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Phosphorylation and activation of cAMP-dependent protein kinase by phosphoinositide-dependent protein kinase

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ABSTRACT **Although phosphorylation of Thr-197 in the activation loop of the catalytic subunit of cAMP-dependent protein kinase (PKA) is an essential step for its proper biological function, the kinase responsible for this reaction *in vivo* has remained elusive. Using nonphosphorylated recombinant catalytic subunit as a substrate, we have shown that the phosphoinositide-dependent protein kinase, PDK1, expressed in 293 cells, phosphorylates and activates the catalytic subunit of PKA. The phosphorylation of PKA by PDK1 is rapid and is insensitive to PKI, the highly specific heat-stable protein kinase inhibitor. A mutant form of the catalytic subunit where Thr-197 was replaced with Asp was not a substrate for PDK1. In addition, phosphorylation of the catalytic subunit can be monitored immunochemically by using antibodies that recognize Thr-197 phosphorylated enzyme but not unphosphorylated enzyme or the Thr197Asp mutant. PDK1, or one of its homologs, is thus a likely candidate for the *in vivo* PKA kinase that phosphorylates Thr-197. This finding opens a new dimension in our thinking about this ubiquitous protein kinase and how it is regulated in the cell.**

Protein phosphorylation is one of the most important processes for cellular regulation and signal transduction in eukaryotic cells. The enzymes responsible for catalyzing this reaction, the protein kinases, are predicted to account for >1% of all the proteins encoded for by the human genome; approximately one in every three proteins in mammalian cells is phosphorylated (1–3). In addition, large families of proteins are being discovered that contribute to localization and assembly of kinase complexes (4). In addition to phosphorylating other proteins, many protein kinases are themselves phosphoproteins, and their biological function and activity are frequently regulated by phosphorylation. One of the most dynamic regions of the protein kinase core, the activation loop, typically contains one or more critical phosphorylation sites. In the absence of phosphorylation, this loop is either disordered or in a conformation that is not optimal for catalysis (5–6). The activity of many protein kinases is regulated by the phosphorylation state of this activation loop. For example, the activation of the cell cycle-dependent protein kinase 2 is mediated by the addition of a single phosphate at Thr-161 in the activation loop. Once phosphorylation takes place, the anionic moiety is positioned by a set of basic residues such that the network of interactions at the active site cleft is poised for catalysis (7). In the case of the mitogen-activated protein kinase, phosphorylation at Thr-183 and Tyr-185 in the activation loop by mitogen-activated protein kinase kinase serves as a switch that not only shifts the equilibrium to an active conformation of the core but also leads to additional conformational changes that create a new dimer interface (8).

Unlike cell cycle-dependent protein kinase 2 and mitogen-activated protein kinase, the catalytic (C) subunit of cAMP-dependent protein kinase (PKA) normally is assembled as an active enzyme with a fully phosphorylated activation loop (9, 10). The regulation of the catalytic subunit of PKA is typically through interaction with an inhibitory regulatory subunit, which sequesters the C subunit in an inactive state under physiological conditions. Activation then is achieved by the generation of cAMP that binds to the regulatory subunit thereby reducing its affinity for the C subunit and leading to activation of the complex (11, 12). Although it is not clear at present whether there is an *in vivo* regulatory mechanism involving phosphorylation/dephosphorylation of Thr-197 in the activation loop of the catalytic subunit, phosphorylation of Thr-197 is a necessary step for the maturation and optimal biological activity of PKA (13, 14). If the normal processing of the C subunit is impaired such as in the kinase-negative S49 mouse lymphoma cells, the catalytic subunit accumulates in an insoluble, unphosphorylated, and inactive form (15). When the catalytic subunit is overexpressed in *Escherichia coli*, the enzyme is capable of autophosphorylating Thr-197 in the activation loop (16–18). A recent report, using the above described kinase negative mutant of S49 mouse lymphoma cells, demonstrated the presence of a heterologous protein kinase that is capable of phosphorylating and activating the catalytic subunit *in vitro* (19). The positive identity of this heterologous protein kinase has yet to be revealed.

