Precision Targeting of Protein Kinases

AN AFFINITY LABEL THAT INACTIVATES THE cGMP- BUT NOT THE cAMP-DEPENDENT PROTEIN KINASE*

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Although the cAMP-dependent (PKA) and cGMP-dependent protein kinases (PKG) usually participate in unrelated biological processes, their enzymological properties are decidedly similar. Based upon the multitude of comparative studies conducted to date, it appears that these two enzymes exhibit very similar peptide substrate specificities. Furthermore, most inhibitors that have been reported for PKG serve in a nearly equal capacity for PKA. Consequently, the task of distinguishing between these enzymes, especially under in vivo conditions, has proved to be daunting. However, we recently have found that PKA will only phosphorylate non-amino acid residues whose ω-configuration corresponds to that found in l-amino acids, whereas PKG will catalyze the phosphorylation of residues corresponding to both l- and d-amino acids (Wood, J., Mendelow, M., Yan, X., Corbin, J. D., Francis, S. H., and Lawrence, D. S. (1996) J. Biol. Chem. 271, 174-179).

Based upon these results, we have designed a potent affinity label for PKG (K<sub>I</sub> = 21.1 ± 4.7 μM), that has no measurable activity toward PKA. This represents the first example of an peptide-based inactivator that fully distinguishes between these two closely related enzymes. These results suggest that a similar strategy may provide highly specific inactivators for other protein kinases as well.

The discovery of signal transduction pathways has revealed how external stimuli elicit biochemical responses from target cells (1-2). Additionally, it has provided a fundamentally new approach for therapeutic intervention in various disease states (3). Protein kinases are the most abundant members of these signaling pathways, and aberrant forms of these enzymatic species have been directly linked to abnormalities in cell growth and differentiation. In short, protein kinase inhibitors may ultimately spawn a new generation of potent antineoplastic agents. However, the design of inhibitors that are targeted for specific protein kinases is a daunting undertaking because of the size of this enzyme family, the relatively general nature of the reaction catalyzed by these species, and the conservation of common catalytic features. This challenge is exemplified by the multitude of studies that have sought to distinguish the cGMP-dependent protein kinase (PKG) from its cAMP-dependent counterpart (PKA) (4-14). These studies have primarily sought to exploit relatively minor differences exhibited by these enzymes in their preferences for specific peptide substrates and ATP analogs. Unfortunately, those synthetic peptides identified as substrates for PKG serve in the same capacity for PKA.

In addition, although some ATP analogs act as powerful inhibitors of PKG, the specificity with which they target PKG, without interfering with the activity of PKA, is subtle at best (14). Finally, although PKG and PKA are activated by different effector molecules, cGMP is essential for the activity of PKG as well as other intracellular cGMP receptors. Consequently, cGMP cannot be readily eliminated or replaced. In short, it is clear that some kinases can be distinguished by their substrate sequence specificity, their ability to accommodate ATP analogs, and/or their reliance upon different effector molecules. Unfortunately, it is extremely unlikely that all protein kinases can be uniquely identified based on these particular traits.

We have recently observed dramatic differences in the active site specificity of protein kinases (15-17). Although all serine/threonine-specific protein kinases phosphorylate serine and threonine in peptide and protein substrates, these enzymes exhibit profound differences in their abilities to catalyze the phosphorylation of unnatural alcohol-bearing residues (18-23). Analogous discrepancies in the active site specificities of tyrosine-specific kinases have been encountered as well (16). Consequently, it should be possible to design protein kinase-specific inhibitors based upon active site properties that are unique to individual enzyme targets. We report herein that the disparate active site specificities of PKA and PKG have been utilized to construct the first example of a PKG-specific peptide-based inactivator, a species that has no measurable effect on the catalytic activity of PKA.

MATERIALS AND METHODS

All chemicals were obtained from Aldrich, except for [γ-<sup>32</sup>P]ATP (DuPont NEN), cGMP and cAMP (Sigma), bovine serum albumin (Sigma), protected amino acid derivatives and Rink resin (Advanced ChemTech), and Liquiscint (National Diagnostics). cAMP-dependent and cGMP-dependent Protein Kinase Preparations—Both PKA (catalytic subunit) and PKG-Iα were isolated and purified as described previously (24-25).

