

Winter 2006

Structure-Function Studies of the cAMP-Dependent Protein Kinase *In Vitro* and in Intact Cells

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**STRUCTURE-FUNCTION STUDIES OF THE cAMP-DEPENDENT
PROTEIN KINASE *IN VITRO* AND IN INTACT CELLS**

by

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A Dissertation Submitted to the Faculty of
Eastern Virginia Medical School and Old Dominion University in Partial Fulfillment of
the Requirement for the Degree of

DOCTOR OF PHILOSOPHY

BIOMEDICAL SCIENCES

OLD DOMINION UNIVERSITY

December, 2006

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ABSTRACT

STRUCTURE-FUNCTION STUDIES OF THE cAMP-DEPENDENT PROTEIN KINASE *IN VITRO* AND IN INTACT CELLS

Gary Z. Morris
Old Dominion University, 2006
Director: Dr. Stephen J. Beebe

There are 518 protein kinase genes in the human genome; this constitutes about 1.7% of all human genes. The cAMP-dependent protein kinase (PKA) serves as the prototypic model for the study of kinases because it contains a conserved catalytic core shared with all eukaryotic kinases, it is the simplest kinase, and it is one of the best-characterized serine/threonine kinases. PKA is ubiquitous in mammals and regulates multiple physiological mechanisms such as the cell cycle, apoptosis, cell motility, energy metabolism, and gene transcription through a well-defined intracellular signaling pathway. While PKA clearly has a central physiological role it is still unclear how PKA mediates multiple physiological mechanisms at the cellular level. Four approaches were used to explore this question using two PKA catalytic subunits, C α and C γ , which share 83% identity in primary structure but differ in function. The first approach sought to identify differences in primary structure between C γ and C α , which may define functional differences between them. To this end chimeras were generated, swapping the carboxyl and amino termini between C α and C γ and were evaluated for functionality

through CREB-mediated reporter assays. Wild type C α and C γ induced CREB-mediated transcriptional activation, but the chimeras failed to exhibit any activity. The second approach sought to characterize phosphorylation differences between purified PKA-C γ and PKA-C α that defines their physiological function. Two novel phosphorylation sites were identified on both isoforms by tandem mass spectrometry analysis (C γ S14 and C α /C γ S259). It was also determined that C γ expressed in Sf9 insect cells, like C α expressed in mammalian cells, is phosphorylated at T197 and S338 and the modification at T197 is important to the function of both isoforms. The third approach sought to characterize the kinetic mechanism of PKA-C γ through determination of the rate for the reaction-limiting step, which was found to be 9-times slower than that of C α . The final approach sought to identify C γ expression in the cell through the use of a new C γ -specific antibody. C γ expression was identified following differentiation of U-937 cells suggesting a novel function for C γ in the cell.

This dissertation is dedicated to my wife Athena, my sons Kevin and Sebastian and my daughter Isabel.

ACKNOWLEDGMENTS

This undertaking could not by any stretch of the imagination have been accomplished alone and so I have many to thank in regard to the work summarized between these covers. I would like to thank my dear friends and colleagues from the Center for Pediatric Research, Eastern Virginia Medical School, Old Dominion University, the University of Puerto Rico, and Tidewater Community College, including Neel Krishna, Stephen Buescher, Kenneth Sommers, Michael Ward, Suzan Cartwright Sampson, Nell, Reece, Emily Hall, Roy Williams, Mark Elliott, Fernando González, Watt Jones, Carrie Gordon, Fred Stemple, and Joel McCormick. All have had a hand, in one form or another, in my completing this Dissertation.

I would like to thank Gerald Pepe who helped me obtain an American Physiology Porter Fellowship and the Porter Development Committee for awarding me the fellowship. The members of my committee –Peter Blackmore, Richard Drake, Julie Kerry, and Howard White– provided me with guidance throughout this endeavor and maintained an open-door policy, allowing me to interrupt them frequently with my endless stream of questions.

I also need to thank my running partners from Running Etc. –Doug, Deb, Connie, Jim, Rich, Mike and John– especially, Doug, who was a mentor and dear friend who stuck by my side through thick and thin on the endless roads runners follow.

I am especially grateful to Paula Fox, the lab manager of Dr. Beebe's laboratory when I started the Ph.D. program. The best description for her role is that of "lab mom." She patiently trained every one of us who came into the lab to be proficient and independent. She was always there to help, never complained and always had a positive

outlook on life.

I thank Dr. Stephen Beebe—or Boss as I frequently called him— for giving me this opportunity and for his faith in the prospect that I would be an acceptable graduate student in his lab. He has been a mentor in science and in life, guiding my growth in both while also becoming a dear friend.

I would not have started or completed the path toward my Ph.D. without the values and ethics instilled in me by my father and mother, Marshall and Juanita, and my grandparents, Guane, Ray and Rae. All have been role models and enthusiastic supporters of this Ph.D. I would also like to thank my brother and sister-in-law, Doug and Dorothy, for helping the family when help was needed.

Finally I thank my wife, Athena, who has lovingly and patiently given me unwavering support throughout this process; my sons Kevin and Sebastian who have had the kind of faith in me that only a son can give his father and in doing so became a source of strength to draw on; and my daughter Isabel, who like the hurricane, came into my life with a force sufficient to redefine my role as a father and a man.

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CHAPTER I

SIGNIFICANCE, SPECIFIC AIMS AND BACKGROUND

SIGNIFICANCE

There are 518 protein kinase genes in the human genome (2); this constitutes about 1.7% of all human genes (2). Kinases allow physiologic extracellular stimuli to elicit specific cellular response through signal transduction. The cAMP-dependent protein kinase (PKA) serves as the prototypic model for the study of kinases because it contains a conserved catalytic core shared with all eukaryotic kinases; it is the simplest kinase; and it is one of the best-characterized serine/threonine kinases (3). PKA is ubiquitous in mammals (4-6) and regulates multiple physiological mechanisms such as the cell cycle (7), apoptosis (8), cell motility (9), energy metabolism (10), and gene transcription (11) through a well-defined intracellular signaling pathway (12,13). Deregulation of PKA is found in several diseases including Carney complex (14,15), Systemic Lupus Erythematosus (16,17), breast cancer (18), and prostate cancer (19). PKA knockout mice demonstrate deficiencies in neuronal pathways including decreased sensitivity to nociceptive responses and alterations to synaptic plasticity (4). While PKA clearly has a central physiological role it is still unclear how PKA mediates multiple physiological mechanisms at the cellular level.

The model for this dissertation is the Journal of Biological Chemistry.

SPECIFIC AIMS

cAMP-dependent protein kinase (PKA) mediates multiple physiological functions including hormone secretion, energy metabolism, apoptosis, and cellular differentiation in response to physiological stimuli. Thus, it is clear that PKA function is crucial to normal cell physiology and homeostasis. Therefore understanding the roles of PKA in the cell is a focus of ongoing scientific research. To this end, there is a large body of scientific literature focusing on two of the PKA catalytic (C) subunits, C α and C β . However less research has been done on the third PKA C-subunit, C γ . Recently we established that C α and C γ , while sharing sequence homology (83% identity in protein primary sequence), do not mediate transcription through the same promoter sequence or transcription factors and differ in their phosphorylation efficiency for multiple substrates tested (20). Research outlined in this dissertation was performed to determine structure-function differences between C α and C γ that explain how they mediate transcription through different mechanisms and why they differ in their catalytic measurements.

Specific Aim 1: To identify differences in primary structure between PKA-C γ and PKA-C α that defines their physiological function in intact cells. Our hypothesis is that differences in C α and C γ primary structures are responsible in part for differential transcriptional regulation. Co-transfections of wild-type and mutated C-subunits with a PKA-selective reporter-vector will be used to characterize and correlate the C-subunit structure with their function.

Specific Aim 2: To characterize post-translational differences between purified PKA-C γ and PKA-C α that defines their physiological function. Our hypothesis is that differences in K_m and V_{max} for physiological substrates are attributed in part to post-

translational differences between C α and C γ . Liquid chromatography-tandem mass spectrometry analysis of purified recombinant histidine-labeled C α and C γ will be used to characterize post-translational phosphorylation differences between the C-subunits.

Specific Aim 3: To characterize the kinetic mechanism for PKA-C γ through determination of the rate constants for each catalytic step of the phosphotransferase reaction. Our hypothesis is that C γ functions through the same kinetic mechanism as C α but has slower rate constants. Stopped-flow analysis of purified recombinant proteins will be used to characterize pre-steady state kinetics necessary for establishing rate constants for each catalytic step.

The results presented in this dissertation suggest that one mechanism by which PKA mediates multiple physiological mechanisms within the cell is through expression of multiple isoforms. Here we focused on the two most divergent catalytic subunits, C α and C γ to study this mechanism. The following is a brief background introducing the kinase enzyme along with some background on PKA that 1) coincide with the specific aims outlined in this section, 2) coincide with work proposed for my Porter Physiology Fellowship and 3) coincide with different manuscripts that have either been published, submitted or in preparation for submission. Each of the following chapters will contain a more specific background pertaining to the focus of that chapter.

BACKGROUND

Protein kinases are an evolutionarily conserved protein family– A kinase is an enzyme that catalyzes the hydrolysis of the γ -phosphate (the terminal phosphoryl group) on a nucleotide triphosphate (NTP) and the covalent binding of this phosphate to a target substrate. The *Enzyme Commission of the International Union of Biochemistry and*

Molecular Biology name for a kinase is a phosphotransferase. The term kinase is a lay-term derived from the Greek word *kinein*, which means to move towards¹. In the context of the kinase the name refers to the movement of the phosphoryl group from NTP to the target substrate by the kinase. Figure 1 shows a timeline identifying important events related to the study of the kinase enzyme in the past century, including the “purification” of phosphorylase kinase in 1943, the first characterization of a kinase, casein kinase (now casein kinase 2), in 1954 and the description of the human kinome in 2002. The human genome contains at least 663 kinase genes (21). Of these, 518 are protein kinases, about half the number initially predicted (2, 22). Thus, almost 2% of the human genome is dedicated to the expression of this protein family suggesting that the function of this protein is very important to mammals (2). Protein kinases have been divided into eight distinct groups based on their structure and function (AGC: containing PKA, PKG, PKC families; CK1: casein kinase 1 group; STE: homologous to yeast sterile 7, sterile 11, sterile 20 kinases group; TKL: tyrosine kinase-like group; TK: tyrosine kinase group; CMGC: containing CDK, MAPK, GSK3, CLK families, and CAMK: calcium/calmodulin-dependent kinase group) (2). Figure 1 also shows how the study of kinases has increased in an exponential manner over the last forty years, based on the number of yearly publications found in Entrez PubMed (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>). Research seems to be nearing a plateau, which may indicate that the rate at which scientist are able to study kinases, generate results and publish them using current technology is reaching a limit. The time line presented in Figure 1 also suggests that key basic science discoveries in the kinase field seem to be made about every nine years.

¹ Based on a personal email communication from Tony Hunter. Please see Appendix A.

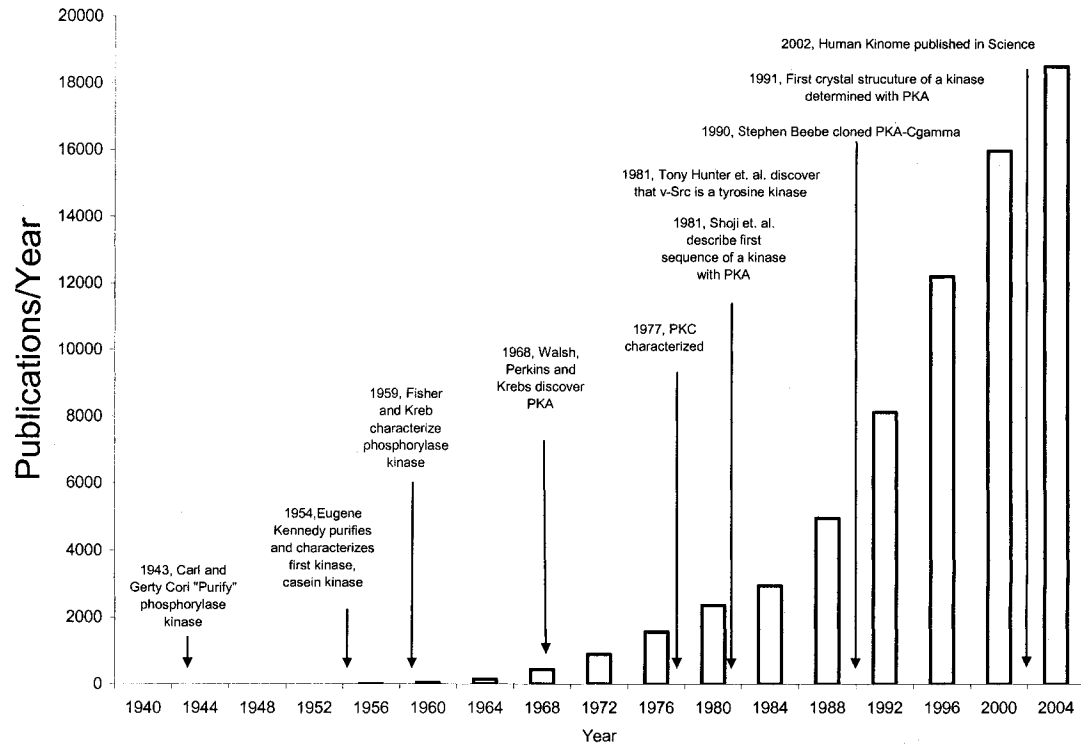


FIG. 1. Time line showing discovery and publication trends in the kinase field. Arrows point to important contributions to the kinase field on the time line, which covers 64 years (x-axis). The bars show publications within a given year, sampled every four years (y-axis).

Kinases *utilize an evolutionarily conserved mechanism* and are found in organisms of all three Domains of life: Archaea, Bacteria and Eucarya (23-25). Kinases are placed in broad groups that include protein kinases, which phosphorylate proteins, and lipid kinases, which phosphorylate lipids generating secondary messengers in the cell. Some kinases, referred to as 'small molecule kinases,' do not fit in any of these groups such as hexokinase (phosphorylates glucose), choline kinase (phosphorylates choline) or ethanolamine kinase (phosphorylates ethanolamine) (26). Kinases are also classified based on the nature of their substrate. These classifications include serine/threonine kinases, tyrosine kinases, histidine kinases, and antibiotic kinases, which are bacterial kinases, associated with bacterial antibiotic resistance and found to be closely associated to eukaryotic serine/threonine kinases (27). The prevalent kinases found in the Eukaryotes are the serine/threonine and tyrosine protein kinases (2). In recent years serine/threonine protein kinases have also been identified in bacteria and archaeobacteria (25,28). Histidine protein kinases are found in bacteria, archaeobacteria, plants, fungi, and slime mold as part of a two-component system, where the kinase autophosphorylates on a histidine residue (the first component), transfers this phosphoryl to an aspartate on the effector domain (the second component) leading to the response to extracellular stimuli (25,28,29).

While the structure and chemical nature of the substrates differ between the kinase groups, most protein kinases share sequence and structural homology through their catalytic core, which consists of approximately 250-300 amino acids (23). Of the 518 protein kinases in the human genome, 478 share homology in this region (2). Mitochondrial pyruvate dehydrogenase kinases have been found to share sequence and structural homology with prokaryotic histidine kinases (30). Histidine kinases and

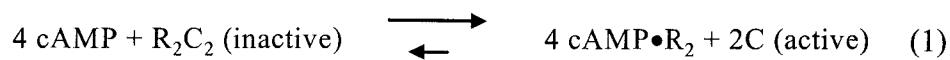
eukaryotic kinases share some sequence homology through the nucleotide binding domain and structural homology through the small lobe (29), suggesting that all kinases evolved from a 'protokinase' present prior to the divergence of the three domains of life(29). This hypothesis depends on the methods and criteria used to classify sequence homology and not all literature agrees that there is homology between the atypical prokaryotic kinases and eukaryotic kinases (23). Furthermore, not all eukaryotic kinases share sequence homology as demonstrated by a group of 40 kinases in the human kinome classified as atypical kinases because they lack sequence homology with the rest of the kinases (2). However all of these kinases have been reported to have phospho-transferase activity and many share structural homology with typical eukaryotic kinases. The homology of kinases among organisms and Domains will be easier to discern once kinomes are identified for all organisms used in these comparison studies. Such work is presently underway in archeabacteria (25). The presence of homologous kinases among Domains is more likely to be due to horizontal transfer between organisms than to independent evolutionary pathways (30). The transfer of a phosphate group as a signal in the organism has been conserved through the diverse groups of kinases found in all three life Domains, making the theory of a protokinase very attractive. The evolutionarily conserved nature of the protein kinase family allows for structure-function relationship characterization of this group through use of structurally simple members of this family.

The best-characterized protein kinase is the cAMP-dependent protein kinase (PKA, EC 2.7.1.37) (31). About 5% of the nearly 340,000 papers published on kinases found on Entrez PubMed are on PKA and, as Figure 1 demonstrates, PKA was the first for many developments in the kinase field. PKA was the first kinase for which a linear structure was

determined through a molecular biology approach, allowing for the generation of recombinant PKA and its subsequent characterization through recombinant protein technology (32). PKA was also the first kinase to be crystallized, allowing for its structure to be elucidated (33). PKA has a relatively simple structure, consisting of 350 amino acids including the 280 residues that constitute the conserved kinase catalytic core along with 70 more amino acids, which also participate in the catalytic function of the enzyme. The PKA catalytic subunit consists of a bilobal structure shared by all protein kinases (33). The combined knowledge of PKA kinetics and structure have contributed to the present understanding of the kinase catalytic mechanism (34-38). Because of this wealth of information the PKA catalytic subunit serves as a template for the structural classification of all kinase domains (23,39,40) and an excellent model to study kinase structure-function relationships.

PKA is very important to organism physiology– PKA is present at varying concentrations in all tissue types (41-43) and exists in the cell as an inactive tetrameric holoenzyme composed of a regulatory (R) subunit dimer and two monomeric C subunits (31,44). The holoenzyme (R_2C_2) can be classified into types I and II PKA determined by the R subunit isoform present in the holoenzyme. There are four known *Homo sapiens* genes for the R subunit: RI α , RI β , RII α , and RII β . Classically, PKA type I elutes from a DEAE column at concentrations below 0.1 M NaCl and type II elutes at concentrations greater than 0.1 M NaCl (44). There are four genes for the C-subunit expressed in *Homo sapiens*: C α , C β , C γ and PrKX (45,46). The PKA catalytic subunits are members of the AGC serine/threonine kinase group (2), and phosphorylate hundreds of substrates involved in numerous physiological processes (1). Each of the C-subunit isoforms is expressed from

an independent gene: C α , 19p13.1; C β , 1p36.1; C γ , 9q13; and PrKX, Xp22.3 (47,48) and they all exhibit different selective tissue expression. C α is ubiquitously expressed, C β is expressed in neuronal tissue, C γ has only been properly identified in the testes, and PrKX is expressed ubiquitously (4,41,45,46). Human C α and C β share the highest degree of homology in their primary amino acid sequence (93% identity) and have multiple splice variants (49-52). C α and C γ are also highly homologous exhibiting 83% identity while C β and C γ exhibit 79% identity (45). PrKX shares the least amount of homology with C α (53% identity). Activation of PKA C-subunits is cAMP-dependent. The level of cAMP in a cell is controlled through G-protein coupled protein receptors, which stimulate or inhibit adenylyl cyclase, modulating the synthesis of cAMP from ATP in response to hormone agonist (Figure 2) (53). cAMP levels in the cell are also regulated by cyclic nucleotide phosphodiesterases (PDEs) (Figure 2). PDEs hydrolyze cyclic nucleotides (e.g. cGMP and cAMP) to 5'-nucleotide monophosphates, eliminating the functional structure of the second messenger required to bind to its target (e.g. PKG and PKA). There are 11 known PDE families (PDE1-11), which vary in their specificities for cAMP and cGMP. Of the eleven



family members, PDE4, 7 and 8 are specific for cAMP. Once cAMP binds the R subunits (2 cAMP molecules: 1 R subunit), which have a high affinity for cAMP (54-56), the R-subunits' affinity for the catalytic (C) subunits decreases by six orders of magnitude (13). Thus, at very low levels of cAMP, the steady state favors formation of the holoenzyme (Equation 1). In the presence of increasing levels of cAMP, the steady state shifts toward dissociation of the holoenzyme (Equation 1).

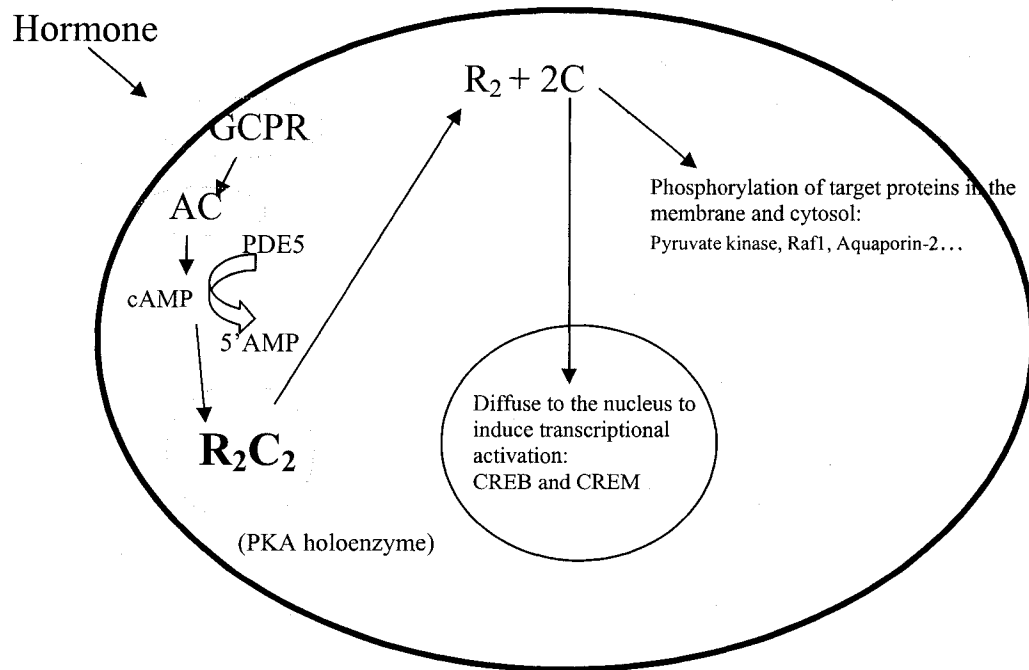


FIG. 2. **PKA intracellular signal transduction pathway.** Hormones that bind G-protein coupled receptors (GPCR) either activate or inhibit adenylyl cyclase (AC) which generates cAMP, which binds the R-subunits of the PKA holoenzyme, releasing the C-subunits, which are now free to propagate the signal transduction through phosphorylation of select substrates (1).

Kinases are catalytic so only a few active kinases are required to propagate a signal for a given agonist and in most cases the duration of this activity is transient, lasting for as long as the agonist is bound to its receptor or that ligand-receptor complex is functional. A disease state is often associated with deregulation of a kinase either because the kinase cannot be activated or inactivated in response to its biological trigger. Determining the role or impact of PKA on organism development and disease through knock out (KO) models has proven tricky as there are multiple C-subunit isoforms, thus the KO of one gene often leads to compensation by other isoforms. However, 73% of $C\alpha$ KO mice do not survive beyond weaning, suggesting that this isoform is very important to the organisms development (57).

The remaining 27% that survived did so in part through compensating for the loss of $C\alpha$ with increased $C\beta$ expression, but still suffered from developmental defects (4). While KO of $C\alpha$ is deleterious in most cases, KO of $C\beta$ has no deleterious effect on mouse development (58), suggesting that this isoform has a different function from $C\alpha$ and that its role is not as dominant in the organism's development. Similar trends are observed with the R-subunits, where KO of $R\alpha$ isoforms leads to developmental defects and KO $R\beta$ isoforms have little or no impact on the organism's development (4), but often leads to the onset of neurological dysfunctions (59,60). These animal models demonstrate that each PKA isoforms has different functions in the development of the organism, where $C\alpha$ has a dominant role in development and $C\beta$ has tissue specific functions and compensates for the loss of the dominant isoforms. Studies of $C\gamma$ in KO mice cannot be performed as this is a primate specific isoform (45). Thus it is difficult to extrapolate from these studies the role $C\gamma$ may have in organism development. While KO models have not been used to study PrKX, it has been demonstrated that PrKX is also important in neuronal differentiation (61).

Diseases that manifest deregulation of PKA are rare, but include colon, breast and prostate cancer (18,62-64), systemic lupus erythematosus (16,17), Cushing's syndrome (65), acromegaly, Carney complex (66), XX male and Swyer's syndromes (48). What is observed in cancer is an increase in R-subunit expression (64), which would lead to increased suppression of C-subunit activity in response to cAMP. This may explain why extracellular C-subunit is detected in transformed tissue culture cell lines (62) where it is likely that the cell is trying to compensate for diminished cAMP-response due to the increase in RI expression by increasing C-subunit expression. This process may continue until it becomes deregulated. But caution should be exerted when interpreting these results, as they were performed with tissue culture cell models where extracellular detection of the C-subunit may be due simply to the inadvertent lysis of a few cells and not to the secretion of the C-subunit from intact cells, something that has not been properly addressed in the published work on the subject. Nevertheless, these studies do imply that overexpressed, cAMP-independent deregulation of the C-subunit may contribute to the progression of cancer. Further evidence for this hypothesis is that ectopic expression of the C-subunit in a prostate cancer cell line, LNCaP, is sufficient to induce neuroendocrine differentiation associated with the progression of prostate cancer (63).

Cushing's, acromegaly, and Carney complex are endocrine diseases associated with mutations to RI α that render expression of a non-functional R-subunit (67). This mutated RI subunit would decrease regulation of C α in the cell, making C α -function cAMP-independent. XX male and Swyer's syndromes are associated with ectopic recombination of PrKX in the X and Y chromosomes during development (48), which could lead to the incorrect expression of this gene during development. Each of these diseases clearly

illustrates that, as with any kinase, the PKA catalytic subunits need to be carefully regulated within the cell and that deregulation of this protein has detrimental effects on the organism's homeostasis.

PKA is regulated through transcription, translation, post-translation and pseudosubstrates— PKA function is regulated by intracellular levels of cAMP, but PKA is also regulated through other mechanisms. The promoters for all the C-subunits are TATA-less, GC rich promoters with multiple start sites (47), which suggest that regulation of these enzymes is under “housekeeping” mechanisms (68,69) associated with cell and tissue specific expression (70). Expression of both PKA R- and C-subunits is also regulated at the mRNA and protein level. C α and C β gene expression is also regulated through splicing as each has several splice variants, C α 1, Cs/C α 2, C β 1, C β 2 and C β 3, which are expressed in a cell type selective manner (52,71,72). Unlike C α , C β , or PrKX, C γ is an intronless retroposon gene that appeared in the human genome forty million years ago (73), thus C γ is not expected to have any splice variants and makes C γ the only known example of a primate specific PKA gene.

The regulation of PKA also occurs through the R-subunits at the mRNA level, which has been studied selectively in T- and B-cells, sertoli, L6 myoblast and lymphoid cells stimulated to undergo cAMP-induced differentiation. RI mRNA is constitutively expressed in L6 myoblast cells, thus regulation of this isoform occurs at the protein level (74). In T and B cells low levels of RI-mRNA are observed along with high levels of RI-protein, and high levels of RII-mRNA and protein expression are observed suggesting different regulatory mechanisms for mRNA for each R-subunit isoform in this cell type (75). Regulation of RII in sertoli and lymphoid cells differs between RII α and RII β ,

where RII β mRNA levels do not change between differentiated and undifferentiated cells, but RII α mRNA levels increase during cAMP-induced differentiation (76-78). Overall, regulation of RI expression is favored at the protein level and RII expression regulation seems to vary depending on the isoform and tissue type.

The R and C subunits are stabilized by formation of the holoenzyme, as the half-lives in the cell for each is twice as long as in the absence of cAMP (in the holoenzyme), than in its presence (79,80). This suggests that degradation of the protein by ubiquitin-mediated pathways is another regulatory mechanism of cAMP-dependent pathways (74). Both C and R subunits also undergo post-translational modifications, which modify their function. RII, but not RI, is phosphorylated at RII α S95 or RII β S112 decreasing its affinity for the C-subunit (reviewed in (81)). C α is phosphorylated at T197 and myristylated at glycine 1 (82,83). Phosphorylation on the catalytic loop at C α T197 is a necessary modification for kinase function, which is conserved among most kinases (84-86). In the case of C α this modification increases its catalytic activity 100-fold (87). Myristylation of C α increases the stability of the enzyme, as the myristate moiety folds into a C α hydrophobic pocket, which has the effect of increasing the protein's melting temperature, a measure of increased stability (83).

Another regulatory mechanism for PKA is through pseudosubstrates, competitive inhibitor proteins that contain the PKA phosphorylation sequence motif (R-x-x-S/T), but have an alanine instead of a serine or threonine and thus cannot be phosphorylated by the C-subunit. The R-subunits and protein kinase inhibitor (PKI) are the two known pseudosubstrates for the C-subunits. RII is phosphorylated by the C-subunit following dissociation of the holoenzyme, so it still serves as C-subunit inhibitor.

Interaction of the C-subunits with the R-subunits to form the holoenzyme sequesters and maintains the C-subunits localized in the cell cytosol (88). In the presence of cAMP, the C-subunits dissociate from the holoenzyme. Once released the C-subunits are free to phosphorylate available substrates in the cell membrane and cytosol and to diffuse into the nucleus (88) where they can phosphorylate transcription factors (Figure 2) (1). PKI inhibits and exports the C-subunits from the nucleus terminating its regulation of transcription factors (89).

There are several mechanisms by which PKA may regulate multiple cellular functions— By catalyzing the transfer of the NTP gamma-phosphate to a protein substrate, the kinase is modifying and altering the phosphoacceptor's function, propagating and/or modifying signal cascades within the cell and regulating the cell's function in response to extracellular signals (90). Kinases are central to most, if not all, of the cell's functions. Examples of this include cyclin-dependent kinases, which regulate the cell cycle (91), receptor associated tyrosine kinases, such as JAK which allow the cell to respond to interleukins during inflammatory responses of the immune system (92), and the MAPK kinase family, one of the best studied kinase signal cascades which regulate the cell's response to growth factors (93). In all of these examples, the kinase is part of a system involving ligand (hormones), receptor and effector proteins (kinases), which are able to regulate multiple cellular functions. Kinases are central to the cell's ability to function as both the unit that is the cell and as part of the collective that forms organs and the organism. With all the variations of this molecule in the human genome the challenge remains to understand how the kinase is able to function selectively in response to a given stimuli.

While PKA responds specifically to levels of cAMP, there are several factors that give PKA diverse physiological roles. One factor is the presence of multiple C- and R-subunit isoforms, which can be expressed ubiquitously or with tissue- or cell-type selectivity (6,13). This suggests that C- and R-subunit isoforms have specific physiological roles. Thus, specific functions in the cell can be addressed by either expressing or activating specific isoforms. Functionally different PKA holoenzymes can be formed and localized in combinations of functionally distinct C- and R-subunit isoforms, some of which have been identified *in vivo* (5,13,94,95) and provide a potential mechanism to diversify and/or fine-tune cAMP signal transduction downstream of the holoenzyme. Such differences have been characterized between C α and C β as pure proteins and in the intact cell (95,96). Comparative studies have also been done between C α and C γ from partially purified cell extract (5).

Functionally distinct PKA holoenzymes can be fine-tuned through differential association of the varying isoforms to form hetero- or mono-isoform composed holoenzymes that contain different catalytic or regulatory subunit isoforms within a given holoenzyme. Thus, not only do the classic Type I PKA or Type II PKA holoenzymes form in the cell, which contain only RI or RII isoforms, respectively, but also holoenzymes containing both RI and RII isoforms can form. Such holoenzymes have recently been observed in T-cells (97). Also, holoenzymes containing multiple C-subunit isoforms were observed. Thus, multiple C-subunits can be present in a given cellular location within a given holoenzyme allowing for multiple targets to be phosphorylated with different specificities in response to a single signal. Or if required, only one C-subunit and or R-subunit isoform could be present within the holoenzyme, allowing for only one very

specific response to a given stimuli. What structural differences between PKA C-subunits would render them such functional differences that would give the cell the ability to mediate multiple cellular responses through the expression of different isoforms? Our hypothesis is that differences between C-subunits may be due to differences in their primary sequence, which would lead to differences in secondary and tertiary structure between C-subunit isoforms. This hypothesis will be tested under Specific Aim 1, in Chapter II, where chimeric constructs will be generated by swapping regions of low sequence homology between two C-subunit isoforms and tested for changes in function.

A second factor that may give PKA a diverse role in the cell is its regulation by other proteins. Proteins known to regulate PKA include protein kinase inhibitor (PKI), A-kinase anchoring proteins (AKAP), and phosphoinositide-dependent kinase-1 (PDK-1). The pseudosubstrate PKI inhibits and exports $C\alpha$ from the nucleus (98) but $C\gamma$ is insensitive to PKI (5,13). This is due to specific differences in the amino acid sequence between $C\alpha$ to $C\gamma$ (S133Q and Y235F) that form a hydrophobic pocket for PKI-binding (99,100). Since the R-subunits do not enter the nucleus (88), this raises the question of what regulates $C\gamma$ gene transcription activity in the nucleus.

AKAPs represent another PKA-regulating protein (101). AKAPs bind the R-subunit and localize PKA holoenzymes in regions where the protein is required. This leaves the kinase and substrate co-localized in a relatively high concentration, increasing substrate specificity. As $C\gamma$ has a lower affinity for R-subunits than $C\alpha$ (102), $C\beta$ or PrKX differ in their affinities for different R-subunit isoforms (46,95), AKAP proteins may not regulate $C\gamma$ to the same extent they regulate $C\alpha$, $C\beta$ or PrKX. Furthermore, $C\alpha$, $C\beta$ and

PrKX localization may be determined through differential associations with the various R-subunit isoforms.

PKA is also regulated by other kinases (103). In mammalian cells a candidate is PDK-1, which phosphorylates C α at T197, increasing its catalytic activity (104), but it is not known if PDK 1 phosphorylates C β , C γ or PrKX. It is tempting to assume that this mechanism is conserved across the different C-subunit isoforms as the regions required for PDK-1 modification of C α are conserved among all of them. But this has yet to be determined experimentally. In the cell PDK-1 does increase the rate of C γ -mediated transcription but has little effect on C α -mediated transcription (105).

Each of the proteins just described regulate PKA, but with potentially different effects on the function of each. One of the most important regulation mechanisms is the phosphorylation of C α at T197, which increases its catalytic activity 100-fold (87). It has not been tested whether this modification is significant for all of the PKA C-subunits, even though structural similarities between them suggest it might be. Differential regulation of this modification among PKA C-subunit isoforms would provide the cell with another mechanism to fine-tune cAMP-mediated signal transduction pathways. Specific Aim 2, in Chapter III, will test the significance of this modification to the function of two PKA C-subunits.

A third factor that may give PKA diverse physiological roles is the kinetic mechanism of each of the catalytic subunit isoforms. Three of the four catalytic isoforms (C α , C β , and C γ) have been shown to vary in substrate specificity as determined through variance in their catalytic rate constants for the same physiological substrates (20,95). This suggests that each catalytic subunit may have 'preferred' physiological substrates which

they will modify in response to a given stimuli. This selectivity may allow a given stimuli to elicit each catalytic subunit to modify different substrates. In addition, the kinetics of phosphorylation may differ between the isozymes resulting in a temporal difference of phosphorylation-mediated activation or inactivation mechanisms that are important to the sequence of events regulated by cAMP (12). Specific Aim 3, in Chapter IV, will test if there is a divergence in the kinetic mechanism of two PKA C-subunits that could explain differences in steady-state kinetics for a given substrate.

