Identification of electrostatic interaction sites between the regulatory and catalytic subunits of cyclic AMP-dependent protein kinase

ROBIN M. GIBSON,¹ YING JI-BUECHLER,² AND SUSAN S. TAYLOR

Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, California 92093-0654

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Abstract

Two classes of molecules inhibit the catalytic subunit (C) of the cyclic AMP-dependent protein kinase (cAPK), the heat-stable protein kinase inhibitors (PKIs) and the regulatory (R) subunits. Basic sites on C, previously identified as important for R/C interaction in yeast TPK1 and corresponding to Lys213, Lys217, and Lys189 in murine C α , were replaced with either Ala or Thr and characterized for their kinetic properties and ability to interact with RI and PKI. rC(K213A) and rC(K217A) were both defective in forming holoenzyme with RI but were inhibited readily with PKI. This contrasts with rC(R133A), which is defective in binding PKI but not RI (Wen & Taylor, 1994). Thus, the C-subunit employs two distinct electrostatic surfaces to achieve high-affinity binding with these two types of inhibitory molecules even though all inhibitors share a common consensus site that occupies the active site cleft. Unlike TPK1, mutation of Lys189 had no effect. The mutant C subunits that were defective in binding RI, rC(K213A) and rC(K217A), were then paired with three RI mutants, rRI(D140A), rRI(E143A), and rRI(D258A), shown previously to be defective in recognition of C. Although the mutations at Asp140 and Asp258 in RI were additive with respect to the C mutations, rC(K213A) and rRI(E143A) were compensatory, thus identifying a specific electrostatic interaction site between RI and C. The results are discussed in terms of the RI and C crystal structures and the sequence homology between the yeast and mammalian enzymes.

Keywords: cAPK; electrostatic interaction; R/C interaction

Although for proper functioning in a eukaryotic cell it is essential that protein kinases be accurate catalysts, of equal importance is that each kinase must be properly regulated so that it can be rapidly turned "on" and "off" in response to specific signals. For cAMP-dependent protein kinase (cAPK), one of the first protein kinases to be discovered and characterized (Walsh et al., 1968), there are two classes of physiological inhibitors, the regulatory subunits

(Rs) and the heat-stable protein kinase inhibitors (PKIs) (Taylor et al., 1990). Both bind the catalytic subunit (C) with high affinity and render it inactive. The two inhibitors share a common mechanism of inhibition involving an autoinhibitor segment, and variations of this mechanism are utilized by many protein kinases.

There are two major classes of regulatory subunit, type I and type II, a classification originally based on the order of elution of their respective holoenzymes from anion exchange resin (Corbin et al., 1977). Each R-subunit has a well-defined domain structure consisting of an N-terminal dimerization domain, a "hinge" region containing an autoinhibitory sequence, and two tandem cAMP binding domains, A and B (Fig. 1A). The autoinhibitory region of R contains the consensus site "RRXS/T Ψ " defined by peptide substrates, in which X is any amino acid, S/T is the site of phosphorylation (the "P-site"), and Ψ is a hydrophobic group located in the P+1 position. In RI, the P-site is a non-phosphorylatable Ala and is thus referred to as a pseudo-phosphorylation site, while in RII this site is auto-phosphorylated. Conformational changes in R resulting from cAMP binding and release are likely to play a key role in the mechanism that regulates the association and dissociation of R and C (Ogreid & Doskeland, 1981; Herberg et al., 1994, 1995). PKI also has an inhibitory region containing the consensus

Reprint requests to: Susan S. Taylor, Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, California 92093-0654; e-mail: staylor@ucsd.edu.

¹Present address: Department of Pharmacology, University of Washington, Seattle, Washington 98195-7750.

²Present address: EGen Corporation, 3550 General Atomics Court, 02-544, San Diego, California 92121.

Abbreviations: cAPK, cAMP-dependent protein kinase; R, cAMP-dependent protein kinase regulatory subunit; RI, Type Ia regulatory subunit of cAMP-dependent protein kinase; RII, Type IIa regulatory subunit of cAMP-dependent protein kinase; C, cAMP-dependent protein kinase catalytic subunit; β -ME, β -mercaptoethanol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EDC, 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide-HCl; PKI, heat-stable protein kinase inhibitor; PRS, peripheral recognition site; IRS, inhibitor recognition site. GRASP, Graphical Representation and Analysis of Surface Properties.

Fig. 1. Location of mutation sites in the catalytic and regulatory subunits of PKA that affect R/C interaction. Location of residues within the linear sequence of R and C that were targeted by site directed mutagenesis as potential R/C interaction sites. (A) Domain structure of the RI-subunit: N-terminal dimerization domain (hatched box), autoinhibitor region (black box), cAMP binding domains A and B (shaded boxes). Numbered residues represent acidic sites in RI that were protected from chemical modification by EDC in the holoenzyme complex (J. A. Buechler, unpubl. obs.). RI and PKI share a common consensus sequence (box) in their inhibitory domains. Additional determinants for high-affinity binding in PKI and the inhibitor peptide, IP20, lie N-terminal to the consensus site (underlined), while those on R are lie C-terminal to this region. (B) Mutation sites on C-subunit.

site "RRX/STΨ." Like RI, the P-site of PKI is occupied by an Ala. Unlike RII, both PKI and RI require MgATP to achieve high-affinity binding with C (Herberg & Taylor, 1993).

