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Biochemistry

Molecular cloning of a rat testis form of the inhibitor protein of cAMP-dependent protein kinase

(protein kinase inhibitor/cyclic AMP)

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ABSTRACT **The form of inhibitor protein of the cAMP-dependent protein kinase (PKI) that has been most thoroughly studied is a protein purified from rabbit skeletal muscle. Beale *et al.* previously isolated a species of PKI from rat testis that appeared from its amino acid composition to be quite distinct from the rabbit skeletal muscle protein [Beale, E. G., Dedman, J. R. & Means, A. R. (1977) *J. Biol. Chem.* 252, 6322–6327]. The amino acid sequence of a form of rat testis PKI has now been determined both by sequencing overlapping peptide fragments for 95% of the protein and by the isolation of a cDNA clone containing the coding region for the 70-amino acid protein. The sequence of the 70-amino acid testis PKI displays a maximum of only 41% sequence identity with the previously sequenced 75-amino acid rabbit skeletal muscle PKI. However, the two forms have identical potency as inhibitors and the key amino acids of the pseudosubstrate site, shown to be critical for maximal inhibition with the rabbit skeletal muscle PKI, have been conserved in the testis protein. The rabbit skeletal muscle and rat testis PKIs most likely represent distinct isoforms. The nucleotide sequence of the rat testis PKI cDNA suggests that a second form of testis PKI, longer by 8 additional amino-terminal amino acids, might also be produced.**

The cAMP-dependent protein kinase, the prime target of the second messenger cAMP in mammalian cells, is activated by the binding of cAMP to the kinase regulatory subunit, releasing the active catalytic subunit. The heat-stable protein inhibitor of this protein kinase (PKI) acts by binding with high affinity to the substrate binding site of free active catalytic subunit (1). The protein from rabbit skeletal muscle (PKI_{sk}) has been extensively characterized and the central pseudosubstrate amino acid residues have been identified within its 75-amino acid sequence (1, 2). Previous studies identified multiple species of PKI in rat testis (3), and one of these was purified to apparent homogeneity and partially characterized (3, 4). We describe here the cloning of a cDNA containing the coding region of a form of rat testis PKI (PKI_{test}) that shares only minimal amino acid sequence homology with the well-characterized PKI_{sk} isoform, although it has retained the essential pseudosubstrate kinase-binding domain and the purified protein is equally potent as an inhibitor.*

EXPERIMENTAL PROCEDURES

Purification of Inhibitor Protein from Rat Testis. Rat testis PKI was purified from 1.26 kg of frozen rat testis as described by Beale *et al.* (3) up to chromatography on DEAE-cellulose. The ammonium sulfate precipitate was dialyzed overnight against 5 mM potassium phosphate/1 mM EDTA, pH 7.0, adjusted to pH 5.0, centrifuged, and then chromatographed on a DEAE-cellulose column (1.5 × 28 cm), using a 5 mM sodium acetate buffer (pH 5.0) with a 1000-ml linear gradient of 5–350 mM sodium acetate (Fig. 1*A*). The fractions of the first peak of inhibitory activity were pooled, dialyzed against 10 mM Mes (pH 6.8), and lyophilized. Chromatography on a Sephadex G-100 superfine column (2.5 × 90 cm; 10 mM Mes/50 mM NaCl, pH 6.8) eluted >90% of the inhibitory activity as a single peak, separated from significant amounts of higher molecular weight material. The lyophilized protein was then subjected to serial μBondapak (Waters) C_{18} reverse-phase HPLC (3.9 × 30 mm), first in 10 mM ammonium acetate buffer (pH 7) with a linear 8–64% acetonitrile gradient, the second in 0.1% trifluoroacetic acid with a linear 24–56% acetonitrile gradient (Fig. 2). This source material was homogeneous by gel electrophoresis (Fig. 3).