The recently discovered phosphoinositide-dependent protein kinase, PDK1, or one of its homologs, is a good candidate for that heterologous PKA kinase (PKAK) for two reasons (20–23). It recognizes and phosphorylates the activation loop of protein kinase B (PKB) (20, 23) and the p70 ribosomal protein S6 kinase ($p70^{s6k}$) (22) whose sequences in this region are very similar to the catalytic subunit of PKA. In addition, PDK1 is localized to membranes by virtue of its plextrin homology domain, and the cell membrane is likely to be where nonphosphorylated, myristylated catalytic subunit resides before phosphorylation and assembly into holoenzyme (15). We demonstrate here that the catalytic subunit of PKA is also an excellent substrate for PDK1.

MATERIALS AND METHODS

Plasmids and Reagents. Wild-type murine C subunit and polyhistidine-tagged wild-type or mutant T197D catalytic subunit (H_6-C) were subcloned into expression vectors pLWS-3 and pET15b, respectively (24, 25). Plasmids pCMV5 containing Myc-tagged PDK1, and Myc-PDK1-KD, the catalytically inactive or kinase dead mutant of PDK1, were the same as

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Abbreviations: PDK1, phosphoinositide-dependent protein kinase; PKA, cAMP-dependent protein kinase; PKB, protein kinase B; PKC, protein kinase C; C, catalytic; H1, histone 1; PDK1-KD, kinase dead mutant of PDK1; PKAK, PKA kinase.

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reported previously (22). PKI was expressed and purified as described (26). Antibodies that specifically recognize the phosphorylated activation loop of protein kinase C (PKC) were a gift from A. Newton (University of California, San Diego) (27). Antibodies against the catalytic subunit of PKA were as described (18). Antibodies specific to PDK1 were generated by using synthetic peptide derived from the C-terminal 20 amino acids of PDK1. Purified peptide was cross-linked with keyhole limpet hemocyanin (KLH; Pierce) as a carrier before immunization. PDK1 antibodies were purified by affinity chromatography on agarose with the corresponding peptide immobilized. Rabbit and mouse anti-myc antibodies were purchased from Babco (Richmond, CA). Horseradish peroxidase conjugated anti-rabbit IgG (donkey) and ECL Western Blotting Detection Reagents kit were obtained from Amersham.

Expression and Purification of Phosphorylated and Non-phosphorylated Wild-Type and Mutant H_6-C. Wild-type and mutant T197D recombinant H_6-C were expressed from the pET15b vector in *E. coli* BL21 (DE3). When the optical density (600 nm) of the expression culture reached 0.3–0.4, the temperature was shifted from 37°C to 24°C. Expression of the protein was induced by the addition of 0.2 mM isopropyl β-D-thiogalactoside when the optical density reached 0.6–0.8. After 5–6 hr of induction at 24°C, the cells were harvested. The histidine-tagged protein was purified by Talon metal affinity resin (CLONTECH). In brief, cells harvested from a 500-ml culture were resuspended in 20 ml of lysis buffer (50 mM sodium phosphate/100 mM NaCl/5 mM 2-mercaptoethanol, pH 8.0). This suspension was then passed through a French pressure (American Instruments, Silver Spring, MD) cell at 1,000–1,500 psi, and the lysate was clarified by centrifugation (4°C) for 45 min at 15,000 rpm. The supernatant was batch bound to 2.0 ml of Talon (CLONTECH) metal resin suspension for 2 hr. The resin was poured into a small column and then washed with 50 ml of lysis buffer followed by 10 ml of 10 mM imidazole in lysis buffer. The protein was eluted with 100 mM imidazole in lysis buffer and dialyzed extensively against buffer B (50 mM Tris·HCl, pH 7.5/10 mM NaCl/1 mM DTT/10% glycerol/1 mM benzamidine/0.2 mM phenylmethylsulfonyl fluoride). To obtain unphosphorylated catalytic subunit, 40 μM of *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89, from Alexis, San Diego, CA), a potent PKA inhibitor, was added to the bacterial culture at the time of induction as reported (19).

