Consequences of Lysine 72 Mutation on the Phosphorylation and Activation State of cAMP-dependent Kinase*

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Most protein kinases are themselves phosphoproteins that contain an essential phosphate in the activation loop of the enzyme (1-5). The activation loop is a conserved motif in the kinase family. The catalytic subunit (C-subunit) of cAMP-dependent kinase (PKA)1 has an essential phosphorylation site in the activation loop at Thr197, in addition to Ser338 in the C-terminal tail (6, 7). Fig. 1, A and B, highlight the structure of the activation segment of PKA, including the phosphate on Thr197 and the environment surrounding the Ser338 phosphate. The phosphate on Thr197 in the activation loop of the C-subunit interacts with His87 of the C helix, Arg160 adjacent to the catalytic base, Asp166, Thr195 on the activation loop, and Lys189 in b-strand 9, which positions Arg190 to interact with the A helix. These interactions act in a synergistic fashion with the phosphate, helping to bring these residues to their proper spatial orientation, but they also anchor the phosphate and consequently the activation segment in a conformation required for activity (8). Mutagenesis of Thr197 and Ser338 demonstrated their importance for full activity (9, 10). Many protein kinases, the structures of which have been solved in the unphosphorylated inactive form, show that the position of this loop differs from that of the phosphorylated enzyme (Fig. 1C) (8, 11-15). Additionally, studies monitoring the fluorescence of an endogenous Trp on the insulin receptor kinase activation loop showed a change in fluorescence intensity upon phosphorylation of the activation loop and reflect the dynamic properties of the loop (15).

In many kinases, phosphorylation of the activation loop is a highly dynamic process triggered by a specific signal. In contrast, although PKA is also a phosphoprotein, it is not activated by phosphorylation of its activation loop in response to a signaling event. Instead, the fully phosphorylated enzyme is assembled with inhibitory regulatory subunits. Its activity in cells is thus controlled primarily by regulatory subunits (R-subunits) that bind the C-subunit with high affinity in the absence of cAMP (16). This mode of activation in which the regulatory and catalytic moieties are separate proteins is unusual in the protein kinase family. Although the C-subunit can be readily phosphorylated in vitro by 3-phosphoinositide-dependent protein kinase-1 (PKD-1), it also undergoes autophosphorylation when it is expressed in E. coli (17). Furthermore, mutant forms of the C-subunit that are defective in autophosphorylation are readily phosphorylated in mammalian cells, whereas mutants that are defective in recognition by PKD-1 are not phosphorylated (18). However, nothing is known about the conformation of the C-subunit in its unphosphorylated state, and very little is known about the process by which the inactive dephosphorylated protein is converted into a fully phosphorylated protein prior to its association with regulatory subunits.

In addition to these essential phosphorylation sites, there are various key residues in the kinase core that play a significant role in the stability and the functional organization of the kinase. One such residue, Lys72 located in subdomain II in the Hanks classification (19), represents one of the most conserved residues in the protein kinase core. This was the first residue to be identified in the active site of a protein kinase (Fig. 1D). The absolute conservation of this residue in every protein kinase

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† The abbreviations used are: PKA, cAMP-dependent kinase; PKC, protein kinase C; FSBA, p-fluorosulfonylbenzoyl 5'-adenosine; HA, hemagglutinin; MAPK, mitogen-activated protein kinase.
subsequently reinforced its importance. This residue was first found to be important for kinase function of the C-subunit using an ATP affinity analog FSBA. The alkylating group on FSBA occupies the region of the protein that recognizes the phosphates of ATP (20, 21), and treatment of the C-subunit with FSBA resulted in inactivation that was protected in the presence of MgATP. Peptide sequencing later identified the modified residue as Lys72 (22). Treatment with a hydrophobic carbodiimide, dicyclohexylcarbodiimide, in the absence of MgATP also irreversibly inhibited the C-subunit, due to cross-linking of Lys72 to Asp184, another conserved residue (23).