Together these factors present a model wherein a cell can mediate different cellular mechanisms in response to the same physiological stimuli through differential expression, localization, and substrate-specificity by different isoforms of the same protein kinase. Characteristics of this model seem most striking when comparing two PKA C-subunits that have the greatest divergence in function while maintain relatively high structural homology, $C\alpha$ and $C\gamma$. These two isoforms serve as a simple model for structure-function characterization of kinases in order to determine what structural components in these two C-subunits provides them diverse functions, and how this translates to functional divergence in other protein kinases.

The conserved structural homology and functional divergence between $C\alpha$ and $C\gamma$ lend them as an ideal model for protein kinase structure-function studies— While both $C\alpha$ and $C\gamma$ share 83% identity in primary structure they differ in gene transcription regulation and substrate and pseudosubstrate specificities (5,20,105,106). Differences in gene transcription regulation were observed through transient transfections in luciferase-reporter assays where $C\alpha$ induced a three to ten-fold higher activation of CREB-response elements containing promoters than $C\gamma$ (105). Differences in pseudosubstrate and

substrate recognition have been observed where $C\alpha$ is inhibited by PKI while $C\gamma$ is not (13,20). Another striking difference between $C\gamma$ and $C\alpha$ is that while both share similar K_m values (steady-state constant associated with affinity by the enzyme for a given substrate) for most substrates, suggesting similar affinities, they have very different V_{max} values (steady-state measure of the enzyme's reaction rate for a given substrate), with $C\gamma$ at least ten-times slower than $C\alpha$ (20). These differences between $C\alpha$ and $C\gamma$ suggest that while both enzymes are cAMP-dependent, they may fulfill different functions within the cell. Unlike $C\alpha$ or $C\beta$, $C\gamma$ is only found in primates (45,73) and is believed to have entered the primate gene pool approximately 40 million years ago as a $C\alpha$ -retroposon (73). Thus $C\gamma$ is transcribed as an intronless message and has no known splice variants, as occur for $C\alpha$ or $C\beta$ (58,71). Presently the literature considers $C\gamma$ to be structurally and kinetically homologous to $C\alpha$ and $C\beta$ (e.g. undergoing the same post-translational modifications, such as myristylation and phosphorylation), when in fact very little is known about the $C\gamma$ kinase. Thus, there is a clear need to express and characterize a homogeneous $C\gamma$ enzyme. However identification, quantification and characterization of $C\gamma$ has been a challenge due to its apparent low abundance in primate cells and tissues, the difficulty in readily expressing sufficient levels of the recombinant enzyme, and the absence of $C\gamma$ -specific antibodies.

$C\gamma$ is unique to the PKA catalytic subunit isozymes. The $C\gamma$ mRNA has only been identified in human testes where it was cloned from a human testes cDNA library (45). PKA Cs, or $C\alpha_2$, has recently been identified as a testes specific isoform, a splice variant of $C\alpha$ expressed in mature sperm (52,107), but unlike $C\gamma$ this isoform is not human specific. Identification of $C\gamma$ in other tissues has been difficult because of the high

homology between $C\gamma$ and $C\alpha$ and the absence of isozyme-specific antibodies. One recent publication describes the identification of $C\gamma$ in mouse pancreatic β -cells through immunohistochemistry (108). But this work was done with an antibody that is not selective for $C\gamma$ and cross-reacts with other C-subunits (105), and since $C\gamma$ is not present in the mouse, it is likely the antibody detected the prevalent isoforms expressed in most cell lines, $C\alpha$. The antibody in question was also used to characterize C-subunit expression in rat cells, where it was reported to identify $C\gamma$ (a primate specific gene) (109). Since $C\gamma$ was originally identified in testes, a tissue with a high concentration of cells undergoing differentiation, it is possible that $C\gamma$ has a function in cellular differentiation. Furthermore, sperm undergo differentiation that involves specific isoforms of the PKA catalytic subunits (52). In addition, $C\gamma$ is an intronless (45) gene, and many intronless genes play a role in cellular differentiation (110-116). This suggests that PKA may have novel role in cellular differentiation through a unique isoform that has not been described before.

The divergence between $C\alpha$ and $C\gamma$ function lends these two isoforms as a good model system to study the effect structural divergence between protein kinases has on functional differences. Furthermore, studying structure-function differences *in vitro* and in intact cells may help further our understanding of the role and function of $C\gamma$ in the cell. Each of the following chapters (II-IV) will coincide with each of the specific aims outlined in this chapter. Chapter V is the result of work I did in addition to the work outlined by my specific aims. This work was necessary to demonstrate that $C\gamma$ was not a test-tube anomaly, but instead an isoform that has slipped through the cracks of the PKA research field, because, as described here in detail, $C\gamma$ is limited in species expression,

has proven difficult to isolate and it shares high homology with other isoforms expressed in the same cell models studied.

CHAPTER II

IDENTIFY DIFFERENCES IN PRIMARY STRUCTURE BETWEEN PKA-C γ AND PKA-C α THAT DEFINE THEIR PHYSIOLOGICAL FUNCTION IN INTACT CELLS

ABSTRACT

The cAMP-dependent protein kinase (PKA) catalytic subunits C α and C γ share 83% identity in primary structure but differ in gene transcription regulation. The C α structure has been well characterized, however physical characterization of C γ 's structure has not been forthcoming. Both C α and C γ share 86% identity in their catalytic cores (amino acids 40-300), 57% identity in their amino terminal (a.a. 1-39) and 83% identity in their carboxyl terminal (a.a. 301-350). The C α amino terminus is important to structure stability and the carboxyl terminus is important to substrate recognition by the kinase. The divergence in homology in the amino and carboxyl terminals makes these likely regions to be responsible for structure-function differences between C α and C γ . This hypothesis was tested here with chimera constructs wherein the first 50 and terminal 70 amino acids were swapped between C α and C γ and tested through transient transfections with CREB-mediated reporter assays. While both wild type (*wt*) isoforms, C α and C γ , induced transcriptional activation, the chimera transfections failed to exhibit any activity in COS7 cells, but exhibited some activity in Kin8 cells. This difference in cell-type dependent activity may suggest that there are cell-type specific factors that are required for the proper expression of the chimeras. Immunoblot analysis demonstrated

that each of the chimeras was expressed in COS7 cells and sequence analysis of the constructs suggests that the expressed chimeras should have the expected primary sequence. While it is difficult to interpret the results obtained from the chimera studies, the amino and carboxyl terminals remain important regions of divergence between $C\alpha$ and $C\gamma$ that warrant further study.

INTRODUCTION

The catalytic subunits for the cAMP-dependent protein kinase (PKA) propagate extracellular signals through intracellular pathways by phosphorylating protein substrates at serine or threonine amino acids located within specific sequences (R-x-x-S/T and R-R-x-S/T are two common ones (1)) in response to an increase in intracellular concentrations of cAMP. PKA modifies substrates in the membrane, the cytosol, and the nucleus (1). One of the cellular functions regulated by PKA is gene expression through modification of transcription factors in the nucleus such as the cAMP-response element binding protein (CREB), the cAMP-response element modulator (CREM), and the Nuclear Factor- κ B (NF- κ B), or through modification of transcription factor regulators in the cytosol such as the NF- κ B inhibitor (I κ B) (1).

Of the four human PKA catalytic subunits ($C\alpha$, $C\beta$, $C\gamma$ and PrKX), the structure-function differences between $C\alpha$ and $C\gamma$ are the most striking (5,105). $C\gamma$ is a putative retrotransposition of $C\alpha$ inserted into the human genome some 40-million years ago (73), which maintains 83% identity with $C\alpha$ in primary sequence but differs in transcriptional regulation through CRE-containing promoters (5,105). Differences in the transcriptional regulation range depend on the cell-type used, but $C\alpha$ -regulated, CREB-mediated transcriptional activity tends to be 3-10 times greater than that of $C\gamma$ (105). There is some

evidence that $C\gamma$ may favor transcriptional activity through a different transcription factor, Sp1 (105), but this work remains to be corroborated by others. There is independent work demonstrating that PKA regulates Sp1-mediated transcriptional activity measured in a human cell line (HL60 cells), however the catalytic subunit(s) responsible for Sp1-mediated transcriptional activity were not identified (117).

As biochemical reactions in the cell follow the structure-equals-function adage, it is possible that differences in function between $C\alpha$ and $C\gamma$ can be attributed to differences in structure. The $C\alpha$ structure has been well characterized through crystallography, circular dichroism, and NMR analysis (33,118-120). However, physical characterization of $C\gamma$'s structure has not been forthcoming because its isolation in sufficient quantities required for this type of characterization has proven difficult (20). It may be possible to predict which regions of the protein confer $C\alpha$ and $C\gamma$ functional differences based on differences in their primary sequence. $C\alpha$'s sequence can be divided into three regions that correspond to structural domains in the folded protein. These include the amino terminus (amino acids 1-39), the catalytic core (amino acids 40-300), and the carboxyl terminus (amino acids 301-350) (118,121,122).

$C\alpha$ and $C\gamma$ share 86% identity in their catalytic core, which is conserved in most of the 518 protein kinases in the human genome and has been used as template to define this region in the protein kinase family (123). The $C\alpha$ amino terminus is important to the enzyme's stability and its interaction with the PKA pseudosubstrates. Deletion of the first 14 amino acids yields a functional isozyme that loses thermostability (a decrease in T_m) but maintains kinetics similar to *wt* $C\alpha$ (121). Deleting the first 39 amino acids yields a non-functional isozyme (121). Interestingly, both isozymes increase the thermostability

of the holoenzyme (121). $C\alpha$ and $C\gamma$ share approximately 57% sequence identity in the first 40 amino acids, making this a likely region responsible for structure-function differences between the two isoforms. It is also possible that this region is less important to the overall function of the enzyme and therefore there can be more variance in the amino-terminus of the enzyme, with little effect to the enzymes catalytic function. Nine of the first 39 amino acids are not conserved between $C\gamma$ and $C\alpha$ (at positions 6, 9, 10, 11, 12, 24, 28, 31, and 34), which constitutes 29% of the amino acids in this region, lending credence to the later.

The $C\alpha$ carboxyl terminus is important to substrate recognition by the kinase (122). Through alanine substitutions four of the 49 amino acids in this region have been shown to be important to affinity with ATP (K317, K319, T330, and E332) and six are important to affinity with the phospho-acceptor substrate (I315, T330, V337, I339, K345, and G346) (122). The carboxyl terminal region evidences relatively high homology in both isoforms (83% identity between $C\alpha$ and $C\gamma$), but two residues important to $C\alpha$ affinity for ATP and Kemptide are mutated in $C\gamma$ (K319T and V337I), which may be important to functional differences observed between the isoforms.

Here we decided to test the importance of the amino and carboxyl terminal regions to the function of each isoform, $C\alpha$ and $C\gamma$, by generating chimeras of these regions. It was hoped that placing either the $C\alpha$ amino or carboxyl terminus on $C\gamma$ would confer $C\gamma$ $C\alpha$ -like functions and that the inverse would hold true for $C\alpha$. With this in mind, the first 50 amino acids and the terminal 70 amino acids were swapped between each isoform and these isozymes were then tested in transient transfections for CREB-mediated reporter activity through a reporter vector consisting of a α -glycoprotein

hormone promoter (which contains two CREB response elements) upstream of a fire fly luciferase open reading frame. Unfortunately both the amino and the carboxyl terminal chimeras were expressed as non-functional proteins, exhibiting a total loss of transcriptional activity in COS7 cells relative to the *wt* isozymes. Loss of activity was not due to a lack of isozyme expression as was demonstrated through immunoblot analysis, but may be due to numerous other factors that were not tested here. An alternative to the approach used here would be to express the chimeras for purification and compare their *in vitro* kinase activity to that of the wild type subunits. But this approach defeats the purpose of measuring chimera activity through transient transfections, which was to avoid the complications inherent to protein purification of an active enzyme, especially one of limited stability such as $C\gamma$. However, transient transfection reporter assays with C-subunit chimeras did not address the question as to the significance of the amino and carboxyl regions to structure-function differences observed between $C\alpha$ and $C\gamma$, but that does not eliminate the study of these regions as a potential source for functional divergence between $C\alpha$ and $C\gamma$.

MATERIALS AND METHODS

Tissue Culture Conditions – Kin8 mouse adrenal cells were maintained in Hams F-10 media with 10% heat inactivated horse serum and 1% neomycin as previously described (5). COS7 cells were grown in DMEM with 10% FBS. Both cell lines were maintained in 5% CO₂ at 37°C.

Chimera constructs – The Chimeras were gifts from Wei Qing Zhang, who constructed both the amino and carboxyl $C\alpha$ and $C\gamma$ chimera expression vectors (Appendix B). Primers were ordered through Invitrogen. Sequencing of chimeras was done in-house

using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's instructions, on a 3100 *Avant* Genetic Analyzer (Applied Biosystem/Hitoch) automatic sequencer.

Plasmids– pCMV-C α was a gift from Dr. Michael Uhler (University of Michigan, Ann Arbor). The pCMV-C γ expression plasmid was created with the addition of *Bam*HI linkers to the *Eco*RI ends of the original C γ cDNA (45) and subcloning the product into the *Bgl*II site of the same pCMV expression plasmid as used for C α . The α -glycoprotein hormone firefly luciferase reporter plasmid (pGH α -fluc) (124) was a gift from Dr. G. Stanley McKnight (University of Washington, Seattle). The pCMV-RII α -GFP expression plasmid used to determine transfection efficiency was a gift from Dr. Suzan S. Taylor (University of California, San Diego). A pUC19 vector was used as a filler vector in all transient transfection experiments.

Reporter Assay Studies– Kin8 and COS7 cells were added at 325,000 cells per well in 6-well plates (9.6 cm²/well) (Corning Inc). Cells were allowed to grow for 24 hours to approximately 60% confluence before transiently co-transfecting with expression, reporter, and/or filler plasmids. The total amount of DNA loaded into the cell was tested in the ranges of 0.5-3.0 μ g using FuGENE 6 (Roche Molecular Biochemicals) at a 3 μ L Fugene: 1 μ g DNA ratio, before arriving at the levels described for each experiment. After 24 hours, the transfected cells were harvested in 500 μ L/well of Cell Culture Lysis Reagent (Promega). Firefly luciferase activity was measured using a Luciferase Assay System (Promega). Briefly, a 100 μ L of sample lysate was added in duplicate wells of a 96-well plate, followed by 100 μ L of firefly luciferase substrate, luciferin, in "Luciferase Assay Buffer." The plate was then shaken for 2 seconds and the number of photons released measured for 10 seconds using a flash assay setting on an

MLX Microtiter Luminescence Detection system (Dynex Technologies), recorded in relative luminescence units.

Immunoblot Analysis – Lysate from samples analyzed by luminometry was assayed with BioRad's Protein Assay reagent to determine protein concentration. A total of 1 μ g protein/transfection condition was loaded in duplicate on a single gel and separated on a 4% stacking, 7.5% running SDS-PAGE gel (125), then transferred to a PVDF membrane using a semi-dry blotting apparatus (BioRad, according to the manufacturer's instructions). The membrane was cut in half and probed with either a rabbit polyclonal antibody (pAb, Santa Cruz Biotechnology, catalogue # sc903, lot E088, diluted 5000-fold), which cross-reacts with both C α and C γ or a rabbit monoclonal antibody (BD Transduction Laboratories catalogue # 610980, lot 6, diluted 1000-fold), which selectively recognized C α , but not C γ . Immunoblots of chimeras were analyzed using the pAb to C α . The PVDF membranes were then incubated with a goat anti-rabbit pAb or anti-mouse pAb conjugated with horseradish peroxidase (Amersham-Pharmacia Biotech). The membranes were exposed to ECL reagents (Amersham-Pharmacia Biotech) and imaged on film (Hyperfilm, Amersham-Pharmacia Biotech) or through digital imaging on a VersaDoc (BioRad).

RESULTS

Chimera Transient transfections– Transfections were carried out in both Kin 8 mouse adrenal cells and COS7 monkey kidney cells, because *wt* pCMV-C α and C γ provide good reporter assay signals above background (105). COS7 cells are a good model system for testing C γ because this is the only primate specific catalytic subunit identified to date (73), and transcription co-factors that may be required to mediate C γ -regulated

transcription are likely to be present in a primate cell line. Kin8 cells were used as a model because they lack any endogenous $C\gamma$, thus any differences observed in an in-cell reporter assay are likely to be due specifically to the ectopic expression of $C\gamma$. Transient transfections were carried out here using 0.1 μg expression vectors and 0.5 μg reporter vector, as these concentrations have previously been demonstrated to be optimal in reporter assay experiments (105). Under these conditions, in COS7 cells, the *wt* pCMV- $C\alpha$ signal was 100-fold above background of a pUC19 empty vector, and the *wt* pCMV- $C\gamma$ signal was ten-fold lower than this signal (Figure 3). This difference in $C\alpha$ and $C\gamma$ reporter activity is consistent with previous experiments in COS7 cells (105). The amino terminal chimeras consisted of swapping the first 50 amino acids between each C-subunit and the terminal 70 amino acids for the carboxyl terminal chimeras. Sequencing of the chimera constructs demonstrated the expected sequence, however no luciferase activity was measured in the COS7 cell transfections with the chimera isozymes. Higher concentrations were tested (0.01-3.0 μg DNA) with no apparent change in the signal measured (data not presented).

The difference in reporter activity between $C\alpha$ and $C\gamma$ is not as striking in Kin8 cells. A higher amount of expression vector was necessary to obtain robust signals from transfections in Kin8 cells (105), thus all transfections in Kin8 cells were done with 1.0 μg expression vector. The signal measured for wild type $C\alpha$ was 14-fold lower in Kin8 cells and about 5-fold lower for $C\gamma$ (Figure 4), which is only a 3.5 fold difference in reporter activity between $C\alpha$ and $C\gamma$. Furthermore, unlike transfections in COS7 cells, transfections with amino terminal chimeras in Kin8 cells did not eliminate all

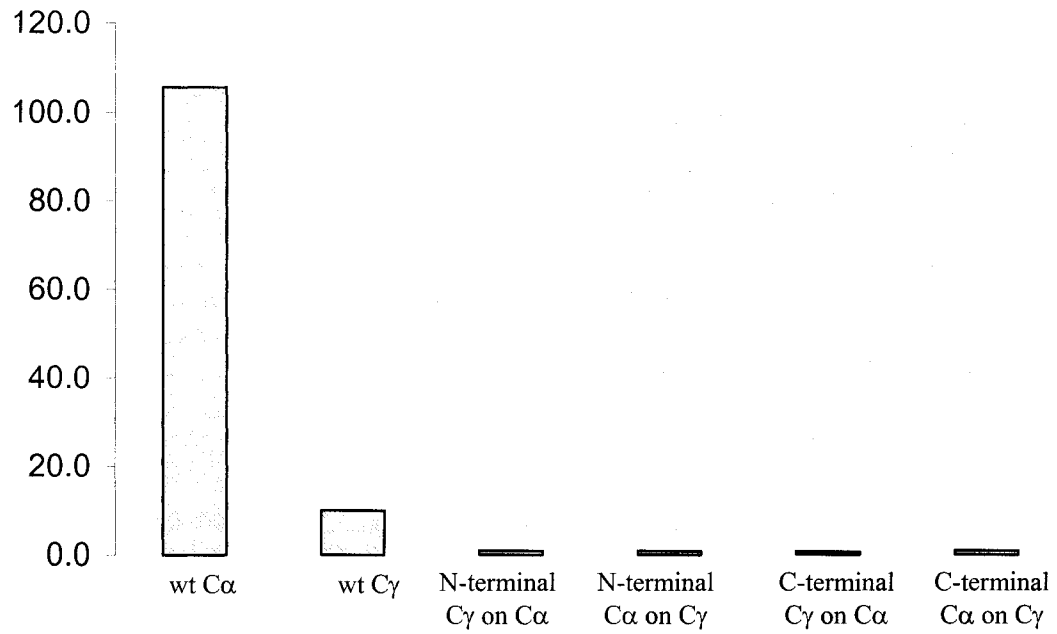


FIG. 3. **Transient transfection in COS7 cells with chimeras.** Co-transfections with wild type (*wt*) or chimera expression vectors and pGH α (CRE4) reporter vector in COS7 cells, $n=3$, *y-axis* expressed as Fold Lums Above Background.

activity measured from the *wt* isozymes. There was about a 1.75-fold decrease from *wt* C α with the C γ amino-terminal chimera and the C α -amino terminal chimera activity did not decrease from *wt* C γ activity (Figure 4). It should be noted that there was not much *wt* C γ activity to begin with (2-fold above background). The carboxyl terminal chimeras were not tested in Kin8 cells.

Immunoblot analysis of chimera transfections in COS7 cells– Extracts from COS7 transfections were analyzed by immunoblot to determine whether the lack of chimera-mediated activity was due to a lack of expression of the isozymes during transfection. Two different antibodies were used selectively to distinguish C α expression from C γ expression. The first was a polyclonal antibody (pAb) to the carboxyl terminus of C α that cross-reacts with C γ . The second was a monoclonal antibody that is selectively immunogenic against the C α catalytic core and does not cross-react with C γ (105) (copyright permission for use of Figure 5A in Appendix C).

Through use of these antibodies it was demonstrated that C α and C γ proteins were expressed after eighteen hours (Figure 5A). The polyclonal antibody cross-reacts with a nonspecific band that is larger than 40 kDa and is present at similar levels in control cells, where no C-subunit was identified following 18 hours. As the harvest of transfected cells was done following a 24-hour incubation, the immunoblot demonstrated that this is sufficient time for the ectopic expression of each isoform. Once it was established that both wild-type isozymes were being expressed, the polyclonal antibody was used to measure the expression of the amino terminal (Figure 5B) and carboxyl

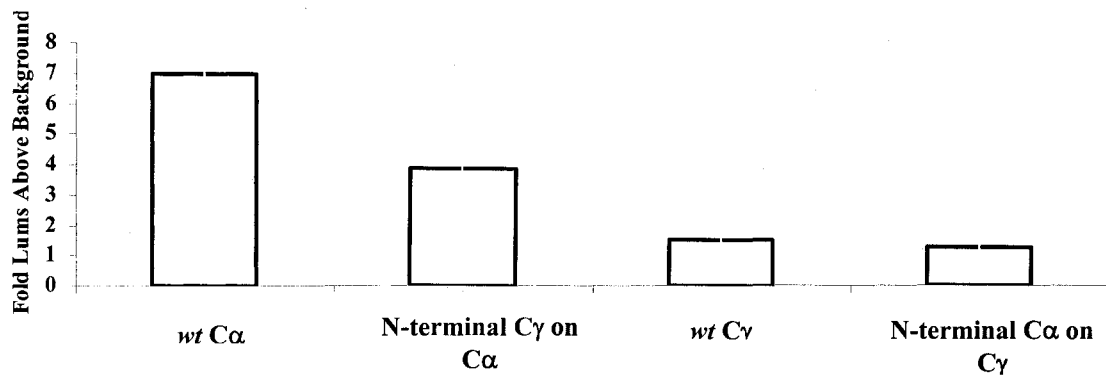


FIG. 4. **Transient transfection in Kin8 cells with chimeras.** Co-transfections with wild type (*wt*) or chimera expression vectors and pGH α (CRE4) reporter vector in Kin8 cells, n=2.

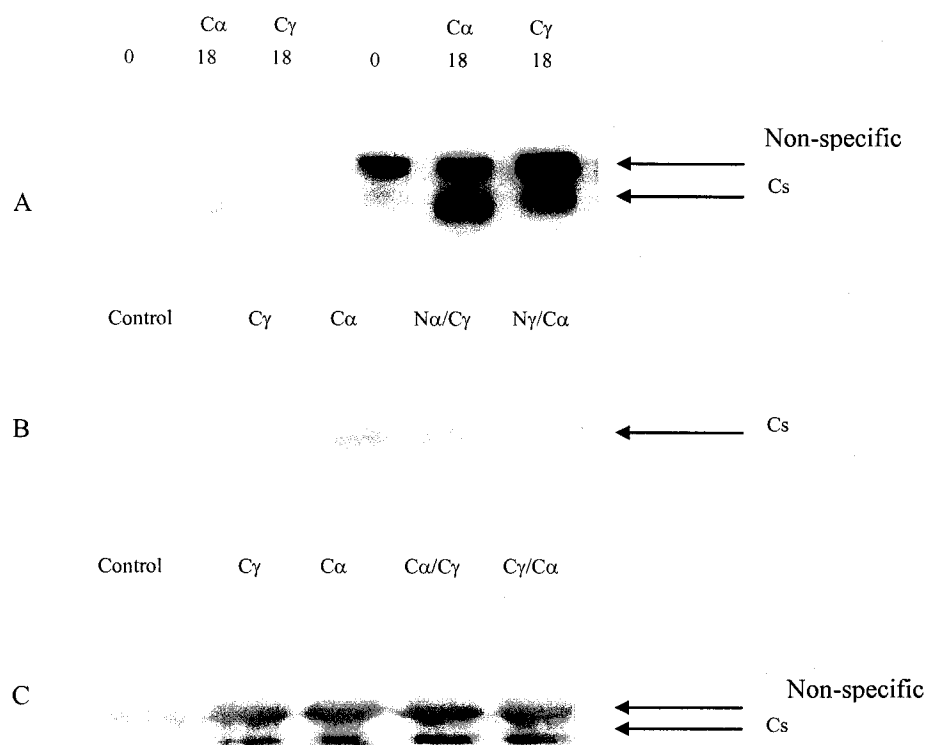


FIG. 5. **Immunoblot analysis of transfections in COS7 cells.** *A*, shows the use of a monoclonal antibody to C α (left panel) or a polyclonal antibody to C α (right panel) in immunoblots of extracts from COS7 transfected with *wt* pCMV-C α and pCMV-C γ analyzed at 0 and 18 hours after transfections (indicated above each lane). *B*, shows immunoblot of amino terminal chimeras treated with pAb to C α following a 24-hour transfection. *C*, shows immunoblot of carboxyl terminal chimeras treated with pAb to C α following a 24-hour transfection. Arrows point at the position of the catalytic subunit (Cs) and the non-specific band.

terminal chimeras (Figure 5C) and it appears that the chimera isozymes were expressed during the 24-hour transfection.

DISCUSSION

Protein folding and its tertiary structure are determined by the sequence of its primary structure (126-129) and protein function is dependent on its structure². Regions of high divergence between C α and C γ at their amino and carboxyl terminals could explain functional differences measured between them. Functional differences between C α and C γ include differential regulation of CREB-mediated transcription and differences in steady-state kinetics for physiological substrates. In both instances C γ -function is less efficient or slower than that of C α . Differences in transcriptional regulation can be linked directly to differential regulation of physiological mechanisms, as transcriptional regulation is significant to the regulation of multiple cellular events.

Both C α and C γ are 350 amino acids long and maintain a high degree of homology throughout the kinase catalytic core (amino acids 40-300, 86% identity) but they diverge significantly in primary sequence in the first 40 amino acids (57% identity) and less so in the terminal 50 amino acids (83% identity). Each of these three regions is associated with specific functions for C α . Several approaches could be taken to study the effect these differences have on C α and C γ function. The approach taken here was to swap entire sections of each isoform, generating two new isozymes with the expectation that placing the C α -terminals on C γ would make C γ function more like C α and vice versa. The chimeras were extended slightly beyond the amino and carboxyl terminal regions, the first 50 instead of 40 amino acids and the terminal 70 instead of 50 amino

acids, in part because this was where primers for generating the chimeras would work best. The homology between C α and C γ does not change significantly with the added length exchanged between the chimeras. The sequencing of the chimera constructs agreed with the expected sequence, thus, based on the sequence, no further alterations to the protein structure were expected in the products from the chimera expression vectors. While the *wt* C-subunit expression vectors behaved as expected in the luciferase reporter assays no measurable activity was detected from any of the chimera expression vectors in the COS7 cells transfections. Chimera transfections in Kin8 cells did have activity, but in this cell line the signal of the *wt* isoforms was not very robust. Differences in activity between COS7 and Kin8 suggests that there may be cell type specific factors that modify transcriptional activity, but it is not clear why no activity was measured at all in COS7 cells. Sequencing of the expression vectors and immunoblot analysis of cell lysate suggests that the protein was being expressed with the correct primary sequence in the cell. Thus, the difference in activity between Kin8 and COS7 cell transfection experiments may be due to factors involved in protein stability. It may be that Kin8 cells have better repair mechanisms for misfolded protein than do COS7 cells. If so, this would suggest that the reason that no activity was measured in COS7 cells was that the chimeras expressed as miss folded proteins and were not rescued by a mechanism to help re-fold them properly. However, misfolded proteins are normally found in the insoluble fraction of cell lysate and the immunoblot analysis of COS7 cell lysate where the ectopically expressed C-subunits were identified (Figure 5) were of the soluble fraction. This would suggest that the proteins expressed were properly folded. So while the likely explanation

² “Structure-equals-function” was our daily chant in Biochemistry at Old Dominion University as taught by Mark Elliott, Ph.D.

is that Kin8 cells express factors that facilitate the function of the chimeras, the nature of these factors is not clear. The approach of co-expressing the chimeras with protein chaperons associated with helping in protein folding (reviewed in (130)) could be used to test this hypothesis.

It may also be possible to trace the degradation of the chimeras in the cell through S^{35} -labeling the ectopic proteins and compare the half-lives of the chimeras to the half-lives of wild-type isoforms when expressed in COS7 and Kin8 cells. This approach has been used successfully to measure the half-life of *wt* C α in mouse derived cell lines (79). A shorter half-life for the chimera relative to *wt* isoforms would suggest that the chimeras are misfolded and being degraded more rapidly than *wt* isoforms in the cell (79,131). Another approach to determine whether the chimeras are not folded properly would be to isolate the protein (through immunoprecipitation) and analyze the protein for structural differences between wild-type and chimera proteins using circular dichroism, which would show changes in secondary structure that could be associated with protein misfolding (119,132-134).

In retrospect, it is possible that the approach utilized here was not the best one to address structural differences that attribute C α and C γ functional differences in the cell. A better approach might have been to generate select point mutations throughout the regions of interest (the amino and carboxyl domains) that would make one isoform resemble the other more closely. There are 17 amino acids that are not conserved in the C γ amino terminus. Each of these amino acids could be mutated to generate 17 independent isozymes for each isoform. Two more isozymes could be generated, one of C α and one of C γ , each containing all 17 of the mutations. While this approach may not

work, the modifications made here to each *wt* C α isoform in generating the chimeras were dramatic and the single point mutant approach may allow for the characterization of the effect each difference has on the overall enzyme structure and function. A similar approach has been used to study the C α carboxyl domain, where each amino acid of interest was mutated to an alanine and each isozyme characterized for function and stability (122). This approach provided some insight into which amino acids in the carboxyl group were important to C α -function. The C-subunit amino terminus still remains a region of interest that should be investigated further. This region evidences the highest divergence in homology and is important to overall protein and holoenzyme stability.

One problem with characterizing purified recombinant C γ is that it lacks the same stability as C α (20, 135), so another explanation for differences in function between C α and C γ may be that C γ is less stable in the cell and therefore less abundant. Differences in amino-terminal region may also confer on C γ a different tertiary structure than that of C α . Until studies are done comparing this domain in C α and C γ , it will remain unclear what effect differences in these regions have on differences in function between isoforms.

CHAPTER III

CHARACTERIZATION OF PROTEIN KINASE A CATALYTIC SUBUNITS α

AND γ PHOSPHORYLATION

ABSTRACT

Of the four human catalytic subunit isoforms— $C\alpha$, $C\beta$, $C\gamma$ and PrKX— $C\alpha$ has been studied extensively and serves as model for all protein kinases but very little is known about $C\gamma$, the only primate specific PKA catalytic subunit. While both $C\gamma$ and $C\alpha$ share 83% identity in primary structure, they differ in steady-state kinetics. A striking kinetic difference between $C\gamma$ and $C\alpha$ is that, while both share similar K_m values for most substrates, suggesting similar affinities, they have very different V_{max} values, with $C\gamma$ at least ten times slower than $C\alpha$. One explanation for the difference observed in their V_{max} is that $C\gamma$ and $C\alpha$ are not phosphorylated to the same extent. Phosphorylation at T197 is key to $C\alpha$ steady-state kinetics, and increases its catalytic rate 100-fold. Experimental evidence suggests that $C\gamma$ is either not phosphorylated, or not phosphorylated to the same extent as $C\alpha$ at conserved residues S139, T197 or S338. Differences in phosphorylation between $C\alpha$ and $C\gamma$ were studied here through reporter assays using $C\gamma$ and $C\alpha$ T197 point mutants, immunoblot analysis using an anti-phospho T197 selective antibody and LC-MS/MS phosphomapping of purified recombinant proteins. Two novel phosphorylation sites were identified on both isoforms by LC-MS/MS analysis ($C\gamma$ pS14

and C α and C γ pS259). It was also determined that C γ is phosphorylated homologously to mammalian expressed C α (pT197 and pS338) and the modification at T197 is important to the function of both isoforms, suggesting that differences in post-translational modification are not the reason for kinetic differences observed between these two isoforms.