Although the binding of the auto-inhibitor sequence to the active site cleft is essential for the inhibition of C, interaction with this site alone is not sufficient to achieve high-affinity binding. Peptide substrates consisting solely of the consensus site bind to the C-subunit with μ M affinity (Zetterqvist et al., 1990), while the larger inhibitory molecules, PKI and the type I and II R-subunits, bind to C with

affinities in the sub-nanomolar range (Hofmann, 1980; Walsh et al., 1990; Herberg & Taylor, 1993). Using peptide analogs, determinants for high-affinity binding on PKI were mapped to regions that lie N-terminal to the consensus site (Fig. 1A) (Cheng et al., 1986; Scott et al., 1986; Walsh et al., 1990). The high-affinity binding of the R-subunit, on the other hand, requires sites that lie C-terminal to the consensus site (Weber & Hilz, 1979; Weldon & Taylor, 1985). Deletion mutagenesis that removed the B-domain as well as the N-terminus of RI established that the B-domain is also not required for high-affinity binding. Thus, the determinants for high affinity binding of R involve regions that extend from the autoinhibitor site through the A-domain (Saraswat et al., 1988; Ringheim & Taylor, 1990; Herberg et al., 1994).

Because we do not yet have a crystal structure of a holoenzyme complex, the molecular details of R/C interaction are not known. Charge-to-Ala scanning mutagenesis in the yeast homolog of the C-subunit, encoded by TPK1, identified specific regions in addition to the consensus recognition site that were important for regulation (Gibbs et al., 1992) (Table 1). One mutant, corresponding to the double mutation, R133A/R134A, in C α , was located N-terminal to the consensus recognition site. When the mutations R133A and R133A/R134A were made in recombinantly expressed murine C α , it was found that although neither mutation disrupted recognition of RI α or RII α , both mutants were defective in binding PKI (Wen & Taylor, 1994). These results were highly consistent with the position of Arg133 and Arg134 in the crystal structure of C bound to the inhibitory peptide, PKI(5–24) (Knighton et al., 1991a, 1991b). The remaining sites in the yeast charge-to-Ala scan, corresponding to Lys189, Lys213, and Lys217, in mammalian C, were mapped to a highly basic surface of C, which lies C-terminal to the consensus sequence recognition site. Additional sites, corresponding to the critical phosphorylation site, Thr197, Trp196, Leu198, and His87, on this surface were identified previously by genetic screens both in mammalian cells and in yeast as important for R/C interaction (Levin et al., 1988; Levin & Zoller, 1990; Orellana & McKnight, 1992; Orellana et al., 1993).

To characterize interaction sites between R and C in the mammalian enzyme, two of the basic residues, Lys213 and Lys217,

Table 1. Mutation in yeast and mammalian C-subunits that affect R:C interaction

Mutation in rC α	Effect of mutation on formation of holoenzyme with RI	Equivalent mutation in yeast C ₁ subunit	Effect of mutation in TPK1 IC ₅₀ (mutant/wild type) ^a
K189T	No effect on RI recognition	K223A	2.1
K213A	Defective: pairs with E243 on RI	K257A	2.5
K217A	Slightly defective	K261A	4.2
N.D.	N.D.	K257A/K261A	7.9
R133A/R134A	Mutation had no effect on R/C interaction, but disrupted interaction with PKI. ^b	R1771/K178A	4.3
R133A	Equally defective as the double mutant (R133!/R134A) in PKI recognition: no effect on R/C interaction. ^b	R177A	3.1

^aThese numbers are taken from Gibbs et al. (1992).

^bTaken from Wen and Taylor (1994).

Table 2. Kinetic parameters of mutant C-subunits^a

	k_{cat} (s ⁻¹)	K_m (pep.) (μ M)	k_{cat}/K_m (pep.) (s ⁻¹ /mM ⁻¹)	K_m (ATP) (μ M)	k_{cat}/K_m (ATP) (s ⁻¹ /mM ⁻¹)
rC(WT)	18	21	857	18	1,000
rC(K213A)	18	20	900	20	900
rC(K217A)	17	14	1,210	18	944

^aSteady-state kinetic parameters for the peptide substrate Kemptide (pep.) and MgATP were measured using the coupled enzyme spectrophotometric assay described in Materials and methods.

were replaced with Ala in the recombinant murine C-subunit. In addition, Lys189 was replaced with Thr. The mutant proteins were then characterized for their ability to recognize wild-type RI and PKI. After showing that Lys213 and Lys217 were important for holoenzyme formation, the mutant C-subunits were then paired with the acidic-to-Ala mutant RI subunits, rRI(D140A), rRI(143A), and rRI(D258A), shown previously to be defective in forming holoenzyme with wild-type C (Gibson et al., 1997). By combining pairs of mutant RI and C subunits, additive and compensatory sites were identified (Wells et al., 1987; Gibbs & Zoller, 1991a).

Results

Residues corresponding to Lys189, Lys213, and Lys217 in the catalytic subunit of cAPK were identified by charge-to-Ala scanning mutagenesis in the yeast homolog of C as residues important for interaction with the regulatory subunit (Gibbs et al., 1992). Based on these results, the corresponding mutations were engineered in recombinantly expressed murine C α to test whether these sites were also important for regulation in the mammalian enzyme (Table 1). The mutant C-subunits, rC(K213A), rC(K217A), and rC(K189T), were all expressed as soluble proteins in *E. coli* at 25°C and purified to homogeneity as described in Materials and methods. rC(K213A) and rC(K217A) eluted at a lower salt concentration (50–100 mM potassium phosphate) than rC(WT), which typically elutes from P-11 resin at 230–270 mM potassium phosphate. This difference in elution is consistent with the removal of basic residues on the surface of the enzyme. rC(K189T) purified exactly as rC(WT).