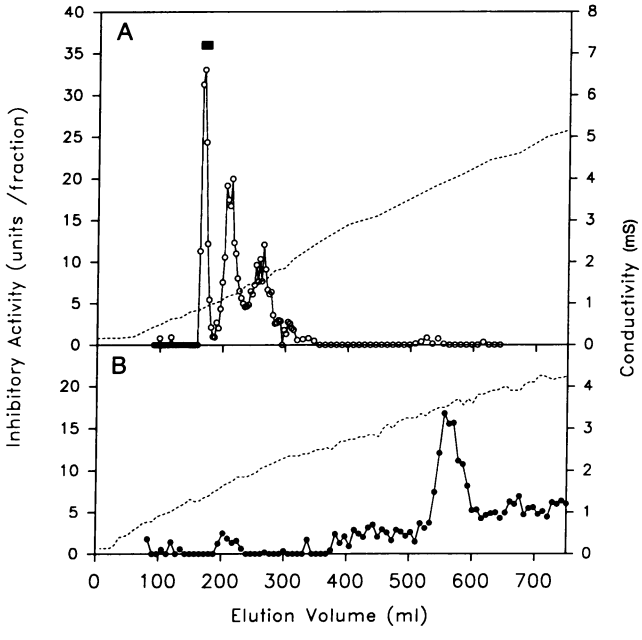

FIG. 1. DEAE-cellulose column chromatography of PKI_{test} and PKI_{sk}. Rat testis (1.26 kg) (*A*) and rabbit skeletal muscle (250 g) (*B*) were the tissue sources (see *Experimental Procedures*). The tissues were treated in an identical manner. The horizontal bar in *A* reflects fractions of PKI_{test} that were pooled for further purification.

Proteolytic Digestions. For the three conditions of digestion, 30 μg of PKI_{test} was incubated with either 16.7 units of Arg-C protease [0.1 M ammonium acetate (pH 8.5), 3 hr at 30°C], 1.5 μg of Glu-C protease [25 mM ammonium acetate (pH 4.0), 18 hr at 25°C], or CNBr (10 mg/ml in 70% formic acid, 16 hr at 20°C). The peptide mixtures generated by these

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Abbreviations: PKI, inhibitor protein of cAMP-dependent protein kinase; PKI_{sk}, rabbit skeletal muscle PKI; PKI_{test}, rat testis PKI.
*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M64092).

procedures were purified by reverse-phase HPLC using an acetonitrile gradient in 0.1% trifluoroacetic acid. Arg-C and Glu-C proteases were from Boehringer Mannheim.

Isolation of PKI_{test} cDNA. A segment of the PKI_{test} cDNA was amplified from a rat testis cDNA library (Stratagene, no. 936503) by polymerase chain reaction (PCR) using upstream and downstream oligodeoxynucleotide primers: 5'-AAYG-CIYTICCIGAYATHCA-3' and 5'-GGAATTCCCCYTGRT-CYTTYTCYTCRTTYTTCAT-3' (where I is deoxyinosine; H = T, C, or A). These primers correspond to Asn^{20} through Gln^{26}, and to Met^{52} through Gly^{60} plus an added *Eco*RI site, respectively. The predicted 130-base-pair (bp) PCR product was isolated as the ethidium bromide-staining band in a 3% NuSieve/1% SeaKem agarose minigel and confirmed by DNA sequencing (6). A 70-bp exact sequence probe from the center of the 130-bp PCR product was used to screen the rat testis cDNA library at high stringency (50% formamide/50 mM sodium phosphate, pH 7.4/75 mM sodium chloride/5 mM EDTA/0.08% Ficoll 400/0.08% polyvinylpyrrolidone/0.08% bovine serum albumin/0.1% SDS at 42°C). Forty-two positives were detected from a screening of 1.5×10^6 plaques. The largest clone explored was 1.35 kbp by agarose gel electrophoresis and 1364 bp by direct sequencing. The entire sense strand and 80% of the antisense strand (including the entire coding region) were sequenced. Over 80% of this sequence was confirmed from two other positive clones.