INTRODUCTION

In the human genome 518 kinases are known to exist (2), but few have been studied as extensively as cAMP-dependent protein kinase (PKA). In the absence of cAMP, PKA exists as a tetrameric holoenzyme composed of a regulatory subunit dimer and two catalytic subunit monomers. The regulatory subunits bind cAMP inducing the release of the catalytic subunits from the holoenzyme. Once released, the catalytic subunits are active and able to phosphorylate multiple substrates, propagating intracellular signals that regulate multiple cellular events (1). Of the four human catalytic subunit isoforms— C α , C β , C γ (45) and PrKX (46) —C α has been studied extensively and serves as a model for all protein kinases (23) but very little is known about C γ , the only primate specific PKA catalytic subunit (45). While both C γ and C α share 83% identity in primary structure (Figure 6), they differ in chromosomal location, gene structure, gene transcription regulation, kinetics, and specificity for peptide substrates and pseudosubstrates (5,20,105,106). A striking kinetic difference between C γ and C α is that while the two share similar K_m values for most substrates, suggesting similar affinities, they have very different V_{max} values, with C γ at least ten times slower than C α (20).

```

C-gamma : P K DTEQE N RG YR GN A SS E LR : 50
C-alpha : A A KGSEQ K KE KK ET S QL D IK : 50

C-gamma : M R Q T G N M V I ID : 100
C-alpha : T K K S N D L I T VN : 100

C-gamma : Q Y L P R Q V C V : 150
C-alpha : E N M A H R I R I : 150

C-gamma : AVQ H L : 200
C-alpha : TFE Y I : 200

C-gamma : V F Y : 250
C-alpha : A Y F : 250

C-gamma : R KL H S R G : 300
C-alpha : K HF D N K N : 300

C-gamma : S EK YT A L I A S : 350
C-alpha : D QR FK T I V G T : 350

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FIG. 6. Sequence alignment of cAMP-dependent protein kinase catalytic subunits, $C\alpha$ and $C\gamma$. Mus musculus $C\alpha$ and Homo sapiens $C\gamma$. Both enzymes share 81% identity in primary amino acid sequence (shaded) with a 58% identity at the amino-terminus (residues 1-40), 83% identity at the carboxyl-terminus (residues 280-350) and 85% identity in the conserved catalytic core (residues 40-280). The dots (•) identify putative $C\alpha$ phosphorylation sites at serine 10, serine 139, threonine 197 and serine 338.

One explanation for the difference observed in their V_{max} is that $C\gamma$ and $C\alpha$ undergo different post-translational modifications. $C\alpha$ undergoes several post-translational modifications, which include myristoylation at glycine 1 (136), deamidation at asparagine 2 (137), and phosphorylation at serines 10, 139, 338 and threonine 197 (82). deamidation and myristoylation at the amino terminus help stabilize $C\alpha$ (83,119), and phosphorylation at T197 is key to $C\alpha$ steady-state kinetics (87), increasing the phosphoryl transfer rate between ATP and the phospho-acceptor 100-fold (138). Experimental evidence suggests that $C\gamma$ is either not phosphorylated, or not phosphorylated to the same extent as $C\alpha$. Specifically, while both isoforms are approximately 40 kDa, $C\gamma$ has a smaller Stoke's radius than $C\alpha$ and has slower kinetics than $C\alpha$ (20), both of which agree with differences observed between phosphorylated and unphosphorylated $C\alpha$ (87,139,140).

Differences in phosphorylation between $C\alpha$ and $C\gamma$ were tested here using a CREB-mediated luciferase reporter assay with $C\gamma$ and $C\alpha$ T197 point mutants, through immunoblot analysis using an anti-phospho T197 selective antibody, and through phosphomapping of purified recombinant his_{10} - $C\alpha$ and his_6 - $C\gamma$ with liquid chromatography coupled to tandem mass spectrometry. We found that recombinant $C\gamma$ is phosphorylated homologously to mammalian expressed $C\alpha$, which is phosphorylated at T197 and S338 (141) and that the modification at T197 is important to the function of both isoforms suggesting that differences in post-translational modification are not the reason for kinetic differences observed between these two isoforms.

MATERIALS AND METHODS

Tissue Culture— All mammalian cells were maintained in 5% CO_2 at 37°C. COS7 cells were grown in DMEM with 10% FBS and 2 mM L-glutamine. Sf9 insect cells were

maintained in a spinner flask (BELLCO Glass Inc.) in serum-free ExCell 420 media at 27°C.

Plasmids- pCMV-C α was a gift from Dr. Michael Uhler (University of Michigan, Ann Arbor). The pCMV-C γ expression plasmid was created with the addition of *Bam*HI linkers to *Eco*RI ends of the original C γ cDNA (45) by subcloning it into the *Bgl*II site of the same pCMV expression plasmid as used for C α . The α -glycoprotein hormone firefly luciferase reporter plasmid (pGH α -*fluc*) (124) was a gift from Dr. G. Stanley McKnight (University of Washington, Seattle). The pCMV-RII α -GFP expression plasmid used to determine transfection efficiency was a gift from Dr. Suzan S. Taylor (University of California, San Diego). A pUC19 vector was used as a filler vector in all transient transfection experiments. The pET16b-his10-C α was generated by Sean Collins and was a gift from Dr. Michael Uhler. Homologous recombination to produce pVL1393-his6-C γ baculovirus was done as described elsewhere (20).

Protein Expression and Purification- Recombinant murine his₁₀-C α was expressed from a pET16b expression vector from IPTG (0.40 mM final) induced BL21(DE3)pLysS (Novagen) transformed competent cells grown overnight in the presence of ampicillin (50 μ g/mL) at 37°C. For 1L of transformed cells (~3g cells), cell suspension was centrifuged at 11, 500 g (JA14 rotor, Beckman J2-MI), at 4°C, for 2h. The supernatant was discarded and the cell pellet stored at -80°C. Cells were harvested in 20 mL binding buffer (50 mM NaPO₄, pH [7.9], 0.5 M NaCl, 10% glycerol) with 2 mM PMSF. The cell suspension was sonicated 5 x 6 sec (mid-setting on a 550 Sonic Dismembrator, Fisher Scientific), while on ice, with 1 min between sonications. Extract was loaded onto a pre-equilibrated 7 mL Ni-IMAC column (ProBond, Invitrogen) and washed with 10 CV wash

buffer (binding buffer with 60 mM imidazole). Initially the enzyme was eluted with an 80 mL 0.06-1.0 mM imidazole gradient in binding buffer, while collecting 1 mL fractions, with the elution peak at about 350 mM imidazole, followed by separation on a Sephadex S300 gel filtration column. It was subsequently found that similar results were obtained if the protein was eluted with a 20 mL 0.06-1.0 mM imidazole gradient off a 2 mL Ni-IMAC column, while forgoing the gel filtration step (while loading the same amount of cell lysate). Both approaches provided about 5.5 U/mg of pure his₁₀-C α . Unphosphorylated his₁₀-C α was obtained by expressing pET16b in BL21(DE3)pLysS cells in the presence of membrane permeable, C α -ATP inhibitor H89 (100 μ M final, 10 mM stock in DMSO) and purified through Ni-IMAC chromatography.

For protein expression and purification of human his₆-C γ , Sf9 cells were grown to 70% confluence in spinner flasks (approximately 2 x10⁶ cells/mL in 1L of growth media), infected with his₆-C γ -baculovirus (80 pfu/cell) and incubated for three days at 27°C. Infected Sf9 cells were centrifuged (1,500 x g for 5 min) and the pellets stored at -80°C as 500 x10⁶ cell/aliquots. Aliquots were homogenized in 15 mL binding buffer (50 mM KPO₄, pH [7.9], 250 mM KCl) with 2 mM PMSF using a ground glass dounce homogenizer (Pyrex). The homogenate was centrifuged (15,800 x g for 30 min at 4°C) and the supernatant was applied to a 15 mL Ni-IMAC column (\approx 3x10⁷ cells/ml resin). The column was washed with 3 CV of binding buffer, 10 CV of wash buffer (binding buffer with 60 mM imidazole) and eluted with an 80 mL 60-600 mM imidazole gradient in elution buffer (binding buffer adjusted to pH [6.4]), while collecting 1 mL fractions. Fractions were assayed for kinase activity. Fractions exhibiting the highest specific activity were pooled and concentrated with Amicon Ultra-15 Centrifugal Filter Devices

(Millipore, 10, 000 MWCO). The protein peak elution occurred at ~ 350 mM imidazole. The purified protein had a SA of 0.5 U/mg and was analyzed for purity by SDS-PAGE subjected to silver staining, coomassie staining, or Sypro Ruby Staining (Molecular Probes).

Point Mutants– Site-directed point mutants T197D and T197A were incorporated directly into pCMV-C α and pCMV-C γ expression vectors using QuickChange II XL site directed mutagenesis kit, according to the manufacturer's instructions (Stratagene). Primer sequences used for point mutants are shown in Table I. Sequencing of point mutants was done using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's instructions, on a 3100 *Avant* Genetic Analyzer (Applied Biosystem/Hitoch) automatic sequencer.

Reporter Assay Studies– COS7 and Sf9 cells were added at 325,000 cells per well in 6-well plates (9.6 cm²/well) (Corning Inc). Cells were allowed to grow for 24 hours to approximately 60% confluence before transiently co-transfecting with expression, accessory, reporter, and/or filler plasmids (0.5-3.0 μ g total DNA range) with FuGENE 6 (Roche Molecular Biochemicals) using a 3 μ L Fugene: 1 μ g DNA ratio. After 24 hours, the transfected cells were harvested in 500 μ L/well of Cell Culture Lysis Reagent (Promega). Firefly luciferase activity was measured using a Luciferase Assay System (Promega). Briefly, 100 μ L of sample lysate was added in duplicate wells of a 96-well plate, followed by 100 μ L of firefly luciferase substrate, luciferin, in "Luciferase Assay Buffer." The plate was then shaken for 2 seconds and the number of photons released measured for 10

TABLE I
Primer Sequences used for Point Mutants

Mutant	Forward	Reverse
C _γ T197D	GGGCCGCACTTGGGACT	GGTCCCGCACAAAGTCC
	TGTGCGGGACC	CAAGTGCGGCCC
C _γ T197A	GCCGCACTTGGGCCTTGT	TCCCGCACAAAGGCCCA
	GCGGGA	AGTGCGGC
C _α T197D	AAAGGCCGTACTTGGGA	GGGGTCCCACACAAGT
	CTTGTGTGGGACCCC	CCCAAGTACGGCCTTT
C _α T197A	GGCCGTACTTGGGCCTTG	GTCCACACAAGGCC
	TGTGGGAC	AAGTACGGCC

seconds using a flash assay setting on an MLX Microtiter Luminescence Detection system (Dynerx Technologies) and recorded in relative luminescence units. To determine transfection efficiency control, cells were co-transfected with 0.5 μg pCMV-RII α -GFP, incubated 24 h, trypsin treated, and mounted on a slide (centrifuged at 7.06 g, 7 min on a Shandon Cytospin III centrifuge). Transfection efficiency was determined as a percent by the ratio of fluorescent cells counted under fluorescent light over total cells counted under white light in the same field (x1000 magnification, under oil emersion, on a Ziess Axioscope fluorescent microscope). A minimum of 200 cells/transfection experiment were counted.

Immunoblots– Samples were separated in a 9% SDS-PAGE gels with a 4% stacking gel, transferred and treated with antibodies as described previously (105). Three different antibodies were used: a rabbit polyclonal antibody to C α , which detects both C α and C γ (Santa Cruz Biotechnology sc-903, 1:1000); a rabbit monoclonal antibody to phospho-T197, which was a gift from Dr. Alexandra Newton's laboratory at the University of California at San Diego (p500 antibody, 1:1000, generated by Dr. Joanne Johnson (142,143)); and a rabbit polyclonal antibody to PKA substrate phospho-serine/threonine (Cell Signaling, cat # 9621, 1:1000). Following primary antibody treatment and washes, the membranes were incubated with HRP-conjugated anti-mouse or anti-rabbit polyclonal antibodies (Amersham Biosciences, 1:1000), then washed. The membranes were developed using Immun-Star HRP substrate (BioRad) and imaged electronically (BioRad's VersaDoc imaging system).

Sf9 cells were harvested to determine PDK1 expression (10 mM potassium phosphate pH [6.9], 1 mM EDT, 0.5% triton-X 100 and 2 mM PMSF). PDK1 expression was measured with a rabbit polyclonal antibody (Cell Signaling cat # 3062, 1:1000).

Kinase assay– In a standard kinase assay, the activity of the C-subunits was determined by the transfer of γ -³²P-ATP (200 μ M) to peptide (65 μ M Kemptide, LRRASLG) in the presence and absence of the protein kinase inhibitor peptide (amino acids 5-24) PKI₅₋₂₄ (2 μ M), as previously described (5). Briefly, a 50 μ L reaction mixture contained 30 μ L test mix (50 mM Tris·HCl (pH 7.0), 200 μ M ATP, 20 mM magnesium acetate, 10 mM DTT, and γ ³²P-ATP (300 cpm/pmol)) plus 10 μ L substrate. The reactions was initiated with the addition of 10 μ L of the enzyme and incubated at 30⁰C for ten minutes, spotted on P-81 paper, and placed in 75 mM phosphoric acid to terminate the reaction. The strips were washed in phosphoric acid 4-5 times, for ten minutes each wash, washed once in ethanol and allowed to dry before being placed in liquid scintillant. Specific activity measurements were incubated for 2, 4, 6, 8, and 10 minutes.

To measure phosphorylation of C γ by a PKA kinase, 128 nmoles his₆-C γ were co-incubated with 0.01 U his-PDK1 (Upstate) in a standard kinase assay using kemptide as a C γ -substrate. C γ activity was measured at regular intervals over a 30-minute time course. PDK1 activity was determined in a separate experiment using PDKtide (KTFCGTPEYLAPEVRREPRILSEEEQEMFRDFDYIADWC, Upstate) as a substrate in a standard kinase assay.

Phosphopeptide mapping and analysis– Protein bands were excised from polyacrylamide gels. Gel slices were cut into 1–2 mm cubes, washed 3 X 500 μ L Ultra-pure H₂O, and incubated in 100% acetonitrile for 45 minutes. If the gel was silver stained, the stain was

removed with SilverQuest™ destaining solution following the manufacturer's instructions. The material was dried in a speed-vac and rehydrated in 12.5 ng/μL modified sequencing grade trypsin solution (Promega) and incubated in an ice bath for approximately 45 minutes. Excess trypsin was removed and replaced with 40 – 50 μL of 50 mM ammonium bicarbonate, pH 8.0. Proteins in solution were digested using the ProteoExtract™ Trypsin digest kit (Calbiochem) following the manufacturer's instructions, with the exception that Promega trypsin was used instead of the trypsin provided with the kit. All trypsin digests were allowed to proceed overnight at 37°C. Peptides were then extracted 2X with 25 μL 50% acetonitrile, 5% formic acid, then dried in a speed-vac, and resuspended in 20 μL Buffer A (5% Acetonitrile, 0.1% Formic Acid, 0.005% heptafluorobutyric acid (HFBA)). Digests (3–6 μL) were loaded onto a 12-cm X 0.075 mm fused silica capillary column packed with 5 μM diameter Magic™ C-18 beads (The Nest Group, Southboro, MA) using a N₂ (g) pressure vessel at 1100 psi. Peptides were eluted by applying a 55 or 150 minute, 0 – 80% linear gradient of Buffer B (95% Acetonitrile, 0.1% Formic Acid, and 0.005% HFBA) at a flow rate of 130 μL/min with a pre-column flow splitter, resulting in a final flow rate of ~200 nL/min directly into the source. The LCQ-DecaXP (ThermoFinnigan) was run in automatic collection mode with an instrument method composed of a single segment and four data-dependent, scanned events with a full MS scan, followed by 3 MS/MS scans of the highest intensity ions. Normalized collision energy was set at 30, activation Q was 0.250, with the minimum full scan signal intensity set at 5×10^5 and a minimum MS2 intensity at 1×10^4 . Dynamic exclusion was turned on utilizing a three-minute repeat count of 2, with the mass width set at 1.50 Da. Sequence analysis was performed with Sequest™ using an

indexed human subset database of the non-redundant protein database from NCBI (PKA-C γ accession number: NM_002732, PKA-C α accession number: NP_032880).

Protein Phosphatase Treatment– Samples containing 50 pmol (2 μ g) pure his₁₀-C α were incubated with 0.75 U of bovine kidney PP2A₁ (0.5 μ g, >90% pure, Sigma) in 50 μ L reaction buffer (20 mM Tris-HCL, pH [7.5], 2 mM MgCl₂, and 1 mM DTT) at 37°C for 30 minutes. The reaction was quenched with 20% Trifluoroacetic acid. Spin-Out™ GT-600 desalting columns were used to replace the reaction sample buffer with 50 mM Ammonium Bicarbonate, pH [8.0]. Samples were digested as described above.

RESULTS

Effect of Point Mutants on the C-subunit's function- It has previously been shown that COS7 cells are optimal for comparing C α and C γ -mediated reporter activity through CRE-containing promoters, such as the α -glycoprotein promoter (105). Sf9 cells were also used as a model because they are the same cell line from which recombinant C γ is purified (20). Dose response studies with 0.5 μ g pGH α -*fluc* and 0.01-1.0 μ g pCMV-C α *wt* and pCMV-C α T197D determined that 0.1 μ g and 1.0 μ g were the most effective doses for transfections with Fugene6 in COS7 and Sf9 cells, respectively (data not shown) with a transfection efficiency of 22 ± 5 % (average \pm standard deviation) (Appendix D). Ectopic expression of pCMV-C α *wt* provides a robust signal through the pGH α -*fluc* reporter vector (78-fold above background) in COS7 cells and a weaker signal in Sf9 cells (3.5-fold above background) (Figure 7). The C α T197A point mutation knocks out 98% of pCMV-C α *wt* mediated reporter activity (3-fold above background) in COS7 cells and 25% of activity in Sf9 cells. Some 13% of pCMV-C α *wt* activity is restored with the C α T197D point mutant in COS7 cells and 10% activity is restored by

this mutant in Sf9 cells. As previously reported (105), ectopic expression of pCMV-C γ *wt* mediated pGH α -*fluc*-activity was about 52% and 33% lower than that of pCMV-C α *wt* in COS7 and Sf9 cells respectively (37-fold and 2-fold above background respectively). However similar trends were observed with the pCMV-C γ point mutants, with C γ T197A mutant knocking out all activity and T197D restored activity back to pCMV-C γ *wt* levels in both COS7 and Sf9 cells. Although not shown here, cell density affected C γ -mediated reporter activity, where at high cell density levels (>80% confluence) little or no reporter activity was measured in the presence of C γ in both cell lines tested here. This observation did not hold true for C α transfections.

Effect of PKA Kinase on Recombinant C γ - C α is phosphorylated at T197 through autophosphorylation in prokaryotic cells (140,144) and through another kinase in S49 mouse lymphoma cells (103). Studies done in the intact cell and *in vitro* suggest that phosphoinositide-dependent kinase-1 (PDK1) is a candidate for physiological regulation of PKA (86,104,145). As recombinant C γ is expressed in and purified from a eukaryotic cell line (Sf9 cells) it is possible that C γ has low activity because this cell line lacks a PDK1 homologue to phosphorylate C γ and C γ may not be able to autophosphorylate. To determine whether Sf9 cells expressed PDK1, Sf9 cell extract was tested through immunoblot analysis using a polyclonal antibody to PDK1 (Figure 8). The antibody detected a band doublet of which the major band had an apparent MW of 63 kDa (as determined through extrapolation of its R $_f$) that coincides with the MW reported for PDK1 (146,147). The band doublet may represent two different PDK1 isoforms expressed in this cell line or two different modification states of the same isoform.

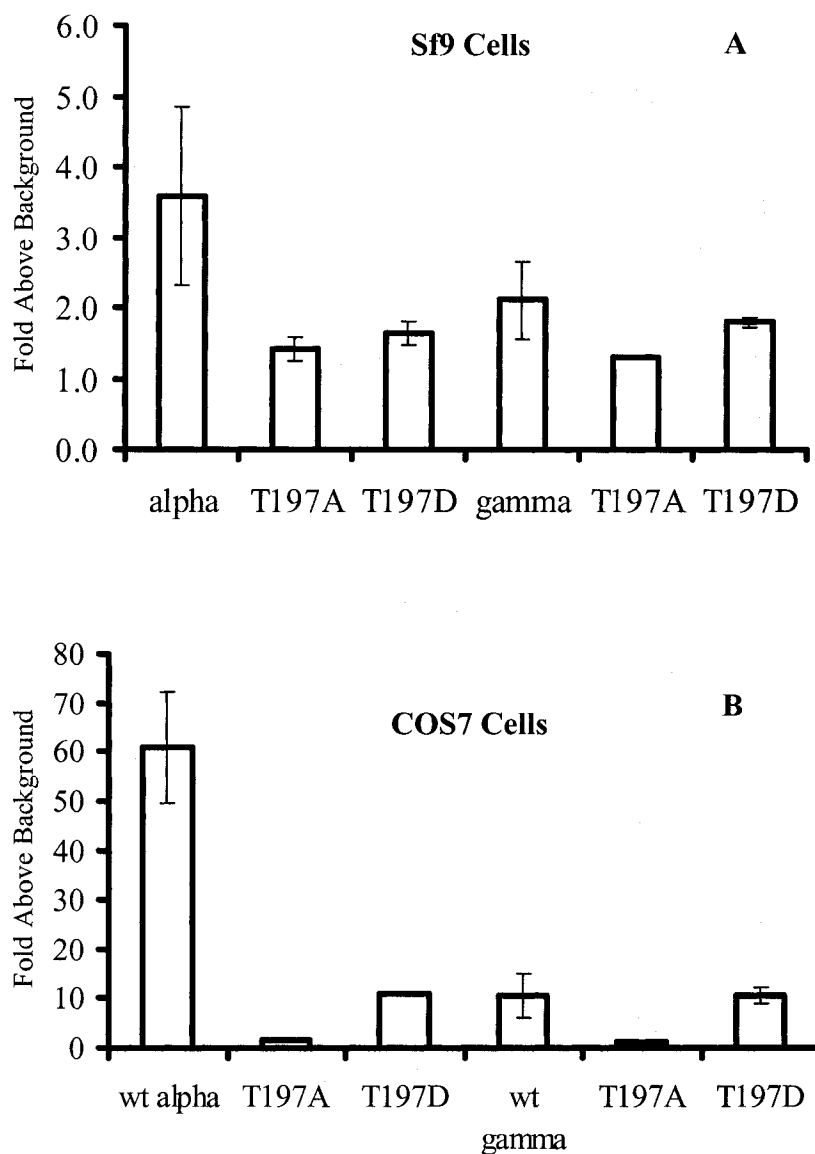


FIG. 7. **T197 point mutant transient transfections demonstrate its importance to α and γ function.** Co-transfection of pGH α -*fluc* with pCMV-Cs *wt*, pCMV-Cs T197D and pCMV-Cs T197A in *A*, Sf9 cells and *B*, COS7 cells using 0.1 μ g or 1.0 μ g expression vector, respectively. Values are expressed as fold above background (transfections with pUC19).

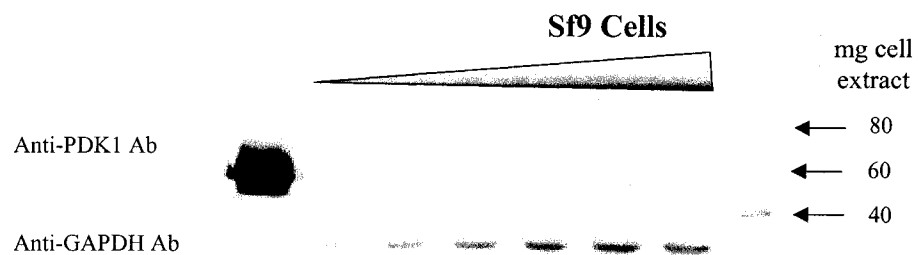


FIG. 8. **Immunoblot analysis of Sf9 cell extract identifies an immunoreactive band.** Pure recombinant his₆-PDK1 (40 ng of truncated PDK1₅₂₋₅₅₆, at left), Sf9 cell extract (5.8-174 μ g, upper panel) and GAPDH (loading control) were run on a 9% gel and analyzed with an anti-PDK1 rabbit polyclonal antibody. The apparent molecular weights of each band are indicated by the arrows to the right of the gel.

Purified PDK1 was co-incubated with recombinant C γ in a standard kinase assay to test whether PDK1 could modify C γ activity (Figure 9A). It was found that C γ steady-state activity did not change in the presence of PDK1 suggesting that purified recombinant C γ is already phosphorylated at T197. PDK1 activity was tested independently with a PKB based peptide substrate, PDKtide, and Kemptide and demonstrated to be active and to have about three fold higher activity with PDKtide (Figure 9B).

Measurement Phosphorylation through Immunoblot Analysis- Immunoblot analysis of both purified isoforms was used to directly measure C-subunit phosphate content. Phosphate content was tested through the use of two antibodies, one that specifically detects phosphorylation at T197 (142,143) and a second antibody that detects phosphorylation at arginine-directed serines or threonines (R-directed Ab), such as the PKA phosphorylation consensus sequences (R-x-x-S/T). Figure 10 shows that both antibodies detect phosphorylation in both pure C α and C γ , and C α expressed in the presence of H89, a membrane permeable inhibitor that selectively binds the C-subunit ATP binding site, was not phosphorylated. The absence of phosphate on C α (H89) supports the mechanism of autophosphorylation for *E. coli* expressed C α . Both antibodies detect phosphorylation at T197, but the R-directed Ab may also detect phosphorylation at the other sites on both isoforms. C α expressed in *E. coli* is reported to be phosphorylated at four residues, S10, S139, T197 and S338 (140), but phosphorylation of C γ has not previously been studied.

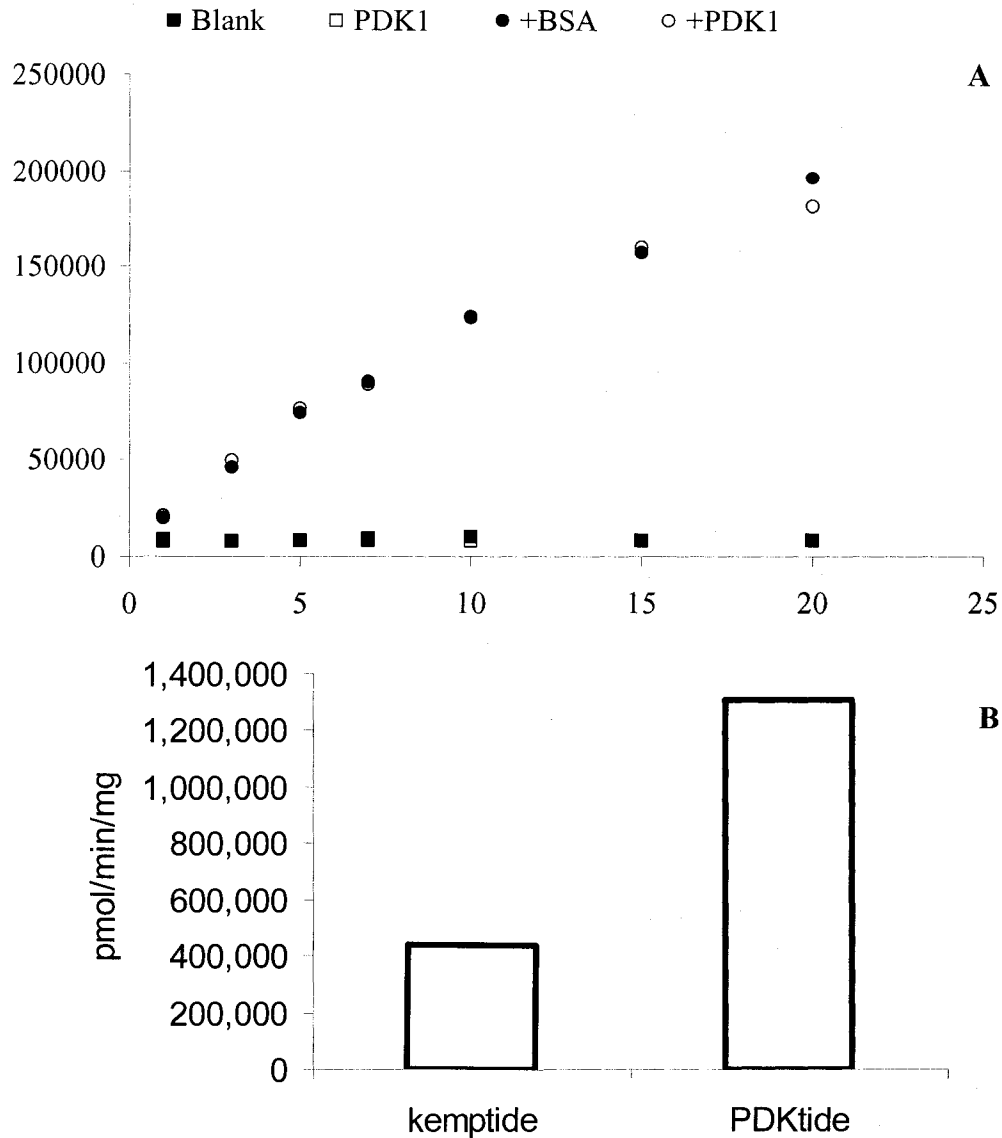


FIG. 9. Co-incubation of pure PDK1 with pure C γ does not increase C γ steady-state kinetics. *A*, Pure recombinant his₆-C γ and his₆-PDK1 were co-incubated in a standard kinase assay with kemptide as a C γ -substrate, stopping the reaction at select time points (x-axis, minutes). BSA was used as a protein control in the absence of PDK1 and activity was left as CPM (y-axis) as total protein levels did not vary between conditions. *B*, PDK1 activity with protein kinase-B based peptide substrate, *PDKtide*, and pyruvate kinase based peptide substrate, *Kemptide*.

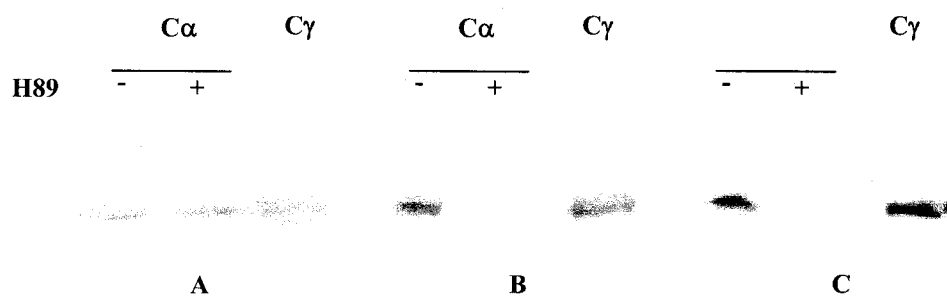


FIG. 10. Immunoblot analysis of pure C α and C γ identifies both isoforms as phosphoproteins. Pure C α expressed \pm H89 and C γ were analyzed with *A*, anti-C α rabbit polyclonal antibody, *B*, anti-RxxpS/T antibody, or *C*, p500 antibody, which is specific for phosphorylation at T197.

LC-MS Phosphomapping of the Catalytic Subunits- Both recombinant isoforms were subject to LC-MS/MS analysis to identify which residues were phosphorylated. Criteria for considering a peptide to be phosphorylated included identification by MS/MS of known phosphorylation sites on C α , identified peptides must have >70% of the b or y ions series, the phosphorylated peptide must have been seen in at least two independent runs, and the total number of ions present for a given scan need to be low enough such that the probability of randomly assigning ion masses is statistically negligible. Three forms of C α were analyzed: (1) native C α , (2) C α treated with PP2A and (3) C α expressed in the presence of H89, a C α competitive inhibitor that binds the C α -ATP binding site and blocks C α -autophosphorylation when C α is expressed in its presence. What was expected in this experiment was the identification and then subsequent removal of phosphates from C α .

Three phosphopeptides were identified on recombinant C α by MS/MS base peak neutral loss analysis (Figure 11). The phosphorylation position on the peptide was identified by manual spectral analysis at K₈GpSEQESVKEFLAK₂₁ (790.9 *m/z*) for S10 (49 *m/z* loss), I₁₃₅GRFpSEPHAR₁₄₄ (1170.32 *m/z*) for S139 (98 *m/z* loss), and G₁₉₃RTWpTLC₁₉₉ (894.04 *m/z*) for T197 (98 *m/z* loss) (Figure 11). Incubating C α with PP2A resulted in phosphate removal only at S139 (Figure 11B) and C α (H89) lacked phosphates at all three sites (Figure 11C). Base peak neutral loss chromatograms were normalized (NL) to base peak amplitude and a decrease in signal was observed between conditions (C α *wt* NL = 1.21×10^9 , C α (PP2A) = 8.1×10^8 , C α (H89) = 7.66×10^5).

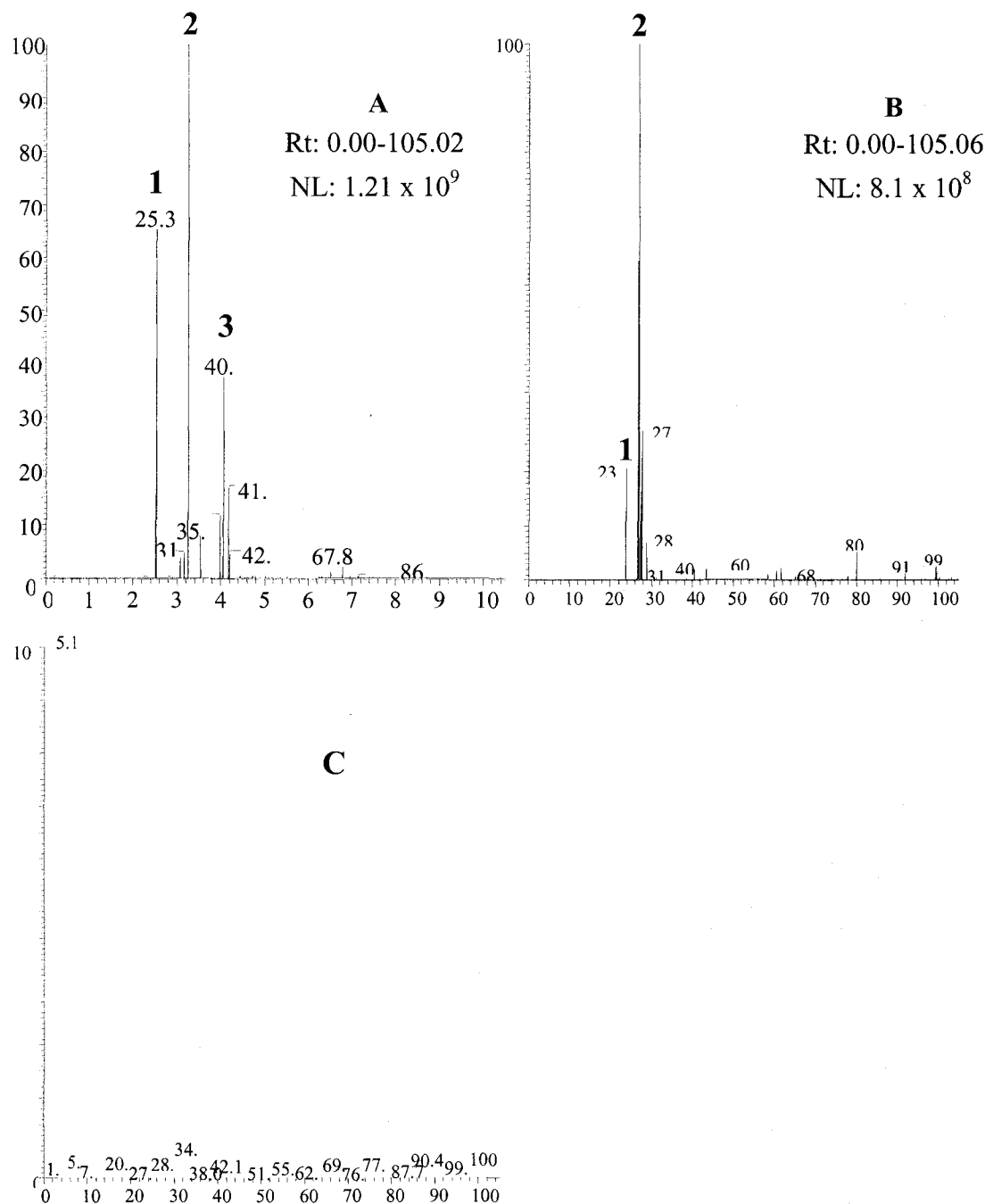


FIG. 11. LC-MS/MS base peak chromatogram of neutral loss from recombinant C α reveals three phosphorylated ions. PKA C α A, untreated, B, PP2A treated and C, expressed in the presence of H89, were analyzed by LC-MS/MS. The identified phosphopeptides were: 1. KGSEQESVKEFLAK, m/z 790.9 (2+), (S10); 2. GRTWTLC, m/z 894.04 (1+), (Thr197); 3. IGRFSEPHAR, m/z 1170.32 (1+), (Ser140). The y-axis represents relative abundance normalized (NL) to base peak and the x-axis is the retention time (Rt).

There may have been removal of phosphate at T197, however our approach was not quantitative, thus we could only measure the presence or absence of a phosphate. Subsequent scans using a longer gradient (150 min 0-80% buffer B) identified phosphates at V₃₃₇pSINEKCGKEFTEF₃₅₀ (1711.8 *m/z*, X_{corr}= 1.8, ΔC_n= 0.5) for S338 and at F₂₅₇PpSHFSSDLK₂₆₆ (1245.3 *m/z*, X_{corr}= 1.9, ΔC_n= 0.2) for S259.

Mapping of C_γ identified phosphopeptides at four distinct sites:

D₈TEQEEpSVNEFLAK₂₁ (820.41 *m/z*) for S14 (Figure 12),

T₁₉₅WpTLCGTPEYLAPEIILSK₂₁₃ (1144.309 *m/z*) for T197 (Figure 13),

