Structural Basis for the Low Affinities of Yeast cAMP-Dependent and Mammalian cGMP-Dependent Protein Kinases for Protein Kinase Inhibitor Peptides†

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ABSTRACT: Affinities of the catalytic subunit (C_1) of *Saccharomyces cerevisiae* cAMP-dependent protein kinase and of mammalian cGMP-dependent protein kinase were determined for the protein kinase inhibitor (PKI) peptide PKI(6–22)amide and seven analogues. These analogues contained structural alterations in the N-terminal α-helix, the C-terminal pseudosubstrate portion, or the central connecting region of the PKI peptide. In all cases, the PKI peptides were appreciably less active as inhibitors of yeast C_1 than of mammalian Cα subunit. K_i values ranged from 5- to 290-fold higher for the yeast enzyme than for its mammalian counterpart. Consistent with these results, yeast C_1 exhibited a higher K_m for the peptide substrate Kemptide. All of the PKI peptides were even less active against the mammalian cGMP-dependent protein kinase than toward yeast cAMP-dependent protein kinase, and Kemptide was a poorer substrate for the former enzyme. Alignment of amino acid sequences of these homologous protein kinases around residues in the active site of mammalian Cα subunit known to interact with determinants in the PKI peptide [Knighton, D. R., Zheng, J., Ten Eyck, L. F., Xuong, N-h, Taylor, S. S., & Sowadski, J. M. (1991) *Science 253*, 414–420] provides a structural basis for the inherently lower affinities of yeast C_1 and cGMP-dependent protein kinase for binding peptide inhibitors and substrates. Both yeast cAMP-dependent and mammalian cGMP-dependent protein kinases are missing two of the three acidic residues that interact with arginine-18 in the pseudosubstrate portion of PKI. Further, the cGMP-dependent protein kinase appears to completely lack the hydrophobic/aromatic pocket that recognizes the important phenylalanine-10 residue in the N-terminus of the PKI peptide, and binding of the inhibitor by the yeast protein kinase at this site appears to be partially compromised.

Peptides corresponding to the active portion of the heat-stable inhibitor protein of cAMP-dependent protein kinase (EC 2.7.1.37) are highly potent and selective competitive inhibitors of the catalytic subunit of that enzyme (Walsh et al., 1990; Walsh & Glass, 1991). These peptides inhibit both the Cα[1] and Cβ isoforms of mammalian catalytic subunit with equal potency (Olsen & Uhler, 1989). PKI(6–22)amide is the minimal peptide structure that retains high potency (K_i = 1–2 nM) for inhibition of mammalian C subunit (Glass et al., 1989a). The structural requirements underlying this function of PKI(6–22)amide have been extensively studied and include an N-terminal amphiphilic α-helix containing Phe[10], the C-terminal pseudosubstrate site containing Arg[18], Arg[19], and Ile[22], and a central connecting region, as depicted in Chart I (Cheng et al., 1986; Scott et al., 1986; Glass et al., 1989a,b; Reed et al., 1989).

PKI peptides are extremely specific for inhibition of cAMP-dependent protein kinase (Scott et al., 1985). Among a number of other protein kinases examined, only a weak interaction of PKI peptides with the cGMP-dependent enzyme has been detected (Glass et al., 1986). In marked contrast to the determinants required for potent activity toward the cAMP-dependent protein kinase, inhibition of the cGMP-dependent enzyme by PKI peptides is actually somewhat enhanced by the complete removal of the N-terminal helical region to leave only the pseudosubstrate site. Also, inhibition of cGMP-dependent protein kinase is only minimally influenced by the individual substitution of each of the arginines in this pseudosubstrate region by lysine (Glass et al., 1986).

Chart I

Thr[6]-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile[22]amide

amphiphilic α-helix — pseudosubstrate site

In yeast, three genes (*TPK1*, *TPK2*, and *TPK3*) code for the C_1, C_2, and C_3 catalytic subunits of the cAMP-dependent protein kinase (Toda et al., 1987). These yeast enzymes are 75–88% identical to one another, and each has over 50% sequence identity with the mammalian Cα and Cβ isoforms (Toda et al., 1987). C_1 has been the best studied of the yeast catalytic subunits (Zoller et al., 1988; Levin et al., 1988; Levin & Zoller, 1990; Gibbs & Zoller, 1991a,b). Zoller et al. (1988)

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[1] Abbreviations: C, catalytic subunit; PKI, heat-stable inhibitor protein of cAMP-dependent protein kinase; Kemptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; Mes, 2-(*N*-morpholino)ethanesulfonic acid; IC_{50}, concentration producing 50% inhibition; Nal, L-(1′-naphthyl)alanine.

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first reported that the peptide PKI(5–24) was over 30-fold less potent in inhibiting yeast C_1 as compared to the mammalian enzyme. Since PKI(5–24) contains a number of different structural determinants important for binding to Cα, it remains to be resolved as to which of these features contribute to the lowered affinity of the inhibitor peptide for yeast C_1.