Other Methods. The preparation of PKI_{sk} and its assay were as described previously (1, 7), except as noted in Fig. 1*B*. Amino acid analyses were performed on samples hydrolyzed for 24 and 48 hr. The products of peptide synthesis were characterized by amino acid composition and mass spectrometry.

RESULTS

Isolation of PKI_{test}. At least three forms of PKI_{test} were separated by anion-exchange chromatography on DEAE-cellulose (Fig. 1*A*). These forms were eluted at a considerably lower conductivity than rabbit skeletal muscle PKI (Fig. 1*B*). These differences in elution fully conform with past data for both proteins (3, 8). The form of PKI_{test} that eluted at the lowest conductivity was purified to homogeneity as described in *Experimental Procedures*. The purified protein migrated as a single 10.5-kDa protein in SDS/polyacrylamide gel (Fig. 3) and did not comigrate with PKI_{sk}. The amino acid composition of this purified PKI_{test} (Table 1) was significantly different from that of the previously sequenced PKI_{sk}, with proline and methionine present in PKI_{test} and histidine and tyrosine absent. This form of PKI_{test} was also notably different from the rat testis inhibitor protein previously isolated by Beale *et al.* (3), but it is not known which of the forms separated by DEAE (Fig. 1*A*) was purified in their studies.

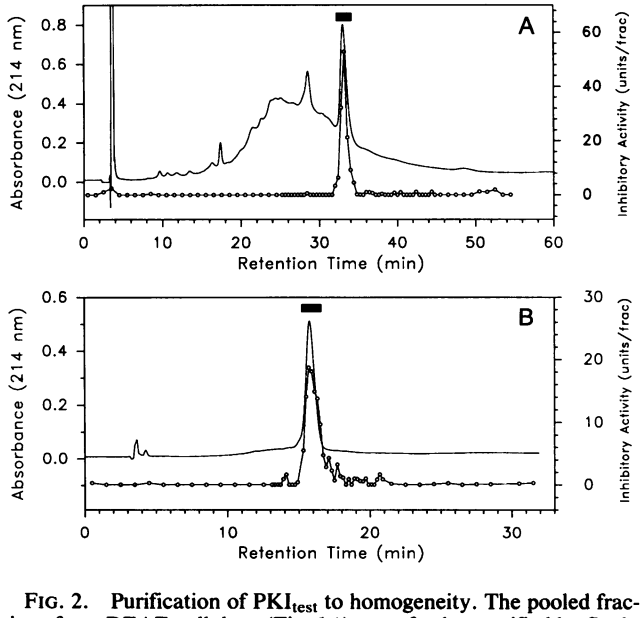

FIG. 2. Purification of PKI_{test} to homogeneity. The pooled fractions from DEAE-cellulose (Fig. 1*A*) were further purified by Sephadex G-100 gel filtration followed by reverse-phase HPLC first with ammonium acetate at pH 7 (*A*) and then with trifluoroacetic acid (*B*). Conditions are described in *Experimental Procedures*. Solid lines, absorbance; open circles, inhibitory activity. Pooling of fractions from each column is indicated by the horizontal bar.

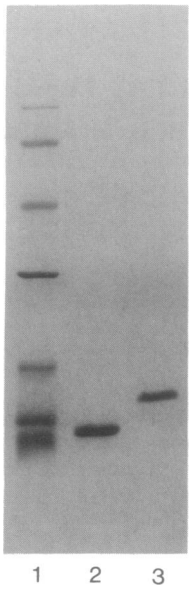

FIG. 3. SDS/polyacrylamide gel electrophoresis of purified PKI_{test}. Samples were electrophoresed in an SDS/10–15% polyacrylamide gradient gel by the method of Laemmli (5) and stained with Coomassie brilliant blue R-250. Lane 1, molecular weight standards (in order of increasing mobility: phosphorylase, 97,400; bovine serum albumin, 68,000; ovalbumin, 42,690; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 22,000; lysozyme, 14,400; and aprotinin, 6500); lane 2, purified PKI_{test} (2 μg); lane 3, purified PKI_{sk} (2 μg).