F₂₅₇PpSKLSSDLK₂₆₆ (601.86 *m/z*) for S259 (Figure 14), and

Y₃₁₆TGPGDASNFDYEEEEELRIpSINEK₃₄₂ (1487.069 *m/z*) for S338 (Figure 15).

Phosphorylation at C_γ S139 was not identified in any of the LC-MS/MS runs performed.

However, identification of phosphorylation at S259 on both isoforms is a new observation. Phosphorylation at C_α S14 was not identified in any of the runs.

DISCUSSION

Autophosphorylation is an important mechanism for regulating protein kinases (81). *Escherichia coli* expressed PKA-C_α autophosphorylates at four residues, S10, S139, T197, and S338 (140) which are phosphatase resistant (141,144). Eukaryotic expressed C_α is phosphorylated at T197 and S338 by a PKA kinase (148) of which pT197 can be removed by a PP2A-like phosphatase in S49 cells (148). However, phosphatase removal of pT197 is enhanced under denaturing (148) and oxidation conditions (149), suggesting that this is a very stable modification. Analysis of the C_γ Stoke's radius, in gel apparent

PR04-106_c18_ms2#779 RT: 19.96 AV: 1 NL: 3.38E7
 T: + c NSI d Full ms2 820.41@35.00 [215.00-1655.00]

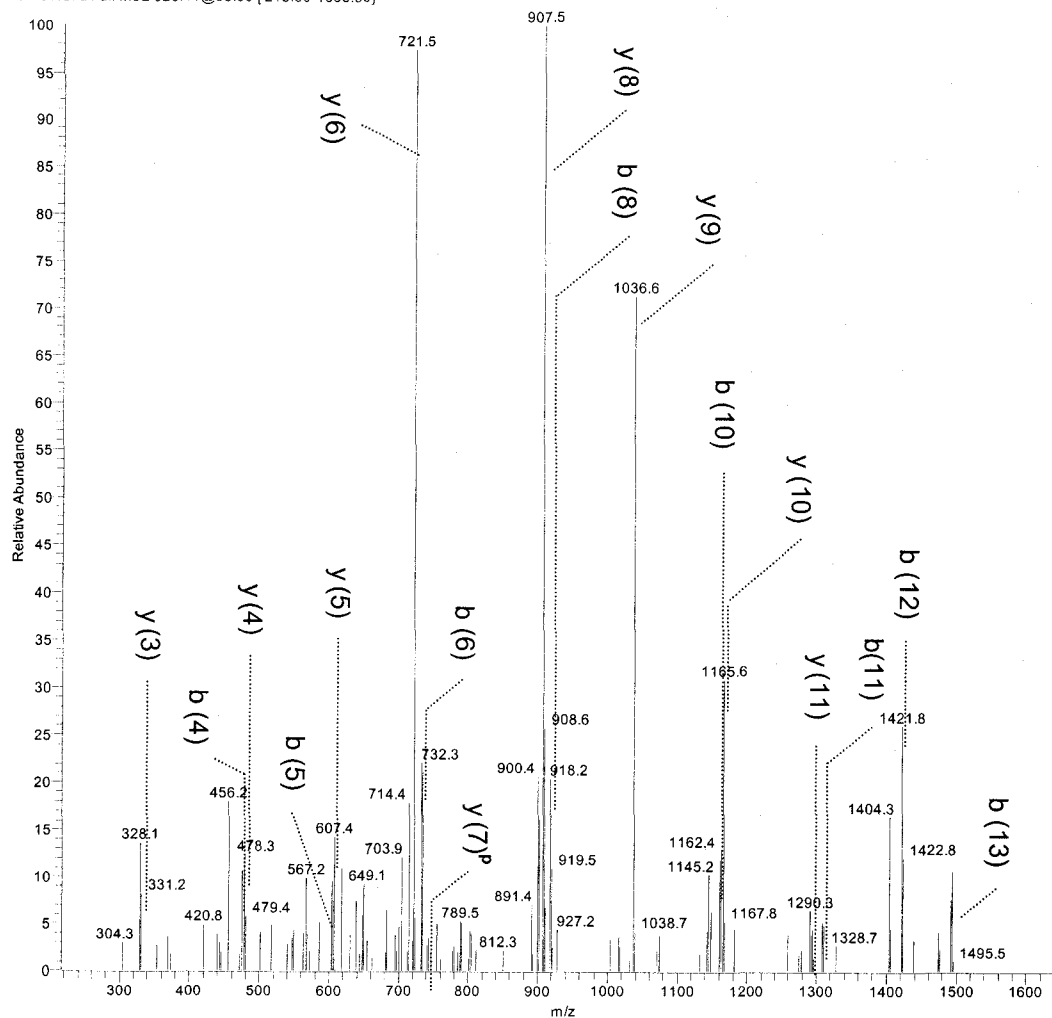


FIG. 12. LC-MS/MS scan of phosphopeptide $D_8TEQEEpSVNEFLAK_{21}$ from recombinant PKA-C γ trypsin digest. Parent ion for phosphorylation at C γ S14, m/z 820.41 (2+).

PR04-265_ms2 #1551 RT: 37.79 AV: 1 NL: 1.73E6
 T: + c NSI d Full ms2 1108.39@35.00 [295.00-2000.00]

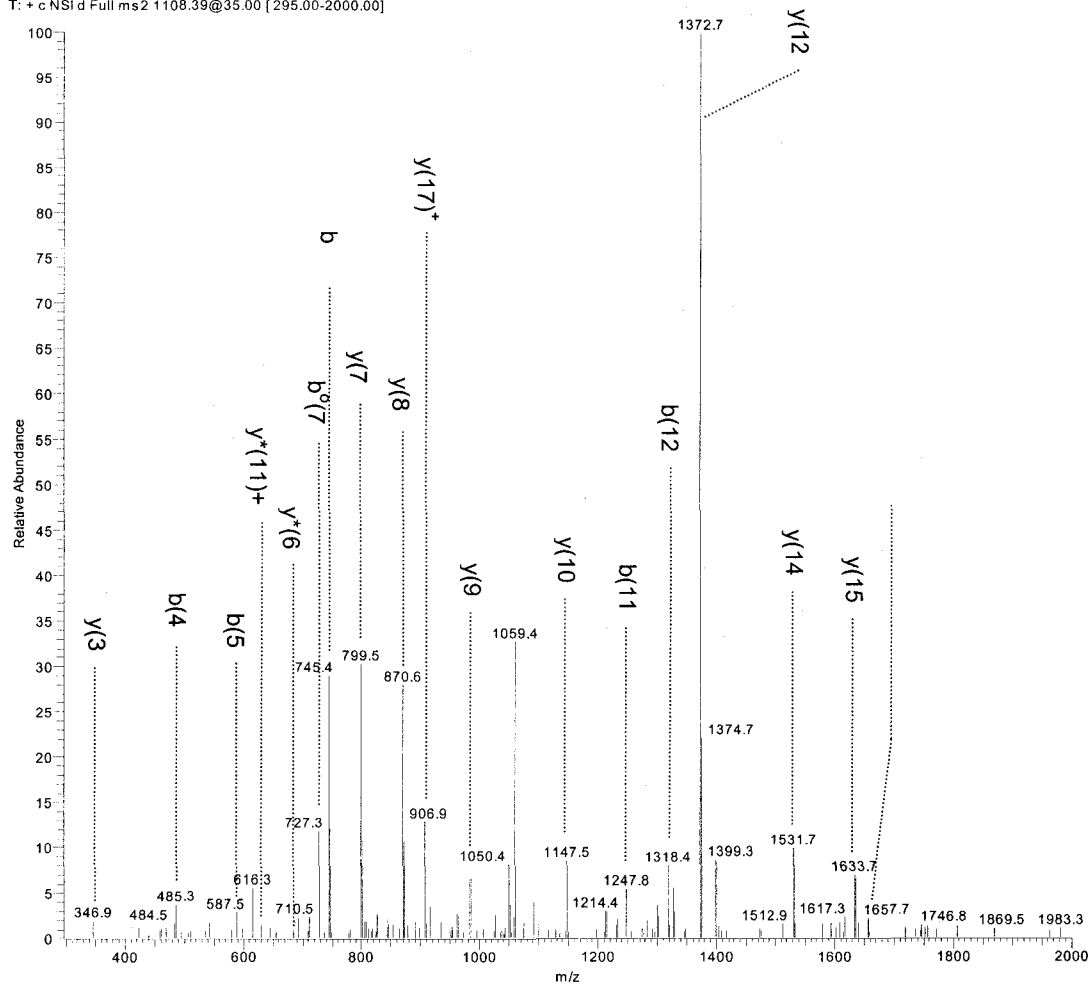


FIG. 13. LC-MS/MS scan of phosphopeptide $T_{195}WpTLCGTPEYLAPeILSK_{213}$ from recombinant PKA-C γ trypsin digest. Parent ion for phosphorylation at C γ T197, m/z 1144.309 (2+).

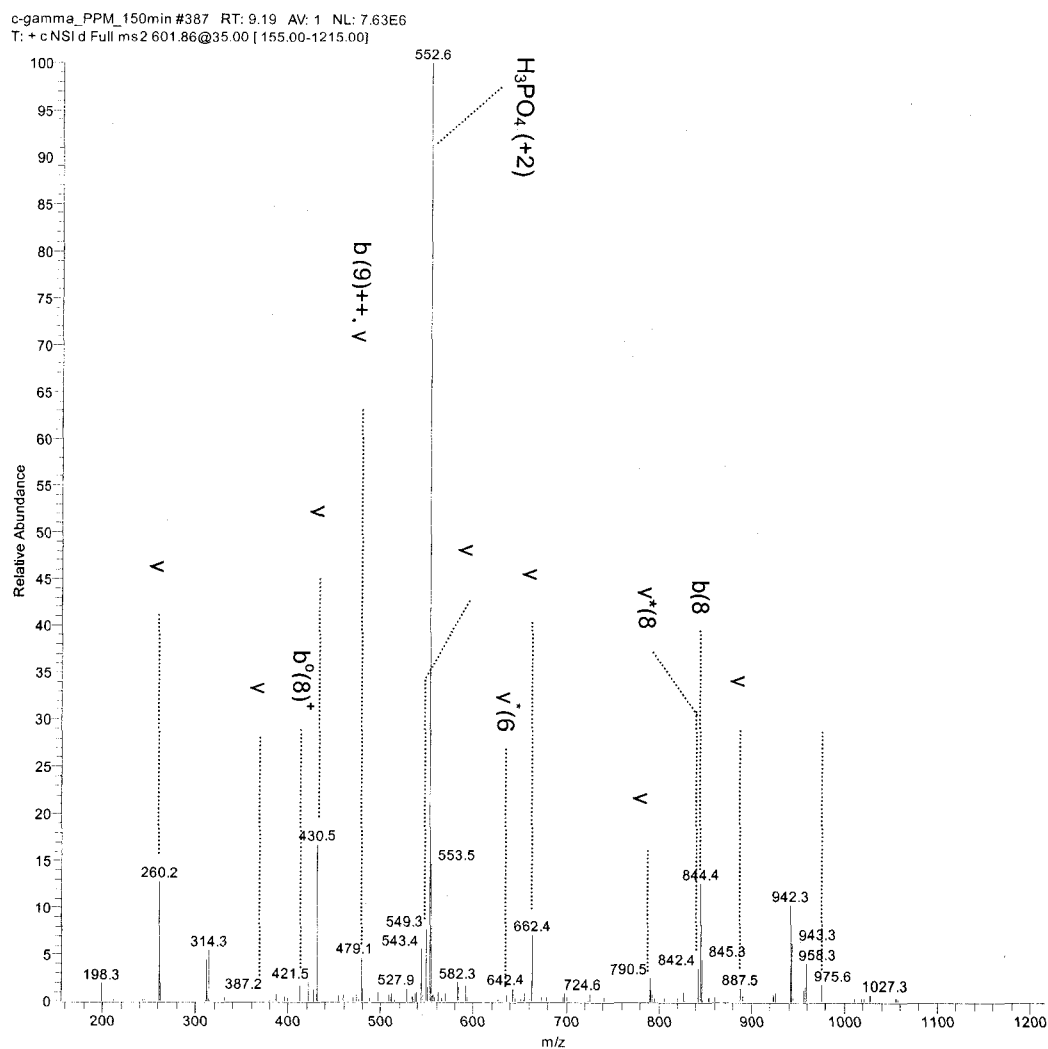


FIG. 14. LC-MS/MS scan of phosphopeptide $F_{257}PpSKLSSDLK_{266}$ from recombinant PKA-Cy trypsin digest. Parent ion for phosphorylation at Cy 259. m/z 601.86 (2+).

PR04-106_c18_ms2 #832 RT: 21.11 AV: 1 NL: 3.30E6
 T: + c NSI d Full ms2 1487.06@35.00 [395.00-2000.00]

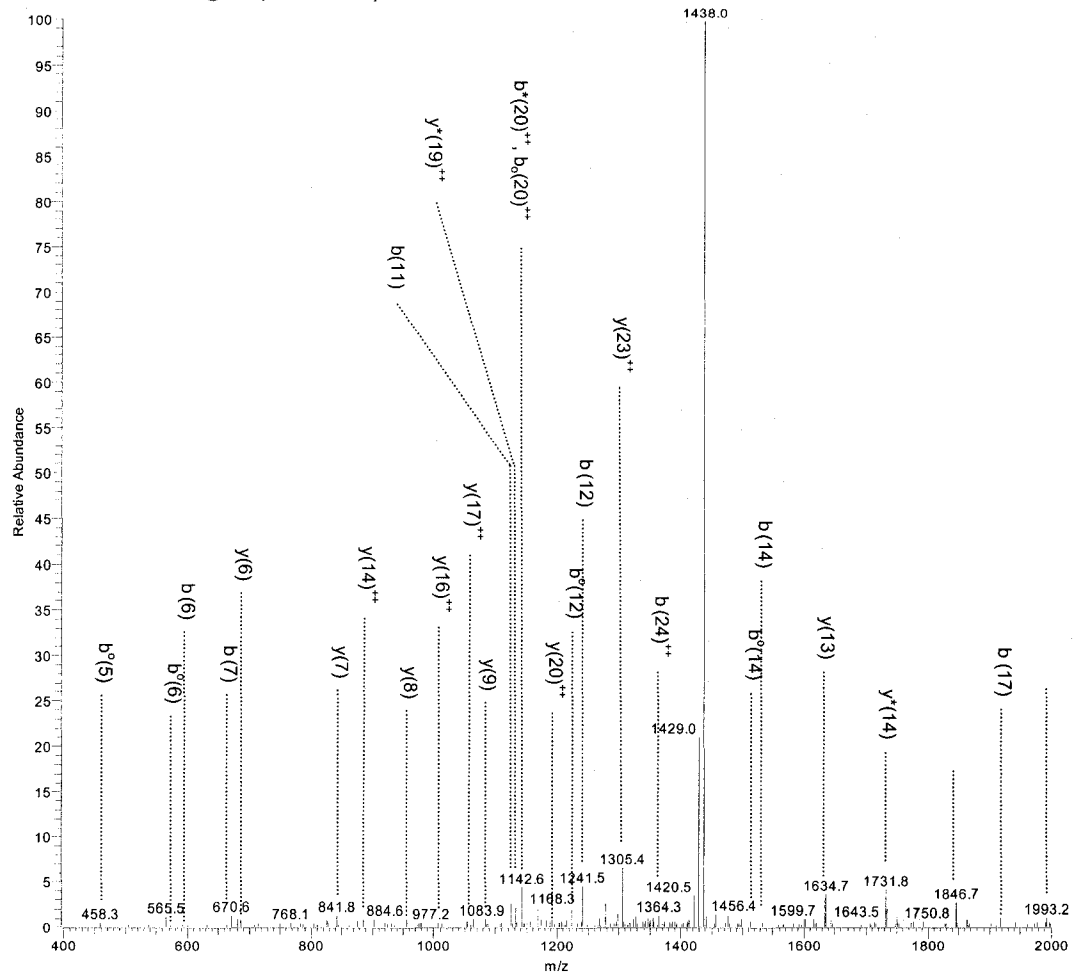


FIG. 15. LC-MS/MS scan of phosphopeptide
Y₃₁₆TGPGDASNFDDYEEEEELRIPsINEK₃₄₂ from recombinant PKA-C γ
trypsin digest. Parent ion for phosphorylation at C γ S338, m/z 1487.069 (2+).

molecular weight and steady-state kinetics suggest that unlike C α , C γ may not be phosphorylated (20).

Characterization of PKA phosphorylation had previously been done through tryptic digest-analysis of P³²-labeled enzyme, mass spectroscopy, analytic isoelectric focusing and chromatography analysis (139). Only recently has liquid chromatography coupled to tandem mass spectroscopy (LC-MS/MS) technology been successfully used to identify phosphorylation sites on recombinant C α (150), but such characterization had not been tried with C γ in part because its expression and isolation has been a challenge (20). Recombinant C γ expresses as an insoluble protein in *E. coli*, but can be expressed in eukaryotic cells including murine Kin8 cells (5) and insect Sf9 cells (20). Both expression systems provide a low yields relative to C α expressed from *E. coli*, and once pure the C γ half-life at -80°C or in liquid nitrogen is much shorter than that of C α (months vs. years at -80°C). Recombinant his-C γ has been expressed here to ease its purification and characterization. This approach allowed for sufficient C γ to be purified for its characterization through immunoblot and LC-MS/MS analysis.

Two of the four C α putative phosphorylation sites, T197 and S338, were identified on C γ via LC-MS/MS and immunoblot analysis, while phosphorylation at all four putative sites was identified on C α . Two novel modifications were also identified on C γ at S14 and at S259 on both isoforms. The modification at C γ S14 may be analogous to phosphorylation at C α S10, which is not conserved in C γ (Figure 6). The sequence surrounding C γ S14 (QEESSV) does not fit a PKA phosphorylation site but was identified as a potential casein kinase 2 (CK2) phosphorylation sequence (OMIGA software). Since Sf9 cells contain a CK2 homolog (151) this modification may be an artifact of expressing

C γ in Sf9 cells. However, CK2 is present in all eukaryotes, so it is also possible that phosphorylation at this residue is important to C γ function and that this modification is present on C γ expressed in human tissue and C γ ectopically expressed in murine Kin8 cells. The function of phosphorylation at C α S10 is still not clear (119) therefore it is difficult to speculate what difference phosphorylation at S14 instead of at S10 will have on the enzyme's function. Phosphorylation at S259, which was observed on both C α and C γ , may also be an artifact of overexpressing a recombinant protein and may not have any biological relevance. Such is the case for modifications on C α S10 and S139, which are only observed on C α expressed in bacteria, yet have no known biological function. Since this modification has not been previously described for C α it may not be that C α is not abundantly modified at S259 and thus the modification is only present on a small fraction of the enzyme purified and thus not detected until now. There are at least three major isozymes of recombinant C α identified from *E. coli* expression that are distinct in the number of phosphates each contains (2, 3, and 4) (139), but there is some evidence that suggests the presence of more (personal communication from Drs. Suzan Taylor and Mike Deal).

The fact that both isoforms purified from different expression systems contain this modification suggests that the mechanism for phosphorylation at S259 may be the same on both enzymes. The sequence surrounding S259 (RFPS) agrees with a PKA phosphorylation sequence (R/KxxS/T (1)) but was also identified as a PKC phosphorylation site (OMIGA software). Since the modification occurred in both prokaryotic and eukaryotic cells it likely that phosphorylation occurred through an

autphosphorylation mechanism for which there is a precedent in *E. coli* expressed C α (140,144,152).

Modifications to S10 and S139 were not identified on C γ . Myristylated C α exhibits reduced phosphorylation at S10 and S139 (82), however it remains to be determined if Sf9 expressed C γ is myristylated. Identification of phosphorylation to C α S139 has been problematic for others (150) yet C α pS139 was one of the first modifications identified here. This discrepancy may stem from differences in expression conditions which may lead to differences in the phosphate content in the isozymes expressed (153). Phosphorylated C α S139 was identified here by base peak neutral loss analysis and found to be the only phosphate completely removed by PP2A, suggesting it is a very labile modification relative to phosphorylation at T197. Phospho-T197 may have only been partially removed by PP2A and therefore not observed by LC-MS/MS analysis.

Sf9 expressed C γ contains phosphorylation modifications analogous to mammalian expressed C α (141). The mechanism for phosphorylation of C γ is not known at this time, but it may be through PDK1, a known PKA kinase (86,104,145). Since C α is a PDK1 substrate in eukaryotic systems and both C α and C γ share sequence homology at three of the four putative phosphorylation sites (Figure 6) and C γ contains the PDK1-interacting fragment (F-x-x-F), located at the C terminus of the PDK1 substrate (154), it is likely that C γ also serves as a PDK1 substrate. An immuno-reactive 63 kDa band was identified in Sf9 cells suggesting PDK1 is present in this cell line making PDK1 phosphorylation of C γ T197 a likely mechanism. Phosphates on C α are very stable as they are not easily added or removed once the kinase is translated (139,140,144) which

may explain why co-incubation of PDK1 did not increase $C\gamma$ steady-state kinetics. Since Sf9 cells express endogenous $C\alpha$ it is also possible that $C\alpha$ phosphorylates $C\gamma$. Bacterial expressed $C\gamma$, like $C\alpha$ (T197A), is insoluble and inactive (20,87) so it was thought that co-expression of $C\alpha$ and $C\gamma$ may increase $C\gamma$ stability, however this was tested and found not to be true (data not shown), suggesting that either *E. coli* expressed $C\gamma$ is already phosphorylated or that $C\alpha$ cannot modify $C\gamma$.

The use of point mutants here demonstrated that phosphorylation at T197 is important to $C\gamma$ function. The T197A point mutant makes the site inaccessible to modification and is deleterious to $C\alpha$ and $C\gamma$ mediated transcriptional activation while the T197D mutation, which mimics phosphorylation at this site, restores some ($C\alpha$) or all ($C\gamma$) of the lost activity. Cell-type differences were observed in the amplitude of the reporter signal measured between COS7 and Sf9 cells for $C\alpha$ and $C\gamma$ transient transfections. In both cell lines $C\gamma$ -mediated reporter activity was lower than that of $C\alpha$ as has been reported elsewhere (105) but $C\gamma$ -mediated reporter activity was higher in COS7 cells than in Sf9 cells. This difference may be due to differences in the cellular protein profile and in the regulation of the C-subunit isoform. While $C\gamma$ expressed from Sf9 cells is phosphorylated at T197, it may be that modification of the catalytic subunits is more efficient in mammalian COS7 cells than in insect Sf9 cells or that Sf9 cells have efficient phosphatases not present in COS7 cells.

While recombinant $C\gamma$ is phosphorylated at T197 and this modification is important to the isoforms function the question still remains as to what causes the functional differences observed between $C\alpha$ and $C\gamma$. Another possibility is that sequence

differences in the C α and C γ amino and carboxyl termini (Figure 6) contribute to functional differences measured between them. Studies done with C α and C γ amino and carboxyl terminal chimeres resulted in the expression of non-functional isozymes in COS7 cells. However, the amino terminal chimeres had little effect on their function when expressed in Kin8 cells (Chapter II). Another possible explanation for functional differences between C α and C γ may be in the binding and release rates of the nucleotide substrates. The release of ADP is the rate-limiting step for C α (34,35). It may be that overall structural differences between C α and C γ induce differences in the rate of the rate-limiting step. This hypothesis is explored in Chapter IV.

CHAPTER IV

**CHARACTERIZE THE KINETIC MECHANISM FOR PKA C α AND C γ
THROUGH DETERMINATION OF THE RATE CONSTANTS FOR EACH
CATALYTIC STEP OF THE PHOSPHOTRANSFERASE REACTION**

ABSTRACT

Human PKA catalytic subunits C α and C γ share high homology in their primary structure (83% identity) but differ in biochemical function. The hypothesis explored here is that C α and C γ differ in their catalytic mechanisms, possibly in their substrate binding-rates, and that differences in their mechanisms translate to differences in their biochemical function. To this end recombinant C α nucleotide binding rates were determined and compared to the C γ ADP off-rate through stopped-flow analysis, using mant-ATP and mant-ADP. The C α -ATP on and off rates were determined ($1.6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and 174 s^{-1} respectively) and found to be in good agreement with the literature. The C α ADP dissociation rate (33 s^{-1}) was slower than the ATP on-rate at physiological ATP concentrations, which suggests that ADP release is the rate-limiting step. However, a second slower rate was measured for mant-ATP binding (19 s^{-1}), which may suggest that mant-ADP dissociation is not the rate-limiting step. Recombinant his₆-C γ was used to measure its mant-ADP-off rate, a step where divergence in the catalytic mechanism from C α could explain differences in biochemical function. The signal measured for the C γ ADP off-rate was reproducible but very low (4 s^{-1}). The measured ADP-off rate for C α is

about 9-times faster than that of $C\gamma$, a result that is in agreement with differences in their steady-state kinetics. The physiological ramifications of biochemical difference between $C\alpha$ and $C\gamma$ still needs to be determined through studies of the isoforms *in vivo* and in the intact cell.

INTRODUCTION

Human PKA catalytic subunits $C\alpha$ and $C\gamma$ share high homology in their primary structure (83% identity) but differ in their function. Differences include CREB-mediated transcription regulation and steady-state kinetics (5,105), where $C\alpha$ -regulated transcriptional activation is 3-10 times higher than that of $C\gamma$ and $C\gamma$ -steady-state kinetics are 10-times slower than that of $C\alpha$ for most substrates tested (20). It is of interest to establish what factors contribute to these differences, which may manifest themselves physiologically. Several approaches have been attempted with little success to elucidate structural differences between $C\alpha$ and $C\gamma$ that could be responsible for their functional differences (Chapters II and III). The hypothesis explored here is that $C\alpha$ and $C\gamma$ differ in their catalytic mechanisms, either in their substrate binding-rates or product dissociation rates, and that differences in their mechanisms translate to differences in their biochemical function.

The $C\alpha$ catalytic mechanism has been well characterized through stopped-flow analysis, viscosometric analysis, characterization of point mutants and steady-state kinetic characterization (34,87,155,156). The mechanism is described as a sequential or ordered Bi-Bi mechanism (Figure 16) where ATP binds PKA first, causing a conformational change that allows for the binding of the phosphoacceptor, followed by an associative, inline transfer of the γ -phosphate from ATP onto the acceptor (reviewed

in (157)). Once phosphorylated, the phospho-substrate is released, followed by the release of ADP, which has been proposed to be the rate-limiting step of this mechanism (34). Here it was decided to compare recombinant C α and C γ binding rates for ATP and ADP because the binding rates of the phospho-acceptor substrate vary depending on its nature and proper progression of the reaction depends on ATP binding the active site before the phospho-acceptor (Figure 16). The proper binding order of the substrates is important to the progression of the catalytic mechanism as illustrated by the fact that when the phospho-acceptor binds C α before ATP the enzyme becomes locked-up in this species (C α -S-ATP) and contributes little to the total phospho-substrate (p-substrate) generated (157) (Figure 16).

Analysis of the C α and C γ catalytic mechanism was done here through stopped-flow analysis, using ATP and ADP conjugated to a N-methylanthraniloyl (mant) fluorophore through the ribose ring (Figure 16). This fluorophore is smaller than most fluorophores decreasing the perturbation in the enzyme reaction. Furthermore the absorption and emission properties of mant are distinct from those of proteins and nucleic acids, has a long fluorescent half-life, and has high sensitivity to small changes in its environment.

Using stopped-flow the C α -ATP on and off rates were measured and found to be in good agreement with the literature (155). The homogenous purification of recombinant his $_6$ -C γ proved to be difficult, but enough was attained to measure the C γ ADP-off rate, a step where divergence in the catalytic mechanism of these two isoforms could explain differences in biochemical function

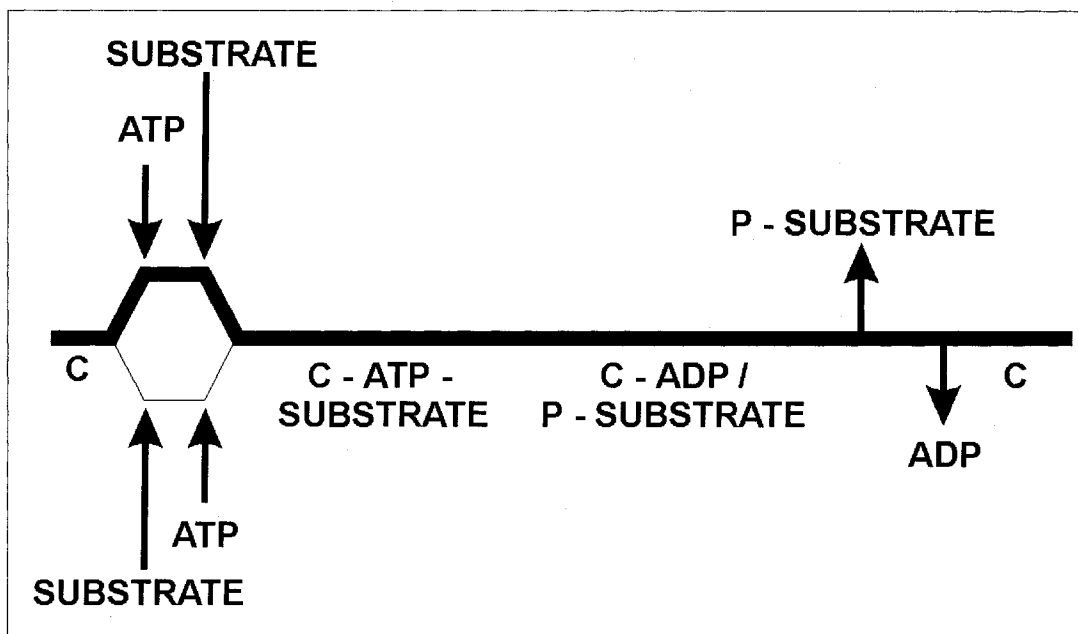


FIG. 16. Diagram depicting the Cleland shorthand notation for the $C\alpha$ catalytic mechanism. The reported mechanism for $C\alpha$ (outlined with the bold line) is an ordered Bi Bi mechanism where ATP and then the phospho-acceptor (substrate) bind $C\alpha$, followed by the transfer of the ATP γ -phosphate to the substrate, the release of the phospho-substrate and finally release of ADP (the reported rate-limiting step). The phospho-acceptor can also bind before the ATP (outlined with the thin line), but this forms a catalytically slow intermediate that cannot contribute to the overall mechanism.

. The measured ADP-off rate for C α was about 9-times faster than that of C γ , a result that is in agreement with steady-state kinetics measurements, but one that needs to be repeated for validation. Unfortunately making this measurement required the use of the entire stock of purified C γ and purification of recombinant C γ still remains a challenge, so at present repeating this measurement cannot be done. The result from this experiment provides a tempting explanation to the biochemical differences observed between C α and C γ , suggesting that C α releases its product (ADP) more rapidly than C γ during the rate-limiting step of the reaction. Thus, since C γ cannot catalyze a reaction any faster than the slowest step of its catalytic mechanism and this step is nine times slower than that of C α , it is possible to outline a mechanism based on their rate differences for the rate-limiting step to explain difference in their biochemical function, which is explored here.

MATERIALS AND METHODS

Plaque Assay and Viral Stock Amplification – To determine the titer of recombinant baculovirus stock, Sf9 cells from a spinner flask were added to 60 mm plates (1.5×10^6 /plate) and treated with 0.5 mL of viral stock dilutions (initially 10^{-1} - 10^{-3}) for one hour, room temperature, with continuous rocking. After an hour the virus stock was removed and the cells were covered with a 5 mL overlay of 0.5% agarose in growth media (SeaPlaque Agarose, low gelling temperature, FMC) and incubated at 27°C for five days. The cells were then stained with viral dye overlay by covering each plate with 3.3 mL 0.06 mg/mL Neutral Red (3.3mg/mL stock, Sigma) in 0.5% agarose and incubated at 27°C overnight (Figure 18). Plates were analyzed by counting number of plaques/plate on a white light transilluminator and calculating plaque forming units (pfu)

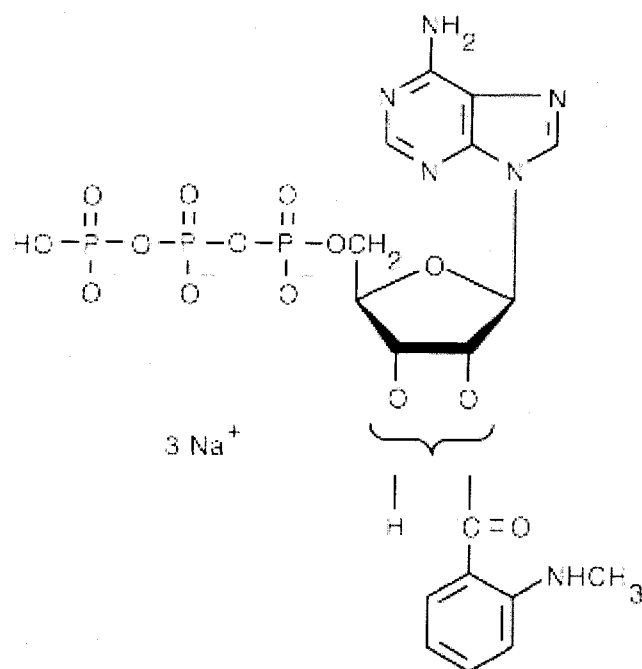


FIG. 17. Structure of 2'-(or-3')-O-(N-methylantraniloyl)adenosine 5'-triphosphate, trisodium salt (mantATP).

as follows: $pfu/mL = (average\ plaques/plate) \times 2 \times (viral\ stock\ dilution)$.

Viral stocks were amplified by adding 0.25 mL viral stock to 15×10^6 Sf9 cells in a T162 cm² flask, rocking flask for one hour at RT, adding 15 mL growth media and incubating at 27°C for five days. Supernatant was harvested and characterized through plaque assay to determine final titer. Optimal titer range is 10^6 - 10^7 pfu/mL.

Expression of his-tagged Catalytic Subunits – The 1.2 kb C γ cDNAs (158) was cloned separately into the *Bam*HI site of baculovirus transfer vector pVL1393 (Invitrogen). Homologous recombination to produce C γ baculovirus stocks occurred through cotransfection of 2×10^6 Sf9 cells for 4 hours at 27°C with 2 μ g baculovirus DNA (AcNPV DNA) and 3 μ g of pVL1393-C γ transfer plasmids. Recombinant C γ stocks were amplified in Sf9 cells (Gibco) and plaque purified according to the manufacturer's protocols (Invitrogen). Clones were selected based on positive kinase activity and the highest expressing recombinant C-subunit baculovirus stocks were chosen for further studies. Isolates were further amplified, quantified through plaque assays (expressed as pfu/mL) and then used to infect Sf9 insect cells for protein expression and purification.

For expression of C γ in Sf9 insect cells (Gibco) cells were cultured at 27°C in ExCell 420 media (JRH) serum free medium. Sf9 cells were grown in monolayer to 70% confluency (approximately 15×10^6 cells/T150 flask), infected with baculovirus (80 pfu/cell) and incubated for three days at 27°C. Sf9 cells that overexpress kinases, such as C α or C γ , undergo morphological changes that are distinct from the classic rounding morphology induced by *wt* baculovirus infection (159). The morphology of infected Sf9 cells changed from small spherical cells (Figure 19A) to large round cells (1-2 days post-infection) and finally developed single or some times double extend processes, which

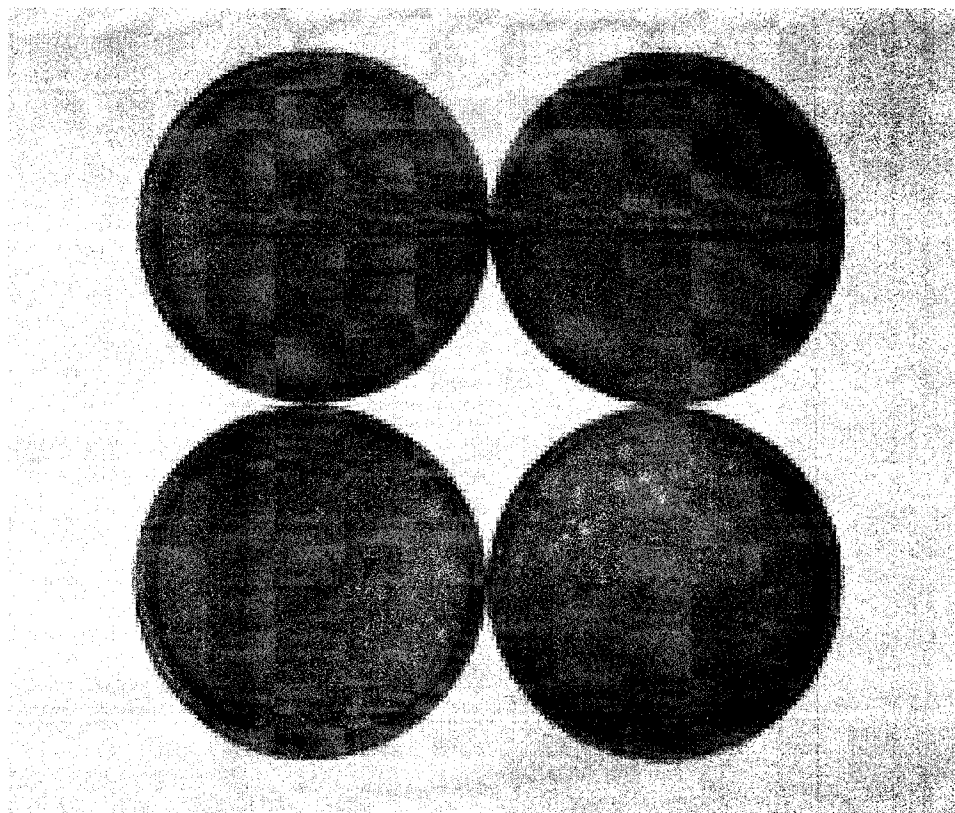


FIG. 18. **Picture of plaques following neutral red overlay.** The four plates in the picture include control (C), along with 10^{-5} (-5), 10^{-6} (-6), and 10^{-7} (-7) dilutions of the his₆-C γ expressing baculovirus stock.

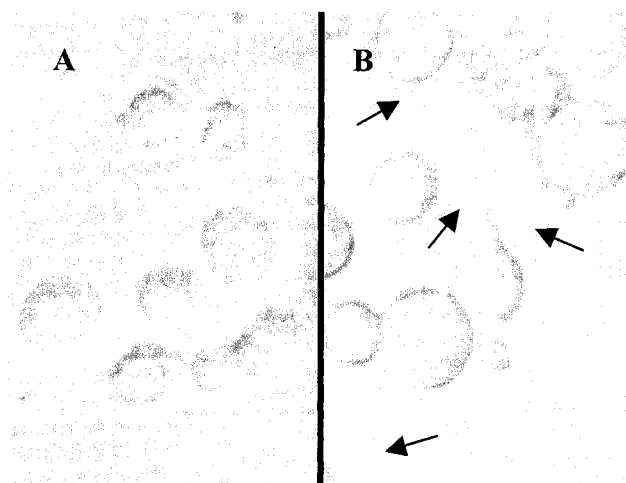


FIG. 19. Micrograph of morphology changes observed in Sf9 Cells after a three-day infection with C γ -gene carrying baculovirus. *A*, A micrograph of uninfected Sf9 cells which exhibit classic Sf9 cell morphology. *B*, A micrograph of Sf9 cells infected three days, expressing C γ , which indirectly causes the polymerization of cytoskeletal proteins. This leads to the formation of structural processes protruding from the cells (marked by the arrows), which resemble tails and serve as markers for C γ expression in the cell. Images were taken on an Olympus IX70 inverted microscope with an Olympus CCD computer-controlled camera (Olympus America, Melville, NY).

resembled tails (2 to 3 days post-infection) (Figure 19B). Similar morphological changes were observed in Sf9 cells expressing recombinant C α , however C α was not purified from Sf9 cells as its expression in this cell line was lytic and resulted in large loss of the protein prior to purification step (20).

For purification of human his₆-C γ , Sf9 cells were grown to 70% confluence in spinner flasks (approximately 2×10^6 cells/mL in 1L of growth media), infected with his₆-C γ -baculovirus (80 pfu/cell) and incubated for three days at 27°C. Infected Sf9 cells were centrifuged (1,500 x g for 5 min) and stored at -80°C as 500×10^6 cell/aliquots. Aliquots were homogenized in 15 mL binding buffer (50 mM KPO₄, pH [7.9], 250 mM KCl) with 2 mM PMSF using a ground glass dounce homogenizer (Pyrex). The homogenate was centrifuged (15,800 x g for 30 min at 4°C) and the supernatant was applied to a 15 mL Ni-IMAC column ($\approx 3 \times 10^7$ cells/ml resin). The column was washed with 3 CV of binding buffer, 10 CV of wash buffer (binding buffer with 60 mM imidazole) and eluted with an 80 mL 60-600 mM imidazole gradient in elution buffer (binding buffer adjusted to pH [6.4]), while collecting 1 mL fractions. Fractions were assayed for kinase activity. Fractions exhibiting the highest specific activity were pooled and concentrated with Amicon Ultra-15 Centrifugal Filter Devices (Millipore, 10, 000 MWCO). The protein peak elution occurred at ~ 350 mM imidazole. The purified protein had a SA of 0.5 U/mg and was analyzed for purity by SDS-PAGE subjected to silver staining, coomassie staining or Sypro Ruby Staining (Molecular Probes).

Recombinant murine his₁₀-C α was expressed from a pET16b expression vector from IPTG (0.40 mM final) induced BL21(DE3)pLysS (Novagen) transformed competent cells grown overnight in the presence of ampicillin (50 μ g/mL) at 37°C. For

1L of transformed cells (~3g cells), cell suspension was centrifuged at 11,500 g (JA14 rotor, Beckman J2-MI), at 4°C, for 2h. The supernatant was discarded and the cell pellet stored at -80°C. Cells were harvested in 20 mL binding buffer (50 mM NaPO₄, pH [7.9], 0.5 M NaCl, 10% glycerol) with 2 mM PMSF. The cells suspension was sonicated 5 x 6sec (mid-setting on a 550 Sonic Dismembrator, Fisher Scientific), while on ice, with 1 min between sonications. Extract was loaded onto a pre-equilibrated 7 mL Ni-IMAC column (ProBond, Invitrogen) and washed with 10 CV wash buffer (binding buffer with 60 mM imidazole). Initially the enzyme was eluted with an 80 mL 0.06-1.0 mM imidazole gradient in binding buffer, while collecting 1 mL fractions, with the elution peak at about 350 mM imidazole, followed by separation on a Sephadex S300 gel filtration column. It was subsequently found that similar results were obtained if the protein was eluted with a 20 mL imidazole gradient off a 2 mL Ni-IMAC column, while forgoing the gel filtration step. Both approaches provided about 5.5 U/mg of pure his₁₀-C α . Unphosphorylated his₁₀-C α was expressed in BL21(DE3)pLysS cells in the presence of 100 μ M H89 (10 mM stock in DMSO) and purified through Ni-IMAC chromatography.

Protein assay– total protein was analyzed via three different methods, Protein Assay (BioRad), based on the Bradford reaction assay, NanoOrange (Molecular Probes), a fluorescent protein assay, or through use of the extinction coefficient for each of the proteins. All three methods provided comparable values (provide extinction coefficients for each catalytic subunits).

Stopped-flow kinetic measurements– All transient kinetic measurements were made similar to methods described elsewhere (155,160) using a KinTec stopped-flow

spectrometer. Briefly, samples were mixed 1:1 in the instrument cuvet using two (single-mixing) or three (double-mixing) syringes. The excitation wavelength was 290 nm, and fluorescence emission was measured using a 420 nm cutoff filter. The instrument collected data points for 0 to 12000 ms time frames in each experiment (logarithmic time base). For data analysis, the average of three individual traces was used and the fluorescence data were recorded as volts. Rate constants and amplitudes were obtained by fitting of the data to equations describing a single or double exponential growth/decay using KinTec software. All fluorescence measurements were made in buffers containing 50 mM Mops, pH 7.0, measured at both 4°C and 25°C.