Kinetic characterization of C-subunit mutants

All of the C-subunit mutants exhibited specific activities that were similar to rC(WT) (typically, 20–30 U/mg). As shown in Table 2, the K_m s for peptide and ATP as well as the k_{cat} s for rC(K213A) and rC(K217A) were similar to rC(WT) in all respects, consistent with the location of these sites in regions of the C-subunit that are removed from the active site.

Inhibition with PKI and RI

The two known classes of cAPK inhibitors are the regulatory subunits and the PKIs. Although the binding of R and C is cAMP mediated, the binding of PKI to the free C-subunit is ligand independent. When PKI is incubated with free C, it inhibits linearly and stoichiometrically at typical physiological concentrations of C and PKI. When rC(K213A) and rC(K217A) were incubated with PKI under assay conditions (30 nM C), inhibition was also linear and

stoichiometric, indicating that the K_d s for the mutant C-subunits for PKI were well below 30 nM (Fig. 2). This was in striking contrast to the R133A mutation made previously where no inhibition by PKI was observed (Wen & Taylor, 1994). This set of mutants, originally identified as regulatory deficient mutants in the yeast C-subunit, thus distinguishes two distinct surfaces on C that contribute differently to the recognition of PKI. One surface surrounding Arg133 is important for PKI, the other, surrounding Lys213 and Lys217 is not.

To establish whether Lys213, Lys217, and Lys189 were important for R/C interaction in the mammalian enzyme, the mutant C-subunits, rC(K213A), rC(K217A), and rC(K189T), were dialyzed with the cAMP-bound form of RI, where holoenzyme is formed as cAMP is removed. As shown in Figure 3, both rC(K213A) and rC(K217A) formed holoenzyme more slowly than rC(WT). For the wild-type controls, inhibition was more than 90% complete after 10 h of dialysis. In contrast, rC(K213A) was only 50% after the same length of time and rC(K217A) was 75% inhibited. In contrast, mutation of Lys189 to Thr had no effect on the rate of holoenzyme formation with rRI(WT) (Fig. 3, inset). These results

Fig. 2. Inhibition of the activity of mutant and wild-type catalytic subunits with the heat stable protein kinase inhibitor, PKI. Wild-type or mutant rC-subunit (30–40 nM each) was pre-incubated with increasing amounts of PKI for one minute at 22°C in assay buffer as described in Materials and methods. The remaining activity was then measured upon addition of Kemptide. The percent activity represents the ratio of activity remaining in the presence of PKI compared to the initial activity measured in the absence of PKI.

Fig. 3. Holoenzyme formation of mutant C-subunits with wild-type RI-subunit. C ($2 \mu\text{M}$) and RI ($3 \mu\text{M}$) were dialyzed as described in Materials and methods using a multi-chambered micro-dialysis apparatus. Aliquots were removed at the times indicated and assayed for catalytic activity in the absence and presence of $100 \mu\text{M}$ cAMP to determine the percent of free C-subunit remaining in the mixture. Zero-remaining activity corresponds to 100% holoenzyme. rC(K213A) ($\blacktriangle$), rC-WT ($\bullet$) (upper panel); rC(K217A) ($\blacktriangledown$), rC-WT ($\bullet$) (lower panel). Inset: rC(K189T) ($\blacklozenge$) or rC(WT) ($\bullet$) ($2 \mu\text{M}$) were dialyzed in the presence of RI ($3 \mu\text{M}$). These samples were dialyzed using a standard dialysis apparatus (bag, clips, and beaker) as opposed to the microdialysis chamber used in the other experiments.

again contrast the two surfaces on the C-subunit. The Lys213/Lys217 surface was important for RI binding while the Arg133 surface was not.

When cAMP was removed from RI by urea-stripping prior to incubation with C, the cAMP-free RI inhibited both rC(K213A) and rC(K217A) linearly and stoichiometrically, indicating the K_d for each of these mutant C-subunits and RI was well below 20 nM , the concentration of C used in the assay.

Mapping electrostatic interaction sites between RI and C

As shown previously, rRI(D140A), rRI(E143A), and rRI(D258A) were also defective in forming holoenzyme with rC(WT) in the dialysis assay (Gibson et al., 1997). In an effort to identify specific electrostatic contacts between the two subunits, the two defective C-subunits, rC(K213A) and rC(K217A), were paired with each of the defective R-subunits, rRI(D140A), rRI(E143A),

and rRI(D258A), and characterized for holoenzyme formation. Figure 4 illustrates our strategy for identifying interacting residues by pairing the acidic-to-Ala mutant RI-subunits with the basic-to-Ala mutant C-subunits. If a mutation site on either R or C is important for R/C interaction, pairing this mutant with wild-type subunit (Fig. 4B) will reduce the rate of holoenzyme formation. All of the mutant proteins used here are defective in this regard. Pairing two defective mutants with each other will yield two results. If two sites are different, both will be disrupted and the defects in holoenzyme formation will be additive (Fig. 4D). If the sites interact, the mutations will be compensatory; only a single site will be disrupted and the rate of holoenzyme formation of the two mutants together will be no worse than each of the single mutations (Fig. 4B and C). This strategy was used previously to map specific interactions sites for peptide substrates (Wells et al., 1987) and successfully identified carboxylates that interact specifically with the P-3 and P-2 Args (Gibbs & Zoller, 1991a).

rC(K213A)

