Contribution of Non-catalytic Core Residues to Activity and Regulation in Protein Kinase A*

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Protein kinase A holoenzyme is comprised of two catalytic (C) and two regulatory (R) subunits which keep the enzyme in an inhibited state before activation by cyclic-AMP. The C-subunit folds into a conserved bi-lobel core flanked by N- and C-terminal tails. We report here characterization of a C-tail loss-of-function mutant, C-F327A, and a related suppressor mutant, C-F327A/K285P. Phe-327 is the only residue outside the kinase core that binds to the adenine ring of ATP, whereas Lys-285 is ~45 Å away and lies in an AGC kinase-specific insert. The two mutations were previously identified from a yeast genetic screen, where the F327A mutation was unable to complement cell growth but mutation of K285P in the same allele rescued cell viability. We show that C-F327A exhibits significant reduction in catalytic efficiency, which likely explains the observed loss-of-function phenotype. Interestingly, the additional K285P mutation does not restore kinase activity but reduces the inhibitory interaction of the double mutant with RII subunits. The additional K285P mutation, thus, helps to keep a low but uninhibited PKA activity that is sufficient for cell viability. The crystal structure of C-F327A/K285P further reveals that recruitment of Phe-327 to the ATP binding pocket not only contributes to the hydrophobic pocket, as previously thought, but also recruits its flanking C-tail region to the kinase core, thereby concertedly positioning the glycine-rich loop and ATP for phosphoryl transfer. The study exemplifies two different ways for regulating cAMP-dependent protein kinase activity through non-conserved residues and sheds light on the structural and functional diversity of the kinase family.

Cyclic-AMP-dependent protein kinase (PKA) is involved in a wide range of cellular functions such as metabolism, transport, cell cycle, and gene regulation. The PKA tetrameric holoenzyme is comprised of two catalytic (C) subunits that possess kinase activity and two inhibitory regulatory (R) subunits that each have two tandem cyclic AMP (cAMP) binding domains (1). In the absence of cAMP, the R-subunit binds to the C-subunit and blocks substrate access so as to inhibit kinase activity. Binding of cAMP to the R-subunits releases the inhibitory interactions and unleashes the C-subunit, allowing it to perform substrate phosphorylation. The R-subunit in yeast Saccharomyces cerevisiae is Bcy1p (2).

As the first kinase structure solved in 1991 (3, 4), PKA C-subunit has been a paradigm of protein kinases and is best studied in terms of its structure-function relationship and kinetic properties (5). The conserved kinase core (residues 40–300) of the C-subunit folds into an N-terminal lobe (N-lobe) and a C-terminal lobe (C-lobe) with the ATP binding site formed in between. The N-lobe is comprised of a twisted β sheet formed by five β-strands and two helices (αB and αC). One of the structural features in the N-lobe is the glycine-rich loop between the β1 and β2 strands. This loop senses nucleotide binding in the active site and exhibits different conformations. The C-lobe is mainly helical (αE–αF) with highly conserved αE and αF helices forming a hydrophobic core. The two lobes are connected by the linker region (residues 120–127). The non-conserved PKA-specific N (residues 1–39) and C termini (residues 301–350) each wrap around the kinase core and interact with both lobes (Fig. 1A). The C-subunit has been crystallized in an apo conformation (6) and with different nucleotides and/or peptides bound (3, 6–11). Correspondingly, the structures have been categorized into open and closed conformations of the active site cleft based on the relative positions of N and the C lobes. The relative movement of the two lobes is essential for catalysis (12).