The current study, using a series of PKI peptide analogues, was directed at assessing if the reduced affinities of the yeast cAMP-dependent and mammalian cGMP-dependent protein kinase for the PKI peptide were due to these proteins not retaining some of the recognition sites that have previously been identified for the interaction of this inhibitor with the mammalian cAMP-dependent enzyme (Glass et al., 1989a,b; Gibbs & Zoller, 1991b). The K_i values of both yeast C_1 and the mammalian cGMP-dependent protein kinase were determined for the parent peptide PKI(6–22)amide, for a truncated PKI peptide that only contains the pseudosubstrate site, and for several additional inhibitor analogues containing structural alterations in either the N-terminal, central connecting, or pseudosubstrate regions of the peptide. These results along with an alignment of the sequences of homologous portions of all three protein kinases, evaluated with reference to the recently published crystal structure of the mammalian catalytic subunit·PKI peptide complex (Knighton et al., 1991a,b), allow conclusions concerning specific structural bases that underlie differences in the inhibitory potencies of the PKI peptides.

Materials and Methods

Yeast and Mammalian Cyclic Nucleotide-Dependent Protein Kinases. Yeast C_1 coded by the *TPK1* gene was prepared as described by Zoller et al. (1988) by overexpression of the holoenzyme from a genetically manipulated strain of yeast followed by purification on an anti-regulatory subunit immunoaffinity column. There was no or minimal contamination of the yeast C_1 with yeast regulatory subunit. In two experiments, addition of 10 μM cAMP caused only a 2.6% and 2.5% stimulation of phosphotransferase activity. Mammalian C subunit was purified to homogeneity from bovine heart by the method of Bechtel et al. (1977). This preparation consists of predominantly the Cα form of the enzyme (Showers & Maurer, 1986). Mammalian cGMP-dependent protein kinase (the Iα isoform) was purified from bovine or porcine lung according to Glass and Krebs (1979).

Synthetic PKI Peptides. PKI(6–22)amide and selected analogues were synthesized by solid-phase techniques, purified, and characterized by analytical HPLC, UV spectroscopy, and amino acid analysis as previously described by Glass et al. (1989a,b).

Protein Kinase Assays. Assay conditions for the titrations of yeast C_1 with PKI peptides were essentially as described previously for the mammalian enzyme (Glass et al., 1989a) and were quite similar to those used for yeast C_1 by Zoller et al. (1988). Phosphotransferase reaction mixtures contained 30 mM Mes buffer (pH 6.9), 5 mM $Mg(acetate)_2$, 250 μM [γ-^{32}P]ATP (43–124 cpm/pmol), 100 μM Kemptide as phosphate-accepting substrate, 3 mM 2-mercaptoethanol, 0.4 mg/mL bovine serum albumin, and 0.5 nM (0.023 μg/mL) yeast C_1. Reactions were initiated with C_1 subunit and assayed at 30 °C for 10 min prior to termination by the phosphocellulose paper method (Roskoski, 1983). Selected inhibitor peptides covering a total range of 0.1–215 000 nM were used to determine IC_{50} values which were obtained from log–logit transformations of the titration data. Each IC_{50} was determined twice in independent assays, and the values were within ±14% of the mean. The mean slope (±SEM) from all of the log–logit plots of yeast C_1 was −0.99 ± 0.03 (n = 14). Inhibition of mammalian cGMP-dependent protein kinase by PKI peptides was assayed using the peptide (Ala^{34})histone H2B(29–35) as substrate under the previously described conditions (Glass et al., 1986). Reaction mixtures contained 30 mM Tris-HCl buffer (pH 7.4), 2 mM $Mg(acetate)_2$, 1 μM cGMP, 200 μM [γ-^{32}P]ATP (133–230 cpm/pmol), 20 μM (Ala^{34})histone H2B(29–35), 3 mM 2-mercaptoethanol, 0.3 mg/mL bovine serum albumin, and 2.3 nM (0.35 μg/mL) cGMP-dependent protein kinase. Titration assays were conducted for 2 min at 30 °C with various PKI peptides in concentrations ranging from 2000 to 464 000 nM. The mean slope (±SEM) of the inhibitor plots for cGMP-dependent protein kinase was −1.02 ± 0.03 (n = 13).

K_i Values for PKI Peptides. Inhibition of mammalian Cα subunit by each PKI peptide was competitive versus the substrate Kemptide as determined by appropriate kinetic analyses (Glass et al., 1989a,b; Scott et al., 1986; Cheng et al., 1986). PKI peptides have previously been shown to be competitive inhibitors of the cGMP-dependent protein kinase (Glass et al., 1986). The K_i values of the PKI peptides tested in this study with yeast C_1 and mammalian cGMP-dependent protein kinase were calculated from the experimentally determined IC_{50} data according to the following equation for competitive inhibitors (Cheng & Prusoff, 1973).

$$K_i = \frac{IC_{50}}{1 + [S]/K_m} \quad (1)$$

The fixed concentrations of 100 μM Kemptide and 20 μM (Ala^{34})histone H2B(29–35) were chosen to be close to their respective K_m values with yeast cAMP-dependent and mammalian cGMP-dependent protein kinases, respectively. Under the above assay conditions, the mean K_m of yeast C_1 for the former peptide is reported below in the Results and Discussion, and the K_m of the cGMP-dependent protein kinase for the latter peptide was 24 μM (Glass & Krebs, 1982).

