

## RESEARCH COMMUNICATION

**A dual-specificity isoform of the protein kinase inhibitor PKI produced by alternate gene splicing**Priyadarsini KUMAR<sup>1</sup> and Donal A. WALSH<sup>2</sup>

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We have previously shown that the protein kinase inhibitor  $\beta$  (PKI $\beta$ ) form of the cAMP-dependent protein kinase inhibitor exists in multiple isoforms, some of which are specific inhibitors of the cAMP-dependent protein kinase, whereas others also inhibit the cGMP-dependent enzyme [Kumar, Van Patten and Walsh (1997), *J. Biol. Chem.* **272**, 20011–20020]. We have now demonstrated that the switch from a cAMP-dependent protein kinase (PKA)-specific inhibitor to one with dual specificity arises as a consequence of alternate gene splicing. We have confirmed using bacterially produced pure protein that a single inhibitor species has dual specificity for both PKA and cGMP-

dependent protein kinase (PKG), inhibiting each with very high and closely similar inhibitory potencies. The gene splicing converted a protein with 70 amino acids into one of 109 amino acids, and did not change the inhibitory potency to PKA, but changed it from a protein that had no detectable PKG inhibitory activity to one that now inhibited PKG in the nanomolar range.

**Key words:** cyclic nucleotides, cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), signal transduction.

## INTRODUCTION

The cAMP-dependent protein kinase inhibitor (PKI) was first discovered simultaneously with the discovery of the kinase, and later the skeletal-muscle form was characterized in detail [1]. This form, PKI $\alpha$ , is a 75-amino-acid peptide. It is a totally specific inhibitor of the cAMP-dependent protein kinase (PKA) [2] and so far is only known to exist as a single molecular entity. The determinants for PKA binding of PKI $\alpha$  have been extensively characterized [3–5]. Subsequent experiments discovered a second species of PKI, PKI $\beta$ , that is coded by a gene distinct from that for PKI $\alpha$  [6,7]. PKI $\beta$ , as initially characterized, was a 70-amino-acid protein that was also an entirely specific inhibitor of PKA [6]. PKI $\beta$ -70 shares only a 41% amino acid identity with PKI $\alpha$ , but both contain identical PKA pseudo-substrate high-affinity binding sites. The mRNA sequence of PKI $\beta$  suggested that a 78-amino-acid PKI $\beta$  species might also exist as a consequence of alternate translation initiation. This form, PKI $\beta$ -78, was subsequently shown to occur in both the cerebellum and the testis [8]. PKI $\beta$ -78 is likewise a total specific inhibitor of PKA [8]. Subsequent experiments with antipeptide antibodies generated against the N-terminal and C-terminal sequences of PKI $\beta$ -70 demonstrated that at least four isoforms of PKI $\beta$  exist, each of which can also occur as both phosphorylated and dephosphorylated forms [8]. All forms were readily detected by the antibodies raised against the two terminal PKI $\beta$  sequences. Two of these forms were PKI $\beta$ -70 and PKI $\beta$ -78 (and their monophosphorylated derivatives). The two new forms were designated PKI $\beta$ -X and PKI $\beta$ -Y, in the absence of knowledge concerning their full molecular identity, although their reactivity to both the N-terminal and C-terminal antibodies clearly identified them as additional isoforms of PKI $\beta$ . PKI $\beta$ -X and PKI $\beta$ -Y comprise respectively 23% and 32% of total PKI $\beta$  in rat testis, and 29%

and 55% in rat cerebellum. Of particular note is the fact that both PKI $\beta$ -X and PKI $\beta$ -Y, unlike PKI $\beta$ -70 and PKI $\beta$ -78, inhibited not only PKA, but also the cGMP-dependent protein kinase (PKG), with quite similar  $K_i$  values. This latter finding of dual specificity is compatible with the very early observations of Szmigielski et al. [9], who reported a 'Type II' isoform of PKI that inhibited both PKA and PKG. We have demonstrated here that PKI $\beta$ -X, one of the dual-specificity inhibitors, is a product of the PKI $\beta$  gene that is created by alternate splicing. As a result of the data given in the present paper, PKI $\beta$ -X is now termed PKI $\beta$ -109.

