Heat-Stable Inhibitor Protein Derived Peptide Substrate Analogs: Phosphorylation by cAMP-Dependent and cGMP-Dependent Protein Kinases

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ABSTRACT: The phosphorylation of substrate peptides derived from PKI, the heat-stable inhibitor protein of the cAMP-dependent protein kinase (PKA), has been studied with both PKA and the cGMP-dependent protein kinase (PKG) using a variety of substitution and deletion analogs. On the basis of K_m, k_{cat} and k_{cat}/K_m values, (Ser[21])PKI$_\alpha$(14−22)amide (numbering based upon native PKI$_\alpha$) is the most effective peptide substrate yet discovered for either kinase, although other peptides, while phosphorylated considerably less efficiently by PKG, are more specific. Although the inhibitory peptide corresponding to this sequence (i.e., with an Ala at position 21) is a much more potent inhibitor of PKA than of PKG (~250-fold), PKG actually exhibits a 60% higher k_{cat} than does PKA with the (Ser[21])PKI$_\alpha$(14−22)amide substrate peptide, with only a 20-fold higher K_m value. The two key PKI residues within this peptide which were found to be essential for substrate activity with both kinases were Arg[18] (P^{-3}) and Ile[22] (P^{+1}). The Arg[19] (P^{-2}) residue, which contributes significantly to both PKI-based peptide inhibitors and substrates of PKA, was only a more minor contributor to PKG substrate efficacy. Of particular note, the Phe[10] (P^{-11}) residue, which contributes very substantially to high affinity binding of both PKI and longer PKI peptide inhibitors, neither positively nor negatively affects the kinetics of either PKA or PKG with PKI-based substrates. A Phe[10] equivalent residue is not present in the linear sequence of any natural PKA substrate, and it would appear that the PKA hydrophobic binding pocket that recognizes Phe[10] may serve to uniquely contribute to the specificity of the interaction of PKI with PKA. Despite there being clear overall similarities in kinetics between PKI-based peptides acting as substrates for either PKA or PKG, as would be expected given the high degree of homology between the two protein kinases, several subtle kinetic differences suggest some variance between the catalytic processes in each, and these differences are likely correlated with key amino acid substitutions in the catalytic sites of the two kinases.

The cAMP- and cGMP-dependent protein kinases are two members of a large family of protein kinases which catalyze the transfer of the terminal phosphoryl group from a donor nucleoside triphosphate to protein substrate. PKA[1] represents the protein kinase about which most information is known (Taylor et al., 1993). It is composed of two identical monomeric catalytic subunits and a dimerized pair of identical regulatory subunits. The inactive holoenzyme, R_2C_2, is activated by the binding of two moles of cAMP per mole of regulatory subunit, leading to the apparent release of two free active catalytic subunits from the regulatory subunit dimer (Johnson et al., 1993). The catalytic subunits of PKA phosphorylate a large variety of proteins within the cell in a coordinated manner resulting in cell-specific physiological effects. How this coordination is achieved is not well understood but is likely to be due in part to variations in the phosphorylation sequences of natural substrates (Walsh et al., 1992; Walsh & Van Patten, 1994). PKG is highly homologous to PKA, but in contrast to PKA it is a homodimer composed of subunits that contain contiguous catalytic and regulatory domains on the same polypeptide chain (Scott, 1991; Hofmann et al., 1992). In the absence of cGMP, PKG is essentially inactive. Its allosteric activation by cyclic nucleotide involves a conformational change in the relationship between the regulatory and catalytic domains, but no subunit dissociation.

The initial studies to evaluate the amino acid sequence determinants for substrate phosphorylation by PKA utilized the model peptide substrate Kemptide (LRRASLG) (Kemp et al., 1977; Feramisco et al., 1980; Hjelmquist et al., 1974; Edlund et al., 1975). Its sequence corresponds to that surrounding the phosphorylation site of hepatic pyruvate kinase, and it exhibits a K_m value that is very similar to that of the full-length protein (Pilkis et al., 1980). Studies examining which residues of Kemptide were important for making it an effective substrate for PKA demonstrated a crucial contribution by the pair of arginines that are on the amino-terminal side and separated by one amino acid from the target serine (Kemp et al., 1977; Feramisco et al., 1980; Zetterqvist et al., 1976). These studies also suggested the possible importance of a hydrophobic residue at the P^{+1} position [nomenclature of Knighton et al, (1991b)]. Further information concerning which amino acids contribute to high-affinity interaction at the substrate binding site of PKA has come from studies of the heat-stable inhibitor protein (PKI) of PKA. The PKI$_\alpha$ form of this protein, as originally isolated from rabbit skeletal muscle (Whitehouse & Walsh, 1982; Van Patten et al., 1992), is a potent competitive inhibitor of PKA (K_i = 0.1 nM) (Demaille et al., 1978; McPherson et

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[1] Abbreviations: PKA, the cAMP-dependent protein kinase; PKG, the cGMP-dependent protein kinase; PKI, the thermostable inhibitor protein of the cAMP-dependent protein kinase.