Sequence Alignments of Catalytic Subunits and Domains of Protein Kinases. Multiple alignments of the homologous mammalian and yeast protein kinases were performed using the algorithm in MicroGenie version 7.0 (Queen & Korn, 1984). The entire sequences of the bovine Cα and Cβ and the yeast C_1, C_2, and C_3 catalytic subunit isoforms were aligned with one another and with the catalytic domains of the 1α and 1β isoforms of cGMP-dependent protein kinases (residues 321–670 and 336–685, respectively). Sources of the protein sequences are listed in the legend to Figure 2. Numbering of the yeast sequences included the initiator methionine residue while those of the mammalian enzymes did not.

Results and Discussion

Inhibition of Yeast C_1 and Mammalian cGMP-dependent Protein Kinase by PKI Analogues. Curves from the titration of yeast C_1 with PKI(6–22)amide and selected inhibitor analogues were linearized by log–logit transformation to derive slopes, IC_{50} values, and the K_i values of the PKI peptides toward yeast C_1 (Table I). The titration curves for all of the peptide analogues were parallel to one another. The slopes of negative unity indicated that the PKI peptides interacted with yeast C_1 in a noncooperative fashion, as is the case with the mammalian C subunit (Glass et al., 1989a,b). Kinetic analyses of the inhibition were not performed for all peptides due to the small amount of yeast enzyme available. However, the titration data were consistent with PKI peptides being competitive inhibitors of the yeast protein kinase versus Kemptide. (DAla^{14})PKI(6–22)amide, (Nal^{10})PKI(6–22)-

Table I: K_i Values of Selected PKI Peptides toward C_1 Subunit of Yeast cAMP-Dependent Protein Kinase and Bovine cGMP-Dependent Protein Kinase

Thr[5]-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile[22]amide

	yeast cAMP-dependent protein kinase		bovine GMP-dependent protein kinase	
inhibitor peptide	slope[a]	K_i[b] (nM)	slope	K_i (nM)
PKI(6–22)amide	−0.98	127	−0.98	37 820
PKI(14–22)amide	−1.07	1442	−0.98	8 000
(Gly[22])PKI(5–22)amide	−0.94	13410	−0.94	46 040
(Ala[10])PKI(6–22)amide	−1.05	2022	−0.98	29 320
(Nal[10])PKI(6–22)amide	−0.99	113	−1.18	8 830
(DPhe[10])PKI(6–22)amide	−0.96	1511	−1.00	23 950
(DAla[14])PKI(6–22)amide	−0.98	106	−1.09	42 820

[a] Slopes of the inhibition curves were obtained from log–logit transformations of the titration data. [b] K_i values were calculated from experimentally determined IC_{50} data as described under Materials and Methods.

amide, and the parent peptide PKI(6–22)amide were the most potent inhibitors of yeast C_1, with K_i values slightly greater than 100 nM. The enzyme activity could be completely inhibited by concentrations of these peptides in excess of 10 μM. The least active inhibitor of yeast C_1 was (Gly[22])PKI(5–22)amide, the analogue with a structural modification that omitted the hydrophobic side chain at its C-terminus (Glass et al., 1989a). It was over 2 orders of magnitude less active than the most potent inhibitor peptides listed above.

A similar titration analysis was undertaken with the same PKI analogues as inhibitors of the mammalian cGMP-dependent protein kinase. The K_i values and slopes with this enzyme are also summarized in Table I. There was no evidence of cooperativity in the interaction of the PKI peptides with the cGMP-dependent enzyme. Inhibition patterns were like those observed with yeast cAMP-dependent protein kinase, except that all of the peptides were appreciably less potent as inhibitors. These results with cGMP-dependent protein kinase are consistent with the structure–function relationships of a different set of PKI peptides studied previously with this enzyme (Glass et al., 1986).

Relative Potencies of PKI Peptides toward Three Protein Kinases. The K_i values of PKI peptides for yeast C_1 and mammalian cGMP-dependent protein kinase are compared to those for the Cα subunit of mammalian cAMP-dependent protein kinase in Figure 1, panel A. The data are expressed as ligand selectivity profiles (Goldstein, 1987; Goldstein & Naidu, 1989). Data on PKI(5–24), the peptide used by Zoller et al. (1988), are included. The rank order of potency of the various PKI peptides for inhibition of yeast C_1 was generally the same as for mammalian Cα subunit. However, all of the peptides were appreciably less active, by generally 1–2 orders of magnitude, toward yeast C_1 than against its mammalian counterpart. These results confirm and extend the observation of Zoller et al. (1988). The range of K_i values within the series of inhibitors was over 1200-fold for mammalian Cα but only about 120-fold with the yeast enzyme. The largest difference in affinities between the yeast and mammalian enzymes was seen with one of the most potent peptides, the Nal[10] analogue, while the smallest difference was with one of the least potent inhibitors (Ala[10])PKI(6–22)amide. These observations probably reflect a floor of minimal binding affinity contributed by a limited region of any of the modified peptide analogues. The K_i values depicted in Figure 1A correspond to a free energy difference between binding of a PKI peptide to the

