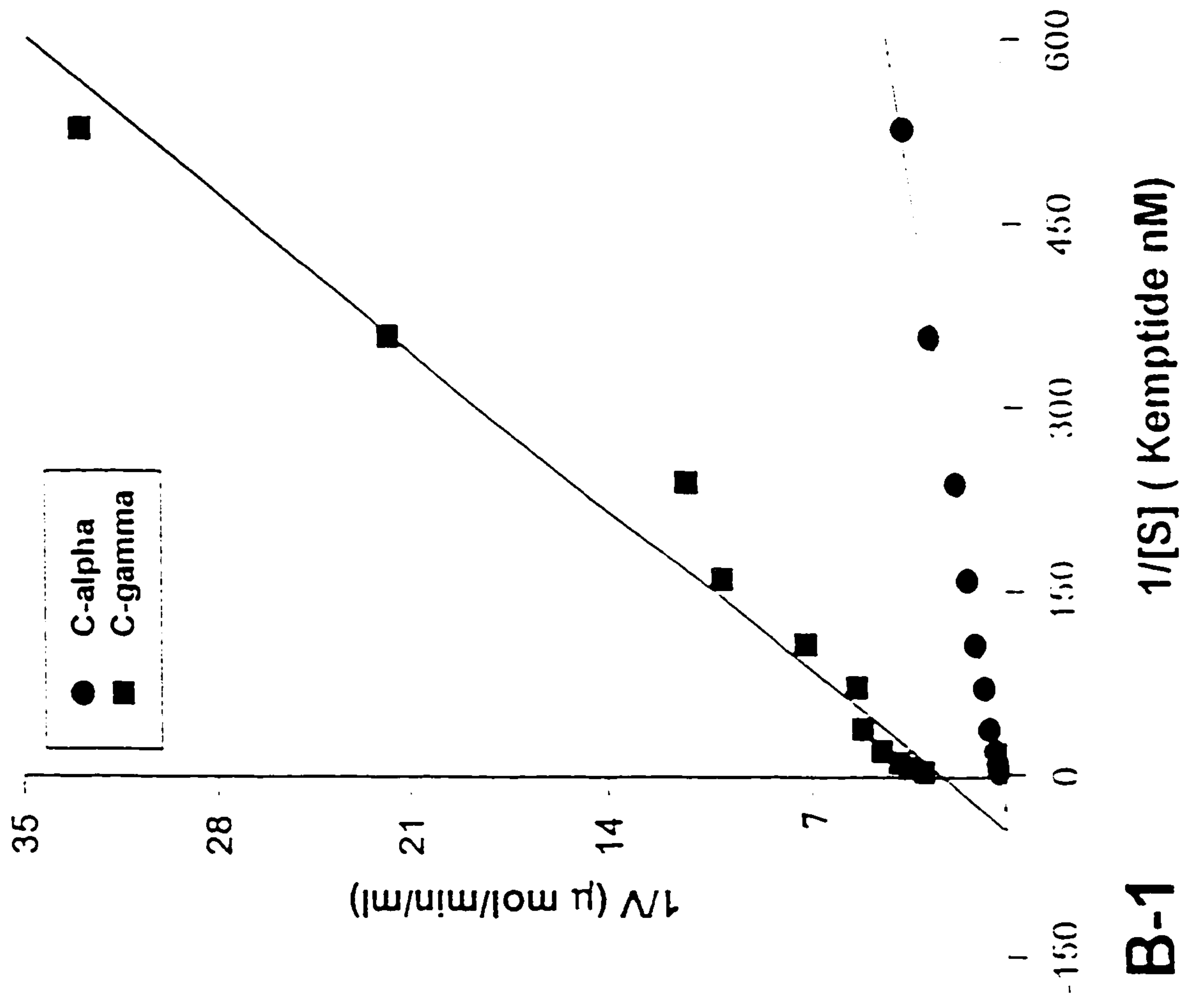

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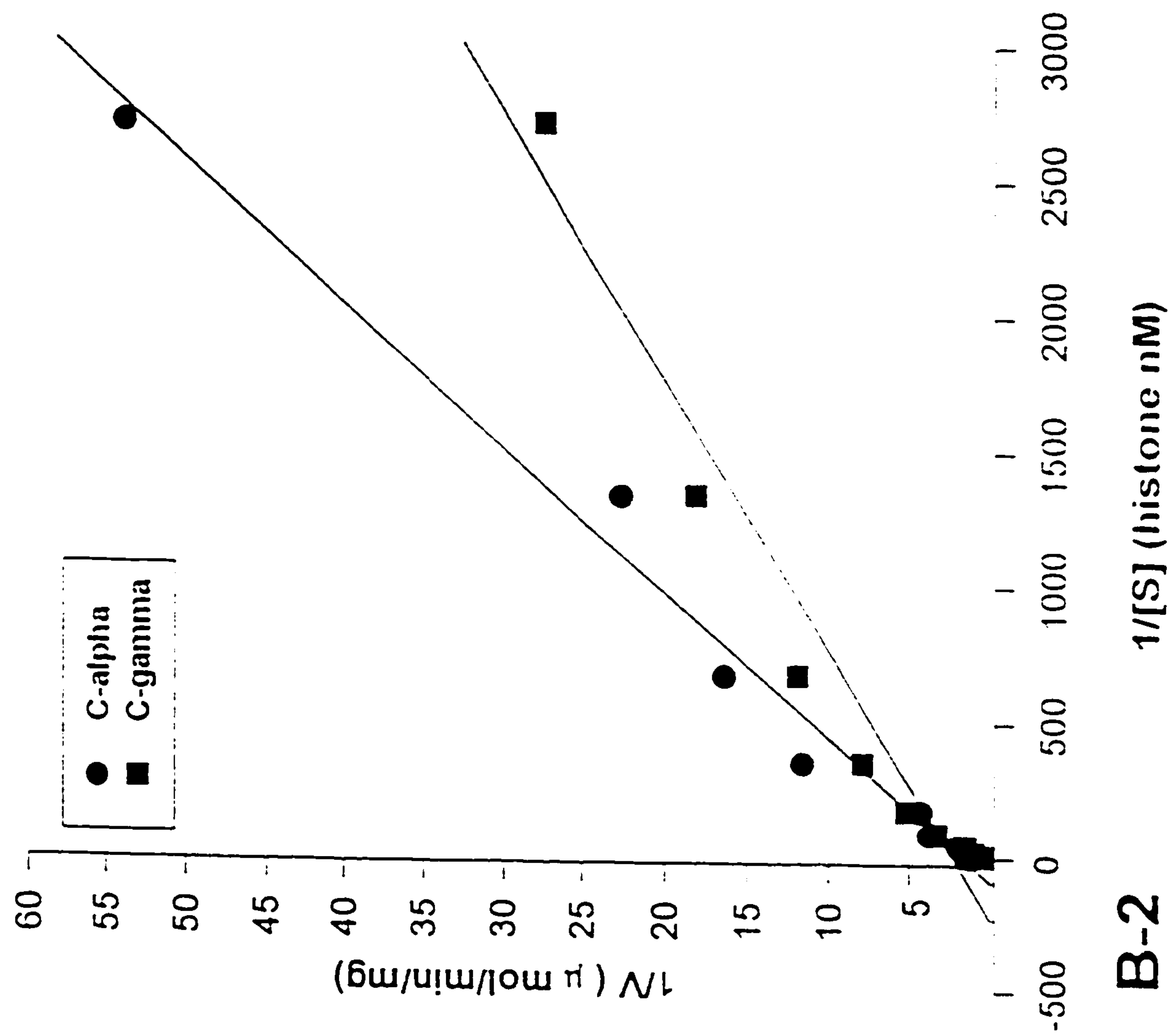

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Table 10 Comparison of Kinetic Parameters of C-subunits for Kemptide and Histone Phosphorylation and Utilization of ATP-Mg^{2+}

C-subunit	Parameter	Kemptide(KT)	Histone(HT)	ATP-Mg^{2+}	
				KT	HT
Cα	Km (μM)	54.50	131.6	25.68	27.47
	Vmax (μM/min/mg)	6.96	3.75	4.91	2.87
	Vmax/Km	0.12	0.03	0.19	0.10
Cγ	Km (μM)	12.91	19.22	12.12	9.99
	Vmax (μM/min/mg)	0.31	0.85	0.64	1.03
	Vmax/Km	0.02	0.06	0.05	0.12

The Km and Vmax were calculated by Double-reciprocal equation based on linear catalytic rate of each reaction.

histone were 22- and 4-fold lower, respectively, with Cγ. Likewise, the Vmax values with ATP as substrate were 8- and 2.7-fold lower with Kemptide and histone as phospho-acceptor substrates, respectively, for Cγ compared with Cα.

Using the Vmax/Km ratio, Cα utilized Kemptide substrate 7-times better than Cγ, but Cγ utilized histone 2-times better than Cα. Similarly, Cα used ATP nearly 4-times better than Cγ with Kemptide as the phospho-acceptor, but Cγ and Cα utilized ATP similarly with histone as the phospho-acceptor.

To extend these studies and obtain a better overview of substrate specificities for both C-subunit isozymes, seven more synthetic peptides were utilized as substrate for kinetic analysis. These seven synthetic peptides were chosen as substrates because they were commercially available, and had been unidentified as substrates for other kinases that had similar or different substrate phosphorylation sequence to Cα.

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2. *Kinetic parameters for other synthetic peptides:* This scanning experiment was designed to provide more direct and accurate evidence to demonstrate the existence of distinct substrate specificity between the Cγ-subunit and Cα-subunit by comparing their kinetic constants on the phosphorylation of following seven synthetic peptide substrates: PKG-1 substrate (Glass & Krebs, 1982); PKG-2 substrate (Glass & Kreb, 1979); PKC peptide substrate (House et al., 1987), SP6 peptide substrate (Pelech et al., 1986), CamKII peptide substrate (Songyang et al., 1994), p34cdc2/cyclin B peptide substrate (Songyang et al.,1994) and Phospho-CREB/GSK3 peptide substrate(Fiol et al., 1994). Compared with Kemptide, these seven synthetic peptides had different changes and/or shifts of amino acid residues around a pair of argnines (P^{-3} and P^{-2}) and phosphorylation target Ser (P^0), which have been identified as conserved or primary substrate phosphorylation site for PKA (Kemp et al., 1977; Zetterqvist et al., 1976) P represents the position (P) of amino acid residue against phosphorylation target ser/thr

Based on the feature of phosphorylation sequence, these seven peptides plus Kemptide could be divided into two categories: One (peptide 1-5) had the RRXSY as phosphorylation sequence just like A-kinase substrates; X represents any amino acid residue. Y represents the amino acid residue with hydrophobic group. The other (peptide 6-8) had the RRXX(X)SY as phosphorylation sequences. The kinetic constants and their ratios for Cγ and Cα are shown in the Table 11 The results from Table 11 can be summarized as following:

1) After the review of all tested peptides, both Cγ and Cα could phosphorylated group-I(peptide 1-5) with RRXSY as common phosphorylation sequence, which was similar to PKA kinase substrate. But, neither Cγ and Cα phosphorylated peptide 6-8 with RRXX(X)SY as common phosphorylation sequence, even when the concentrations of these peptides were increased to 1 mM.

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Table 11 Comparison of Cα and Cγ-subunits on Kinetic Parameters for Synthetic Peptides and Protein Phosphorylation

Peptides	Sequence		Km(μM)	Vmax	Vmax/Km
	P^{-3} P^{-2} P^{-1} P^{0} P^{+1} P^{+2}			(μ mol/min./mg)	
1. Kemptide	Leu-*Arg*-*Arg*-Ala-Ser-Leu-Gly				
		Cα	50.3	4.91	0.098
		Cγ	12.5	0.32	0.026
2. PKG-substrate-1	Arg-*Lys*-*Arg*-Ser-Arg-Ala-Glu				
		Cα	292.5	4.60	0.016
		Cγ	33.3	0.58	0.017
3. PKG-substrate-2	Arg-*Lys*-*Arg*-Ser-Arg-Lys-Glu				
		Cα	500.1	0.025	0.0001
		Cγ	33.3	0.16	0.005
4. S6-substrate	*Arg*-*Arg*-Leu-Ser-Ser-Leu-Arg-Ala				
		Cα	237.7	6.71	0.028
		Cγ	50.3	0.76	0.015
5. PKC peptide	Arg-Phe-Ala-*Arg*-*Lys*-Gly-Ser-Leu-Arg-Glu-Lys-Asn-Val				
		Cα	50.3	6.84	0.136
		Cγ	25.3	0.76	0.030
6. CamKII peptide	*Lys*-*Arg*-Gln-Gln-Ser-Phe-Asp-Leu-Phe				
		Cα	> 1000		not detectable
		Cγ	>1000		not detectable
7. p34cdc2/cyclin B peptide	Arg-*Arg*-*Arg*-Pro-Met-Ser-Pro-Lys-Lys-Lys-Ala				
		Cα	> 1000		not detectable
		Cγ	> 1000		not detectable
8. Phospho-CREB/GSK3	Lys-*Arg*-*Arg*-Glu-Ile-Leu-Ser-Arg-Arg-Pro-Ser(P)-Tyr-Arg				
		Cα	>1000		not detectable
		Cγ	> 1000		not detectable
9. Histone(III)		Cα	100.6	2.18	0.022
		Cγ	20.1	0.84	0.042

The Km and Vmax were calculated by Eadie-Hofstee plot based on linear phosphorylation rate phosphorylation.

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2) For the peptides (1-5) which could be phosphorylated by both C-subunits. Cγ always had 4-10 fold lower Km values and approximately 10 fold lower Vmax values than Cα for the same substrate.

3) Considering the Vmax/Km values of each peptide for both C-subunits. Cα phsphorylated peptide-1 and peptide-5 four-time better than Cγ; For peptide-2 and peptide-4. Cγ and Cα had same phosphorylation efficiency. In contrast, Cγ phosphorylated peptide-3 fifty-fold better than Cα; Based on the Vmax/Km values. histone still was best substrate for Cγ compared to these peptide substrates.

4) Comparing peptide-1 with peptide-3. Cα could phosphorylate peptide-1 nearly 1000-fold better than it could phosphorylate peptide-3. This indicated that Cα could not tolerate the substrate with two basic amino residue at P^{+1} and P^{+2} position. In contrast, Cγ phosphorylated peptide-1 only 5-fold better than phosphorylated peptide-3. suggesting Cγ could tolerate the substrate with two basic amino resides at P^{+1} and P^{+2} position.

This *in vitro* kinetic comparisons for the phosphorylation of synthetic peptides indicates a difference in the substrate specificity between Cγ and Cα. The results clearly demonstrate that Cγ had distinct substrate specificity from Cα. Since results from *in vitro* comparison on kinetic constants for substrate phosphorylation showed that Cγ had different substrate specificity from Cα, it is reasonable to predict that Cγ and Cα exhibited different substrate specificity in the cell. The following experiments were designed to explore this possibility.

3. *Protein phosphorylation in cytosol and nucleus by endogenous and exogenous recombinant C-subunit isozymes:* To demonstrate the existence of C-subunit isozyme-specificity for substrate phosphorylation of cellular proteins, two experiments were designed. One was to determine the phosphorylation of heat-treated cellular proteins from cytosol and nuclear extracts of Kin8 clone by exogenous homogeneous C-subunits. The other one was to examined the phosphorylation of

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native cellular proteins from same cytosol and nuclear extracts of kin8 clone by recombinant C-subunits permanently expressed in the Kin8 clone. The phosphorylated proteins were labeled with ^{32}P-ATP and shown by autoradiography as described in Methods. The results were shown in Fig 28. The results (Figure 28, panel A.) showed that 1) the cellular phosphoprotein profiles in cytosol (Lane 1, 3, 5, 8 and 10) were different from those in nuclear extract (Lane 2, 4, 6, 7 and 10) regardless of cell clones and sources of C-subunits. This result was predicted because of different populations of protein and kinases in cytosol and nucleus; 2) no particular isozyme-specific phosphorylated protein bands were shown in either cytosol (lane 1 & 3) or nuclear extracts (lane 2 and 4) when the heat-denatured cellular proteins were used as substrate for exogenous homogeneous C-subunits; 3) Very few phosphorylated protein bands (about 50-90 kDa region) were revealed in the cytosol and nuclear extract of the Kin8 clone in the presence 1 μM PKI, a specific inhibitor for Cα- and Cβ-subunits of PKA. This result suggests that not much protein was phosphorylated by endogenous non-PKA kinases in the cytosol (lane 5) and nuclear extract (lane 6) of Kin8 cells in this experiment condition; 4) endogenous phosphoprotein profiles of cellular proteins in nuclear extracts (lane 7) and cytosol (lane 8) of Cγ clone were notably different from respective ones (lane 9 and lane 10) in Cα clones. For example, two phosphorylated protein bands (about 60 and 68 kDa) were detected in the cytosol of Cγ clone (lane 8), but these bands were not detected in the Cα clone (lane 10). In contrast, two unique phosphorylated protein bands (about 97 kDa and 30 kDa) were found in the cytosol of Cα clone only (lane 10). In the nuclear extracts, more proteins (66 kDa to 100 kDa) were phosphorylated in Cγ clone (lane 7) than Cα clone (lane 9). One 35 kDa protein was phosphorylated in Cγ clone (lane 7), but not in the Cα clone (lane 9).

Because very few proteins were phosphorylated by non-PKA kinase (lane 5 and Lane 6), the distinctively phosphorylated proteins in nuclear extracts (lane 7, 9) and cytosol (lane 8, 10) of C-subunit expressing clones (Cγ and Cα) might be resulted from the PKA-specific- phosphorylation

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Figure 28. Cγ and Cα Differently Phosphorylate Proteins in the Cytosol and Nucleus of Kin8 Clones

Native or heat-denatured cytosol and nuclear extracts were prepared from Kin8, Cα and Cγ clones stimulated with Zinc(85μM) and cAMP (50 μM) for 18 hours. Extract proteins were phosphorylated with [γ-^{32}P]ATP by recombinant purified Cα (0.3 nmol) (lane: 1 and 2), Cγ (equal amount of Cα Kemptide kinase activity) (lane: 3 and 4) and endogenous C-subunits (lane 5-10) in the presence and absence of 1 μM PKI_{5-24} as indicated in Figure at 30 °C for 10 min. as described in Methods. Equal amounts of protein (25 μg) were run in each lane on two pieces of 9% SDS-PAGE. Molecular weight marker (M.W.) is the same as described in Figure17. Panel A: autoradiography of 9% SDS-PAGE showing phosphoproteins in each lane. Panel B : Coomassie blue-stained 9% SDS-PAGE gel showing protein profiles in each lane.