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Table 1: Phosphorylation of Kemptide and (Ser[21])PKI$_\alpha$(14−22)amide by cAMP- and cGMP-Dependent Protein Kinases[a]

		PKA			PKG		
substrate	peptide sequence	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})
Kemptide	LRRASLG	4.7	640	140	210	1700	8.1
(Ser[21])PKIα(14−22)amide	GRTGRRNSI	0.11	1220	11000	2.5	1900	760

[a] Substrate phosphorylation reactions were carried out as described under Experimental Procedures and as described by Whitehouse et al. (1983) and Glass et al. (1989a). Apparent K_m (at near saturating ATP levels) and V_{max} values were determined from reciprocal plots of $1/v$ versus $1/[S]$. The calculated k_{cat} values for PKG are given per monomer of PKG to allow direct comparison with the k_{cat} of PKA.

al., 1979), and from it highly potent inhibitory peptides have been derived. Studies with PKI$_\alpha$(5−24)amide [numbering is based upon the sequence of full-length PKI (Scott et al., 1985)], and peptides based upon its sequence, have extended our understanding of the determinants for high-affinity binding to the substrate site of PKA (Cheng et al., 1985; Walsh et al., 1990). Amino acid substitution and deletion studies with PKI$_\alpha$(5−24)amide have identified Phe[10] (P^{-11}), Arg[15] (P^{-6}), Arg[18] (P^{-3}), Arg[19] (P^{-2}), and Ile[22] (P^{+1}) in the sequence ^{5}TTYADFIASGRTGRRNAI^{22} as the most critical residues for potent inhibition of PKA (Cheng et al., 1986; Glass et al., 1989a,b). The crystal structure of the catalytic subunit of PKA with the bound inhibitor PKI$_\alpha$(5−24)amide has then further allowed the determination of the docking sites for each of these residues within the structure of the enzyme (Knighton et al., 1991b; Zheng et al., 1993; Bossemeyer et al., 1993).

The substrate specificity of the cGMP-dependent protein kinase (PKG) is similar to that of PKA; however, it is not identical (Glass, 1990). Although PKG and PKA phosphorylate many of the same protein substrates *in vitro*, PKA is clearly much more effective with the majority of these substrates. Furthermore, it would appear that the amino acid determinants required for high-affinity interaction at the substrate binding site of PKG differ to some degree from those of PKA. PKG is only marginally inhibited by PKI-derived peptides, with K_i values 1000−10000-fold higher than those exhibited with PKA, and the full-length PKI does not inhibit PKG at all, even at concentrations in excess of 100 μM (Glass et al., 1986, 1992). To expand upon this comparison between substrate interactions with PKA and PKG, and to explore likely similarities and differences between both inhibitory peptide and substrate peptide binding to the protein kinase catalytic sites, we have evaluated a series of Ser[21]-containing PKI-derived peptides as substrates for the two cyclic nucleotide-dependent protein kinases.

EXPERIMENTAL PROCEDURES

Synthetic Peptides. (Ser[21])PKI$_\alpha$(6−22)amide and selected analogs were synthesized as COOH-terminal amides by solid-phase techniques, purified to homogeneity, and characterized by analytical HPLC and amino acid analysis, as previously described by Glass et al. (1989a). The composition and stoichiometry of amino acids in each of the synthesized peptides were as expected.

Enzyme Purification. PKG and the catalytic subunit of PKA were purified to homogeneity from bovine lung as described by Glass and Krebs (1979) and from beef heart as described by Bechtel et al. (1977), respectively.

Synthetic Peptide Phosphorylation. Phosphorylation of peptides by PKA was carried out as follows. Assays were conducted for 1 min at 30 °C in a reaction volume of 0.08 mL containing 25 mM MES buffer (pH 6.8), 5 mM magnesium acetate, 32 μM [γ-^{32}P]ATP (6000 cpm/pmol), 3.75 mM 2-mercaptoethanol, 0.5 mg/mL bovine serum albumin, a range of concentrations of phospho-accepting substrate, and protein kinase catalytic subunit. The amount of enzyme used per assay was adjusted (1.87 ng/mL−12.5 ng/mL) depending on the degree of substrate utilization to ensure assay linearity. Assays were initiated by the addition of catalytic subunit. Reactions were terminated by the addition of 60 μL of 50% acetic acid and [^{32}P]phosphopeptide was quantitated in 50 μL aliquots of the reaction mixture by the phosphocellulose paper method using an acetic acid washing procedure (Glass et al., 1978; Roskoski, 1983). Phosphorylation of peptides by PKG was carried out as follows. Assays were conducted for 2 min at 30 °C in a reaction volume of 0.08 mL containing 30 mM Tris-HCl (pH 7.4), 2 mM magnesium acetate, 1 μM cGMP, 32 μM [γ-^{32}P]ATP (6000 cpm/pmol), 3 mM 2-mercaptoethanol, 0.25 mg/mL bovine serum albumin, a range of substrate concentrations, and 190 ng/mL PKG (except where noted). Reactions were initiated by the addition of enzyme and terminated by the addition of 60 μL of 50% acetic acid. [^{32}P]-Phosphopeptide was quantitated as above for PKA with the exception of using paper washes of phosphoric acid (Glass et al., 1978; Roskoski, 1983). Kinetic constants were determined from linear regression analysis of the reciprocal plots.