Amino Acid and Nucleotide Sequence of PKI_{test}. The purified PKI_{test} had a blocked amino terminus. Portions of the intact

Table 1. Amino acid composition data for various forms of PKI

Amino acid	No. of residues: Rat testis*	Rat testis†	Rabbit muscle‡	Rat testis§
Ala	9.0	9	10	19
Arg	3.1	3	4	8
Asn	8.8	2	4	19
Asp		7	5	
Gln	9.0	3	3	24
Glu		6	9	
Gly	4.4	4	7	16
His	0	0	1	2
Ile	1.8	2	4	3
Leu	7.0	7	5	9
Lys	8.1	8	3	9
Met	0.8	1	0	1
Phe	1.0	1	1	5
Pro	4.0	4	0	24
Ser	7.4	8	10	23
Thr	2.0	2	6	9
Trp	0	0	0	3
Tyr	0	0	1	0
Val	2.8	3	2	14
Total		70	75	158

*From amino acid composition analysis of the testis protein purified in this study.
†From translation of the cloned cDNA sequence.
‡From amino acid sequence data for the purified protein from rabbit skeletal muscle (2).
§From amino acid composition analysis of the testis protein purified by Beale *et al.* (3).

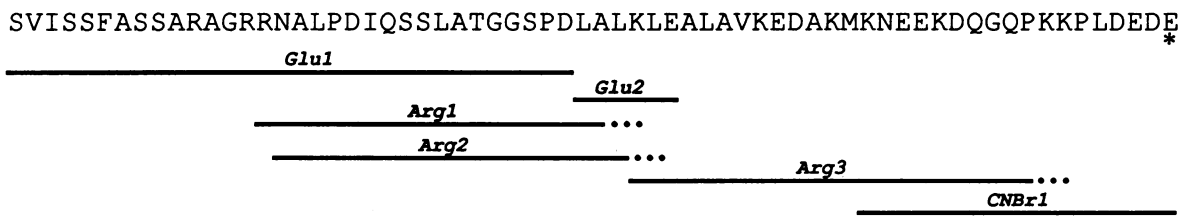

FIG. 4. Partial amino acid sequence of PKI_{test} from the purified protein. Pure PKI_{test} was digested under various conditions and several of the resulting peptides were sequenced from the amino-terminal end as far as the sensitivity of amino acid detection permitted. Digestions, separation of peptides, and sequencing were described in *Experimental Procedures*. *Arg1*, *-2*, and *-3* are peptides from the Arg-C digest; *Glu1* and *-2* are peptides from the Glu-C digest; *CNBr1* is a peptide from the CNBr digest. Asterisk denotes a difference with data from the cDNA, see *Results*.

PKI_{test} were cleaved with CNBr, Arg-C, and Glu-C, and isolated peptides were sequenced from the amino-terminal end as far as possible. A combination of sequence from six of these overlapping peptides allowed for the deduction of 66 amino acids of the protein's sequence (Fig. 4). It appears that there was some cleavage by the Glu-C protease at an aspartic residue (Asp^{36}) to yield the *Glu2* peptide, and there was a rather unexpected cleavage by the Arg-C protease at a leucine-lysine bond (Leu^{39}-Lys^{40}) to yield the *Arg3* peptide. It is likely, given the next amino acid in the PKI sequence, that the complete sequences were obtained for the *Glu1* and *Glu2* peptides, but the *Arg1* and *Arg3* peptides, and possibly the *Arg2* peptide, may have extended beyond the point where sequence was obtained. This partial sequence allowed the design of probes in order to isolate the cDNA.