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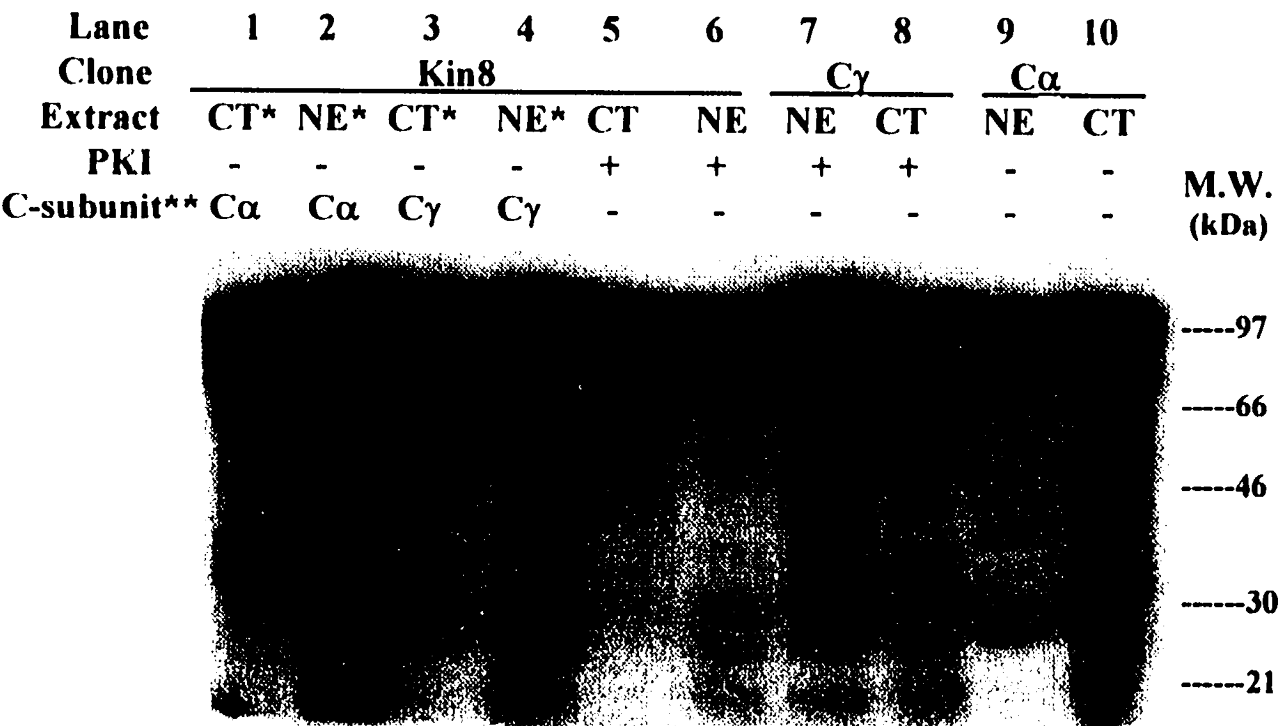

A. CT: cytosol; NE: nuclear extracts; *: boiled sample;
**: purified recombinant C-subunits

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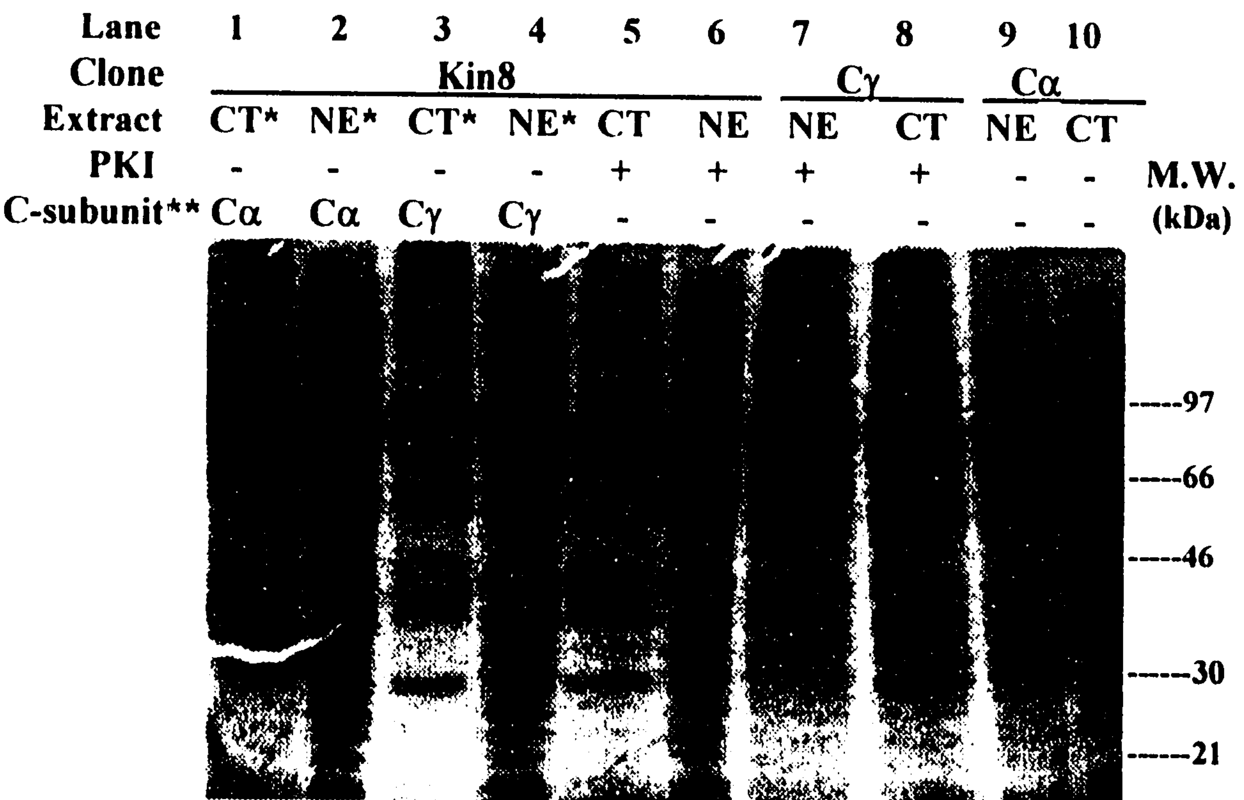

B. CT: cytosol; NE: nuclear extracts; *: boiled sample;
**: purified recombinant C-subunits

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Because there were not detectable differences on denatured cellular protein phosphorylation by exogenous homogeneous C-subunit isozymes, which suggested that the substrate specificity of C-subunits might partially involved in the recognition of protein conformation.

Figure 28 (panel B) showed the Coomassie-blue-stained protein profile of the same SDS-PAGE gel used for autoradiography (Figure 28 Panel A.). No obvious difference of protein profiles were detected in respective lane of Kin8, Cα and Cγ clones, though the protein profiles in the nuclear extracts of Kin8 clone (lane 2, 4, and 6) appeared to be different from that in the cytosol of same clone (lane 1, 3, and 5). These results suggested that Cγ and Cα phosphorylated different cellular proteins in the Kin8 clone.

C. Kinetics of C-Subunits for Pseudosubstrate Specificity

The C-subunits act as the major mediators for cAMP/PKA function, and they have to be precisely regulated in the cell. PKA activity is regulated by two kinds of modulators. One is cAMP as an activator, the other is pseudosubstrate as inhibitors, such as R-subunit and PKI. Pseudosubstrate interacts with C-subunits directly and cAMP interacts with the R-subunit to indirectly regulate C-subunit activity. These pseudosubstrates not only inhibit phosphotransferase activity of C-subunits, but also localize C-subunits in the cell. For example, RII subunits can compartmentalize the C-subunits as holoenzyme by anchoring with specific PKA kinase anchoring protein (Goghlanet al., 1993), which was one kind of cytoskeleton proteins. In addition, R-subunit excludes the free C-subunits from nucleus in the absence of cAMP. Similarly, PKI inhibits C-subunit kinase activity in cytosol and nucleus in the presence of cAMP. PKI also exports the C-subunits from nucleus into cytosol. This terminates C-subunit nuclear function for the activation of cAMP-responsive gene expression (Wen et al., 1995). This information suggests that pseudosubstrates are very important to determine when and where the C-subunits phosphorylate their substrates. Several observations

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suggested that Cγ had different specificity in the interaction with R-subunit and PKI from Cα. Previous findings showed 1) that Cγ kinase activity was not sensitive to PKI-inhibition *in vitro*, which means that Cα, but not Cγ, is modulated by PKI; 2) Cγ-type-I holoenzyme ($C\gamma_2RI_2$) prepared from Cγ/Kin8 clone needed higher cAMP concentration to be dissociated than to dissociate Cα-type-I holoenzyme ($C\alpha_2RI_2$). This suggests that Cγ binds with RI-subunits more tightly than Cα (Beebe et al., 1997). 3) Cγ-type II holoenzyme ($C\gamma_2RII_2$) was detected in permanent Cγ/Kin8 transfect, but no Cα-type-II holoenzyme was detected in the permanent Cα transfected Cα/Kin8 clone (Beebe et al., 1992; 1997). This suggests that Cγ-subunit has higher affinity to bind with RII-subunits than Cα-subunits in the Kin8 cell. These pseudosubstrates manipulate C-subunit function by inhibition and relocalization of C-subunit. The different pseudosubstrate specificity of Cγ and Cα indicate that pseudosubstrate is likely to differentiate functions between Cγ and Cα. This may serves as another important way to diversify the C-subunit function in the whole PKA/cAMP signal transduction pathway.

To determine the pseudosubstrate specificity for Cγ and Cα in detail, the same homogeneous C-subunit preparations were utilized used for substrate specificity studies. Two R-subunit isoforms and two PKI isoforms were expressed in bacteria and purified to homogeneity as described in Methods. These homogeneous pseudosubstrate and synthetic $PKI_{(5-24)}$ peptide, which contains the necessary inhibitory amino acid sequence for Cα and Cβ, were used as inhibitors to titrate the phosphotransferase activity for both C-subunit isozymes using Kemptide and histone as substrate. The IC50 values of each pseudosubstrate inhibition for C-subunit kinase activity with respective substrate were measured to evaluate the inhibition potency or binding affinity of each pseudosubstrate for Cγ and Cα.

1.*Inhibition of C-subunit phosphotransferase activity by PKI pseudosubstrate*. To compare both C-subunit isozymes on the pseudosubstrate specificity for PKI, in this experiment, two isoforms of

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PKI proteins and one PKI_{5-24} peptide were utilized as inhibitors to titrate both C-subunit isozymes kinase activities. The IC50 values for each PKI-inhibition were measured to evaluate distinct interaction between C-subunit isoforms and PKIs. Two isoforms of PKI proteins were expressed in bacteria and were purified by affinity chromatography on Amylose columns to at least 95% homogeneity (Figure 26) as previously described (Baude et al., 1994) and outlined in the Methods Since PKI fusion protein containing the maltose binding protein behaved identically to the PKI without the MBP (Baude et al., 1994), the full length MBP-PKIα and MBP-PKIβ fusion proteins were directly used as inhibitors in the present experiment. Table 12 showed the IC50 values for Cα and Cγ using Kemptide or histone as substrates with each of three PKIs as pseudosubstrates

Table 12 IC_{50} Values (nM) of PKI-Inhibition for C-Subunits Activity

C-Subunit	PKI_{5-24}		MBP-PKIα		MBP-PKIβ	
	KT	HT	KT	HT	KT	HT
Cα	8.3	4.0	0.94	0.22	49.5	13.6
Cγ	>90,000	53,000	34,000	24,290	>90,000	30,000

The data was derived from one representative experiment. The similar results were obtained by three independent experiments

Result (Table12) showed that Cα was inhibited at nanomolar concentrations of each of the inhibitors and the IC50 values for Cα with Kemptide as substrate were in good agreement with the values reported by others (Baude et al., 1994, Gamm et al., 1995). The order of potency for the inhibitors was PKIα > PKI_{5-24} > PKIβ with Kemptide and histone as substrates PKIα had IC50 values that were 3-6-fold lower than IC50 values for PKI_{5-24}. This was in good agreement with the

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results of Baude et al. (1994). For each inhibitor, the IC50 values with Cα were 2-4-fold lower with histone as substrate than with Kemptide as the substrate.

In contrast to the nanomolar IC50 values with Cα, Cγ was very poorly inhibited by all of the PKIs tested and IC50 values were in the micromolar range. When determinations were possible, the IC50 values for the inhibitors were 600-134,000-fold higher with Cγ than with Cα, depending on the inhibitor and the substrate. Although it may be of little practical consequence, PKIα and PKIβ were both better inhibitors than PKI_{5-24} forCγ as a kinase inhibitor. Besides PKI, the R-subunit is the other group of pseudosubstrate inhibitor for C-subunit in the cell.

2. *Inhibition of phosphotransferase activity by R-subunits as pseudosubstrate* Intact cell studies showed that Cγ differed from Cα in the interaction with R-subunits in Cγ versus Cα stably transfected Kin-8 clones which were deficient in the mock-transfected Kin-8 cells (Beebe at al., 1992; Beebe's manuscript, 1997). Higher cAMP concentration was required to dissociate CγRI holoenzyme than CαRI holoenzyme. Cγ could restore typeII holoenzyme in the Kin8 clone, but Cα could not. These observations suggested that Cγ had different affinity in the interaction with RI and RII subunits in the Kin8 cells. To further test this hypothesis, *in vitro* studies were designed to analyze the R-C interaction quantitatively by titrating C-subunit isozymes with homogeneous RI and RII and determining IC50 values as described in Methods.

Figure 29 and 30 illustrated the concentration-dependent inhibition of each C-subunit isozyme with the cAMP-bound RIα-and RIIα-subunit using two different substrates. Table 13 shows the IC50 values derived from the curves in the respective figures.

The figure (Figure 29) showed that the phosphotransferase activity of both C-subunit isozymes with two substrates could be inhibited by nanomolar range of both R-subunit preparation and had similar inhibition titration curves. The IC50 values of this cAMP-bound RIα-subunit preparation

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Figure 29. RIα-Inhibition of C-Subunits

Homogeneous Cγ- and Cα-subunits were prepared as described in Methods. Kinase activity was assayed with 65 μM Kemptide (Panel A) or 85 μM histone (Panel B) in the presence of increasing concentration of purified bacterial recombinant cAMP-bound RIα-subunits. The cAMP-bound-RIα was prepared as described in Methods. The curves were fitted using the average values of triplicate assay points from representative experiments, and the error bars depict the standard deviation from mean. Less than 5% variation of results were obtained in three independent experiments.

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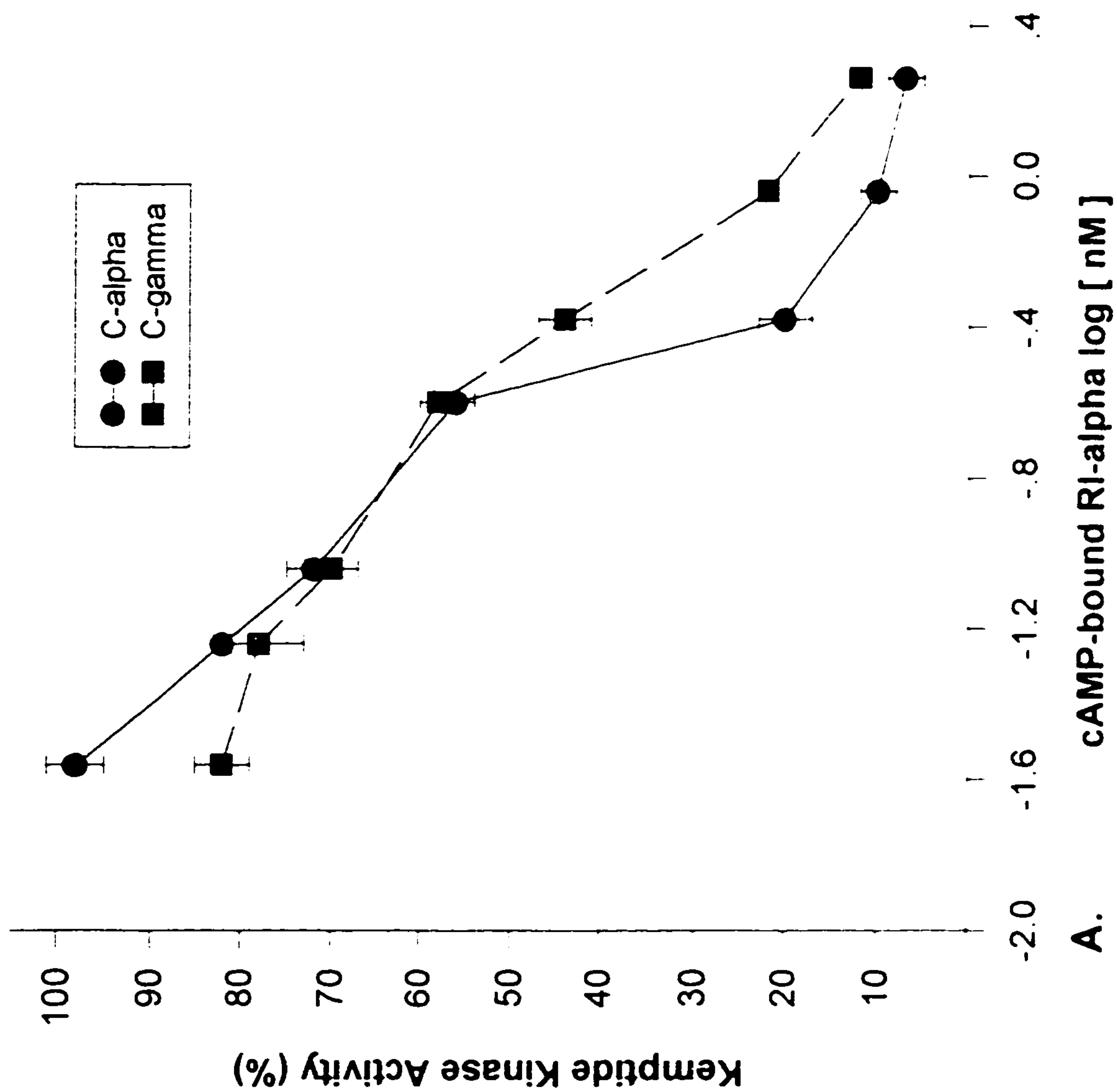

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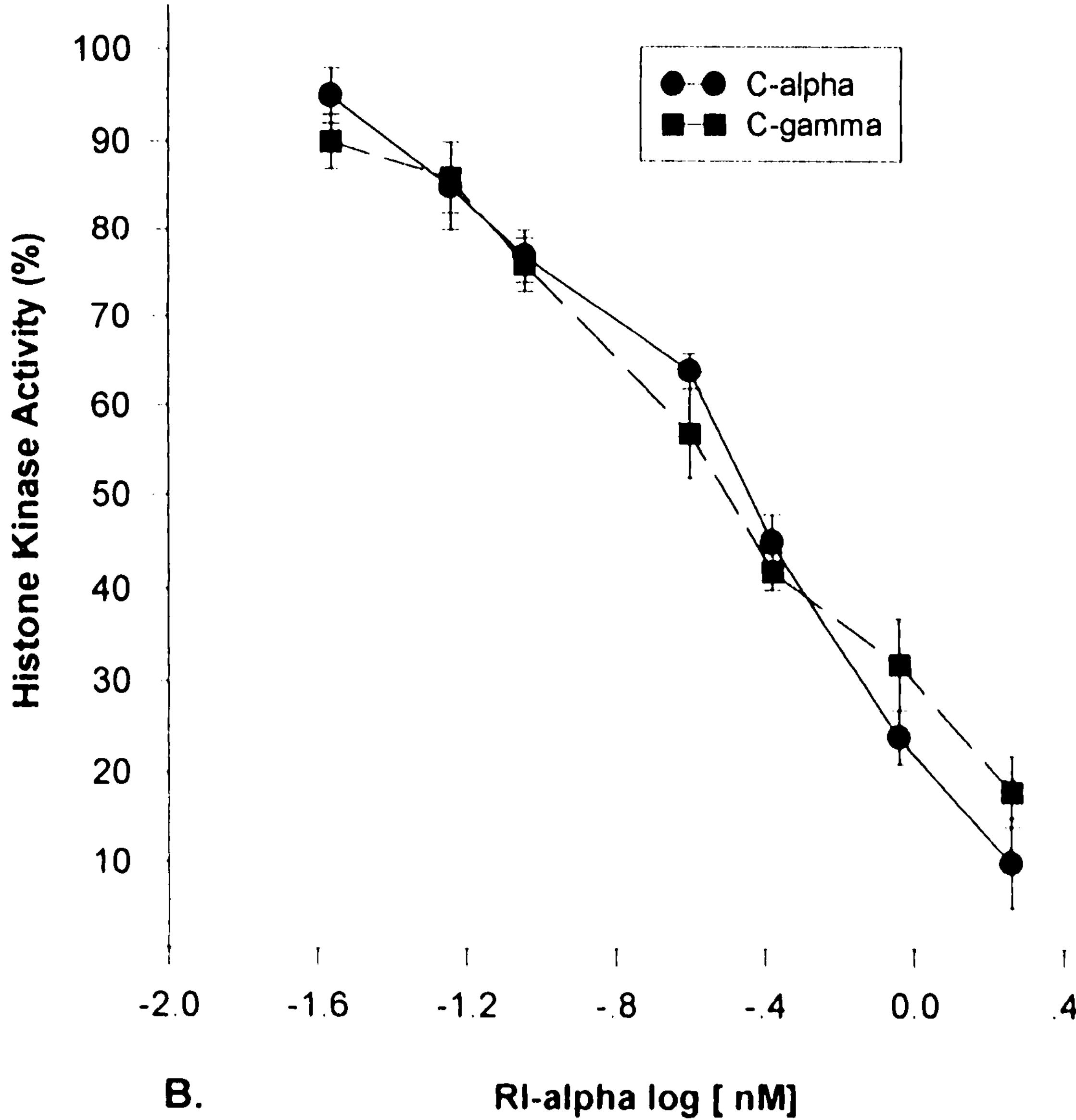

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Figure 30. RIIα-Inhibition of C-Subunits

Homogeneous Cγ- and Cα-subunits were prepared as described in Methods. Kinase activities were assayed with 65 μM Kemptide (Panel A) or 85 μM histone (Panel B) in the presence of increasing concentration of purified bacterial recombinant his_{10}-RIIα-subunits. His_{10}-RIIα was prepared as described in Methods. The curves were fitted using the average values of triplicate assay points from representative experiments, and the error bars depict the standard deviation from mean. Less than 5% variation of results were obtained in three independent experiments.