## MATERIALS AND METHODS

Cloning of PKI $\beta$ -109

A <sup>32</sup>P-labelled probe, generated by PCR using two primers (oligonucleotides 5 and 6; see Table 1) and the PKI $\beta$ cDNA as template [6], was used to screen  $1 \times 10^6$  plaques of a Lambda ZAP II rat cerebellum cDNA library (Stratagene, La Jolla, CA, U.S.A.), according to the manufacturer's protocol. Of the plaques, 12 were identified as positive and were analysed further by PCR using two primers (oligonucleotides 7 and 8; Table 1) that, between them, spanned the entire coding region plus part of the upstream 5'-non-translated region. The predicted product size on the basis of the established  $\beta$ -cDNA sequence was 433 bp [6]. One of the positive plaques, however, gave a base-pair product of approx. 100 bp higher than this expected size. This form was purified further, and the pBluescriptSK(-) phagemid was excised from the Lambda ZAP II vector using a helper phage, as described by the manufacturer's protocol. The clone was subsequently sequenced by automated sequencing, identifying its product as a 109-amino-acid peptide.

Abbreviations used: PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; PKI, protein kinase inhibitor.

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**Table 1** Synthetic oligonucleotides

Identified no.	Sequence (all written 5' to 3')	Basis for sequence	Reference
1	GGAAATCCATATGGGAGGCGGCACGAGCCAGCCAGAAGCCAGCAAGATTCTGTGCATGAGGAC	PKI $\beta$ -2 clone (465–514) plus 5' <i>Nde</i> I site	[14]
2	GTCCTCATGACAGAATCTTGTGGTCTGGGCTCGTGCCGCCCTCCCATATGGAATTC	PKI $\beta$ -2 clone (514–465) plus 3' <i>Bsp</i> HI site	[14]
3	GCGTGTGCATGAGGACAGATTCATCGGA	PKI $\beta$ -78 expression vector (1–20), plus 5' <i>Bsp</i> HI site	[8]
4	GATCAGGGGATCCACCTGTAGCCAG	PKI $\beta$ -78 expression vector (134–109), plus 3' <i>Bam</i> HI site	[8]
5	TCTGTAATCAGCAGCTCCG	PKI $\beta$ clone (271–299)	[6]
6	TGTAGCCAGTGAACCTG	PKI $\beta$ clone (351–334)	[6]
7	GGCGCCGCAAGCGTCTCTAGCGCG	PKI $\beta$ clone (37–61)	[6]
8	TTATTTGTCTTCGTCTAGG	PKI $\beta$ clone (470–458)	[6]

### Expression of PKI $\beta$ -109

To generate an expression vector for PKI $\beta$ -109, the coding sequence of PKI $\beta$ -109 was amplified by PCR, with *Nde*I and *Bam*HI sites at the 5' and 3' ends respectively, and this was subcloned into the pET5a vector. The expression vector thus generated was transfected into the BL21(DE3) Gold-pLysS strain of *Escherichia coli*, glycerol stocks were made in Luria–Bertani broth containing 100  $\mu$ g/ml carbanecillin, and proteins were expressed as described by Studier et al. [10]. Briefly, a 1:1000 dilution of the glycerol stock was made in SOC medium (5 g of bacto-yeast extract/20 g of bacto-tryptone/2 ml of 5 M NaCl/0.19 g of KCl/10 ml of 1 M MgCl<sub>2</sub>/10 ml of 1 M MgSO<sub>4</sub>/3.6 g of D-glucose) containing 100  $\mu$ g/ml carbanecillin and the culture was grown to an absorbance of 0.8. It was then induced with 0.4 mM isopropyl  $\beta$ -D-thiogalactoside, and the cells were grown for an additional 12 h at 30 °C. The cells were harvested, lysed, and the protein was then purified using DEAE and gel-filtration column chromatography, as described previously [8]. The composition of the protein was confirmed by amino acid analysis, and the protein was chromatographed on a one-dimensional SDS/16% polyacrylamide gel (Novex, San Diego, CA, U.S.A.) as described by Laemmli [11]. Coomassie Blue staining and Western-blot analyses were performed as described in [8] using the PKI $\beta$ (5–22) antibody. PKA inhibitory activity assays were performed as described previously [2], using Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) as substrate and PKA catalytic subunit as obtained from bacterial expression [12]. PKG inhibitory activity assays were undertaken as described previously [2,13] using [Ala<sup>34</sup>]H2B-(29–35) as substrate. PKG-1 $\alpha$  was kindly provided by Dr Sharon Francis (Department of Molecular Physiology and Biophysics, Vanderbilt University Nashville, TN, U.S.A.).

