High Affinity Binding of the Heat-stable Protein Kinase Inhibitor to the Catalytic Subunit of cAMP-dependent Protein Kinase Is Selectively Abolished by Mutation of Arg\textsuperscript{133}∗

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The two classes of physiological inhibitors of the catalytic subunit of cAMP-dependent protein kinase are the regulatory subunits and the heat-stable protein kinase inhibitors (PKIs), and both share a common mechanism of inhibition. Each has a similar inhibitor site that resembles a peptide substrate, and this occupies the P-3 to P-1 portion of the peptide recognition site. However, in addition to this consensus site, each inhibitor requires a peripheral binding site to achieve high affinity binding. Arg\textsuperscript{133} and Arg\textsuperscript{134} lie on the surface of the catalytic subunit with Arg\textsuperscript{133} coming close to the amphipathic helix of PKI(5-24) (Knighton, D. R., Zheng, J., Ten Eyck, L. F., Xuong, N.-h., Taylor, S. S., and Sowadaki, J. M. (1991) Science 253, 411-420). Replacement of Arg\textsuperscript{133} and Arg\textsuperscript{134} with Ala selectively abolishes the high affinity binding of PKI. Replacement of Arg\textsuperscript{133} alone is sufficient to give the same phenotype. In the presence of MgATP, the $K_{D_{app}}$ is increased from 0.2 to 105 nM, and in the absence of ATP, the $K_{D}$ is too large to be reliably measured.

Based on the crystal structure, Arg\textsuperscript{133} hydrogen bonds to the P-7 backbone carbonyl of PKI(5-24). However, more importantly, it also contributes to the hydrophobicity of the P-11 binding site in the C-PKI(5-24) complex. We predict that it is the perturbation of this hydrophobic pocket that accounts for the effects of this mutation. In the absence of peptide, Arg\textsuperscript{133} may help to stabilize Glu\textsuperscript{230}, a buried carboxylate that binds to the P-2 Arg in the crystal structure of C-PKI(5-24). Replacement of Arg\textsuperscript{133} and Arg\textsuperscript{134} with Ala has little effect on catalysis using a heptapeptide substrate and has no effect on the inhibition of the catalytic subunit by the regulatory subunit. The results thus demonstrate that these two inhibitor proteins that both bind to the catalytic subunit with a high affinity utilize different sites on the enzyme to achieve tight binding.

The γ isofrom of the catalytic subunit is insensitive to inhibition by PKI and in this isoform, Arg\textsuperscript{133} is replaced with Gln. We predict that this change accounts for the altered inhibitor properties of Cγ.

cAMP-dependent protein kinase (cAPK) is unusual among the protein kinase family in that its activation is mediated by a mechanism that involves the dissociation of regulatory (R) and catalytic (C) subunits. The free catalytic subunit, therefore, has the potential to interact with a variety of different inhibitor proteins. There are actually two classes of protein inhibitors of the C-subunit. First are the R-subunits that bind tightly only in the absence of cAMP (1). Within this class are at least four unique gene products, R1\textsuperscript{1-17}, R1\textsuperscript{20}, R1\textsuperscript{33A}, and R2\textsuperscript{3} (5). The other class of inhibitors are the heat-stable protein kinase inhibitors (PKIs) (6, 7). These are small proteins that bind with high specificity to the C-subunit. Three unique gene products are known: PKI\textsuperscript{α} (8), PKI\textsuperscript{β}1, and PKI\textsuperscript{β}2 (9, 10). PKI\textsuperscript{α} can be expressed as two different proteins due to alternative splicing of the RNA at the NH\textsubscript{2} terminus of the protein (10). So far no physiological mechanism is known for reversing this mechanism, although in vitro cAMP-free R can successfully compete for C when it is complexed with PKI (11).

In contrast to typical substrates, these inhibitor proteins bind to the C-subunit with a high affinity, typically in the subnanomolar range (12, 13). Both classes of inhibitor also use a common mechanism for inhibiting the catalytic activity of the C-subunit. Specifically, both PKI and the R-subunits contain an autoinhibitor site that resembles a peptide substrate (Fig. 1). The minimum consensus site for recognition of a peptide substrate is R-R-X-T/X’, where X’ is a hydrophobic residue. This consensus sequence is common to most substrates and inhibitors of cAPK (14). In both the R-subunit and PKI this consensus region occupies the peptide binding site and renders the enzyme inactive by preventing the binding of other substrates. PKI and the R-subunits are thus competitive inhibitors of peptide substrates.