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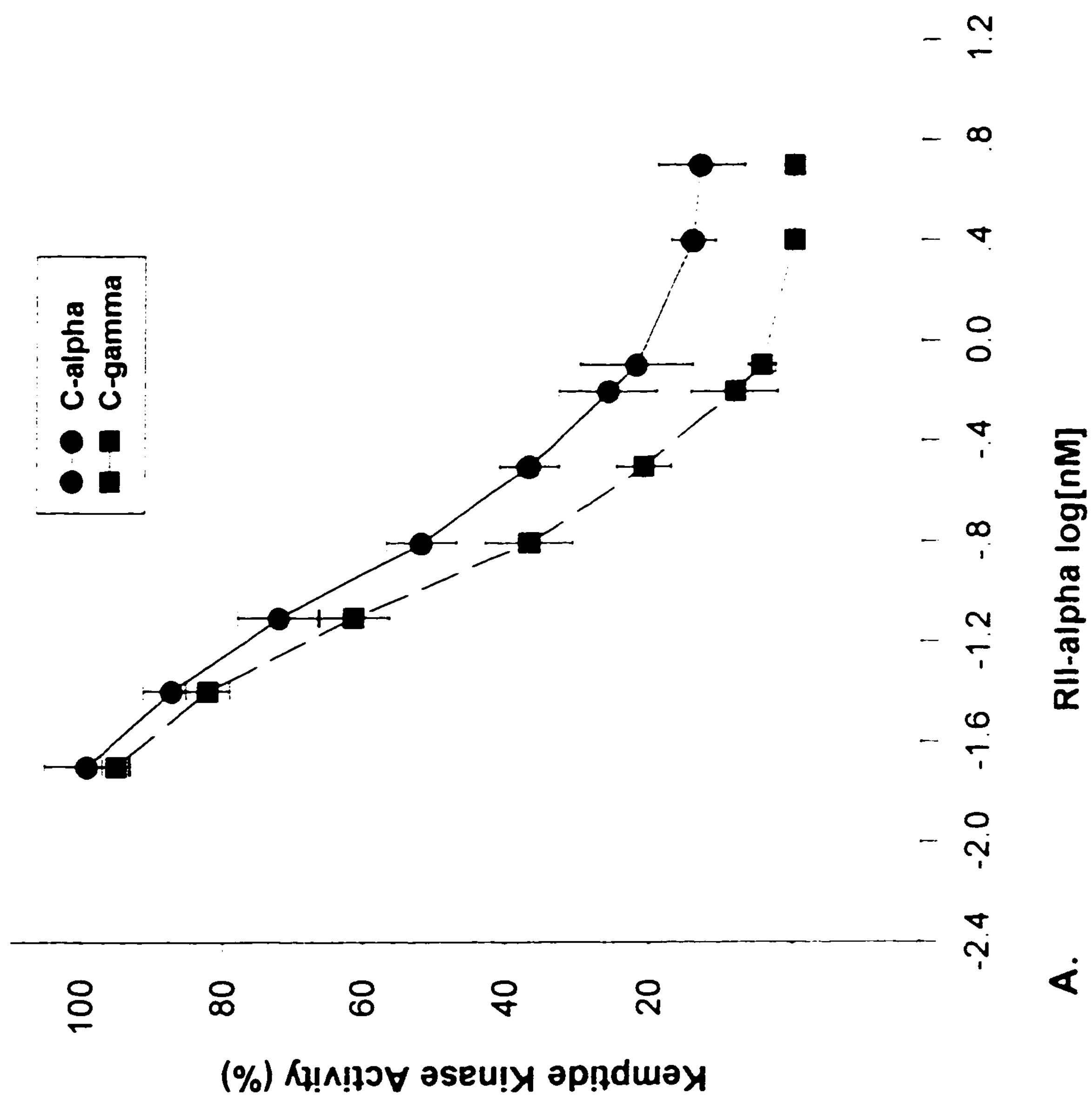

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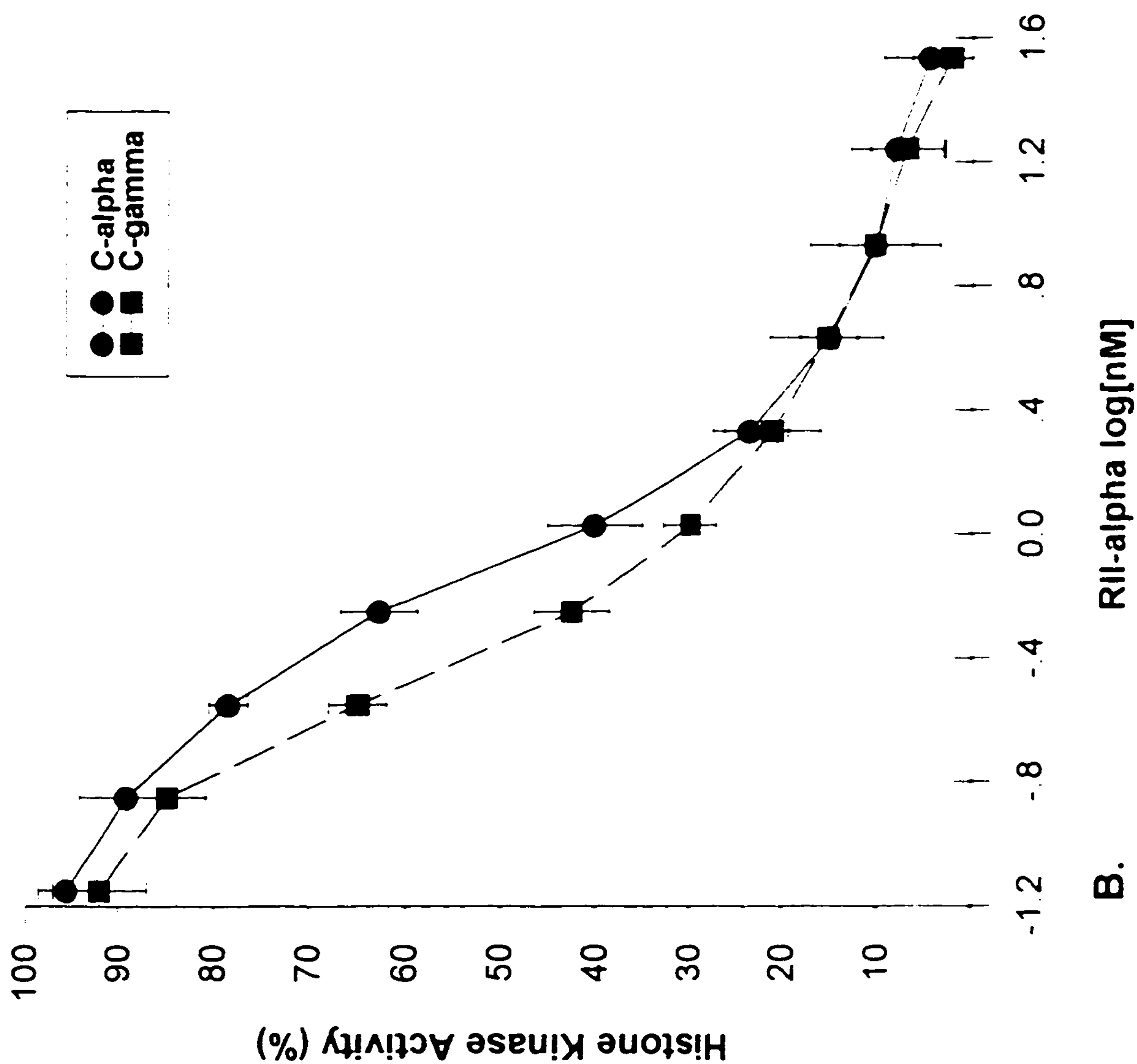

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Table 13 IC_{50} Values (nM) of R-Subunit Inhibition for C-Subunits

C-Subunit	cAMP-bound RIα		cAMP-free RIα		RIIα	
	KT*	HT**	KT	HT	KT	HT
Cα	0.37	0.29	3.6	1.4	0.2	0.8
Cγ	0.33	0.25	4.2	3.1	0.1	0.4

KT: 65 μM Kemptide was use as substrate; HT: 85 μM histone was used as substrate

for Cγ and Cα. were very close with both substrates. However, 2-fold higher concentrations of cAMP-free RIα-subunit were required to inhibit Cγ than that required to inhibit Cα when histone was the substrate (Table 13). The IC50 values of cAMP-free RIα-subunit inhibition for Cγ and Cα were 3.0 and 1.4 nM with histone as substrate, respectively. In contrast, the IC50 values of cAMP-free RIα-inhibition for the two C-subunit isozymes were similar when Kemptide was the substrate, which might suggest that substrates might have little effect on association inhibition of PKA holoenzyme isoforms that differ in their C-subunit isozyme. Although cAMP-free RIα and cAMP-bound RIα were used as inhibitors to investigate pseudosubstrate specificity of both C-subunit isozymes for RI-subunit in this vitro study, there was no detectable differences in their IC50 values between Cγ and Cα subunit with either substrate.

A His_{10}-tag-RIIα-subunit was used as inhibitor to determine the pseudosubstrate specificity of both C-subunit isozymes for RII-subunit. Figure 30 showed the his_{10}-RIIα-inhibition titration curve for both C-subunits with two substrates. The results from these RIIα-inhibition titration curves showed that 85-90% transferase activities of both C-subunit could be inhibited by 2-4 nm RIIα-subunits depending on different substrates, which suggested that the substrate might slightly affect

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the RIIα-inhibition as occurred in cAMP-free RIα-inhibition. But, the RIIα-subunit inhibition curves appeared to be very similar for both C-subunits isoforms. Consequently, the IC50 values of RIIα-inhibition for Cγ and Cα were very close (Table 13), which indicated that inherent sequence difference between Cγ and Cα could not be revealed by this *in vitro* RIIα-subunit inhibition approach.

The inhibition of both C-subunit phosphotransferase activities by these recombinant R-subunits could be released by 1μM cAMP (Table 14), which indicated these recombinant subunits of PKA had the normal conformation as predicated, and these purified recombinant subunits of PKA could be used *in vitro* study to mimic the native R-C interaction in cell.

Table 14 Release of R-Subunit Inhibition for C-Subunit Kinase Activity by cAMP*

C-subunit	No R-subunit	RIα	%**	RIα+cAMP	RIIα	%	RIIα+cAMP
Cα	401	98	76	411	43	89	398
Cγ	185	23	87	172	18	90	196

* 6.1 nM purified cAMP-bound RIα or 0.75 nM purified his_6 RIIα-subunit were used as inhibitor to inhibit C-subunit kinase activity in the absence or presence of 1 μM of cAMP with Kemptide (65 μM) as substrate. **: % of inhibition

The results (Table 13) did not reveal the pseudosubstrate specificity of C-subunit isoforms for R-subunit as observed in intact cell. The explanation for that was that some known factors, including cAMP, autophosphorylation of RII, and ATP-Mg^{2+} (for RI only), affect the C-R interaction in intact cells, but these factors could not be restored *in vitro* exactly as in intact cells

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To simplify the conditions to compare distinct affinity between the R-C interaction, a series of synthetic

RIα peptides with different lengths and mutations in pseudosubstrate binding site were used as inhibitors to further compare Cγ and Cα for RI-subunit specificity.

3. *Effects of lengths and substitutions of amino acids of RIα peptides on the inhibition of C-subunit transferase activity:* A major difference between RI-subunit and RII-subunit is that RII-subunit can be autophosphorylated as substrate by C-subunits, but, RI-subunit can not be phosphorylated by C-subunits, because the phosphoacceptor (Serine at 97) is substituted by Alanine. However, both of them can bind C-subunit to form holoenzymes. The RI-subunit binds with C-subunit as pseudosubstrate, which suggests that the RI-subunit contains specific amino acid sequence which could be recognized and bound by C-subunits in their pseudosubstrate binding domains. It is these pseudosubstrate sequence region of RI-subunits that C-subunits really recognize and interact with. In order to further define specific interaction between the C-R, a series of synthetic RIα peptides (kind gift from Dr.Corbin, Vanderbilt University, Medical school) with substitution in this pseudosubstrate binding region and different length were used as inhibitor for both C-subunit isozyme kinase activities. By comparing the IC 50 values of each RIα-peptide for Cγ and Cα, pseudosubstrate specificity of both C-subunits for RIα were evaluated. In this experiment, three sources of C-subunits were utilized. Recombinant Cα from bacteria and recombinant Cγ expressed in Sf9 cells using baculovirus were utilized in these studies and compared with IC 50 values from native Cα prepared from heart muscle.

The results showed that the IC50 values of all these RIα-peptides for mammalian native Cα-subunit prepared from heart muscle and recombinant Cα-subunit prepared from bacteria as

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TABLE 15 The Effects of Length & Substitution of Amino Acids of RIα-peptide on the Inhibition of C-Subunit Activity

Peptide	Peptide Sequence	*IC50 (μM)			(Cγ/Cα**)
		**Heart-Cα	R-Cα	R-Cγ	
Group-I	***P-5 P-4 P-3 P-2 P-1 P P+1 P+2				
	Arg-Arg-Gly-Ala-Ile-Ser	810	94	2430	26
	Arg-Arg-Arg-Arg-Gly-Ala-Ile-Ser	1 8	7	44	6.3
	Pro-Pro-Pro-Pro-Asn-Pro-Val-Val-Lys-Gly-Arg-Arg-Arg-Arg-Gly-Ala-Ile-Ser	12	10	97	9.7
Group-II					
	Pro-Pro-Pro-Pro-Asn-Pro-Val-Val-Lys-Gly-Arg-Arg-Arg-Arg-Gly-Ala-Ile-Ser	12	10	97	9.7
	Pro-Pro-Pro-Pro-Asn-Pro-Val-Val-Lys-Gly-Arg-Arg-Ala-Arg-Gly-Ala-Ile-Ser	6230	12400	5605	0.45
	Pro-Pro-Pro-Pro-Asn-Pro-Val-Val-Lys-Gly-Arg-Arg-Arg-Ala-Gly-Ala-Ile-Ser	630	210	315	1.5
	Pro-Pro-Pro-Pro-Asn-Pro-Val-Val-Lys-Gly-Arg-Arg-Arg-Arg-Gly-Ala-Ala-Ser	350	145	1950	13
Group III					
	Arg-Arg- Arg- Arg-Gly-Ala-Ile-Ser	18	7	44	6.3
	Ala- Arg- Arg-Arg-Gly-Ala-Ile-Ser	8	19	930	49
	Ala -Ala- Arg- Arg-Gly-Ala-Ile-Ser	203	70	2400	34
Group IV					
	N-Pro-Pro-Pro-Pro-Asn-Pro-Val-ValLys-Gly-Arg Arg- Arg -Arg-Gly-Ala-Ile-Ser-C	12	10	97	9.7
	N-Arg-Arg-Arg-Arg- Gly-Ala- Ile- Ser- Ala-Glu-Val-Tyr-Thr-Glu-Glu-Asp-Ala-Ala-Ser-Tyr-Val-Arg-Lys-Val-Ile-Pro-Lys- Asp-Tyr-Lys-Thr-C	14	15	17	1.1

Ic50 values for heart muscle Cα were derived from Dr. Corbin's unpublished data, P the position of amino acid residue relevant to phosphate acceptor **IC50 values of Cα were derived from recombinant bacterial Cα

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described in Methods (Table 15) were in relatively close agreement. But, the results did show that Cγ-subunit responded to some amino acid residual substitutions in RIα-peptides remarkably differently from both Cα in term of respective IC50 values. The results were presented as following. For comparison, the peptides were divided into four group according to the length and relativity of these peptides.

The results (Table 15) from <u>Group I</u>, which were native RIα peptides with different length, showed that the IC50 value of wildtype RIα-peptide for Cγ was 6-10 fold higher than those for both Cα. Deletion of Arg-Arg (P^{-5} P^{-4}) increased about 45-, 15-, and 55-fold IC50 values of this mutant RIα-peptide for heart Cα, recombinant Cα and Cγ isozymes, respectively. The deletion of residues from the N-terminal amino to the Arg-Arg (P^{-5} P^{-4}) changed IC50 values for all three C-subunits less than two-fold. These results suggested that 1) Cγ had less affinity (6-10 fold) in binding affinity with this pseudosubstrate region of RIα peptide than Cα. 2) Arg-Arg (P^{-5} P^{-4}) was critical amino acid residues in RIα-inhibition for all C-subunits, especially for Cγ. 3) In contrast, N-terminal residues before Arg-Arg (P^{-5} P^{-4}) appeared to be not important in RIα-inhibition for all three C-subunits.

The results from <u>Group II</u>, which were same length (18 amino acid residues) of RIα-peptides with substitutions of some amino acid residues, showed that the mutation of Arg (P^{-3}) into Ala increased IC50 values 500-fold for heart Cα ,1000-fold for recombinant Cα and 56-fold for Cγ in these RIα-peptides inhibition. The mutation of Arg (P^{-2}) into Ala increased near 50-fold IC50 values for both Cα , but only increased 3.5-fold IC50 values for Cγ. The substitution of Ile (P[illegible]) with Ala increased 2-fold IC50 values of this RIα peptide-inhibition for these C-subunit isozymes. These results indicated that 1) Arg (P^{-3}) was the most important amino acid residue for the RIα-inhibition of C-subunit in this pseudosubstrate region. 2) Arg (P^{-2}) was the second most important in the RIα peptide-inhibition for Cα-subunit, but no so important for Cγ. 3)The original difference

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on the IC50 values of wildtype RIα-peptides for Cγ and Cα was 6-10 fold. The substitution of either of Arg^{94} or Arg^{95} with Ala not only dramatically decreased the RIα inhibition-potency for all these C-subunit isozymes, but also covered the original 6-10 fold distinction in the IC50 values of RIα-peptide inhibition between Cγ- and two Cα-subunit isozymes. These results suggested that two basic amino acid residues in RI pseudosubstrate site played so important role in the RI-inhibition that the deletion of either one of them eliminated the normal interaction between the pseudosubstrate region of RIα-peptide and C-subunits. These two basic residues in RI-peptide are corresponding to two basic residues (R-R) at P^{-3} and P^{-2} of PKA substrate phosphorylation site (4). The Ile (P^{+1}) was important for all three tested C-subunit isozymes in the RIα-peptide inhibition.

The results from <u>Group III</u>, which were same length (8 amino acid residues) RIα-peptides with substitution of two amino acid residues at P^{-5} P^{-4}, showed that substitution of Arg (P^{-5}) with Ala increased 2-fold IC50 values of this RIα peptide-inhibition for Cα, but increased 6.5 fold IC50 value for Cγ. The substitution of Arg-Arg (P^{-5} P^{-4}) with Ala-Ala increased 10-fold IC50 values of RIα -inhibition for Cα and 17-fold for Cγ. These results were consistent with results from group I, which indicated that two consecutive Arg (P^{-5} P^{-4}) residues were important and had additive effects for the RIα-inhibition of C-subunits.

The results from <u>Group IV</u>, which were native RI-peptides with different terminal amino acid residues, showed that addition of N-terminal 10 amino acids before Arg (P^{-5}) or addition of C-terminal 23 amino acids after Ser (P^{+2}) did not affect the IC 50 values for Cα-subunit isozymes. But addition of C-termini 23 amino acids deprived the 6-10 fold differences on IC50 values between the Cγ and Cα in RIα peptide-inhibition, which suggested that C-terminal sequence of RIα pseudosubstrate was particularly important to inhibit Cγ, but not Cα. This result implied that primary sequence of RIα pseudosubstrate was important to both C-subunits, but the sequence

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surrounding this pseudosubstrate sequence may serve as a structure to manipulate specificity for R-subunits to interact with different isozymes of C-subunit.

These results clearly demonstrated that Cγ and Cα-subunits shared some identities in the interaction with RIα-peptides, especially in the N-termini to pseudophosphorylation site, but Cγ needs extra C-terminal residues for full RIα-inhibition. This result is consistent with the conclusion made from *in vitro* substrate specificity study for Cγ and Cα. In summary, C-terminal residual sequence to phospho-acceptor site may serves as the structure that differentiates the substrate and pseudosubstrate specificity for Cγ and Cα.

D. Function And Possible Mechanism For C-subunit-Mediated Regulation of CRE-Reporter Gene Activity

The previous results showed that Y1/Kin8 mutant cells permanently transfected with Cγ and Cα exhibited different cell phenotypes (Table1), which suggested that Cγ had distinct cell function from Cα. Among these distinct phenotypes, steroidogenesis, which is regulated by Cα, but not Cγ, is directly associated with cAMP-mediated gene expression. This suggested that Cγ might play a distinct role from Cα in the regulation of cAMP-mediated gene expression. It is important to define the molecular mechanisms underlying the distinct functions of two isoforms for the regulation of cAMP-mediated gene expression. It will help to better define the potential for functional diversification of PKA in the cAMP/PKA signal transduction pathway.

C-subunit plays roles in the cell through the phosphorylation of a variety of substrates. It is hypothesized that the distinct roles of Cγ and Cα in the regulation of cAMP-mediated gene expression and other cell phenotypes in Y1/Kin8 cells were a result of their different substrate and/or pseudosubstrate specificities. All previous in vitro studies were designed to search for such molecular evidence to support this hypothesis. The results generated from in vitro kinetic studies

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clearly indicated the substrate and pseudosubstrate specificity differences between Cγ and Cα, which strongly support the hypothesis in this dissertation. However, molecular mechanism for differences in phenotypes expression requires further studies. Additional strategies were designed to determine the molecular mechanisms underlying the distinct functions between permanently transfected Cα and Cα clones in the regulation of cAMP responsive gene expression.