Figure 5 shows the dialysis curves representing the rates of holoenzyme formation of rC(K213A) when paired with the RI-subunit mutants, rRI(D140A) (Fig. 5, upper panel), rRI(E143A) (Fig. 5, middle panel), and rRI(D258A) (Fig. 5, lower panel). The wild-type control formed greater than 90% holoenzyme after 10 h of dialysis at room temperature. When rC(K213A) was paired with rRI(D140A) (Fig. 5, upper panel, open triangles) the rate of holoenzyme formation was much worse than when either rC(K213A) was paired with rRI(WT) (Fig. 5, upper panel, open circles), or when rRI(D140A) was paired with C-WT (Fig. 5, upper panel, closed triangles). This suggested that the K213A mutation on the

Fig. 4. Mapping electrostatic interaction sites between the regulatory and catalytic subunits of PKA. (A) Wild-type C-subunit, C-WT (hatched rectangle) with two basic sites (+) interacting with two acidic sites (-) on wild-type R-subunit, R-WT (shaded rectangles). (B) Mutation of one site to Ala (A) on either subunit affects one interaction site. (C) A mutant C-subunit (C-M) and a mutant R-subunit (R-M) in which the Ala mutations have affected the same interaction site. (D) A mutant C-subunit and a mutant R-subunit in which the Ala mutations have affected two interaction sites. The dashed line (---) indicates an electrostatic interaction between the subunits.

Fig. 5. Holoenzyme formation of rC(K213A) with rRI(D140A), rRI(E143A), and rRI(D258A). Wild-type C (solid symbols) or rC(K213A) (open symbols) were dialyzed with RI as described in Materials and methods using a multi-welled micro-dialysis apparatus. rC(WT)/rRI(WT), control (●); rC(K213A)/rRI(WT) (○); upper panel: rC(WT)/rRI(D140A) (▲), rC(K213A)/rRI(D140A) (▲); middle panel: rC(WT)/rRI(E143A) (■), rC(K213A)/rRI(E143A) (□); lower panel: rC(WT)/rRI(D258A) (◆), rC(K213A)/rRI(D258A) (◇).

C-subunit and the D140A mutation on the RI-subunit affected two different interaction sites. Similarly, pairing rC(K213A) and rRI(D258A) also resulted in an additive defect (Fig. 5, lower panel, open diamonds), suggesting that these two mutations affected two different interaction sites. In contrast, when rC(K213A) was paired with rRI(E143A) (Fig. 5, middle panel, open squares) the rate of holoenzyme formation was no worse than when either of the mutant subunits was paired with wild type (Fig. 5, middle panel, open circles, and closed squares). Thus, Lys213, on the C-subunit, and

Fig. 6. Holoenzyme formation of rC(K217A) with rRI(D140A), rRI(E143A), and rRI(D258A). Wild-type C (solid symbols) or rC(K217A) (open symbols) were dialyzed with RI as described in Materials and methods using a multi-welled micro-dialysis apparatus. rC(WT)/rRI(WT), control (●); rC(K217A)/rRI(WT) (○); upper panel: rC(WT)/rRI(D140A) (▲), rC(K217A)/rRI(D140A) (▲); middle panel: rC(WT)/rRI(E143A) (■), rC(K217A)/rRI(E143A) (□); lower panel: rC(WT)/rRI(D258A) (◆), rC(K217A)/rRI(D258A) (◇).

Glu143, on the RI-subunit, are likely to interact in the holoenzyme complex.

rC(K217A)

Although the K217A mutation had a smaller affect on the rate of holoenzyme formation compared to the K213A mutation, when rC(K217A) was paired with either rRI(D140A), rRI(E143A), or rRI(D258A), an additive defect was observed in each case (Fig. 6).

Thus, none of these sites on the RI-subunit interact directly with Lys217 on the C-subunit in the holoenzyme complex.

Discussion

Unlike the interaction between C and PKI, the interactions between R and C involve sites that spread over a larger surface area. For PKI, recognition is achieved by a contiguous peptide that includes an amphipathic helix followed by the consensus site inhibition segment. In contrast, the binding of R involves a large surface area that lies C-terminal to the consensus site peptide. The regions of R that contribute to this interaction, furthermore, extend well beyond the region that immediately flanks the consensus site inhibitor segment. The conformational changes that regulate the formation of holoenzyme will occur primarily within the R subunit in response to the cooperative binding and release of cAMP. In the absence of a crystal structure of the holoenzyme complex, the precise molecular interactions that contribute to the high-affinity binding between R and C are not well defined. Several different conformational states of the catalytic subunit have been crystallized (for review, see Cox et al., 1994). More recently, the X-ray crystallographic structure of an RI-subunit deletion mutant, (Δ 1-91) RI, was also solved (Su et al., 1995). The N-terminal 20 amino acid residues including the autoinhibitor region of RI were not resolved in the crystal structure of (Δ 1-91) RI, suggesting that this portion of the molecule is flexible. It is likely that this region will adopt a more stable conformation once it is complexed to C (Su et al., 1995).

Based on the results described here as well as on previous results using proteolysis, deletion mutagenesis, and genetic scanning, the C-subunit clearly employs two distinct surfaces in addition to the inhibitor recognition site at the cleft interface, to achieve high affinity binding to its two inhibitors, PKI and the regulatory

subunits. We define these two surfaces here as peripheral recognition sites as distinct from the consensus recognition site that is common to all substrates and inhibitors of cAPK. Peripheral recognition site 1 (PRS1) is essential for PKI while peripheral recognition site 2 (PRS2) is critical for the R-subunits.