Bioinformatics analysis revealed that the C-tail is a hallmark of the AGC subfamily of kinases (PKA, PKG, PKC family) that provides intramolecular regulation of the kinase (13). Kannan et al. (13) defined three sites conserved in AGC kinases that tether the C-tail to the kinase core: the N-lobe tether, the active-site tether (AST), and the C-lobe tether. The N-lobe tether docks the very C terminus FXXF motif (single amino acid code, X represents any amino acid) to a hydrophobic pocket for PKA that consists of residue 5–24 of protein kinase inhibitor (PKI); AGC, protein kinase A, kinase G and kinase C; AST, active-site tether; Bicine, N,N-bis(2-hydroxyethyl)glycine; MOPS, 3-(N-morpholino)propanesulfonic acid.
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above the αC helix in the N-lobe. This interaction has been shown to be conserved in the AGC kinases and plays an important role in regulating kinase activity (14). The C-lobe tether links the beginning of the C-tail (300–318) to the αE-helix and the β8 strand in the C-lobe, the αC-B4 loop in the N-lobe, and the linker connecting the two lobes. The AST is the most dynamic part of the C-tail. In the apo structure when no nucleotide is bound, the tail loses contact at the AST site, causing most of the residues in this region to become disordered (Fig. 1A). An important feature of the AST is the interaction of Phe-327 with the ATP binding pocket (13), where Phe-327 contributes to closing the hydrophobic pocket for the adenine moiety of ATP (Fig. 1B).

Many efforts have focused on the development of drug-like inhibitors targeting the ATP binding pockets of protein kinases. It has proved challenging due to the conserved features of the inhibitors targeting the ATP binding pockets of protein kinases. Kennedy et al. (16) developed a yeast genetic screen to further elucidate the function of ATP site residues and their connections to other parts of the molecule. The PKA C-subunits in S. cerevisiae are encoded by three TPK genes (TPK1, TPK2, and TPK3). PKA activity is essential for cell survival, and a nonviable phenotype was observed in a tpk-null strain (17). However, the mammalian mammalian C-subunit can replace the yeast counterparts to maintain cell viability (18). Utilizing this property, Ala mutants of eight residues that form the adenosine binding pocket (Leu-49, Val-57, Val-104, Met-120, Tyr-122, Leu-173, Thr-183, and Phe-327, Fig. 1B) were each introduced into a tpk-null strain to determine whether these mutants could maintain viability. Of the mutants tested, only the F327A mutant and L173A mutant failed to do so. Random mutations were then introduced to the same mutant gene harboring either the F327A or L173A mutation, and these mutagenic libraries were screened for suppression of the non- viable phenotype. Several suppressors were identified. Although some of these suppressor mutations were located near the ATP binding site, the majority were distal to the ATP pocket. One such suppressor for the F327A phenotype acquired an additional K285P mutation.

Lys-285 is localized in the loop connecting the αH- and α1-helix (αH-α1 Loop, Leu-272—Val-288, Fig. 1A) at the end of the C-lobe of the kinase, 45 Å away from the ATP pocket (Fig. 1A). This loop is part of a recently identified long-range network that connects the catalytic loop and peripheral sites on the C-lobe, including the αG-helix that contributes directly to interactions with the R-subunit (19–21). Bioinformatics analysis also highlighted a five-residue insert (AGC-specific insert, Fig. 1A) within this region (Gly-282—Gly-286) that is specific to the AGC subfamily of kinases. Although they all have the insert, the sequence is different for each AGC kinase (13).

To provide a biochemical explanation of why F327A, a residue that lies outside the conserved kinase core, failed to maintain cell growth and why an additional distal site mutation of K285P rescued cell viability and, hence, to understand the function of these two uniquely located sites, we purified the mutants, compared their enzymatic properties, and solved the crystal structure of CF327A/K285P. We showed that the catalytic efficiency of F327A was reduced by ~50-fold, and this might explain why it failed to maintain cell growth. Structural analysis further suggested that Phe-327 plays a key role in ATP binding as well as in further engaging its flanking C-tail region to interact with the kinase core to correctly orient the glycine-rich loop and ATP for catalysis. On the other hand, K285P had no effect on kinetic properties, but it affected the inhibitory interaction with the R-subunit mechanism. Our data showed that additional K285P mutation rendered the double mutant CF327A/K285P a low PKA kinase activity that was not inhibited by Bcy1p, which appeared to be sufficient for cell growth. This provided a mechanism for suppression of the F327A phenotype by a distal site mutation K285P.