Protein Kinase Assays And Steady State Kinetic Determinations – In the standard kinase assay, the activity of the C-subunits was determined by the transfer of γ -³²P-ATP (200 μ M) to peptide (65 μ M Kemptide, LRRASLG) in the presence and absence of the protein kinase inhibitor peptide (amino acids 5-24) PKI₅₋₂₄ (2 μ M), as previously described (20).

Steady-state kinetic constants (K_m and V_{max}) for purified C α and C γ were determined using a modified protein kinase assay. Briefly, a 50 μ L reaction mixture contained 30 μ L test mix (50 mM Tris·HCl (pH 7.0), 200 μ M ATP, 20 mM magnesium acetate, 10 mM DTT, and γ ³²P-ATP (300 cpm/pmol)) and 10 μ L substrate. The reactions was initiated with the addition of 10 μ L of the enzyme and incubated at 30°C for ten minutes, spotted on P-81 paper and placed in 75 mM phosphoric acid to terminate the reaction. The strips were washed in phosphoric acid 4 or 5 times, for ten minutes each wash, washed once in ethanol, and allowed to dry before being placing in liquid scintillant. Specific activity measurements were incubated for 2, 4, 6, 8 and 10 minutes. Various concentrations of the substrates kemptide (1.9-435 μ M) were used to determine

K_m and V_{max} values. The determination of apparent K_m for the phosphate donor substrate ATP was done using a non-radioactive kinase assay (Appendix H).

Immunoblots – Samples were separated on a 9% SDS-PAGE gels with a 4% stacking gel, transferred and treated with antibodies as described previously (105). A rabbit polyclonal antibody to C α from Santa Cruz Biotechnology (sc-903, 1:1000) that cross-reacts with both C α and C γ was used to identify the purified recombinant proteins. Following primary antibody treatment and washes, the membranes were incubated with HRP-conjugated anti-rabbit polyclonal antibody (Amersham Biosciences) at a 1:1000 dilution and washed. The membranes were developed using Amersham's ECL detection kit and imaged electronically (BioRad's VersaDoc imaging system).

RESULTS

Purification and characterization of his-C α and C γ – Pure recombinant C γ had previously been obtained using a non-conjugated protein, requiring two chromatographic steps for purification, first with carboxymethyl (CM) ion-exchange chromatography followed by gel filtration chromatography (20). The advent of LC-MS/MS technology (please see Chapter III) provided one more method to characterize purified recombinant C γ . LC-MS/MS analysis of in-solution digest of purified C γ (method described in Chapter III) identified peptides to six proteins (Figure 20): (1) baculovirus p48 (YFNQSLIVDSK, Xcorr 3.97, Δ Corr= 0.16; YSDENINLLIHK Xcorr 3.78, Δ Corr= 0.25; SSFENQANNTDNIHNVK, Xcorr 3.32, Δ Corr= 0.41), (2) serum albumin precursor (LGEYGFQNALIVR, Xcorr 4.13, Δ Corr= 0.31; DAFLGCFLYEYSR, Xcorr 3.28, Δ Corr= 0.42; HLVDEPQNLIK, Xcorr

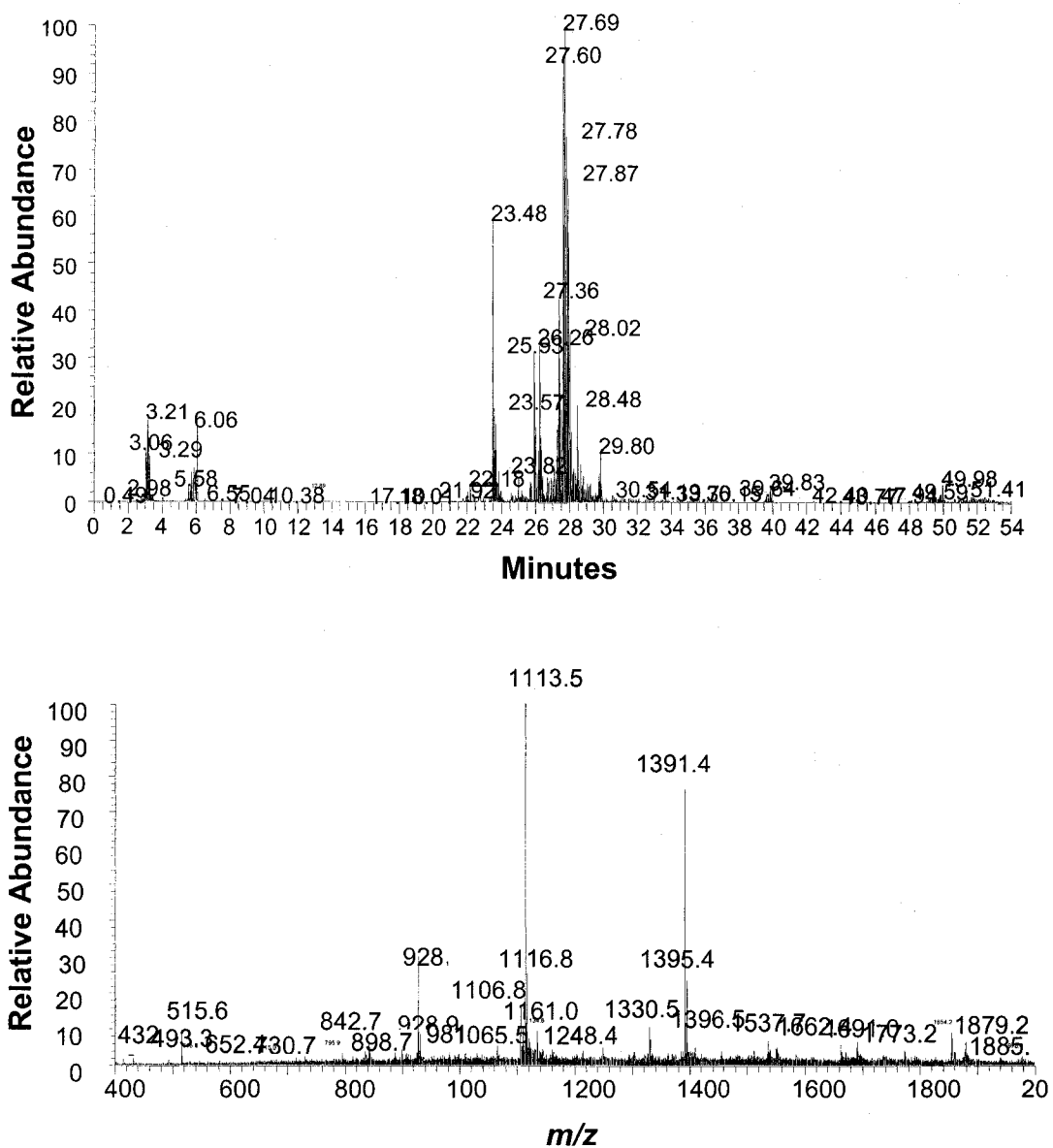


FIG. 20. Liquid chromatography coupled to mass spectrometry analysis of sample containing purified recombinant C γ . A, chromatography coupled to B, mass spectrometry (mass/charge or m/z) analysis of trypsin digested C γ containing sample revealed peptide fragments that matched sequences of various proteins, including bovine serum albumin, human keratin, and baculovirus protein p48. None of the fragments were matched the C γ sequence.

2.99, $\Delta\text{Corr}= 0.25$; KVPQVSTPTLVEVSR, $X_{\text{corr}} 2.83$, $\Delta\text{Corr}= 0.14$), (3) human keratin type II cytoskeletal 1 (SLDLDSIIAEVK, $X_{\text{corr}} 2.79$, $\Delta\text{Corr}= 0.17$; TLLEGESR, $X_{\text{corr}} 2.54$, $\Delta\text{Corr}= 0.12$), (4) GDP dissociation inhibitor (LSAIYGGTYMLDKPIDEIVLGEGGK, $X_{\text{corr}} 5.77$, $\Delta\text{Corr}= 0.48$; SPYLYPMYGLGELPQGFAR. $X_{\text{corr}} 5.43$, $\Delta\text{Corr}= 0.42$), (5) chain A crystal structure of caspase-8/p35 complex (DYSDQMDGFHDSIK, $X_{\text{corr}} 5.77$, $\Delta\text{Corr}= 0.48$); and (6) E chain, leech-derived tryptase inhibitor (IITHPNFNGNTLDNDIMLIK, $X_{\text{corr}} 4.30$, $\Delta\text{Corr}= 0.40$). None of the peptides identified corresponded to $C\gamma$, suggesting that $C\gamma$ was not the most abundant protein isolated from its purification. This became a major concern because all of the enzyme assays performed to characterize $C\gamma$ are dependent on an accurate measure of its concentration in the reaction, which becomes a problem when a contaminant is present in the sample.

Another approach was devised to purify baculovirus-expressed recombinant $C\gamma$ from Sf9 cells to improve resolution during purification and to minimize the chromatographic step required for purification. The $C\gamma$ ORF was inserted into a vector that would express it with the addition of six histidines to its amino terminus to allow for immobilized metal affinity chromatography (IMAC) purification using Ni^{2+} - or Co^{2+} -chelated resin (Described in Materials and Methods). After comparing Ni and Co resin for $C\gamma$ purification, it was found that $C\gamma$ had a higher affinity for the Ni-chelated resin (eluting at 350 mM imidazole) than for Co-chelated resin (eluting at 150 mM imidazole), making Ni-resin favorable to minimize the loss of $C\gamma$ during binding and washing steps of the purification. But this approach still proved difficult in part because it was difficult to optimize $C\gamma$ expression in Sf9 cells. Also, Sf9 cells contain a population of proteins that

bind the Ni resin (Figure 21). Because of this, resolution of endogenous Sf9 proteins from baculovirus expressed C γ was also difficult (Figure 21). Fractions containing C γ had to be selected after protein levels in the elution profile dropped, leading to a loss of C γ in the shoulder where overlapping occurred between C γ and Sf9 cell-protein (Figure 21). Once expression and purification conditions were optimized C γ was expressed and purified from Sf9 cells with yields of 2mg of recombinant protein that were >80% pure (Figure 22). High-yield, homogeneous purification of C α ectopically expressed in bacteria is relatively straightforward and well documented (96,139,161). His₁₀-C α was purified here so that both isoforms could be compared containing the same type of modification.

Recombinant his₆-C γ and his₁₀-C α were characterized through steady-state kinetics following purification using Kemptide as a substrate, and were analyzed with Eadie-Hofstee plots (Figure 23). Results from this characterization suggest that C γ 's affinity for Kemptide is about 5 times higher than previously reported with the non-his tagged isozyme ($K_m = 4 \pm 2 \mu\text{M}$, average \pm standard error, $n = 9$), while his₁₀-C α exhibited similar affinity for Kemptide as measured with the non-his tagged isozyme ($K_m = 23 \pm 10 \mu\text{M}$, average \pm standard error, $n = 7$). These averages represent measurements made from several different preparations over the past five years. An average for their V_{max} could not be made, as many of the preparations made were not pure enough for an accurate determination of C γ protein concentration in the preparation.

Stopped-flow characterization of C α -ATP binding rates– The ATP binding rates were determined with C α , but not C γ , because of the high amounts of C α readily obtained

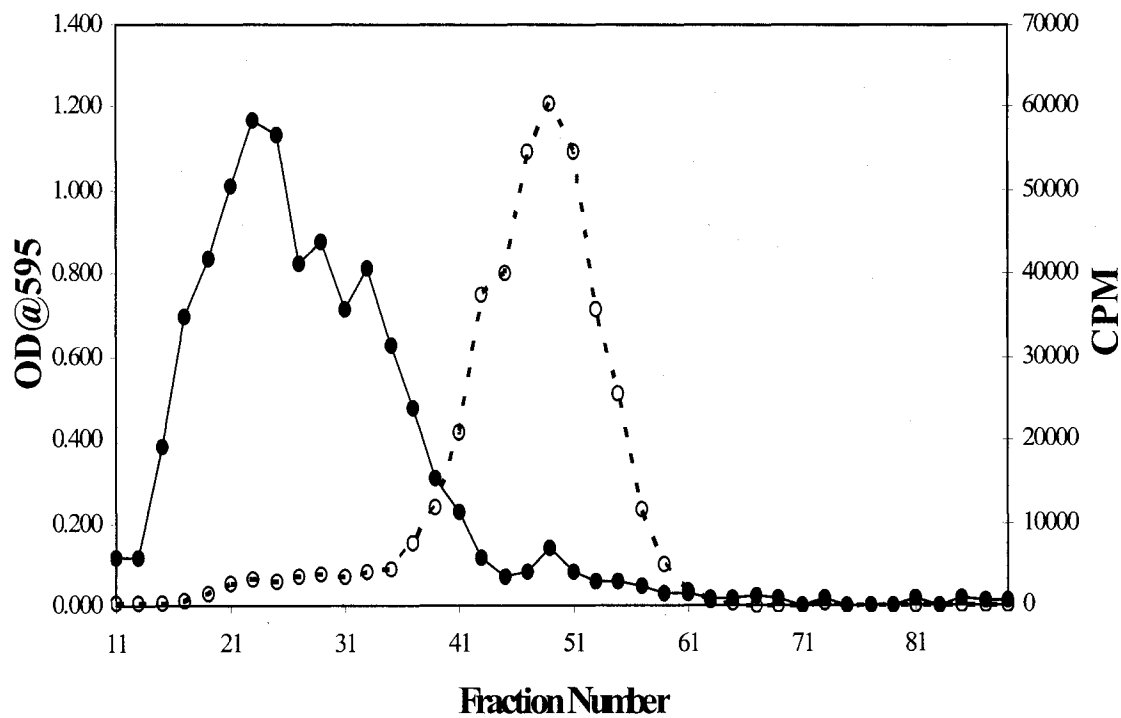


FIG. 21. **Cy nickel IMAC purification profile.** This profile represents the best resolution obtained between cellular lysate proteins (closed circles) and Cy activity (open circles). In this experiment fractions 45-57 were pooled together for the concentration step to avoid inclusion of contaminant proteins.

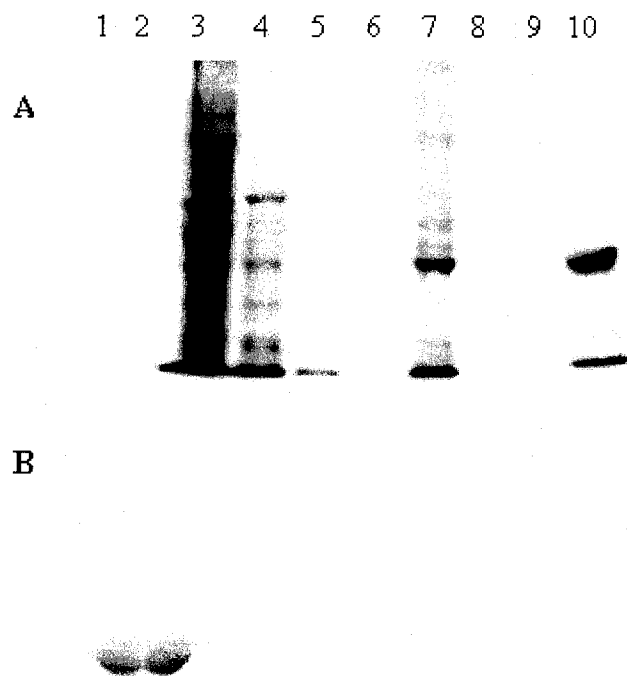


FIG. 22. **SDS-PAGE analysis of C-subunit purification.** Purification of both C-subunits was followed by silver stain gel analysis. *A*, purification profile for his₆-C γ , which corresponds to the purification profile in Figure 20. Lane 1, molecular weight marker; 2, empty; 3, extract; 4, flow through; 5, wash 1; 6, wash 2; 7, elution of fractions 37-44; 8, elution of fractions 45-57; 9, empty; 10 pooled and concentrated fractions 45-57. *B*, gel showing purified his₁₀-C α .

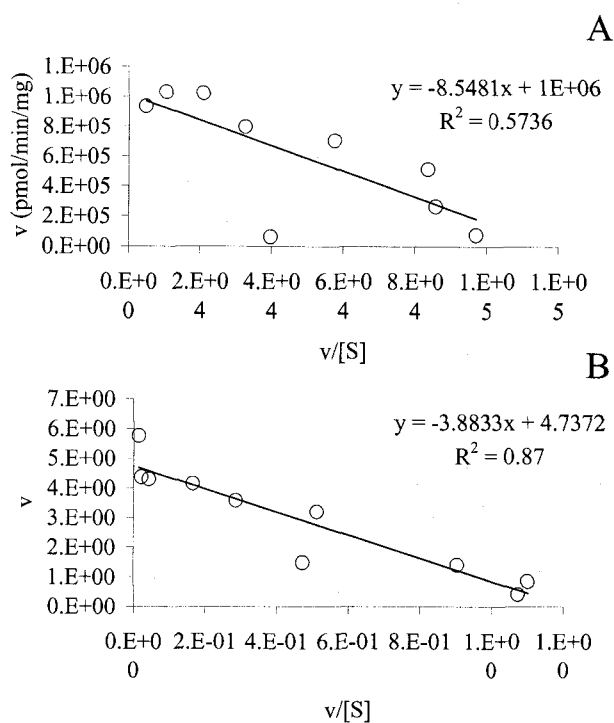


FIG. 23. **Eadie-Hofstee plots for steady-state kinetics with Kemptide.** Steady-state kinetics were determined through standard kinase assays. The slope of each plot was used to determine and compare K_m values, which were, A, $8.5 \mu\text{M}$ for $C\alpha$ and B, $3.8 \mu\text{M}$ for $C\gamma$.

through expression and purification from bacteria and the low expression levels of C γ in Sf9 cells. Stopped-flow characterization of C α has been done using several approaches (38,155). The approach used here employs fluorescence resonance energy transfer (FRET) to measure the binding and release of ATP from C α 's active site (155). The principal of this approach is that exciting tryptophans in the C α active site at 290 nm, releases energy (320-340 nm) exciting mantATP as it comes into proximity with the C α active site, re-emitting energy at >420 nm as it does so, which is measured by the stopped-flow fluorometer. Thus, as mantATP binds the C α active site an increase in fluorescence is measured, recorded as volts, and plotted. The binding rate (or rates) is determined by the equation used to fit the hyperbolic curve measured.

Two rates were measured for C α -mant-ATP binding at 25°C. The first rate (k_1) is a very rapid increase in fluorescent signal followed by the second rate (k_2), which is a slower decrease in signal (Figure 24). Rates k_1 and k_2 were measured at several concentrations of mantATP and plotted (Figure 25). The slope for the first rate, k_1 , was found to be mantATP concentration-dependent (1.6×10^6 M/sec). The slope for k_2 did not change with changing concentrations of mant-ATP (19 s^{-1}). The slope of the k_1 linear-curve corresponds to C α 's binding rate for mant-ATP, which is within experimental error of the value reported in the literature. As reported, this rate is very rapid, but not as rapid as diffusion-limited reaction rate (10^8), where the reaction occurs as rapidly as the molecules can encounter.

The mant-ATP binding was also measured at 4°C. At this temperature several interesting things were observed. First was that the mant-ATP binding rate is similar at both temperatures ($k_{1(4^\circ\text{C})} = 1.9 \times 10^6 \text{ M}^{-1}\text{sec}^{-1}$). The second observation is that three rates were measured at 4°C ($k_{2(4^\circ\text{C})} = 0.3\text{sec}^{-1}$, $k_{3(4^\circ\text{C})} = 0.002\text{sec}^{-1}$). The second and third rates at

4°C are mant-ATP concentration independent. The second rate measures an increase in fluorescent signal ($\text{amplitude}_{200 \mu\text{M mant-ATP}} = -0.0662$) and the third rate measures a decrease in fluorescent signal ($\text{amplitude}_{200 \mu\text{M mant-ATP}} = 0.0165$).

The mant-ATP binding was also measured at 25°C in the presence of Kemptide to determine whether the presence of the phospho-acceptor substrate affected the observed binding rate of the nucleotide substrate (Appendix J). This was done by mixing 13 μM $\text{C}\alpha$ from one syringe with 100 μM mant-ATP and varying concentrations of Kemptide (25-400 μM) in the other syringe. The slopes for the two rates measured at 25°C, k_1 and k_2 , decreased very slightly with increasing amounts of Kemptide ($k_{1\text{Kemptide}}: y = -0.1961x + 296.43, R^2 = 0.3865$; $k_{2\text{Kemptide}}: y = -0.0063x + 11.038, R^2 = 0.0278$), but it is not clear if these slopes fall within the error measured for each rate. A similar trend is observed in the amplitudes for each rate ($\text{amplitude}_{1\text{Kemptide}}: y = -0.0001x - 0.1012, R^2 = 0.5346$; $\text{amplitude}_{2\text{Kemptide}}: y = -7 \times 10^{-6}x + 0.0123, R^2 = 0.085$). The correlation values for these curve fits are not very good, suggesting that there is little difference between the measured rates or amplitudes at each Kemptide concentration. However, there is a decrease in the amplitude of k_2 relative to the amplitude measured in the absence of Kemptide, suggesting that Kemptide affects the step corresponding to k_2 . The ATP-off rate for $\text{C}\alpha$ was determined through two independent approaches. The first was simply the y-intercept of Figure 25, the slowest rate of the mantATP binding rate curve, 174sec^{-1} . The second approach was through double-mixing stopped-flow (Figure 26) In this approach mant-ATP was first mixed with $\text{C}\alpha$, followed by a second mix with a high concentration of ATP (3 mM). The ATP binds the $\text{C}\alpha$ active site, displacing mant-ATP as mant-ATP dissociates from the active site.

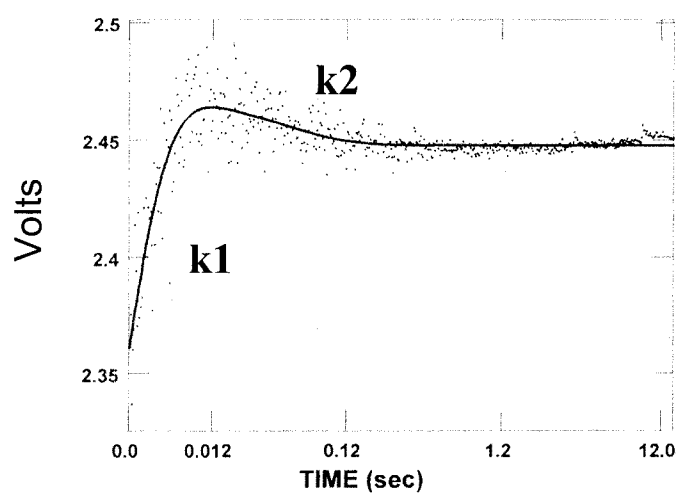


FIG. 24. **Binding of mant-ATP to $C\alpha$ measured using stopped-flow spectroscopy.** Time-dependent change (x-axis) in fluorescence in volts (y-axis) upon mixing $4\ \mu\text{M}$ $C\alpha$ and $400\ \mu\text{M}$ mant-ATP at 25°C . Two fluorescent signals are observed, k_1 , which increases with time and k_2 , which decreases with time.

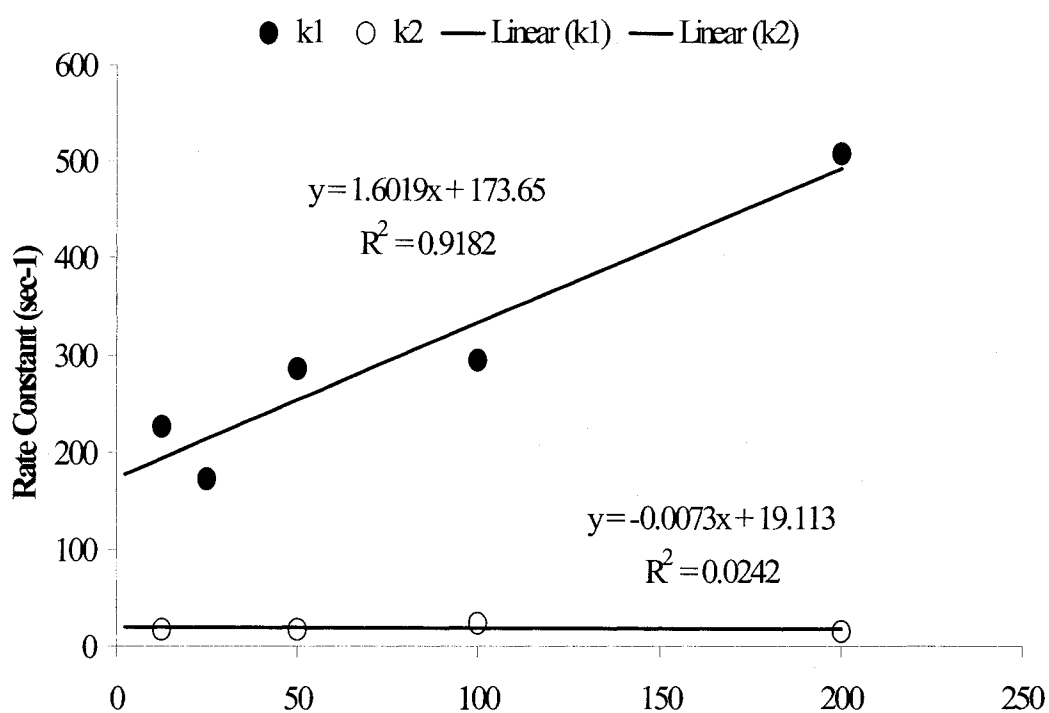


FIG. 25. Effects of total mant-ATP concentration (12.5-200 μM) at 25°C . The plot shows rates k_1 and k_2 measured against several concentrations of mant-ATP (x-axis). A rate was measured for 400 μM mant-ATP, but this rate fell off the linear curve and did not fit a hyperbolic curve with statistical confidence.

This rate is independent of ATP concentration and is measured as a decrease in fluorescence, which corresponds to the mant-ATP off-rate (174sec^{-1}). As can be seen the value from this measure is in excellent agreement with the value obtained from the y-intercept in Figure 25 (173.65sec^{-1}) and this value is in good agreement with the value reported in the literature (190sec^{-1})(155).

Stopped-flow characterization of C α and C γ ADP dissociation rates– Once satisfactory measures were made with mant-ATP and C α , it was of interest to measure the mant-ADP off-rates for C α and C γ . The release of ADP is reported to be the slowest step in C α 's mechanism (Figure 15), thus it is described as its rate-limiting step. This measure was done here through double-mixing experiments where the isoforms were pre-mixed with mant-ADP, followed by the addition of high concentrations of ATP (3mM). The C α /mant-ADP off rate (35sec^{-1}) was determined from a fit of the curve measured as the fluorescent signal decreases while the mant-ADP is trapped off the catalytic subunit (Figure 26A), and is in agreement with the literature (75sec^{-1}) (38,155). A very low signal was obtained when the same experiment was done with C γ (Figure 26B), but a reproducible rate was measured ($4.1 \pm 0.3 \text{ sec}^{-1}$; Avg. \pm Std. Error, $n = 3$). The rate measured for the C γ mant-ADP off-rate is nine times slower than the C α rate. Unfortunately, this approach used the entire stock of purified C γ , and complications of C γ expression prevented repeating the experiment.

DISCUSSION

Biochemically, C α and C γ differ in their steady-state kinetics for classic PKA substrates, including histone and Kemptide (20), specifically the C γ V_{max} is ten-times lower than that of C α . One possible explanation for this difference is that C γ has slower substrate binding

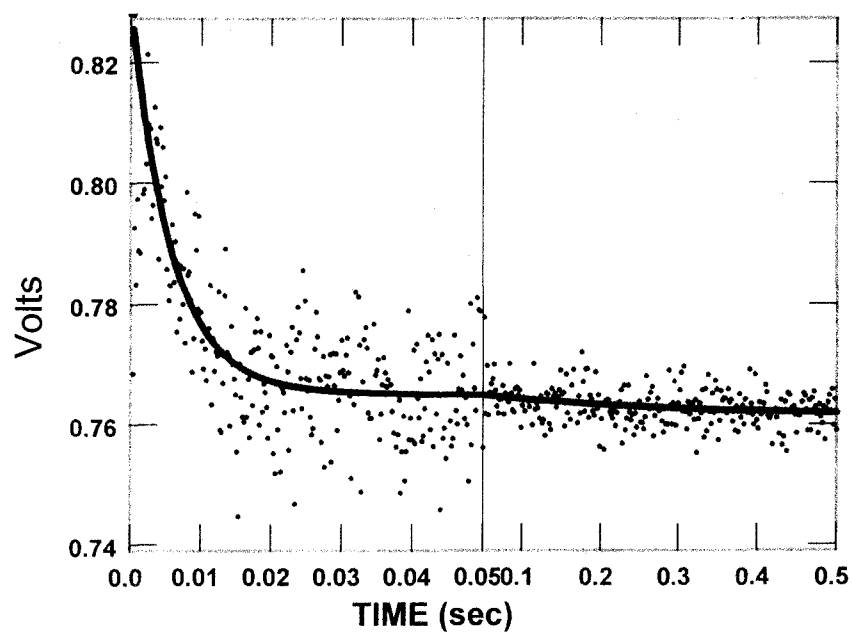


FIG. 26. **Double-mixing experiment trapping mant-ATP from $C\alpha$ at 20°C.** 8 μM $C\alpha$ was pre-mixed with 200 μM mant-ATP in one syringe, and then rapidly mixed with 3 mM Mg-ATP. The data was fit with a double-exponent function to obtain the rates $k_1 = 174 \text{ s}^{-1}$ and $k_2 = 3.8 \text{ s}^{-1}$.

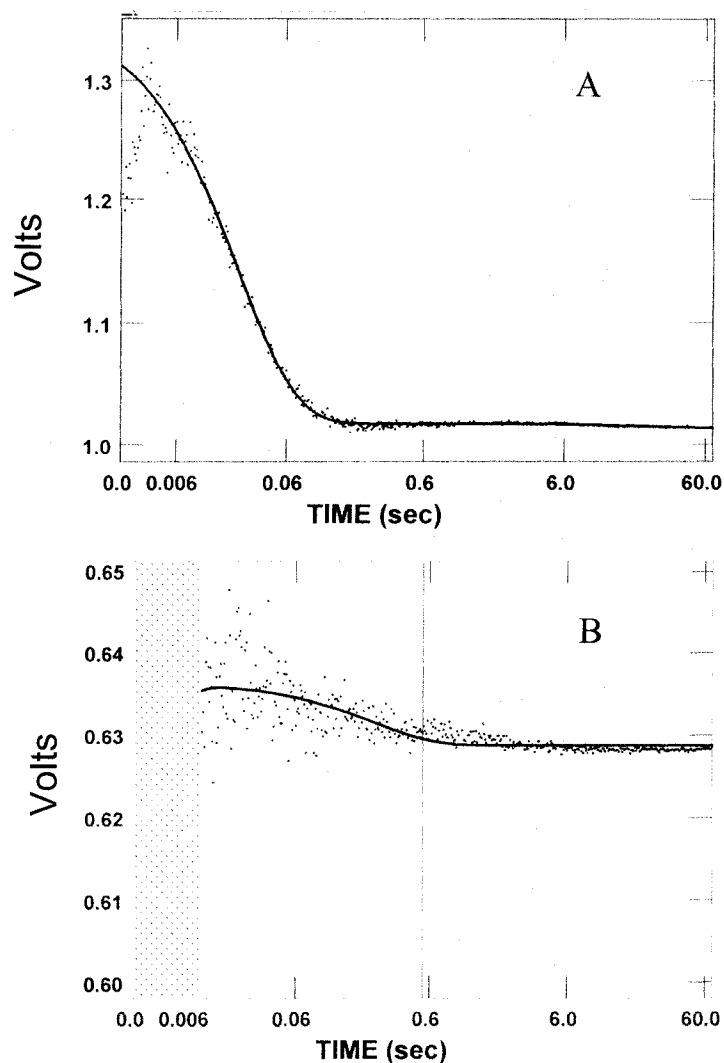


FIG. 27. **Double-mixing experiment trapping mant-ADP from $C\alpha$ and $C\gamma$ using ATP at 20°C.** *A*, 12 μM $C\alpha$ was pre-mixed with 200 μM mant-ADP in one syringe and then rapidly mixed with 3 mM Mg-ATP. The data was fit with a double-exponent function to obtain the rate for $k_1 = 34.5 \text{ s}^{-1}$ (amplitude = 0.277). *B*, 6 μM $C\gamma$ was pre-mixed with 100 μM mant-ADP in one syringe and then rapidly mixed with 3 mM Mg-ATP. The data was fit with a double-exponent function. The first component was ignored here as it reflects an increase in mant-ADP. The rate for $k_2 = 4.0 \text{ s}^{-1}$ (amplitude = 0.00749) is the rate for the dissociation of mant-ADP from $C\gamma$.

and/or release rates, slowing down the rate of its catalytic mechanism. If true, this would explain why $C\gamma$ exhibits lower CREB-mediated reporter activity, as this mechanism depends on the C-subunit phosphorylating CREB at S133 (162). Thus $C\gamma$ would phosphorylate 0.1 of the CREB molecules that $C\alpha$ would, causing a ten-fold difference in reporter-activity measured, which is what is observed in some cell types (105). Here we studied the catalytic mechanisms of both $C\alpha$ and $C\gamma$ through stopped-flow analysis using fluorescent-labeled ATP and ADP substrates, which are the first substrates to bind and last product to leave the C-subunit catalytic active site (34,155).

Two general classifications serve to describe the kinase kinetic mechanism based on the data from steady and pre-steady-state kinetic studies and crystal structure analysis of more than thirty kinases (163). Mono-substrate kinetics is a relatively simple model used to teach enzyme kinetics and is easily described by the Michaelis-Menten equation (164). However kinases catalyze the reaction between two substrates (a nucleotide triphosphate and a phosphoacceptor), making the reaction more complex. However, when the concentration for one substrate is held constant the Michaelis-Menten equation still holds true for the second substrate (165), providing an approach to study the two-substrate mechanism of an enzyme. The *steady-state* kinetic characterization is determined once all of the reaction components (enzyme, co-enzymes and substrates) have reached equilibrium. This provides a lot of information but is limited because this type of characterization is not able to easily differentiate the binding and dissociation rates for individual ligands. Discerning binding rates for each ligand in an enzymatic reaction helps to develop a model for the enzyme's reaction mechanism. A technique often used to measure the catalytic rates is stopped-flow, which measures the rates for each ligand, when possible, before

equilibrium between enzyme and substrates is established (166). Thus, this technique measures an enzyme's pre-steady state kinetics.

One mechanism used by kinases is the *Bi-Bi* mechanism (Figure 15) where the order in which the NTP and the phospho-acceptor substrates bind the enzyme may (ordered Bi Bi) or may not (random Bi Bi) matter. The key to this mechanism is that no phospho-enzyme intermediate is formed instead the enzyme directly transfers the NTP phosphoryl group to the phospho-acceptor substrate. Hexokinase is an example of an enzyme that follows this mechanism; serine-threonine kinases also follow this mechanism (157,163,167). The second mechanism for kinases is the *Ping Pong Bi Bi* mechanism wherein the kinase binds an NTP and catalyzes the transfer of the NTP phosphoryl group to its own active site. The remaining NDP is then released from the kinase, leaving behind a phospho-kinase enzyme intermediate, which then binds the final phospho-acceptor substrate, catalyzing the transfer of the phosphoryl group to the substrate. Examples of this mechanism include alkaline phosphatase, tyrosine phosphotransferases, nucleotide diphosphate kinase, and P-ATPase (168). A point of controversy concerning protein kinase that utilize the ordered Bi Bi mechanism is whether the phosphoryl transfer occurs through an associative (S_N2) mechanism, where there is no phosphoryl intermediate formed between the enzyme and substrates within the active site of the enzyme or a dissociative (S_N1) mechanism where a phosphoryl intermediate forms prior to phosphoryl transfer (26,169,170).

The mechanism described for $C\alpha$ is an ordered Bi Bi mechanism (Figure 15) (34,171,172), where the literature favors the in-line associative transfer of the phosphate, and the release of ADP as the rate-limiting step (34,35,155). The results presented here,

while far from being conclusive, seem to support the published mechanism for $C\alpha$, where the ATP binding rate is very rapid (Figure 24) and the ADP release is much slower (Figures 26). However, the results presented here and published elsewhere (155) make it clear that the $C\alpha$ catalytic mechanism is not as straightforward as depicted in Figure 15 (171,172). Stopped-flow analysis of the ligand binding rates for ATP and ADP were studied here through use of fluorophore conjugates nucleotides, mantATP and mant-ADP, which serve as good analogues to study ATP and ADP binding to $C\alpha$ (155). Stopped-flow analysis presented here of mant-ATP binding $C\alpha$ at 25°C measures two rates, k_1 which is a rapid increase, mant-ATP concentration-dependent rate, and k_2 which is a slow decrease, mant-ATP concentration-independent rate. This suggests that there are at least two steps involved in the binding of mant-ATP. Ni et al. measure three rates for this step (155), the first two match the ones measured here, and the third one is also ligand concentration-independent but slower than k_2 , suggesting that there may be a third step to $C\alpha$ binding mant-ATP. Results presented here of mant-ATP binding $C\alpha$ at 4°C measure three rates, but unlike the published work at 25°C the second rate also measures a slow increase in fluorescent signal, thus at 4°C there are two rates that measure an increase in signal and a third that measures a decrease in signal. The third rate measured at 4°C would coincide with k_2 measured at 25°C, but it is much slower than would be predicted by estimating a two-fold decrease every 10°C, suggesting that this rate is very temperature dependent, which may mean it is measuring a conformational change in $C\alpha$. Between the work presented here at 4°C and 25°C and the published work there are four measured rates, suggesting that there may be three steps in the binding of mant-ATP. The mant-ATP off-rate for $C\alpha$ was arrived at via two independent approaches, which were in

very good agreement with each other (174 sec^{-1}) and in good agreement with the published work (165 sec^{-1} and 190 sec^{-1}) (155).

The $C\alpha$ mant-ATP on-rate was also measured in the presence of Kemptide, which is a second substrate for $C\alpha$, to determine if having both substrates present affected either of the mant-ATP rates measured at 25°C . A common trend was observed in this experiment for both rates measured and their corresponding amplitudes, and that was that there seemed to be a decrease in both rates and amplitude as the Kemptide concentrations increased. This would suggest that high concentrations of the phosphoacceptor would slow down the ATP binding-rate. One interpretation of this may be that at higher phosphoacceptor concentrations two enzyme species are formed, one wherein the ATP bound before the phosphoacceptor and a second where the phosphoacceptor bound the kinase before ATP. It is not clear without further experiments whether the later species described would undergo FRET, but if the phosphoacceptor were to bind the kinase first this may lead to a change in the amplitude of second signal. However the correlation values for these experiments are very low suggesting that either there were outliers in the experiment or that there really is no trend and having Kemptide present does not affect the ATP binding rate. At this point the experiment has not been repeated enough times to statistically discern between these two possibilities.

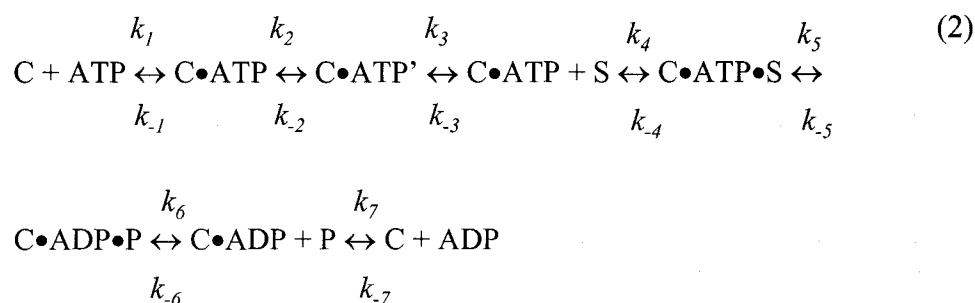
The ADP off-rate for both $C\alpha$ and $C\gamma$ was measured through double mixing experiments, in which the kinase was first mixed with mantADP and then mixed with ATP. The results presented here agree with the release of ADP as the being slower than ATP on or off-rates, and thus would fit as the rate-limiting step for $C\alpha$. The $C\alpha$ mant-ADP off-rate was approximately five-times slower than the ATP off-rate and ten-times

slower than the measured ATP-on rate. These rates are also in good agreement with the reported mechanism for $C\alpha$, where ATP binds first, followed by binding of the phosphoacceptor, the phosphorylation of this substrate and the release of the phosphorylated substrate, leaving ADP in the active site, which is released last (Figure 15). Furthermore, the results presented here for $C\alpha$ are in good agreement with the values reported in the literature (155). However, this mechanism (outlined in Equation 2) does not take into account the second rate (k_2) measured for mant-ATP binding $C\alpha$, which was measured here as being slower than the mant-ADP off-rate (k_7), but was reported elsewhere not to be statistically different from the mant-ADP off-rate (155). This raises the question, is mant-ADP dissociation truly the rate-limiting step for this reaction? The discrepancy rests with which experimental values are accepted to be true.