Materials. [γ-^{32}P]ATP was synthesized as described by Glynn and Chappell (1964) as modified by Reimann et al. (1971). Phosphocellulose paper was purchased from Whatman. Kemptide was purchased from Peninsula Laboratories.

RESULTS

Phosphorylation of Substrate Peptides by cAMP-Dependent Protein Kinase. Shown in Table 1 are the K_m, k_{cat}, and k_{cat}/K_m values for the phosphorylation of Kemptide and (Ser[21])PKI$_\alpha$(14−22)amide by PKA, as well as the corresponding data for PKG which will be discussed in a later section. Consistent with previous studies of the phosphorylation of peptide substrates by PKA, (Ser[21])PKI$_\alpha$(14−22)-amide is a very high affinity substrate for the kinase (Glass et al., 1989a) and in fact is a much better substrate for the enzyme than is Kemptide by a factor of 81-fold by the criterion of k_{cat}/K_m (Table 1). It is kinetically the best peptide substrate for PKA so far identified. We have evaluated the importance of a number of the individual amino acid residues for substrate phosphorylation in a series of PKI-derived substrate analogs containing substitutions and additions of amino-terminal residues, specifically in reference to the known effects of those residues on the efficacy of inhibition of PKA.

Table 2: Effect of Amino Acid Substitution on Phosphorylation of PKI-Derived Substrate Peptides by cAMP- and cGMP-Dependent Protein Kinases[a]

substrate	peptide sequence	PKA K_m (μM)	PKA fold k_{cat}	PKA fold change in k_{cat}/K_m	PKG K_m (μM)	PKG fold k_{cat}	PKG fold change in k_{cat}/K_m
(Ser^{21})PKI$_\alpha$(14-22)amide	GRTGRRNSI	0.12	1.0	1.0	2.4	1.0	1.0
(Ala^{14}Ser^{21})PKI$_\alpha$(14-22)amide	**A**RTGRRNSI	0.13	1.2	1.1	2.3	1.6	1.67
(Lys^{15}Ser^{21})PKI$_\alpha$(14-22)amide	G**K**TGRRNSI	0.22	1.1	0.60	5.8	1.4	0.58
(Ala^{16}Ser^{21})PKI$_\alpha$(14-22)amide	GR**A**GRRNSI	0.14	1.4	1.2	3.8	1.2	0.75
(Leu^{17}Ser^{21})PKI$_\alpha$(14-22)amide	GRT**L**RRNSI	0.19	1.2	0.79	4.5	0.43	0.23
(Lys^{18}Ser^{21})PKI$_\alpha$(14-22)amide	GRTG**K**RNSI	21.2	0.59	<0.01	140.0	0.10	<0.002
(Lys^{19}Ser^{21})PKI$_\alpha$(14-22)amide	GRTGR**K**NSI	3.3	0.60	0.02	7.4	0.70	0.22
(Ala^{20}Ser^{21})PKI$_\alpha$(14-22)amide	GRTGRR**A**SI	0.15	1.7	1.3	3.6	0.60	0.39
(Gly^{22}Ser^{21})PKI$_\alpha$(14-22)amide	GRTGRRNS**G**	1.6	1.0	0.09	15.0	0.20	0.03
(Ser^{21})PKI$_\alpha$(11-22)amide	IASGRTGRRNSI	0.18	1.4	1.0	2.7	0.74	0.64
(Ser^{21})PKI$_\alpha$(6-22)amide	TYADFIASGRTGRRNSI	0.32	0.56	0.21	7.0	0.79	0.26
(Ala^{7}Ser^{21})PKI$_\alpha$(6-22)amide	TY**A**DFIASGRTGRRNSI	0.34	1.0	0.34	12.0	0.50	0.09
(Ala^{10}Ser^{21})PKI$_\alpha$(6-22)amide	TYADF**A**IASGRTGRRNSI	0.51	0.84	0.20	16.0	0.88	0.12
(Trp^{10}Ser^{21})PKI$_\alpha$(6-22)amide	TYADF**W**IASGRTGRRNSI	0.23	0.57	0.29	ND	ND	ND

[a] Substrate phosphorylation reactions were carried out as described under Experimental Procedures, and the data were calculated as described in Table 1. Fold k_{cat} and k_{cat}/K_m values, for both PKA and PKG, are expressed relative to the k_{cat} and k_{cat}/K_m values for (Ser^{21}) PKI$_\alpha$(14−22)amide with each enzyme, respectively. ND, not determined.