EXPERIMENTAL PROCEDURES

Materials—Inhibitory peptide IP20 (TTYADFIASGRT-GRRNAIHD, residues 5–24 from the heat stable PKA inhibitor PKI) was synthesized on a Milligen peptide synthesizer and purified by high performance liquid chromatography. Pre-packed Mono S 10/10 ion exchange column and Superdex 75 gel filtration column were purchased from GE Health. Crystallization reagents 2-methyl-2,4-pentanediol, Bicine, glycerol, and ammonium acetate were obtained from Fluka. Phosphocellulose paper p81 was from Whatman. Radioisotope labeled [γ-32P]ATP was from GE Healthcare. Other reagents were from Qiagen, Invitrogen, and Sigma-Aldrich.

Purification of Yeast and Mammalian R-subunit—Recombinant yeast (Bcy1p) and mammalian (RII) R-subunits were cloned into a pRSET vector at the NdeI site to remove the polyhistidine tag. Each contained the two CAMP binding domains A and B and the inhibitory sequence without the N-terminal dimerization domain. Specifically, they were yeast Bcy1p (136–416) and rat RIIβ (108–402). Proteins were overexpressed in Escherichia coli BL21 (DE3) cells and purified to homogeneity using CAMP-agarose affinity resin. Cell pellets were resuspended in lysis buffer containing 50 mM MOPS, pH 7.0, 500 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2 mM dithiothreitol, plus protease inhibitors (5 mM benzamidine, 0.5 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 1 mM pepstatin A, 1 mM leupeptin, 30 μM each of Nα-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and Nα-tosyl-L-lysine chloromethyl ketone (TLCK)). Isobutylmethylxanthine, a nonspecific phosphodiesterase (PDE) inhibitor was also included to protect CAMP on the resin from being degraded by cellular PDEs. After passing through a French press at 1000 p.s.i., the lysate was centrifuged at 30,000 × g for 45 min at 4 °C. Approximately 50 ml of cell lysate supernatant (from 2 to 6 liters of culture) was either batch-wise incubated with 2–5 ml of CAMP-agarose resin for 1 h at 4 °C or passed through a 5-ml prepacked CAMP-agarose column at 1 ml/min through a Profinia affinity purification system (Bio-Rad). The resin was washed with 700 mM NaCl in lysis buffer. The protein was eluted with 6 ml of 25 mM cGMP in above lysis buffer, with pH adjusted to 5.5 to ensure efficient elution. Eluted protein was concentrated and applied to a Superdex 75 gel filtration column for further purification. The column was equilibrated with 50 mM MOPS, pH 7.0, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2 mM dithiothreitol plus 5 mM benzamidine. Protein was eluted with the same

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buffer. This step further purified the protein to homogeneity and efficiently removed the cGMP molecules from the buffer. The next step involved the use of a Mono S 10/10 column (GE Healthcare), followed by cation exchange chromatography on pre-packed Superdex 75 gel filtration column and eluted with 50 mM Bicine, 200 mM ammonium acetate, pH 8.0, with 2 mM dithiothreitol. The protein was concentrated to

| TABLE 1 |
|------------------|
| **Data collection and refinement statistics** |
| **Space group** | P2_12_1 |
| **Cell dimensions** | |
| a (Å) | 57.565 |
| b (Å) | 78.50 |
| c (Å) | 100.63 |
| **Number of molecules per asymmetric unit** | 1 |
| **Number of unique reflections** | 12,647 |
| **Redundancy** | 2.6 |
| **Resolution range (Å)** | 50.0-2.5 (2.50-2.59) |
| **Rsym** | 0.095 (0.440) |
| **Data completeness (%)** | 87.0 (91.0) |
| **R-factor (R_free)** | 0.212 (0.265) |

**Root mean square deviation from ideality**

- Bond lengths (Å): 0.008
- Bond angles (%): 1.4

* Rsym = 2(I-<I>)/<I>, where I is observed intensity, <I> is the average intensity from multiple observations of symmetry-related reflections.

The numbers in the parentheses correspond to the highest resolution shell.