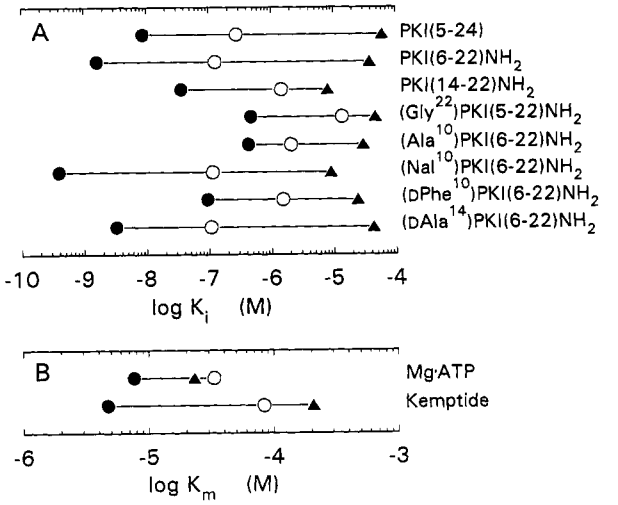

FIGURE 1: Comparison of (A) the K_i values for PKI peptides and (B) the K_m values for substrates exhibited by yeast C_1 (○), mammalian Cα subunit (●), and mammalian cGMP-dependent protein kinase (▲). The inhibitory potencies of the PKI peptides and the Michaelis constants for Mg·ATP and Kemptide are graphed as ligand selectivity profiles (Goldstein, 1987; Goldstein & Naidu, 1989). (A) The data for mammalian C subunit are from Glass et al. (1989a,b). The K_i of PKI(5–24) for yeast C_1 is taken from Zoller et al. (1988) and is compared to data on the C-terminal amide of PKI(5–24) with mammalian C subunit and the cGMP-dependent protein kinase (Glass et al., 1989a). (B) The K_m of the yeast enzyme for Mg·ATP is taken from Zoller et al. (1988). The K_m data for cGMP-dependent protein kinase are taken from Glass and Krebs (1979) and Glass et al. (1981).

yeast or to mammalian C subunits of approximately 8–14 kJ/mol, depending on the exact analogue chosen for comparison.

All of the peptides inhibited the mammalian cGMP-dependent protein kinase over a quite narrow range (only 6-fold) of very high concentrations, and the rank order of potency of the analogues for this enzyme was different than for either of the catalytic subunits of the cAMP-dependent protein kinase (Table I). Consistent with our previous observations with the cGMP-dependent enzyme using PKI(5–24)amide as the parent peptide (Glass et al., 1986), the short pseudosubstrate peptide PKI(14–22)amide was, in fact, more active than the full-length PKI(6–22)amide. The potency of the latter inhibitor was only modestly improved by substitution of Phe[10] with naphthylalanine but was essentially unaffected by substitution at this position with alanine or D-phenylalanine. The difference in free energies between interaction of the PKI peptides with the homologous cGMP-dependent and cAMP-dependent protein kinases ranged between 13 and 26 kJ/mol.

The inhibition data for yeast C_1 and the mammalian cGMP-dependent protein kinase were somewhat similar to one another in that both enzymes were more poorly inhibited by all of the PKI peptides, regardless of their structural modifications, than was mammalian Cα subunit (Figure 1A). Yeast C_1 and the mammalian cGMP-dependent protein kinase are alike in that both the C_1 subunit of the former and the catalytic domain of the latter contain an N-terminal extension as compared to the 350-residue mammalian C subunit. An N-terminal truncation mutant of yeast C_1 would more closely resemble mammalian C subunit in this regard. However, it is improbable that all of the PKI peptides would exhibit appreciably more potent inhibition toward such an altered enzyme, because this truncation mutant has kinetic constants for Kemptide similar to those of wild-type C_1 (Kuret & Pflugrath, 1991).

Relative Kinetic Constants of Protein Kinases for Substrates. The mean K_m value (±SEM) of yeast C_1 for

Kemptide was 83.0 ± 9.0 μM, and the mean k_{cat} was 1306 ± 339 min^{-1} under the fixed Mg^{2+} and ATP concentrations described above. This K_m is close to that previously reported for C_1 by Zoller et al. (1988). K_m and k_{cat} values for our recent preparations of bovine heart Cα subunit with Kemptide as substrate under these conditions were 4–5 μM and about 980 min^{-1}, respectively. Thus, while the homologous yeast and mammalian protein kinases had similar catalytic constants, the former enzyme exhibited a nearly 20-fold lower apparent affinity for the peptide substrate. The mammalian cGMP-dependent protein kinase is also known to have a quite high K_m value for Kemptide, but the k_{cat} of this enzyme for Kemptide is similar to that of the cAMP-dependent protein kinase (Glass & Krebs, 1979).

