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

Received for publication, July 7, 2004, and in revised form, December 15, 2004
Published, JBC Papers in Press, December 22, 2004, DOI 10.1074/jbc.M407586200

Ganesh H. Iyer‡, Michael J. Moore‡§, and Susan S. Taylor¶

From the Howard Hughes Medical Institute and Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, California 92093-0654

General strategies to obtain inactive kinases have utilized mutation of key conserved residues in the kinase core, and the equivalent Lys 
 in cAMP-dependent kinase has often been used to generate a “dead” kinase. Here, we have analyzed the consequences of this mutation on kinase structure and function. Mutation of Lys 
 to histidine (K72H) generated an inactive enzyme, which was unphosphorylated. Treatment with an exogenous kinase (PKD-1) resulted in a mutant that was phosphorylated only at Thr 
 and remained inactive but nevertheless capable of binding ATP. Ser 
 in K72H cannot be autophosphorylated, nor can it be phosphorylated in an intermolecular process by active wild type C-subunit. The Lys 
 mutant, once phosphorylated on Thr 
, can bind with high affinity to the RIα subunits. Thus a dead kinase can still act as a scaffold for binding substrates and inhibitors; it is only phosphoryl transfer that is defective. Using a potent inhibitor of C-subunit activity, H-89, Escherichia coli-expressed C-subunit was also obtained in its unphosphorylated state. This protein is able to mature into its active form in the presence of PKD-1 and is able to undergo secondary autophosphorylation on Ser 
. Unlike the H-89-treated wild type protein, the mutant protein (K72H) cannot undergo the subsequent cis autophosphorylation following phosphorylation at Thr 
. Using these two substrates and mammalian-expressed PDK-1, we can elucidate a possible two-step process for the activation of the C-subunit: initial phosphorylation on the activation loop at Thr 
 by PDK-1, or a PDK-1-like enzyme, followed by second cis autophosphorylation step at Ser 
.

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) has an essential phosphorylation site in the activation loop at Thr 
, in addition to Ser 
 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 Thr 
 and the environment surrounding the Ser 
 phosphate. The phosphate on Thr 
 in the activation loop of the C-subunit interacts with His 
 of the C helix, Arg 
 adjacent to the catalytic base, Asp 
 on the activation loop, and Lys 
 in β-strand 9, which positions Arg 
 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 Thr 
 and Ser 
 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, Lys 
 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...
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 alkylation 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 the H-89 and the Lys72 mutants are excellent substrates for PDK-1.

**EXPERIMENTAL PROCEDURES**

**Materials**—Reagents were obtained as follows: pRSETB expression vector (Invitrogen), [γ-32P]ATP (PerkinElmer 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 (Genosys-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 antibody to the Thr197 sequence was synthesized at the Peptide and Oligonucleotide Facility at the University of California, San Diego on a Millagen 9050 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 phosphorylated from unphosphorylated PKA C-subunit in the bacterial expression vector pRESTB. The antibody to the unphosphorylated Thr197 (α-Thr197-OH) was generated using a peptide corresponding to the sequence around Thr197: KGRTWTLGOTPEYLA. This peptide was sent to Cocalico Biologicals, Inc. (Reamstown, PA) for antibody generation in rabbit. The α-Ser338-P and α-Thr197-OH antibodies were affinity-purified by conjugating wild type and K72H respectively to Bio-Rad Affi-Gel and following the manufacturer’s protocol.

**Site-directed Mutagenesis of the PKA Catalytic Subunit**—cDNA for the murine PKA Cα-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
epitope tag added at the C terminus of the protein. All mutations were made using the Muta-Gen 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 r.p.m. in a Beckman JA20 rotor at 4 °C for 40 min. The proteins were purified via their His tag using Talon metal affinity resin (Clontech). In brief, supernatant was batch-bound to 1.0-ml resin/500-ml culture for 2 h at 4 °C. The resin was then incubated 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 Lys-Arg-Ala-Ser-Leu-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 × 106/10-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. 48 h after transfection, the cells were trypsinized and resuspended in buffer A (50 mM Tris-Cl, pH 7.5, 50 mM NaCl, 10 mM NaF, 10 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 0.5 mM EGTA, 1 mM dithiothreitol, 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100, 10 μg/ml aprotinin) as described (25). The cells were then subjected to three rounds of freeze-thaw cycle followed by centrifugation at 50,000 r.p.m. in a Beckman TL100 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).

Immunoprecipitation and PDK-1 Phosphorylation Assays—For the immunoprecipitation experiments, 2 μl of mouse anti-Myc antibody was mixed with the cell extract from 1% of a 10-cm dish of 293 cells transfected with PDK-1 in 25 μl of buffer A for 2 h on ice. The immunocomplex was then transferred to 10 μl (bed volume) of protein G-Sepharose resin and mixed for 1 h on a rotating wheel at 4 °C. The immunoprecipitates then were washed at room temperature five times: twice with buffer A, twice with buffer A plus 0.5 mM NaCl, and once with buffer B (50 mM Tris-Cl, pH 7.5, 10 mM NaCl, 1 mM dithiothreitol, 10% glycerol, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride). For the kinase assay, 0.25 μg of (H-89)-C was mixed with 25 μM ATP, 5 μCi of [γ-32P]ATP, and 10 μM MgCl2 in 25 μl of buffer B and incubated with the immobilized PDK-1 for 45 min at 30 °C with frequent gentle mixing.