**Purification of C-subunit Mutants**—C-subunit mutations of F327A, K285P, or the double mutant were introduced to the murine PKA C subunit in pRSET vector using the QuikChange kit by Stratagene (Agilent Technologies). All three mutants and the wild type protein were expressed in E. coli BL21 (DE3) cell and purified to homogeneity on P11 resin followed by cation exchange chromatography on pre-packed Mono S 10/10 column (GE Healthcare), as described previously (22). Expression of mutant protein C^C285P was similar as that of the wild type protein. Both C^F327A and C^F327A/K285P were coexpressed with a 3-phosphoinositide-dependent protein kinase 1 (PKC) kinase-expressing vector pGEX-PDK1 to ensure phosphorylation of Thr-197.

**Crystallization of C^F327A/K285P**—For crystallization, C^F327A/K285P from a Mono S column was further purified on a Superdex 75 gel filtration column and eluted with 50 mM Bicine, 200 mM ammonium acetate, pH 8.0, with 2 mM dithiothreitol. The protein was concentrated to ~5 mg/ml and mixed with MgCl₂, ATP, and IP20 at a molar ratio of 1:10:10:10 before setting up the crystallization trials. Hanging-drop vapor diffusion was used; each drop consisted of 1 μl of the protein mixture and 1 μl of well solution. Crystals appeared after 1–2 weeks at 4 °C at conditions of 8–12% 2-methyl-2,4-pentanediol, 100 mM Tris-HCl, pH 7.5, 10 mM dithiothreitol.

**Data Collection and Refinement**—A 2.5 Å data set was collected at the x-ray crystallography facility at University of California, San Diego equipped with Rigaku FR rotating anode generators. Data collection was performed under a liquid nitrogen stream. The cryo-protectant consisted of 15% 2-methyl-2,4-pentanediol, 1% 2-mercaptoethanol, 0.5–2 nM C-subunit, and varied concentrations of either ATP or Kemptide at 30 °C for 15 min. Reactions were quenched with 30% acetic acid. Specific activity of [γ-32P]ATP was in the range of 500–2000 cpm/pmol. An ascending chromatography on phosphocellulose p81 paper (Whatman) was used to separate unreacted [γ-32P]ATP from the protein-bound radioactivity as described previously (26). Briefly, 20 μl of the quenched reaction mix was spotted on pre-marked samples on a 20 × 20 cm square sheet of p81 paper. The whole p81 sheet was then placed into a chromatography development tank containing 20 mM phosphoric acid, pH 2–2.5, where peptides or proteins did not migrate, but the unreacted [γ-32P]ATP moved upward. After separation, the spot areas were cut out, dried, and put into scintillation vials for radioactivity detection by Cerenkov counting on a Beckman LS 6000SC liquid scintillation system.

**Inhibition Assay by the R-subunit**—Inhibition of the wild type or mutant C-subunits by Bcy1p or RII subunit was carried out under similar conditions as above. Either wild type or mutant C-subunit was incubated with varying concentrations of the R-subunit for 30 min at 30 °C. A mixture of Kemptide, ATP, and [γ-32P]ATP was then added to start the reaction. Final concentrations were 1–2 nM C-subunit, 0.5 mM ATP, and 0.5–1 mM Kemptide, and each R-subunit ranging from 2 μM to 1 mM. Similarly, as described above, the reaction was quenched by 30% acetic acid, and phosphate incorporation into Kemptide was quantified. Curve fitting and IC₅₀ calculations were performed with GraphPad Prism 5 software (GraphPad, San Diego, CA).

**RESULTS**

**F327A Caused Significant Reduction of the Catalytic Efficiency of the Enzyme**

Residues forming the hydrophobic binding pocket for the adenine ring in PKA are shown in Fig. 1B. Phe-327 is unique in several ways. It is the only residue that lies outside the conserved core and the only one that exhibits large conformational changes in the presence and absence of the nucleotide (5). In the yeast genetic screen study by Kennedy et al. eight adenosine complex of Y204A mutant, PDB ID 1RDQ) were tested as the searching model, with the latter structure producing a better solution. This model was refined to an R-factor of 21.2% and R_free of 26.5%. The final model included one molecule of C-subunit from residues 14–350 (the N-terminal 13 residues were disordered), one molecule of IP20, one molecule of ADP, and 24 water molecules. As assessed by Procheck (25), 84.4% of the main chain φ-ψ torsion angles were in the most favored region in the Ramachandran plot, with none falling in the non-allowed regions.