### Expression and purification of 'PKI $\beta$ -2'

A 92-amino-acid species of PKI, termed PKI $\beta$ -2, has been suggested previously by Scarpetta et al. [14], on the basis of an identified cDNA sequence. Based on this DNA sequence, this form would be identical with PKI $\beta$ -78, except that it would contain a 14-residue N-terminal extension. The cDNA sequence corresponding to the first 45 bp of the coding region of the proposed mouse PKI $\beta$ -2 species [14] was synthesized as single-stranded sense and antisense oligonucleotides (oligonucleotides 1 and 2; Table 1), with an *Nde*I site at the 5' end. A *Bsp*HI site was introduced at the 3' end by changing the base at position 42 from G to C. These oligonucleotides were made double-stranded by incubating at 65 °C in TEN buffer [10 mM Tris/HCl (pH 7.5)/1 mM EDTA/50 mM NaCl] for 5 min and at room temperature for 2 h. The PKI $\beta$ -78 sequence was amplified with oligonucleotides 3 and 4 (Table 1) with *Bsp*HI and *Bam*HI sites at the 5' and 3' ends respectively, and PKI $\beta$ -78 expression vector as template [8]. These two pieces of DNA were restriction-

digested with *Bsp*HI, ligated, and gel-purified. The 199 bp product was then digested with *Nde*I and *Bam*HI before ligating to an *Nde*I–*Bam*HI double-digested PKI $\beta$ -78 expression vector [8]. The colonies were screened by PCR using two sets of primers within the coding region of PKI $\beta$ -78. Seven positive clones were obtained. One of the positive colonies was used for expression of the protein using the procedures described previously for PKI $\beta$ -70 and PKI $\beta$ -78, and purified to homogeneity using the same chromatographic steps as used for PKI $\beta$ -70 and PKI $\beta$ -78, except that a Sephacryl S-100 gel-filtration column was used, equilibrated in 10 mM Mes/200 mM NaCl buffer, pH 6.8 [8]. The purified protein was checked for homogeneity using Coomassie Blue staining, amino-acid analysis and sequencing of the first 18 N-terminal amino acids.

### RESULTS

As a strategy to determine the identity of the higher-molecular-mass PKI $\beta$ -X and PKI $\beta$ -Y isoforms, a rat cerebellum library was screened with a probe from within the coding region of PKI $\beta$ -70, and then, by using multiple primer combinations from within non-translated and the coding regions of the PKI $\beta$ -70 cDNA, the positive clones from the initial screening were screened by PCR. This led to the isolation of a clone that gave a higher base-pair product than anticipated from the PKI $\beta$ -70 cDNA sequence. This clone was identical in sequence for both the coding and non-coding regions with the cloned cDNA first identified for PKI $\beta$ -70 [6], except that it contained an insert of 105 bp (Figure 1; denoted by single underlining). The insert occurs 8 bp upstream of the ATG initiation site for PKI $\beta$ -78, and it included the insertion of a further upstream ATG that would result in a predicted protein of 109 amino acids. The 109-amino-acid sequence contained within it the full sequence of PKI $\beta$ -70 (Figure 1). This new ATG has a strong Kozak consensus sequence of GCC<sup>-3</sup>ACCATGG<sup>+4</sup>, which would promote efficient translation. The clone was characterized further by subcloning into an *E. coli* expression vector, and subsequent expression and purification of the protein in *E. coli*. SDS/PAGE and Western-blot analyses using the PKI $\beta$ (5–22) antibody of this expressed protein confirmed the identity of PKI $\beta$ -X as PKI $\beta$ -109 (Figures 2a and 2b).

This expressed PKI $\beta$ -109 protein was tested as an inhibitor of the PKA and PKG (Figure 2c). As had been previously observed [8] with the PKI $\beta$ -X protein isolated from rat cerebellum, PKI $\beta$ -109 was found to be a high-affinity inhibitor of not only PKA, but also PKG. Unlike the situation with PKA, however, PKI $\beta$ -109 inhibits PKG only to 50% of its control activity. The concentration of PKI $\beta$ -109 that resulted in its half-maximal effect with both enzymes was very similar (PKG, 4 nM; PKA, 3.5 nM). PKI $\beta$ -109 was thus a highly potent inhibitor of PKG, and was 10000-fold more potent than any other peptide inhibitor