Shown in the top half of Table 2 are the K_m values for phosphorylation of the PKI$_\alpha$(14−22)amide-based substrate analogs, along with the fold changes in both k_{cat} and k_{cat}/K_m values relative to the parent peptide (Ser^{21})PKI$_\alpha$(14−22)-amide. Each of the amino acids of this substrate sequence (other than the target serine) was tested using the same substitution as used in the previous studies that examined their effects on inhibitory potency (Glass et al., 1989a). Except in three cases, the effect of these amino acid substitutions on the K_m values with PKA was minimal (not greater than 2.5-fold). The substitution of either Arg^{18}, Arg^{19}, or Ile^{22}, however, increased the K_m value dramatically. These changes mirror the effects observed upon their substitution in inhibitory peptides (Cheng et al., 1986; Glass et al., 1989a). Figure 1 shows a comparison between fold changes in K_m and K_i values of the substituted peptides versus the corresponding parent peptides, (Ser^{21})PKI$_\alpha$(14−22)amide and PKI$_\alpha$(14−22)amide, respectively (Cheng et al., 1986; Glass et al., 1989a). It is important to note that whereas a K_i value directly correlates with inhibitor binding affinity, also encompassed within a K_m value are elements of the rate parameters of the catalyzed reaction. Quite clearly, however, the substitutions in the pseudosubstrate region (residues 14−22) of the peptide that produced the greatest effect on K_i also had the greatest effect on K_m, suggesting considerable similarities in the residues that are critical for both substrate and inhibitor efficacy. The substitution of Arg^{15} by lysine appeared to have a somewhat greater effect upon the inhibitory potency of PKI peptides, with a 8-fold effect on the K_i value with PKA (Cheng et al., 1986), whereas the K_m value for peptide (Lys^{15}, Ser^{21})PKI$_\alpha$(14−22)amide was only 2-fold higher than the parent substrate peptide.

In addition to the changes in the pseudosubstrate region of (Ser^{21})PKI$_\alpha$(14−22)amide, we have evaluated the importance of additional amino-terminal residues, which in the case

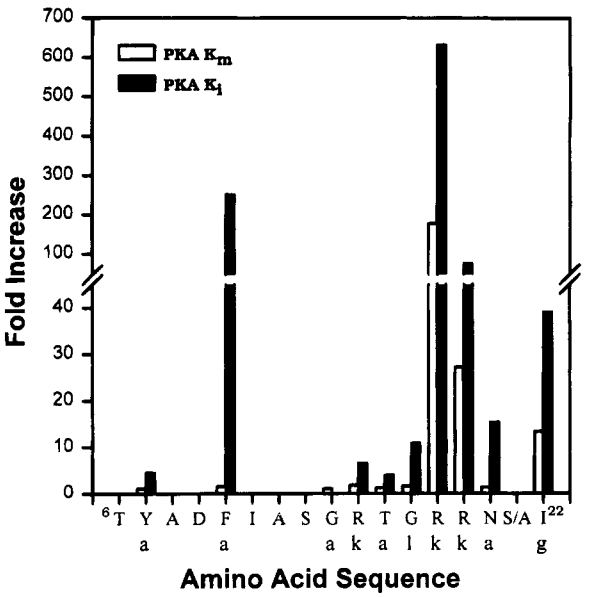

FIGURE 1: Comparison of the effects of amino acid substitution in PKI peptides on their phosphorylation by and inhibition of the cAMP-dependent protein kinase. Fold changes in K_m values (open bars) are expressed relative to either (Ser^{21})^{1}PKI$_\alpha$(6−22)amide or (Ser^{21})PKI$_\alpha$(14−22)amide parent peptides (Table 2), in comparison to K_i values (closed bars) for equivalent substitutions in corresponding inhibitor peptides (Glass et al., 1989a,b; Cheng et al., 1986). The lowercase letters represent the one-letter code for the substituted amino acid.

of the longer inhibitory peptide, PKI$_\alpha$(6−22)amide, contribute substantially to the binding to PKA. Residues 6−11 within PKI$_\alpha$(6−22)amide form an amphipathic α-helix (Reed et al., 1987, 1989) that contributes to the positioning of Phe^{10} of PKI within an aromatic/hydrophobic binding pocket on the large lobe of PKA (Knighton et al., 1991a; Zheng et al., 1993; Bossemeyer et al., 1993). Clearly Phe^{10} is critical for