**Kinetic Assays**—Kinetic analysis of the wild type and mutant C-subunits were performed by radioisotope [γ-32P]ATP labeling using LRRASLG (Kemptide) as substrate. To measure the Kₘ for ATP, Kemptide was maintained at a fixed concentration of 0.5–1.0 mM, and the concentration of ATP was varied from 2 μM to 1 mM. To determine the Kₘ for Kemptide, the ATP concentration was fixed at 0.5 mM, and the Kemptide concentration was varied from 2 μM to 1 mM. Reactions were quenched with 30% acetic acid. Specific activity of [γ-32P]ATP was in the range of 500–2000 cpm/pmol. An ascending chromatography on phosphocellulose p81 paper (Whatman) was used to separate unreacted [γ-32P]ATP from the protein-bound radioactivity as described previously (26). Briefly, 20 μl of the quenched reaction mix was spotted on pre-marked samples on a 20 × 20 cm square sheet of p81 paper. The whole p81 sheet was then placed into a chromatography development tank containing 20 mM phosphoric acid, pH 2–2.5, where peptides or proteins did not migrate, but the unreacted [γ-32P]ATP moved upward. After separation, the spot areas were cut out, dried, and put into scintillation vials for radioactivity detection by Cerenkov counting on a Beckman LS 6000SC liquid scintillation system.
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Wild type C

| Enzyme       | \( K_{\text{Kemp}} \) (\( \mu M \)) | \( k_{\text{cat}} \) (s\(^{-1}\)) | \( K_{\text{ATP}} \) (\( \mu M \)) | \( k_{\text{cat}}/K_{\text{Kemp}} \) |
|--------------|---------------------------------|-----------------|-----------------|-----------------|
| Wild type C  | 11 ± 1                          | 20 ± 2          | 20 ± 3          | 1.8             |
| C\( ^{K285P} \) | 96 ± 1                          | 26              | 299             | 0.04            |
| C\( ^{F327A,K285P} \) | 1206 ± 51                      | 25 ± 5          | 181 ± 31        | 0.02            |

TABLE 2
Kinetic analysis of C-subunit mutants

Binding residues were mutated to Ala and assessed for their ability to maintain cellular viability in a tpk-null strain. The F327A mutant was one of only two mutants that could not maintain cellular viability. 4 We purified the C\( ^{F327A} \) mutant to homogeneity and assessed its kinetic properties. Kinetic data for the wild type enzyme in this study were comparable with previous studies (27), with a \( k_{\text{cat}} \) of 20/s and \( K_m \) for ATP and Kemptide of 20 and 11 \( \mu M \), respectively. Compared with the wild type enzyme, C\( ^{F327A} \) had a similar \( k_{\text{cat}} \) but the \( K_m \) for ATP and Kemptide increased 10- and 60-fold, respectively, reflecting a ∼50-fold decrease in catalytic efficiency (Table 2). Decrease of enzymatic activity of the mutant is consistent with the loss-of-function phenotype seen in the yeast viability screen, as PKA activity is required for cell growth.

K285P Mutation Does Not Alter Kinetic Properties of the Enzyme

The genetic screen showed that although C\( ^{F327A} \) mutant failed to maintain cell viability in the tpk-null strain, the additional, distal suppressor mutation K285P restored cell growth. 4 We then looked at the effect of the K285P mutation by itself and in the double mutant. C\( ^{K285P} \) had similar kinetic parameters as the wild type enzyme, with a \( k_{\text{cat}} \) of 20.9/s, and the \( K_m \) for ATP and Kemptide of 21.2 \( \mu M \) and 10.0 \( \mu M \), respectively (Table 2). C\( ^{F327A,K285P} \) had a \( k_{\text{cat}} \), comparable with the wild type, and the \( K_m \) for ATP or Kemptide was 10- or 100-fold higher, respectively, than that of the wild type enzyme. Its kinetic properties were similar to that of C\( ^{F327A} \). Thus, the K285P mutation did not alter kinetic properties of the enzyme, and it did not restore the reduced catalytic efficiency caused by the initial F327A mutation (Table 2).

