A chimeric mechanism for polyvalent *trans*-phosphorylation of PKA by PDK1

Robert A. Romano,¹ Natarajan Kannan,² Alexandr P. Kornev,³ Craig J. Allison,³ and Susan S. Taylor^{1,3,4*}

¹Departments of Chemistry and Biochemistry, University of California San Diego, La Jolla, California 92093-0654

²Department of Biochemistry and Molecular Biology and Institute of Bioinformatics, University of Georgia, Athens, Georgia 30602

³Howard Hughes Medical Institute, University of California San Diego, La Jolla, California 92093-0654

⁴Department of Pharmacology, University of California San Diego, La Jolla, California 92093-0654

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Abstract: Phosphorylation on the activation loop of AGC kinases is typically mediated by PDK1. The precise mechanism for this *in-trans* phosphorylation is unknown; however, docking of a hydrophobic (HF) motif in the C-tail of the substrate kinase onto the N-lobe of PDK1 is likely an essential step. Using a peptide array of PKA to identify other PDK1-interacting sites, we discovered a second AGC-conserved motif in the C-tail that interacts with PDK1. Since this motif [FD(X)₁₋₂Y/F] lies in the active site tether region and in PKA contributes to ATP binding, we call it the Adenosine binding (Ade) motif. The Ade motif is conserved as a PDK1-interacting site in Akt and PRK2, and we predict it will be a PDK1-interacting site for most AGC kinases. In PKA, the HF motif is only recognized when the turn motif Ser338 is phosphorylated, possibly serving as a phosphorylation “switch” that regulates how the Ade and HF motifs interact with PDK1. These results demonstrate that the extended AGC C-tail serves as a polyvalent element that *trans*-regulates PDK1 for catalysis. Modeling of the PKA C-tail onto PDK1 structure creates two chimeric sites; the ATP binding pocket, which is completed by the Ade motif, and the C-helix, which is positioned by the HF motif. Together, they demonstrate substrate-assisted catalysis involving two kinases that have co-evolved as symbiotic partners. The highly regulated turn motifs are the most variable part of the AGC C-tail. Elucidating the highly regulated *cis* and *trans* functions of the AGC tail is a significant future challenge.

Keywords: PKA; PDK1; AGC kinases; C-tail; phosphorylation; Ade motif; HF motif; turn motif

Introduction

Protein kinases represent one of the largest gene superfamilies,¹ and the AGC kinases constitute one of the major Ser/Thr kinase subfamilies. Although a number of AGC kinase structures have been solved recently, the catalytic (C) subunit of cAMP-dependent protein kinase (PKA) still serves as a prototype for the other AGC kinases for understanding catalysis² and more recently for understanding protein:protein interactions within this branch of the kinome. The catalytic

subunit of PKA contains 350 amino acids, and the conserved bilobal core is formed by residues 40–300. The small lobe of the core (N-lobe, residues 40–120) is made up of mostly β -sheets and is responsible for trapping ATP at the active site cleft. The mostly helical large lobe (C-lobe, residues 121–300) facilitates substrate recognition and provides the catalytic machinery for phosphoryl transfer.^{2–5} In the N-lobe, the glycine-rich loop binds ATP and coordinates with the C-helix to position the γ -phosphate of ATP for transfer to a protein substrate. The C-lobe contains a magnesium positioning loop, an activation loop, the catalytic loop, and the P+1 loop that are all necessary elements for catalysis. The activation segment is comprised of all but the catalytic loop. Only when the activation

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*Correspondence to: Susan S. Taylor, 9500 Gilman Drive, La Jolla, CA 92093-0654. E-mail: staylor@ucsd.edu

segment is phosphorylated is it anchored to the catalytic loop via the HRD motif. The N- and C-lobes are also linked non-covalently by two hydrophobic spines, a catalytic spine that is completed by the adenine ring of ATP and a regulatory spine that is formed upon activation.^{6,7}