potent inhibition of PKA, as its substitution with Ala causes a dramatic reduction (>250-fold) in inhibitory potency (Glass et al., 1989a,b). In contrast, Phe[10] appears to make little contribution to the catalytic interactions of PKI-based substrate peptides (Figure 1 and Table 2). Its substitution in (Ser[21])PKI$_\alpha$(6−22)amide by either alanine, which would remove the aromatic moiety, or tryptophan, which would increase the aromaticity, produced little change in either k_{cat} or K_m values. In addition, the longer peptide (Ser[21])PKI$_\alpha$-(6−22)amide containing Phe[10] was only slightly poorer as a substrate than was (Ser[21])PKI$_\alpha$(14−22)amide. These results are in contrast to what is observed with these types of substitutions or deletions with inhibitory PKI-peptides (Glass et al., 1989a,b) and suggest that, in comparison to the significant contribution that Phe[10] makes to the binding energy of inhibitory PKI peptides with PKA, such a residue has little role in dictating substrate efficacy. Also examined in these studies was the intermediate length peptide, (Ser[21])-PKI$_\alpha$(11−22)amide. In the case of inhibitory peptides this 12-residue peptide was 50-fold worse as an inhibitor than PKI$_\alpha$(6−22)amide and even 2-fold worse as an inhibitor than the shorter peptide PKI$_\alpha$(14−22) amide. This has been suggested to indicate that when residues 11, 12, and 13 are not tethered as a consequence of the Phe[10] binding interaction, they can partially disrupt the binding by the residues of the pseudosubstrate domain (Glass et al., 1989a). In contrast, there was little difference between (Ser[21])PKI$_\alpha$(6−22)amide, (Ser[21])PKI$_\alpha$(11−22)amide, and (Ser[21])PKI$_\alpha$(14−22)amide as PKA substrates.

Comparison of Phosphorylation of Substrates Peptides by cGMP- and cAMP-Dependent Protein Kinases. A comparison of the kinetics of phosphorylation of Kemptide and (Ser[21])PKI$_\alpha$(14−22)amide by both PKA and PKG is presented in Table 1. (Ser[21])PKI$_\alpha$(14−22)amide is a most effective substrate for PKG, markedly better than Kemptide by about 2 orders of magnitude mainly due to a lower K_m value. By the criterion of k_{cat}/K_m, (Ser[21])PKI$_\alpha$(14−22)amide is in fact the most efficient peptide substrate for PKG yet identified (Glass, 1990; Colbran et al., 1992; Butt et al., 1994). It is important to note that by the criterion of $[k_{cat}/K_m]_{PKG}$ / $[k_{cat}/K_m]_{PKA}$ other peptide substrates have been identified that, although exhibiting lower k_{cat} and higher K_m values, are nevertheless more specific for PKG as compared to PKA (Colbran et al., 1992; Butt et al., 1994). Although both (Ser[21])PKI$_\alpha$(14−22)amide and Kemptide have lower K_m values for PKA than they do for PKG, both substrates exhibit higher k_{cat} values with PKG. This greater catalytic capability of PKG, even with substrates based upon peptides more physiologically relevant to PKA than to PKG, suggests that despite being highly homologous proteins there are at least some subtle differences between the actual catalytic processes of the two enzymes. The difference in K_m values of only ~20-fold observed with (Ser[21])PKI$_\alpha$(14−22)amide as a substrate for PKG versus PKA is of note, given that there is an approximately 250-fold difference between the two enzymes in their K_i values for PKI$_\alpha$(14−22)amide (Glass et al., 1986, 1989a, & 1992).

The role of the individual amino acids in dictating the substrate efficacy for PKG was further determined using PKI-based peptide substrate analogs (Table 2). Similar to PKA, the substitutions which produced the most noticeable increases in the K_m values of the substrate peptides for PKG were at positions Arg[18] and Ile[22] and, to a lesser degree,

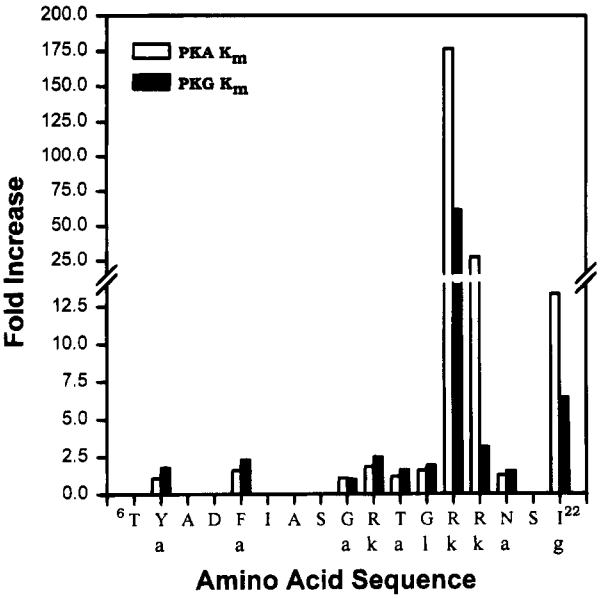

FIGURE 2: Effects of amino acid substitution in PKI peptides on their phosphorylation by cAMP and cGMP-dependent protein kinases. Comparison of the fold changes in the K_m values for either PKA (open bars) or PKG (closed bars), expressed relative to either (Ser[21])PKI$_\alpha$(6−22)amide or (Ser[21])PKI$_\alpha$(14−22)amide parent peptides (Table 2). The lowercase letters represent the one-letter code for the substituted amino acid.