As shown in Figure 7, in the X-ray crystal structure of the C-subunit complexed with the inhibitor peptide, PKI(5-24), the N-terminal region of PKI(5-24) forms an amphipathic helix. This helix interacts with the surface of C that corresponds to PRS1 (Knighton et al., 1991a, 1991b). Arg133 is an integral part of this PRS1 surface. Replacement of Arg133 with Ala abolished high-affinity binding with PKI but had no effect on R1-binding (Wen & Taylor, 1994). The C-terminal portion of PKI(5-24) contains the consensus site that is common to nearly all substrates and inhibitors of cAPK. This portion binds to the active site cleft of C and, based on cross-linking studies in type II cAPK (First et al., 1988) and mutagenesis studies of the consensus site Args in RI (Buechler et al., 1993) and RII (Wang et al., 1991), it is likely that the consensus site of R will bind to this region of C in a similar fashion. The surface of C required for high-affinity binding of the R-subunit, referred to here as PRS2, is also indicated in Figure 7. Lys213 and Lys217 contribute to this surface. In contrast to Arg133, replacement of these residues with Ala interfered with R1-binding but had little effect on PKI binding.

Figure 8 highlights that the electrostatic features of surfaces on the C-subunit corresponding to PRS1 and PRS2 are quite different. Figure 8A and B shows the surface of C that interacts with PKI. As indicated in Figure 8B, this surface, including PRS1 and the consensus recognition site, is very acidic. The importance of this acidic surface in the recognition of the positively charged P-6, P-3, and P-2 Args of PKI and inhibitory peptides has been demonstrated genetically, by peptide mapping, and by combinatorial mapping, and all of these results are fully consistent with the crystal structure

Fig. 7. X-ray crystal structure of the C-subunit. Blow-up of the activation segment of the catalytic subunit bound to the inhibitor peptide, IP20 (shaded ribbon), and ATP (black) (Zheng et al., 1993).

Fig. 8. Crystallographic models of the catalytic and regulatory subunits of PKA. **(A)** Space-filling model of C bound to the inhibitor peptide PKI(5–24) (red). The position of Arg133 is indicated (arrow). A version of this figure was used in Wen and Taylor (Wen & Taylor, 1994). **(C)** C-subunit rotated 180° around z-axis, highlighting the PRS2 surface important for recognition of R-subunit. The position of Lys213 that interacts with Glu143 on RI is indicated (arrow). **(E)** Model of (Δ1-91)RI (Su et al., 1995). One cAMP molecule (yellow) occupies each domain, A (turquoise) and B (royal blue), acidic residues protected from chemical modification in the holoenzyme are indicated in white. The arrow indicates the position of Glu143. **(B,D,F)** The electrostatic features of C and (Δ1-91)RI were modeled using the GRASP program. Versions of B and D were used in Tsigelny et al. (1996). Negative charge is indicated in red, positive charge in blue, and neutral charge in white. **(B)** Surface of C important for binding PKI and inhibitory peptides (PRS1). **(D)** Surface of C important for high affinity binding of R (PRS2). **(F)** Electrostatic surface of (Δ1-91)RI.

(Glass et al., 1989; Gibbs & Zoller, 1991a, 1991b; Knighton et al., 1991a, 1991b). Figure 8C and D shows a view of the C-subunit, rotated 180° around the z-axis. This surface (PRS2) contains several basic residues, including His87, Lys189, Lys213, Arg194, and Lys217, which surround the negatively charged phosphothreonine, Thr197-P, in the activation loop. Mutations in this surface generate mutant proteins that are defective in regulation by R. These mutants do not, however, interfere with binding to PKI. It is this surface that is important for binding to the R-subunit. In contrast to this basic surface on C, the A-domain of RI, which must dock to that surface, is negatively charged (Fig. 8F).

Although the sites corresponding to Lys189, Lys213, and Lys217 were all identified as important R/C interaction sites in yeast, when mutated in mammalian C only Lys213 and Lys217 were

defective in forming holoenzyme, with the K213A mutation being somewhat more defective than the K217A mutation. This is the opposite of what was observed when the equivalent mutations were introduced in TPK1 (Table 1) (Gibbs et al., 1992). These results are consistent, however, with the chemical modification of these residues and their position on the crystal structure of the murine C-subunit. Chemical modification of the C-subunit by acetic anhydride demonstrated that Lys217 was less reactive than Lys213 (Buechler et al., 1989). In the crystal structure of C, unlike Lys213, which is exposed, Lys217 interacts with the backbone carbonyl of Gly193, a residue located on a loop containing Lys189, which, in turn, is involved in a hydrogen bond network surrounding P-Thr197 (Zheng et al., 1993). The different effects of mutating the homologous residues, Lys189, Lys213, and Lys217 in the

murine and yeast catalytic subunits suggests that even though these molecules are 40% homologous (Toda et al., 1987), the precise molecular interactions between TPK1 and BCY1 in yeast, and the molecular interactions between RI and C α in the mammalian system, are likely to vary. It should also be emphasized that R/C interaction in the yeast PKA is several orders of magnitude weaker ($K_d = 20$ nM) than the interaction of the mammalian subunits ($K_d = 0.2$ nM) (Levin & Zoller, 1990; Herberg et al., 1994). The yeast C-subunits also are not inhibited by PKI.