Regulation of activity in most AGC kinases, including PKA, is governed in part by phosphorylation of the activation segment at a conserved Thr or Ser. This phosphorylation is mediated by upstream kinases, typically PDK1, and the addition of this one phosphate optimally positions the activation segment for substrate recognition and catalysis. Once activated, the kinase toggles between open and closed conformations as it goes through a catalytic cycle. This transition between open and closed conformations in PKA is governed, in part, by the binding of ATP and peptide or protein substrate; however, to have a fully active enzyme requires also specific interactions of the N- and C-tails with both lobes of the core.^{8,9} Although the N-terminal regions of AGC kinases are important in unique ways for the function of the core,¹⁰ they are not conserved throughout the family. In contrast, the C-tails are a conserved structural element of all AGC kinases, and the conformation of the C-tail fluctuates between static and dynamic states that correlate with opening and closing of the active site cleft. Sequence analysis, furthermore, shows that the unique features conserved in the core of the AGC kinases are there to functionally integrate the core with the C-tail.¹¹ This functional integration had previously suggested a *cis* regulatory role of the C-tail.⁸ In particular, three regions of the C-tail were highlighted to be important for interacting with the kinase core: the N-lobe tether (NLT), active site tether (AST), and the C-lobe tether (CLT) (refer Fig. 1). In PKA residues 340–350, at the C-terminus, form the NLT which wraps around and anchors to the small lobe in both the open and closed conformations.^{8,12} This NLT contains a hydrophobic (HF) motif at the C-terminus that interacts with a hydrophobic pocket on the small lobe of the AGC kinases. Mutation of F350 to alanine in PKA gives a kinase dead phenotype.^{8,13,14} Residues 320 through 339 constitute the AST, and they contribute directly to the ATP binding pocket and to flexibility as the kinase toggles between the open and closed conformations. In PKA, this region contains a string of acidic amino acids flanked by hydrophobic residues (FDDYEEEEI).¹² The large lobe of PKA is stabilized by the amino-terminal residues of the C-tail (300–319) classified as the CLT, and this is the only element of the C-tail that is anchored stably to the large lobe. The C-tail also contains motifs such as the PXXP motif that are proposed to interact with other proteins. Most recently, the PXXP motif in PKC β II was shown to bind to two chaperones Hsp90 and cdc37 and contribute to protein stability.¹⁵ Thus, the C-tail can also function as a *trans*-regulatory element by facilitating interactions with other proteins. PKA can serve as a paradigm for the other kinases

because there are many structures of closed and open conformations and because the entire C-tail was scanned by Ala mutagenesis.

One of the most studied conserved docking motifs in the C-tail is the HF motif at the end of the C-tail tether. PDK1, also in the AGC family, is the activating kinase for most AGC kinases.¹³ Unlike other AGC kinases, PDK1 has only the remnant of a C-tail [Fig. 1(B)]; thus, it lacks a conserved HF motif. Instead, it is proposed that each AGC kinase uses its own HF motif to dock to PDK1 by binding to the hydrophobic pocket on the N-lobe of PDK1. PDK1 can then phosphorylate the activation loop of the heterologous kinase to generate an active enzyme. Akt was the first substrate found for PDK1 followed by many other AGC kinases including PKA,^{16,17} Akt,¹⁸ and PKC.¹⁴

Our goal here was to identify potential motifs in PKA that could interact with an activating kinase, PDK1. To identify linear interacting motifs between the catalytic subunit of PKA and its activating kinase, PDK1, we used a peptide array strategy. By overlaying PDK1 with arrayed peptides from the catalytic subunit, we identified two major regions that contained PDK1-interacting peptides. These peptides were clustered in the activation segment that contains the phosphorylation site and in the C-tail. We show here that the conserved FD(X)₁₋₂Y/F motif in the AST region of the C-tail is a major docking site for PDK1. This motif is also a key recognition element for Akt and PRK-2 and, based on its strict conservation, is likely a PDK1-interacting motif for most AGC kinases. When Ser338 in the turn motif of PKA is phosphorylated, the hydrophobic motif becomes the dominant recognition element for PDK1. A comparison of arrays of PKA, Akt, and PIFtide show that many residues in the CLT are important for binding to PDK1, not just the hydrophobic phenylalanines. Modeling of the C-tail of PKA onto the structure of PDK1 allows us to predict an extended chimeric interface between PDK1 where the FD(X)₁₋₂Y/F motif completes the ATP binding pocket and positions the ATP phosphates for transfer to a protein substrate while the hydrophobic motif anchors the C-helix, which also helps to coordinate the positioning of the γ -phosphate for transfer to a protein substrate. The combined contributions of both motifs create a PDK1 that is optimally poised for transfer of the phosphate from ATP to the activation loop of its cognate substrate kinase. The turn motif that joins these two docking sites is highly variable and is frequently regulated by phosphorylation.