In this work, we have addressed two questions. First, we ask what are the functional consequences of mutation of lysine 72? Second, can this “dead” kinase be used to probe the pathway whereby the C-subunit is activated by phosphorylation? To achieve this, Lys72 was replaced with His, Arg, Ala, and Met. Additionally, to provide a suitable comparison with the inactive C-subunit, the wild type C-subunit was expressed in the presence of a PKA inhibitor, H-89. This C-subunit is not phosphorylated. Using phospho-specific antibodies, we show that both H-89 and the Lys72 mutants are excellent substrates for PDK-1.

Second, can this “dead” kinase be used to probe the pathway whereby the C-subunit is activated by phosphorylation? To achieve this, Lys72 was replaced with His, Arg, Ala, and Met. Additionally, to provide a suitable comparison with the inactive C-subunit, the wild type C-subunit was expressed in the presence of a PKA inhibitor, H-89. This C-subunit is not phosphorylated. Using phospho-specific antibodies, we show that both H-89 and the Lys72 mutants are excellent substrates for PDK-1 at Thr197; however, only wild type C-subunit can be phosphorylated at Ser338 and thus converted into an active enzyme. These results define a two-step activation process dependent on an intramolecular autophosphorylation at Ser338. In addition, we show that phosphorylation at Thr197 is sufficient for RIα binding, thus defining a new role for the activation loop independent of its role in coordinating the active site conformation. Thus the dead enzyme is still capable of forming a holoenzyme complex once it is phosphorylated.

EXPERIMENTAL PROCEDURES

Materials—Reagents were obtained as follows: pRSETB expression vector (Invitrogen), β-[γ-32P]ATP (PerkinsElmer Life Sciences), Escherichia coli strains BL21(DE3) (Novagen, Madison, WI), H-89 (LC Laboratories, Woburn, MA), Muta-Gene site directed mutagenesis kit and Affi-Gel (Bio-Rad), horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences), Gammabind G-Sepharose (Amersham Biosciences), pcDNA-3 eukaryotic expression vector (Invitrogen), SuperSignal West Pico chemiluminescent substrate detection kit (Pierce), oligonucleotides (Geniosis-Sigma), the PepTag PKA activity assay kit (Promega, Madison, WI), and Effectene transfection kit (Qiagen, Valencia, CA). Mouse monoclonal anti-Myc antibodies (Covance, Princeton, NJ), antibodies that specifically recognize the phosphorylated activation loop of protein kinase C (PKC), were a gift from A. Newton (University of California, San Diego, CA) (24). Antibodies against the catalytic subunit of PKA were described previously (10). Plasmid pCMV5 containing Myc-tagged PDK1 were the same as described previously (25), and DNA sequencing was performed with the ABI Prism 310 Genetic Analyzer from PE Applied Biosystems. The peptide to the Thr197 sequence was synthesized at the Peptide and Oligonucleotide Facility at the University of California, San Diego on a Millagen 9050 PepSys peptide synthesizer using standard Fmoc (N-(9-fluorenyl) methoxycarbonyl) methodology activator and purified by high performance liquid chromatography.

Mass Spectrometry—Electrospray/mass spectrometry was performed using a Hewlett-Packard 59887A electrospray mass spectrometer. Protein was desalted prior to analysis by narrow bore chromatography.

Site-specific Antibodies—Antibodies were generated to distinguish the phosphorylation state of residues at several sites in the C-subunit. The antibody to the unphosphorylated Thr197 (α-Thr197-OH) was generated using a peptide corresponding to the sequence around Thr197; the peptide to the Thr197 sequence was synthesized at the Peptide and Oligonucleotide Facility at the University of California, San Diego on a Millagen 9050 PepSys peptide synthesizer using standard Fmoc (N-(9-fluorenylmethoxycarbonyl) methodology activator and purified by high performance liquid chromatography.