A crystal structure of the C-subunit of cAPK was obtained by co-crystallizing with a 20-residue inhibitor peptide, PKI(5-24), derived from PKI(15-17). The structure of the ternary complex containing ATP as well as PKI(5-24) describes the total inhibitor complex (18-21). These structures illustrate how the consensus sequence binds to the active site of the enzyme. They also explain in part the high affinity binding of PKI. An essential feature for the high affinity binding of PKI is an amphipathic helix that precedes the consensus site and occupies a hydrophobic patch on the surface of the large lobe of the C-subunit. Like the R-subunits, the high affinity binding of PKI requires the synergistic binding of MgATP (11, 13, 22).

The R-subunits will presumably occupy the consensus site of the C-subunit in a manner that is analogous to PKI. However, there is evidence to suggest that the R-subunits do not utilize the same mechanism as PKI to achieve high affinity binding. For example, based on sequence alone, it is unlikely that the R-subunit of cAPK; c1, recombinant catalytic subunit; R2, type I recombinant regulatory subunit; R3, type II recombinant regulatory subunit; R133A, Arg\textsuperscript{133} in C-subunit substituted with Ala; R133A, L33A, Arg\textsuperscript{33} and Arg\textsuperscript{74} in C substituted with Ala; R209K, Arg\textsuperscript{209} in R-subunit substituted with Lys.
segment of R1 and RII that precedes the consensus site forms an α-helix (23, 24). Proteolysis also supports this conclusion since cleavage just before the consensus site of the RII-subunit does not eliminate high affinity binding of the RII-subunit to the C-subunit (25, 26). A mutant form of the R1-subunit where residues 1–91 are deleted further supports this conclusion.

In an effort to identify specific regions beyond the consensus recognition site that bind to the R-subunit, Gibbs et al. (28) used a family of mutants that bind to the yeast C-subunit, TPKI. These mutants were generated by selectively replacing all of the charged residues in TPKI with Ala. They then screened for mutants that were catalytically intact in terms of phosphorylating the haptapeptide, LRRASLG, but were not regulated properly by the R-subunit (29). Three mutants were isolated from this screen. One mutant, R133,134A, was located on the N-terminus of the consensus site. The rest, K189A, K212H and K213A,217A were located on the surface that lies just beyond the COOH-terminal side of the consensus sequence close to the auto phosphorylation site, Thr197. In order to better understand these mutant proteins, the same mutations were introduced into the mammalian C-subunit. We describe here the consequences of replacing Arg133 and Arg134 with Ala in the mammalian C-subunit. The single mutant where only Arg133 is replaced with Ala shows the same phenotype as the double mutant.

EXPERIMENTAL PROCEDURES

Materials—MOPS was from Sigma; fluorescein 5-isothiocyanate (FITC) was from Molecular Probes; Centricon 30 was from Amicon; and Mono S HR 10/10, Mono S HR 10/10, PD10, and NAP 10 columns were from Pharmacia LKB Biotechnology Inc. The peptide substrate, Kemptide, was from Biomedical Library, UCSD.

Site-Directed Mutagenesis—The mutations in the C-subunit were introduced as described by Kunkel (30, 31). The following oligonucleotides were synthesized with a DNA synthesizer (Applied Biosystems Inc. model 390B).

R133,134A: 5'-GCTGAACCTTCCAATCCGTGCTAGGTGGG-3' Sequence 1
R133A: 5'-GCTGAACCTTCCAATCCGTGCTAGGTGGG-3' Sequence 2

Bases differing from the wild-type cDNA are indicated in bold. All mutations were confirmed by DNA sequence analysis. The mutants were expressed in Escherichia coli BL21 (DE-3) as described previously (30). The rC-subunit was purified with a 35-fold molar excess of FITC for 30 min at 22 °C in the labeling buffer containing 100 mM Hepes (pH 8.0), 8 mM MgCl2, 4 mM ATP. The labeled rC-subunit was further purified using Superdex G-75 gel filtration chromatography in the following buffer (50 mM Tris (pH 7.4), 150 mM KCl, 2 mM EDTA).