Results

Recognizing that there are many AGC-conserved peptide motifs embedded within the C-terminal tail of the PKA catalytic subunit, we sought to determine whether we could identify potential interacting sites using a peptide array strategy. By arraying peptides on a grid, one can potentially identify linear interacting motifs

Figure 1. The C-tail of AGC kinases is a conserved *cis*-regulatory element. The C-tail of AGC kinases is conserved and contains three major segments that have been classified as the NLT, the CLT, and the AST.¹¹ (A) In PKA, the C-tail includes residues 300–350 while residues 300–319 constitute the CLT. The AST (residues 320–339) is mostly disordered in the apo (open) state, but becomes ordered in the closed state when ATP and substrate peptide are bound. The structure of the C-tail in its closed conformation shows how Tyr330 and Phe327 come close to the adenine ring of ATP. (B) Unlike other AGC kinases, PDK1 has only a remnant of the C-tail. (C) The C-tail of PKA functions as a *cis*-regulatory element, where conserved motifs in the C-tail interact with AGC-specific conserved residues in the core. (D) Alignment of four AGC kinases with PDK1, the AGC-specific activating kinase. Several conserved motifs are highlighted including the PXXP motif, the Ade motif, and the HF motif. Also, highlighted in turquoise are a conserved phosphorylation site at the turn motif and a phosphorylation site following the HF motif.

Figure 2. Peptide array of the catalytic subunit of PKA overlaid with PDK1. Peptides representing the catalytic subunit of PKA were arrayed as 18mers and staggered by two residues. The first peptide corresponding to residues 1–18 is indicated by N while the last corresponding to residues 332–350 is designated as C. Blank sites are indicated as B. Two sets of PDK1-interacting peptides were identified. One corresponds to the activation segment. The sequence is shown at the top and the sequences of the spots that indicate PDK1-interacting peptides are underlined beneath the sequence. The second set of PDK1-interacting peptides correspond to the AST region of the C-tail, and the sequence is indicated below with the sequence of the individual peptides underlined. On the right, each AST interacting peptide is correlated with the illuminated spot.

that may be shielded in the full-length protein depending on whether it is active or inactive or interacting with another protein or protein domain. In its active conformation, PKA interacts with many substrate proteins as well as its inhibitory regulatory subunits. However, like many protein kinases, PKA is also activated by phosphorylation. Thus, it must be recognized by an activating kinase prior to assuming its fully phosphorylated active conformation. PDK1 is an activating kinase for PKA.^{16,19} We thus asked whether peptide arrays might allow us to identify PDK1-interacting regions in the catalytic subunit.

Identification of PDK1-interacting peptides in the PKA catalytic subunit

To identify linear peptides in the catalytic subunit that interact with PDK1, we synthesized a peptide array of the catalytic subunit and then overlaid with myc-tagged PDK1 that had been expressed in HEK-293 cells. We then imaged the array by chemiluminescence to identify PDK1-interacting peptides. Initially, we used 18mers of the catalytic subunit beginning with residues 1–18 and ending with residues 332–350. The array contained 112 peptides that covered the entire sequence. The results showed two clear clusters of PDK1-interacting peptides (refer Fig. 2). The first and strongest set of PDK1-interacting peptides were located in the region corresponding to the activation segment where the site of phosphorylation is located. The second cluster was located in the region corresponding to the C-terminal tail (C-tail). In this study, we focus on PDK1-interacting peptides in the C-tail region.

Identification of the PDK1-interacting residues in the C-tail of PKA correspond to a novel conserved FD(X)₁₋₂Y/F motif

While we expected to find PDK1-interacting peptides in the C-tail region, we were surprised to find that the interacting peptides were located in the AST region and not in the region corresponding to the hydrophobic motif. To localize more specifically the amino acids in the C-tail that were important for interacting with PDK1, we expanded the array of this region by generating peptides that were staggered by a single residue. This array [Fig. 3(A)] confirmed that the interacting peptides were in the AST region. By using an Ala scan array to walk through this region, we found, furthermore, that the residues that reduced binding corresponded to the site around the conserved F-D-D-Y motif (residues 327–330) as shown in Figure 3(B). As was seen in Figure 1, this is a highly conserved motif in the AGC family and corresponds to residues that contribute to the ATP binding pocket in PKA. We subsequently define this motif for the family as the FD(X)₁₋₂Y/F motif or, for simplicity, the adenosine binding (Ade) motif.