One approach used here to try and answer this question was to determine the turn-over rate or k_{cat} for the purified $C\alpha$ used in these experiments and compare it to the different rates measured here. While the significance of k_{cat} to a mechanism depends on the nature of the mechanism it should coincide with the rate-limiting step of the reaction. Upon calculating the turn-over rate for the pure $C\alpha$ used in these experiments it was found to be about ten-times slower (3.9 sec^{-1}) than the slowest rate measured or any published turn-over rates which range from 52 sec^{-1} to 20 sec^{-1} (37,87,156). This suggests that there was about five-to-ten-times less active $C\alpha$ in the prep used here as the calculation depends on the total concentration of enzyme used ($k_{cat} = V_{max}/[E_{tot}]$). This observation may explain why a third rate (k_3) was not measured at 25°C , as here we may have had less available enzyme in the reaction as in the published work (155). A decrease

in the amount of enzyme would cause a decrease in the amplitude of the signal measured, making it more difficult to fit the curve to the low signal of a slow rate.

Based on the work presented here and published elsewhere there are at least seven different steps to the C α catalytic mechanism. A mechanism is displayed in Equation 2 that attempts to consolidate all of the rates measured or published for C α , where k_1 ($1.6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$), k_{-1} (174 s^{-1}), K_2 (19 s^{-1}) and k_7 (35 s^{-1}) were determined here. Rate k_3 is based on published work (155). Rate k_4 , while not measured will depend on the catalytic



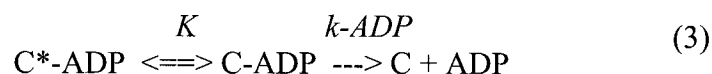
subunit (C) affinity for the phosphor-acceptor (S), k_5 is the rate of the chemical phosphor-transfer and k_6 will depend on the dissociation rate of the phosphorylation product (P). It is not clear from the experiments done here if the C α K_m for Kemptide ($23 \mu\text{M}$) is equivalent to k_6 in Equation 2.

This is the first time pre-steady state kinetics have been measured with C γ . The challenge for this experiment was in isolating sufficient protein to make the measurements. Here sufficient C γ was isolated to measure its binding rate with one nucleotide. As the release of ADP is the rate-limiting step for C α , it was felt that this was a likely step where C α and C γ mechanisms might diverge and thus chose to use what sample was available from purifying C γ to study its ADP off-rate. The signal measured for this step was very weak and did not provide a curve that was very distinct from the

mantADP background signal. However, the signal obtained from double mixing mantADP and ATP to measure the off-rate provided a reproducible exponential curve fit for $C\gamma$ with a slope nine-times slower than that of $C\alpha$. Because the signal was so low it is not clear if the slope measured is accurate or if the weak signal is due to insufficient quantities of functional protein. The latter is a likely possibility because pure recombinant $C\gamma$ is not very stable at -80°C or in N_2 (*l*), so it is possible that only a portion of the protein in the purification preparation was functional, which would yield a lower signal in the stopped-flow experiments. However, the concentrations of enzyme used for $C\alpha$ and $C\gamma$ were calculated to be the same in these experiments and the steady-state kinetic measures with this $C\gamma$ prep allowed for the highest dilution of $C\gamma$ made to date, 100x, suggesting that there was more enzyme than usual obtained from this purification, but this does not mean that there was enough active $C\gamma$ for this type of experiment. Once sufficient $C\gamma$ can be purified to repeat these experiments the enzyme should be tested in binding assays with pure recombinant R-subunits, an approach that will stoichiometrically identify the concentration of active $C\gamma$ in the preparation, as only functional $C\gamma$ will bind the R-subunit (173). This measure will provide a starting point for an accurate measure of each isoforms tested in the stopped-flow experiments.

If the values measured here for $C\alpha$ and $C\gamma$ are accepted as accurate, then the difference in rates for the release of ADP can be used to outline an equation that could explain why $C\gamma$ is kinetically slower than $C\alpha$ (Equation 3), wherein if the equilibrium rate between the different species of C-ADP (K) or the dissociation rate of C and ADP ($k\text{-ADP}$) is very different between the two catalytic subunits the equation could explain differences observed (Appendix E).

Differences in reaction rates may be due to overall differences in their tertiary structure, as illustrated by $C\gamma$ having a smaller stoke's radii (20) (Appendix F), suggesting an overall different shape than $C\alpha$. This may affect interactions in the active site making $C\gamma$ less efficient at phosphorylating, as demonstrated by $V_{\max}:K_m$ ratios for Kemptide and



Histone (20). The difference in Stoke's radius between the two catalytic subunits may also reflect that purified recombinant his- $C\gamma$ is improperly folded. But this is unlikely because the measurement of the radius is dependent on determining where the protein elutes off size-exclusion chromatography and this is determined by kinase activity. This is, the measured radius is based on an active enzyme, which should be folded sufficiently to be active. It is also possible that his- $C\gamma$ is miss-folded, still active, but not as active as endogenous $C\gamma$. But this is difficult to confirm as endogenously expressed $C\gamma$ has yet to be isolated from any tissue and characterized.

There are several key residues that are important to the inline-transfer between ATP and the phosphoacceptor, including K72, the G-triad, G50, G52 and G55, D166, K168, N171 and D184 (118,157,174). All of these are conserved between $C\alpha$ and $C\gamma$ and depend on the distances within the active site for proper proximity to the substrates. It is possible that these distances are disrupted in $C\gamma$, affecting the interaction between the residues and the substrates, and this effect is manifested as a slower catalytic rate for $C\gamma$. This model would be difficult to test through point-mutants as it depends on determining the position of each residue within $C\gamma$'s active site. The best way to test this is through determining the

crystal structure of $C\gamma$, an approach that requires high quantities of highly pure protein, something that remains elusive in the characterization of $C\gamma$.

The results presented here however support the hypothesis that differences in kinetics between $C\alpha$ and $C\gamma$ are in part due to differences in the rates of their rate-limiting step as the differences observed in this rate-limiting step agree with differences observed in steady-state kinetics and transient reporter assays. The physiological ramifications of the biochemical difference between $C\alpha$ and $C\gamma$ still needs to be determined through studies of the isoforms *in vivo* and in the intact cell.

CHAPTER V

DIFFERENTIAL EXPRESSION OF cAMP-DEPENDENT PROTEIN KINASE

C α AND C γ ISOFORMS IN U-937 CELLS

ABSTRACT

The cAMP-dependent protein kinase (PKA) catalytic subunits C γ and C α share 83% identity in primary structure but differ in chromosomal location, gene structure, gene transcription regulation, kinetics, and specificity for peptide substrates and pseudosubstrates. In contrast to the ubiquitous expression of C α , C γ is primate-specific and mRNA has been identified only in human testes. For the first time we present the selective identification of endogenous C γ protein and mRNA expressed in human U-937 cells. Increases in protein kinase inhibitor (PKI)-insensitive, cAMP-dependent kinase activity following 8-CPT-cAMP-induced differentiation of U-937 cells suggested an increase in C γ expression during differentiation, while total PKA catalytic activity did not change. A new antibody identified a 40-kDa band following differentiation of U-937 cells and RT-PCR/RFLP analysis demonstrated that C γ mRNA was present before and after differentiation. DEAE chromatographic analysis of U-937 cells showed that the PKA profile changed from similar levels of type I and type II PKA in undifferentiated cells to 100% type I in differentiated U-937 cells. Furthermore, 60% of PKI-insensitive, cAMP-dependent activity in U-937 cells was associated with type I PKA suggesting C γ favors association with type I PKA in this cell line. These results expand our

understanding of structure-function roles for PKA isoforms in cell differentiation and function. Furthermore the increase in $C\gamma$ expression during U-937 differentiation suggests a novel function for $C\gamma$ in the cell.

INTRODUCTION

The cyclic AMP-dependent protein kinase (PKA, EC 2.7.1.37) is a member of the AGC serine/threonine kinase family (2), which phosphorylates hundreds of substrates involved in numerous physiological processes, such as energy metabolism, cell division, proliferation, apoptosis, and differentiation (1). PKA is present at varying concentrations in all tissue types (42,43) and exists as an inactive holoenzyme consisting of a regulatory subunit (R) dimer and two catalytic subunit (C) monomers in the absence of cAMP. Two types of PKA exist in the cell, type I and type II, determined by the R subunit isoform present in the holoenzyme. There are four known *Homo sapiens* genes for the R subunit: $RI\alpha$, $RI\beta$, $RII\alpha$, and $RII\beta$. Classically, PKA type I elutes from a DEAE column at lower NaCl concentrations than type II (175). There are three genes for the C-subunit expressed in *Homo sapiens*: $C\alpha$, $C\beta$, and $C\gamma$ (45). Human $C\alpha$ and $C\beta$ share the highest degree of homology in their primary amino acid sequence (93% identity) and have multiple splice variants (49-52). $C\alpha$ and $C\gamma$ are highly homologous exhibiting 83% identity while $C\beta$ and $C\gamma$ exhibit 79% identity (45). Unlike the $C\alpha$ and $C\beta$ gene, the $C\gamma$ gene is an intronless retroposon gene, which appeared in the human genome an estimated forty million years ago (73). Thus, $C\gamma$ is the only known example of a primate specific PKA gene.

$C\alpha$ and $C\gamma$ differ in gene transcription regulation and substrate and pseudosubstrate specificities. Differences in gene transcription regulation were observed

through transient transfections and luciferase-reporter assays where $C\alpha$ induced transcriptional activation through CREB and $C\gamma$ did not (105). Differences in pseudosubstrate and substrate recognition have been observed where $C\alpha$ is inhibited by protein kinase inhibitor (PKI) while $C\gamma$ is not (13,20) and $C\gamma$ exhibits a lower V_{\max} than $C\alpha$ for most substrates tested (20). These differences between $C\alpha$ and $C\gamma$ suggest that while both enzymes are cAMP-dependent, they may fulfill different functions within the cell.

The $C\gamma$ mRNA has been identified only in human testes where it was cloned from a human testes cDNA library (45). PKA Cs, or $C\alpha 2$, has recently been identified as a testes specific isoform, a splice variant of $C\alpha$ expressed in mature sperm (52,107), but this isoform is not human specific. Identification of $C\gamma$ in other tissues has been difficult because of the high homology between $C\gamma$ and $C\alpha$ and the absence of isozyme-specific antibodies. Since $C\gamma$ was identified in testes, a tissue with a high concentration of cells undergoing differentiation, it is possible that $C\gamma$ has a function in cellular differentiation. Furthermore, sperm undergo differentiation that involves specific isoforms of the PKA catalytic subunits (52). In addition, $C\gamma$ is an intronless (73) gene, and many intronless genes play a role in cellular differentiation (110-116). U-937 myeloid leukemia cells were selected as a model system to investigate this possibility. This cell line can be induced to differentiate when treated with several cAMP-elevating reagents including forskolin and dibutyryl cAMP.

We present evidence for the first time that $C\gamma$ is expressed in U-937 cells using DEAE-Sepharose chromatography separation, kinase assays in the presence and absence

of PKI, immunoblot analysis with C-subunit isozyme-selective antibodies, and RT-PCR coupled with restriction fragment length polymorphism (RFLP) analysis. Three days after 8-CPT-cAMP induced differentiation, analysis of mRNA, protein, and catalytic activity indicated increases of C γ expression. These observations suggest a previously undefined function for C γ in cellular differentiation and pose new questions concerning regulation of human cell differentiation by PKA catalytic and regulatory isozymes.

MATERIALS AND METHODS

Tissue Culture Conditions – U-937 myeloid leukemia cells were maintained in RPMI 1640 with 10% FBS and L-glutamine at a density of 5×10^5 cells/mL, incubated at 37°C and 5% CO₂. For chromatography analysis, kinase assays, immunoblot analysis, and RFLP analysis, 2.4×10^6 cells/mL in 85 mL of growth media were induced to differentiate with 50 μ M 8-(4-chlorophenylthio) adenosine- 3', 5'- cyclic monophosphate (8-CPT-cAMP, 10 mM stock in H₂O) for three days.

Cell Harvest – Following a three-day treatment with 8-CPT-cAMP, 204×10^6 U-937 cells were washed with one volume of isotonic KP buffer (10 mM potassium phosphate, 1 mM EDTA, 250 mM sucrose, pH [6.9]), sedimented (200 x g, 15 minutes), the supernatant discarded, and the cell pellet stored at -80°C until used for chromatography analysis. When appropriate, cells were thawed on ice, homogenized with a Dounce homogenizer in 2 mL KP buffer without sucrose (low ionic strength KP) containing protease inhibitors (10 μ g/mL leupeptin, 20 μ g/mL pepstatin A, 2 mM phenylmethylsulfonyl fluoride, 2 μ g/mL trypsin inhibitor and 2 μ g/mL aprotonin), 10 mM 1,4-dithiothreitol, and 50 mM potassium fluoride (a phosphatase inhibitor). Cell lysis was assessed microscopically using trypan blue. The homogenate was sedimented

(15,800 x g, 10 minutes, 4°C), the supernatant removed and kept on ice. One aliquot of supernatant was combined with Laemmli sample buffer (1:3, sample buffer: sample), incubated at >90°C for 10 minutes and stored at -20°C. The remainder of the cell extract was used for kinase assays and chromatographic separation.

Proliferation Assay – Proliferation rate decreases as cells differentiate. To use proliferation as a differentiation marker, 3000-5000 U-937 cells/well in a 96 well plate were treated with 50 µM 8-CPT-cAMP for three days and compared to untreated (undifferentiated) cells. Following treatment, cells were incubated 30 minutes with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS, Cell Titer 96 Aqueous One Solution Cell Proliferation Assay, Promega), according to the manufacture's instruction. The optical density was measured at 492 nm for each well in a Tecan plate reader, where an increase in absorbance corresponds to an increase in cell viability. Data are presented as the mean ± standard error (n=5), with significance shown relative to undifferentiated, as determined by a Student's t-test.

Nitrobluetetrazolium Assay – Nitrobluetetrazolium (NBT) reduction by U-937 cells was used to determine respiratory burst activity in cells differentiating toward monocyte-like cells. The respiratory burst was also used as a differentiation marker because it includes wide range of sophisticated functions that are acquired during differentiation and absent in undifferentiated cells. NBT reduction was measured in undifferentiated cells and cells treated for 3 days with 50 µM 8-CPT-cAMP. Following three days ± treatment, 1×10^6 cells were incubated at 37°C for 45 minutes with 0.1% NBT and phorbol 12-myristate 13-acetate (20ng/mL final), applied to a glass slide (Cytospin III centrifuge, Shandon

Southern; Sewickley, PA), stained with 0.5% safranin, and analyzed microscopically under oil immersion. A minimum of 200 cells/slide was counted. Data are presented as the mean \pm standard error (n=5), with significance shown relative to undifferentiated cells, as determined through a Student's t-test.

DEAE Chromatography Analysis of Cell Extracts – Chromatographic separations were performed at 4°C by loading 1.8 mL of cell extract (an average of 7.5 mg/ml protein in undifferentiated cells and 0.9 mg/mL in differentiated cells as determined by BioRad's Protein Assay) onto a 4 mL diethylaminoethyl (DEAE)-Sepharose fast-flow resin (Pharmacia Biotech) column, that had been pre-equilibrated with 10 column volumes of low ionic strength KP buffer. The flow-through was passed over the column three times. The extract was eluted with a 50 mL, 0-500 mM NaCl gradient in KP buffer, while collecting 1 mL fractions, which were analyzed for kinase activity.

Kinase Assay – Enzyme activity was measured in crude cell extract and from chromatography samples by measurement of P^{32} - γ -ATP incorporation into Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Glu, Bachem Bioscience Inc.) as described previously (24). All reactions contained 78 μ M Kemptide, \pm 10 μ M cAMP, \pm 1 μ M protein kinase inhibitor (PKI). Activity was corrected for the total protein in the cell extract and expressed as *pmol of ATP incorporated/minute/mg total protein* in cell extract.

Immunoblot analysis – Samples were separated on a 9% SDS-PAGE gels with a 4% stacking gel, transferred and treated with antibodies as described previously (105). Three different antibodies were used at the indicated dilutions: a rabbit polyclonal antibody to $C\gamma$ from Santa Cruz Biotechnology (sc-905, lot# A297, 1:1000), a polyclonal antibody to $C\gamma$ from Abgent (cat# AP7014a, lot# SH020924K, 1:500), and a $C\alpha$ -selective monoclonal

antibody (Transduction Laboratories, cat# P73420, lot# 6, 1:1000). Following primary antibody treatment and washes, the membranes were incubated with HRP-conjugated anti-mouse or anti-rabbit polyclonal antibodies (Amersham Biosciences) at a 1:1000 dilution and washed. The membranes were developed using Amersham's ECL detection kit and imaged electronically (BioRad's VersaDoc imaging system).

U-937 RNA isolation and RT-PCR/ Restriction Fragment Length Polymorphism (RFLP) analysis— **This work was done by Dr. Rana C. Morris.** Because of the high homology between $C\alpha$ and $C\gamma$, including the C' un-translated region, it was not possible to specifically amplify either of the isoform mRNAs without inclusion of the other. However, we were able to differentiate the two based on differences in restriction enzyme sites in amplified sequences that included both $C\alpha$ and $C\gamma$. Untreated and treated U-937 cells were isolated on days 0, 1, 2 and 3. Cell pellets (2×10^6 cells) were lysed with Trizol reagent (Gibco BRL/Invitrogen) and total cellular RNA (RNA_{tot}) was isolated via phenol/chloroform extraction followed by isopropanol precipitation, washed twice with 70% ethanol and dissolved in DEPC-treated dH_2O . Purity and quantity of both types of RNA samples was assessed via UV spectrophotometer at 260 and 280 nm. The A_{260}/A_{280} ratios for all RNA isolates were consistently between 1.86 and 1.96.

Reverse transcription was done following manufactures instructions (AMV reverse transcriptase, Promega). Five micrograms of RNA_{tot} was subjected to first-strand synthesis by 30 units of AMV-RT with an 2.5 μg oligo (dT) primer in a total volume of 25 μL at 48°C for 60 minutes.

PCR primers were generated using sequences from the human cAMP-dependent protein kinase catalytic subunits ($C\alpha$: NM_002730 and $C\gamma$: NM_002732) to amplify a

portion of PKA-C α and -C γ based on a highly conserved region between C α and -C γ , that significantly diverges from human C β (NM_002731, NM_207578, NM_182948). The primer set amplifies a 370 base pair region of both C α (284-654nt) and C γ (256-626nt) transcripts (forward primer: 5'-CCACTACGCCATGAAGATCCTC-3' and reverse primer: 5'-CGCGCTTGGCGAAACCGAAGTC-3').

Five microliters of the RT reaction was added to a standard PCR reaction mixture containing 5 units Taq polymerase (Promega) and 2.5 μ g of each the forward and reverse C α /C γ -specific primers in a total volume of 25 μ L. Negative controls were run to determine if any genomic and reagent contamination was present along with positive controls, generated by “doping” additional PCR reaction tubes with 1 μ g of plasmid-based expression vectors for C α and C γ transcripts. A 10-minute 94°C hot start began the 45-cycle reaction (30 second 94°C dissociation step, 45 second 58°C annealing step and 90 second 74°C extension step) with a final 10-minute 74°C extension step added after the 45th cycle. PCR products were analyzed by separating 5 μ L of each reaction mixture in a 2% Tris-acetate-EDTA agarose gel before performing the restriction digestion analysis.

Five microliters of the PCR reaction were added to subsequent restriction digestions containing 1 unit of RsaI, PstI or XhoI (Promega) in appropriate reaction buffers and incubated for 1 to 2 hours at 37°C. Standard glycerol-based DNA sample buffer was added to the entire restriction digestion mixture. The samples were applied to 15% polyacrylamide gels with a 3% stacking gel and separated (30 mA, 4 hours) using a Tris-borate-EDTA buffering system. The gels were then rocked at room temperature for 5 minutes in 200 mL ethidium bromide solution (0.25 μ g/mL), and imaged using a UV transilluminator and a digital camera.

RESULTS

Differentiation of U-937 Cells with 8-CPT-cAMP – The human U-937 myeloid leukemia cell line differentiates in response to increases in intracellular levels of cAMP induced with forskolin (176), but not with agonist to G-protein coupled receptors such as histamine and dimaprit (176,177). Alternatively, the cells can be treated directly with membrane permeable cAMP analogues such as dibutyryl cAMP (dbcAMP) (178,179) to bypass adenylyl cyclase. However, dbcAMP breaks down into butyrate (180,181), which can also induce differentiation of U-937 cells (180). Another analogue that may be more suitable for a cAMP-specific effect is 8-(4-chlorophenylthio) adenosine- 3', 5'- cyclic monophosphate (8-CPT-cAMP). To the best of our knowledge there are no published reports showing U-937 cells differentiate in response to 8-CPT-cAMP. This analogue has no known biologically active byproducts, has a higher affinity for PKA (182,183) and is less susceptible to phosphodiesterase (PDE) hydrolysis than dbcAMP (10,184). Dose response studies demonstrated that the 8-CPT-cAMP EC_{50} was 50 μ M for differentiation of U-937 cells (data not shown).

Markers for U-937 cellular differentiation include a decrease in cellular proliferation and DNA synthesis, increase in cell adherence and its associated extracellular markers CD11 and CD18, increase in phagocytosis and its associated extracellular marker Fc γ RII, increase in respiratory burst activity, superoxide production, and their associated marker CD14 (176,178,179,185-188). For this study we selected two of these markers, which include diverse cell functions and have been well established for U-937 cellular differentiation: cellular proliferation, which was tested here with an MTS assay, and an increase of respiratory burst activity, which was tested with nitroblutetrazolium (NBT).

Using these methods, a 2.5-fold increase in respiratory burst (Figure 28A) and a 38% decrease in cellular proliferation (Figure 28B) were observed in cells treated with 8-CPT-cAMP for 3 days. A 30% decrease in cell number was observed in treated cells relative to untreated cells by hemocytometer analysis (data not shown), which is in agreement with the values obtained from the MTS assay (Figure 28B). There was also no apparent difference in cellular viability between treated and untreated cells after three days as determined by trypan blue exclusion under a hemocytometer (20% decrease in viability in both treated and untreated cells, data not shown). The results from the MTS and NBT assays demonstrate that a three-day treatment with 50 μ M 8-CPT-cAMP is sufficient to initiate differentiation in U-937 cells and these results are in good agreement with published work showing U-937 differentiation in the presence of forskolin and dbcAMP (176). *Kinase Activity in Whole-Cell Extract* – While $C\gamma$, $C\alpha$, and $C\beta$ all bind to the regulatory subunit and can be specifically differentiated from other kinases by their cAMP-dependence, a key difference among them is that $C\alpha$ and $C\beta$ are inhibited by PKI ($IC_{50} = 5-12$ nM (95)) and $C\gamma$ is not ($IC_{50} > 90,000$ nM (20)). The conditions set for the kinase assay make the assay very specific for PKA. These include measurement of activity in the presence of cAMP and the use of a PKA-selective substrate used at a selective concentration. The use of cAMP in this assay alone makes this assay specific for PKA, for of the 518 kinases identified in the human kinome, only one has been described as cAMP-dependent, and that is PKA. The difference in PKI-sensitivity between the isoforms was exploited here to identify $C\gamma$ -associated kinase activity in the cell. *C γ -associated* kinase activity is defined here as cAMP-dependent, PKI-insensitive kinase activity measured in the presence of the PKA selective substrate,

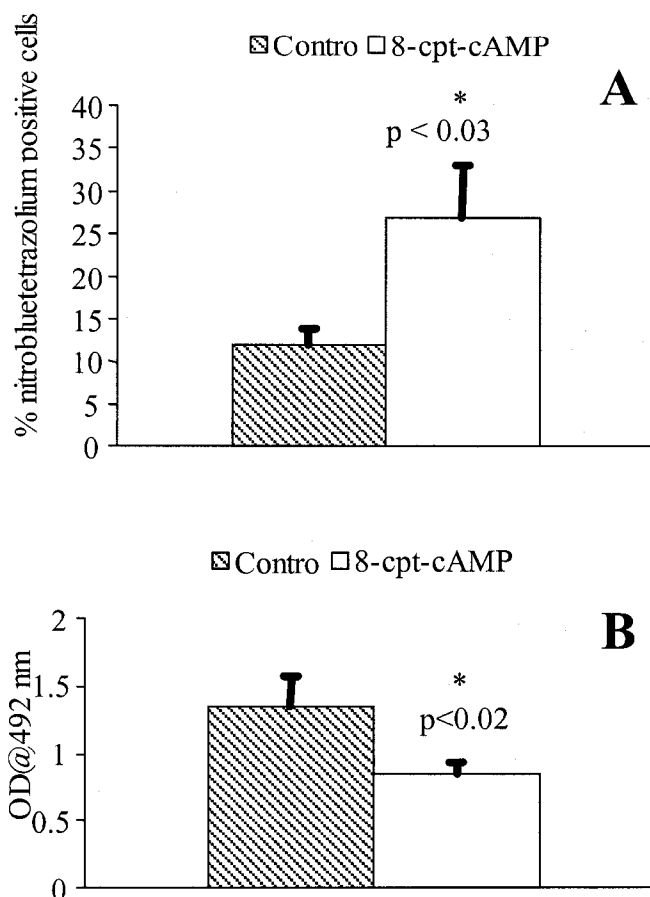