Fluorescent Labeling of C-subunit and PKI—Both the rC-subunits and PKI were labeled with fluorescein 5-isothiocyanate (FITC) using procedures that were described previously (40). The rC-subunit was labeled with a 35-fold molar excess of FITC for 30 min at 22 °C in the labeling buffer containing 100 mM Hepes (pH 8.0), 8 mM MgCl2, 4 mM ATP. The labeled rC-subunit retained more than 97% of its initial phosphotransferase activity and was able to form holoenzyme with the R1-subunit in a manner similar to unlabeled rC-subunit.

PKI was labeled under the same conditions as described for the rC-subunit except that no MgATP was included in the labeling buffer. Labeled and unlabeled PKI showed no difference in their capacity to inhibit the rC-subunit based on the spectrophotometric assay. PKI Activity Assays—All activity assays were performed using the spectrophotometric method described by Cook et al. (41). This assay couples the production of MgADP to pyruvate kinase and lactate dehydrogenase, and the resulting decrease in absorbance at 340 nm due to the oxidation of NADH is followed. Kemptide, LRRASLG, was used as substrate.


8424 cAPK, Importance of Arg133 for PKI Binding

FIG. 1. Physiological inhibitors of the catalytic subunit. Sequences of the high affinity binding site regions of PKI and the R1- and R1I-subunits are compared with a commonly used haptapeptide substrate. The sequence NH2 terminus to the conserved site is important for the high affinity binding of the PKI (7), whereas the region COOH terminus to the consensus site appears to be important for the R-subunit binding (25, 26).2

have a significant impact on catalytic activity. Quite comparable with the wild-type R'-subunit.

Extent of holoenzyme formation in the presence and absence of MgATP can be achieved. For these studies, wild-type R-subunit was used, not the mutant R. Holoenzyme was prepared by dialyzing the mixture of FITC-labeled rC-subunit and rR-subunit against the buffer in the absence of MgATP.

Analytical Gel Filtration—In order to measure binding of R and PKI to C without depending on an activity assay, analytical gel filtration was performed according to the methods described by Herberg and Taylor (11). All the experiments for R-C interaction were carried out using a Superose 12 HR10/30 column, whereas all the experiments for PKI-C interaction were carried out using a Superdex G-75 column. Various concentrations of holoenzyme or PKI-C complex were preincubated for 30 minutes and then injected using a 25 µl sample loop unless otherwise specified. Protein was detected in a double detector system connecting a UV monitor (Pharmacia LKB) with a fluorescence detector (Hitachi fluorescence spectrophotometer) using an absorption wavelength of 493 nm and an emission wavelength of 520 nm. The percentages of holoenzyme and PKI-C-subunit complex were calculated based on the peak areas corresponding to the complex and free protein. The values of the holoenzyme peak area were corrected by a quench factor of 0.77 in the presence of MgATP, since the formation of holoenzyme quenched the fluorescence signal. The correction factors for C-PKI complex peak was 1.1 in the presence of MgATP.

### RESULTS

#### Basic Kinetic Parameters of R133,134A—
Before considering its capacity to be inhibited by PKI and the R-subunits, the kinetic properties of the mutant protein were characterized. These are summarized in Table I. The mutant did show a 2–3-fold increase in its $K_m$ for both peptide and ATP compared with the wild-type R-subunit using the heptapeptide LR-RASLG. However, these changes were not major and the $k_{cat}$ was slightly increased. These mutations, therefore, did not have a significant impact on catalytic activity.