Figure 3. Peptide array of the C-tail of PKA and an alanine scan of the binding region both overlaid with PDK1. To further validate that the AST region in the C-tail of PKA would recruit PDK1 as suggested in Figure 2, 18mer peptides were synthesized at every amino acid in the region already identified to bind PDK1. (A) The Ade motif is shown in green and a phosphorylation site necessary for an active enzyme Ser338, is shown in red. (B) An alanine scan of the region was synthesized to understand which amino acids within the sequence were important for the interaction. The resulting array shows that, when the amino acids that comprise the Ade motif are substituted with an alanine, PDK1 no longer bound to the peptide. We classify binding as strong (●), weak (*), or null (✕). (C) To confirm the importance of AST residues for the interaction of the catalytic subunit and PDK1, we pulled down PKA catalytic subunit with GST-PDK1, that had both been bacterially expressed. The results confirmed that mutation of D328 and Y330 in the AST as well as F350 in the C-terminus, blocked interaction with PDK1.

Importance of the Ade motif for interaction of full length PKA and PDK1

To determine whether residues in the Ade motif are important for interaction of the full length kinase with PDK1, we introduced Ala mutations into key residues in the C-tail of the catalytic subunit and then asked whether these mutations interfered with the ability of GST-tagged PDK1 to pull down the catalytic subunit. The pull-down assays for several C-tail mutants of the catalytic subunit are shown in Figure 3(C). Mutation of Tyr330 and Asp328 was sufficient to significantly reduce the pull-down by PDK1 whereas mutation of the two prolines in the PXXP motif (residues 313–316) in the CLT region did not interfere with the pull-down of PDK1 (data not shown). We showed previously that Phe347 and Phe350 are important recognition motifs for phosphorylation of the C-subunit by PDK1, and these residues were also defective in the pull-down assay.⁸ The Phe350Ala mutant was not pulled down whereas the amount of the Phe347Ala that was pulled down was reduced compared to wild type PKA.

The Ade motif is a PDK1-interacting motif in other AGC kinases

To determine whether this Ade motif is conserved as a PDK1-interacting motif in other AGC kinases, we arrayed the C-tail of Akt and PKC β II. The C-tail of Akt is known to be important for interacting with PDK1, and the activation of Akt is a highly regulated process where recruitment to membranes through PH domains and phosphorylation by MTOR all participate.^{20,21} For the array, we used only the C-tail (residues 425–482) and arrayed the tail as contiguous 18mers. As seen in Figure 4(A), peptides from the AST region of Akt also interacted strongly with PDK1. To determine which specific residues were important for interacting with PDK1, we did an Ala scan. This Ala scan [Fig. 4(B)] confirmed that each of the residues in the Ade motif (Phe-Asp-Glu-Glu-Phe in Akt) was important for recognition by PDK1. In addition, there were residues flanking the Ade motif that also seemed to be important for recognition of PDK1 by the C-tail of Akt.

Phosphorylation of Ser338 enhances binding of the N-lobe tether

We were surprised initially to find that peptides in the NLT region containing the hydrophobic motif (Phe-Thr-Glu-Phe) did not appear as strong binders on the original array of the PKA catalytic subunit. To determine whether phosphorylation of Ser338 was important for recognition of this region in the C-tail, we carried out an array where Ser338 was phosphorylated. The results, shown in Figure 5(A), were striking and suggest that phosphorylation of Ser338 may serve as a switch mechanism for different motifs in the C-tail to interact with PDK1. When Ser338 was phosphorylated, the NLT now interacted strongly with PDK1 but only

when the peptide included both the Ser(P) and the terminal two residues, Glu349 and especially Phe350. Because the arrayed peptides are attached to the filter through their C-terminal residue, we wanted to determine whether the peptides could bind better if there was a glycine linker between the peptide and the filter. As seen in Figure 5(A), the addition of a glycine linker did not restore binding of the C-tail peptides in the absence of phosphorylated Ser338.

An Ala scan of this phosphorylated region of the C-tail [Fig. 5(B)] showed that several residues were important for interacting with PDK1, not just the hydrophobic motif. In fact, other residues, especially the acidic residues, seemed to be more important than the two phenylalanines at the C-terminus. The penultimate residue, Glu349, as well as Glu346 and the Cys-Gly (residues 343–344) motif all appeared to be the important determinants for binding to PDK1 as well as Arg336 and Tyr330 in the Ade motif. The shorter peptides in the walk through shown in Figure 5(A) did not contain both the Ade motif and the hydrophobic motif and in that case, no binding was observed until Glu349 and Phe350 were included.