Expression of Myc-PDK1 and Myc-PDK1-KD in 293 Cells. Human 293 cells were propagated at 2×10^6 per 10-cm dish in DMEM plus 10% fetal bovine serum. DNA (25 μg) for plasmids encoding either PDK1 or PDK1-KD was used for each transfection with the calcium phosphate method (28). Two days 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-glycerol phosphate/10 mM Na pyrophosphate/0.5 mM EGTA/1 mM DTT/1 mM benzamidine/0.5 mM phenylmethylsulfonyl fluoride/0.1% Triton X-100/10 μg/ml aprotinin) as described (22). The cells were then subjected to three rounds of freeze–thaw cycle, followed by centrifugation at 50,000 rpm for 30 min to collect the supernatant (S100) containing the over-expressed proteins.

Immunoprecipitation and Kinase Assays. Cell extracts equivalent to 1% of a 10-cm dish were used for all the experiments. For the immunoprecipitation experiments, 2 μl of mouse anti-myc antibody (Babco, Richmond, CA) were mixed with the cell extract in 25 μl of buffer A for 2 hr on ice. The immunocomplex was then transferred to 10 μl (bed volume) of protein G beads (Sigma) resuspended in 25 μl of buffer A and mixed for 1 hr on a rotating wheel. The immunoprecipitates then were washed at room temperature five times: twice with buffer A, twice with buffer A plus 0.5 M NaCl, and once with buffer B. For the kinase assay, 0.25 μg of (H89)-C was mixed with 25 μM ATP, 5 μCi [γ-^{32}P]ATP, and 10 mM $MgCl_2$ in 25 μl of buffer B and incubated with the immobilized PDK1 for 45 min at 30°C with frequent gentle mixing. For the PKA activity assay, the above reaction mixture was then separated from the PDK1 beads by centrifugation and transferred to a new tube containing 1 μg of histone 1 (H1). Incubation was carried for 10 min at room temperature. All reactions were stopped by boiling in SDS sample buffer. Protein samples were then separated on 10% or 12.5% SDS/PAGE or 10% NuPAGE gel (NOVEX, San Diego). The phosphorylation reaction was analyzed by either autoradiography or Western blot analysis by using antibodies specifically recognizing phosphorylated Thr-197.

RESULTS

Phosphorylation of the Catalytic Subunit of PKA by PDK1. To determine whether the catalytic subunit of PKA was a substrate for PDK1, it was necessary to generate a nonphosphorylated form of the catalytic subunit that could be used in the phosphorylation assay. Because the purified mammalian catalytic subunit as well as the recombinant catalytic subunit expressed in *E. coli* already are fully phosphorylated at Thr-197 and are very resistant to dephosphorylation by phosphatases *in vitro* (10, 29), neither can be used as a substrate. To prevent autophosphorylation of the recombinant catalytic subunit, the enzyme was expressed in *E. coli* in the presence of H89, a highly specific inhibitor of PKA, to produce unphosphorylated catalytic subunit, designated as (H89)-C as described by Cauthron, *et al.* (19). Both the histidine-tagged and nontagged protein were expressed, purified, and then used as substrates for the PDK1 phosphorylation assay.

Both Myc-tagged PDK1 and the catalytically inactive mutant of PDK1, Myc-PDK1-KD, where the essential Lys-61 was replaced with Gln, were expressed independently in 293 cells. Extracts from cells expressing active Myc-PDK1 were immunoprecipitated with Myc antibodies. When this immunoprecipitate was incubated with purified H_6-(H89)-C in the presence of MgATP, linear and time dependent phosphorylation of the C subunit was observed (Fig. 1). To determine whether this phosphorylation was due specifically to the overexpressed