Interaction with Regulatory Subunits—Having established that the kinetic properties of mutant C-subunit were comparable with the wild-type catalytic subunit, inhibition by the two R-subunits was investigated. The interaction of the C-subunit with the R'-subunit was first determined by measuring the ability of the R'-subunit to inhibit the activity of the C-subunit using the spectrophotometric assay. A mutant form of the R'-subunit, R209K, that exhibits lower affinity for CAMP by approximately an order of magnitude (35), was used since this mutant R'-subunit is able to bind C-subunit rapidly. A fixed amount of wild-type or mutant C-subunit was incubated with increasing amounts of R209K R'-subunit for 1 min at 22 °C. Kemptide was then added to initiate the kinase reaction. As shown in Fig. 2, the activity of the R133,134A C-subunit mutant (Fig. 2, panel A) could be titrated readily by R209K R'-subunit. Linear titration line was obtained, when R133,134A was used at 43 nm, indicating that the affinity of double mutant C-subunit for R'-subunit is at least 30 times lower than the concentration of mutant C-subunit used in this assay condition. In other words, the dissociation constant ($K_d$) for R133,134A holoenzyme is less than 4.3 nm in the presence of ATP. This is quite comparable with the wild-type R'-subunit.

To further investigate the R-C interaction, analytical gel filtration was employed. This assay was described in a previous paper (11). Using this method, the direct measurement of the extent of holoenzyme formation in the presence and absence of MgATP was performed. A mutant form of the R133,134A/C-subunit was used, not the wild-type R-subunit. Holoenzyme was prepared by dialyzing the mixture of FITC-labeled rC-subunit and rR-subunit against the buffer in the absence of MgATP.

#### Table I: Kinetic properties of the R133,134A mutant

<table>
<thead>
<tr>
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<th>Wild type</th>
<th>R133,134A</th>
<th>R133,134A/ wild type</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ (µM)</td>
<td>38</td>
<td>93</td>
<td>2.4</td>
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<tr>
<td>$k_{cat}$ (s⁻¹)</td>
<td>20</td>
<td>33</td>
<td>1.6</td>
</tr>
<tr>
<td>$K_m$ (µM)</td>
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<td>3.0</td>
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</tr>
<tr>
<td>$k_{cat}$/$K_m$</td>
<td>0.9</td>
<td>1.6</td>
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#### Fig. 2

Inhibition of the activity of mutant and wild-type catalytic subunits by the mutant (R209K) regulatory subunit. In the panel A, either the wild-type rC-subunit (●) or mutant (R209K) subunit (○) (43 nm each) was preincubated with increasing amounts of the mutant RI-subunit, rR(R209K), for 1 min at 22 °C in the assay buffer as described under “Experimental Procedures.” Kemptide was added to initiate the kinase reaction. The percent activity represents the ratio of remaining C-subunit activity assayed in the presence of rR(R209K) compared with the initial activity assayed in the absence of rR(R209K). Panel B shows the inhibition of activity of the C-subunit (●) and rC(R133A) (○) (78 nm each) by rR(R209K).

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Mutant +ATP

\begin{figure*}[h]
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\includegraphics[width=\textwidth]{fig3.png}
\caption{Concentration dependence of dissociation of wild-type and mutant holoenzymes. In panel A, holoenzymes containing rC (\textcircled{1}), rC(R133A,R134A) (\textcircled{2}), and rC(R133A) (\textcircled{3}) were prepared as described under "Experimental Procedures." Serial dilutions of each holoenzyme in the presence (\textcircled{1}) and the absence (\textcircled{3}) of MgATP were applied to the Superose 12 column. When MgATP was present, 100 \textmu M ATP and 1 mM MgCl\textsubscript{2} were used. The percentage of holoenzyme at various concentrations was calculated based on the peak areas of holoenzyme and free C-subunit. Panel B showed the concentration dependence of dissociation for the type II holoenzymes formed with the wild type (\textcircled{1}) and R133,134A (\textcircled{3}). The type II holoenzymes were run in the absence of MgATP.}
\end{figure*}

To test whether the R133,134A rC-subunit can still interact with R\textsuperscript{II}-subunit, analytical gel filtration was performed under the same conditions as those used for measuring the \(K_d\) of the type I holoenzyme. The apparent \(K_d\) values for wild-type and R133,134A mutant type II holoenzyme are close to 0.5 nM in the absence of MgATP (Fig. 3, panel B), indicating that the R133,134A mutant also interacts normally with R\textsuperscript{II}-subunit.

Taken together, the R133,134A mutant is able to bind to both type I and type II R-subunits with an affinity similar to that of wild-type C-subunit.