F327A and K285P Exhibited Reduced Inhibition by the R-subunit

The above data suggested that the rescue of cell growth by additional K285P mutation was not due to a direct restoration of the kinase activity. This led us to the examination of the inhibitory interaction of the mutants with the R-subunits. We found that whereas the wild type C-subunit was readily inhibited by the yeast R-subunit Bcy1p with an IC\(_{50} \) of 4 nM, both mutations caused significant reductions in their inhibitions by Bcy1p. Although the concentration of Bcy1p required for 50% inhibition of C\( ^{F327A} \) was ∼300 nM, C\( ^{K285P} \) was only inhibited by ∼15% at a concentration of Bcy1p up to 1 \( \mu M \). The double mutant C\( ^{F327A,K285P} \) responded similarly to Bcy1p as the C\( ^{K285P} \) (Fig. 2A). In yeast cells the concentration of Bcy1p might be high enough to cause significant inhibition of the F327A mutant but left the double mutant much less inhibited. Thus, although the catalytic activity of C\( ^{F327A,K285P} \) was not restored, the K285P mutation appeared to release the double mutant from inhibition by the Bcy1p, leading to constitutively low yet uninhibited PKA activity that may be sufficient to sustain viability in the tpk-null strain.

It, thus, appeared that although both Phe-327 and Lys-285 contributed significantly to Bcy1p interaction, the K285P mutation profoundly disrupted this interaction. To test whether the same effect would apply for the mammalian R-subunits, we examined the inhibition profile of the mutants by the mammalian RII subunit, the isoform that shares most similarity with Bcy1p. RII subunit inhibited the wild type C-subunit with an IC\(_{50} \) at 3.6 nM. Both C\( ^{F327A} \) and C\( ^{K285P} \) mutants were inhibited by RII with an IC\(_{50} \) of ∼16 nM. Notably, the concentration of RII for 50% inhibition of C\( ^{F327A,K285P} \) was ∼500 nM, which was more than just an additive effect from the single mutations (Fig. 2B), suggesting a possible synergistic correlation between the two sites.

The Crystal Structure of C\( ^{F327A,K285P} \) Further Defines the Roles of Phe-327 and the C-tail in Regulation of Kinase Activity

To obtain structural insights into the effects caused by the mutations, the C\( ^{F327A,K285P} \) mutant protein was crystallized. The structure was solved at a resolution of 2.5 Å as a complex with ADP and the inhibitory peptide IP20 (Table 1). The overall structure was comparable with that of the wild type protein (Fig. 3A, left); the root-mean-square distance was 0.56 Å when compared with the whole molecule with a similar ternary complex of wild type C-subunit with ADP and SP20 (A21S mutant...
of IP20) (PDB ID 1JBP) (9). However, as indicated in Fig. 3B (orange arrows), three major differences were observed; 1) the C-tail moved away from and had fewer contacts with the core, 2) the glycine-rich loop moved away from the nucleotide, and 3) ADP, especially the ribose and phosphate moieties, shifted from their canonical positions in the active site and made no contacts with the glycine-rich loop. The K285P site also exhibited a modest shift in its main chain position (Fig. 3A).

Reduced Interaction of the C-tail with the Kinase Core—When in the apo-form where no ligands or substrates bind, the C-subunit assumed an open conformation where Phe-327 and the adjacent C-tail region were often disordered and not interacting with the kinase core. In the ternary complex the enzyme always assumed a closed conformation, where the C-tail anchored to the kinase core. Although as a ternary complex, the C-tail of CF327A/K285P assumed an intermediate conformation between the open and closed states. As seen in Fig. 3A, the C-tail detached itself from the core at the Phe/Ala-327 site. The most significant displacement occurred near residues Asn-326 and Phe/Ala-327, with a maximum shift of 3–6 Å away from where it resides when it is anchored to the core. The side chains of residues Asn-323—Asp-326 were disordered in CF327A/K285P, and interactions between the C-tail and core residues were significantly reduced (Fig. 3B).