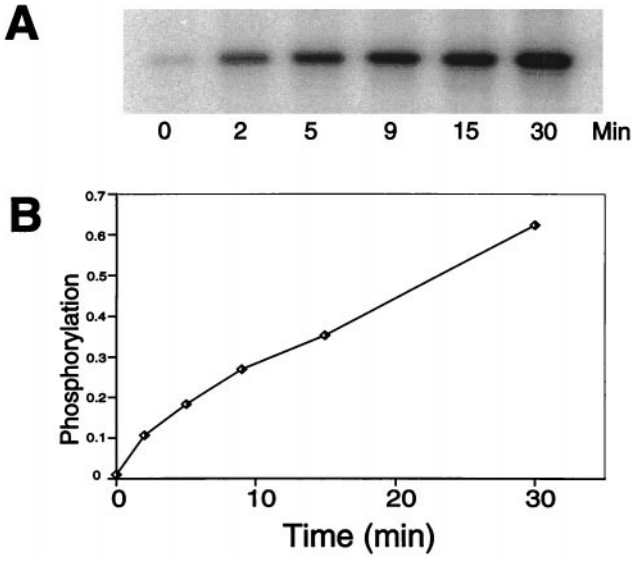

FIG. 1. Time course of phosphorylation of the catalytic subunit of PKA by immobilized Myc-PDK1. The immunoprecipitation kinase assays were performed as described in *Materials and Methods*. The reactions were stopped by addition of 20 mM EDTA, and samples were analyzed by SDS/PAGE. The ^{32}P-labeled proteins were visualized by autoradiography. Phosphorylation was quantitated by phosphoimaging. Units are arbitrary.

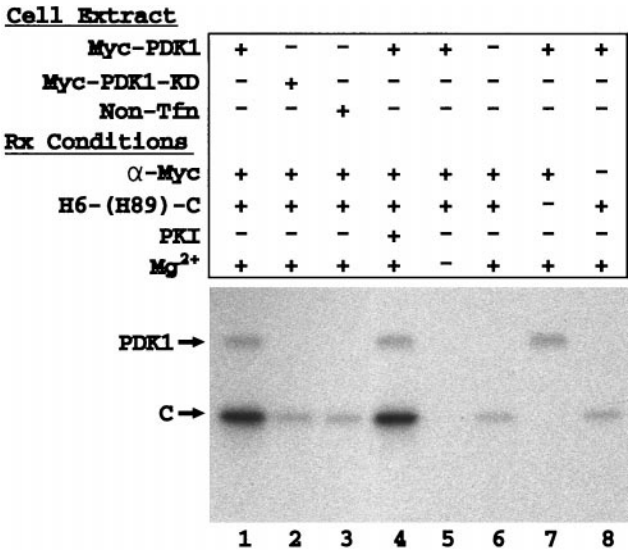

FIG. 2. Phosphorylation of catalytic subunit of PKA by PDK1 under various conditions. The concentration of the heat stable protein kinase inhibitor, PKI, was 0.3 μM. The amount of Myc-PDK1 and Myc-PDK1-KD used was equivalent based on Western blot analysis. Positions of PDK1 and C subunit migration are indicated by arrows.

PDK1, a Myc antibody immunoprecipitate from cells that over-expressed the kinase dead form of PDK1, Myc-PDK1-KD, was incubated with the catalytic subunit. Under these conditions, little phosphorylation was observed (Fig. 2, lane 2). No phosphorylation of the band corresponding to PDK1 under these conditions indicated that the basal level of PDK1 phosphorylation, as observed in lane 1 where the immunoprecipitate of active PDK1 was used, was due to PDK1 autophosphorylation. The low level of catalytic subunit phosphorylation observed with the kinase dead mutant was comparable to what was observed when immunoprecipitates from nontransfected 293 cells were used (Fig. 2, lane 3). Because no Myc-tagged PDK1 or PDK1-KD were expressed in 293 cells, this low basal level of phosphorylation of (H89)-C was caused by autophosphorylation. The phosphorylation of H_6-(H89)-C by PDK1 was not inhibited by PKI and also was dependent on Mg^{2+} (Fig. 2, lanes 4 and 5). Experiments using partially purified nontagged (H89)-C gave identical results as H_6-(H89)-C (data not shown).

Depletion of Myc-PDK1 with Anti-PDK1 Antibodies Proportionately Reduced PKA Phosphorylation. To further confirm the correlation of PDK1 and PKA kinase activity, an immunodepletion assay was performed by using PDK1 antibodies. These antibodies should recognize any endogenous PDK1 as well as overexpressed Myc-PDK1. When the expressed Myc-PDK1 was immunoprecipitated with anti-Myc antibodies, PKAK activity was observed as expected (Fig. 3, middle lanes). Only a low level of basal autophosphorylation activity was observed when the PDK1 expressing 293 extract was immunoprecipitated with nonspecific antibodies to the hemagglutinin epitope (Fig. 3, lanes 1 and 2). In contrast, when the Myc-PDK1-transfected 293 cell extract was preincubated with an antibody against PDK1 before the immunoprecipitation, the observed phosphorylation of PKA by expressed Myc-PDK1 was reduced significantly (Fig. 3*A*). The level of PKA phosphorylation correlated with the amount of Myc-PDK1 present in the cell extract after treatment with the PDK1 antibodies, and depletion of the enzyme led to a proportional drop in activity (Fig. 3 *B* and *C*). This observation established a qualitative correlation between the level of PKA phosphorylation activity and the amount of Myc-PDK1 and further confirmed the direct involvement of PDK1 in PKA phosphorylation.