In the case of Akt, unlike PKA, the original walk through shown in Figure 4(A) indicated that PDK1-interacting peptides were also found in the NLT regions of the C-tail. However, the interacting peptides did not seem to correlate precisely with the HF motif; at least the presence of the HF motif alone (residues 461–480) was not sufficient to bind to PDK1. Ala scans of the NLT region could help to decipher the importance of individual residues, but the initial walk through is sufficient to say that each AGC kinase will be different in detail. In addition, the results shown here are also sufficient to demonstrate that many residues in the C-tail of Akt will contribute to interactions with PDK1. The hydrophobic phenylalanines may contribute but they are not necessarily the most important residues for docking. So far all of the AGC kinases that we have tested have revealed PDK1-interacting motifs in the Ade region, but there is considerable variability in the NLT region that contains the HF motif.

PRK2 has two clusters of PDK1-interacting peptides, one corresponding to the Ade region and the other localized to the HF motif in the C-tail

PRK2 is another kinase that is phosphorylated and activated by PDK1. The HF motif was first recognized as an important motif in PRK2, and the C-terminus of PRK2 was used to generate PIFTide, a very good peptide mimetic for phosphorylation by PDK1.^{13,22} To determine whether the C-terminus of PRK2 also had other PDK1-interacting motifs, we arrayed a portion of the C-tail (residues 933–984) and then overlayed with PDK1. As seen in Figure 4(C), there were two clusters of PDK1-interacting peptides. The first and strongest cluster correlated with the region that contains the

Figure 5. Phosphorylation of PKA residue Ser338 acts as a PDK1 binding switch that recruits PDK1 to either the Ade or the hydrophobic motif in a phosphorylation dependant manner. Since the phosphorylation of Ser338 in PKA is an essential step in activating PKA, we asked whether the phosphorylation of this residue would then recruit PDK1 to the hydrophobic motif as was previously known but not seen in the peptide array experiments. (A) 18mer peptides, corresponding to the C-terminal PKA hydrophobic motif region were synthesized with a phospho-serine at position 338. When Ser338 was phosphorylated, the NLT bound tightly to PDK1. (B) An alanine scan of the phospho-Ser338 containing region was generated as 25mer peptides. The array shows that a phosphorylated Ser338 peptide is necessary to recruit PDK1 to the hydrophobic motif containing region. Notably, phospho-Ser338 is able to rescue the need of the Ade motif to recruit PDK1 to the amino acid sequence. We classify binding as strong (●), weak (*), or null (X).

Ade motif and further confirms our hypothesis that this motif in most AGC kinases will be recognized by PDK1. The region that contained the hydrophobic motif also interacted with PDK1, but the interactions were much weaker.

We next did an Ala scan of the C-terminal 25 residues of PRK2 to determine which residues contribute most to PDK1 binding. This 25mer corresponds to PIFtide and does not include the Ade motif. The

results [Fig. 4(D)] indicated that many residues both N-terminal and C-terminal to the HF motif contribute to binding. In contrast to the walk through, the 25mer includes the C-terminal region after the HF motif, and five strong determinants are located in this segment. Several strong determinants also lie N-terminal to the HF motif including Leu966 and Ser967. None of the 18mers used in the walk through contained both the C-terminal residues and the Leu-Ser. The Leu-Ser motif corresponds to the turn motif in PRK2, and the array indicates that replacement of either the Leu or the Ser with Ala abolishes interaction with PDK1. Although we did not do an array with the phosphorylated Ser in the turn motif, the above results are sufficient to confirm that the turn motif may play a key role in determining how individual AGC kinases interact with PDK1.

Modeling Ade motif from the C-tail of PKA onto the N-lobe of PDK1 creates a chimeric site that creates a complete ATP binding site for PDK1

Analysis of the PDK1 structure²² reveals not only that PDK1 lacks a complete C-tail, but also that it lacks a complete ATP binding site. Thus, the ATP binding pocket in PDK1 has an empty space that would be filled by Phe327 in the C-tail of PKA when PKA is a substrate or by the comparable residue in the C-tail of another AGC kinase. To confirm that this site could be a docking site for the AST region of PKA, we aligned structures of PKA and PDK1 by their F-helices. It was demonstrated earlier that the F-helix is an organizing scaffold that positions the protein kinase structural elements.⁷ After such alignment, the phenylalanine of the Ade motif and both phenylalanines of the HF motif in the C-terminal tail fit very well into the corresponding pockets on the surface of the N-lobe of PDK1. Figure 6 shows how the last 25 residues of the C-tail of PKA (shown in red) models onto the small lobe of PDK1. This model strongly suggests that the binding sites for these motifs are conserved in these protein kinases. On the basis of this model, the resulting site that is created for binding the adenine ring of ATP is a chimeric site comprised of residues from the core of PDK1 and from the Ade motif in the C-tail of PKA.