Site-directed Mutagenesis of the PKA Catalytic Subunit—cDNA for the murine PKA Ca-subunit in the bacterial expression vector pRESTB was used as a template for Kunkel-based site-directed mutagenesis as described previously (26, 27). cDNA for the C-subunit transfected into COS cells was engineered in the pcDNA-3 expression vector, with a HA

FIG. 1. Location of Lys72, Thr197, and Ser338 in the active C-subunit. The diagram highlighting the Thr197-P interactions (A) and Ser338-P interactions (B) was taken from the crystal structure of the mouse C-subunit bound to IP20 and ATP (1ATP.pdb). C shows the superimpositions of the unphosphorylated activation loops of several different kinases (insulin receptor (IR), cdk2, and MAPK (39)) as compared with the phosphorylated activation loop of the C-subunit and Lys72 interactions (D) in the structure of mouse C-subunit.
epitope tag added at the C terminus of the protein. All mutations were made using the Muta-Gene kit as per the manufacturer's recommendations. DNA sequencing analysis instruments confirmed the presence of the correct mutation.

Expression of Murine PKA Catalytic Subunit—Histidine-tagged wild type and mutant C-subunits were expressed in the E. coli strain BL21 (DE3). Cells were grown in YT medium containing 100 μg/ml ampicillin at 37 °C to an optical density at 600 nm of 0.5–0.8, induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside for an additional 6 h at 24 °C, collected by centrifugation, and stored frozen. To obtain unphosphorylated C-subunit, 50 μM H-89 was added to the cultures from a 1,000× stock of H-89 in Me2SO at the time of induction. Cells from 500 ml of culture were resuspended in 10-ml lysis buffer (50 mM sodium phosphate, 100 mM NaCl, 5 mM 2-mercaptoethanol, pH 8.0) and lysed by one pass in a French pressure cell at 1,000 p.s.i. Insoluble material was removed by centrifugation at 15,000 rpm in a Beckman JA20 rotor at 4 °C for 40 min. The lysates were purified via their His tag using Talon metal affinity resin (Clontech). In brief, supernatant was batch-bound to 1-ml resin/500-ml culture for 2 h at 4 °C. The resin was washed twice with lysis buffer, a wash with 10 mM imidazole in lysis buffer followed by two 100 mM imidazole elutions and a final 500 mM elution.

Catalytic Activity Assays—The PepTag assays were performed according to the manufacturer's instructions. This qualitative assay uses the PepTag-pArg-Apa-Sep-Lys-Gly (Kemptide) peptide substrate tagged with a fluorescent dye. Upon phosphorylation, the net charge of this peptide changes from +1 to +1, which then alters the migration of the peptide when run on an agarose gel. Briefly, lysed bacterial supernatant expressing the wild type or mutant proteins was incubated with the tagged Kemptide substrate and activator buffers at 30 °C, and the reaction was run on a 1% agarose gel at 100 V. Active protein was detected by its substrate migrating toward the anode.

Expression of Myc-tagged PDK-1 in 293 Cells—Human 293 cells were propagated at 2 × 10^6/10-cm dish in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum. pCMV5 vector containing Myc-tagged PDK-1 was transfected using Qiagen Effectene transfection kit as per the manufacturer's protocol. Cells were resuspended in Buffer A (50 mM Tris-Cl, pH 7.5, 50 mM NaCl, 10 mM NaF, 10 mM 48 h after transfection, the cells were trypsinized and resuspended in buffer A, twice with buffer A plus 0.5M NaCl, and once with buffer B (50 mM Tris-Cl, pH 7.5, 10 mM NaCl, 1 mM dithiothreitol, 10% fetal bovine serum, 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100, 10 μg/ml aprotinin) as described (27). The cells were then subjected to three rounds of freeze-thaw cycle followed by centrifugation at 50,000 rpm in a Beckman TLA 100 rotor for 30 min at 4 °C. Purified recombinant PDK-1 was used when indicated and was a gift from A. Newton (University of California, San Diego).

Phosphorylation State of Transfected C-subunit Constructs—Wild type, T197A, and S338A in the pcDNA-3 expression vector HA-tagged at the C terminus were transfected into COS cells using the Effectene transfection kit as per the manufacturer's protocol. Cells were resuspended in Buffer A and lysed by three rounds of freeze-thaw followed by centrifugation at 4 °C for 20 min 18,000 rpm. Transfected C-subunit was isolated by immunoprecipitation using a mouse monoclonal anti-HA antibody. These samples were then immunoblotted with the indicated rabbit generated antibodies to determine expression and phosphorylation state.