Degenerate oligonucleotides were synthesized that corresponded to two segments of this amino acid sequence and used for PCR amplification of a segment of the PKI_{test} cDNA from a rat testis cDNA library. A piece of DNA of the predicted size was PCR-amplified from library DNA and this piece was sequenced, revealing that it did in fact code for the expected section of the PKI_{test} protein. A central 70-bp piece of this DNA was then used to identify 42 positive plaques. The initial sequence of PKI_{test} cDNA was derived from one clone containing a 976-bp insert (nucleotides 159–1134 of the sequence shown in Fig. 5) and was then extended using two other clones. The largest clone contained the 1364-base sequence shown in Fig. 5. Inspection of the DNA sequence shows the presence of three possible ATG translation initiation codons at nucleotide positions 72–74, 235–237, and 256–258. Initiation at the third ATG codon (nucleotides 256–258) would produce a 70-amino acid protein (calculated molecular weight, 7455) that includes the amino acid sequence deduced from our purified PKI_{test} plus an additional four amino acids at the amino terminus: Thr^1-Asp^2-Val^3-Glu^4. This sequence accurately matches the amino acid composition data obtained for the purified testis protein (Table 1) and is consistent with the *Glu1* peptide (Fig. 4) being produced by a Glu-C cleavage at the amino-terminal side of Ser^5. Peptide sequence data for *CNBr1* indicated that the carboxyl-terminal amino acid was glutamic acid, whereas the DNA sequence indicated that this amino acid was lysine. A codon for lysine at this position was confirmed by DNA sequencing of three distinct cloned inserts. The peptide sequence data are more likely to be in error, since obtaining the last carboxyl-terminal amino acid of a long peptide has difficulties inherent in the sequencing technique. The presence of lysine at this position is in accord with the amino acid composition for the intact protein (Table 1) as well as amino acid composition data for the *CNBr1* peptide (data not shown). As