Phe-327 is in the middle of the most dynamic region of the C-tail spanning from Asp-323 to Ile-335. The interaction of the Glu-332—Ile-335 region with the core remained largely unchanged in the mutant. Although MgCl₂ and ATP were included in the crystallization buffer, the electron density map clearly indicated the absence of any Mg²⁺ ions, and there was density only for ADP instead of ATP in the CF327A/K285P structure. In addition, the mode of ADP interactions with the protein was different from what has been previously observed for nucleotide binding in the wild type C-subunit structures. The presence of MgCl₂ and its concentration in the crystal was later confirmed by quantitative flame atomic spectrometry (data not shown).

Even though ADP still occupied the nucleotide binding site in the CF327A/K285P structure, notable displacements were observed starting from the adenine ring position; the deviation became more significant for the orientation of the phosphate group (Fig. 3C). The mutant structure showed that the β-phosphate group of ADP shifted away from the glycine-rich loop by 3–4 Å. As a result, the interactions that are normally seen between the nucleotide and the glycine-rich loop in wild type protein were lost. The phosphate moiety turned to interact with Asn-171 and the P-2 Arg of the IP20 peptide; these interactions were not previously observed. Notably, the temperature factors for the ADP molecule and glycine-rich loop were also signifi-

FIGURE 2. Inhibition of the wild type and mutant C-subunits by yeast Bcy1p and RII subunit. Purified wild type (wt) and mutant C-subunits were incubated with varying amounts of Bcy1p (A) or RII subunit (B) as indicated. Kinase activity toward Kemptide was measured as described under "Experimental Procedures." The data are plotted as a percentage of the activity in the absence of Bcy1p (A) or RII (B). IC₅₀ of Bcy1p (A) or RII (B) was deduced from curve-fitting using GraphPad Prism. Data are shown as a representative of duplicate experiments.
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A

B

C

FIGURE 3. Alterations in C-tail-core interactions and nucleotide binding in the C327A/K285P mutant. A, superimposition of C327A/K285P and the wild type C-subunit that complexed with ADP and SP20 peptide (PDB ID 1JBP (9)). Structures are rendered in schematic representation. Structural segments that exhibit changes in the mutant are highlighted. Those are the C-tail, glycine-rich loop, the ADP molecule, and the αH-αI loop from the wild type (blue) and the mutant (red) structures. The Cα atoms of Phe/Ala-327 in the C-tail, Ser-53 in the tip of the glycine-rich loop, and Lys/Pro-285 in the αH-αI loop are rendered as spheres. The IP20 peptide is in tan. The ADP binding pocket of the C327A/K285P mutant is rendered as a van de Waal surface model; the orientation is the same as that of the wild type protein in Fig. 1B. ADP molecules for the mutant structure (tan) and the wild type (pink) are drawn as sticks. F327A is in red, and Leu-49, which exhibits the largest positional shift, is colored in light blue. B, comparison of the interactions between the C-tail and the kinase core. The C-tail is colored red in the mutant structure (right) and in red in the mutant structure (left). The glycine-rich loop is colored in light gray, and the linker region is in teal. Three orange arrows (right) in the mutant structure indicate the positional shifts relative to the wild type structure of the C-tail, glycine-rich loop, and the ADP molecule. Hydrogen-bond interactions are shown as dashed lines. Detailed interactions between the C-tail and core residues are listed below the figures. Plausible hydrogen bond distances are shown in the last two columns for the wild type and mutant structures. ϕ indicates hydrophobic interactions among listed residues. — indicates disruption of the interaction. C, comparison of the mode of ADP binding to the active site in the wild type (left) and mutant (right) structures. Hydrogen bond interactions are shown as dashed lines. Detailed interactions are listed below the figures.
Phe-327 and the C-tail Dynamically Organize ATP Binding and Catalysis—Role of Phe-327 as a part of the ATP binding pocket has been recognized earlier. The crystal structure of C\(^{\alpha F\text{-327A/K285P}}\) showed that the function of Phe-327 may be more than that. In the mutant structure the C-tail is disordered, and the active site glycine-rich loop and the bound nucleotide were in different positions as seen in the wild type protein. Altogether they support a role of Phe-327 and the C-tail in organizing the active site into the correct conformation for catalysis. Binding of Phe-327 to ATP appears to drive a coherent mechanism to create an extended network of additional contacts between the C-tail and the core to position the glycine-rich loop and, hence, the phosphate moiety of ATP for phosphoryl transfer. The flexibility of the C-tail (6, 30) allows it to be anchored and released from the kinase core easily depending on whether ATP is bound or not. Of the eight residues that form the ATP binding pocket, mutation of the gatekeeper residue Met-120 to Gly only caused a decrease of 3-fold in catalytic efficiency, and mutation of Leu-173 to Ala appeared not to bring much change to the catalytic activity after the activation loop Thr-197 is phosphorylated by phosphoinositide-dependent protein kinase 1. In contrast, F327A mutation exhibited a ~50-fold reduced catalytic efficiency even when Thr-197 is phosphorylated. These data are in line with additional roles of Phe-327 than just binding the ATP as discussed above. Given the conservation of the sequences in this region (13), several AGC kinases, especially PKB and PKC, may also utilize the C-tail to regulate kinase activity in similar ways.