**RESULTS**

Purification of Wild Type C-subunit and Lys72 Mutants—Lys 72 is conserved throughout the protein kinase family and is critical to kinase activity. When this residue is mutated, activity is dramatically decreased. Mutation of the equivalent Lys in other kinase family members often serves as the traditional “kinase-dead” mutant. To probe the function of Lys72 and to explore its phosphorylation state, four different mutations (K72A, K72H, K72M, and K72R) were made at the Lys72 position. The mutant proteins were purified by the addition of a polyHis tag at the N terminus followed by affinity chromatography. A qualitative PepTag activity assay was used to determine whether these proteins were active. Although all proteins were purified in equivalent amounts, only the wild type subunit was active (Fig. 2B). Based on densitometry and the coupled kinase assay, the activity of these mutants was less than 1% of the wild type C-subunit.

Mass spectrometry of the mutant proteins indicated that their mass was consistent for each mutant protein in an unphosphorylated state (Table I). To determine whether these dead kinases could be phosphorylated by a heterologous protein kinase, the Lys72 mutant proteins were all incubated with PDK-1 and [32P]ATP. As indicated in Fig. 2A, all four mutant proteins were phosphorylated by PDK-1, in contrast to the wild type C-subunit, which is already fully phosphorylated prior to incubation with [32P]ATP. However, although these mutant proteins were good substrates for PDK-1, activity was not re-
stored upon phosphorylation (data not shown).

Phosphospecific Antibodies—To specifically characterize the phosphorylation state of the C-subunit, antibodies were generated that could distinguish the phosphorylation at Thr197 and Ser338. It was established previously that an antibody generated against a phosphorylated peptide corresponding to Thr197 in the activation loop of protein kinase C was able to discriminate the phosphorylation state of Thr197 in the activation loop of the C-subunit (28). This segment is highly conserved in PKA and PKC. This antibody will be referred to here as α-Thr197-P. Additional peptides were synthesized to generate antibodies specific to other phosphorylation sites. Peptides were synthesized corresponding to the unphosphorylated Thr197 of the activation loop as well as the phosphorylated site in the C-terminal tail at Ser338 and used as the antigen for generation of antibodies in rabbits. These antibodies are designated as α-Thr197-OH and α-Ser338-P.

Autophosphorylation of Wild Type and Mutant C-subunit—The ability of the proteins to undergo autophosphorylation following phosphorylation with PDK-1 was tested using the antibodies specific to the two phosphorylation sites, Thr197 and Ser338. To compare wild type with the Lys72 mutants, we needed a wild type control protein that is not phosphorylated. To obtain dephosphorylated C-subunit, the wild type protein was expressed in the presence of an inhibitor of the C-subunit. The addition of H-89, an ATP analog, at the time of induction inhibits the C-subunit and prevents autophosphorylation (29).

Both K72H- and H-89-treated wild type were incubated with PDK-1, and samples of each were tested in Western blot analysis. All three antibodies indicated that neither protein was phosphorylated before PDK-1 treatment (Fig. 3). Following incubation with PDK-1, each substrate protein was phosphorylated, but phosphorylation was not equivalent. The α-Thr197-P antibody indicated that both proteins were phosphorylated at this site. The antibody to the unphosphorylated Thr197 demonstrated, furthermore, that phosphorylation was essentially complete. Although phosphorylation of wild type (H-89) protein was complete, over 80% of K72H was phosphorylated. A significant difference between the two proteins was phosphorylation at the Ser338 site. Indeed, after PDK-1 treatment, only the wild type (H-89) protein underwent this second phosphorylation event. Only 2% of Lys72 mutant was phosphorylated, whereas over 74% of wild type (H-89) protein was phosphorylated at Ser338. A blot using an antibody to the C-subunit showed that all three proteins were of equal intensity before and after phosphorylation.