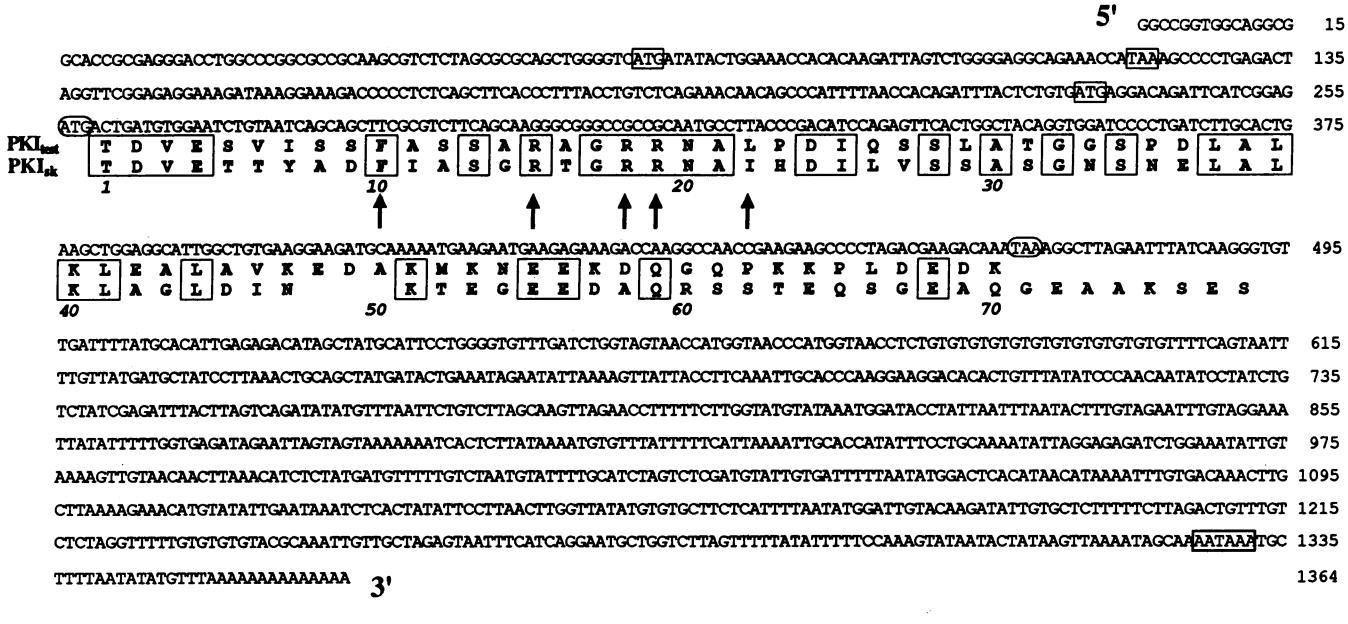

FIG. 5. Nucleotide sequence of the cloned cDNA for PKI_{test}. The 1364-nucleotide sequence of the cloned cDNA is shown along with the predicted amino acid sequence of a 70-amino acid open reading frame corresponding to PKI_{test}. The three ATG initiation signals denoted by the boxes occur at nucleotide positions 72–74, 235–237, and 256–258 from the 5′ end. Shown below the PKI_{test} sequence is the previously elucidated PKI_{sk} sequence (10). A 3-amino acid gap has been introduced into the PKI_{sk} sequence to maximize amino acid identity between the two sequences. Identity between residues of PKI_{test} and PKI_{sk} are shown by boxes. The role of each amino acid in the region of PKI_{sk} residues 5–22 has been examined extensively by means of amino acid substitution (1). The key residues conserved in the pseudosubstrate domain are denoted by arrows.

noted, the ATG codon at nucleotides 256–258, which would synthesize the 70-amino acid PKI, is preceded by two other possible protein synthesis initiation sites. The first initiation codon, at 72–74, is followed by an in-frame stop codon at 120–122 and thus would not produce a long protein that contained within it the PKI sequence. Both this stop codon and the second stop codon, at nucleotides 214–216, are in frame with the PKI coding sequence. Their presence eliminates the possibility that PKI is derived from a large protein and that we simply have not identified a large-enough cDNA. A second potential initiation site, the ATG at 235–237, is in frame with the sequence for PKI with no intervening stop codons. If initiation began at this point, an alternative species of PKI could be produced with a 7 (or 8)-residue additional amino-terminal extension (dependent upon the processing of the initial methionine). The close identity between the amino acid composition of the purified protein, which was obtained from two separate composition analyses, and that of the 70-amino acid PKI protein deduced from the coding sequence (Fig. 1) strongly indicates that the protein which we have purified from rat testis is not a 77 (or 78)-amino acid form of PKI. For it to be so, the composition analysis would have to be in error by one residue each for Arg, Asp/Asn, Gln/Glu, Met, and Thr and by two residues for Ser.

Inhibitory Activity of PKI_{test}. Kinetic analyses (Fig. 6), performed in accord with the method of Henderson (11) for high-affinity inhibitors, demonstrated that PKI_{test}, like PKI_{sk}, is a competitive inhibitor of the cAMP-dependent protein kinase, with a K_i value of 0.14 nM. This is, within error, identical to that for PKI_{sk} (Table 2). Previously it has been shown (1, 2) that a very substantial amount of the inhibitory activity of PKI_{sk} is retained by the peptide PKI_{sk}-(5–22)-amide, which contains the pseudosubstrate site for the protein kinase. The equivalent peptide from PKI_{test} [i.e., PKI_{test}-(5–22)-amide], in contrast, was a poor inhibitor relative to the full-length parent protein (Table 2).