Preparation of Leu-Arg-Arg-Arg-Arg-Phe-Ala-Phe-Cys[3-nitro-2-pyridinesulfenyl-(Npys)]-amide (1 and 2) — Fmoc-Leu-Arg[4-methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr)]-Arg[Mtr]-Arg[Mtr]-Arg[Mtr]-Phe-Ala-Phe-Cys[triphenylmethyl (Trt)]- and Leu-Arg-Arg-Arg-Arg-Phe-Ala-Phe-Cys[Trt]- were prepared on the 4(2, 4-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin (Rink resin, 0.3 mmol/g substitution level) with Fmoc-amino acids (26). A standard Fmoc-amino acid/benzotriazolyl-3-oxazolid-N-oxide(dimethylamino)
phosphonium hexafluorophosphate (Bpp) peptide synthesis protocol was employed using a fully automated Biosearch 9600 peptide synthesizer. The Leu-Arg(Mtr)-Arg(Mtr)-Arg(Mtr)-Arg(Mtr)-Arg(Phe)-DL-Ala-Phe-Cys(Tt)-Rink resin was treated with NpysCl (10 equivalents) in DMF for 24 h at room temperature. Excess NpysCl was removed via extensive washing with DMF. The peptides were subsequently cleaved from the resin and deprotected with 9:1 trifluoroacetic acid/thioanisole and subsequently precipitated from solution with ethyl ether. The solids were collected, dissolved in water, and purified on an ion exchange column (SP-Sephadex C-25; 0.6–1.2 M KCl gradient in 50 mM NaOAc, pH 3.5, 300 ml total volume). The peptides were desalted and further purified via preparative high performance liquid chromatography as described previously (17).

Inactivation Assays—Prior to enzyme inactivation, diithiothreitol or β-mercaptoethanol was removed from enzyme stock solutions by dialysis (48 h, argon, 4°C). Inactivation of the protein kinases was performed in the presence (100 mM MOPS, 150 mM KCl, 0.125 mg/ml BSA, 50 mM MgCl2, 10 mM ATP, pH 7.1, 5°C) and absence (100 mM MOPS, 150 mM KCl, 0.125 mg/ml BSA, pH 7.1, 5°C) of ATP. Inactivation of PKA was accomplished by incubating the enzyme (500 μg to 2 μg) with a minimum of five different concentrations (encompassing the Kᵢ₈) of peptide inactivator. At selected time intervals, aliquots were removed and diluted 100-, 500-, or 1000-fold into an ice-cold enzyme dilution buffer containing 100 mM MOPS, 150 mM KCl, and 0.125 mg/ml BSA (pH 7.1). No further inactivation occurred after dilution of the inactivation reaction mixtures. Residual PKA activity was determined by addition of 10 μl of diluted enzyme to 40 μl of an assay solution (17). Inactivation of PKG (250 μM) was performed as described for PKA except that 20 μM cGMP was present during inactivation and subsequent assay of residual PKG activity.

RESULTS

The inhibitory efficacies of peptides 1 and 2 (see Structures 1 and 2) toward PKA and PKG are summarized in Table 1. No time-dependent loss in PKA activity is observed with peptide 2 (at concentrations as high as 1 mM) in the presence of MgATP. Time-dependent loss in PKA activity is observed with peptide 1 and 2) toward PKA and PKG are summarized in Table I. No methylbenzenesulfonyl.

2 PKG also contains a threonine that, according to sequence alignments, should maintain an active site position analogous to that of Thr-201 in PKA. One possible explanation for the differences in the active site substrate specificities of these two enzymes is that the orientation of the threonine residue in PKG does not impede ω-residue occupancy of the active site. However, other factors may be responsible for the dramatic substrate specificity differences exhibited by these two enzymes. See Ref. 17 for a more complete discussion.