The second messenger, cAMP, regulates some cell physiological functions by a mechanism activating the expression of some genes. It is well known that the 5'-flanking region of many cAMP responsive genes contains cAMP responsive elements (CRE) as promoter that will be recognized and bound by trans-activating transcription factors in CREB/ATF family. After being activated by cAMP in the cell, C-subunits can induce these CRE-containing gene expression sufficiently in response to cAMP-stimulation (Grove et al., 1987; Day et al., 1989; Mellon et al., 1989; Maure et al., 1989), presumably by activating CREB/ATF transcription factors via phosphorylation in the nucleus. The best known trans-activating transcription factor whose function is regulated directly by C-subunit phosphorylation is CREB (cAMP responsive element binding-protein) (Gonzalez et al., 1989; Habener et al., 1990; 1991). The phosphorylation of CREB and other co-transcription factors by C-subunit is necessary for the transcriptional activation in the response to elevated intracellular cAMP levels in the cell (Figure 2). The *in vitro* and in intact studies showed that Cγ differed from Cα in substrate and pseudosubstrate specificities, which suggests that it is possible that Cγ and Cα phosphorylate CREB and/or other CREB/ATF transcription factors differently. This may be due to their unique substrate specificity and/or cellular location in the cell. Different phosphorylation of transcription factors by Cγ and Cα may account for distinct regulation of gene expression by Cγ and Cα in Y1 Kin8 cells.

In addition, Cγ is non-sensitive to PKI-inhibition, a potent, heat stable, PKA-specific inhibitor. As it was mentioned before, PKI not only inhibits C-subunit kinase activity in the cytosol and

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nucleus, but also negativity regulated the Cα-mediated stimulation of cAMP-regulated gene transcription by exporting C-subunits from the nucleus (Wen et al., 1995). Cγ-subunit Cα kinase activity is insensitive to PKI inhibition (Table 12). Because PKI inhibits C-subunit kinase activity as a competitive inhibitor, it is predicated that Cγ doesn't bind PKI because Cγ kinase activity is insensitive to PKI-inhibition.

Arg[133], which has been defined as an essential basic residue for the complementary interaction between Cα and PKI, is conspicuously substituted by Gln, which served as another evidence to support the predication that Cγ-subunit does not bind PKI (Wen et al., 1994). PKI-insensitivity may provide another way that may differ Cγ from Cα in the regulation of CRE-mediated gene expression. The phosphorylation patterns and cellular physiological effects for Cγ and Cα would be different in the presence or absence of PKI.

To specifically determine if Cγ, like Cα, could regulate gene transcription through CRE element in cAMP-regulated genes, CRE-reporter gene system was utilized as a model. The regulation of CRE-reporter gene expression by C-subunit isozyme were evaluated based on the reporter gene activities in transiently transfected HEK 293 and Kin8 cells with CRE-luc reporter gene, which was from α-glycoprotein hormone as described in Methods, and two C-subunit isozyme expression vectors : pMT and pCMV. pMT C-subunit expression was inducible by Zinc in a methionine promoter. The pCMV vectors were constitutive expression vector with CMV as promoter.

1. *Cγ and Cα regulate the CRE-reporter gene activity distinctively and cooperatively.* To determine the function of Cγ and Cα in the regulation of cAMP-mediated gene expression, the CRE-luciferase vector was co-transfected with pMTCγ or pMTCα or their combination in HEK 293 cells. The Table 16 showed the results from these experiments.

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Table 16 Cγ and Cα Had Distinct and Cooperative Function in the Regulation of CRE-Luciferase Gene Activity

pMT Cα /Cγ	*Zinc / 8-CPT-cAMP	ALU**
Cα	-	4305
Cα	+	51850
Cγ	-	2169
Cγ	+	5180
Cα+Cγ	-	2189
Cα+Cγ	+	635,880

*:Treatment by zinc for the expression of pMT C-subunit expression vectors driven by metallothionein promoter. Treatment by cAMP for the activation of C-subunits * Luciferase activity was determined in a luminoometer. ALU: arbitrary light units.

The result (Table 16) showed that Zinc induction and cAMP treatment of Cα clone induced luciferase reporter gene activity 10-fold. In contrast, Cγ induced the CRE-luciferase activity only two-fold. Interestingly, cotransfection with Cα and Cγ induced luciferase reporter gene activity 10-fold more than the sum of transfection with Cα and Cγ alone This suggested that Cγ synergistically enhanced Cα-regulated reporter gene activity The same results were obtained in Kin8 cells (not shown). Cγ did not regulate CRE-reporter gene activity, but profoundly enhanced Cα-mediated CRE-reporter gene activity This result was the first evidence to show that two-isozymes of one kinase could have synergetic interaction in the regulation of gene expression More interestingly, this enhancement on the CRE-reporter regulation of gene activity by Cγ and Cα turned into attenuation when the amount of both C-subunit expression vectors used for co-

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transfection were higher than a certain point (Figure 31). The Figure31 showed that if Cα was cotransfected with low amount of Cγ DNA (< 3.0μg), reporter gene activity was enhanced about 5-fold. In contrast, with relatively high amount of Cγ (≥ 3.0 μg) for cotransfection, the reporter gene activity was attenuated about 3-7 fold. Similarly, the Cγ alone could not stimulate reporter gene activity. This result suggested that Cγ alone weakly effected the CRE-reporter gene expression, but Cγ could manipulate the Cα function in the regulation of CRE-reporter gene activity positively and negatively.

It has been proven that C-subunit phosphorylates CREB in nucleus. The phosphate-group on the CREB provides a signal for CBP and other co-transcription factors to bind to them, finally to activated CRE-mediated gene expression. In order to determine the molecular mechanisms for Cγ vs Cα-mediated regulation of reporter gene expression, the a series of experiments were designed to answer the following questions: 1) Are active and free C-subunits levels increased in the cytosol and nucleus of both C-subunit expressing Kin8 clones after Zinc/cAMP stimulation? 2) Do Cγ like Cα phosphorylate CREB in nucleus ? 3) Do Cγ and Cα-subunit induce the increases of CREB protein levels ? 4) Do the nuclear extracts from Cγ and Cα nuclear extracts exhibit differences for CRE-binding elements, and if they do, what are components in these CRE-binding complexes from respective nuclear extracts?

2.Catalytic Cγ-subunits are elevated in Cγ-clone as Cα-subunits in Cα-clone by Zinc/cAMP-stimulation: The result showed the Cγ alone weakly could activate CRE-reporter gene activity (Table 16). One easy explanation for this is that Cγ expression levels were too low in the permanently transfected Cγ clone to induce CRE-reporter gene expression. Cγ and Cα expressions in these C-subunit expressing clones were determined before. But, these cells are at high passage number. It is required to determine the stability of C-subunit expression in these clones.

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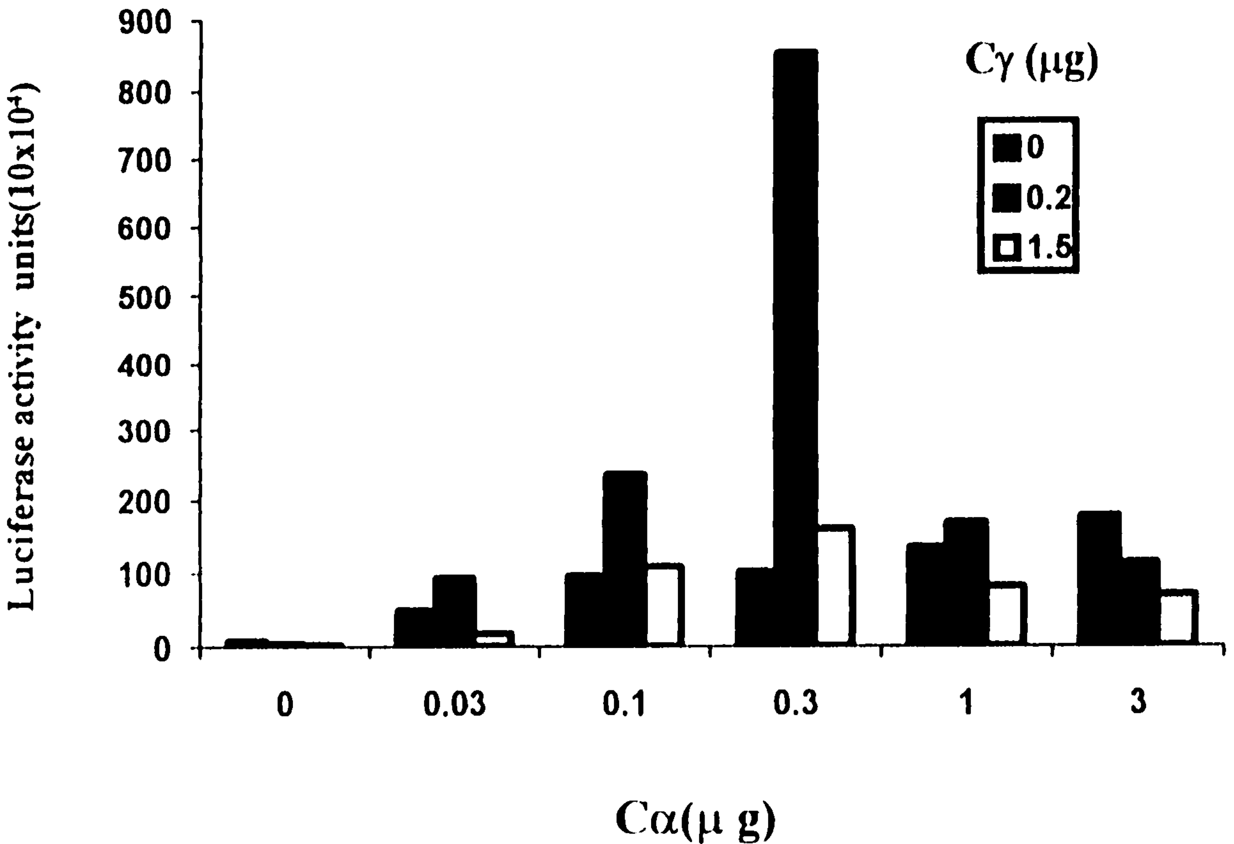

Figure 31 Cγ Modulates Cα-Mediated Regulation of CRE-Luciferase Reporter Gene Activity Positively and Negatively
HEK 293 cells from six wells (with 70% confluent) were cotransfeted with 1 μg CRE-luc (α-luc-168) and varying amounts of pCMV-Cγ and/or pCMV-Cα or their combination as indicated in the figure by calcium-phosphate coprecipitation The luciferase activities were assayed from transient transfected cells after 4 hours transfection and 18 further growing as described in Methods. The data shown were derived from one representative experiment. The same experiments were performed three times with less than 15% variation

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To explore this possibility, the specific kinase activity for Cγ and Cα were detected in Cγ Vs Cα stably transfected clones and their parent clone (Kin8) with or without stimulation with zinc/cAMP. The zinc treatment was to stimulate the expression of C-subunit expression vectors driven by metallothionein promoter (Glanville et al., 1981). The cAMP treatment was to active C-subunits via dissociating the holoenzymes.

Table 17 Elevation of Cγ- and Cα-kinase activity in Cγ Venus Cα Transfected Clones

Clones		cAMP/Zn^{2+}*		Increasing (fold)
		-	+	
Kin 8	PKI-Sen.	630	1070	1.70
	PKI-insen.	71	77	1.08
Cα2	PKI-Sen.	1035	2748	2.65
	PKI-insen.	189	217	1.15
Cγ7	PKI-Sen.	840	1500	1.79
	PKI-insen.	570	1620	2.84

* Zinc: 80 μM; cAMP: 50 μM. Stimulation for 18 hours. Kemptide(65 μM) was used as substrate for kinase assay. The data was derived from one representative experiment. The similar results were obtained by three individual experiments

The results from Table 17 indicate that active kinase levels of both Cγ and Cα-subunits are elevated by Zinc/cAMP stimulation in respective clones. Table 17 showed that Cγ had about 4-fold higher basal (no Zinc/cAMP treatment) PKI-insensitive kinase activity if compared with the average value of same kinase in Kin8 and Cα clones, which probably was a result of leaky expression in the Cγ expression vector. But, the PKI-insensitive kinase activity was increased another 2.8-fold by the stimulation of Cγ clone with Zinc/cAMP. The final PKI-insensitive kinase

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activity in the Cγ clone was 7 and 20- fold higher if compared with those in Cα2 and Kin8 clones, respectively. Zinc/cAMP stimulation increased the PKI-sensitive kinase activity in the Cγ clone as much as in Kin8 control clone. Similarly, the Zinc/cAMP stimulation did increased about 2.6-fold PKI-sensitive kinase activity in the Cα clone, but did not affect much PKI-insensitive kinase activity level in Cα and Kin8 clone. These results suggested that catalytic Cγ-subunit was efficiently expressed in the Cγ clone by Zinc/cAMP stimulation as Cα-subunit in the Cα clone. The results from Table 17 are similar to a previous report (Beebe et al., 1992), which indicates that both C-subunit expressions are still stable in respective Kin8 clones.

The results (Table 17) showed that the catalytic Cγ-subunit level was increased specifically in the Cγ clone. PKI-sensitive Cα-subunit level was specifically elevated in the Cα clone after Zinc/cAMP stimulation. These results suggested that different kinase activity level in Cγ- and Cα-clones was not a reason to account for the distinction in the induction of CRE-reporter gene expression by both C-subunit isoforms. The next anticipation was to determine if the Cγ-subunit was translocated into the nucleus like Cα after its dissociation from R-subunits by cAMP.

3.*Cγ translocates into nucleus like Cα*. Table 17 showed that both Cγ and Cα activity were increased in both Cγ /Cα clones after Zinc/cAMP stimulation, but CRE-reporter gene activity only was activated by Cα, but not Cγ (Table 16). This suggested that something must happen after the expression and activation of C-subunits in the cell. Previous data (Wen et al., 1995) showed that Cα translocated into the nucleus activated CRE-containing gene expression by CREB phosphorylation. To determine if Cγ activity appeared in the nucleus, the kinase activity assay and immunoblot analysis were applied to detect both C-subunit in the nuclear extracts of permanently Cγ vs Cα transfected clones after zinc/cAMP stimulation.

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Table 18 Elevation of Specific Kemptide Kinase Activity of C-Subunits in the Nuclear Extracts of Three Kin8 Clones by Zinc/cAMP Stimulation*

Clone		Zinc/cAMP		Elevation
		-	+	(fold)
Kin8	PKI-Sen.	520	1260	2.4
	PKI-insen	42.2	55	1.3
Cα2	PKI-Sen.	810	2606	3.2
	PKI-insen.	184	262	1.4
Cγ7	PKI-Sen.	480	1640	3.4
	PKI-insen.	760	2400	3.2

* Zinc: 80 μM; cAMP: 50 μM. Stimulation for 18 hours. Kemptide(65 μM) was used as substrate for kinase assay. The data was derived from one representative experiment. The similar results were obtained by three individual experiments

The results from Table 18 indicated that PKI-insensitive kinase activity was only increased specifically in the nucleus of Cγ clone. PKI-sensitive kinase activities were increased in all three clones, but the specific kinase activity in the nucleus of Cα was higher than that of the other two clones. Table18 showed that the stimulation of Cγ clone with Zinc/cAMP increased 3.2-fold specific Cγ PKI-insensitive kinase activities in nucleus. In contrast, no notable changes in PKI-insensitive kinase were detected in the nuclear extracts of Cα and Kin8 clones, which suggested the elevation of PKI-insensitive kinase activity was Cγ-clone specific after Zinc/cAMP stimulation. The Zinc/cAMP treatment not only elevated 3.2-fold PKI-sensitive kinase activity in nuclear extracts of the Cα clone, but also increased 2.4 and 3.4 PKI-sensitive kinase activities in the nuclear extracts of Kin8 and Cγ clones, respectively. This suggested that Zinc/cAMP-treatment

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caused the free Cα-subunits to translocate into nuclei in the Kin8 and Cγ clones However, the absolute value of PKI-sensitive specific kinase activity in the Cα clone was still 1 6-2 fold higher than those in the Kin8 and Cγ clones. This result suggests that Cγ translocates into nucleus after Zinc/cAMP treatment.

The kinase activity assay quantitatively demonstrated the active Cγ-subunit was not only elevated as Cα, but also translocated into nucleus in the Cγ clone. The same result was confirmed by immunoblot analysis using C-subunit specific antibody. Figure 32 showed that 39kDa mammalian Cγ specific band (Beebe et al., 1992) which differed from 41 kDa Cα band in the Kin8 clone, was much intensified in the nuclear extract of the Cγ clone after Zinc/cAMP treatment than no-treatment, but no 39 kDa Cγ band could be detected in the nuclear extracts of the Cα and Kin8 clones regardless of Zinc/cAMP treatment (not shown data). This further supports the identity of 39 kDa band as Cγ subunit. Results from kinase assay and immunoblot analysis clearly demonstrated that the nuclear translocation of C-subunits is not an reason accounting for distinct regulation of CRE-reporter gene expression by different C-subunit isozymes

4.Cγ phosphorylats CREB as Cα in nucleus: CREB is major mediator for PKA to regulate cAMP-responsive gene expression. CREB has to be phosphorylated by Cα-subunit to activate CRE-containing gene expression cooperatively with other co-transcription factors To investigate if Cγ-subunit can phosphorylate CREB in the nucleus, the phospho-CREB were detected in the nuclear extracts of Cαand Cγ clones after Zinc/cAMP treatment by immunoblot analysis using phospho-CREB (Ser133) antibody as described in Methods

Results showed that both Cγ and Cα expression by Zinc/cAMP stimulation elevated the level of immuno-reactive phospho-CREB protein as shown by immunoblot analysis using phospho-CREB specific antibody (Figure 33), which suggested that Cγ could phosphorylate CREB as Cα

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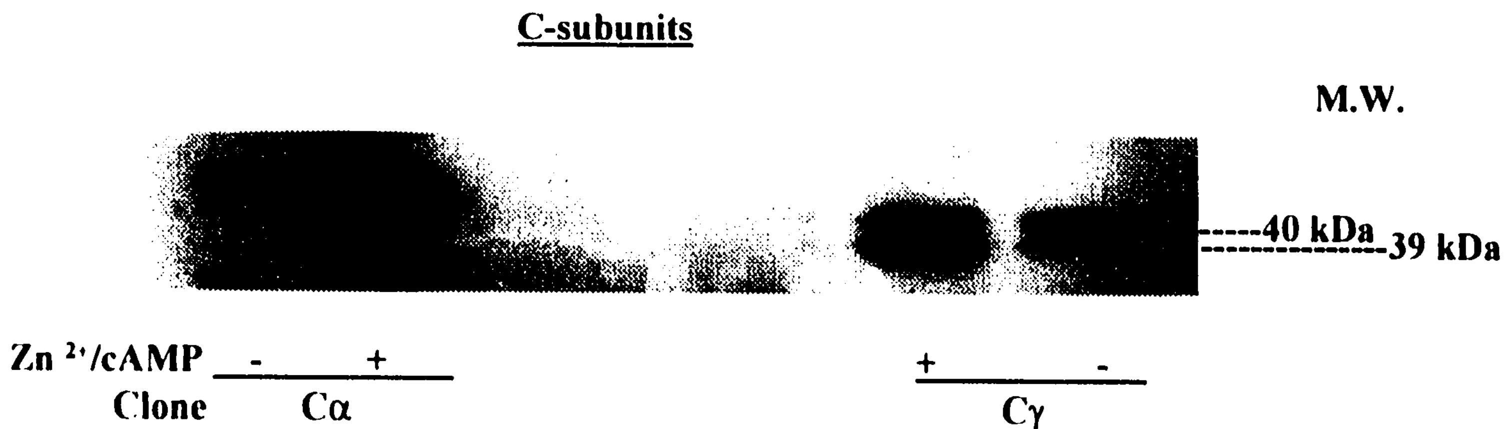

Figure 32 Detection of C-Subunit in Nuclear Extracts of Cα and Cγ Clones after Zinc/cAMP Stimulation by Immunoblot Analysis

Nuclear extracts were prepared from Cγ and Cα clones which were stimulated in the presence or absence of 85μM zinc plus 50μM cAMP as described in Methods Nuclear extracts (5 μg) were subjected to 9% SDS-PAGE as indicated in figure C-subunit level was detected by immunoblot analysis using C-subunit-specific antibody

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It was noted the elevated phospho-CREB level by C-subunit phosphorylation after cAMP-stimulation was attenuated to basal level rapidly by phosphatase-2-mediated dephosphorylation in the cell (Wadzinski et al., 1993), which suggested transcriptional induction of CRE-containing gene by CREB was transient and precisely regulated by the phospho-CREB level at the particular time after cAMP-stimulation. It was anticipated that kinetic profile of phospho-CREB level may be different in Cγ and Cα clones because of their different substrate specificity, different cellular location and unique kinetic features. To explore this possibility, the kinetic profiles of phospho-CREB were examined in the nuclear extracts of Cγ, Cα and control kin8 clones after varied-time cAMP exposure.

The results (Figure 33, Panel A) showed that phospho-CREB level in Kin8 control clone was not changed much along the 50 min time course of cAMP stimulation. The kinetics of CREB phosphorylation differs between Cγ- and Cα clones. The phospho-CREB level in Cα clone started to elevate by 5 min. or earlier, and was attenuated to basal level after 25 min. The duration of elevated phospho-CREB was about 20 min. after cAMP stimulation. In contrast, the phospho-CREB level in Cγ clone started to elevate at 10 min and was attenuated to basal level after 20 min. The relatively high level of phospho-CREB in Cγ was lasted about 10 min after cAMP stimulation. This result indicated that 1) Cα rapidly and sustainly phosphorylated CREB. In contrast, Cγ appeared to phosphorylate CREB slowly and transiently, 2) Basal level of phospho-CREB in Kin8 clone did not change along the course of cAMP-treatment.