Role of Lys-285 in Regulation of the Inhibitory Interaction with the R-subunit—The side chain of Lys-285 is typically solvent-exposed and often disordered or highly flexible in the structure of the C-subunit when it is not bound to a regulatory subunit, so its functional importance was not appreciated until the recent structure of the RII subunit in complex with the C-subunit, where it showed that Lys-285 directly interacted with the R-subunit (31). Our data independently confirm that Lys-285 plays a key role in the inhibitory interaction of the C-subunit with Bcy1p and RII subunit (Fig. 2).

The location of Lys-285 also adds further significance why we chose to study the mutant. It is one of the five residues (Gly-282—Gly-286) that were shown by bioinformatics analysis to be an AGC-specific insert (13). This insert lies in the \(\alpha H\)-αl loop at the very end of the conserved kinase core. The \(\alpha H\)-αl loop was highlighted by recent findings that a conserved mutation of an Arg-280 in the loop has been linked to several disease states (32). Furthermore, Deminoff et al. (33) showed that when the residue corresponding to Arg-280 was mutated to Ala in the yeast C-subunit Tpk1p, the enzyme exhibited reduced activity and increased binding to some substrates. We showed that the mutation in mammalian C-subunit resulted in more than a 10-fold reduction in \(k_{\text{cat}}\). Arg-280 forms an ion pair interaction with Glu-208 in the substrate binding \(p + 1\) loop. This ion pair was shown to be a unique conserved feature among eukaryote protein kinases; it is not found in the simpler eukaryotic-like kinases (32). More studies are needed to further elucidate the role of the loop.

Connection between the ATP Binding Site (Phe-327) and the \(\alpha H\)-αl Loop (Lys-285)—As shown in Fig. 2B, a single mutation of K285P or F327A alone had a modest effect on inhibition by the RII subunit; however, the double mutant \(\alpha F^{\text{K285P/F327A}}\) appeared to synergistically bring an enhanced effect. Long-range communication between the \(\alpha H\)-αl loop and other parts of the molecule, including structural motifs in the active site, was previously observed by dynamic studies using hydrogen/deuterium exchange (19, 34). This study may extend the network to include the non-catalytic core regulatory motif. In a recent bioinformatics study, Kornev and co-workers (35) showed that the adenine ring of the nucleotide complete a “catalytic spine” connecting the C- and N-lobe of the kinase core, and Phe-327 interacts with the residues in the spine. The catalytic spine is a stretch of residues that create a hydrophobic interaction network starting from the C-lobe kinase core to the N-lobe by residues that are conserved among several diverse kinases (35). Perhaps this could be part of a pathway connecting the \(\alpha H\)-αl loop to the active site. Communication between the ATP binding site and distal regulatory sites have been documented in other kinases through different mechanisms (15, 36–38).

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