5' GGCACGAGGCAGCTCGGTGCCGAGAGGGCCGGTGGCAGGCGGCACCGCGAGGGACCTGGCCCCGGCGC 68  
 CGCAAGCGTCTCTAGCGCGCAGCTGGGGTTCATGATATACTGGAAACCACACAAGATTAGTCTGGGGAG 136  
 CAGAAACCATAAAAGCCCCCTGAGACTAGGTTCGGAGAGGAAAGATAAAGGAAAGACCCCCCTCAGCTT 204  
 CACCCCTTACCTGTCTCAGAAACAACAGCCCATTTTAACCACAGATTTACCTGGGCCACAGGCCACC 272

ATGGATGTGCACCCTGATGCCCTGGGGGTCAAAGAAAAACAGTGGAGAACGGCATGGGAGCGGC 338  
M D V H P D A P G G Q K K T V E N G M G G G  
 1 10 20

AGCCAGGAGCCAGCAAGACTCTGTGATGAGGACAGATTTCATCGGAGATGACTGATGTGGAATCT 404  
S P G A Q Q E S V M R T D S S E M T D V E S  
 30 40

GTAATCAGCAGCTTCGCGTCTTCAGCAAGGGCGGGCCGCGCAATGCCTTACCCGACATCCAGAGT 470  
 V I S S F A S S A R A G R R N A L P D I Q S  
 50 60

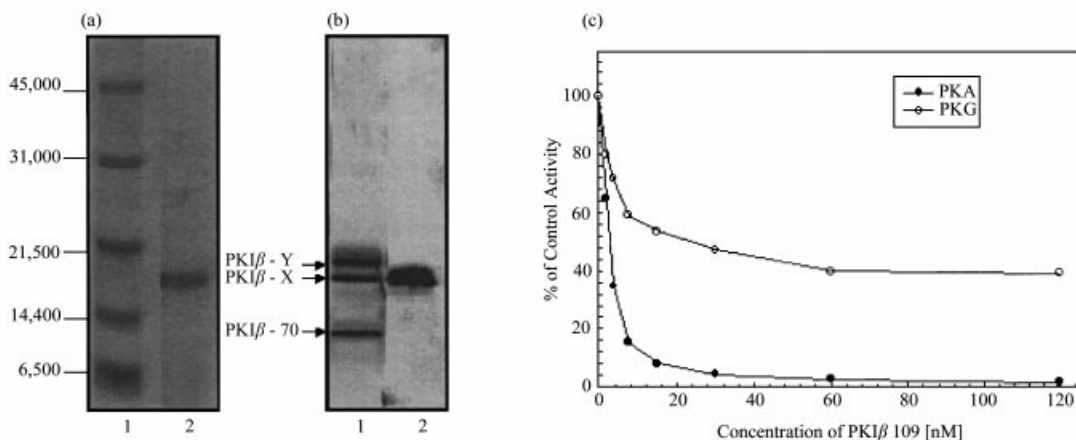
TCACTGGCTACAGGTGGATCCCCTGATCTTGCAGTGAAGCTGGAGGCATTGGCTGTGAAGGAAGAT 536  
 S L A T G G S P D L A L K L E A L A V K E D  
 70 80

GCAAAAATGAAGAATGAAGAGAAAGACCAAGGCCAACCAAAAAAGCCCCCTAGACGAAGACAAAATAA 602  
 A K M K N E E K D Q G Q P K K P L D E D K  
 90 100 109

AGGCTTAGAATTTATCAAGGGTGTGATTTTATGCACATTGAGAGACATAGCTATGCATTCTGGGGTG 671  
 TTTGATCTGGTAGTAACCATGGTAACCCATGGTAACCTCTGTGTGTGTTTGTGTGTGTTTTCAGT 740  
 AATTTGTATGATGCTATCCTTAAACTGCAGCTATGATACTGAAATAGAATATTTAAAAGTTATTCCTT 809  
 ACAAAATGCACCCAAGGAAAGGACACACTGTTTATATCCCAACAATATCCTATCTGTCTATCTCGGGAT 878  
 TTACTTAGTCAGATATATGTTTAAATCTGTCTTAGCAAGTTAGAACCCTTTTCTTGGTATGTATAAATG 947  
 GATACCTATTAATTTAATACTTTGTAGAATTTGTAGAAAATPATATTTTGGTGAGATAGAATTAGTAG 1016  
 TAAAAAATCACTCTTATAAAATGTGTTTATTTTTCATTTAAAATTCACCATATTTTCTGCAAAAATAT 1085  
 TAGGAGAGATCTGGAAATATTTGTAAGTTGTAACAACCTTAAACATCTCTATGATGTTTTTGTCTAATG 1154  
 TATTTTGCATCTAGTCTGGATGTATTTGTGATTTTTTAAATATGGACTCACATAACATAAAATTTGGGACA 1223  
 AACTTGCTTAAAAGCTCGTGCC 3' 1245