Stoichiometry of Phosphorylation—The rate of phosphorylation of the Lys72 mutant proteins by PDK-1 was then compared with phosphorylation of the wild type (H-89) protein. Both proteins are rapidly phosphorylated, but more 32P was incorporated into the wild type (H-89) substrate as compared with the K72H mutant protein, despite an equivalent amount of protein (Fig. 4A). It was established previously that in vitro, the only site of phosphorylation by PDK-1 is Thr197 (30), yet there is another phosphorylation event necessary to produce the fully phosphorylated and active C-subunit. This other phosphorylation event is likely brought about by autophosphorylation, which the mutant protein is incapable of undergoing.

<table>
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<tr>
<th>Table I</th>
<th>Mass spectroscopy of Lys72 mutant proteins</th>
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<tr>
<td>Mutant</td>
<td>Theoretical mass (two phosphates)</td>
</tr>
<tr>
<td>H6K72A</td>
<td>42,704.7</td>
</tr>
<tr>
<td>H6K72H</td>
<td>42,770.8</td>
</tr>
<tr>
<td>H6K72M</td>
<td>42,764.8</td>
</tr>
<tr>
<td>H6K72R</td>
<td>42,789.8</td>
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To monitor the efficiency of phosphorylation at the Ser338 site and to probe whether the plateau in phosphorylation is due to the complete phosphorylation of the substrate, loss of PDK-1 activity, or inefficiency of the event, PDK-1 phosphorylation was followed using site-specific antibodies. The α-Thr197-P antibody shows an equivalent increase in intensity of bands for both the wild type (H-89) and the K72H substrates, indicating that both get phosphorylated equally well (Fig. 4B). The α-Thr197-OH antibody shows a corresponding decrease in band intensity with time. For the α-Ser338-P antibody, only the wild type (H-89) shows an increase (Fig. 4). The increase in intensity at this second site closely follows the time course of Thr197 phosphorylation, suggesting that there is little lag time before the second phosphorylation occurs.

Time Course of Activation of Substrate C-subunits—This processing of the C-subunit from its unphosphorylated form to its phosphorylated form is done to achieve a conformation that is necessary for activity, the final measure of any enzyme. It has been shown previously that the wild type (H-89) protein is active toward a histone substrate after treatment with PDK-1 (28). To address how long it takes activity to follow the initial phosphorylation event, aliquots were removed at various times from a PDK-1/C-subunit reaction, and the activity was assayed at these times. Although the activity appears to immediately follow the initial phosphorylation events for the wild type enzyme, the mutant substrate remains inactive (Fig. 4C). This is a final piece of evidence distinguishing these two very different substrates of PDK-1. Although both appear to be equally good substrates, the mutant cannot go through the complete process of activation and thus could represent an intermediate in the processing pathway.

Comparison of PDK-1 and PKA as the PKA Kinase—The mechanism used by E. coli-expressed C-subunit to achieve its fully phosphorylated and active form is autophosphorylation (31), and it has been suggested that this is the mechanism employed in mammalian cells. When expression of mutants that are defective in autophosphorylation is compared with mutants that are defective in PDK-1 phosphorylation, only the autophosphorylation-defective mutants were phosphorylated in mammalian cells. A comparison was made here, under the
same in vitro conditions, of the PKA kinase activity of PDK-1 and wild type active PKA C-subunit. The K72H mutant was used as the substrate, removing any potential activity from the activated substrate. The time course comparison clearly shows that, although equivalent amounts of PDK-1 and C-subunit were used, PDK-1 is an overwhelmingly better kinase for the substrate C-subunit (Fig. 5).