DISCUSSION

The PKI_{test} cDNA that we have isolated contains three potential ATG initiation codons (Fig. 4). The first of these, at nucleotides 72–74, begins an open reading frame that is very short and would not lead to a protein containing within it the PKI sequence; nor, because of the context of its sequence (9),

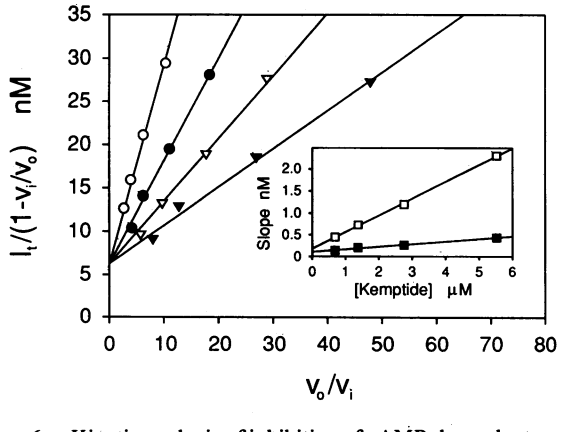

FIG. 6. Kinetic analysis of inhibition of cAMP-dependent protein kinase activity by inhibitor proteins. Protein kinase activity was determined in the presence and absence of inhibitor protein at Kemptide concentrations of 5.6 μM (○), 2.8 μM (●), 1.4 μM (▽), and 0.7 μM (▼); ATP concentration was 8 μM. Data are plotted in accordance with Henderson (11). PKI_{test} concentrations ranged from 7.9 to 26.6 nM; catalytic subunit concentration was 0.8 nM. (*Inset*) Replots of the slopes of the Henderson analyses versus Kemptide concentration for both PKI_{test} (□) and PKI_{sk} (■), assayed under identical conditions. K_i values obtained are reported in Table 2.

would it block initiation at a downstream site. The amino acid composition analysis of the pure PKI_{test} that we have isolated (Table 1) indicates it to be the 70-amino acid protein (Thr^1 to Lys^{70}) denoted in Fig. 5. The ATG codons at 235–237 and at 256–258 are in frame with this sequence and the existence of these two sites raises the issue of what is the initial product of protein synthesis. The isolated 70-amino acid PKI_{test} could have been synthesized directly, by removal of the initial methionine and addition of the amino-terminal blocking group. Alternatively, it could have been derived by co- or posttranslational modification of the 78-amino acid protein that contained 8 additional amino-terminal amino acids. Although protein synthesis most usually begins at the first initiation site, neither the ATG at 235–237 nor the ATG at 256–258 is positioned within a strongly favorable nucleotide sequence for protein synthesis initiation (9). In accord with the rules proposed by Kozak (9), there is quite a high likelihood that the initiation site at 235–237 will be leaky, allowing direct initiation at 256–258 and, consequently, the direct synthesis of the 70-amino acid PKI (plus the initial methionine). Even so, synthesis might also initiate at 235–237 to produce a second form with a 7 (or 8)-residue amino-terminal extension (Met-Arg-Thr-Asp-Ser-Ser-Glu-Met), and this might explain the multiple species observed by DEAE chromatography (Fig. 1). The presence of the initiation codon at 235–237 was identified in five separate clones, suggesting that there are not two separate PKI cDNA species, one with and one without the initiation site at 235–237. With the potential exception of the addition of the 8 amino acids at the amino terminus, the isolation of PKI_{test} cDNA proves that PKI is not derived by processing of a larger protein, a question that has been raised in consideration of the harsh denaturing conditions used in its purification.