Chemical modification of the type I holoenzyme identified several acidic residues on RI that were protected from modification by the water-soluble carbodiimide, 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDC) in the holoenzyme complex (J. A. Buechler, unpubl. obs.). Charge-to-Ala mutagenesis of recombinantly expressed RI confirmed that three of these sites, Asp140, Glu143, and Asp258, were important for holoenzyme formation (Gibson et al., 1997). The positions of these residues are indicated in Figure 8E. Three mutant RI-subunits, rRI(D140A), rRI(E143A), and rRI(D258A), were paired with basic-to-Ala mutant C-subunits to address the role of the electrostatic features of R and C in the formation of holoenzyme. The mutant pair, rC(K213A) and rRI(E143A), formed holoenzyme no worse than when either of the single mutants were paired with wild type, indicating an interaction between these two residues. The space-filling models of RI and C (Fig. 8C and E) show the position of Lys213 on the C-subunit and Glu143 on the RI-subunit.

The precise contacts between the regulatory and catalytic subunits in the holoenzyme complex will not be fully elucidated until a high-resolution crystal structure of the holoenzyme is solved. However, in the absence of a crystal structure, this electrostatic contact provides a basis for analyzing the docking surfaces on these two proteins. Using the crystal structures of rRI(Δ 1-91) and C, we are now attempting to model a docked complex based on the Lys213–Glu143 interaction pair. Because the R-subunit is thought to undergo significant conformational changes upon releasing cAMP and binding to C, however, a simple docking process is unlikely.

Materials and methods

Materials

Reagents were purchased as follows: adenosine 5'-triphosphate (ATP), 2-(N-morpholino)ethanesulfonic acid (Mes), MOPS, tris(hydroxymethyl)aminomethane (Tris), (Sigma, St. Louis, MO); restriction endonucleases, T4 ligase, T7 polymerase, (United States Biochemical, Cleveland, OH, or GIBCO/BRL, Grand Island, NY); radioactive nucleotides (Amersham, Arlington, IL, or NEN Dupont, Boston, MA); media supplies (Difco). The peptide substrate, LRRASLG, was synthesized at the Peptide and Oligonucleotide Facility at the University of California, San Diego. Oligonucleotides were synthesized with an Applied Biosystem DNA synthesizer, Model 380B. The following bacterial strains were used: *E. coli* DH5 α , *E. coli* JM101 (ATCC, Rockville, MD), *E. coli* BL21-DE3 (gift from William Studier of Brookhaven National Laboratories, Upton, NY), and *E. coli* E222. The following vectors were used: phagemid pUC119 (ATCC), PRSET-B (Invitrogen, San Diego, CA), and PLWS-3 (Slice & Taylor, 1989).

Mutagenesis

Mutations in the regulatory and catalytic subunits were introduced as described by Kunkel (Kunkel, 1985). Lys213 and Lys217 were

mutated to Ala in the C-subunit in the vector PRSET-B to generate the mutants, rC(K213A) and rC(K217A). Lys189 was mutated to Thr in the vector pUC119. The K189T mutant C α gene was then sub-cloned into the expression vector PLWS-3 according to Slice and Taylor (1989).

Purification of catalytic subunits

Wild-type or mutant C-subunits were purified from 4L of BL21(DE3) *E. coli* by phosphocellulose chromatography as described previously (Yonemoto et al., 1991). Batch elutions were performed with 150 mM potassium phosphate. Protein fractions were pooled and concentrated using an Amicon concentrator with a YM30 filter disk. Following P-11 purification, rC(WT) and rC(K189T) were further purified by FPLC ion exchange chromatography on Mono-S (Pharmacia, Piscataway, NJ) (Herberg et al., 1993). Isoform II was used for the following experiments. Purified C-subunits were stored at 4 °C at a concentration of 1 mg/mL. The purity of the proteins was confirmed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Concentration was determined by absorbance at 280 nm using an extinction coefficient of 1.2.

Purification of regulatory subunits

Wild-type RI and mutant regulatory subunits, rRI(D140A), rRI(E143A), and rRI(D258A), were purified from *E. coli* 222 cells as described previously (Gibson et al., 1997) using anion exchange chromatography (DE52) followed by FPLC gel filtration on Superdex 200 (Pharmacia). The concentration and purities of the RI-subunits were determined by absorbance at 280 nm using an extinction coefficient of 1.0 and by SDS-PAGE. The purified RI-subunits were approximately 95% pure as judged by SDS-PAGE and analytical gel filtration.

The cAMP binding sites in the purified R-subunits are typically fully saturated with cAMP and dialysis is not sufficient to remove the cAMP. To prepare cAMP-free R-subunits, the RI-subunit was treated with urea as described previously (Buechler et al., 1993).

Kinetic characterization of mutant C-subunits

The specific activities of the C-subunits were determined using the spectrophotometric assay of Cook et al. (1982) and the peptide substrate, Kempptide (LRRASLG). The K_m s for ATP and peptide substrate were determined using Michaelis-Menten kinetics. Typically, 20 nM C-subunit was pre-equilibrated with buffer containing 1 mM ATP, 10 mM MgCl₂, 1 mM phosphoenolpyruvate, 0.3 mM NADH, lactate dehydrogenase (12 units), and pyruvate kinase (4 units) in a final volume of 1 mL. Reactions were initiated by adding varying amounts of Kempptide (LRRASLG). To determine the K_m for ATP, C-subunit was pre-equilibrated in assay mix containing saturating amounts of Kempptide (200 μ M) and the reaction was initiated by adding varying amounts of ATP. K_m s and V_{max} were determined using a curve-fitting program, KaleidaGraph™.