3 We are indebted to Professor Susan S. Taylor for pointing out the close proximity between the P + 2 residue of PKI(5–24) and Cys-199.
activity (Fig. 1a). In contrast, PKA is unable to accommodate, at the P subsite, residues that contain an \(\alpha\)-configuration corresponding to d-amino acids. Consequently, the configuration associated with the d-Ala peptide 2 should preclude an orientation in the active site that would allow for Cys-199 modification (Fig. 1b).

PKG also contains a cysteine residue that, on the basis of dose primary sequence homology, should occupy a position analogous to that of Cys-199 in PKA (36). However, unlike PKA, PKG can productively incorporate \(\alpha\)-residues within the catalytic site (17). In short, whereas peptide 1 should inactivate both PKA and PKG, peptide 2 should be effective only against PKG. We have established the validity of this premise and have also obtained some unexpected results with respect to the inactivation of PKA. These results are as follows.

The L-Ala-based Affinity Label 1 Is Unprecedented in That MgATP Does Not Block PKA Inactivation—The L-Ala-containing species 1 potently inactivates PKA in a time-dependent fashion (Table I). Interestingly, the \(k_i\) and \(k_2\) values obtained from these inactivation experiments are MgATP-dependent. The \(k_i\) for peptide 1 is approximately 4-fold better in the presence than in the absence of MgATP. One possible explanation for this behavior is that ATP produces a conformational change that reorients critical active site functionality, a notion consistent with previously reported physical measurements of PKA (37). However, the ATP-induced improvement in \(k_i\) is offset by a 6-fold drop in \(k_2\) (the unimolecular rate constant for covalent modification of the enzyme). In short, while the individual \(k_i\) and \(k_2\) values are MgATP-dependent, the inhibitory efficacy (i.e. \(k_i/k_2\)) of 1 does not dramatically differ in the presence or absence of MgATP. In this sense, the behavior of peptide 1 is unlike that of any other cysteine-directed inactivator of PKA. ATP has been previously shown to protect the Cys-199 sulfhydryl against covalent modification (27, 29–35). Originally, it was suggested that the \(\gamma\)-phosphate of ATP must be in close proximity to the sulfhydryl moiety since ATP is more effective than ADP in impeding the inhibitory behavior of cysteine-targeted inactivators. However, the ternary structure of PKA-ATP-PKI(5–25) reveals that the \(\gamma\)-phosphoryl group is too far from Cys-199 to directly interfere with covalent modification (28). Consequently, the protective influence of ATP most likely occurs via an indirect mechanism, such as a change in active site conformation. This structural alteration may preclude ready access of cysteine-directed modifying agents to Cys-199. Nonetheless, the inhibitory behavior of peptide 1 is clearly distinct from that of previously described affinity labels in that it inactivates PKA, even in the presence of MgATP. This may very well be due to the location of the electrophilic Npys disulfide moiety on the catalytic site-directed peptide. The structure of the PKA-MgATP-PKI(5–24) ternary complex reveals that the P + 2 residue on the active site-bound PKI(5–25) peptide is in close proximity to Cys-199.

The L-Ala-based Affinity Label 1 Is a Potent Inhibitor of PKG—PKG, like its cAMP-dependent counterpart, utilizes L-amino acids, as well as configurationally analogous species (i.e. 4) as substrates (17). Consequently, the L-Ala-containing peptide 1 should bind to the active site of PKG in a fashion that results in enzyme inactivation. Indeed, inactivation is observed

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**Table I**

| Inactivator | cAMP-dependent protein kinase | cGMP-dependent protein kinase |
|-------------|-------------------------------|------------------------------|
|             | With ATP | Without ATP | With ATP | Without ATP |
| LRRRRF AFC(Npys) (1) |       |             |       |             |
| \(k_i (s^{-1})\) | 25.4 ± 1.9 | 104 ± 19 | 15.1 ± 2.2 | 12.7 ± 2.3 |
| \(k_2 (s^{-1} \cdot M^{-1})\) | 228 ± 10 | 380 ± 110 | 1090 ± 56 | 2600 ± 190 |
| LRRRRF(o) AFC(Npys) (2) |       |             |       |             |
| \(k_i (s^{-1})\) | 108 ± 12 | 21.1 ± 4.7 | 80 ± 12 |             |
| \(k_2 (s^{-1} \cdot M^{-1})\) | 791 ± 32 | 249 ± 30 | 1070 ± 47 |             |