Figure 1, panel B, compares the K_m values of the three protein kinases for Mg·ATP and the peptide Kemptide. The kinetic constants of the enzymes for the substrates followed their relative affinities for the PKI peptides, with the yeast C_1 exhibiting K_m values intermediate between those observed for mammalian Cα and the cGMP-dependent protein kinase. In fact, the apparent affinities of yeast C_1 for its substrates were most similar to those of the cGMP-dependent protein kinase. Recent work by Denis et al. (1991) compared the abilities of yeast and mammalian C subunits to phosphorylate a kinetically favorable model peptide corresponding to the phosphorylation site in an endogenous yeast protein substrate, the transcriptional activator ADR1. Although ADR1 is a naturally-occurring yeast protein, ADR1(217–234) was phosphorylated with a 23-fold lower K_m value by mammalian C subunit as compared to the yeast enzyme. Thus, the results with this ADR1 peptide substrate were quite similar to those for Kemptide. Although the homologous protein kinases from the two species have similar substrate specificities, the mammalian enzyme apparently has a greater inherent affinity for substrates than does yeast C_1. Accordingly, as recently reported by Zoller et al. (1991), mammalian Cα expressed in yeast was able to functionally substitute for the disrupted yeast C subunits.

Sequence Alignment of Protein Kinases around PKI Recognition Residues. Both yeast C_1 and the cGMP-dependent protein kinase have decreased affinities as compared to the homologous mammalian Cα subunit for an entire series of PKI peptide analogoues as inhibitors as well as for Kemptide as a substrate. Thus, these lower affinities appear to be inherent properties of the two enzymes. The extremely tight binding of PKI peptides to mammalian Cα subunit [for example, 0.4 nM K_d for (Nal[10])PKI(6–22)amide] is due to multiple points of contact between the enzyme and this ligand over the entire length of the peptide (Walsh et al., 1990; Knighton et al., 1991b). The major determinants of this high-affinity binding are Phe^{10} in the N-terminal amphiphilic α-helix and the residues Arg^{18}, Arg^{19}, and Ile^{22} in the pseudosubstrate portion of the PKI peptide (Cheng et al., 1986; Scott et al., 1986; Glass et al., 1989a,b). Arg^{15} in the central connection region also contributes to the overall favorable interaction (Prorok & Lawrence, 1989). Some of the specific hydrophobic/aromatic interactions, electrostatic and hydrogen bonds, and van der Waals contacts involved in the association of the PKI peptide with C subunit have recently been delineated. Gibbs and Zoller (1991b) identified three of the acidic residues in yeast C_1 that recognize the two basic amino acid determinants in Kemptide. The three-dimensional crystal structure of the mammalian Cα subunit of cAMP-dependent protein kinase has recently been solved by Knighton et al. (1991a,b). This structure of the binary complex of Cα subunit with a PKI peptide bound at its active site has identified amino acids in the protein kinase that interact with Phe^{10}, Arg^{15}, Arg^{18}, Arg^{19}, and Ile^{22} in the inhibitor peptide (Knighton et al., 1991b). Therefore, the probable structural bases that underlie the disrupted bonding interactions between PKI(6–22)amide and either yeast C_1 or the mammalian cGMP-dependent protein kinase can be inferred by aligning the sequences of these homologous enzymes with the known PKI recognition residues in the Cα subunit.

Binding of Phe^{10} in PKI peptides by mammalian Cα involves residues Tyr^{235}, Pro^{236}, and Phe^{239} in one region of the linear sequence of the enzyme (Knighton et al., 1991b). This area is located in subdomain IX described by Hanks et al. (1988). As shown in Figure 2A, yeast C subunits contain the two aromatic residues corresponding to Tyr^{235} and Phe^{239} but have a threonine substitution for those residues corresponding to Pro^{236}. This places a hydrophilic amino acid in the otherwise hydrophobic binding pocket for Phe^{10} of PKI. The yeast enzyme contains two tyrosine residues instead of the one tyrosine and one phenylalanine present in mammalian Cα. Interestingly, the human Cγ isoform has the conserved sequence Val-Gly-**Phe-Pro**-Pro-Phe-**Tyr**-Ala-Asp (Beebe et al., 1990), but its binding affinity for the PKI peptide is not known. These sequence alignments are consistent with the Phe^{10} residue being important for binding of the parent peptide to the yeast protein kinase because its substitution with alanine resulted in an appreciable loss in inhibitory activity. However, while the aromatic pocket for recognition of the N-terminus of PKI is retained in yeast C_1, its affinity is probably reduced compared to that of the mammalian enzyme due to the threonine substitution for Pro^{236}. This appears to be especially true for the (Nal[10])PKI(6–22)amide analogue. In contrast to the yeast enzyme, the corresponding recognition sequence for Phe^{10} in the mammalian cGMP-dependent protein kinase, while retaining the residue corresponding to Pro^{236}, totally lacks the two aromatic residues, having two hydrophilic serines in their places. Thus, the cGMP-dependent protein kinase appears not to have retained the hydrophobic/aromatic binding pocket that recognizes Phe^{10} in the N-terminus of PKI. The absence of this site is clearly one reason for the lower affinity of cGMP-dependent protein kinase for the PKI peptides. It also readily accounts for the shorter PKI(14–22)amide being the most potent inhibitor toward this protein kinase. It is curious, however, that substitution of Phe^{10} by naphthylalanine in the full-length inhibitor enhanced peptide binding, although the same substitution by alanine was without effect. Perhaps the naphthylalanyl residue can interact with the aromatic Phe^{557}, adjacent to the missing Phe^{558} in cGMP-dependent protein kinase (Figure 2A), or with some other modest hydrophobic region located elsewhere on the surface of the enzyme.