Inhibition of mutant C-subunits

Two methods were used to study the inhibition of C. For the first method, a fixed concentration of C (20–30 nM) was incubated with increasing concentrations of either cAMP-free RI or PKI in 1 mL of assay mix for 1 min at room temperature prior to the

addition of Kempptide substrate. The remaining activity based on the spectrophotometric assay was then determined. For cAMP-free R-subunit and PKI with wild type C, the inhibition curves are linear and stoichiometric because the K_d s for the wild-type R and PKI are well below the 20 nM concentration of C used in the assay.

Equilibrium dialysis was also used to evaluate R/C interaction. For this method, cAMP-saturated RI (R_2cAMP_4) (3 μ M) was incubated with C (2 μ M) (vol. = 0.8 mL) and dialyzed against buffer A (25 mM potassium phosphate, 100 mM ATP, 500 mM $MgCl_2$, 5% glycerol, 5 mM β -ME, pH 6.5) at 22°C. Aliquots (30 mL) were removed at the indicated times and assayed for catalytic activity in the absence and presence of 100 μ M cAMP. The total activity in the presence of cAMP did not change over the course of the dialysis. The experiments were carried out with a small excess (1.5-fold) of $R_2(cAMP)_4$ to ensure that the end point of the reaction would theoretically correspond to zero catalytic activity in the holoenzyme mixture, indicating complete holoenzyme formation. In the dialysis method, the C-subunit must compete with cAMP for the regulatory subunit before an inhibited complex can form. Although at 4°C the release of cAMP is very slow and little holoenzyme forms, at 22°C, wild-type holoenzyme forms an inhibited complex after about 10 h of dialysis. The data generated from this method are very reproducible and are always carried out in duplicate along with wild-type controls.