Although this result suggested the existence of different kinetics of CREB-phosphorylation in the Cγ clone and the Cα clone, it was possible that cAMP stimulation itself might induce a time-dependent expression of CREB differently in the Cγ and Cα clones. In other words, different kinetic profiles of phospho-CREB level in Cγ and Cα clone might be a result of distinct induction of CREB level upon the time of cAMP stimulation rather than different CREB phosphorylation by

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Figure 33. Kinetics of CREB Phosphorylation in the Nuclear Extracts of Kin8, Cα and Cγ Clones after cAMP Stimulation

The nuclear extracts were prepared from Kin8, Cα2 and Cγ clones which were pre-stimulated by Zinc (80 μM) for 18 hour, then stimulated with cAMP (50μM) for various time (5, 10, 15, 20, 30, and 50 min.) as described in Methods. Nuclear extracts (10 μg) were loaded in each lane on three different gels and subjected to 9% SDS-PAGE. One gel was probed for phospho-CREB level by immunoblot analysis using (p-Ser^{133}) phospho-CREB antibody (Panel A). The second gel was probed for total CREB level by immunoblot analysis using CREB-specific antibody (Panel B.). The third gel was probed for protein levels by Coomassie blue stained SDS-PAGE (Panel C-1 and Panel C-2.). Clone 1: kin8 cells; Clone 2: Cα 2 cells; and Clone 3: Cγ 7 cells.

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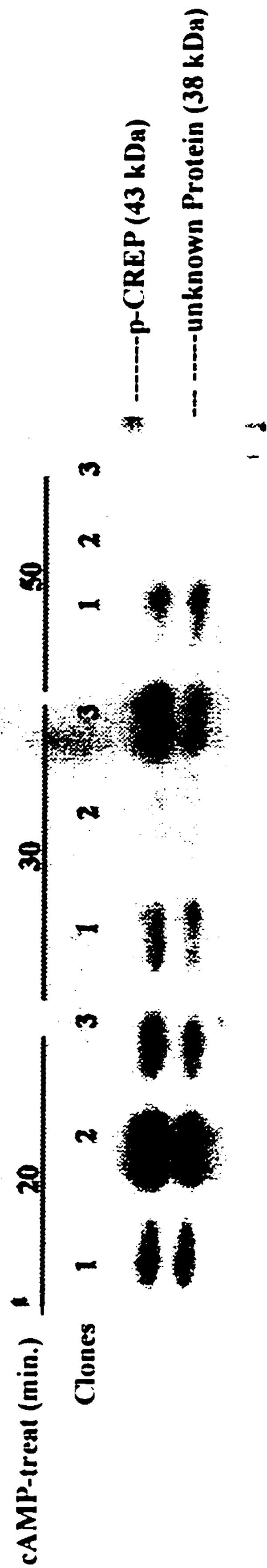

A. 1: Kin8; 2: Cα; 3: Cγ

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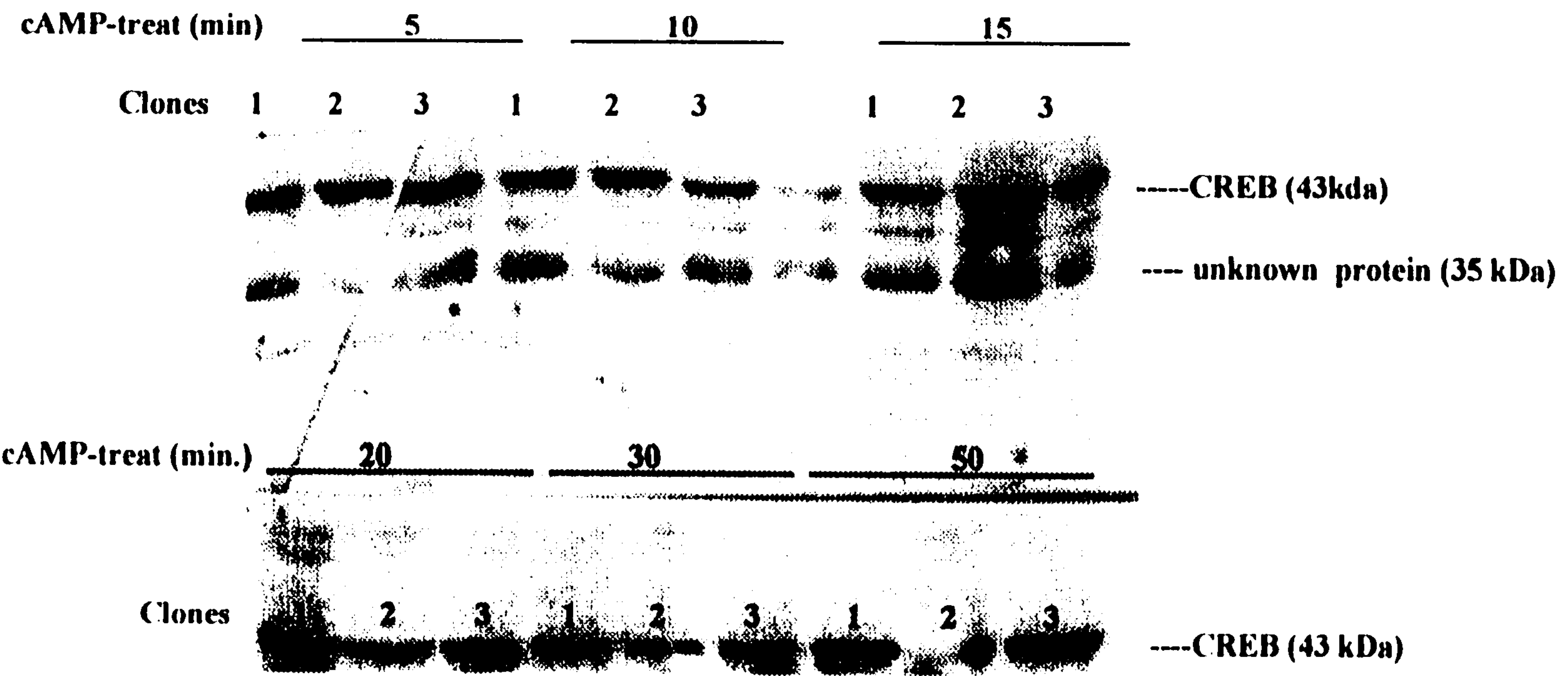

B. 1:kin8 ; 2: Cα ; 3: Cγ

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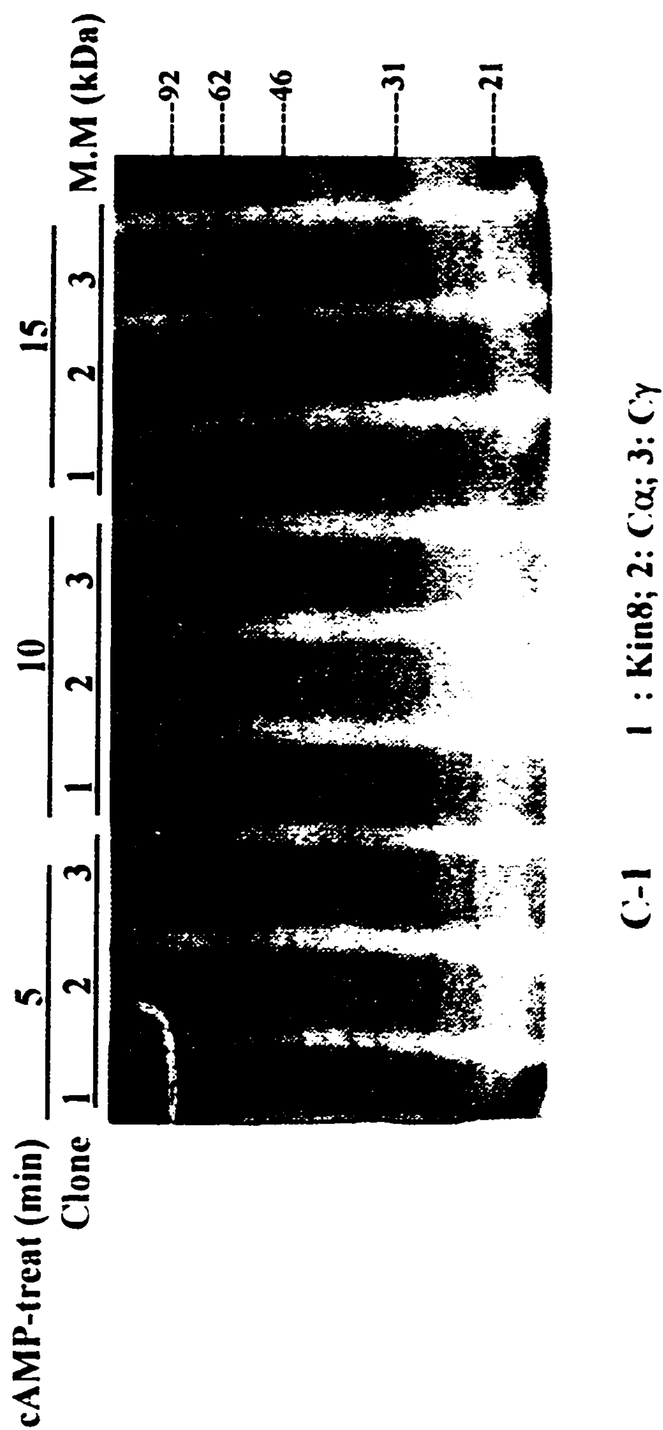

C-1 1 : Kin8; 2: Cα; 3: Cγ

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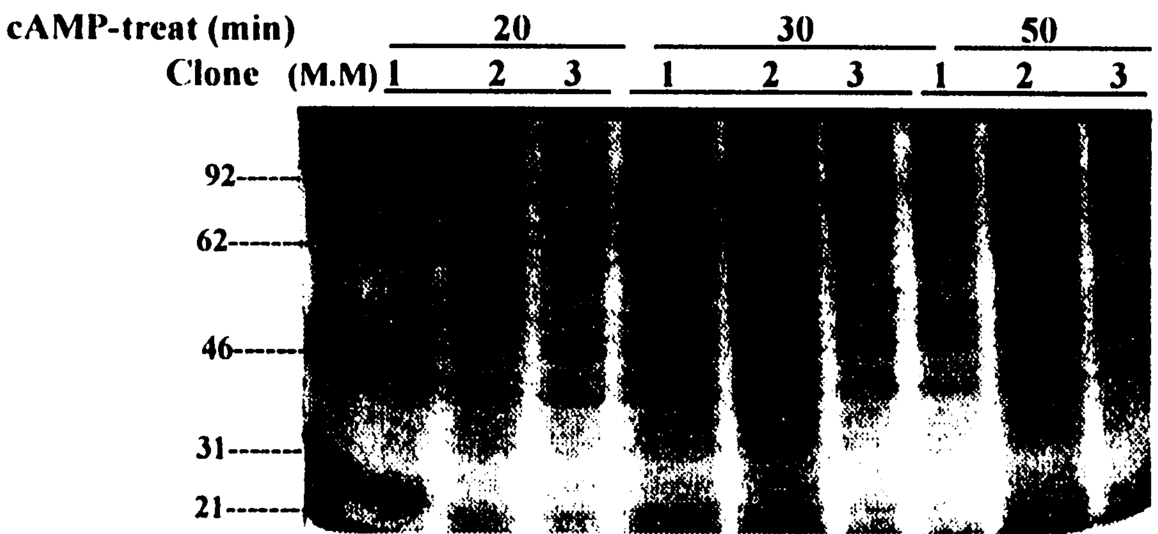

C-2. 1: Kin8; 2: Cα; 3: Cγ

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C-subunit isozymes in these three tested clones. To demonstrate if CREB level was change during the time course of cAMP-stimulation, the total CREB level was detected by immunoblot analysis using normal CREB antibody along the time of cAMP stimulation in these three clones. The results (Figure 33, Panel B) showed that the total CREB level was not changed in any three clones during 50 min cAMP stimulation. Similarly, the result from Coomassie blue stained-same SDS-PAGE gel showed that cAMP stimulation did not remarkably induce the time-dependent production of new nuclear proteins in these three clones (Figure 33, Panel C-1 and C-2). This also demonstrated that protein samples for these immunoblot gels were loaded equally. All these data indicated that Cγ can phosphorylate CREB in Ser^{133} like Cα, which was verified by specific phospho (Ser^{133})-CREB antibody, but both C-subunits had different kinetics for CREB phosphorylation. It is unclear yet how to correlate this result with distinct regulation of CRE-reporter gene expression by Cγ and Cα.

In some cell types, it has been suggested that Cα-subunit regulates CREB gene expression, but this is not universally present. The CREB gene promoter exhibits 3 CRE elements as well as other cis elements (Walker et al., 1995), so it is possible for C-subunit to regulate CREB gene expression. Therefore, an experiment was designed to determine whether Cγ, like Cα, affects the expression of CREB. This is another possible mechanism to account for the observed distinct regulation of CRE-reporter gene expression by Cγ vs Cα.

5. *Cγ and Cα stimulate the elevation of immunoreactive CREB protein level.* To explore the possibility that Cγ and Cα differently regulate total CREB protein levels in the nucleus, Cγ- and Cα-clones were cultured in the presence or absence of Zinc/cAMP. The total CREB levels were detected by CREB specific antibody. The results (Figure 34) showed the immuno-reactive total CREB protein levels were remarkably increased in the nuclear extracts of both clones after

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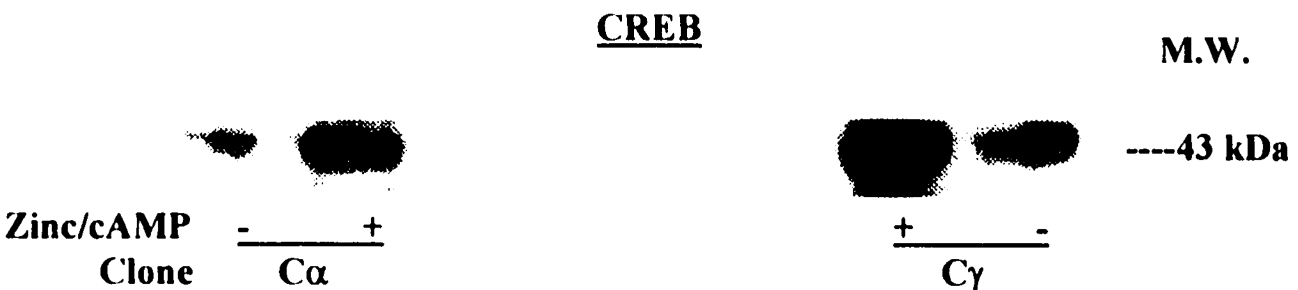

Figure 34. Induction and Activation of Cα and Cγ with Zinc and cAMP Result in Elevation of Immunoreactive CREB Levels in Kin8 Clones

Nuclear extracts were prepared from Cγ and Cα clones stimulated with Zinc (85μ M) plus cAMP (50μM) for 18 hours. Equal nuclear extracts (10 μg) as indicated in figure were run on a 9% SDS-PAGE. Total CREB protein level was determined by immunoblot analysis using CREB-specific antibody as described in Methods

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Zinc/cAMP which are activated by C-subunit- stimulation. In parallel, immunoreactive both C-subunits were increased after Zinc/cAMP stimulation (Figure 32). However, immuno-reactive CREB protein level was not increased in Kin8 nucleus after Zinc/cAMP stimulation (not shown data). This result suggested that the expression of both C-subunits by Zinc/cAMP stimulation specifically resulted in the elevation of immuno-reactive CREB protein level. Therefore, different effects of Cγ and Cα on CREB protein level is not likely to account for observed distinction in the regulation of CRE-reporter gene expression by C-subunit isozymes.

There are some other transcription factors which belong to ATF/CREB family. Cγ and Cα may have different actions to activate them. To explore this possibility, the gel-mobility shift assay was utilized to detect possible different transcription complex in the nuclear extracts of Cγ and Cα clone using consensus CRE sequence as probe as described in Methods.

6. *Similar CRE-mobility shifts were found in the nuclear extracts of Cγ, Cα and Kin8 clones*

Stimulation of CRE-containing gene expression by phospho-CREB is modulated by adjacent-associated proteins and many of them are substrates for C-subunits (Miller et al., 1993). Because Cγ and Cα have different substrate specificity, it is possible that Cγ and Cα differently manipulate the interactions between phospho-CREB and co-transcription factors by distinct activation, which mediates the observed distinction. Transcription factors in CREB/ATF family, which are activated by C-subunit-mediated phosphorylation, can bind CRE-DNA element as a promoter in cAMP-responsive genes directly or indirectly. To explore the possibility that Cγ and Cα differentially manipulate the interaction between phospho-CREB and its co-factors. Gel mobility shift assays were applied to detect the predicted unique complex in the nuclear extracts of Cγ and Cα clones after Zinc/cAMP-stimulation. Different CRE-mobility shift patterns on non-denaturing polyacrymide gels after they bind ^{32}P-labled CRE-probe would suggest that different elements of

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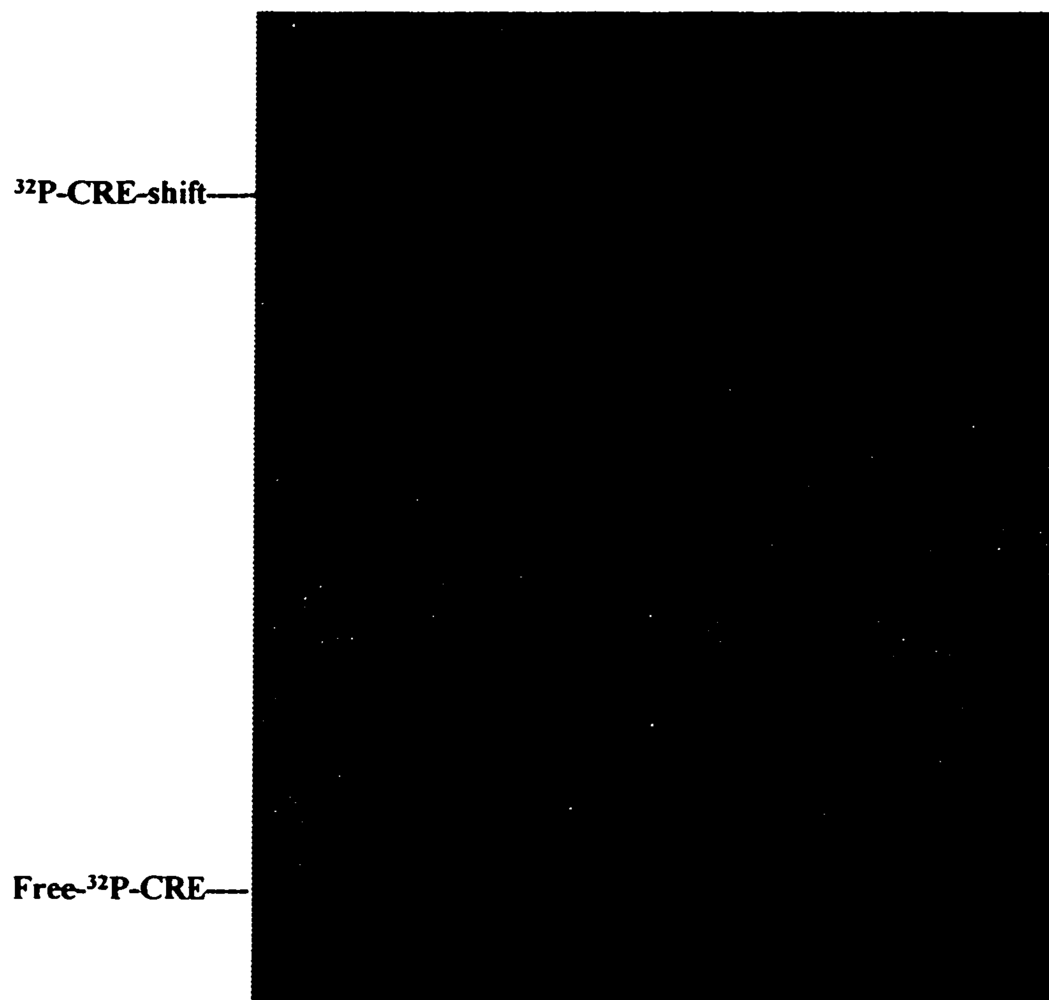

1: Kin8; 2: Cα; 3: Cγ

Figure 35. Gel Mobility Shift Analysis of CRE-Binding Transcription Factors in the Nuclear Extracts of Kin8, Cα, and Cγ Clones after Zinc/cAMP Stimulation. Nuclear extracts were prepared from Kin8, Cα and Cγ clones that were stimulated with Zinc(85 μM) plus cAMP(50 μM) for 18 hours as described in Methods. Equal amount of nuclear extracts (15 μg proteins) as indicated in figure were incubated with 0.5-1.0 ng ^{32}P-labeled CRE-consensus oligonucleotide as probe and analyzed by gel mobility shift assay as described in Methods. 5-fold and 10-fold molar excess of unlabeled CRE probe as specific competitor were added to demonstrate CRE-specific binding.