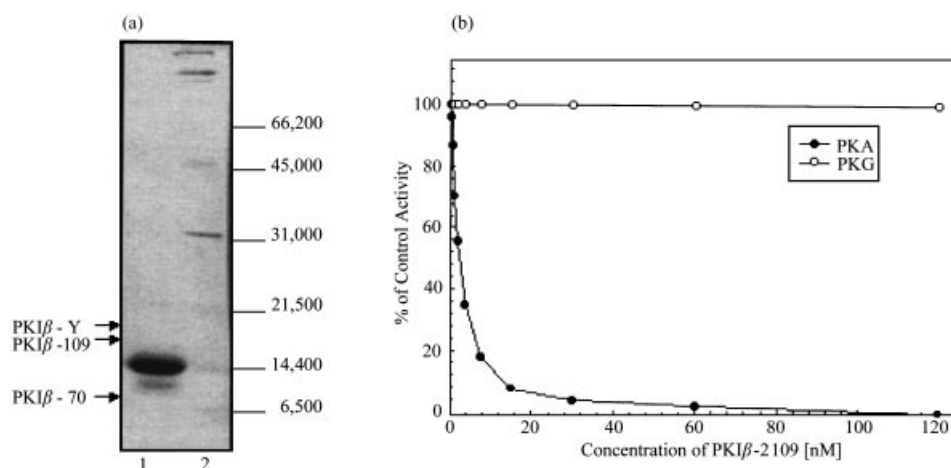
**Figure 1** Nucleotide sequence of the cloned cDNA for PKI $\beta$ -109

With Genbank® accession no. A413572, the 1245 bp nucleotide sequence of the cloned cDNA is shown along with the predicted amino acid sequence of a 109-amino-acid open reading frame corresponding to PKI $\beta$ -X. The 105 bp insert due to alternate splicing is underlined. The amino acids that had been added on to the N-terminal end of PKI $\beta$ -70 by alternate splicing are denoted by double underlining.



**Figure 2** Characterization of PKI $\beta$ -109

(a) Coomassie Blue-stained gel of purified expressed PKI $\beta$ -109: lane 1, molecular-mass standards; lane 2, purified PKI $\beta$ -109. (b) Western blot using PKI $\beta$ (5–22) antibody: lane 1, heat-treated 60-day-old rat testis extract; lane 2, bacterially expressed PKI $\beta$ -109 protein. (c) Titration of PKA and PKG with PKI $\beta$ -109 protein. Phosphotransferase assays of PKA (●; 6.4 nM PKA catalytic subunit with Kempide as substrate) and PKG (○; 1.6 nM enzyme with [Ala<sup>34</sup>]H2B-(29–35) as substrate) were performed in the presence of the indicated concentrations of PKI $\beta$ -109 diluted in 0.5 mg/ml BSA in Mes, pH 6.8. The assays were performed as described in the Materials and methods section.



**Figure 3** Purification and characterization of PKI $\beta$ -2

(a) Coomassie Blue-stained gel of purified expressed PKI $\beta$ -2: lane 1, purified PKI $\beta$ -2; lane 2, molecular-mass standards. (b) Titration of PKA and PKG with PKI $\beta$ -2 protein. Phosphotransferase assays of PKA (●) and PKG (○). The assays were performed as described in the Materials and methods section, and in the legend for Figure 2.

that has been investigated [2]. The reason why PKI $\beta$ -109 inhibits PKG to only 50 % of the control activity remains to be elucidated. It is noteworthy that, unlike PKA, the cyclic-nucleotide-dependent activation of PKG does not involve the separation of the two catalytic subunits. Inhibition to only 50 % of the control activity might thus be accounted for by PKI $\beta$ -109, for some reason yet to be discerned, only inhibiting one of the two catalytic sites in the dimeric protein. This set of experiments demonstrates that, by the process of alternate splicing, the PKI $\beta$  gene can direct a product that is either a specific inhibitor of PKA or is a dual inhibitor of both PKA and PKG. Remarkably, the difference between PKI $\beta$ -109 and PKI $\beta$ -78 of 31 residues results in a change from a protein that has no detectable PKG inhibitory activity to one that inhibits PKG in the nanomolar range.