Since yeast C_1 exhibited a lowered affinity both for the truncated inhibitor PKI(14–22)amide and for Kemptide, the depressed interaction with the yeast enzyme must also involve the pseudosubstrate site of the PKI peptide, possibly altered recognition of the important –Arg^{18}–Arg^{19}– moiety contained therein. Recognition of Arg^{18} of PKI by mammalian Cα involves electrostatic interactions with residues Glu^{127}, Asp^{329}, and Glu^{331} present in two separate areas of the linear sequence of the protein (Figure 2B) (Gibbs & Zoller, 1991b; Knighton et al., 1991b). Glu^{127} is located in subdomain V (Hanks et al., 1988) while Asp^{329} and Glu^{331} are very close to the C-terminus of the protein. Glu^{127} and the residues around it are conserved both in the yeast C subunits and in cGMP-dependent protein kinase. Therefore, the differences in affinity

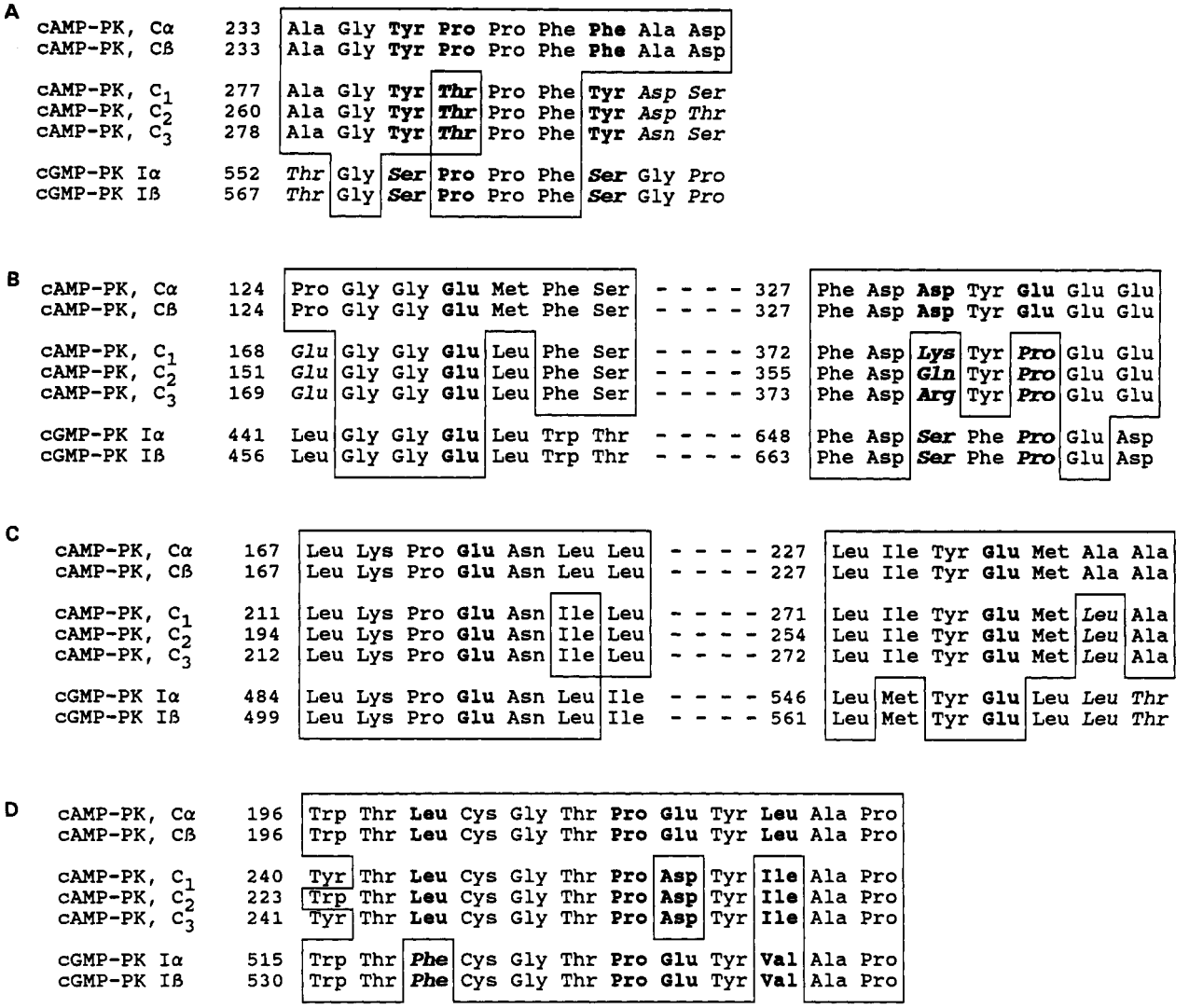

FIGURE 2: Multiple alignment of amino acid sequences from bovine and yeast catalytic subunits and the catalytic domains of bovine cGMP-dependent protein kinases around the residues involved in recognition of Phe[10], Arg[15], Arg[18], Arg[19], and Ile[22] in PKI peptides. (A) Tyr[235], Pro[236], and Phe[239] (in boldface type) in bovine Cα form a hydrophobic/aromatic pocket that binds Phe[10] in PKI (Knighton et al., 1991b; Glass et al., 1989b). (B) Glu[127], Asp[329], and Glu[331] (in boldface type) in bovine Cα undergo electrostatic interactions with Arg[18] in the pseudosubstrate portion of PKI (Gibbs & Zoller, 1991b; Knighton et al., 1991b). (C) Glu[170] and Glu[230] (in boldface type) in bovine Cα undergo electrostatic interactions with Arg[19] in the pseudosubstrate portion of PKI (Gibbs & Zoller, 1991b; Knighton et al., 1991b). (D) Glu[203] (in boldface type) in bovine Cα electrostatically bonds with Arg[15] in the central portion of PKI (Knighton et al., 1991b). Leu[198], Pro[202], and Leu[205] (in boldface type) form a hydrophobic site for interaction with Ile[22] of PKI (Knighton et al., 1991b). The number of the first residue in each segment is indicated. Boxes enclose identical residues. Conservative substitutions are in normal type, and nonconservative substitutions are in italics. Dashes indicate a gap between two separate regions of the linear sequence. Groupings of amino acids for this purpose are Val, Leu, Ile, Met, Pro, and Cys (hydrophobic); Phe, Tyr, and Trp (aromatic); Gly and Ala (small nonpolar); Ser, Thr, Asn, and Gln (uncharged polar); Asp and Glu (negatively charged); and Lys, Arg, and His (positively charged). The sequences of bovine Cα (350 residues) and Cβ (350 residues) isoforms of catalytic subunit are from Showers and Maurer (1986), and those of the yeast C_1 (397 residues), C_2 (380 residues), and C_3 (398 residues) isoforms of catalytic subunit are from Toda et al. (1987). The sequences of the catalytic domains (the C-terminal 350 residues) of the Iα and Iβ isoforms of bovine cGMP-dependent protein kinase are from Takio et al. (1984), Wernet et al. (1989), and Sandberg et al. (1989). PK, protein kinase.

for PKI among the enzymes are probably not accounted for by this residue. Asp[329] and Glu[331], however, are not at all conserved in the yeast C subunits or in mammalian cGMP-dependent protein kinase (Figure 2B). Asp[329] is changed to a basic amino acid in yeast C_1 and C_3 and to neutral hydrophilic residues in yeast C_2 and cGMP-dependent protein kinase. The Glu[331] of Cα is converted to proline in all other protein kinases, although in all the enzymes there is an immediate neighboring glutamic acid residue. A diminished binding of Arg[18] thus likely contributes to the lessened affinities of both yeast cAMP-dependent and mammalian cGMP-dependent protein kinases for PKI peptides as well as for the substrate Kemptide.