Autophosphorylation of Ser804–1 μM H8-K72H was used as a substrate for PDK-1 as described above. After a 90-min incubation with PDK-1, 0.2 μM active untagged wild type C-subunit was added for an additional 60 min. Aliquots with and without the addition of wild type enzyme were added to SDS-gel loading buffer and subjected to SDS-PAGE followed by immunoblotting using the α-subunit and β-α Ser804-P antibodies to determine the phosphorylation state of Ser804. To evaluate the concentration dependence of autophosphorylation, (H-89)-C subunit in varying amounts (1–10 μμg) was mixed with 25 μM ATP, 5 μCi of [γ-32P]ATP, and 10 mM MgCl2 in 25 μl of buffer B and incubated for 30 min at 37 °C. The reaction was stopped by the addition of Laemmli loading buffer (4×) and boiled for 5 min. Proteins were separated by SDS-PAGE (10%), and the gel was dried and exposed to x-ray film.

Binding to Regulatory Subunit—6 μg of mutant proteins were incubated for 60 min with 5 μg of R1α regulatory subunit (in presence of 5 mM Mg2+ and 5 mM ATP), which act as pseudo substrates in vivo, and run on a non-denaturing Tris-glycine gel. The H8-K72H did not bind to R1α, and a band was observed corresponding to the free R-subunit. The PDK-1-treated H8-K72H catalytic subunit formed a complex with the R1α. The bands ran slightly higher than the wild type catalytic subunit since the mutant had a polyHis tag.

RESULTS

Purification of Wild Type C-subunit and Lys72 Mutants—Lys72 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 spectroscopy of the mutant proteins indicated that their mass was consistent for each mutant protein in an unphosphorylated state (Table 1). 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 also 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 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

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Theoretical mass (two phosphates)</th>
<th>Theoretical mass (unphosphorylated)</th>
<th>Actual mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₇K72A</td>
<td>42,704.7</td>
<td>42,544.7</td>
<td>42,543.6</td>
</tr>
<tr>
<td>H₇K72H</td>
<td>42,770.8</td>
<td>42,610.8</td>
<td>42,611.1</td>
</tr>
<tr>
<td>H₇K72M</td>
<td>42,764.8</td>
<td>42,604.8</td>
<td>42,604.8</td>
</tr>
<tr>
<td>H₇K72R</td>
<td>42,789.8</td>
<td>42,629.8</td>
<td>42,628.4</td>
</tr>
</tbody>
</table>
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 Ser338 Autophosphorylation—Both the unphosphorylated wild type C-subunit and Lys72 mutant proteins are good in vitro substrates for PDK-1. However, unlike the wild type protein, in which phosphorylation by PDK-1 on Thr197 is followed rapidly by the autophosphorylation of Ser338, the K72 mutant proteins are only phosphorylated on Thr197. To determine whether this is because Ser338 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 \( \text{H}^{32} \)-Ser338-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 P32 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 phosphorylation site.

Functional Consequences of Mutation at the Lys72—Mutation of the conserved Lys72 completely abolishes the kinase activity of the protein, and the activity is not restored by phosphorylation at the activation loop Thr197. 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-denatur-

![Fig. 4. Phosphorylation and activity of C-subunit substrates by PDK-1 as a function of time. A, equivalent amounts of wild type (W) (H-89) and K72H (0.25 μg) were incubated with immunoprecipitated Myc-PDK-1 (5 nM) in the presence of MgATP and [γ-32P]ATP. Aliquots were removed at the indicated times and, after SDS-PAGE, 32P incorporation was visualized by autoradiography. B, PDK-1 was incubated with both wild type (H-89) and K72H (1 μM). Aliquots were removed at the indicated times, subjected to SDS-PAGE, and then immunoblotted with the α-Thr197-P, α-Thr197-OH, and α-Ser338-P antibodies. C, a time course was also performed using the PepTag assay on aliquots taken from reactions at the indicated times. Experiments were performed in triplicate with similar results.](image)

![Fig. 5. Comparison of PDK-1 and PKA as a C-subunit kinase. H6K72H (1 μM) was used as substrate to compare phosphorylation by catalytic amounts (5 nM) of PDK-1 or wild type C-subunit as a function of time. Multiple reactions were performed in the presence of [γ-32P]ATP, and aliquots were subject to SDS-PAGE. 32P incorporation was visualized by autoradiography. The figure shows the results from one such experiment.](image)
epitope tag at the C terminus of the protein. These constructs, along with wild type C-subunit, were transfected into COS cells. The transfected cells were lysed and subject to immunoblotting using rabbit-generated antibodies to HA epitope tags engineered at their C terminus. These proteins were then subject to immunoblotting using rabbit-generated antibodies to the C-subunit, Thr197-P, or Ser338-P as indicated. Wt, wild type.