**Mechanism of Ser^{338} Autophosphorylation**—Both the unphosphorylated wild type C-subunit and Lys^{72} mutant proteins are good in vitro substrates for PDK-1. However, unlike the wild type protein, in which phosphorylation by PDK-1 on Thr^{197} is followed rapidly by the autophosphorylation of Ser^{338}, the K72 mutant proteins are only phosphorylated on Thr^{197}. To determine whether this is because Ser^{338} is shielded from solvent and thus unavailable for phosphorylation, the mutant C-subunit was incubated with active C-subunit following incubation with PDK-1. Western blot analysis of the reaction components using the α-Thr^{197}-P antibody identified only a faint band, which corresponds to the untagged active wild type C-subunit that was added after the initial PDK-1 incubation (Fig. 6B). The concentration dependence of the phosphorylation was also examined using a P^{32} incorporation assay on the (H-89)-purified C-subunit (Fig. 6C). The rate of phosphorylation was independent of concentration, which indicates that autophosphorylation of this site occurs by an intramolecular mechanism that requires the C-subunit molecule to possess catalytic activity and catalyze phosphoryl transfer to its second phosphoryl acceptor site.

**Functional Consequences of Mutation at the Lys^{72}**—Mutation of the conserved Lys^{72} completely abolishes the kinase activity of the protein, and the activity is not restored by phosphorylation at the activation loop Thr^{197}. To ascertain whether the mutation interferes with other properties besides phosphoryl transfer, binding of the mutants to the regulatory subunit was evaluated using the RIα subunit. Following incubation of the mutants with RIα and running the samples on a non-denaturing Tris/glycine gel, no bands corresponding to the holoenzyme were detected for the unphosphorylated K72H. Under these conditions, a band was seen corresponding to the holoenzyme complex for the wild type. Surprisingly, a holoenzyme band was also observed for the PDK-1-treated H^{6}K72H (Fig. 7). The fact that phosphorylated K72H can bind to the RIα subunit demonstrates several points. First, it demonstrates that the protein has retained some of its functional properties, although it is unable to transfer the phosphate from ATP to substrates. Secondly, it demonstrates that the binding of RIα to the C-subunit is dependent on phosphorylation of the activation loop. Since holoenzyme formation for RIα is ATP-dependent (32), the results suggest that the mutant is also capable of binding ATP.

**In Vivo Phosphorylation State of Phosphorylation Site Mutants**—In vitro experiments using K72H and wild type (H-89) unphosphorylated proteins demonstrate the possible pathway the unphosphorylated C-subunit undergoes to achieve its fully phosphorylated and active form. The two phosphorylation sites required for an active protein are at Thr^{197} and Ser^{338}. Alanine mutant proteins engineered at these sites were constructed in a eukaryotic expression vector with the addition of an HA
Mutation of Lys$^{72}$ in PKA

The catalytic subunit of PKA, which is one of the essential residues at the active site of all protein kinases, has at least two apparent roles. It interacts with the C-subunit, Thr$^{197}$-P, or Ser$^{338}$-P as indicated. Wt, wild type.

**DISCUSSION**

Protein kinase A was first identified as the enzyme that phosphorylated and activated phosphorylase kinase in vivo. Phosphorylation state of phosphorylation site mutant proteins. The above proteins were transfected into COS cells followed by immunoprecipitation using mouse antibodies specific for the HA epitope tags engineered at their C terminus. These proteins were then subject to immunoblotting using rabbit-generated antibodies to the C-subunit, Thr$^{197}$-P, or Ser$^{338}$-P as indicated. Wt, wild type.

**Fig. 6.** Autophosphorylation of Ser$^{338}$. A, H$_{4}$K72H (1 μM) substrate was used to probe Ser$^{338}$ autophosphorylation. Lanes 1 and 2 represent the untagged wild type and the H$_{4}$K72H standards, respectively. Lane 3 represents an aliquot from a reaction of PDK-1 and H$_{4}$K72H, whereas lane 4 had active untagged wild type C-subunit (0.2 μM) added after 90 min. These samples were subjected to immunoblotting using α-C-subunit (A) and α-Ser$^{338}$-P (B) antibodies. Data indicated are representative of three different experiments.