Binding of Arg[19] in PKI peptides by mammalian Cα involves residues Glu[170] and Glu[230] in two separate areas of the linear sequence (Gibbs & Zoller, 1991b; Knighton et al., 1991b). Glu[170] is located in subdomain VI and is within one of the two conserved motifs that are characteristic of protein kinases with serine/threonine specificity (Hanks et al., 1988). Glu[230] is in subdomain IX of Hanks et al. (1988) and is closely N-terminal to the region that recognizes Phe[10] of PKI (see above). As shown in Figure 2C, both Glu[170] and Glu[230] and their surrounding residues are highly conserved in all three yeast C subunits and in the mammalian cGMP-dependent protein kinases. Therefore, these residues probably do not underlie the differences in affinity for PKI peptides among the various enzymes. Arg[15] in the PKI peptide interacts predominantly with Glu[203] in Cα (Knighton et al., 1991b). As shown in Figure 2D, this acidic residue is also present in the cGMP-dependent protein kinases. The corresponding amino

acids in yeast C_1, C_2, and C_3 are conservatively changed to an aspartate residue (Figure 2D), the shorter side chain of which may make a minor contribution to the disparity between the mammalian and yeast C subunits in binding PKI(6–22)-amide.

It is clear that Ile^{22} is also important for binding of the parent peptide to the yeast protein kinase because its substitution with glycine resulted in a 2 order of magnitude loss in inhibitory potency. Recognition of the hydrophobic side chain of Ile^{22} in PKI by mammalian Cα involves residues Leu^{198}, Pro^{202}, and Leu^{205} in one region of the linear sequence (Knighton et al., 1991b). These residues are located in subdomain VIII and are part of the second conserved motif that is diagnostic for serine/threonine kinase specificity (Hanks et al., 1988). This is the same region of the enzyme that contains Glu^{203} (see above). As indicated in Figure 2D, Pro^{202} of Cα is conserved in all of the other enzymes. Leu^{198} is conserved in the yeast C subunits and is substituted with the similar phenylalanine in the mammalian cGMP-dependent protein kinases. Leu^{205} is conservatively substituted with isoleucine and valine in yeast C subunits and in the cGMP-dependent protein kinases, respectively. Therefore, the binding site for Ile^{22} of PKI appears to be retained, and these residues probably do not contribute greatly to the observed differences in affinity for PKI among the protein kinases. Curiously, the substitution of Ile^{22} with glycine had little effect on the activity of the inhibitor toward cGMP-dependent protein kinase, raising doubts as to how important the interaction with Ile^{22} is for this enzyme even though the binding site appears to have been retained as judged by the linear amino acid sequence. Finally, note that the mammalian Cα and Cβ subunits have identical residues within all of these amino acid sequences that recognize and bind the inhibitor peptide (Figure 2). This observation is fully consistent with the equally potent inhibition of these two enzyme isoforms by PKI(5–22) (Olsen & Uhler, 1989).