Why is PDK1 in an inactive conformation?

When we mutated Phe327 to Ala in PKA, for example, the catalytic efficiency of the enzyme was reduced by 40-fold due to an increase in the Km for both ATP and peptide.²³ A structure of this mutant shows not only that the AST region of the tail is displaced but also that ATP is bound in a catalytically incompetent conformation. On the basis of these results, we conclude that PDK1, which has a truncated tail, is catalytically defective with regard to its ATP binding site. It cannot properly bind the adenine ring, and it cannot on its own orient the phosphates of ATP for efficient transfer to a protein substrate. Only when an Ade

Figure 6. Modeling of the PKA C-terminal tail onto PDK1. The docking of the PKA C-tail onto the PDK1 structure is shown in the center. On the right top are the regions of the adenosine binding pocket that are contributed by PDK1 while the chimeric site that includes Phe327 and Tyr 330 from the C-tail of PKA is shown on the bottom right. On the left top is the hydrophobic pocket on PDK1, on the bottom left is the PKA HF motif docked into this pocket. Hydrophobic parts of the HF and Ade binding pockets in PDK1 are shown as yellow surfaces. The C-terminal tail of PKA is colored dark red. The N-lobe of PDK1 is colored white; the C-lobe is colored tan. Pdb#: PKA (1atp); PDK1 (1h1w).

motif from a substrate kinase docks onto the N-lobe of PDK1, would there be a complete site that resembles the active ATP binding pocket of PKA (refer Fig. 6). In PKA, the residues that flank Phe327 help to position the glycine loop through interactions with both β strands 1 and 2. Thus, we propose that the C-tail of a substrate kinase such as PKA or Akt serves as a *trans*-regulatory element for PDK1. Docking of the Ade motif to the N-lobe of PDK1 not only tethers the substrate kinase but also increases the catalytic efficiency of PDK1.

Docking of a heterologous HF motif to the C-helix of PDK1 creates a second chimeric site

When we docked the C-tail of PKA onto the N-lobe of PDK1, the HF motif creates another well-packed chimeric site that serves to position the C-helix in a conformation that would be optimal for catalysis (refer Fig. 6). Thus, the C-tail of each substrate kinase is polyvalent and has the potential to activate PDK1, by at least two mechanisms: it positions the C-helix for catalysis via the HF motif and uses the Ade motif to bind ATP and position the glycine-rich loop for catalysis. This is an excellent example of substrate-assisted catalysis and suggests that the two kinases have co-evolved as partners.

We were surprised initially to find that PDK1 was in an active conformation in the crystal structure even though the activation loop was highly disordered. For example, the link between Lys111 in β strand 2 and Glu130 in the C-helix is present and the regulatory spine, another hallmark of active kinases,⁶ is in place.

We thus investigated whether a symmetry-related molecule might account for this ordering of the N-lobe. Surprisingly, we found that Tyr288 from a symmetry related molecule fills the hydrophobic pocket in a manner that is entirely analogous to the HF motif (refer Fig. 7). This tyrosine is located in the G-helix,

Figure 7. Symmetry related molecule in the PDK1 crystal occupies the HF binding pocket and mimics the HF motif. G-helix of the symmetry mate is colored dark red. Hydrophobic surface of the HF binding pocket is shown as yellow surface. N-lobe is white, C-lobe is tan, symmetry related molecule is gray.

Figure 8. Polyvalent docking motifs from the C-tails of AGC kinases provide a polyvalent, dynamic, and highly regulated mechanism for interacting with PDK1. On the left are several AGC kinases that were aligned based on the F-helix. The CLT and the NLT are superimposed well as is the Ade motif when the kinase is in a closed conformation. The turn motifs, however, are very different. On the basis of our model, we predict that the AST of the substrate kinase will bind to the adenine binding pocket of PDK1 when PDK1 is active. The turn motif will be unique for each kinase and will be regulated in novel ways, often by yet another kinase. On the right is the conformation of the C-tail in the closed and open conformation of PKA. When ATP is bound in the closed conformation, the AST is recruited to the adenine binding pocket where it assists in catalysis. Pdb#: PKA (1atp); PDK1 (1h1w); Akt (1o6l); ROCK1 (2esm); PKCθ (2jed).

an important docking motif for many protein kinases.^{24–27} For example, the homologous Tyr in PKA, Tyr247, as well as the entire G-helix, is an important docking site for the regulatory subunits. Thus, in the absence of the correct partner, two important docking surfaces interact with each other to help form a well packed crystal structure. There is no evidence to suggest, however, that this is a physiologically relevant interaction site.