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CRE-binding complex exist. There are several variations surrounding consensus CRE sequence that affect specificity for transcription factors binding. In this experiment, ^{32}P-labled-consensus CRE (27 bp) sequence(Santa Cruz) was used as a probe to screen CRE-binding factors in the nuclear extracts of Kin8, Cα and Cγ clones as described in Methods. Gel mobility shift experiment (Figure 35) showed that transcription factors in Cγ (lane 3) and Cα clones (lane 2) bound to the CRE-probe resulting in a similar gel shift pattern. This shifted CRE-band was attenuated competitively by unlabeled CRE probe (4-9 lane). This indicated that this band-shift was CRE-specific.

It has been shown that elevated phospho-CREB levels induced by cAMP-stimulation would be attenuated into basal level in Cγ and Cα clones. In the basal level, there was not a detectable difference on the phospho-CREB level (Figure 33 Panel A). There is the correlation between the activation of transcription factors and co-factors by C-subunit-mediated phosphorylation and their CRE-binding affinity (Lundblad et al.,1995). Because the nuclear extracts used for gel-shift assay (Figure 35) were prepared from the cells which were stimulated by Zinc/cAMP for 18 hr, it was possible all differentially activated transcription factors by Cγ and Cα phosphorylation were returned to basal dephosphorylated form by phosphoprotein phosphatase in the cell. Therefore, we analyzed time-dependent effects of CRE-binding transcription factors in the nuclear extracts of all three clones after cAMP stimulation.

Figure 36 showed that the kinetic profiles of CRE-binding complex levels in the nuclear extracts of three clones after cAMP-stimulation. The formation of CRE-binding complex level appeared to change with the time of cAMP stimulation in the Cγ and Cα, but not in the Kin8 clone. The CRE-binding complex level in the Kin8 clone was not changed along 50 min cAMP treatment (lane 1, 4, 7, 10, 13, and 16). But, the CRE-binding complex level from nuclear extracts of the Cα clone started to increase at 5 min (lane 2), and reached to peak at 10 min (lane 5). After 30

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Figure. 36 Kinetic Analysis for CRE-binding Transcription Factors in the Nuclear Extracts of Kin8, Cα and Cγ Clones after cAMP Stimulation by Gel Mobility Shift Assay

The nuclear extracts were prepared from Kin8, Cα2 and Cγ clones which were pre-stimulated by zinc (85 μM) for 18 hours, then stimulated by cAMP (50 μM) for various time (5, 10, 15, 20, 30, and 50 min.) as described in Methods. Nuclear extracts (15 μg proteins) as indicated in the figure were analyzed for kinetic profiles of CRE-binding transcription factors by gel mobility shift assay using a ^{32}P-labled CRE-consensus oligonucleotide probe as described in Methods. Clone 1: kin8 ; Clone 2: Cα; and Clone 3: Cγ.

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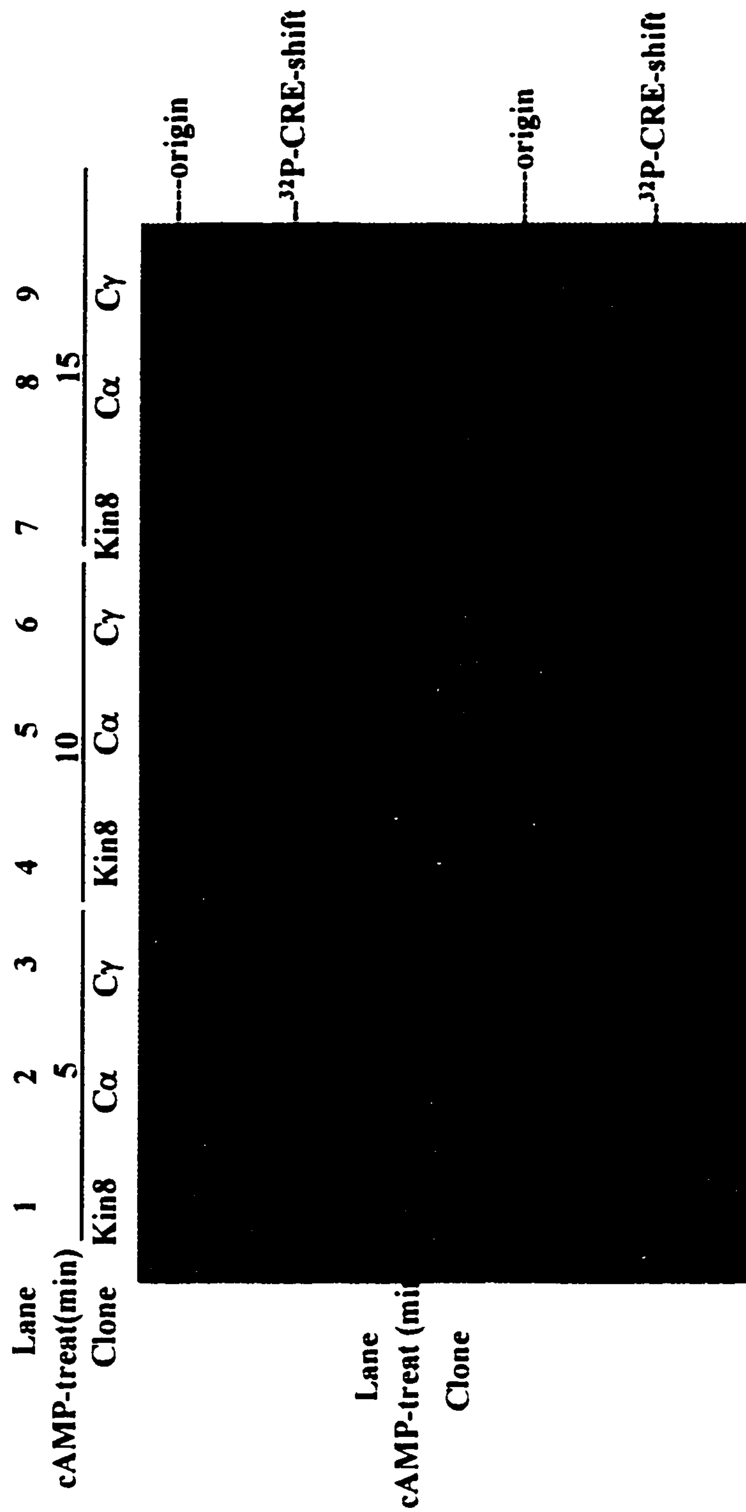

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min. the CRE-binding complex started to be attenuated (lane 17). In contrast, CRE-binding complex in Cγ nuclear extract appeared by 10 min. (lane 3), reached peak levels at 15 min (lane 9), then returned to basal levels after 20 min (lane 15). The kinetic profiles of CRE-binding complex levels (Figure 36) for Cγ and Cα were very similar to the kinetic profile found from CREB-phosphorylation (Figure 33). This suggested that phospho-CREB might be a major component in this CRE-binding complexes.

To explore if phospho-CREB was one component in the CRE-shift band from Cγ and Cα clone, phospho-CREB (Ser^{133}) antibody was utilized to detect the presence of phospho-CREB in the CRE-complex by super gel mobility shift assay as described in Methods. The phospho-CREB antibody specifically binds to phospho-CREB in the nuclear extracts. Phospho-CREB bound with anti-phospho-CREB becomes bigger in molecular weight than free phospho-CREB, which results in further retardation (super-shift) of CRE-probe on non-denatured polyacrymide gel. The gel (Figure 37) showed that anti-phospho-CREB resulted in similar super CRE-band shift in the nuclear extracts of same three clones. This suggested that phospho-CREB was at least one component of these CRE-binding complexes. Results from gel mobility shift assay did not show notable difference, which suggested this approach may be not sensitive enough to identify the differences occurred on the transcription factors in these three clones, because the results from CRE-reporter gene studies (Table 16) and intact cell studies in Y1/Kin8 cells (Table 1) demonstrated the existence of such differences. This distinct role of Cγ and Cα in the regulation of CRE-reporter gene activity has been observed in two CRE-reporter gene system (not shown data). One CRE-element was from the promoter of α-glycoprotein gene (α-luc-168). The other CRE was from the promoter of enkinphreon gene. This suggested that CRE was not so specific and might be not necessary to conduct distinct role on the regulation of CRE-reporter gene expression by C-subunit isozymes.

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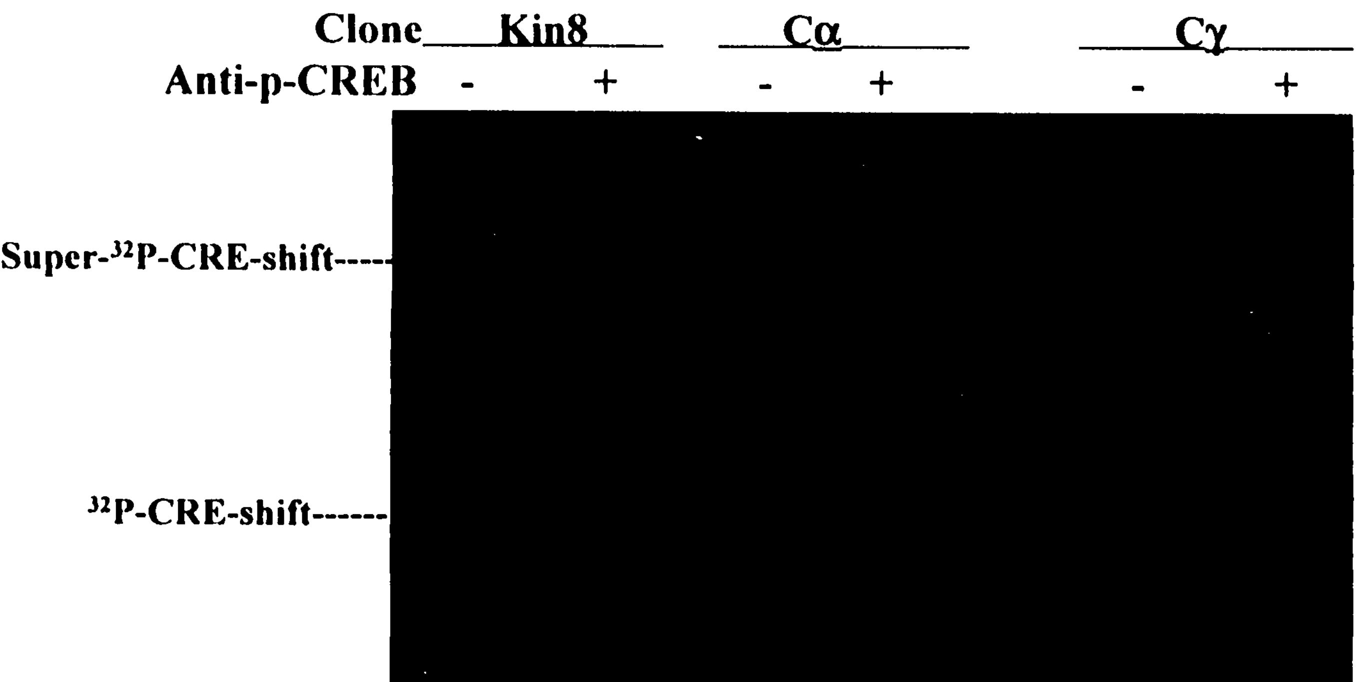

Figure 37. Phopho-CREB is one component of CRE-binding transcription factors in the nuclear extracts of Kin8 , Cα and Cγ after Zinc/cAMP stimulation.

The nuclear extracts were prepared from Kin8, Cα and Cγ clones which were stimulated with Zinc (85 μM) for 18 hour and cAMP(50 μM) for 15 min. as described Methods. Nuclear proteins (10 μg proteins) was incubated with 0.5-1 ng ^{32}P-labled CRE-consensus oligonucleotide probe in the presence and absence of 2 ng phospho-CREB(Ser^{133})-specific antibody as indicated in figure, then subjected to super-gel mobility assay as described in Methods.

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In addition, increasing number of isoforms of transcription (co)factors in CREB/ATF family and cross-reaction among them have been reported, which make it harder to inspect them one by one only by CRE-gel mobility approach. This situation promoted us to find a efficient way to point toward our target, or at least to rule out some possibilities. Gal4 reporter gene system was utilized to determine if CRE and dimerization of CREB are required for or involved in the distinct and cooperative regulation of CRE-gene

activity for this purpose.

7.CRE sequence and dimerization of CREB are not necessary to show the distinction and cooperative interaction between the Cγ and Cα in the regulation of CRE-reporter gene activity

In the Gal4 reporter gene experiment, two Gal4 expression vectors were utilized. Gal-4 protein binding DNA sequence is used to replace the CRE sequence as a promoter in a luciferase reporter gene vector to form Gal-4 luc (kind gift from Dr. Quinn, University of Pennsylvania). The DNA sequence for Gal4 DNA binding domain is fused with the DNA sequence encoding for CREB activation domain ($CREB_{1-277}$) to form Gal4-CREB fusion protein expression vector (kind gift from Dr. Quinn). It was shown that CRE sequence itself and dimerization of CREB were not necessary for Cα to regulate the reporter gene activity, as long as phospho-CREB(1-277) binds to the upstream of cAMP-responsive gene (Figure38) (Quinn 1993, 1994). This prompted us to determine if CRE and dimerization of phospho-CREB are necessary to show the distinct function between Cγ and Cα in the regulation of CRE-reporter gene activity. If Gal-4 reporter gene and CRE-reporter gene activity were regulated by Cγ and Cα similarly, it will help us to rule out some possibilities which are associated with varied CRE-elements and complicated cross-interaction among transcription factors

To conduct this experiment, Gal-4 luc and Gal-4 CREB were transiently cotransfected into HEK 293 cells with pCMV Cγ or pCMV Cα and their combination as described in Methods The

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results (Figure 35) showed that 1) Cα could stimulate 5-20 fold of Gal-4 luciferase reporter gene activity in concentration-dependent way ; 2) Cγ alone could not stimulate Gal-4 luciferase gene activity (less than control); 3) cotransfected with low amounts of Cα (≅0 3μg) and low amount Cγ (0.3 μg) Gal-4 luciferase reporter gene activities were enhanced 5-9 fold greater than the sum of the efficiency of Cγ and Cα. This result was similar to the result from cotransfection experiment with α-luc CRE-luciferase reporter gene system (Fig 30). The Gal-4 results (Figure 39) indicated that CRE and dimerization of CREB were not necessary for the presence of distinction and interaction between Cγ and Cα in the regulation of CRE-containing gene expression. This result also predicts that the distinction and cooperative interaction between Cγ and Cα in the regulation of CRE-reporter gene activity is mediated by a intra-CREB-molecular(1-277) mechanism or in the domain of CBP or other co-transcription factors, which complementary interacts with trans-activation domain of phospho-CREB.

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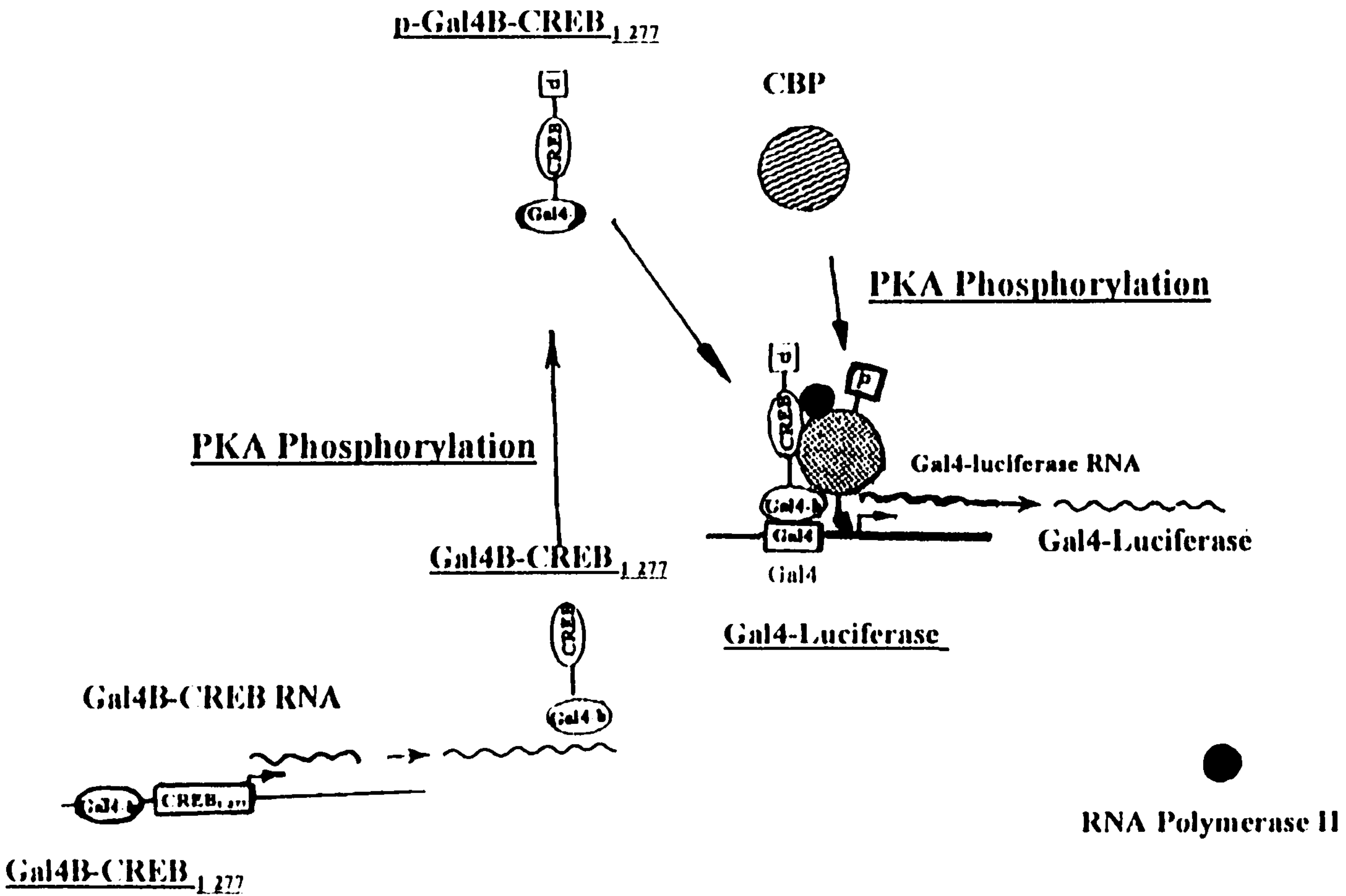

Figure 38 Diagram For the Gal4-CREB and Gal-4-Luciferase Reporter Gene Expression System

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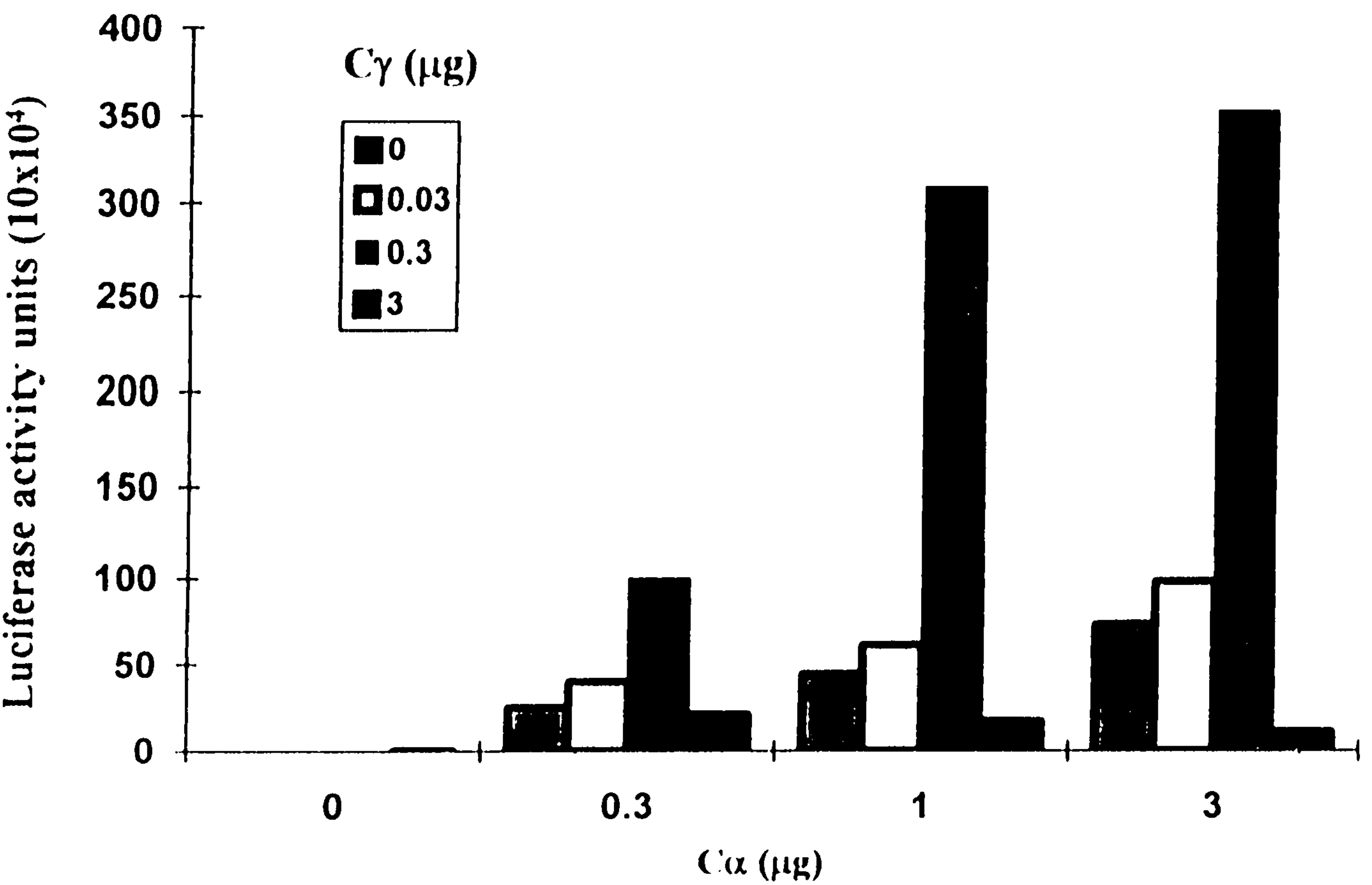