Arg[19]. A comparison of the effects of residue substitution on PKG- and PKA-catalyzed phosphorylation is illustrated in Figure 2. For both PKG and PKA the substitution that produced the greatest effect by far was that of Arg[18], producing 60- and 180-fold increases in K_m values, respectively. Interestingly, while substitution of this arginine residue by lysine in (Ser[21])PKI$_\alpha$(14−22)amide caused a dramatic change in the K_m for both kinases, with PKG a very pronounced reduction in k_{cat} was also obtained, resulting in an overall decrease in k_{cat}/K_m value for this enzyme of >500-fold. This result suggests some differences between the catalytic processes in the two kinases. A difference in kinetic effects is also seen with PKG and PKA with substitution of the P^{+1} substrate residue. With both protein kinases, the elimination of the hydrophobic side chain of the isoleucine residue at P^{+1} causes significant increases in the K_m values, but with PKG there is also a marked decrease in the k_{cat} value. The large overall effect of the P^{+1} substitution on kinetic constants for substrate peptides with PKG appears somewhat in contrast to the much lesser effect observed with similar substitutions with inhibitory peptides (<2-fold); however, the parent peptides are themselves only weak inhibitors of PKG (Glass et al., 1992; unpublished data). The substitution of the P^{-2} Arg[19] residue by lysine caused a ~3-fold increase in the K_m value for PKG and an overall 5-fold decrease in the k_{cat}/K_m (Table 2). This seems in contrast to the more significant effect that this residue contributes to either substrate (Table 2) or inhibitory (Cheng et al., 1986) efficacy with PKA. There is only a very minimal effect of such a substitution on the inhibition of PKG by PKI peptides (Glass et al., 1986). The P^{-6} Arg[15] replacement by lysine showed only minor effects on the interaction of PKG with either PKI-derived peptide substrates (Table 2) or inhibitors (Glass et al., 1986).

As with substrates of PKA, Phe[10] appears to contribute minimally to the interaction and/or catalytic process of PKI-based substrate peptides with PKG. The lengthening of the

peptide from (Ser[21])PKI$_\alpha$(14−22)amide to (Ser[21])PKI$_\alpha$(6−22)amide increased the K_m value ~3-fold with little effect on the k_{cat} (Table 2). Similarly, the substitution of phenylalanine with alanine in this latter peptide caused no significant effect with PKG on the k_{cat} and just a 2.3-fold increase in K_m value (Table 2). The Phe[10] substitution by alanine also does not influence the weak inhibitory activity of PKI-based peptides with PKG (Glass et al., 1992; unpublished data). The equivalent substitution in inhibitory peptides with PKA results in a >250-fold increase in the K_i value (Glass et al., 1989b).

DISCUSSION

The results presented here, defining the residues critical to the efficiency of PKI-based substrate peptides of PKA, reflect to a substantial degree the conservation of amino acids flanking the phosphorylation sites of natural substrates of PKA (Walsh et al., 1992; Kemp & Pearson, 1990; Zetterqvist et al., 1990). Physiological substrates of PKA have a high degree of conservation of the P^{-2}−P^{-3} pair of arginines and the P^{+1} hydrophobic residue. The P^{-6} arginine is present at considerable less frequency in natural substrates, although this residue may be of more importance when the pair of arginines is compromised by either the absence of one of them or their substitution by the much less favored lysine (Zetterqvist & Ragnarsson, 1982). Both latter situations arise in several natural substrates (Walsh et al., 1992). Clearly from the data, Arg[18], Arg[19], and Ile[22] are the most important determinants for PKI-derived substrate peptide binding and catalysis by PKA, with only small contributions provided by the remaining residues. By inspection of the crystallographic binary and ternary complexes with PKI$_\alpha$(5−24)-amide bound to PKA (Knighton et al., 1991a; Zheng et al., 1993; Bossemeyer et al., 1993), it would appear that Arg[18], Arg[19], and Ile[22] may in fact be more than sufficient to dock the sequence surrounding the acceptor serine of substrates into the catalytic site. The side chain of Arg[18] bridges the small and large lobes of the kinase, while Arg[19] docks the peptide to the large lobe of the kinase, and Ile[22] would appear to fix the phosphorylatable residue at the catalytic center. It is now also clear why substitution of Arg[18] would have the most dramatic effect on both inhibitor and substrate binding. The Arg[18] side chain, in addition to bringing the peptide into the interface between the two lobes of PKA and being bound to both, is hydrogen bonded to the ribose of ATP, and it is likely also to be a key determinant of cleft closure (Olah et al., 1993). One could imagine that deletion or distortion of these interactions of Arg[18] would have dramatic effects on both binding and catalysis.