Discussion

By comprehensively analyzing the AGC family of protein kinases, we were able to predict not only that the C-tail is a conserved element for all members of this kinase subfamily but also that it functions as a *cis*-regulatory element for each kinase.¹¹ As seen in Figure 8 where four AGC kinases are superimposed, the C-tail is anchored stably to the C-lobe by the CLT and to the N-lobe by the NLT and these regions are conserved. The AST that links these two segments is dynamic and is only recruited to the core in the presence of nucleotide. It then facilitates catalysis by helping to organize the glycine-rich loop and position the γ -phosphate for transfer to the protein substrate.²³ Even though this dynamic AST lies outside the conserved catalytic core, it is an essential part of the active site and appears to have co-evolved with the core. In the closed state, docking of the Ade region is also conserved. Our previous analysis of the C-tail showed, in addition, that a

number of well-defined motifs were embedded within the C-tail, such as the PXXP motif, that can also serve as docking sites for interaction with other proteins. The PXXP motif in PKC β II, for example, was recently shown to be a docking site for Hsp90 and cdc37.¹⁵ Thus, the C-tail appears to be a polyvalent element for mediating protein interactions with motifs that function both *cis* and *trans*.

PDK1 plays a unique role in the AGC family. It is an activating kinase for most of the AGC kinases. Its structure in the family is also unique because it lacks a functional C-tail; it has only the remnants of a C-tail. To understand how PDK1 recognizes a substrate kinase, we used a peptide array strategy and asked whether we could identify linear PDK1-interacting motifs. In addition to recognizing the activation segment that contains the site that PDK1 phosphorylates, the array also identified the C-tail as a region that interacts with PDK1. By dissecting the PDK1-interacting sites in the C-tail of PKA, we demonstrated that the Ade motif, located in the AST, is recognized by PDK1. PDK1 also recognizes this motif in the C-tail of Akt, PRK2, and PKC β II (data not shown), and we predict that it will be recognized by most AGC kinases. Although others have identified the hydrophobic motif as an important docking site for PDK1, we expanded the interaction region here and predicted that an extended region of the C-tail from a substrate kinase will *trans*-interact with PDK1 and contribute actively

to its own phosphorylation. Modeling suggests that docking of the C-tail of PKA to PDK1 can easily generate a chimeric enzyme that would be optimally poised for catalysis. The C-tail of the substrate kinase is thus not just docking to PDK1 but also serving as a *trans*-regulatory element for PDK1 by providing an extended polyvalent mechanism for substrate-assisted catalysis. This symbiotic relationship between two AGC kinases clearly needs to be validated by solving crystal structures of complexes of the two proteins, and this is a future challenge for structural biologists.

Although PDK1 will likely recognize both the HF motif and the Ade motif in most of its substrate kinases, our preliminary comparison of interaction sites in Akt, PRK2, and PKA suggests that each kinase will show differences in the importance of flanking residues and how access to the conserved motifs is regulated. The turn motif that links the Ade and the HF motifs, for example, typically contains a critical phosphorylation site and is tightly regulated. This region can assume multiple conformational states depending not only on the activation state of the kinase but also on how this region interacts with flanking domains or other proteins. Since these flanking domains are typically missing in most of the AGC kinase structures that are available, this "turn" region is often disordered. In some cases, the turn motif is thought to be *cis*-autophosphorylated while in other cases there appears to be yet another kinase that is responsible for its phosphorylation.²⁸ Recent evidence for Akt suggests that the turn motif is phosphorylated by yet another heterologous kinase and this event actually primes Akt for phosphorylation by PDK1. Thus, there are likely to be many levels of cross-talk between an AGC kinase and its interactions with PDK1 that lead to its subsequent activation. We demonstrated earlier that the phosphorylation of the turn motif in PKA (Ser338) is likely due to *cis*-autophosphorylation,²⁹ and the results presented here suggest that this phosphorylation might "prime" the C-tail for docking of the HF motif. While the detailed kinetic mechanisms for regulation, as well as the timing for each phosphorylation event, will be unique for each kinase, this is a challenge that needs to be resolved. As seen here, there will be some common themes, but each kinase will be unique.