In summary, the reduced affinities of both yeast C_1 and the mammalian cGMP-dependent protein kinase for the PKI peptide are likely due to several primary structural differences in the enzymes that result in lessened bonding interactions with at least two parts of the inhibitor peptide, the Phe^{10} residue in the N-terminal amphiphilic α helix and Arg^{18}, one of the two basic residues in the C-terminal pseudosubstrate region. However, the precise orientations of specific amino acid side chains around these nonconserved residues in each of the protein kinases remain unknown. A better understanding of the exact structural bases for these differences in affinity for inhibitor and substrate peptides must await the crystal structure of the yeast protein kinase or modeling of the primary sequences of yeast C_1 and of cGMP-dependent protein kinase onto the coordinates of the structure of the mammalian Cα subunit.

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Modulators of the Glucocorticoid Receptor Also Regulate Mineralocorticoid Receptor Function†

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ABSTRACT: Modulators are proposed to be novel ether aminophosphoglycerides that stabilize unoccupied and occupied glucocorticoid receptor steroid binding and inhibit glucocorticoid receptor complex activation. Two isoforms, modulator 1 and modulator 2, have been purified from rat liver cytosol [Bodine, P. V., & Litwack, G. (1990) *J. Biol. Chem. 265*, 9544–9554]. Since the mineralocorticoid receptor is relatively resistant to activation, modulator's effect on rat distal colon mineralocorticoid receptor function was examined. Warming of unoccupied receptor decreased residual specific [^{3}H]aldosterone binding by 86 ± 2%. Both modulator isoforms completely prevented this destabilization with K_m's of 2 ± 1 μM modulator 1 and 24 ± 5 μM modulator 2. Warming of occupied mineralocorticoid receptors decreased [^{3}H]aldosterone binding by 56 ± 3%. Modulator only partially stabilized occupied receptor binding with K_m's of 10 ± 2 μM modulator 1 and 68 ± 8 μM modulator 2. Modulator inhibited receptor activation with K_m's of 3 ± 1 μM modulator 1 and 33 ± 10 μM modulator 2. Double-reciprocal analysis showed linear kinetics, and mixing modulator isoforms together had additive effects on unoccupied and occupied receptor steroid binding stabilization and activation inhibition. Colon cytosol contained a low molecular weight, heat-stable factor(s) which inhibited receptor activation and stabilized occupied receptor steroid binding. Molybdate completely stabilized unoccupied mineralocorticoid receptor steroid binding and inhibited activation with half-maximal effects at 3–4 mM but only stabilized occupied receptor binding by ~40%. These data indicate that (i) apparent physiologic concentrations of modulator stabilize mineralocorticoid receptor steroid binding and inhibit receptor activation, (ii) an aldosterone-responsive tissue contains a modulator-like activity, and (iii) molybdate mimics the effects of modulator. Thus, modulator affects both mineralocorticoid and glucocorticoid receptor function. Differences observed suggest that in vivo both modulator 1 and 2 could stabilize unoccupied mineralocorticoid receptor binding whereas only modulator 1 stabilizes unoccupied glucocorticoid receptor binding and that the modulator isoforms have additive effects on occupied mineralocorticoid receptor binding and activation but act synergistically on occupied glucocorticoid receptor binding and activation.

The steroid hormone receptors are a family of hormone-dependent transcriptional regulatory factors (Gustaffson et al., 1987). The unactivated steroid-binding receptor form is an oligomer containing receptor protein and the 90-kDa heat shock protein (hsp-90) (Joab et al., 1984). Binding of hormone to receptor induces dissociation of hsp-90, exposing the receptor DNA-binding domain, a process termed activation or transformation (Munck et al., 1990). The activated receptor then binds to specific hormone regulatory elements on DNA and alters transcription of steroid-responsive genes. The receptor subsequently dissociates from chromatin in a form unable to bind hormone and may be either degraded or recycled back to the oligomeric unactivated steroid-binding receptor form (Bodine & Litwack, 1990a).

Studies from many laboratories have demonstrated that each step in the steroid receptor cycle is influenced by other cellular factors (Bodine & Litwack, 1990a). Several endogenous steroid receptor regulators have been identified by their effects on a specific steroid receptor (Schmidt & Litwack, 1982; Dahmer et al., 1984), but it is unknown whether these factors affect all steroid hormone receptors or are specific for individual receptors. In contrast, a low molecular weight, heat-stable factor which stabilizes glucocorticoid receptor (GR) hormone binding and inhibits GR activation was subsequently reported to be present in many tissues and also to regulate estrogen and androgen receptor function (Dahmer et al., 1984; Cake et al., 1976; Leach et al., 1982; Sato et al., 1980). This

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