The lack of a role for Phe[10] in PKI substrate−peptide efficacy was in part a surprising finding. On the one hand this phenylalanine in PKI peptides clearly contributes very significantly to the energy of binding to PKA as inhibitors. On the other hand, a phenylalanine at the P^{-11} position has not been found in the linear sequence of any natural substrates of PKA (Walsh et al., 1992), other than in an autophosphorylation site in the kinase itself (Toner-Webb et al., 1992). The only difference between the substrate and inhibitory PKI analogs is the presence of a phosphorylatable hydroxyl group that distinguishes the alanine from the serine at position 21 in the peptide sequence. The crystal structure resolution of PKA has shown, however, that in the PKA-PKI-ATP·Mg ternary complex Ser[53] of PKA interacts with the peptide backbone carbonyl of the Ala[21] residue of PKI-peptide (Bossemeyer, 1994). It has been suggested that when a substrate is bound rather than an inhibitor, this interaction of Ser[53] with the peptide backbone would facilitate the orientation of the phosphoryl acceptor hydroxyl toward the donor γ-phosphate of ATP (Bossemeyer, 1994). In fact, in the structure of the enzyme crystallized with a serine-containing substrate peptide and Mg·ADP, the acceptor hydroxyl has been shown to become ideally positioned for transfer of the γ-phosphate (Mudhusdan et al., 1994). Such interactions with the acceptor seryl hydroxyl could lead to other changes in the kinase that would not arise with bound Ala[21]-containing inhibitor peptides. Of note, differences are apparent in the circular dichroism spectra of the PKA-Kemptide versus PKA-Ala-Kemptide complexes (Reed et al., 1985), and these changes, seen with the substrate but not the inhibitory peptide, have been suggested to arise as a consequence of a repositioning of an intrinsic PKA tyrosine residue when a substrate serine hydroxyl group is present (Reed et al., 1985). One result of the changes prompted by the presence of the acceptor serine hydroxyl in a ternary enzyme complex could be some distortion of the conformation of the PKA Phe[10]-binding pocket such that its contribution to the binding affinity of peptide substrates is minimized (Baude et al., 1994; Wen & Taylor, 1994). Our data suggest that there has been no selective pressure to retain the P^{-11} aromatic residue in physiological substrates since its role in modulating substrate efficacy is at most limited. An intriguing question which this study has brought to the surface is, if the Phe[10] aromatic binding pocket has no required function in the binding of substrates to PKA, why is it present? Phe[10] appears to be a key binding determinant for both the α and β forms of PKI (Van Patten et al., 1992). We would suggest that, in serving as a high-affinity docking site, the Phe[10] binding pocket of PKA is what creates the specificity for high-affinity PKI binding and that it is uniquely conserved in PKA for this purpose. Its presence may be particularly important for successful competition with substrates. The residues that bind Phe[10] in the large lobe of PKA (Knighton et al., 1991b; Bossemeyer et al., 1993) are absent in PKG (Glass et al., 1992) and, as a likely consequence, PKG is not inhibited by PKI (Kuo et al., 1976; Glass et al., 1986). The Phe[10] binding pocket on PKA also does not appear to have a role in the binding of PKA regulatory subunit since there is no phenylalanine at the equivalent positions in any of the subunit isoforms. The Phe[10] binding pocket is conserved in both the mammalian C_α and C_β isoforms of PKA catalytic subunit, and clearly also to some extent in yeast PKA (Glass et al., 1992). Interestingly, the C_γ isoform of PKA, in which the Tyr[235] and Phe[239] residues of the Phe[10] binding pocket have been changed to phenylalanine and tyrosine, respectively, is resistant to PKI$_\alpha$(5−24)amide inhibition (Beebe et al., 1992).

PKA and PKG are highly homologous proteins (Scott, 1991). The crystal structure of PKG has yet to be determined; however, molecular modeling of its catalytic domain, as based upon the X-ray coordinates of the binary complex of PKA with PKI(5−24)amide, suggests that the domains of the two kinases are structurally very similar (Hofmann et al., 1992). Thus it is not surprising that there would be considerable similarity between them in their interaction with PKI-derived substrate peptides (Figure 2). This similarity is most apparent in the observed increases in K_m values as a

consequence of substitution of the P^{-3} and P^{+1} residues of $(Ser^{21})PKI_{\alpha}(14-22)$ amide. Such parallel effects can be readily explained by the conservation in PKG of the two known PKA binding determinants for Arg^{18} (Glu^{127} and Asp^{328}, numbering based upon PKA; Knighton et al., 1993) and the conservation of the hydrophobic character of binding determinants for Ile^{22} (Glass et al., 1992).