Figure 39 CRE and Dimerization of CREB Were Not Necessary for the Presence of Distinction and Cooperation between Cγ and Cα in the Regulation of CRE-Reporter Gene Activity

HEK 293 cells from six wells (with 70% confluent) were cotransfeted with 1 μg Gal4-luc, 1μg Gal4-CREB, and various amounts of pCMV-Cγ and/or pCMV-Cα or their combinations as indicated in the figure by calcium-phosphate coprecipitation The luciferase activities were assayed from each transient transfected cells after 4 hours transfection and 18 further growing as described in Methods The data shown were derived from one representative experiment The same experiments were performed three times with less than 15% variation

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CHAPTER IV

DISCUSSION

A. Expression and Purification of Recombinant Proteins for *In Vitro* Study

Cγ was expressed as inactive and insoluble proteins in bacteria after intensive attempts to active it. This result suggested that Cγ needed to be modified or re-folded by some eukaryotic cherpone used for maturation or activation after translation. This function was absent in the bacteria. Considering the facts that Cα and Cβ were successfully expressed as active proteins in bacteria (Gamn et al., 1996), it is suggested that Cγ expression differs from other two isoforms of C-subunit and is eukaryote-specific or modulated in different way. Interestingly, two isoforms of C-subunits from *Aplysia* were expressed as insoluble and less-active proteins in bacteria under a variety of growth conditions and attempts to naturalize these proteins failed to yield fully active kinase (Cheley et al., 1992). The results suggested that Cγ appeared to be closer to the C-subunit from an insect cell than other mammalian C-subunit isoforms in terms of the requirement for eukaryotic factors for activation. These results also suggested that some Cγ-specific requirements for the expression of active enzymes in bacteria are not well understood, and some specific requirements for the activation of C-subunits expressed in bacteria varied with different C-subunit isozymes.

There are several differences between eukaryotic and prokaryotic systems in the expression of recombinant proteins. However, posttranslational modifications are the most important ones. At least two posttranslational modifications have been characterized for C-subunits, which are myristylation and phosphorylation

Myristylated proteins can be separated into two categories, those that are soluble and those that associate with membrane (Towler et al., 1988). In the protein associated with membrane, myristylation is believed to provide a hydrophobic anchor for the protein to associate with membrane (Chow et al., 1987). The structure of the mammalian C-subunits served as the first

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example of how a myristyl group can contribute to the stability of a soluble protein. It is less likely for Cγ expressed in bacteria to be myristylated, because bacteria do not have this metabolic enzymes for the myritylation. In addition, if Cγ was transcripted from the second AUG cordon, Cγ does not exhibit N-terminal free glycine, which is required for the acylation with myristic acid for recombinant Cα (Carr et. Al., 1982).

Recombinant Cγ-subunits expressed in eukaryotic systems have the potential to be myristylated, but it is still not clear about the myristylation of recombinant Cγ. One thing is clear that myristylation only stabilizes Cα in eukaryotic cells, but does not affect the substrate specificity or kinetic behaviors for C-subunits. For example, when C-subunits were first expressed in *E. coli*, it was not myristylated because E. coli., lacks *N*-myristyl transferase (NMT). But the unmyristylated C-subunit was kinetically indistinguishable from the myristylated form in mammalian cells though the myristylated C-subunits was more labile to heat denaturalization in the monomeric state and in the holoenzyme complex (Slice et al., 1989; Yonemoto et al., 1993). It is unlikely that myristylation of the isozymes will alter the substrate-specificity for these recombinant C-subunits.

Compared with recombinant Cα-subunit, the recombinant Cγ-subunit was less stable in vitro and in intact cell. Myristylation stablized C-subunits, which suggested that recombinant Cγ-subunit might not be myristylated. Recombinant Cγ always exhibits lower stability than Cα regardless of cell system, which suggested that decreased stability was another unique feature for Cγ.

Cγ has an intronless gene structure (Beebe at al., 1990). The proteins with intronless genes have been shown to conserve some identities, including low-stability, limited tissue-specific-distribution and low expression levels. All the previous data in this laboratory indicated that Cγ shared these common features for intronless proteins. The function of intronless proteins has been associated with the cell differentiation, which implies that Cγ may be transiently expressed and can phosphorylate the substrates which are associated with cell differentiation. This is consistent with

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fact that Cγ exists in human testis. There are undifferentiated cells in human testis The low level expression and less-stability of Cγ may correlate its transient specific function The results from U937 cell studies supported this hypothesis that Cγ is present in undifferentiated cells and involved in the cell differentiation of U937 cells. When U937 cells were induced to differentiate by cAMP-stimulation, two types of PKA holoenzymes were separated by chromatography Results showed that the type II holoenzyme was decreased, but the type-I did not changed much. This suggested that type-II holoenzyme was associated with physiological change of U937 cells from proliferation to differentiation. It was reported before that the type-II holoenzyme mediated some cell differentiation (Beebe and Corbin, 1986). Interestingly, Cγ-like PKI-insensitive kinase activity was increased and located in type-II holoenzyne of differentiated U937 cells (Table 4) Cγ-like PKI-insensitive kinase activity were further identified as cAMP-dependent histone kinase with 39 kDa mammalian Cγ-specific immunoreactive bands (Figure 10, Table 4) All these features are consistent with known criteria for Cγ-subunits. Result from U937 studies suggested that Cγ-like protein expression was specifically associated with the differentiation of U937 cells Of course, more experiments are needed to further confirm this hypothesis. For example, it will be important to demonstrate the presence of Cγ-specific mRNA only in differentiated U937 cells, but not in undifferentiated cells.

Autophosphorylation, which is required for full activity of Cα (Toner-Webb et al., 1992), is the other posttranslational modification that could affect either kinase, or mobility shift of recombinant C-subunit bands on SDS-PAGE, but not C-subunit substrate specificity The autophosphorylation of C-subunits is believed to be a critical step in the final maturation of C-subunit in eukaryotic cells, (Steinberg et al., 1993) Cγ has been expressed in three cell systems and resolved as two close immunoreactive protein bands There are several possible explanations for the presence of more than two Cγ bands in the immunoblot analysis Alternative uses of two in-frame AUG start

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cordon (Beebe et al., 1990) in the transcription of Cγ-cDNA was thought as a possible reason, which resulted in two active Cγ bands with nine amino acid difference. But, it becomes less likely because Cγ expression vector containing only one AUG start cordon was also expressed as more than two Cγ immuno-reactive proteins in bacteria.

As it was mentioned above, autophosphorylation of Cα caused the up-shift of Cα-band on SDS-PAGE (Steinberg et al., 1993).Therefore, an other possible explanation is that Cγ may become a substrate for Cα or other kinase. This autophosphorylation of Cγ may be necessary for activation of recombinant Cγ. Consequently, the autophosphorylation of Cγ might cause the two close immunoreactive Cγ protein bands. The lower band was unphosphorylated Cγ and less active, the higher band was active phosphorylated Cγ. There are two pieces of evidence to support this hypothesis: 1) recombinant Cγ expressed in bacteria was inactive and had relatively smaller apparent molecular weight compared with those active Cγ expressed in eukaryotic cells due to absence of posttranslational autophosphorylation. 2) when recombinant Cγ expressed in Sf9-baculovirus system was purified by CM-Sepharose chromatography, two kinase peaks were revealed in elution profiles. The peak-II contained only PKI-insensitive kinase activity, and exhibited two immunoreactive C-subunit bands, but the final homogenous active Cγ contained only higher Cγ band. Therefore, it is possible that autophosphorylation of Cγ isofom might be important to activate Cγ and account for the presence of two immunoreactive Cγ-bands in all three cell systems, but it requires further studies to prove it.

The reason for the large differences in expression levels between Cγ and Cα in Sf9 cell using baculovirus is not clear. Morphological observation showed that Cα-baculovirus infected Sf9 cells stopped growing after two day post-infection (not shown data). In contrast, recombinant Cγ-baculovirus infected Sf9 cells kept growing in seven day post-infection. These results suggested that the expression of both C-subunit isozymes differentially induced the inhibition of cell

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proliferation. In Kin8 cells, transfection of Cγ or Cα caused the inhibition of cell proliferation (Table 1). This result suggested that eukaryotic cells have limited ability to tolerate recombinant C-subunits.

The viral lysis curves of infected Sf9 cells with both recombinant C-subunits in baculovirus appeared to be similar based on the observation that both recombinant C-subunits started to be released from infected Sf9 cells at similar time (Figure 10 and Figure 12). Although exact mechanism underlying this distinction of both C-subunits on the inhibition of infected Sf9 cell's proliferation is unknown, a possible explanation is that both C-subunits expressed in Sf9 cells are active, but the differences in the modification for activation after expression and substrate specificity of both recombinant C-subunits in Sf9 cells cause the distinct inhibition of infected cell growth. For example, Cα probably induced the early inhibition of cell proliferation due to lack of a requirement for the modification to induce its activation after translation and/or specific phosphorylation of some regulatory substrates in host cells, which initiated the signal to stop cell proliferation. In contrast, Cγ might need some time to be modified for full activation after its translation and/or it could not phosphorylate such regulatory substrates in host cells as to initiate signal for the inhibition of infected Sf9 cell growth. In other words, it is predicted that this delay for Cγ-activation and/or the deficiency of Cα-mediated specific phosphorylation of regulatory substrate in host cells resulted in the distinct effects on infected cell proliferation. The relatively low expression level of C1-subunits from insect cells (*Aplysia*) in same system has been reported to be due to slowed Sf9 cell growth after infection by C1-baculovirus (Cheley et al., 1992). Interestingly, although Cγ and Cα-subunits had differences in the expression level and in the effects of infected cell's proliferation, Sf9 cells were induced to extend long neturite-like tail only by both recombinant C-subunit baculovirus infection, but not wildtype baculovirus infection (not shown data). The same long neturite-like structure was observed in the Sf9 cells infected by

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Aplysia C1-recombinant baculovirus (Cheley et al., 1992), which has been identified as a result from host p-10 protein phosphorylation by recombinant *Aplysia* C1-subunits. The protein p-10 is a 10 kDa polypeptide which is heavily phosphorylated at the same time when C-subunit baculovirus infected Sf9 insect cells by extended neturite-like structures (Cheley et al., 1992). The fact that the same long neturite-like structure in the infected Sf9 cells was induced by both C-subunit isozymes suggested that Cγ and Cα could phosphorylate p-10 protein.

In this project, the recombinant Cγ and Cα have been purified to homogeneity with 0.85 (μ mol ^{32}P-incorp./min./mg with histone as substrate) and 6.96 (μmol ^{32}P-incorp./min./mg with Kemptide as substrate) as specific kinase activities respectively. This is the first time for the recombinant Cγ to be purified to homogeneity. The specific kinase activity of Cα is close to that derived from bacterial recombinant Cα prepared by Baude (1994). It was predicted that the specific kinase activity of both recombinant C-subunits could be improved if denatured C-subunits can be removed by affinity chromatography.

Compared with Cα, Cγ appeared to have relatively low specific kinase activity. One possible reason is that there are some denatured Cγ-subunits which were not able to be removed. Because recombinant Cγ has lower stability than recombinant Cα, this may become a major reason for reduced specific activity. Lower turnover number of Cγ for substrate phosphorylation (Table 10, 11) may serve as another reason for low specific kinase activity of Cγ. In addition, the histone III used as a substrate for Cγ kinase activity assay may be not as good a substrate as Kemptide for Cα. For example, Cγ phosphorylated PKG-substrate-2 (peptide-3) 20-fold better than Cα. This implies that specific kinase activity for Cγ-subunit isozyme is varied with different substrates.

Present expression study indicated that recombinant Cγ expression differed from recombinant Cα in expression level and , posttranslational modification. Cγ-subunits always were expressed as two close immunoreactive bands in all three systems regardless their activity and expression levels

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In contrast, Cα was expressed as one active C-subunit band in all three cell systems. Active Cγ-subunit expression was eukaryotic cell specific. Cα was expressed as active protein in prokaryotic and eukaryotic expression system, but Cγ could be efficiently expressed only in eucaryotic cells. All these distinctions between Cγ- and Cα-expression suggested that cell systems were not the only reason to account for these differences. Some inherent differences between Cγ and Cα were observed in distinctive expression levels, kinase activities and protein modifications. This research also shows that optimal expression of each recombinant C-subunit isozymes may depend on cell systems, but expression system did not effect the substrate and pseudosubstrate specificity for either of recombinant C-subunits. All the recombinant C-subunits used for this research exhibit similar substrate and pseudosubstrate specificities no matter what expression systems was used, their expression levels and their specific activities. Therefore, it is no problem to use the homogeneous C-subunits expressed in different cell systems for kinetic comparison.

B. Kinetic Analysis of C-Subunits for Substrate Specificity

The results from kinetic comparison for both C-subunits on substrate phosphorylation (Table 10) indicated that Cγ and Cα had different substrate specificity. The substrate specificity of Cγ-subunit was predicted based on different cell phenotypes in permanently Cγ/Cα transfected Kin8 clones (Beebe et al., 1992). To confirmed this prediction, recombinant C-subunits expressed in different systems were first compared for their substrate and pseudosubstrate specificity with crude cell lysate (Table 3) and further extended by the kinetic analysis for substrate phosphorylation with homogeneous C-subunits.

Compared with Cα, Cγ always had lower Km values for substrate and Mg^{2+}-ATP binding, but turned over those bound-substrates much more slowly. This kinetic comparison provides molecular explanation for previously observed substrate specificity for both C-subunit isozymes. Results

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(Table 11) generated from kinetic comparison of both C-subunits on the phosphorylation of seven other synthetic peptides provide more detailed evidence to demonstrate the existence of substrate specificity between Cγ and Cα. All these results from kinetic comparisons provide some important information about unique catalytic feature of Cγ for the first time. These are summarized as follows:

1. *Cγ and Cα shared the primary PKA kinase substrate specificity*: The initial investigations exploring the nature of C-subunit's substrate phosphorylation site were focused on the target phosphorylation site from hepatic pyruvate kinase. Kemptide was synthesized peptide based on the phosphorylation site of pyruvate kinase, and now was treated as efficacious a substrate as its parent protein, pyruvate kinase (Pilkis et al., 1980). Those studies demonstrated that the importance of the pair of arginine residues on the amino-terminal side (P^{-3} and P^{-2}) of the phosphor-accetator, serine, with apparently poor tolerance for deviation from this strict motif. Shifting the pair of arginine either closer to, or further from, the target serine residue or their conservative substitution by lysine, markedly compromised the peptide as substrate for the C-subunit (Kemp et al., 1975; 1977). This unique phosphorylation site sequence (RRXSY) has been considered as primary substrate specificity for PKA kinase. Present results (Table11) showed that both C-subunits could phosphorylate peptides(1-5) with RRXSY phosphorylation sequence, but not phosphorylate peptide (6-8) with RRXX(x)SY as phosphorylation sequence. This result indicates that Cγ and Cα shared primary substrate specificity for PKA kinase. Therefore, Kemptide may be the best substrate for C-subunits, and can be used to differentiate PKA kinases from other kinases. Being insensitive to PKI-inhibition, Cγ can be differentiated from other C-subunits (Cα and Cβ) for substrate phosphorylation by PKI.

The results from this kinetic analysis also indicated closely related protein kinases (such as A-, C-, and G-kinases) have their particular substrates in terms of its low Km and high Vmax values

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The convergent evolution forces them to phosphorylate multiple substrate with overlapping specificity with other kinase (Walsh et al., 1994). For example, both C-subunits could phosphorylate PKC-peptide as well as Kemptide in term of Vmax/Km values. The same phenomenon may apply to Cγ- and Cα-subunit isozymes. The amino acid residue sequence of Cα-subunit isozyme shared a high degree of similarity (83% identity) with those from Cγ-subunit isozyme. Therefore, both C-subunit isozymes shared the primary substrate specificity as observed in this research. But, the inherent sequence difference (17%) between the both isozymes provides the structural basis for them to sense substrate structure differently.

2. *Cγ-subunits had unique catalytic feature for substrate phosphorylation.* Cγ shared some substrate specificity with Cα, especially for primary phosphorylation site of substrate to PKA kinase. But, inherent differences in structure between Cγ and Cα determined that Cγ had unique substrate specificity and catalytic features. The results from these kinetic comparisons(Table 10 and 11) suggested that Cγ generally exhibits as good or better a Km value, but a lower Vmax value for substrate phosphorylation if compared with Cα. It is envisioned that enzymatic reactions take place in cell micro-environments and these kinetic differences may be significant in cells, especially if there is a temporal effect of the phosphorylation reaction on cell function. The Vmax/Km ratios differ between Cγ and Cα, which suggests that one or more steps in transferring phosphate group from ATP to peptide/protein substrate differs. Cγ appeared to bind substrate with higher affinity than Cα, but turn-over rate was much slower. These kinetic features of Cγ predicate that Cγ can act as an inhibitor for some Cα-mediated functions by occupying the substrate faster than Cα, especially at some region where the available substrates become a limited factor. These kinetic differences may provide a mechanism to fine-tune cAMP signaling and under some conditions may determine the presence or absence of phosphorylation.

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3. *C-terminal amino residues to phospho-acceptor site (Ser/Thr) might serve as the structure to differentiate substrate specificity between Cγ and Cα.* This kinetic comparison of both C-subunits for substrate specificity not only showed the unique catalytic feature for Cγ, but also provide evidence to define the structure which differentiates substrate specificity for Cγ- and Cα-subunit. Results (Table 11) showed that the only difference in sequence between the PKG-substrate-1 and PKG-substrate-2 was that the Ala at P^{+2} in PKG-substrate-1 which was substituted by basic Lys in PKG-substrate-2. One more basic amino acid at P^{+2} position eliminated the ability for Cα to phosphorylate PKG-substrate-2 (1000-fold decreasing in Vmax/Km value), but Cγ still could phosphorylate this peptide (5-fold decreasing in Vmax/Km value). This result indicated that the structure for Cγ and Cα to sense substrates differently was located at the C-terminal to phospho-acceptor Serine.

4. *Cγ and Cα phosphorylate native cellular proteins differently.* The results derived from cellular protein phosphorylation in Kin8 clone by recombinant C-subunits (Figure 27) suggested that both C-subunt isozymes might phosphorylate natural proteins differently. Several explanations could be made to account for this observed distinct phospho-protein profile in Cγ and Cα clones. 1) Zinc/cAMP stimulation induced the production of new proteins in respective cells. The isozyme-specific phospho-protein bands was resulted from distinct protein composition of both C-subunit clones rather than C-subunit isozyme-mediated specific protein phosphorylation. In other words, Cγ and Cα indirectly caused different protein phosphorylation. 2) The protein composition in C-subunit expressing clones were similar, distinct phospho-proteins in respective C-subunit expressing clones were resulted from C-subunit isozyme-specific phosphorylation. The total protein profile in the cytosol and nuclear extracts of these clones were detected by Coomassie-stained-SDS-PAGE. The result (Figure25, Panel b) showed that total protein profiles in each respective lane were similar. These results suggested that different phosphorylation profiles in Cγ

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and Cα clone were most likely a result of distinct substrate specificity for both C-subunit isozymes.

The results also suggested that such substrate specificity exhibited by Cγ and Cα partially depended on the presence of native conformation of the proteins substrates, because homogeneous Cγ and Cα phosphorylated the heat-denatured proteins in cytosol and nuclear extracts similarly (Figure 28). It is still not known which kind of proteins were phosphorylated distinctively by Cγ and Cα in the cell. How much do these distinct substrate phosphorylation account for the exhibited distinct phenotype in respective C-subunit expressing clones (Table 1). But, the data presented here provides additional evidence to support the original hypothesis that substrate specificity of the C-subunit isozyme accounts for the observed distinct Y1/Kin8 cell phenotype which are permanently transfected with Cγ and Cα.