Methods

Bacterial protein expression/purification

Histidine-tagged (H6) WT catalytic subunit in the pET15b vector was expressed in *E. coli* [BL21 (DE3)] and purified using TALON metal affinity resin from Clontech. All bacteria were grown in YT media with 100 µg/mL ampicillin at 37°C. Cells were induced using 500 µM IPTG (final concentration) after reaching an optical density at 600 nm of 0.6–0.8. Cells were grown for an additional 6 h at 24°C and har-

vested by centrifugation at 5000 rpm for 15 min. The cell pellets were resuspended in 10 mL lysis buffer per gram of cell pellet (50 mM KH₂PO₄, 20 mM Tris-HCl, 100 mM NaCl, 5 mM β-ME, pH 8.0). Cells were lysed by passing twice through a French press at 1000 psi each and then centrifuged at 15,000 rpm for 45 min at 4°. The supernatant was then incubated with 1 mL (bed volume) TALON resin per L of supernatant that was pre-equilibrated with lysis buffer. After batch binding for 2 h at 4°C, the resin was spun down at 3000 rpm and the non-binding fraction was removed. The resin was washed three times with lysis buffer (pH 7) with the first wash containing 10 mM imidazole. The protein was then eluted from the resin using four elutions, each two times the resin bed volume, at each of the following imidazole concentrations: 50, 100, 200, and 500 mM imidazole. Samples containing the most protein were further purified on a superdex S200 size exclusion column using buffer containing 50 mM KH₂PO₄, 20 mM Tris-HCl, 100 mM NaCl, 5 mM β-ME, pH 8.0.

Catalytic subunit mutants [PKA(F327A), PKA(D328A), PKA(Y330A), PKA(Y330F), PKA(F347A), and PKA(F350A)] were expressed in *E. coli* [BL21 (DE3)]. All proteins were grown in YT media with 100 µg/mL ampicillin. Cells were induced as described above and grown for an additional 16 h at 16°C. After centrifugation, the cell pellets were resuspended in 10 mL lysis buffer per gram of cell pellet, lysed as described above, and reserved for pull-down experiments.

GST-PDK1 was expressed in *E. coli* [BL21 (DE3)] and purified using glutathione sepharose beads from Amersham. All Bacteria were grown in YT media with 100 µg/mL ampicillin at 37°C. Cells were induced using 500 µM IPTG (final concentration) after reaching an optical density at 600 nm of 0.6–0.8. After growing for an additional 6 h at 24°C and harvested by centrifugation at 5000 rpm for 15 min, the cell pellets were resuspended in 10 mL PBS per gram of bacterial pellet. Cells were lysed as described above. The supernatant was then incubated with 1 mL glutathione sepharose beads per L of supernatant that was pre-equilibrated with PBS. After batch binding for 2 h at 4°C, the resin was spun down at 3000 rpm, washed three times with PBS and reserved for pull-down experiments.

Site directed mutagenesis of the PKA catalytic subunit

cDNA for the *M. musculus* PKA catalytic subunit in the bacterial expression vector (with N-terminal poly histidine tag) pET15b was used as the parental DNA for site directed mutagenesis using the Quick-Change mutagenesis kit (Stratagene) as per the manufacturer's protocol. GeneAmp PCR system 9700 was used for the reaction steps and subsequent mutated cDNA was sequenced to confirm the mutation (Eton Bioscience).

Pull-down of catalytic subunit mutants with GST-PDK1

Lysates containing the PKA mutants of the catalytic subunit were incubated with crudely purified GST-PDK1 bound to glutathione sepharose beads. The solutions were allowed to incubate rotating for 2 h at 4°C. The resulting complexes were pulled out of solution using 10 µL (bed volume) glutathione sepharose beads, washed three times with PBS, then eluted using a low pH buffer. The samples were then run on a SDS-Page gel and transferred for immuno-blotting using an antibody specific to PKA_C (anti-PKA_C from BD transduction laboratories, clone 5B Mouse IgG2b) and imaged by chemiluminescence.

Peptide array synthesis/analysis

Filter bound peptide arrays were synthesized according to Intavis AG Parallel Peptide Synthesis SPOT protocol. In brief, the peptides were synthesized on cellulose membranes derivatized with β-alanine by an ester bond for covalent anchoring of peptides as distinct spots. The arrays were incubated with HEK 293 cellular lysates transfected to overexpress ^{myc}PDK1. The arrays were analyzed by Western blot, using antibodies that recognized the myc epitope: anti-9E10 Monoclonal Antibody, Cat. # MMS-150R LN#14865002, IgG1.

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