FIG. 28. **8-CPT-cAMP induces differentiation in U-937 cells.** U-937 cells were treated for three days with 50 μ M 8-CPT-cAMP and markers for differentiation were determined. *A*, acquisition of respiratory burst activity was determined by reduction of Nitrobluetetrazolium and *B*, proliferation was determined using MTS proliferation assays as described in Materials and Methods. Bars represent the average of five experiments with (*) indicating statistical significance relative to control in each experiment. Error bars represent the standard error for each condition.

Kemptide. This is in contrast to *C α -associated* activity, which is defined as cAMP-dependent, PKI-sensitive kinase activity measured in the presence of Kemptide. Measurement of kinase activity in cell extract showed total cAMP-dependent activity did not change appreciably in differentiated cells (723 pmol/min/mg) relative to undifferentiated cells (703 pmol/min/mg) (Figure 29). However, cAMP-dependent, PKI-insensitive activity increased from 186 pmol/min/mg in undifferentiated cells (26% of total activity) to 270 pmol/min/mg in differentiated cells (37% of total activity) (Figure 29). This result suggests an increase in *C γ -associated* kinase activity during or following differentiation.

DEAE Chromatography Profiles of Undifferentiated and Differentiated U-937 Cells – To determine the effect of the cAMP-analogue on U-937 cell's PKA profile, the extracts of treated and untreated cells were separated by DEAE column chromatography. Kinase activity profiles were generated using four different conditions (\pm cAMP, \pm PKI) for each fraction collected from a 50 mL, 0-500 mM NaCl gradient elution. The profile shows that U-937 cells underwent a shift from 36% type I, 64% type II PKA (Figure 30A) in undifferentiated cells to 100% type I PKA in differentiated cells (Figure 30B) after three days of treatment with 8-CPT-cAMP. The total activity in undifferentiated cells under peaks I and II was 6111 ± 1267 pmol/min/mg, while in treated cells the activity shifted completely to type I, 7271 ± 1922 pmol/min/mg (\pm SE, n=3). The total PKI-insensitive activity eluted from the DEAE column from undifferentiated cells was 738 ± 146 pmol/min/mg, while from treated cells this activity was 828 ± 405 pmol/min/mg (\pm SE, n=3).

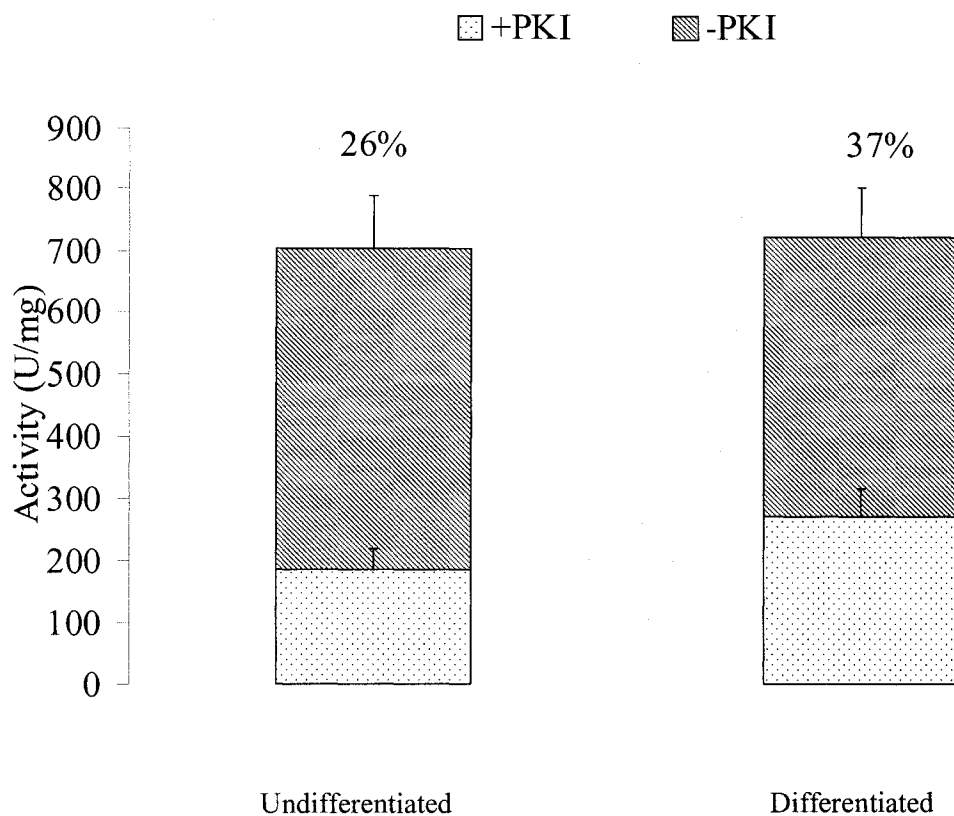


FIG. 29. **8-CPT-cAMP-induced differentiation increases $C\gamma$ -associated PKI-insensitivity, cAMP-dependent activity in U-937 cells.** U-937 cells were differentiated as described in the legend to Figure 1 and Materials and Methods. Protein kinase activity was determined with Kemptid as substrate in the presence and absence of cAMP and PKI (see Materials and Methods). Each bar is divided into PKI-sensitive (dotted section of bar) and PKI-insensitive (stripped section of bar) activity from total cAMP-dependent kinase activity. PKI-insensitive activity is also indicated as a percent of total activity above each corresponding condition. Each bar represents an average of five experiments and the error bars represent the standard error for each condition.

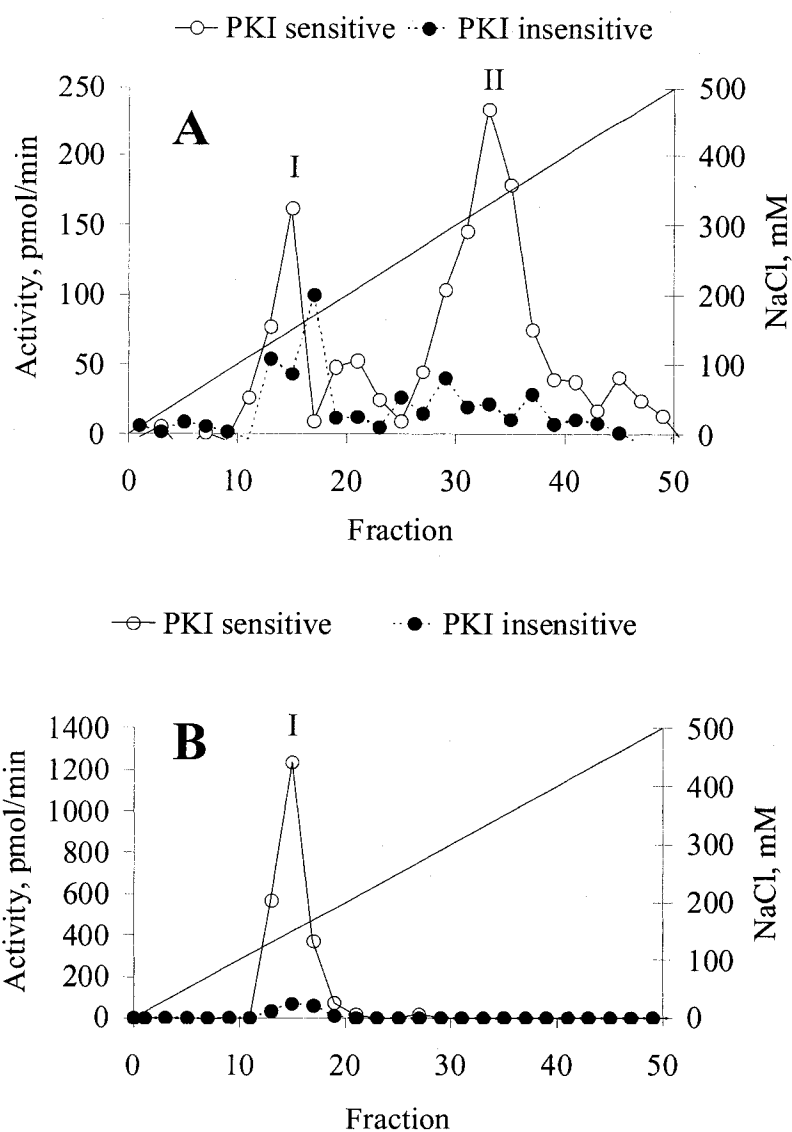


FIG. 30. 8-CPT-cAMP-induced differentiation of U-937 cells shifts the PKA profile to the type I isoform. U-937 cells were differentiated as described in the legend to Figure 28 and in the Materials and Methods section. Cell extracts were separated by DEAE-sepharose chromatography, *A*, before or, *B*, after treatment with 8-CPT-cAMP and analyzed for Kemptide kinase activity in the presence of cAMP (10 μ M) and the presence and absence of PKI (1 μ M). The profiles are representative of three independent experiments.

The DEAE profile also shows that in undifferentiated cells approximately 60% of cAMP-dependent, PKI-insensitive activity is associated with type I PKA and remains associated with type I PKA in differentiated cells (Figure 30A).

Immunoblot Analysis of U-937 Cells – A difficulty with selectively identifying $C\gamma$ expression in cells and tissues has been the absence of an antibody that selectively detected this isoform. Both PKA catalytic subunits $C\alpha$ and $C\gamma$ share 83% identity, but only 58% identity at the amino terminus (amino acids 1-40). Santa Cruz Biotechnology and Abgent developed polyclonal antibodies (pAb) to this region of $C\gamma$. These antibodies were tested here for selectivity on serially diluted (20-1.25 ng) purified $C\alpha$ and $C\gamma$ (each of 40kDa apparent molecular weight). The antibody from Santa Cruz detected both purified recombinant isoforms, while the antibody from Abgent only detected recombinant $C\gamma$ at all protein levels tested except at 1.25ng (Figure 31A). The only discernible difference between $C\alpha$ and $C\gamma$ with the Santa Cruz antibody is that $C\gamma$ provides a better signal (darker band) than $C\alpha$, which allowed for cell extracts, the antibody, or both to be titrated to the point where the antibody only detected $C\gamma$ (data not shown). However, this approach was not practical since $C\alpha$ is expressed in all tissue types, and $C\gamma$ expression in any tissue type has yet to be characterized other than in human testes (45). The Santa Cruz antibody has recently been used to incorrectly identify expression of human specific $C\gamma$ in rat cells (109), indicating the importance of demonstrating the selectivity and limitations of these antibodies. Based on our result the Abgent antibody was used to probe membranes with extract from differentiated and undifferentiated U-937 cells (Figure 31B).

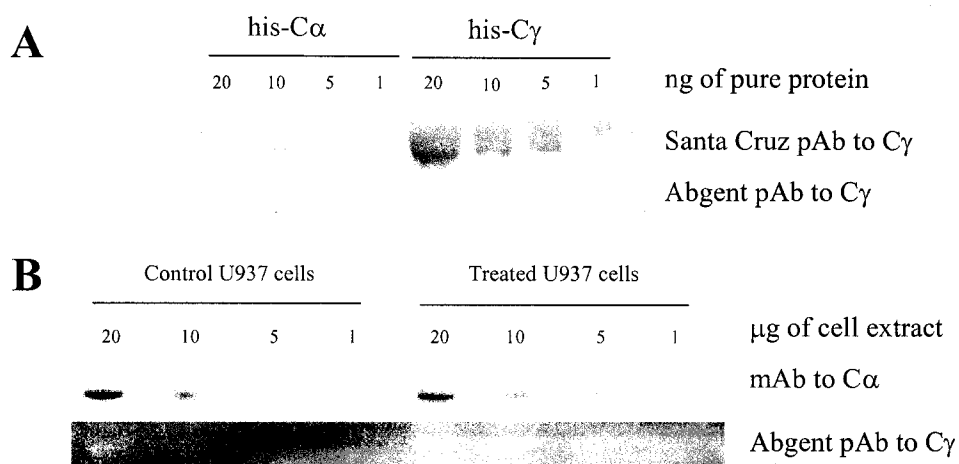


FIG. 31. Immunoblot analysis demonstrates expression of both C α and C γ in U-937 cells. *A*, two commercially available polyclonal antibodies made against C γ -peptides were tested against purified recombinant C γ and C α . Lanes were loaded with between 1.25-20 ng of protein as indicated by the shaded bar over each blot. *B*, the Abgent anti-C γ -antibody was tested against blots with differentiated and undifferentiated U-937 cell extract. The lower panel in this figure is presented as an inverted image to aid visualization of the C γ -band.

The antibody detected a 40kDa band only in extract from differentiated U-937 cells suggesting $C\gamma$ expression increases during or following differentiation. An anti- $C\alpha$ selective monoclonal antibody (105) was used to determine $C\alpha$ expression levels before and after U-937 differentiation (Figure 31B). This antibody detected similar levels of $C\alpha$ expressed before and after differentiation, suggesting $C\alpha$ protein levels do not change during the period tested.

RT-PCR/RFLP Analysis of U-937 Cells – Because of the high homology between $C\alpha$ and $C\gamma$, it has not been possible to design primers that selectively amplify only one mRNA from the two isozymes, something we have attempted numerous times with no success. However, the specificity of this RT-PCR allowed for the selective amplification of only $C\alpha$ and $C\gamma$ transcripts in human cells. The primer set used for the PCR step amplifies a 370 base pair region of both $C\alpha$ (284-654nt) and $C\gamma$ (256-626nt) transcripts and was designed to prevent the amplification of the $C\beta$ isoform. No bands other than the expected 370 bp amplicons were observed.

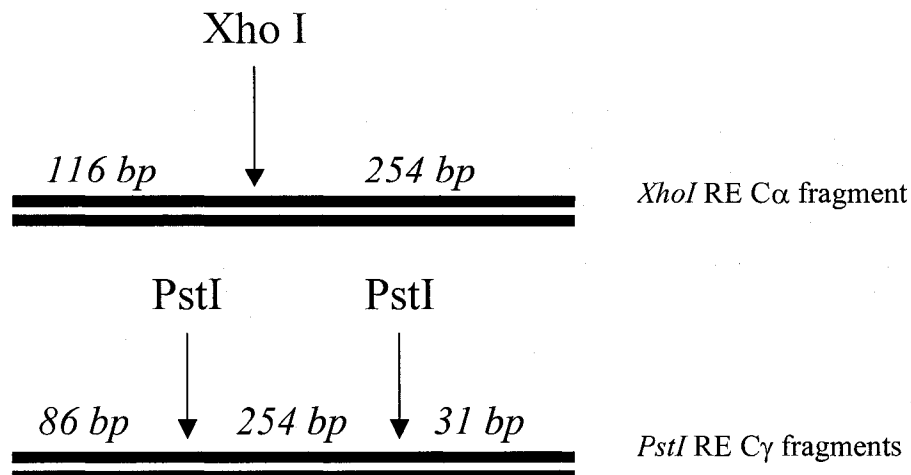
To differentiate the amplicons, an adaptation of the restriction fragment length polymorphism (RFLP) protocol was developed (Figure 32A). The $C\alpha$ amplicon sequence does not contain a PstI digestion sequence, but PstI cleaves the $C\gamma$ amplicon into fragments of 254, 86 and 31 base pairs. The restriction enzyme XhoI does not cut the $C\gamma$ amplicon but cleaves the $C\alpha$ amplicon into fragments of 255 and 116 base pairs. In addition, the restriction enzyme RsaI cleaves both amplicons, yielding different fragments of 207 and 164 base pairs for $C\alpha$, with 149, 106, and 101 base pairs for $C\gamma$. The RsaI reaction would also serve to identify any potential amplification of the $C\beta$

isoforms, since expected patterns for this fragment are bands of 165 and 106 base pairs. These contaminating bands were not observed.

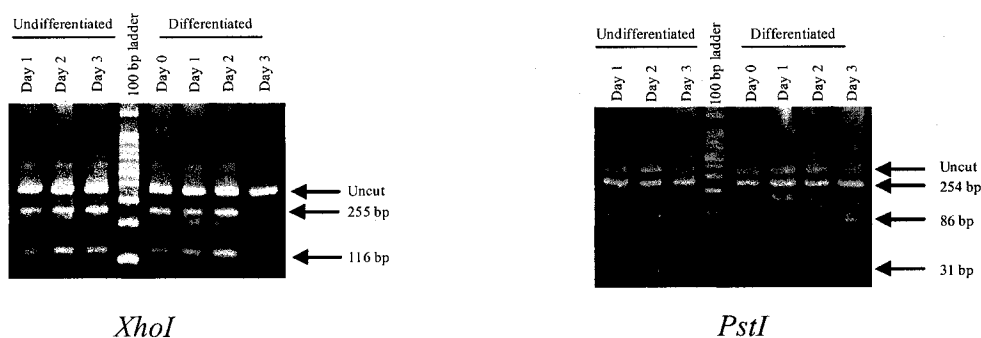
Both $C\alpha$ and $C\gamma$ RT-PCR/RFLP fragments were detected in control U-937 cells. $C\alpha$ -specific fragments were seen as *RsaI* digests of 206 and 165bp (data not shown), *XhoI* digests of 255 and 116bp, and the band not cut by *PstI* (Figure 32B). The $C\gamma$ -specific fragments were seen as *RsaI* digests of 148, 106, and 101bp (data not shown), *PstI* digests of 254, 86bp and 31bp, and the band not cut by *XhoI* (Figure 32C). The RT-PCR/RFLP analysis of undifferentiated and differentiated U-937 cells demonstrated that both $C\alpha$ and $C\gamma$ RT-PCR fragments are seen on days zero through day two in untreated and treated U-937 cells (Figure 32B). However, by day three, $C\gamma$ RT-PCR/RFLP fragments were still visible while no apparent $C\alpha$ transcripts were detected in treated U-937 cells (Figure 32C).

DISCUSSION

Until now $C\gamma$ was believed to be a testes-specific PKA isoform, identified only through the cloning of its mRNA. Here for the first time we demonstrate the expression of active endogenous $C\gamma$ protein from an *in-cell* system. The hypothesis put forth in this paper was that $C\gamma$ expression occurs in cells undergoing differentiation, regardless of the cell's tissue source. Here we present, for the first time, evidence that $C\gamma$ is expressed in cells from tissues other than the testes. Furthermore, because $C\gamma$ mRNA is present before differentiation, and protein expression and kinase activity increases following differentiation, it is possible that $C\gamma$ has a function in cellular differentiation. U-937 cells are a human histiocytic lymphoma cell line (189),



A



B

C

FIG. 32. Differential expression of C α and C γ mRNA in U937 cells during 8-CPT-cAMP induced differentiation. Control and 50 μ M 8-CPT-cAMP-treated U-937 cells were isolated on days 0, 1, 2, and 3 and total RNA was isolated as described in Materials and Methods. *A*, scheme of expected fragments from restriction enzyme digest of RNA for each isoform as indicated to the right of the figure. Restriction enzyme digests of RT-PCR products was done using, *B*, Xho I or, *C*, Pst I for analysis of C α and C γ expression, respectively. Each gel is representative of three independent experiments.

a transformed pre-monocytic cell line that has retained some pre-monocyte characteristics but capable of fully differentiating. About 20-30%

of the cells in culture exhibit differentiation markers at any given time, such as phagocytic activity and the expression of Fc receptors (189). However this cell line is considered by many to be a useful model for cellular differentiation because of its increased response to pro-differentiation agents such as TPA, IFN γ and cAMP, while not becoming terminally differentiated. We utilized the cAMP-PKA pathway to induce U-937 cell differentiation and investigate C γ expression during cellular differentiation. Cellular differentiation was determined through two well-characterized differentiation markers that include highly specialized functions exhibited by differentiated cells. These included cell growth arrest and increased respiratory burst activity. U-937 cells exhibit differentiation markers when treated with cAMP-signaling pathway agonist such as forskolin and dbcAMP (176,185,190), but there are inherent problems with each of these reagents, such as metabolic byproducts and degradation of endogenous cAMP by PDE. Therefore, we opted to use 8-CPT-cAMP, another membrane permeable, cAMP-analogue, which was less susceptible to degradation by cAMP PDE (10). Three days of treatment with 8-CPT-cAMP induced reduced cell growth rate without decreasing cellular viability, while increasing the cell's respiratory-burst activity. Both markers are used to identify pre-monocytic cellular differentiation to macrophages. While differentiation of U-937 cells with a cAMP analogue has been described before, this is the first time it has been demonstrated with 8-CPT-cAMP and the first time that C γ expression at the mRNA and protein level have been detected in any cell.

An increase in cAMP-dependent, PKI-insensitive kinase activity was measured in U-937 cells treated three days with 8-CPT-cAMP, suggesting an increase in $C\gamma$ protein expression following differentiation. While the apparent increase in $C\gamma$ activity may appear modest, changes in cAMP-dependent functions do not require large changes in cAMP or PKA enzyme activity. Furthermore, total cAMP-dependent kinase activity did not increase. Since the maximal velocity of $C\gamma$ is significantly lower than that of $C\alpha$ (20), the real change in $C\gamma$ activity may be underestimated based on a comparison with $C\alpha$. In addition, $C\gamma$ does not bind PKI and therefore will not be exported from the nucleus by the same mechanism for $C\alpha$. Thus the real impact of $C\gamma$ expression on transcription is uncertain. While transcriptional regulation by the two isozymes is different, the real role for $C\gamma$ -regulated transcription remains to be determined, but difference may be related to cell events such as cellular differentiation. While it has been demonstrated that $C\gamma$ appears to be more PKG-like, preferring substrates with differences on the carboxyl-termini of the phosphorylation site (20), the physiological meaning for differences in substrate specificity between the isozymes *in vivo* remains to be determined. What remains perplexing is a potential role for $C\gamma$ that is specific to primates.

A more readily recognized change following U-937 cell differentiation was the PKA profile, which shifted from a type I: type II ratio of 0.64 in undifferentiated cells to 100% type I in differentiated cells, with $C\gamma$ activity prevalently associated to type I PKA. A similar trend has been observed in Chinese hamster ovary (CHO) cells treated with dbcAMP (7), although the loss of type II PKA following treatment with dbcAMP was not as dramatic in CHO cells. This is mostly likely due to differences in cell types. CHO is a hamster epithelial cell line and U-937 is a human hematopoietic cell line. Increases in

RI α in response to treatment with cAMP analogues have also been observed in cultured fibroblast and Rat-1 cells (7). This implies that an increase in intracellular cAMP serves as a positive feedback signal for RI α expression, and that RI α is part of the cell's differentiation mechanism. However, PKA function in cellular differentiation is still not clear.

The stable level of total kinase activity present in the cell before and after differentiation suggests that a basal pool of potential PKA activity is maintained in the cell irrespective of the cell's differentiation status. Maintenance of total PKA activity levels in the cell may be achieved through compensation by differential expression of the catalytic subunit isoforms. An example of this is seen in C β 1 knockout mice, where total kinase activity does not decrease in the absence of C β 1 (58), suggesting that C α compensates for the loss of C β 1. Thus, an increase in C γ expression in U-937 cells during differentiation may be offset by a decrease in C α expression to maintain a constant kinase activity level in the cell. This is supported by the result showing C α , but not C γ , mRNA decreased following three days of treatment with 8-CPT-cAMP, while C α protein levels did not decrease based on immunoreactivity with the putative C α antibody. The possible presence of C α protein in the absence of its RNA may be due to the stability of the PKA catalytic subunit, which has a 9.2 hour half-life when bound to the regulatory subunit (79). Alternatively, it is possible that the cAMP-dependent, PKI-sensitive activity observed post-differentiation is due to C β , since immunoreactivity between C α and C β with these antibodies has not been verified. While there is no precedent for C β compensation for a loss in C α , C-subunit isozyme shifts have not been closely analyzed in a differentiation paradigm. However, a compensation of C γ for C α activity would

require a considerable increase in $C\gamma$ catalytic activity since the V_{\max} is at least 10 times lower than the $C\alpha$ V_{\max} (20). Nevertheless, about 90% of PKI-sensitive activity in U-937 cells was present in the holoenzyme form (activity ratios were 0.04 in untreated cells and 0.13 in treated cells, data not shown). Based on these results, further analysis of C-subunit isozymes during U-937 cell differentiation is warranted. It is especially noteworthy that $C\gamma$ catalytic activity and immunoreactivity increases after 8-CPT-cAMP-mediated differentiation in U-937.

One major advance and important development demonstrated here is the identification of an antibody that is relatively specific for $C\gamma$ that has allowed the unequivocal differentiation of the $C\gamma$ - and $C\alpha$ -subunit isoforms. Antibodies to specifically identify $C\beta$ remain unavailable. The $C\gamma$ antibody detected a 39-40 kDa band that appeared following, but not prior to, differentiation and was concurrent with the increase in $C\gamma$ -associated kinase catalytic activity. The antibody did not detect this band prior to differentiation, possibly because $C\gamma$ expression levels were below the detection limit of the antibody. The kinase assay used is very sensitive (10) and thus, likely to detect $C\gamma$ -catalytic activity before an antibody detects the protein. While immunoblot analysis with different antibodies does not allow quantification of C-subunits, the increase in cAMP-dependent, PKI-insensitive kemptide kinase activity following differentiation provides a valuable tool to differentiate and quantify changes in $C\gamma$ activity. The low PKI-insensitive specific activity relative to PKI-sensitive, cAMP-dependent activity may be due to low $C\gamma$ expression levels and/or lower $C\gamma$ V_{\max} relative to $C\alpha$ (20). Thus, while it is clear that $C\gamma$ expression increases following differentiation,

because the two isozymes are so different kinetically, it is problematic to compare them on an activity basis. It is intriguing that the homology of the two isozymes is so high, yet specific identification of their respective mRNAs is problematic. Thus, it has not been possible readily to differentiate and quantify the mRNA levels of $C\gamma$ and $C\alpha$. While $C\alpha$ and $C\beta$ have higher homology than $C\gamma$ and $C\alpha$, there are differences that complicate specific $C\gamma$ identification. First, differences between $C\gamma$ and $C\alpha$ do not occur in clusters, but are spread out through the sequence, making it difficult to find specific primers. In addition, $C\alpha$ and $C\beta$ can be easily differentiated in their 3' un-translated sequences, which are highly homologous in $C\gamma$ and $C\alpha$. Clues to differences in transcription targets, which are likely to reveal potential roles for $C\gamma$ in differentiation, have also been difficult. However, potential differences in compartmentalization provide further avenues for investigation. Because $C\gamma$ appears preferentially to associate with the RI subunit, the type I PKA is often predominant in differentiated tissues, and the different localization of type I and type II (191), suggest that compartmentalization of type I- $C\gamma$ is likely to play a functional role that remains to be determined. It is interesting in this regard that activation of type I PKA is sufficient to inhibit T lymphocyte proliferation (192).

The presence of $C\gamma$ in the primate genome for 40 million-years suggests a potential role for this cAMP-dependent, PKI-insensitive kinase in human function and the result here suggest a role in differentiation. However, it cannot be ruled out that it is a human genome remnant that has not experienced evolutionary selective pressure. The function of $C\gamma$ in the cell remains to be unequivocally elucidated but the results presented here and elsewhere (13,20,105) suggest that differences observed between $C\gamma$ and $C\alpha$ in stable transfections (5) and *in vitro* biochemical experiments (20) may manifest

themselves during cellular processes such as cellular differentiation. $C\gamma$ -association with type I PKA, the only isoform present following differentiation, the increase in $C\gamma$ -associated activity following differentiation, and the presence of $C\gamma$ mRNA before and after differentiation all support the idea that $C\gamma$ may have a function in cellular differentiation that is distinct from the other isoforms.

Identification of functional $C\gamma$ protein expressed in the cell completes the $C\gamma$ central dogma (i.e. gene expresses the messenger RNA, which in turn expresses the functional protein) where the gene (106) and mRNA (45) had previously been identified in the cell. Presented here are the concurrent identification of both the mRNA and the protein in the U-937 cell line. The presence of $C\gamma$ mRNA before and after differentiation hints at the possibility for $C\gamma$ post-transcriptional regulation, something suggested for $RI\alpha$ (reviewed in (193)). This would add another regulatory step for this isoform; other steps include transcriptional (through a CG-rich promoter) and post-translational (via phosphorylation) regulation. Now that an antibody selective for $C\gamma$ is available, it may be possible to study the dynamics of $C\gamma$ expression and localization in the cell in order to understand regulation of this isoform. While this work raises many questions about $C\gamma$ it clearly demonstrates for the first time that functional $C\gamma$ is expressed in human cells during a very specific cellular process.

The results indicated here suggest that there is a switch in both R and C-subunit isoforms during the cAMP differentiation program in U-937 cells. However, the regulation of R- and C-subunit levels is obscure. The C-subunits are downstream of TATA-less promoters, which are often regulated as housekeeping genes and associated with constitutive expression. Thus, the levels of the subunits themselves may be regulated

at the level of mRNA and/or protein. $C\alpha$ protein has a half-life of less than 10 hours, suggesting that the protein is turned over rapidly, but turnover before and after differentiation in U-937 cells has not been analyzed. It is interesting that three days after initiation of differentiation by 8-CPT-cAMP, the $C\alpha$ mRNA is not observed while the presence of cAMP-dependent, PKI-sensitive activity is present. It is not clear at this time if this is due to $C\alpha$ and/or $C\beta$ activity, but it is possible that the $C\alpha$ mRNA is turned over rapidly as it is in S49 cells (79, 80), while $C\gamma$ continues to be expressed.

CHAPTER VI

SUMMARY AND CONCLUSIONS

SUMMARY

The ideas for the work addressed in this dissertation came from two sources. The first one was from a National Institute of Health Grant (GM44777) awarded to Dr. Stephen J. Beebe designed to study C α and C γ in order to understand protein kinases structure-function relationships in this protein family, which is one of the largest groups of evolutionarily conserved protein families in the human genome. The second source was an American Physiological Society Pre-Doctoral Porter Fellowship for Minorities awarded to this Ph.D. candidate. Within this fellowship three specific aims were outlined to address this dissertation, which came out this Ph.D. candidate understanding the theory and specific aims described and outlined in Dr. Beebe's NIH Grant. The three specific aims addressed in this dissertation were: 1) to identify differences in primary structure between PKA-C γ and PKA-C α that defines their physiological function in intact cells (Chapter II); 2) to characterize post-translational differences between purified PKA-C γ and PKA-C α that defines their physiological function (Chapter III); and 3) to characterize the kinetic mechanism for PKA-C γ through determination of the rate constants for each catalytic step of the phospho-transferase reaction (Chapter IV). The results from a fourth objective were presented here, which was to address the question, is C γ expressed in differentiating cells? (Chapter V). The work presented in chapters III and V is currently being submitted for publication, thus each chapter was presented in this dissertation in the

format of an independent manuscript to facilitate extracting each chapter for publication purposes. This work also follows up on research initiated by Dr. Wei Qing Zhang done for his dissertation under the guidance Dr. Stephen J. Beebe. To the best of my knowledge there is currently nobody else doing research that focuses on characterizing the PKA-C γ catalytic subunit. Following are summaries of the findings presented in this dissertation (Chapters II-V). The summaries are derived from the abstracts presented at the beginning of each chapter.