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References

- Buechler JA, Vedvick TA, Taylor SS. 1989. Differential labeling of the catalytic subunit of cAMP-dependent protein kinase with acetic anhydride. *Biochemistry* 28:3018–3024.
- Buechler YJ, Herberg FW, Taylor SS. 1993. Regulation defective mutants of type I cAMP-dependent protein kinase: Consequences of replacing Arg94 and Arg95. *J Biol Chem* 268:16495–16503.
- Cheng H-C, Kemp BE, Pearson RB, Smith AJ, Misconi L, Van Patten SM, Walsh DA. 1986. A potent synthetic peptide inhibitor of the cAMP-dependent protein kinase. *J Biol Chem* 261:989–992.
- Cook PF, Neville ME, Vrana KE, Hartl FT, Roskoski JR. 1982. Adenosine Cyclic 3',5'-monophosphate dependent protein kinase: Kinetic mechanism for the bovine skeletal muscle catalytic subunit. *Biochemistry* 21:5794–5799.
- Corbin JD, Sugden PH, Lincoln TM, Keely SL. 1977. Compartmentalization of adenosine 3':5'-monophosphate and adenosine 3':5'-monophosphate-dependent protein kinase in heart tissue. *J Biol Chem* 252:3854–3861.
- Cox S, Radzio-Andzelm E, Taylor SS. 1994. Domain movements in protein kinases. *Curr Opin Struct Biol* 4:893–901.
- First EA, Bubis J, Taylor SS. 1988. Subunit interaction sites between the regulatory and catalytic subunits of cAMP-dependent protein kinase: Identification of a specific interchain disulfide bond. *J Biol Chem* 263:5176–5182.
- Gibbs CS, Knighton DR, Sowadski JM, Taylor SS, Zoller MJ. 1992. Systematic mutational analysis of cAMP-dependent protein kinase identifies unregulated catalytic subunits and defines regions important for the recognition of the regulatory subunit. *J Biol Chem* 267:4806–4814.
- Gibbs CS, Zoller MJ. 1991a. Identification of electrostatic interactions that determine the phosphorylation site specificity of the cAMP-dependent protein kinase. *Biochemistry* 30:5329–5434.
- Gibbs CS, Zoller MJ. 1991b. Rational scanning mutagenesis of a protein kinase identifies functional regions involved in catalysis and substrate interactions. *J Biol Chem* 266:8923–8931.
- Gibson RM, Buechler YJ, Taylor SS. 1997. Interaction of the regulatory and catalytic subunits of cAMP-dependent protein kinase. *J Biol Chem* 272:16343–16350.
- Glass DB, Cheng H-C, Mende-Mueller L, Reed J, Walsh DA. 1989. Primary structural determinants essential for potent inhibition of cAMP-dependent protein kinase by inhibitory peptides corresponding to the active portion of the heat-stable inhibitor protein. *J Biol Chem* 264:8802–8810.
- Herberg FW, Bell SM, Taylor SS. 1993. Expression of the catalytic subunit of cAMP-dependent protein kinase in *E. coli*: Multiple isozymes reflect different phosphorylation states. *Protein Eng* 6:771–777.
- Herberg FW, Dostmann WRG, Zorn M, Davis SJ, Taylor SS. 1994. Crosstalk between domains in the regulatory subunit of cAMP-dependent protein kinase: Influence of amino terminus on cAMP binding and holoenzyme formation. *Biochemistry* 33:7485–7494.
- Herberg FW, Taylor SS. 1993. Physiological inhibitors of the catalytic subunit of cAMP-dependent protein kinase: Effect of MgATP on protein/protein interaction. *Biochemistry* 32:14015–14022.
- Herberg FW, Taylor SS, Dostmann WRG. 1995. Active site mutations define the pathway for the cooperative activation of cAMP-dependent protein kinase. *Biochemistry* 35:2934–2942.
- Hofmann F. 1980. Apparent constants for the interaction of regulatory and catalytic subunit of cAMP-dependent protein kinase I and II. *J Biol Chem* 255:1559–1564.
- Knighton DR, Zheng J, Ten Eyck LF, Ashford VA, Xuong N-h, Taylor SS, Sowadski JM. 1991a. Crystal structure of the catalytic subunit of cAMP-dependent protein kinase. *Science* 253:407–414.
- Knighton DR, Zheng J, Ten Eyck LF, Xuong N-h, Taylor SS, Sowadski JM. 1991b. Structure of a peptide inhibitor bound to the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* 253:414–420.
- Kunkel TA. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc Natl Acad Sci USA* 82:488–492.
- Laemmli UK. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.
- Levin LR, Kuret J, Johnson KE, Powers S, Cameron S, Michaeli T, Wigler M, Zoller MJ. 1988. A mutation in the catalytic subunit of cAMP-dependent protein kinase that disrupts regulation. *Science* 240:68–70.
- Levin LR, Zoller MJ. 1990. Association of catalytic and regulatory subunits of cyclic AMP-dependent protein kinase requires a negatively charged side group at a conserved threonine. *Mol Cell Biol* 10:1066–1075.
- Ogreid D, Doskeland SO. 1981. The kinetics of the interaction between cyclic AMP and the regulatory moiety of protein kinase II. *FEBS Lett* 129:282–286.
- Orellana SA, Amicux PS, Zhao X, McKnight GS. 1993. Mutations in the catalytic subunit of the cAMP-dependent protein kinase result interfere with holoenzyme formation without disrupting inhibition by protein kinase inhibitor. *J Biol Chem* 268:6843–6846.
- Orellana SA, McKnight GS. 1992. Mutations in the catalytic subunit of cAMP-dependent protein kinase result in unregulated biological activity. *Proc Natl Acad Sci USA* 89:4726–4730.
- Ringheim GE, Taylor SS. 1990. Dissecting the domain structure of the regulatory subunit of cAMP-dependent protein kinase I and elucidating the role of MgATP. *J Biol Chem* 265:4800–4808.
- Saraswat L, Ringheim GA, Bubis J, Taylor SS. 1988. Deletion mutants as probes for localizing regions of subunit interaction in cAMP dependent protein kinase. *J Biol Chem* 263:18241–18246.
- Scott JD, Glacum MB, Fischer EH, Krebs EG. 1986. Primary-structure requirements for inhibition by the heat-stable inhibitor of the cAMP-dependent protein kinase. *Proc Natl Acad Sci USA* 83:1613–1616.
- Slice LW, Taylor SS. 1989. Expression of the catalytic subunit of cAMP-dependent protein kinase in *Escherichia coli*. *J Biol Chem* 264:20940–20946.
- Su Y, Dostmann WRG, Herberg FW, Durick K, Xuong N-h, Ten Eyck L, Taylor SS, Varughese KI. 1995. Regulatory subunit of protein kinase A: Structure of deletion mutant with cAMP binding domains. *Science* 269:807–813.
- Taylor SS, Buechler JA, Yonemoto W. 1990. cAMP-dependent protein kinase: Framework for a diverse family of regulatory enzymes. *Annu Rev Biochem* 59:971–1005.
- Toda T, Cameron S, Sass P, Zoller MJ, Wigler M. 1987. Three different genes in *S. cerevisiae* encode the catalytic subunits of the cAMP-dependent protein kinase. *Cell* 50:277–287.
- Tsigelny I, Grant BD, Taylor SS, Ten Eyck LF. 1996. Catalytic subunit of cAMP-dependent protein kinase: Electrostatic features and peptide recognition. *Biopolymers* 39:353–365.
- Walsh DA, Angelos KL, Van Patten SM, Glass DB, Garetto LP. 1990. *The inhibitor protein of the cAMP-dependent protein kinase*. Boca Raton, FL: CRC Press.
- Walsh DA, Perkins JP, Krebs EG. 1968. An adenosine 3',5'-mono-phosphate-dependent protein kinase from rabbit skeletal muscle. *J Biol Chem* 243:3763–3765.
- Wang YH, Scott JD, McKnight GS, Krebs EG. 1991. A constitutively active holoenzyme form of the cAMP-dependent protein kinase. *Proc Natl Acad Sci USA* 88:2446–2450.
- Weber W, Hiltz H. 1979. Stoichiometry of cAMP binding and limited proteolysis of protein kinase regulatory subunits RI and RII. *Biochem Biophys Res Commun* 90:1073–1081.

- Weldon SL, Taylor SS. 1985. Monoclonal antibodies as probes for functional domains in cAMP-dependent protein kinase II. *J Biol Chem* 260:4203–4209.
- Wells JA, Powers DB, Bott RR, Graycar TP, Estell DA. 1987. Designing substrate specificity by protein engineering of electrostatic interactions. *Proc Natl Acad Sci USA* 84:1219–1223.
- Wen W, Taylor SS. 1994. 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 Arg133. *J Biol Chem* 269:8423–8430.
- Zetterqvist ÖZ, Ragnarsson U, Engstrom L. 1990. *Substrate specificity of cyclic AMP-dependent protein kinase*. Boca Raton, FL: CRC Press.
- Zheng J, Knighton DR, Ten Eyck LF, Karlsson R, Xuong N-h, Taylor SS, Sowadski JM. 1993. Crystal structure of the catalytic subunit of the cAMP-dependent protein kinase complexed with MgATP and peptide inhibitor. *Biochemistry* 32:2154–2161.