**Fig. 7.** Holoenzyme formation. The ability of the catalytic subunit and K72H mutants to form holoenzyme was tested using RIα subunit as described under "Experimental Procedures." RIα (6 μM) was preincubated with K72H, PDK-1-treated K72H (pK72H), and wild type (WT) C-subunit (6 μM each), in the presence of MgCl$_{2}$/ATP, and subjected to non-denaturing PAGE. The experiments were carried out in the presence and absence of 0.1 mM cAMP. Identical results were obtained from experiments carried out in triplicate.

**Fig. 8.** In vivo phosphorylation state of phosphorylation site mutant proteins. The above proteins were transfected into COS cells followed by immunoprecipitation using mouse antibodies specific for the HA epitope tags engineered at their C terminus. These proteins were then subject to immunoblotting using rabbit-generated antibodies to the C-subunit, Thr$^{197}$-P, or Ser$^{338}$-P as indicated. Wt, wild type.
**Mutation of Lys^{72} in PKA**

**Importance of Lys^{72}**—The effects of mutagenesis of Lys^{72} reiterate its critical role in function. Even when the Lys was changed to residues that conserved charge, Arg and His, or approximate size, Met, there was no distinction from the Ala mutant protein. Structural information from many inactive kinases indicates that the orientation of the C-helix is typically altered, disrupting many of its interactions (11, 13, 36, 37). A common interaction observed in the inactive form occurs between Glu^{91} of the C-helix and Arg^{165} of the catalytic loop. Most likely, after phosphorylation on Thr^{197}, the mutant residues at the 72 position are not capable of competing Glu^{91} away from Arg^{165}. It could also be that, for the Arg and Met mutations, these bulkier residues introduce a steric hindrance that prevents the C-helix from assuming its proper conformation. To understand these details will require a crystal structure of the unphosphorylated protein and these mutants.

The ability of the phosphorylated K72H to bind to the regulatory subunit RIα demonstrates that the kinase function, in terms of protein–protein interactions, is somewhat restored in the phosphorylated form. Phosphorylation of Thr^{197} restores its capacity to serve as a scaffold for the RIα. However, the inability to carry out phosphorylation restricts its function as a catalyst. Thus docking to another protein is phosphorylation-dependent and independent of catalytic activity. This observation may be relevant to a number of protein kinases in human kinome that are predicted to lack protein kinase activity due to the absence of an essential active site residue (38). These proteins can still serve as docking sites for other proteins, and hence lack of catalytic function does not mean that such proteins are inert and devoid of function.

**Autophosphorylation at Ser^{338}**—In both K72H- and (H-89)-treated C-subunit, the autophosphorylation that occurs when the enzyme is expressed in E. coli is abolished. Phosphospecific antibodies confirmed that both proteins are excellent substrates for phosphorylation of Thr^{197} by PDK-1, but only the wild type C-subunit can go on to phosphorylate Ser^{338} at the C-terminus. This observation shows that not only does the K72H mutant protein serve to characterize phosphorylation by PDK-1, it can also serve as a possible model for the intermediate in the processing pathway. Knowing that the active C-subunit has two phosphates and that PDK-1 only phosphorylates Thr^{197}, there must be another mechanism that accounts for phosphorylation at Ser^{338}. Because the K72H is inactive and unphosphorylated at Ser^{338}, we conclude that this occurs via autophosphorylation, which is rapid and occurs after phosphorylation at Thr^{197}.

Furthermore, since exogenous wild type C-subunit does not phosphorylate Ser^{338} in the K72H mutant and since the rate of phosphorylation of the C-subunit is concentration-independent, the autophosphorylation must be intramolecular or cis (39, 40). Without a structure for the C-subunit in its unphosphorylated state, we can only speculate that Ser^{338} either must be positioned close to the active site before phosphorylation at Thr^{197} occurs or is freely mobile and capable of docking to the active site cleft. Such a position could direct phosphoryl transfer to the Ser as would be done in a substrate, in which the following rearrangement of the phospho-Ser^{338} would remove it from the active site. Mutagenesis of Ser^{338} demonstrated that the S338A mutant was unstable (10). Mutagenesis to Glu, and not Asp, was able to confer wild type kinetic values for $K_m$ for ATP and Kemptide but a 3-fold decrease in $k_{cat}$. When this site was mutagenized along with a series of sites to characterize the C-terminal tail, the Ala, Asn, and Asp mutants were examined (41). Here, S338A had elevated $K_m$ values for both ATP and Kemptide. This mutant also had a decreased thermostability, suggesting structural contributions. The phosphate may be stabilizing the tail to aid in substrate recognition. Biophysical tools are currently underway to assess the conformational consequences of an unphosphorylated Ser^{338}.