Despite the similarities between PKA and PKG, there appear to be several subtle but key distinctions between the two enzymes. One difference is that whereas both (Ser^{21})-$PKI_{\alpha}(14-22)$amide and Kemptide are more efficacious as substrates for PKA than PKG, as based upon K_m, they are less effective based upon k_{cat}. A second apparent distinction is that the substitution of either the P^{-3} or P^{+1} residue markedly diminishes the k_{cat} of the reaction with PKG, while having little to no effect upon that with PKA. Since the K_i value is a direct measurement of binding affinity, whereas the K_m value encompasses not only the binding interaction of substrate to enzyme but also some of the components of the catalytic process, a direct comparison of K_i and K_m values is inappropriate. An examination of the relative fold differences between the sets of data for the inhibitors and substrates, however, provides valid insights of the differences between the two protein kinases. In contrast to the large difference observed between the effectiveness of PKI peptides with PKA and PKG, the PKI-based substrates were much more equal in their effectivness with the two enzymes. The PKI-based substrate peptide, $(Ser^{21})PKI_{\alpha}(14-22)$amide, is an effective substrate for PKA, and the inhibitory Ala^{21}-containing peptide clearly interacts extremely tightly with PKA with a noteably low K_i value (Glass et al., 1989a). The PKI-based inhibitory peptides, on the other hand, only poorly inhibit PKG, being some 1000-fold less effective with it than with PKA (3−40 nM vs 8−31 μM; Glass et al., 1986, 1989a, 1992), yet the PKI-based substrate peptide $(Ser^{21})PKI_{\alpha}(14-22)$amide is the most effective peptide substrate yet identified for PKG as based upon the criterion of k_{cat}/K_m (Tables 1 and 2; Colbran et al., 1992; Butt et al., 1994). A similar trend of marked differences as inhibitors of the two protein kinases, but similarities as substrates, is also observed in the changes that occur with residue replacements. The substitution of either the P^{-3} or P^{+1} residues has the greatest effects in the modulation of the inhibition of PKA (Cheng et al., 1986; Glass et al., 1989a), has pronounced effects as substrate determinants for both PKA and PKG, but only minimally alters the peptides as inhibitors of PKG (Glass et al., 1992).

There are a number of possible explanations for some of the differences between PKA and PKG with respect to the relationship between PKI-derived substrate and inhibitory peptides. One obvious distinction between PKA and PKG is that, in the assays of peptide efficacy, PKA assays are done using free catalytic subunit whereas the PKG assays have utilized the PKG holoenzyme, containing both catalytic and regulatory domains, albeit activated. It is possible that the presence of the regulatory domain of PKG might produce some effects which would not be the case with free catalytic subunit of PKA. There are also some key differences between PKA and PKG in the residues involved in substrate binding and the catalytic reaction, which may well be the basis for some of the observed differences between the two enzymes. As detailed above, the P-loop Ser^{53} of PKA, via its interaction with both the substrate peptide backbone and the γ-phosphate of ATP, appears to assist in the positioning of the target seryl hydroxyl (Bossemeyer et al., 1993; Mudhusdan et al., 1994). The importance of the nature of the residue at this position has also been emphasized by the studies of phosphorylase kinase. In the catalytic γ subunit of this enzyme, the residue in the P-loop equivalent to Ser^{53} is a valine. Its mutation to a serine, i.e., restoring it back to what is present in PKA, results in enhanced ternary catalytic complex formation, but coupled to a markedly reduced V_{max} (Lee et al., 1992). In the P-loop in PKG, the residue equivalent to Ser^{53} in PKA is a glycine, a substitution which clearly cannot contribute in the same manner to the catalytic reaction as does Ser^{53} in PKA. In PKA, Thr^{51} of the P-loop interacts with the 3′-hydroxyl of the ribose moiety of ATP via Arg^{18} of PKI-peptide (Bossemeyer et al., 1993; Bossemeyer, 1994) and therefore is a likely contributor to the cleft closure necessary for effective catalysis. In PKG the residue in an equivalent position to Thr^{51} is a valine. There are also substitutions in the adenine base hydrophobic pocket of the two kinases. Tyr^{122}, Val^{123}, and Leu^{173} of PKA are replaced in PKG by alanine, cysteine, and isoleucine, respectively, and likely correlated with such changes are noted differences in the ATP analog specificity of the two enzymes (Flockhart et al., 1984). All together, these various changes in central residues in PKG in comparison to those in PKA, and likely others as well, are candidate contributors to the differences observed in this current study between the two kinases and their interactions with PKI-based substrates and inhibitors. An important tool to resolve the subtle but consequential distinctions between the two kinases in their modes of interaction with substrates will be the crystallographic resolution of PKG in binary and ternary complexes with $(Ser^{21})PKI_{\alpha}(14-22)$amide. Such a type of assessment is important for an understanding of the difference in substrate specificities between the two enzymes that exist physiologically. The PKI-derived peptides have provided a sound basis for comparison of PKA and PKG, given that they are excellent substrates for both enzymes.

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