Specific Aim 1: To identify differences in primary structure between PKA-C γ and PKA-C α that defines their physiological function in intact cells (Chapter II)– This hypothesis was tested here with chimera constructs wherein the first 50 and terminal 70 amino acids were swapped between C α and C γ and tested through transient transfections with CREB-mediated reporter assays. While both isoforms induced transcriptional activation, the chimera transfections failed to exhibit any activity in COS7 cells, but exhibited some activity in Kin8 cells. This difference in cell type dependent activity may suggest that there are cell type specific factors that are required for the proper expression of the chimeras. Immunoblot analysis demonstrated that each of the chimeras was expressed in COS7 cells and sequence analysis of the constructs suggests that the expressed chimeras should have the expected primary sequence. While it is difficult to interpret the results obtained from the chimera studies the amino and carboxyl terminals remain important regions of divergence between C α and C γ that warrant further studies.

Specific Aim 2: To characterize post-translational differences between purified PKA-C γ and PKA-C α that defines their physiological function (Chapter III)– Differences in phosphorylation between C α and C γ were studied here through reporter assays using C γ

and C α T197 point mutants, immunoblot analysis using an anti-phospho T197 selective antibody and LC-MS/MS phosphomapping of purified recombinant proteins. Two novel phosphorylation sites were identified on both isoforms by LC-MS/MS analysis (C γ pS14 and C α and C γ pS259). It was also determined that C γ is phosphorylated homologously to mammalian expressed C α (pT197 and pS338) and the modification at T197 is important to the function of both isoforms suggesting that differences in post-translational modification are not the reason for kinetics differences observed between these two isoforms.

Specific Aim 3: To characterize the kinetic mechanism for PKA-C γ through determination of the rate constants for each catalytic step of the phosphotransferase reaction (Chapter IV)– Here recombinant C α and C γ binding rates for ATP and ADP were compared through stopped-flow analysis, using ATP and ADP conjugated to a N-methylanthraniloyl (mant) fluorophore. Using this approach the C α -ATP on and off rates were determined and found to be in good agreement with the literature. The C α release of ADP was slower than the release of ATP, which is in agreement with ADP release being the rate-limiting step. However, several other slow rates were measured for both the mant-ATP on and off rates, which may suggest that mant-ATP is not the rate-limiting step. But the work presented here was unable to clarify this question. The homogenous purification of recombinant his₆-C γ proved to be difficult but enough was attained to measure the C γ ADP-off rate, a step where divergence in the catalytic mechanism of these two isoforms could explain differences in biochemical function. The signal measured for the C γ ADP off-rate was reproducible but very low and needs to be repeated in another independent experiment. However, the measured ADP-off rate for C α

is about 9-times faster than that of $C\gamma$, a result that is in agreement with differences in their steady-state kinetics. While it is still not clear what structural differences between $C\alpha$ and $C\gamma$ lead to functional differences it is encouraging to identify a difference in their catalytic rate. The physiological ramifications of the biochemical difference between $C\alpha$ and $C\gamma$ still needs to be determined through studies of the isoforms *in vivo* and in the intact cell.

Is $C\gamma$ expressed in differentiating cells? (Chapter V)– For the first time we present the selective identification of endogenous $C\gamma$ protein and mRNA expressed in human U-937 cells. Increases in protein kinase inhibitor (PKI)-insensitive, cAMP-dependent kinase activity following 8-CPT-cAMP-induced differentiation of U-937 cells suggested an increase in $C\gamma$ expression during differentiation, while total PKA catalytic activity did not change. A new $C\gamma$ -specific antibody identified a 40-kDa band following differentiation of U-937 cells and RT-PCR/RFLP analysis demonstrated that $C\gamma$ mRNA was present before and after differentiation. DEAE chromatographic analysis of U-937 cells showed that the PKA profile changed from similar levels of type I and type II PKA in undifferentiated cells to 100% type I in differentiated U-937 cells. Furthermore, 60% of PKI-insensitive, cAMP-dependent activity in U-937 cells was associated with type I PKA suggesting $C\gamma$ favors association with type I PKA in this cell line. These results expand our understanding of structure-function roles for PKA isoforms in cell differentiation and function. Furthermore the increase in $C\gamma$ expression during U-937 differentiation suggests a novel function for $C\gamma$ in the cell.

CONCLUSIONS

In order to identify causes for biochemical differences between C α and C γ both the primary structure and modified structure of C α and C γ were compared and characterized. The two isoforms were also characterized for differences in their catalytic mechanism and analyzed for expression patterns in the cell. The results from the chimera studies, where portions of the amino or carboxyl terminals were swapped between C α and C γ , remain inconclusive. This makes it hard to interpret the significance of the amino and carboxyl terminal regions to the function of the catalytic subunits. Studies of these regions have been done successfully with C α through the characterization of point mutants and have demonstrated that these regions are important to the enzymes stability and substrate recognition. As C γ seems to be more labile than C α but still functional, it seems likely that differences in these regions will eventually help address physical differences between C α and C γ that contribute to their functional differences. The amino terminus is of particular interest because this region is where there is the greatest divergence from homology between C α and C γ and among C-subunit isoforms among species. Furthermore the amino terminus contributes to overall structural stability of C α . Because the C α amino and carboxyl domains fall outside of the conserved kinase catalytic core it is difficult to draw from studies on these regions in other kinases. In most kinases these regions are a lot more extensive than in C α and C γ and the functions for these domains are very diverse, ranging from protein-protein interactions, localization signals, and regulatory functions, depending on the kinase.

A feature conserved amongst most kinases is the phosphorylation of the catalytic loop. Most, if not all, kinases need to be phosphorylated in this region to be active. And

many of them need to be phosphorylated on the catalytic loop to be fully functional. Studying the phosphorylation state of C α and C γ addresses an important question: can a kinase be active in the absence of phosphorylation on its catalytic loop? Because phosphorylation differences on the catalytic loop were not found between C α and C γ , which is the only modification shown to date to be of significance to the function both isoforms, functional differences between C α and C γ do not seem to be due to phosphorylation differences. Two novel phosphorylation modifications were identified on C γ and C α and it will be of interest in future studies to determine if these modifications are important to the function of either catalytic subunit. It will also be of interest to compare these modifications with other kinases to determine if these are conserved among other kinases. Thus, the studies presented in the Chapters II and III have not demonstrated physical differences between the catalytic subunits to which functional differences can be attributed.

A likely explanation for functional differences was observed upon studying the catalytic mechanisms of each C-subunit. The catalytic mechanism for C α has been studied in great detail, and while the literature clearly favors an ordered Bi Bi mechanism with the release of ADP as the rate-limiting step, it is also clear from the same literature that many questions remain about this mechanism. Here it was found that the “rate-limiting” step is much slower for C γ than for C α . Thus, it is very possible that functional differences are due to differences in their ADP off-rates. The simple explanation for this observation is that C α and C γ exhibit differences in their overall tertiary-structure, which may affect the conformation of the isoforms and binding of the substrates. This measurement may explain why C γ exhibits slower kinetic and reporter activities as these

mechanisms depend on phosphorylation by the C-subunits. Thus $C\gamma$ would phosphorylate 0.1 of the substrate molecules that $C\alpha$ would, causing a ten-fold difference in kinetic or reporter-activity measured, which is what is observed in most instances.

The most elusive fact about $C\gamma$ has been the absence of its expression identified in the cell. Here for the first time $C\gamma$ expression has been identified in differentiating cells. The appearance of $C\gamma$ in differentiated U-937 cells suggests a novel role for PKA in this cellular process.

While the results presented here fail to identify specific physical differences that could define functional differences between $C\alpha$ and $C\gamma$, the same results have helped us understand the significance of phosphorylation to the catalytic loop of the enzyme, for without this modification both kinases lost all of their function. Furthermore, $C\gamma$ is a functional kinase that is expressed in the cell during specific cellular events such as differentiation, and has survived 40 million years in the human genome. So while the nature of $C\gamma$ function in the cell remains elusive, it is likely that the manifestation of the functional differences between $C\alpha$ and $C\gamma$ will eventually be observed when the tools to knock out $C\gamma$ or $C\alpha$ without disrupting the other isoform are developed. But the present results suggest that during some cellular events, such as differentiation, it seems important that a cAMP-dependent kinase with slower kinetics and reporter activity than $C\alpha$ be expressed, while $C\alpha$ expression be suppressed.

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APPENDIX

APPENDIX A**PERSONAL COMMUNICATION FROM TONY HUNTER ON THE ORIGIN OF
THE TERM KINASE**

Dear Dr. Hunter,

I am a graduate student in Dr. Stephen Beebe's laboratory characterizing PKA-Cgamma. You may not recall but we met very briefly at a second messenger Gordon Research Conference in 2001. I have enjoyed reading many of your reviews and learned a great deal through these. Recently I have been curious about the history of kinases, when they were first discovered and where the name "kinase" came from as this is not a proper name for an enzyme, according to IUBMB, as kinases are phospho-transferases. Could you recommend a good source or reference from which to obtain this type of information on kinases. The earliest publication I could find in PubMed with kinase in it was 1963, and this was a German paper, I do not recall the authors. I would appreciate any information you could share with me on this matter.

Sincerely, Gary

From: Tony Hunter [mailto:hunter@salk.edu]
Sent: Thursday, January 30, 2003 9:20 PM
To: Morris, Gary Z.
Subject: Re: Question about the origin of the name "kinase" (?)

Gary - You really need to address this question to one of the old timers in the field like Ed Krebs, but I'll give it a shot. Although phosphoproteins had been known since the 1930's, the first protein kinase activity to be detected was casein kinase (actually we now know this was CK2) by Kennedy in 1954 (JBC 207:153; JBC 211:969; see Annu. Rev. Biochem. 61:1). For a history of protein phosphorylation, you might look at Cohen (Nature Cell Biol. 4:E127). The word kinase derives from the Greek kinein, which means to move towards, and in this context it is moving the phosphate from ATP onto an acceptor (e.g. hexose or protein). I hope that this is helpful. Tony

APPENDIX B

CONSTRUCTION OF CHIMERAS

-----Original Message-----

From: zhang98 [mailto:zhang98@uic.edu]

Sent: Monday, November 11, 2002 12:31 PM

To: Beebe, Stephen

Subject: construction of C-subunit chimera

Hi Dr. Beebe:

I am sending you the procedure how to make those chimera as attached file and the following. Any question, please call me. thans.

John

Construction of Ca Chimera containing cDNA encoding aa1 to aa 50 of Cg sequence and Cg Chimera containing cDNA encoding aa1 to aa 50 of Ca sequence:

Human Ca and Cg chimera cDNA were subcloned via the HindIII and EcoRI sites into the pCMV5 (Invitrogen). The splicing sites of the chimeras mapped to highly differentiated regions of PKA catalytic subunits (the first 50 amino acids sequence). The chimeric constructs were then generated by repeated cycles of two-step polymerase chain reaction overlap extension (Horton et al 1989) with the Ca and Cg cDNA containing plasmids as templates, respectively. The cDNAs were fused sequentially at homology regions of splicing site RS with the primers listed in Table I. Primers Ca-A/Cg-A and Ca-D Cg-D provided Hind III and EcoRI linkers, respectively, for subcloning into pCMV5. The correct sequence of the final products was confirmed by automated sequencing.

Table

Oligonucleotide primers for splicing by overlap extension polymerase chain reaction. For the internal primers, the bold primer sequence letter denote the locations of the corresponding splicing sites coding sequences or their reverse complements, respectively.

Name	Sequence	Sense/antisense
Internal primers		
Ca-B	5' CTGGTGCCTCACCAGCATCACTCGCCCAAAGGAGCCGGT	(s)
	Cg sequence for overlapping	
Ca-C	5'ATGCTGGTGAAGCACAAGGAG	(AS)
Cg-B	5' CTTGTGCTTCACCAGCATCACCCGCCCGAAGGAGCCCAT	(S)
	Ca sequence for overlapping	
Cg-C	5'ATGCTGGTGAGGCACCAGGAG	(AS)

External primers

Ca-A 5'-ATATAAGCTTATGGGCAACGCCGCCGCC (S)
Ca-D 5'-ATATGGATCC CTAAAACTCAGTAAACTCCTT (AS)
Cg-A 5'-ATATAAGCTTATGGGCAACGCCCCGCCAAG (S)
Cg-D 5'-ATATGGATCCCTAAAACTCAGAAAACTCCTT (AS)

-----Original Message-----

From: zhang98 [mailto:zhang98@uic.edu]
Sent: Wednesday, September 22, 2004 11:17 AM
To: Morris, Gary Z.
Subject: RE: Hey John

Hey Gary:

Sorry for the delay because thing up here become pretty tough. Now I am sending you the sequence I designed for Chimera at 280 aa after.

1) Cg-a-5' (1)CAAGAACTCCAAGGGTTTTCGCTTGGTGAGGTCCAC
2)Cg-a-5'(2)AAACCCTTGGAGTTCTTGGGGGTCAATGACATCAAG
3)Ca-g-5'(1)CAAGGACTCCAAAGGCTTGGCGCTTGGTGAGATCCAC
4)Ca-g-5;(2)AAGCCTTTGGAGTCCTTGGGGGTGGCGACATCAAG

Please take a look and synthesize them in HPLC scale. Send them to me while you have them. I will let people done as soon as possible.

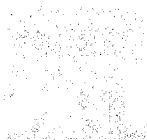
Thanks.

Regards

John

APPENDIX C

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of the House of Elsevier in 1580
and celebrate the establishment
of the Elsevier company in 1880.

16 June 2005

Our ref: HG/mm/jun 05.J063

Mr Neil Reece
ReeceNM@CHKD.COM
For: Gary Morris

Dear Mr Morris

ARCHIVES OF BIOCHEMISTRY & BIOPHYSICS, Vol 403, pp 219-228, Morris et al, "Differential..." 1 Figure only

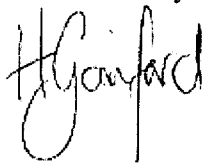
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Yours sincerely

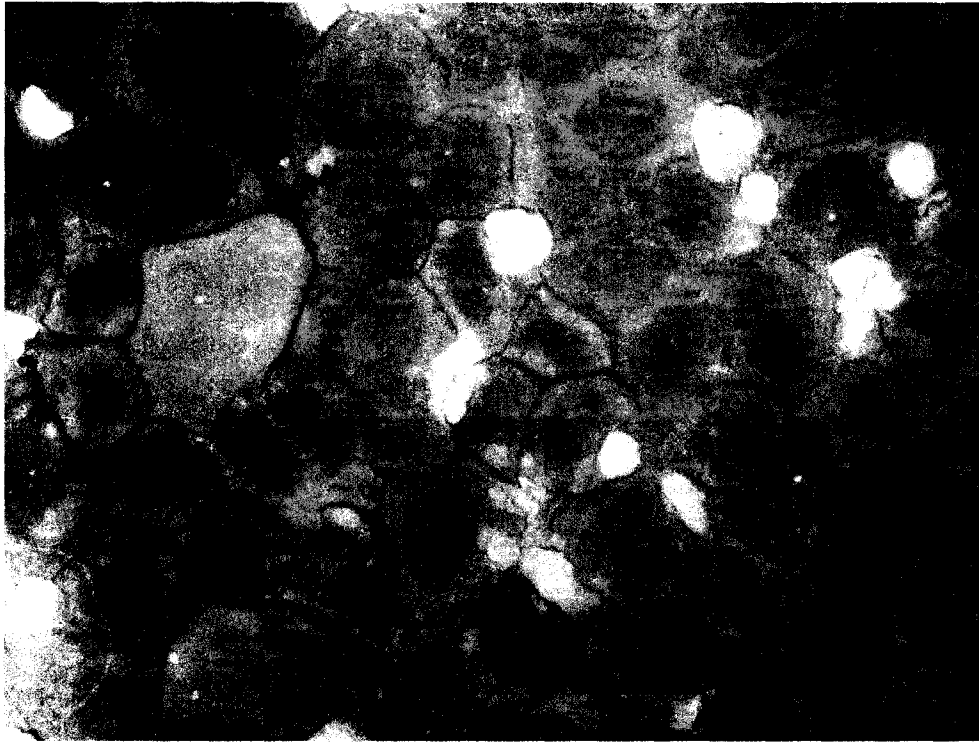


Helen Gainford
Rights Manager

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APPENDIX D

DETERMINATION OF TRANSFECTION EFFICIENCY



Fluorescent image of COS7 cells transfected with a pCMV5 RI-GFP expression vector to determine transfection efficiency using Fugene 6. Transfection efficiency was determined as a percent by the ratio of fluorescent cells counted under fluorescent light over total cells counted under white light in the same field (x1000 magnification, under oil emersion, on a Ziess Axioscope fluorescent microscope). A minimum of 200 cells/transfection experiment were counted. The transfection efficiency was determined to be 22 ± 5 % (average \pm standard deviation).

APPENDIX E

EQUATION TO DEFINE RATE-LIMITING STEP DIFFERENCE BETWEEN $C\alpha$ AND $C\gamma$

-----Original Message-----

From: Howard White [mailto:howard_white@hotmail.com]

Sent: Tuesday, June 14, 2005 3:54 AM

To: MorrisGZ@CHKD.COM

Subject: RE: rate limiting step for PKA

I must say that I didn't recall that we obtained a reasonably ok result with gamma. Assuming that was the case there could be a relationship between the ADP dissociation and steady state rates - but I need to reread the papers. Could you either send me a pdf or the reference to the work from the San Diego lab on mant kinetics? In general the following sort of mechanism would produce parallel dependences of ADP dissociation and steady state rates.



$$k_{\text{steadstate}} = K \times k\text{-ADP}$$

where $K = 0.1$ of some steps prior to ADP dissociation is unchanged and $k\text{-ADP}$ is the measured rate of ADP dissociation. There are other possibilities.

I hope this is of some help.

APPENDIX F

MEASURING STOKES RADII OF PURIFIED RECOMBINANT CATALYTIC SUBUNITS

W. Zhang, G. Z. Morris, S. J. Beebe (2004) *Protein Expression and Purification* (35)
pp.156-169.

The Stokes radii (R_s) and molecular weight (MW) for each purified recombinant catalytic subunit were measured using their elution constant (K_{avg}). The K_{avg} for each subunit was determined as follows: $K_{avg} = (V_e - V_o) / (V_t - V_o)$ where V_e is the elution volume of the protein, V_t is the total volume of the column, and V_o is the column void volume determined by the elution volume of blue dextran. A standard curve of $\sqrt{(-\log K_{avg})}$ vs. Stokes radii of the standard was used to calculate the Stokes radii of each catalytic subunit. The standards used to generate each curve were ferritin (440 kD, 61 Å), aldolase (158 kD 48.1 Å), albumin (67 kD, 35.5 Å), ovalbumin (43 kD, 30.5 Å), chymotrypsinogen (25 kD, 20.9 Å), myoglobin (20.2 kD, 20.2 Å), and cytochrome C (11.7 kD, 17 Å).

The correlation coefficient for the Stokes radii was 0.93. These parameters were determined from a Sephacryl S-300 (320 mL) gel filtration column (Amersham Bioscience). Four mL fractions were collected and analyzed by measuring OD@280 nm (Perkin Elmer Lambda 40 uv/vis spectrophotometer). Results were plotted as elution volume vs OD, and the peak elution for each standard was used as its V_e . Analysis of Co

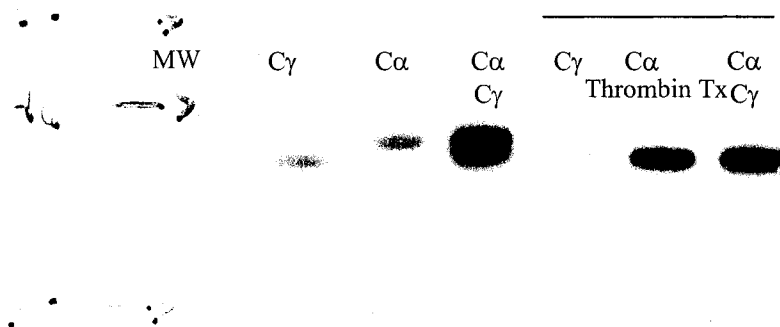
and $C\gamma$ was carried out during purification following the CM-Sepharose chromatography and from crude extract with no difference observed on the measured K_{avg} between the two sample forms. $C\alpha$ and $C\gamma$ fractions were tested for catalytic activity using a standard kinase assay. Total volume loaded onto column for each run was 1mL (~ 0.3% of total column volume).

The Stoke's radii for $C\alpha$ was measured at $27.5 \pm 0.4 \text{ \AA}$ and for $C\gamma$ at $24.2 \pm 0.8 \text{ \AA}$ (the p-value = 0.02).

APPENDIX G

THROMBIN DIGEST CHARACTERIZATION OF C α AND C γ

A thrombin digest of purified recombinant C α and C γ was done with 1ng/ μ L C-subunit and 1U of thrombin (Sigma) in a final volume of 50 μ L of KP10EDTA1 [6.9] and incubated at 37°C for one hour. The reaction was stopped by adding 50 μ L sample buffer and incubating at >90°C for 10 minutes. The samples were analyzed by running 5 ng of C-subunit from each reaction on a 9% acrylamide gel with 4% stacker, transferring to a PVDF membrane, and treating with a rabbit polyclonal antibody against the carboxyl terminal of C α (Santa Cruz Biotechnology) that cross-reacts with both isoforms. The membrane was incubated with ECL reagent (Amersham Biosciences), imaged by exposing film to the ECL treated membrane.



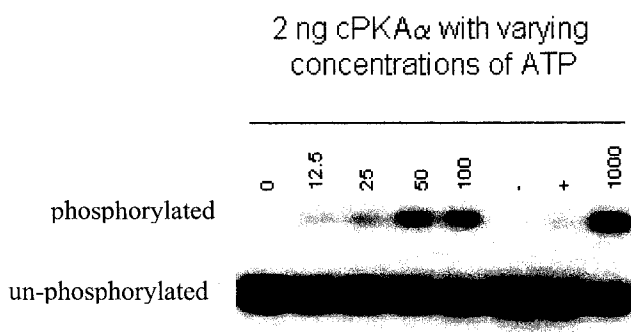
The figure shows that thrombin cleaves the his₁₀-tag off of C α , which contains an RGD thrombin cleavage sequence. But C γ disappears all together with thrombin treatment and it is not clear why, as the antibody recognizes the C-subunit carboxyl terminal and the C γ -thrombin cleavage site is on the amino terminus.

APPENDIX H

DETERMINING $C\alpha$ K_M FOR ATP USING PROMEGA'S NON-RADIOACTIVE KINASE ASSAY

The non-radioactive kinase assay was done according to manufacture's protocol (PepTag® Assay for Non-Radioactive Detection of Protein Kinase C or cAMP Dependent Protein Kinase catalogue # 5340). Briefly, 1 μ L of pure recombinant $C\alpha$ (2 μ g/ml) in PKA dilution buffer (350mM K₃PO₄ (pH 7.5) 0.1mM DTT) was mixed with 5 μ l PepTag® PKA 5X reaction buffer (100mM Tris-HCl (pH 7.4) 50mM MgCl₂), 5 μ l PepTag® A1Peptide, and 9 μ L water in a 0.5ml microcentrifuge tube and kept on ice until 5 μ l of the corresponding ATP concentration was added (0-100 μ M, final concentrations). The reaction was initiated by the addition of ATP and incubating at 30°C for 30 minutes.

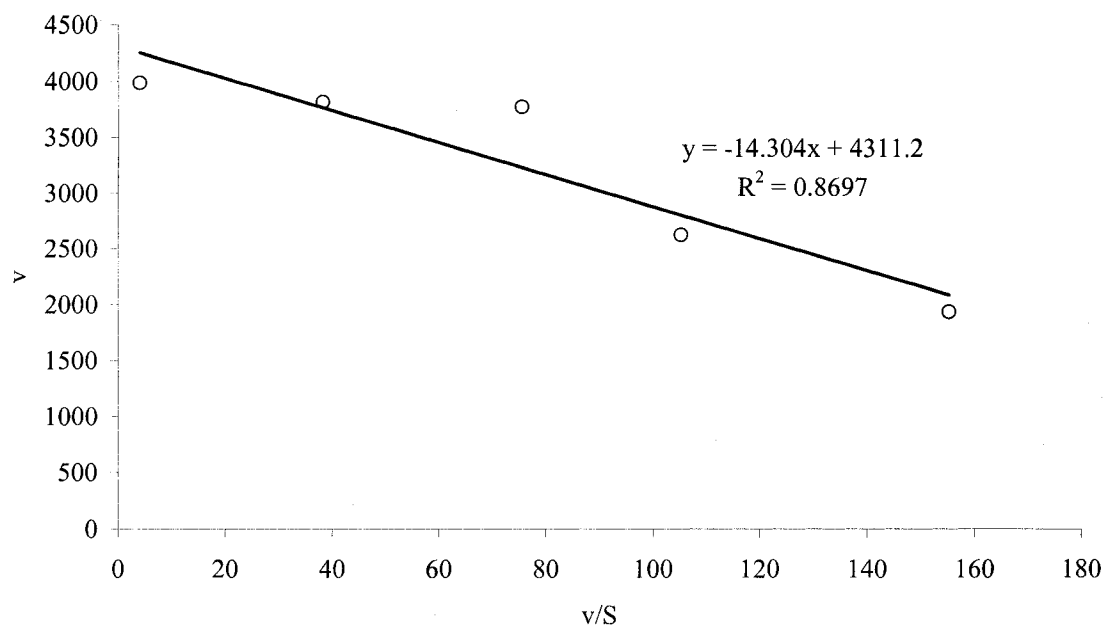
Incubating in a heating block at 95°C for 10 minutes terminated the reaction. Glycerol (1 μ l of 80%) was added to each sample following incubation. Samples were then loaded into a 1% agarose gel and



run at 25V until phosphorylated and non-phosphorylated PepTags were resolved in gel. The gel was imaged on a VersaDoc (BioRad) and the band intensity was determined using Quantity One software (BioRad). An Eadie-Hofstee plot was generated from this data using Band intensity (y-axis) vs. [ATP]/band. The slope of this curve represents the

measured $C\alpha$ K_m for ATP, which was determined here at 14 μ M. This measure is in good agreement with the published value (25 μ M, Zhang et al *Protein Expression and Purification* (2004) 35, 156-169).

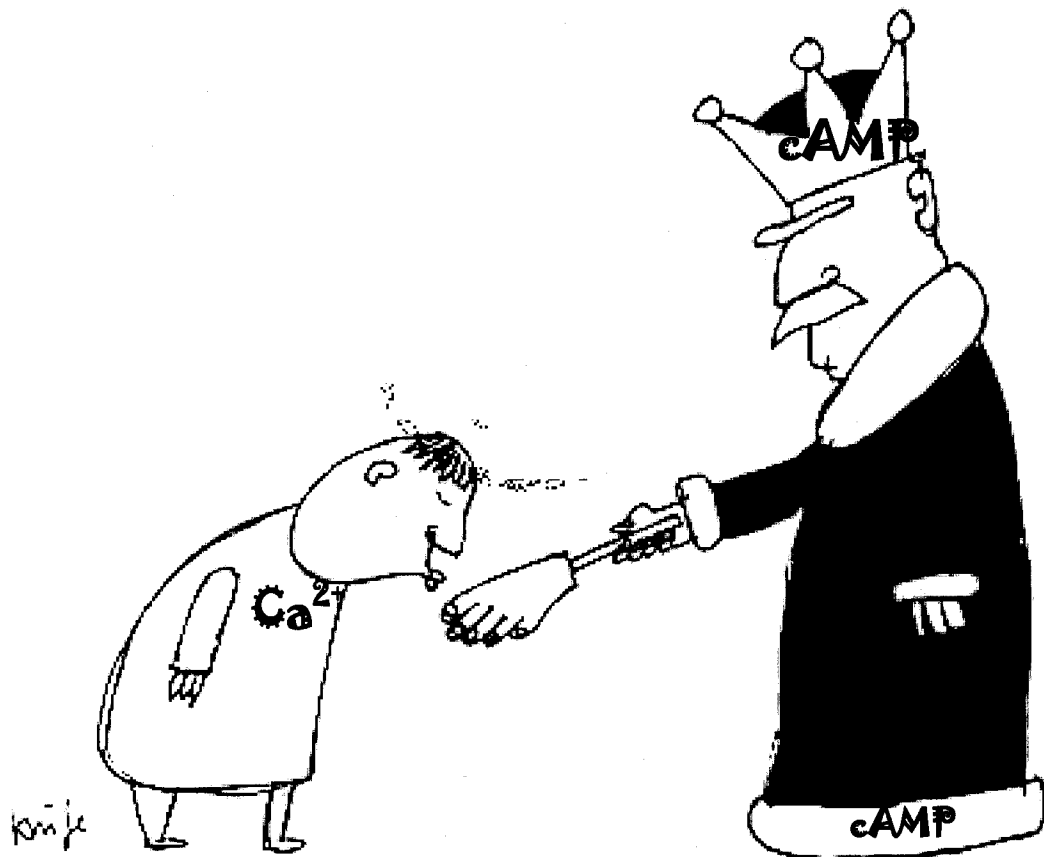
Eadie-Hofstee Plot for cPKA α with ATP, Based on Results from Non-radioactive Kinase Assay



APPENDIX I

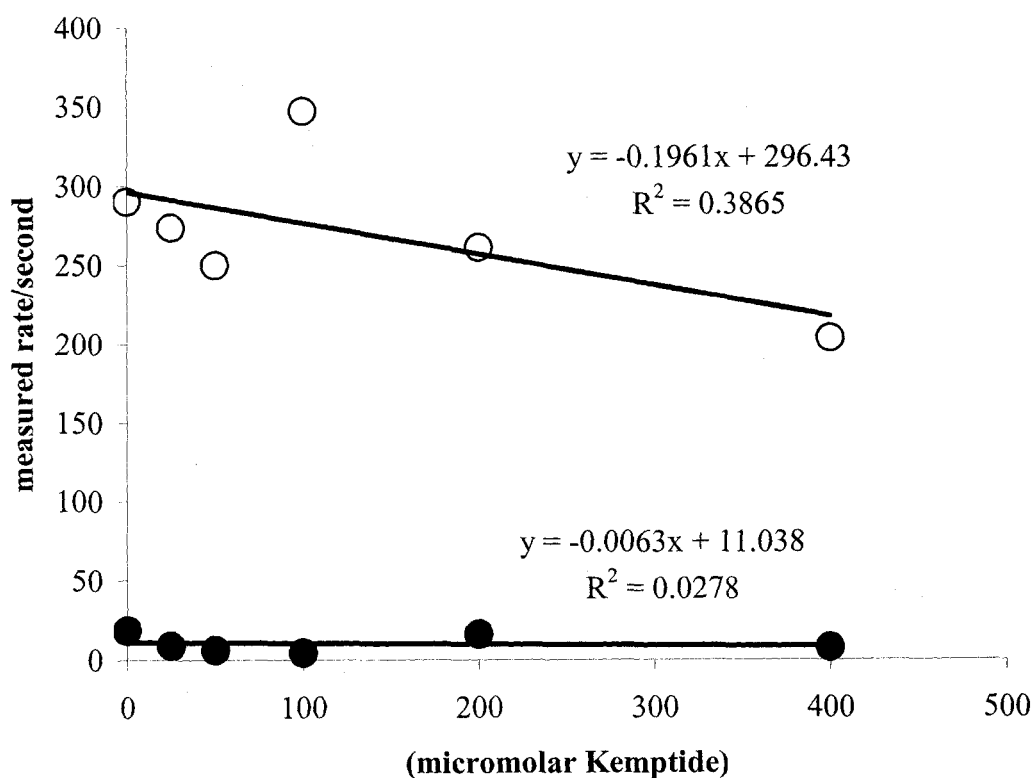
cAMP, KING OF SECOND MESSANGERS

You work with calcium? Take a bow and kiss it!



APPENDIX J

STOPPED-FLOW MEASURE OF $C\alpha$ -MANT-ATP ASSOCIATION RATE IN THE PRESENCE OF THE PHOSPHO-ACCEPTOR SUBSTRATE, KEMPTIDE

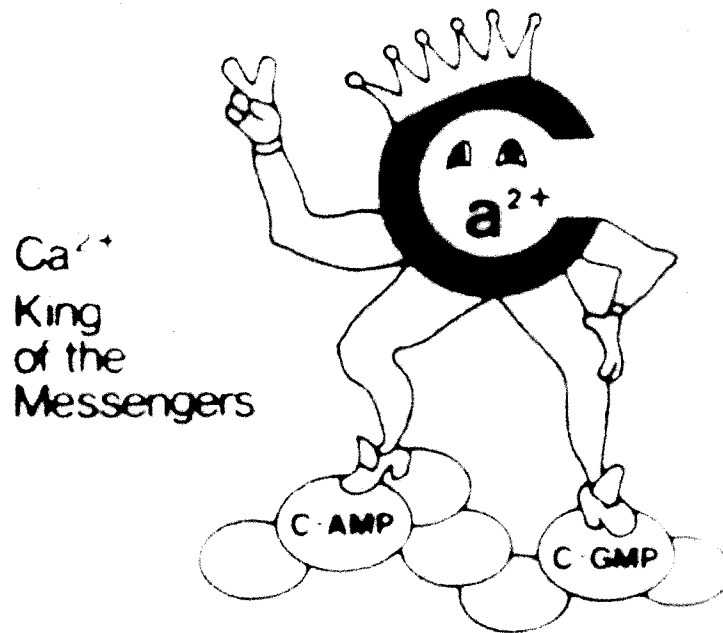


The mant-ATP on rate for $C\alpha$ was measured here at 25°C in the presence of Kemptide to determine whether the presence of the second substrate affected the observed binding rate of the first substrate. This was done by mixing 13 μM $C\alpha$ from one syringe with 100 μM mant-ATP mixed with varying concentrations of Kemptide (25-400 μM) in the other syringe. The slopes for the two rates measured at 25°C, k_1 (open circles) and k_2 , (closed circles) decreased very slightly with increasing amounts of Kemptide (k_1 Kemptide: $y = -$

$0.1961x + 296.43$, $R^2 = 0.3865$; $k2_{\text{Kemptide}}: y = -0.0063x + 11.038$, $R^2 = 0.0278$), but it is not clear if these slopes fall within the error measured for each rate. A similar trend is observed in the amplitudes for each rate ($\text{amplitude1}_{\text{Kemptide}}: y = -0.0001x - 0.1012$, $R^2 = 0.5346$; $\text{amplitude2}_{\text{Kemptide}}: y = -7 \times 10^{-6}x + 0.0123$, $R^2 = 0.085$). The correlation values for these curve fits are not very good, suggesting that there is little difference between the measured rates or amplitudes at each Kemptide concentration.

APPENDIX K

CALCIUM, KING OF SECOND MESSENGERS



VITA

GARY Z. MORRIS

Center for Pediatric Research, Eastern Virginia Medical School

855 West Brambleton Avenue, Norfolk VA 23510

Old Dominion University, Norfolk, VA 23529

EDUCATION

- 2000 Accepted into the Biomedical Science PhD program at Eastern Virginia Medical School-Old Dominion University. Preceptor- Dr. Stephen Beebe.
- 2000 **Masters in Chemistry**, OLD DOMINION UNIVERSITY (Norfolk, VA). Preceptor- R. L. Williams.
- 1999 **Bachelor of Science in Biochemistry**, OLD DOMINION UNIVERSITY (Norfolk, VA).
- 1998 **Summer Internship Program in Chemistry**, UNIVERSITY OF PUERTO RICO (Río Piedras, PR)- NSF Research Experience for Undergraduates. Mentor- Dr. Fernando González.

EMPLOYMENT

- 2000 **Research Assistant**, Center for Pediatric Research (Norfolk, VA).
- 2000 **Adjunct Faculty**, Tidewater Community College (Norfolk, VA.).
- 1999 **Research Assistant**, Enological Research Laboratory in the Department of Chemistry and Biochemistry, Old Dominion University, (Norfolk, VA).
- 1999 **Graduate Teaching Assistant**, Department of Chemistry and Biochemistry at Old Dominion University (Norfolk, VA.).

HONORS AND AWARDS

- 2004 FASEB Travel award to ASBMB meeting in Boston, MA.
- 2004 Renewal of APS Porter Physiology Fellowship, \$18,000 one-year stipend.
- 2003 *Merck Fellow*, Porter Physiology Development Committee
- 2003 Awarded APS Porter Physiology Fellowship, \$18,000 one-year stipend.
- 2000 Winner of the Third Annual Student Research Poster Session in Hampton Roads at Old Dominion University Norfolk, VA.
- 1999 Third place at the 78th Annual Meeting of the Virginia Academy of Science.

PUBLICATIONS

- 2004 Weiqing Zhang, **Gary Z. Morris**, Stephen J. Beebe. *Protein Expression and Purification*, Vol. 35, pp. 156-169 May 2004.
- 2002 Rana C. Morris, **Gary Z. Morris**, Weiqing Zhang, Merica Gellerman and Stephen J. Beebe. *Archives of Biochemistry and Biophysics*, Vol.403, pp. 219-28 July 2002
- 2002 **Gary Z. Morris**, Roy L. Williams, Mark S. Elliott and Stephen J. Beebe. *The Prostate*, Vol. 52, pp. 319-329 September 2002.
- 2001 **Gary Morris**, James Yuan, and Roy Williams. *Journal of Chromatographic Science*, Vol. 39, pp. 321-4 August 2001.