**DISCUSSION**

Protein kinase A was first identified as the enzyme that phosphorylated and activated phosphorylase kinase in response to increased levels of cAMP (33). The regulation of PKA by cAMP is mediated by the unique subunit organization of PKA, in which an independent regulatory subunit (R-subunit) controls the activity of the catalytic subunit (C-subunit) (34).

**FIG. 6. Autophosphorylation of Ser338**. A, H6K72H (1 μM) substrate was used to probe Ser338 autophosphorylation. Lanes 1 and 2 represent the untagged wild type and the H6K72H standards, respectively. Lane 3 represents an aliquot from a reaction of PDK-1 and H6K72H, whereas lane 4 had active untagged wild type C-subunit (0.2 μM) added after 90 min. These samples were subjected to immunoblots using C-subunit (A) and α-Ser338-P (B) antibodies. Data indicated are representative of three different experiments. C, 1, 2, 5, and 10 μM wild type H6-Wt (H-89)-treated PKA was incubated with [γ-32P]ATP for 30 min at 30 °C. Samples from each reaction were subjected to SDS-PAGE, and the gel was exposed to a Bio-Rad imaging screen. The band intensity was divided by the concentration of enzyme to give the rate of autophosphorylation per the amount of enzyme and normalized with the lowest enzyme concentration. The dashed line describes the kinetics in the case in which enzyme activity enzyme activity depends on molecular collisions. The slope obtained is indicative of an intramolecular phosphorylation reaction.

**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 MgCl2/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 via 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, Thr197-P, or Ser338-P as indicated. Wt, wild type.
**Importance of Lys**

The effects of mutagenesis of Lys 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. A common interaction observed in the inactive form occurs between Glu of the C-helix and Arg of the catalytic loop. Most likely, after phosphorylation on Thr, the mutant residues at the 72 position are not capable of competing Glu away from Arg. 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 R2a demonstrates that the kinase function, in terms of protein-protein interactions, is somewhat restored in the phosphorylated form. Phosphorylation of Thr restores its capacity to serve as a scaffold for the R2a. 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. 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**

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 by PDK-1, but only the wild type C-subunit can go on to phosphorylate Ser 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, there must be another mechanism that accounts for phosphorylation at Ser. Because the K72H is inactive and unphosphorylated at Ser, we conclude that this occurs via autophosphorylation, which is rapid and occurs after phosphorylation at Thr.

**Mutation of Lys**

Furthermore, since exogenous wild type C-subunit does not phosphorylate Ser in the K72H mutant and since the rate of phosphorylation of the C-subunit is concentration-independent, the autophosphorylation must be intramolecular or cis. Without a structure for the C-subunit in its unphosphorylated state, we can only speculate that Ser is either mobile or 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 would remove it from the active site. Mutagenesis of Ser demonstrated that the S338A mutant was unstable. 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 mutated along with a series of sites to characterize the C-terminal tail, the Ala, Asn, and Asp mutants were examined. 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.

**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 by PDK-1 or a PDK-1-like enzyme followed by intramolecular autophosphorylation at Ser. 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 is not readily accessible to phosphorylation by either PDK-1 or C-subunit until Thr is phosphorylated. Ser is not 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 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 and Arg that flanks the recognition site for the P-2 Arg. This would position Arg and Ser into the active site cleft. This general model of cis autophosphorylation following phosphorylation of Thr 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 are exposed, whereas Ser on the C-terminal tail is shielded and inaccessible. Upon phosphorylation on Thr 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, and the 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 Thr197 occurs first and is required for further phosphorylation and activation. S338A does get phosphorylated, but only on Thr197, again demonstrating that it is the site where phosphorylation occurs first. The lack of phosphorylation at Ser538 in the T197A mutant protein strengthens the conclusion that it undergoes intramolecular autophosphorylation because neither endogenous C-subunit nor a Ser538 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. PKD-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). PKD-1 binds the newly synthesized unphosphorylated PKC at a terminal hydrophobic site, exposing the activation loop Thr500 for phosphorylation, after which PDK-1 is released. The C-terminal phosphorylation sites presumably lie close to the active site, where intramolecular autophosphorylation occurs. PKC is properly phosphorylated at this point, but activity is now regulated through its C1 and C2 domains. The similarity between these results characterizing PKA phosphorylation with PKC maturation through phosphorylation lends credibility to the PKA model but also points to future work on PKC that may aid in understanding the local consequences of the mutation on the structures of the dephosphorylated protein, is needed to understand clearly the local consequences of the mutation on the activation state of the C-subunit.

Acknowledgments—We thank Drs. Frank Ma, Mira Sastri, and Kenneth Humphries for helpful discussions. We also thank Nina Haste and Dr. Elzbieta Radzio-Andzelm for some of the figure preparation and Siv Garrod for technical assistance.

REFERENCES