*No inactivation was observed after incubation with 1 mM LRRRRF(o) AFC(Npys) (2) for 20 min.*
both in the presence and absence of MgATP (Table I). We previously demonstrated that MgATP offers substantial protection against thiol-specific reagents in PKG (38). Consequently, it appears likely that peptide 1 binds to both PKG and PKA in a manner that favorably positions the electrophilic disulfide moiety adjacent to the active site cysteine.

The D-Ala-based Affinity Label 2 Fails to Inactivate PKA in the Presence of ATP, but Is a Powerful PKG Inactivator—In contrast to the L-Ala affinity label, no time-dependent inactivation of PKA is observed with the D-Ala-containing peptide 2 in the presence of MgATP. This result is consistent with the fact that PKA does not utilize peptide 5 (and other D-amino acid-related species) as substrates. However, 2 does inactivate PKA in the absence of MgATP. Furthermore, the inactivation constants for 1 and 2, when MgATP is not present, are nearly identical. In short, all available evidence suggests that PKA is unable to accommodate D-amino acid residues within the active site region if MgATP is present. In the absence of MgATP, this stereo-preferential behavior vanishes.

Unlike PKA, PKG can accommodate D-residues in the presence of MgATP (e.g. PKG catalyzes the phosphorylation of peptide 5). Consequently, the D-Ala-containing affinity label 2 should bind to PKG in a fashion that promotes Cys modification and concomitant enzyme inactivation. Indeed, it does. The $K_i$ associated with 2 (21.1 ± 4.7 μM with ATP) is only slightly higher than that obtained with 1 (15.1 ± 2.2 μM). The PKG-inhibitory potency of 2 is in marked contrast to its absolute impunity as an inactivator of PKA (i.e. with MgATP present). This behavior is remarkably clean, particularly in light of the notoriously broad overlapping substrate specificity of these protein kinases. Peptide 2 is the first example of a PKG-directed inactivator that has no measurable influence on the catalytic power of PKA. We note that this discriminatory behavior is only observed in the presence of MgATP. However, the predominant in vivo cyclic nucleotide-activated forms of these two protein kinases contain active site-bound ATP, the state required for selective inhibition. Although the reactive disulfide functionality may not be appropriate for in vivo studies (due to high intracellular glutathione concentrations), other suitably appended groups, that can serve as transition state analogs, suicide substrates, or even simple reversible inhibitors, should provide the requisite stability to ensure selective inhibition. In addition, we note that since these peptide-based species are unlikely to be membrane-permeant, in vivo studies would likely necessitate the use of micro injection technology.

We have only recently begun to analyze the ability of protein kinases to recognize and phosphorylate non-amino acid moieties. Nevertheless, a surprising picture has begun to emerge. Members of the same protein kinase family (i.e. serine-threonine-specific or tyrosine-specific) do not necessarily share the same specificity for nonstandard residues. Consequently, it should be possible to generate enzyme-targeted inhibitors by exploiting observed differences in the active site specificities of even closely related enzymes. Although such differences in active site behavior may be surprising, behavioral patterns of this sort are not unprecedented. For example, specific protein kinases can be selectively inhibited with certain ATP analogs, in spite of the fact that all protein kinases utilize ATP (39–40). In the case of naturally occurring substrates, the active sites of different protein kinases accommodate chemically identical moieties (e.g. ATP and serine). Clearly, however, a shared in vivo specificity does not necessarily imply an equally shared affinity for chemical entities that are synthetic in origin.

In summary, we have created the first example of a PKG-specific inhibitor by exploiting the unique active site recognition properties exhibited by this enzyme. If further differences in the active site behavior of protein kinase family members continue to emerge, then a similar strategy may be brought to bear on the general issue of the precision targeting of protein kinases.

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