Interaction with PKI—The interaction of R133,134A mutant C-subunit with PKI was first studied using the spectroscopic method. Unlike the R-subunit, the heat-stable protein kinase inhibitor, PKI, as well as the NH\textsubscript{x}-terminal peptide of the inhibitor, PKI(5–24), do not inhibit the phosphotransferase activity of R133,134A double mutant with a high affinity (Fig. 5, panels A and B). The inhibitory constant (\(K_i\)) values were determined by a Dixon plot shown in the insets. The \(K_i\) of PKI and PKI(5–24) for R133,134A mutant increased by a factor of 430 and 173, respectively, compared with that for wild-type C-subunit in the presence of MgATP.

To determine whether one of the two arginines was primarily responsible for this increased \(K_i\), the single mutant, R133A, was studied. Like the double mutant, R133A behaved like wild-type C-subunit in terms of its inhibition by the R-subunit (Fig. 2, panel B). However, when the \(K_i\) of PKI for this single mutant
C-subunit was measured using the spectrophotometric method, an increase of 525-fold was observed compared with that for wild-type C-subunit (Fig. 5, panel C). This mutant thus demonstrates that Arg133 and not Arg134, is primarily responsible for the decreased affinity of the mutant protein for PKI. All the \( K_i \) values in the presence of MgATP are summarized in Table II.

Since, like the \( R^3 \)-subunit, the interaction of PKI and C is synergistically dependent on MgATP, the increased \( K_i \) in the presence of MgATP may have two explanations. One possibility is that the mutant C-subunit can still recognize PKI normally but that MgATP can no longer facilitate the tight complex formation. Alternatively, this mutant C-subunit may be defective in recognizing PKI both in the presence and absence of ATP. To resolve these questions, analytical gel filtration was used to directly measure the complex formation in the presence and absence of MgATP. The same conditions to those described for Superdex G-75 chromatography. No complex was formed in the absence of MgATP at concentrations up to 60 \( \mu \)M of both C-subunit and PKI (Fig. 6, panel A). This suggested that the R133,134A mutant C-subunit is defective in its recognition of PKI, not just in its capacity to bind ATP with a high affinity. In the presence of 1 mM ATP and 2 mM MgCl\(_2\), labeled PKI forms a complex very poorly with the R133,134A mutant, indicating that the presence of MgATP can stabilize the complex formation (Fig. 6, panel A). To investigate the effect of MgATP on the formation of the PKI-C complex, 60 \( \mu \)M of the rC(R133A,R134A) was incubated with 1 \( \mu \)M of PKI in the presence of various concentrations of MgATP. Samples were then applied onto the Superdex G-75 column to separate the complex and free FITC-labeled PKI. As shown in Fig. 6, panel B, an increased proportion of complex was observed with increasing amount of MgATP. 50% PKI was associated with R133,134A in the presence of 7 mM ATP and 1 mM MgCl\(_2\). Using same amounts of wild-type C-subunit and PKI, all of the wild-type C-subunit binds to PKI even in the absence of MgATP. When 100 \( \mu \)M each of the wild-type C-subunit and PKI were incubated with increasing amounts of ATP, 220 nm of ATP was required to achieve half-maximal complex formation (11); however, under these conditions, no complex formation was observed for the R133,134A mutant C-subunit, even in the presence of 1 mM ATP. Therefore, R133,134A mutant C-subunit also needs higher ATP to stabilize the PKI-C complex. The R133,134A mutant thus is defective in its recognition of PKI as well as PKI(5–24) both in the presence and absence of MgATP.

**DISCUSSION**

Both classes of physiological inhibitors of the C-subunit, the R-subunits, and the PKIs contain an auto-inhibitory site that resembles peptide substrates. This region, extending from the P−3 through the P+1 site, occupies the peptide binding site and renders the enzyme inactive by preventing the binding of other substrates. However, in addition to this region, these two inhibitors require other sites to achieve high affinity binding. To identify those additional sites, Gibbs et al. (29), using charge-to-alanine scanning mutagenesis, identified three mutants in the yeast C-subunit that recognized the consensus sequence but were not regulated properly by the R-subunit. One of these mutants, equivalent to R133,134A in the mouse C-subunit, displayed an IC\(_{50}\) value that was increased 4.2-fold. The catalytic efficiency of this mutant in yeast also was reduced slightly due to a 2.3-fold increase in the \( K_i \) for Kemp tide. In order to characterize this mutation in vitro, Arg133 and Arg134 in the recombinant mouse C-subunit were both replaced with Ala. In addition, a single mutant was made where only Arg133 was replaced with Ala. These mutations did not have a major effect on catalytic activity similar to what was observed for the yeast enzyme (29).