In a previous report, Scarpetta and Uhler [14] identified a cDNA which, if translated, would give rise to another possible isoform of PKI $\beta$  of 92 amino acids, which was termed PKI $\beta$ -2. To evaluate whether PKI $\beta$ -2 was either PKI $\beta$ -X or PKI $\beta$ -Y, a PKI $\beta$ -2 expression vector was created that included the 42 bp extension corresponding to the mouse PKI $\beta$ -2 clone [14] at the N-terminus of the PKI $\beta$ -78 expression vector. The purified protein obtained by *E. coli* expression migrated at a molecular mass of 14.4 kDa, lower than the observed molecular masses of PKI $\beta$ -109 and PKI $\beta$ -Y (Figure 3a). The expressed PKI $\beta$ -2 inhibited PKA, but not PKG (Figure 3b). On the basis of its size and inhibitory activity, PKI $\beta$ -2 is clearly neither PKI $\beta$ -109 (PKI $\beta$ -X) nor PKI $\beta$ -Y. Given its sequence, PKI $\beta$ -2 would be expected to react with our panel of PKI $\beta$  antibodies [8]; however, in none of our past tissue-distribution studies has a form of PKI $\beta$  been observed that would have co-migrated with PKI $\beta$ -2.

## DISCUSSION

PKA and PKG share extensive overall homology in both their catalytic and regulatory domains, have similar catalytic mechanisms and phosphorylate many of the same proteins (*in vitro*) ([15–17]; also see the website [http://www.sdsc.edu/Kinases/pkr/pk\\_catalytic/pk\\_hanks\\_seq\\_align\\_long.html](http://www.sdsc.edu/Kinases/pkr/pk_catalytic/pk_hanks_seq_align_long.html)). Within their

catalytic cores, many of the residues that determine protein substrate binding are identical, or closely similar. PKI inhibits PKA by acting as a pseudo-substrate, binding to many of the same residues in the catalytic core that bind the protein substrates [3–5,18]. Intact PKI $\alpha$ , PKI $\beta$ -70 and PKI $\beta$ -78, however, specifically inhibit PKA, and do not inhibit PKG, even at very high concentrations [2,6,8]. The high affinity of PKI for PKA is clearly a consequence of its pseudo-substrate binding to the catalytic site. Low-molecular-mass peptides derived from PKI $\alpha$ , however, have been shown to inhibit not only PKA, but also to a lesser but still significant extent, PKG [2]. This is not surprising, given the high degree of identity that exists in the core residues of PKA and PKG that dictate protein substrate specificity. It is also, therefore, not surprising that a form of PKI might exist that would inhibit both PKA and PKG, as is now evident for PKI $\beta$ -109. Presumably, PKI $\beta$ -109, because of its additional N-terminal residues, adopts some differences from PKI $\alpha$ , PKI $\beta$ -70 and PKI $\beta$ -78 in terms of its structure that allows it to bind as a pseudo-substrate within the catalytic site of PKG. What is of particular note, with our new findings, is that forms of PKI can be created by alternate gene splicing that are either specific inhibitors of PKA or dual-specificity inhibitors of both PKA and PKG, and, furthermore, these different forms differ not within the pseudo-substrate domain, but elsewhere in the protein. Some features of the sequences and/or structures of the forms of PKI, in addition to their pseudo-substrate domain, must contribute to the high selectivity that they can display towards either PKA or PKG.

To date, the precise physiologically distinct roles of PKA and PKG have yet to be clearly delineated, although clearly they are activated in response to two unique sets of signal messenger cascades. The physiological role of PKI $\beta$ -109, and the factors that dictate the alternate splicing of the PKI $\beta$  gene to produce it rather than PKI $\beta$ -70 or PKI $\beta$ -78, await elucidation. The PKI $\beta$  species are by far the predominant form of PKI in germ cells, and are about equal in concentration to PKI $\alpha$  in cerebellum. PKI $\beta$ -109, in addition to the second dual-specificity PKI $\beta$  form, PKI $\beta$ -Y, the structural identity of which has yet to be determined, constitute more than 50 % of the PKI $\beta$  forms that exist

in both germ cells and cerebellum. Their extensive presence and dual specificity adds yet another level of regulatory complexity to the already-complex signal-transduction pathways. The identity of PKI $\beta$ -Y awaits elucidation, and despite extensive screen efforts no additional gene products have been identified; thus it is likely that PKI $\beta$ -Y contains the same amino acid sequence as PKI $\beta$ -109. The larger molecular mass of PKI $\beta$ -Y may be a consequence of some covalent modification.

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Received 14 December 2001/14 January 2002; accepted 18 January 2002