**Model for Activation of C-subunit**—Characterizing the phosphorylation of the wild type C-subunit and an inactive mutant form of the C-subunit (K72H) has thus elucidated a stepwise pathway for generating the active enzyme (Fig. 9). The mechanism involves initial phosphorylation of Thr^{197} by PDK-1 or a PDK-1-like enzyme followed by intramolecular autophosphorylation at Ser^{338}. Although we do not know the conformation of the unphosphorylated C-subunit, we deduce that the activation loop is fully exposed and accessible to phosphorylation by PDK-1, whereas Ser^{338} is not readily accessible to phosphorylation by either PDK-1 or C-subunit until Thr^{197} is phosphorylated. Ser^{338} is a substrate for trans autophosphorylation and may be shielded or may be quite mobile. If the latter is the case, it is most likely the string of six acidic residues that precede Arg^{336} that prevent it from docking to the active site of a neighboring C-subunit. In contrast, the tethered tail could have access to its own active site. In fact, the same acidic residues could be drawn to the basic patch comprised of Arg^{133} and Arg^{334} that flanks the recognition site for the P-2 Arg (42). This would position Arg^{336} and Ser^{338} into the active site cleft. This general model of cis autophosphorylation following phosphorylation of Thr^{197} is reinforced by the in vivo phosphorylation state of the phosphorylation site mutant proteins. T197A

**Fig. 9. Model for C-subunit activation by phosphorylation.** The unphosphorylated C-subunit, possibly membrane-associated due to its N-terminal myristoylation, is locked into a novel conformation. In this conformation the activation loop and Thr^{197} are exposed, whereas Ser^{338} on the C-terminal tail is shielded and inaccessible. Upon phosphorylation on Thr^{197} by PDK-1, the activation loop assumes the conformation required for activity and displaces the C-terminal tail. This is followed by intramolecular autophosphorylation of Ser^{338}, and the C-subunit is assembled in its active form as seen in the mammalian enzyme. The inactive mutant protein K72H, unable to undergo the second autophosphorylation step, is indicated in the second panel.
disrupts phosphorylation at both sites, indicating that phosphorylation at Thr\(^{197}\) occurs first and is required for further phosphorylation and activation. S338A does get phosphorylated, but only on Thr\(^{197}\), again demonstrating that it is the site where phosphorylation occurs first. The lack of phosphorylation at Ser\(^{338}\) in the T197A mutant protein strengthens the conclusion that it undergoes intramolecular autophosphorylation because neither endogenous C-subunit nor a Ser\(^{338}\) kinase phosphorylates this site in vivo.

This model for activation has also been suggested for PKC (43). PKCβII is larger than the PKA C-subunit with its regulatory domains contained within its primary structure. PDK-1 has been demonstrated to be the in vivo kinase for this enzyme, and a model describing its role in PKC activation has been proposed (24, 25). PDK-1 binds the newly synthesized unphosphorylated PKC at a C-terminal hydrophobic site, exposing the phosphorylation with PKC maturation through phosphorylation and also to elucidate a process for the activation of PKC.

Clearly, the importance of Lys\(^{72}\) for catalysis is confirmed by this work, but the inactive kinase has allowed us not only to elucidate functions of the C-subunit that are independent of catalysis and but also to elucidate a process for the activation of the C-subunit. A more detailed analysis of the conformational changes that occur upon mutation of the Lys\(^{72}\), as well as structures of the dephosphorylated protein, is needed to understand clearly the local consequences of the mutation on the activation state of the C-subunit.

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