To investigate interactions between the C-subunit and its two physiological inhibitors, two methods were used. The interactions were first determined by measuring the capacity of these proteins to inhibit the catalytic activity of the C-subunit in the presence of MgATP using a spectrophotometric activity assay. However, in order to compare the \( K_{i, app} \) values for subunit interactions in the presence and absence of MgATP, it was necessary to use a method that was independent of the activity assay. To accomplish this, analytical gel filtration was used (11).

Using both of these methods, the R133,134A mutant was shown to function like the wild-type C-subunit in terms of forming holoenzyme with either the \( R^3 \)-subunit or the \( R^1 \)-subunit. Furthermore, no significant differences were found for the \( R^3 \)-subunit either in the presence or absence of ATP. This is in contrast to the yeast C-subunit, where the comparable mutation showed a 4.2-fold increase in the IC\(_{50}\). We attribute this to subtle differences between BCY1 and the mammalian R-subunit. BCY1 is a type II R-subunit, and it does not bind to the yeast C-subunit nearly as tightly as the mammalian R-subunit binds to the mammalian C-subunit. The difference in R-C interaction in the presence and absence of cAMP is only a little more than an order of magnitude (42) in contrast to the mam-
malian R- and C-subunits, where the difference is 4–5 orders of magnitude (43). The yeast cAPK may, therefore, be more sensitive to small perturbations. Although the precise explanation for the differences between yeast and mammalian cAPKs is not totally clear, it is unambiguous that the R133,134A mutant in the mammalian C-subunit does not interfere with holoenzyme formation or holoenzyme stability.

These findings with regard to holoenzyme formation are in stark contrast to complex formation with PKI. This mutation severely impairs the interaction of C with PKI, both in the presence and absence of MgATP. The $K_i$ values are increased by over 400-fold, so that under physiological conditions, this C-subunit would no longer be inhibited by PKI. The yeast C-subunit does not have a high affinity binding site for PKI. This can be attributed to alterations in the hydrophobic pocket that binds the critical P-11 Phe in PKI (44, 45). This hydrophobic pocket is comprised of Tyr$^{235}$, Pro-Pro-Phe-Phe with Tyr$^{235}$, Phe$^{239}$, and Pro$^{236}$ interacting predominantly with the P-11 Phe as seen in Fig. 7. In the yeast TPKI, the equivalent of Pro$^{236}$ is replaced with a Thr and Phe$^{239}$ is replaced with Tyr.

**Fig. 7.** A stereo view showing the immediate environment surrounding R133. This diagram was taken from the coordinates of the binary complex of PKI(5–24) and the C-subunit (17).

**Fig. 8.** Space filling model showing the location of Arg$^{133}$ and Arg$^{134}$ in the crystal structure of the catalytic subunit. Residues 15–127, predominantly located in the small lobe, are shown in purple, whereas residues 128–350 are shown in pink. The inhibitor peptide PKI(5–24) is shown in red with P-11 Phe highlighted as turquoise. Arg$^{133}$ and Arg$^{134}$ on the surface of the large lobe are shown with their side chain carbons in green and nitrogens in blue. Glu$^{359}$ that comes close to Arg$^{133}$ and ion pairs with the P-2 Arg is shown in yellow with its oxygens in orange. The hydrophobic P-11 binding pocket is comprised of Tyr$^{235}$, Pro$^{236}$, and Phe$^{239}$, all shown in yellow. Panel A shows the position of Arg$^{133}$ relative to the overall structure of the ternary complex containing MgATP and PKI(5–24). Panel B shows the immediate environment surrounding Arg$^{133}$ with PKI(5–24) removed. Panel C shows the immediate environment surrounding Arg$^{133}$, where Arg$^{133}$ is replaced with Ala.
These differences most likely account for its inability to bind PKI (46, 47).