C. Kinetic Analysis of C-Subunits for Pseudosubstrate Specificity

PKI is a small (80 amino acids), potent, heat-stable and specific inhibitor for the C-subunit (Glass et al.,1986). PKI competitively binds to substrate-binding site of C-subunit as pseudosubstrate with tremendously high affinity, especially in the presence of cAMP when the C-subunit is dissociated from the holoenzyme as free subunit (Walsh et al., 1971) Present results demonstrated that Cγ was totally insensitive to all three tested forms of PKI The crystal structure of C-subunit bound with PKI_{5-24} peptide (Knighton et al., 1991) and model peptide studies of PKIαF10 (Glass et al., 1989a; 1992b) suggested that the hydrophobic pocket of C-subunit was important for C-PKI interactions. The presence of Tyr^{235} and Phe^{239} in this hydrophobic pocket are needed for full inhibitory efficiency of PKIs. Interestingly, Cγ-isozyme has a similar conserved hydrophobic sequence (Beebe et al., 1992). Similarly, PKC is a PKI insensitive kinase, but PKC conserves this hydrophobic pocket sequence. This phenomenon may imply that hydrophobic pocket is important

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for PKI-inhibition, but the kinases with hydrophobic pocket structure do not imply that they have to be inhibited by PKI. Why do these PKI-insensitive kinases still conserve this hydrophobic sequence ? This phenomenon may suggest that some other residues modulate this C-PKI interaction. Wen (1994) demonstrated that mutation of Arg^{133} (beyond hydrophobic pocket) into Ala in Cα selectively abolished PKI high-affinity binding to Cα. In Cγ, Arg^{133} is conspicuously replaced by Gln. It was predicted that this single amino acid difference would abolish the ability of Cγ to bind to PKI, which accounts for the inability of Cγ to be inhibited by PKI. As mentioned before, PKI inhibits C-subunit kinase activity as a competitive inhibitor. It is most likely that Cγ does not bind to PKI, though there is not direct evidence to prove it (Wen et al 1994). In this experiment (Table12), it was also shown that substrates affect the PKI-inhibition for Cα, which may be due to the competitive-binding of PKI and substrate to the C-subunits

In spite of intensive biochemical studies, the physiological roles of PKI remain unclear. It was proposed that PKI provided a mechanism to dampen the kinase response by inactivating low levels of enzyme (Walsh et al., 1990). PKI expression level was correlated with cell division cycle. When the cell entered division cycles, the PKI expression was elevated. This result suggested that the function of PKI might be associated with the regulation of cell proliferation. The association of testicular PKI with microtubules (Tash et al., 1980) and the isolation of several PKI isoforms (Olsen et al., 1991; Van Patten et al., 1991; Scarpetta et al., 1993) that exhibited tissue- and development-specific expression, suggested additional roles for PKI in the cell (Van Patten et al., 1992). Recent studies demonstrated that PKI entered the nucleus following its injection into the cytoplasm (Fantozzi et al., 1994) and exported the C-subunits from the nucleus into cytoplasm, which suggested a specific role for PKI in the regulation of cAMP-mediated gene expression by reducing nuclear staying time of the C-subunits (Wen et al., 1995). It was shown that catalytic Cγ-subunits were elevated in the nucleus by cAMP (Figure 29, Table 18), which suggested that Cγ .

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like Cα, might translocate into the nucleus. PKI-insensitive Cγ has the potential to avoid the PKI-mediated down-regulation of gene expression in nucleus. PKI is a potent inhibitor for Cα and Cβ, but the inhibitory-potency varies with different isoforms of PKI and different isoforms of C-subunit (Gamm & Uhler, 1996), which suggests that different C-subunit isoforms can exhibit different sensitivity to PKI-inhibition. It is possible that Cγ evolved to be a PKI-insensitive enzymes from this differentiation of PKI-sensitivity.

The PKI-insensitivity of Cγ and the PKI-sensitivity of Cα are the most striking difference between the two isozymes, though the role(s) for this inhibitor in cell function is not yet clearly defined. Nevertheless, in the presence of the inhibitor, the effects of Cα, but not Cγ, would be blocked and a different pattern of protein phosphorylation would likely occur. This was supported by the observation that different phosphorylation profiles in the cytosol and nuclear extracts of Cγ and Cα clones were present (Figure 28). It is predicted that the physiological function of PKI is partially correlated with the function of Cγ in the cell.

The results from these *in vitro* studies indicated that the interactions of these purified C-subunit isozymes with purified recombinant R-subunits are indistinguishable, or the difference was subtle. The absence of distinction in RIα- and RIIα-inhibition for Cγ and Cα based on IC50 values of R-inhibition seems to be a discrepancy to the observation in intact cell. In the intact cell, Cγ, but not Cα, restored type-II PKA holoenzymes. This result suggested that Cγ bind to RII with higher affinity than Cα (Beebe et al., 1997). However, intact cell phenomena were not observed *in vitro*. This discrepancy between the *in vitro* results and the observation in intact cell may suggest that determination of IC50 values is not sensitive enough to detect the existence of different affinity between Cγ and Cα for the R-subunit. It has been known that the affinity between R-C in the cells was manipulated by following several factors. 1) The substrate affects the R-C interaction by inducing conformational changes of C-subunits (Rannels et al., 1980, Granot et al., 1980). 2)

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cAMP binds to two specific sites of R-subunit and induces the reorganization of C-subunit binding domain (Granot et al., 1984); 3) Mg^{2+}-ATP interferes the R-C affinity by reducing cAMP-binding affinity of RI-holoenzymes (Ringheim and Taylor, 1990); 4) RII can be phosphrylated by C-subunits. Phosphorylated RII differed from unphosphorylated RII in C-subunit-binding affinity (Bechtel et al., 1975; Ringheim et al., 1990); 5) that Y1 adrenal mutant cell, kin8 cell (Rae et al., 1979), contains RI mutant (Olson et al., 1991) and is defective in cAMP-mediated response, which may make it difficult to correlate the observation in intact cell data with results from *in vitro* Therefore, the C-R interaction is very complicated process (Taylor, 1989). The condition set up for this *in vitro* experiments did not mimic the condition in intact cells. In other words, the approach with more sensitivity has to be applied to determine the binding affinity between C-R. For example, R-C specific interaction is better evaluated by determining distinct appearent activation constant (Ka) of holoenzymes by [^{3}H]cAMP. The different C-R-specific binding affinity is correlated with their Ka values. The Ka values can be precisely measured by spectrophotometer (Herberg et al., 1996). Recently, more sensitive physical approach, surface plasmon resonance (SPR) has been applied to determine the C-R-specific affinity. Different affinity between C-subunits and R-subunits can evaluated by a BIAcore instrument (Pharmacia/Biosensor) SPR was used to detect changes in mass in real time on a sensor chip surface which was prepared by direct coupling of the C-subunit or the R-subunit by primary amines to the CM dextran (Herberg et al., 1994)

The results from RI-peptides study indicated that Cγ-subunit interacted with RI-peptide differently from Cα-subunits. The C-terminal residues of pseudosubstrate are required for Cγ inhibition by RIα. As mentioned before, two R subunit classes exist, which distinguishes the type I from type II PKA holoenzyme. Based on relevant functions, R-subunit can be divided into five distinct domains, including dimerization, localization (RII only), cAMP-binding, C-subunit-binding and hinge which connects the cAMP-binding and C-subunit-binding domains The major domain

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structures of R subunit isoforms are similar. All the R subunits have the C-subunit-binding domains. The striking difference between the RI- and RII-subunit is that the C-subunit-binding domain in RII acts as substrate, but RI acts as a pseudosubstrate (RI) (Beavo et al., 1975) The pseudosubstrate region of RI has been well defined (Taylor et al., 1990) and some mutant RIα-peptides in this region have been synthesized. To further define C-subunit-isozyme-specific interaction with R-subunit, these mutant synthetic RIα-peptides were used as inhibitors to titrate homogeneous C-subunit activity. Theoretically, the designed experiments will provide a more efficient way to define structural determinants which are involved in interaction between R-subunit and C-subunit, and can eliminate some factors which interfere the interaction between the full length of R-subunits and C-subunits. As it was anticipated that Cγ responded very differently to some substitution of amino acids in RIα peptides from both Cα-subunits. Only major difference in conserved phosphorylation sequences between peptide substrates and RIα pseudosubstrates is the substitution of serine in substrate with alanine in RI-pseudosubstrate. The results of kinetic analysis showed that Cγ exhibited similar structural requirement for substrate phosphorylation and pseudosubstrate inhibition. This leads to the conclusion that both C-subunits shared N-terminal sequence specificity, which were that both C-subunits needed two basic arginine residue at N-termini of phosphoacceptor for substrate phosphorylation and pseudosubstrate inhibition. However, Cγ differed from Cα in the interaction with C-terminal amino residues of phosphorylation site.

In vitro, distinct C-subunit residues are involved in the recognition and inhibition of kinase activity by the R-subunits versus the PKI peptide. This result suggested that Cγ had more notable differences in the domain which recognized the PKI than in those which interact with R-subunits. Differences in substrate and pseudosubstrate of Cγ and Cα may account for observed intact cell

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functional differences (Table 1), but it is likely that other undefined factors in intact cells are also responsible, at least in part, for phenotype differences in Cγ and Cα-expressing cells

All these kinetic comparisons provide strong evidence to demonstrate the existence of distinct specificity for Cγ and Cα in substrate and pseudosubstrate, which differentiate Cγ and Cα function in terms of activation and localization in the cell. All these results prompted us to search for the anticipated distinct roles of Cγ and Cα in the cell function. Primary data has shown that Cγ could not turn on cAMP-mediated gene expression for steroidogenesis in Kin8 cell, but Cα could (Table 1). This implies that Cγ and Cα play distinct role in the regulation of cAMP-mediated gene expression. In addition, Cα-subunits have been definitely proved as only one well-defined mediator for cAMP-responsive gene expression. It is logical to explore the possibility whether Cγ and Cα regulate cAMP-mediated gene expression differently.

D. Function and Possible Molecular Mechanism For C-Subunit-Mediated Regulation of CRE-Reporter Gene Activity

It is of great interest to determine how two isozymes of A kinase can play distinct and cooperative role in the regulation of cAMP-mediated gene expression. The results of this research may provide important evidence to show a novel mechanism for PKA to diversify cAMP function in the cell

Cα and Cγ are efficiently expressed as active kinase, translocate into the nucleus to phosphorylate CREB and induce the elevation of CREB protein level in Kin8 cells. The Cγ activity in the nucleus is reported here for the first time. These results serve as evidence to indicate that Cγ participate in the regulation of gene expression and may help to define the *in vivo* function for Cγ in future studies.

Table 18 showed that Zinc/cAMP treatment not only turns on exogenous C-subunit vector to express recombinant C-subunits, but also causes endogenous Cα to move into nuclei in the Kin8

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and Cγ clones. However, no CRE-reporter gene activity or steroidogenesis were detected in Kin8 clone and Cγ clone after zinc/cAMP stimulation. This suggests that some additional things must happen to mediate the interaction between active C-subunits and transcription factors In other words, elevated C-subunit kinase activity by Zinc/cAMP stimulation does not necessarily activate cAMP-regulated gene expression in Y1/Kin8 cells. The changes in PKI-sensitive kinase activity (fold-increase) were similar in Cγ and Cα clones. However, absolute specific PKI-sensitive kinase activity in Cα clone was still 1.6-2 fold higher than Kin8 and Cγ clones This suggests that PKI-sensitive kinase activity has to be elevated beyond a certain level so that the CRE-containing gene could be turned on.

The results suggested that both Cγ and Cα could phosphorylate CREB (Figure 30), but kinetic profiles of phospho-CREB level were different in Cγ and Cα clones Although it is unclear how to correlate this phenomenon with their distinct function in the regulation of CRE-reporter gene activities, it must be associated with unique catalytic feature of Cγ in substrate phosphorylation (low Km and low Vmax) or in the rate of nuclear translocation of C-subunits (Hagiwara et al., 1993) or cellular location by distinct interaction with PKI and or/RII subunits in the cells

Results from gel-mobility shift assay suggest that transcription factors activated by Cγ, just like those activated by Cα, could bind to CRE-probe and phospho-CREB is a major component of these CRE-binding complexes. Gel-mobility shift assay with consensus CRE-sequence as a probe showed that transcription factors in Cγ and Cα clones caused a similar CRE-shift and super-shift by addition of anti-phospho-CREB antibody. It is possible that this approach was not sensitive enough to detect the difference between Cγ and Cα Actually, the results from Gal-4 reporter gene system showed that the presence of CRE was not necessary for the observed C-subunit-mediated cooperative regulation of CRE-reporter gene activity, which suggests that Gel-mobility shift assay

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could not reveal the different components in these transcription factors which are different in the nuclear extracts of Cγ and Cα clones.

Increasing number of isoforms for each member in CREB/ATF family and different forms of CRE, and complicated interaction among these transcription factors or co-factors have been recently reported. For example, at least five isoforms of CREB (Foulkes et al., 1991) and several isoforms of CREM (Foulkes et al., 1991) have been identified. CRE-element can be divided into four domains. Each of CRE-element from different CRE-containing gene differ in these four regions, which account for distinct specificity and binding affinity to respective trans-activating transcription factors (Liu et al., 1991); Consequently, cells evolved to respond to cAMP stimulation differently depending on unique structure of CRE and surrounding sequences. This situation encouraged us to design new strategies to answer the presented question. It has been reported that CRE and dimerization of CREB is not necessary for Cα to stimulate CRE-reporter gene activity by Gal-4 luciferase reporter gene and Gal-4 CREB system (Quinn, 1993). This finding encourages us to determine whether CRE sequence and dimerization of CREB are necessary for the cooperative co-regulation of Cγ and Cα-subunits in reporter gene expression. Gal4 luc is reporter gene expression vector, which was constructed by fusing Yeast Gal-4 protein binding DNA sequence (as promoter) with luciferase gene. Gal4-CREB is CREB (1-277) and Gal4 fusion protein expression vector, which was constructed by connecting Gal4 protein DNA sequence with 1-277 CREB peptide DNA sequence. CREB-Gal-4 fusion protein only contains CREB-trans-activating domain (1-277) and Gal-4 DNA binding domain. The dimerization domain of CREB was deleted. If the Gal-4 study showed that CRE and dimerization is necessary for C-subunit isozyme-mediated cooperative co-regulation of reporter gene activity, it would suggest that the observed distinction and cooperation between Cγ and Cα in the regulation of CRE-reporter gene activity must be involved in specific CRE-elements or specific dimerization of CREB with

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other transcription factors. But, If the results show the CRE sequence and dimerization of CREB are not necessary for the cooperative co-regulation of Cγ- and Cα-subunits in the reporter gene expression, many possibilities can be ruled out. Something must happen in the activation domain of CREB (1-277 amino acids) or the domain of co-transcription factor which interact with CREB activation domain. CBP is a co-transcription factor which specifically binds to the trans-activation domain of phospho-CREB. This suggests that CBP is alternative target for Cγ and Cα to activate differently.

The results (Figure 39) clearly demonstrated that CRE and dimerization of CREB was not necessary for the presence of cooperative co-regulation of Cγ and Cα-subunits in reporter gene expression. This indicated that Cγ and Cα distinctively and cooperatively regulate CRE-reporter gene activity by intra-CREB-molecular mechanism or by complementary interaction between CREB activation domain and CBP or other co-factors. More experiments have been designed to define exact molecular events about how Cγ and Cα modify CREB-trans-activation domain (1-277) or/and the region of CBP which differentially interact with phospho-CREB activation domain.

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CHAPTER V

SUMMARY AND CONCLUSIONS

This research was designed for the following specific aims: 1) To provide pure recombinant C-subunit isozymes for in vitro characterization for substrate and pseudosubstrate specificity; 2) To characterize native recombinant C-subunit isozymes for substrate and pseudosubstrate specificity; 3) To search for molecular mechanisms which underlay the cooperative co-regulation of CRE-reporter gene expression by Cγ and Cα. The results from this dissertation research achieved the above the specific research aims. This dissertation research is the first study to fully compare for Cγ with Cα *in vitro* for substrate and pseudosubstrate specificity and in intact cell for the function in the regulation of cAMP-mediated gene expression.

A. Summary

1. *For in vitro study, optimal expression of Cγ and Cα occurred in the Sf9 cell and bacteria, respectively:* Both C-subunit isozymes were expressed in three expression systems. Stable-transfected C-subunit clones in the Kin8 clone provided a first valuable model to compare the functions of Cγ and Cα in the intact cells,. However, the limited expression level of recombinant proteins in mammalian cell restricts its application to provide a sufficient amount of homogeneous C-subunit preparation. In this research, the bacteria and Sf9 insect cells using baculovirus systems have been successfully utilized to express both C-subunit isozymes. Optimal expression of Cγ and Cα occurs in the Sf9 cell using baculovirus and bacteria, respectively. High efficiency expression of both C-subunits in these respective systems generates abundant recombinant active enzymes. This allows purification to homogeneity in sufficient amount for *in vitro* kinetic analysis by two-step sequential chromatography.

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2. *Substrate and pseudosubstrate specificity of recombinant C-subunits are not changed by expression level and expression system:* Although Cγ and Cα used in this study were prepared from different expression systems, all tested features of these C-subunit isozymes on (pseudo) substrate specificity, kinetic properties and stability were similar to those characterized from respective C-subunit isozymes from Cγ- and Cα mammalian Kin8 clones. In addition, a series of reports clearly indicated that posttranslational modification like myristylation and autophosphrylation (Steinberg et al., 1993) of recombinant C-subunits did not effect the substrate /pseudosubstrate specificities, kinetic characters or the regulation of gene expression of recombinant C-subunits. Myristylation of C-subunits did improve the heat-stability for recombinant C-subunits (Taylor, 1989; Yonemoto et al., 1993; Clegg et al., 1993) The autophosphorylation of C-subunit was also necessary for full activation of naive recombinant C-subunits as well as caused the C-subunits mobility shift on SDS-PAGE (Steinberg et al., 1993) Therefore, expression level and system do not change the substrate and pseudosubstrate specificity and kinetic characters for C-subunit isozymes.

3. *Cγ has distinct specificity for substrate and pseudosubstrate:* The partially purified Cγ and Cα from respective Kin8 clones have been shown to have substrate and pseudosubstrate specificity (Beebe et al., 1992), but kinetic comparison of biochemical properties for Cγ and Cα is not available. This research performed a detailed kinetic comparison of substrate and pseudosubstrate specificity for Cγ and Cα. The results clearly demonstrated that Cγ and Cα had distinct substrate and pseudosubstrate specificity, which confirmed and extended previous data Kinetic analysis also define specific kinetic features for Cγ, including 1) identical requirement of primary conserved phosphorylation sequence for PKA (RRXS/TY); 2) lower Km and Vmax values compared with Cα; 3) the sequence specificity in the C-termini of phosphoacceptor site/pseudophosphorylation

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site for substrate phosphorylation and pseudosubstrate inhibition; and 4) insensitivity of Cγ to all kinds of PKI-inhibition when compared with Cα. Distinct native cellular phosphorylated proteins patterns were exhibited in respective cytosol and nuclear extracts of Cγ and Cα clone after Zinc/cAMP stimulation. This provides the evidence to demonstrate the existence of substrate specificity of both C-subunit isozymes in intact cells.

4. *Cγ and Cα distinctively and cooperatively regulated CRE-reporter gene expression in an intra-CREB-molecular dependent way or by interfering the complementary interaction between the CREB activation domain and CBP:* It is hypothesized that distinct substrate and pseudosubstrate specificities of C-subunit isozymes *in vitro* might imply that Cγ and Cα played the different roles in the cell functions via their differential activation and localization. The results in this research indicated that Cγ alone weakly affects the regulation of cAMP-reporter gene activity. However, Cγ could manipulate the function of Cα in the regulation of cAMP-reporter gene activity positively and negatively in dose-dependent way. After examining the nuclear activities of C-subunits, which are associated with the regulation of CRE-reporter gene activity, Cγ and Cα exhibit similar behaviors in the 1) expression of immuno-reactive proteins levels, 2) a nuclear translocation, 3) the phosphorylation of CREB, 4) the induction of elevation of CREB level in the nucleus, and 5) the CRE-mobility shifting, though Cγ differed from Cα in kinetic phosphorylation of CREB proteins. However, Gal4-luc/Gal4-CREB experiments indicated that Cγ and Cα distinctly and cooperatively regulate CRE-reporter gene activity in an intra-CREB-molecular dependent way or by interfering the complementary interaction between CREB activation domain (1-277) and CBP.

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B. General Conclusion

All these data leads us to make the conclusion that Cγ may diversify cAMP/PKA function by distinct cellular location, which was mediated by distinct interactions with R-subunit and PKI as pseudosubstrate, and/or activation, which was mediated by distinct substrate specificity.

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CHAPTER VI

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