Fig. 5 also provides a comparison of the sequence of *rat* PKI_{test}, as obtained from the cDNA, with that of *rabbit* PKI_{sk}, which had been sequenced previously by Scott *et al.* (10). Both proteins have a blocked amino terminus with the blocking group yet to be identified. The degree of sequence homology between the two forms is remarkably low. In Fig. 5 the two sequences are aligned with a 3-residue gap inserted in PKI_{sk}, the longer of the two proteins, in order to maximize homology. Even with this possible gap the two display only 41% identity. The very low degree of homology between the two, in comparison to other rat and rabbit proteins, favors the argument that PKI_{sk} and PKI_{test} represent different PKI isoforms. One possibility is that the amino acid sequence of the less conserved, carboxyl-terminal portion of PKI_{test} is required for some functional role unique to this form of PKI. Interestingly, 8 of the 10 residues from Lys^{47} through Glu^{56} are homologous to a region of troponin C, and this homology is not shared with PKI_{sk}. This sequence of troponin C, Lys-Glu-Asp-Ala-Lys-Gly-Lys-Ser-Glu-Glu (KEDAKgK-sEE, with residues not conserved in PKI_{test} shown by lowercase letters), is the region of the protein that interacts with target proteins (12). Of note, testis PKI has been suggested to be associated with microtubular proteins (13). An explanation for why two proteins with relatively little homology can inhibit the cAMP-dependent protein kinase with equally high potency may be that there is little structure to be conserved in PKI because the protein is primarily random coil in solution (14).

Table 2. Kinetic constants for inhibitory proteins and peptides

Protein or peptide	K_i, nM
PKI_{sk} (full protein)	0.09
PKI_{test} (full protein)	0.14
PKI_{sk}-(5–22)-amide	0.83
[Ala^{13}]PKI_{sk}-(5–22)-amide	3.2
PKI_{test}-(5–22)-amide	90

Although PKI_{sk} and PKI_{test} show only minimal sequence homology, they share a very high degree of conservation in their mode of interaction with the catalytic site of the protein kinase. Their K_i values are, within error, identical (Table 2). The primary determinants for inhibitory activity in PKI_{sk} lie in the amino-terminal portion of the molecule, referred to as the pseudosubstrate site (1), and a synthetic peptide, representing Thr^5 through Ile^{22} of PKI_{sk}, is a highly potent inhibitor (1, 2). Peptide studies involving single amino acid substitutions within this region of PKI_{sk} have demonstrated the importance of Arg^{19}, Arg^{18}, and Arg^{15} (listed in decreasing order of importance) for maximal inhibitory potency (1). Phe^{10} and Ile^{22} also make a major contribution to the inhibitory activity. Each of these amino acids is present in the identical position of the PKI_{test} sequence reported here, with the exception of Ile^{22} for which there is a conservative replacement by leucine (Fig. 5). Leucine at this position, however, only slightly decreases inhibitory potency in synthetic peptides (2). The position of this entire group of amino acids has been conserved with respect to the blocked amino terminus of PKI_{sk} and PKI_{test}. Surprisingly, a synthetic peptide representing amino acids 5–22 of PKI_{test} is a much poorer inhibitor of the kinase than is the peptide from the corresponding sequence of PKI_{sk} (Table 2). It may be that the remainder of the protein is required to stabilize the structure of the pseudosubstrate site in PKI_{test} whereas this is not the case for PKI_{sk} (15). A similar situation is seen with peptides of various lengths derived from PKI_{sk} (1, 2). Although Ser^{13} is also conserved between PKI_{sk} and PKI_{test}, this amino acid is apparently not essential for normal inhibitory activity (Table 2). It is possible that this serine has some other important role, such as a site of phosphorylation for the control of PKI activity, but there is no evidence for such a phosphorylated form of PKI.

The level of cAMP-dependent protein kinase inhibitory activity has been shown to increase in response to follicle-stimulating hormone in both Sertoli cell-enriched rat testis (16) and isolated Sertoli cells (17). This increase is blocked by cycloheximide and therefore is apparently a result of either transcriptional or translational stimulation. It was not clear from these data which form or forms of testis inhibitor were responsible for the increase. The cloned cDNA described here should be a valuable tool in determining whether the PKI_{test} that we have purified is involved in this hormonal response. Differential genetic regulation could provide another explanation for the existence of multiple isoforms of PKI.

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