In order to determine whether Arg$^{133}$ or Arg$^{134}$ contributed more prominently to the altered properties of this mutant, a single mutant was engineered by replacing Arg$^{133}$ with Ala. Since this mutant behaves in all regards like the double mutant, we conclude that the phenotype of the double mutant is due predominantly to the Arg$^{133}$ replacement.

Does the crystal structure of the C-subunit provide an explanation for the observed phenotype? A space filling model of this region of the C-subunit is shown in Fig. 8 while the specific contacts of less than 4 Å that Arg$^{133}$ makes with other neighboring atoms are summarized in Table III. The proximity of Arg$^{133}$ to the P-11 hydrophobic pocket is particularly clear. Arg$^{133}$ also hydrogen bonds to the backbone α-carbonyl of the P-7 residue. This backbone interaction is not important for the Kemptide, which only occupies the P-4 to P-2 sites, or for the R-subunits; however, it is important for PKI. Arg$^{133}$ is also close enough to ion pair with Glu$^{230}$. Based on its reactivity with the hydrophobic carbodiimide, dicyclohexyl carbodiimide, Glu$^{230}$ was predicted to be in a hydrophobic environment (48, 49), and this is consistent with the crystal structure. In the binary and ternary complexes Glu$^{230}$ ion pairs with the P-2 Arg and thus neutralizes the buried charge (16). How this negative charge is neutralized in the apoenzyme is unclear, but its interactions with Arg$^{133}$ could become more prominent. Perturbation of this interaction may very well account for the 2-fold increase in $K_m$ for the peptide. However, the greatest effect of this mutation with respect to PKI may be to reduce the hydrophobicity of the P-11 pocket. The methylene carbons of Arg$^{133}$ contribute to the hydrophobicity of this site and come within 3.5-4.0 Å of the P-11 Phe. When the P-11 Phe is replaced with dihydro-Phe, an artificial amino acid that is even larger and more hydrophobic than Phe, the affinity of PKI(5-24) for the C-subunit is increased by an order of magnitude. This finding supports the conclusion that this is quite an extended hydrophobic pocket (7, 47). As seen in Fig. 8, replacing Arg$^{133}$ with Ala leaves a deep hole, and we conclude that the C-subunit compensates by re-structuring the hydrophobic P-11 binding pocket.

Does this mutation have any physiological relevance? Although it could obviously be an important tool for probing the physiological role of PKI, the behavior of this mutant in vivo remains to be determined. However, there is another obvious physiological implication of this mutation. Three gene products of the C-subunit have been identified, Ca, C6, and Cy (50-53). Ca is expressed constitutively in all cells, whereas the expression of C6 is much more tissue-specific. These two C-subunits are indistinguishable in their catalytic properties, and both can be inhibited readily by both R and PKI. The Cy-subunit was first identified in human testis and is expressed only at low levels. The only property that has been identified so far that distinguishes Cy from Ca and C6 is that it can no longer be inhibited by PKI (54). In Cy, Arg$^{133}$ is conspicuously replaced by Gin, and we predict that this single amino acid difference accounts for the inability of Cy to be inhibited by PKI. The physiological significance of this observation also remains to be established.

What implications does this mutation have for protein kinase function in general and, in particular, for understanding how protein kinases recognize their substrates and inhibitors? It reflects a rather remarkable flexibility when one compares how this enzyme recognizes its various classes of physiological inhibitors. To recognize all of these inhibitors, as well as its normal substrates, the enzyme utilizes a common consensus recognition site that flanks the site of phosphotransfer. This extends, in general, from the P-3 Arg to the P+1 site and, in some substrates such as PLCγ, may extend as far as the P-6 Arg substrate. However, to achieve high affinity binding requires further interactions. The findings here demonstrate that the C-subunit can use one surface to achieve high affinity binding of PKI. This is the surface that complements the amphipathic helix of PKI that lies NH$_{2}$-terminal to the consensus site. This surface, however, is not required to achieve high affinity binding of the R-subunits. Instead, the high affinity binding of the R-subunit apparently requires the surface of the C-subunit that lies COOH-terminal to the consensus site (29, 55). Whether the C-subunit uses similar flexibility in recognizing different substrates remains to be determined. Whether other protein kinases also display this type of versatility also remains to be established.

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