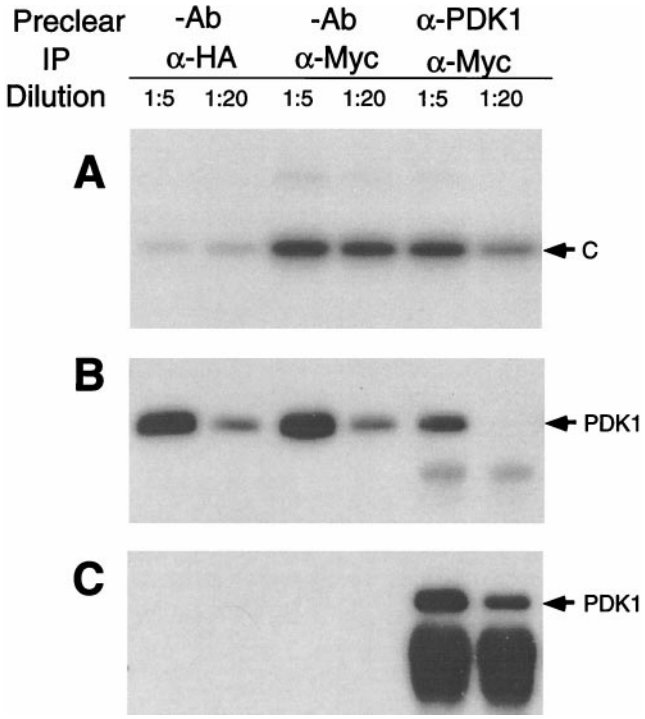

FIG. 3. Myc-PDK1-mediated phosphorylation of (H89)-C is prevented by depletion of Myc-PDK1 with PDK1 antibodies. The S100 fraction of 293 cells transfected with Myc-PDK1 was diluted 5- or 20-fold and then preincubated with or without PDK1 antibodies as described in *Materials and Methods*. Cross-reacting proteins were removed by centrifugation after the addition of protein G beads. (*A*) Autoradiography showing phosphorylation of H89-C by Myc-PDK1 with or without preincubation with the PDK1 antibody. (*B*) Western blot showing the amount of Myc-PDK1 remaining in the cell extracts with and without preincubation with PDK1 antibody. (*C*) Immunoblot displaying the amount of Myc-PDK1 pulled down by pretreatment with anti PDK1.

PDK1 Phosphorylates PKA at Thr-197 in the Activation Loop. The catalytic subunit of PKA purified from animal tissues is phosphorylated at two sites, Thr-197 and Ser-338 (9, 10). Two additional residues, Ser-10 and Ser-139 are found to be phosphorylated *in vitro* in the recombinant catalytic subunit (16–18). Of all these phosphorylation sites, only phosphorylation of Thr-197 is necessary and sufficient for biological activity (13, 14). To verify the site of phosphorylation, Thr-197 in the histidine-tagged catalytic subunit was changed to Asp (14). Mutation at Thr-197 eliminated phosphorylation of the catalytic subunit by PDK1, suggesting that Thr-197 is the specific site that is phosphorylated by PDK1 (Fig. 4*A*). To further confirm that Thr-197 was the site of phosphorylation by PDK1, antibodies that recognize only the Thr-197 phosphorylated C subunit were used. Although these antibodies were originally generated specifically against the phosphorylated activation loop of PKC (27), they also recognize the activation loop of the catalytic subunit of PKA when it is phosphorylated on Thr-197. As shown in Fig. 4*B*, these antibodies cross-reacted well with the wild-type C subunit, which is fully phosphorylated at Thr-197, and other sites but did not react with H_6-(H89)-C, which is not phosphorylated at any of its sites. The antibody also does not cross-react with the mutant T197D (Fig. 4*B*). These antibodies also could be used to monitor the specific phosphorylation of Thr-197 with time by using extracts of 293 cells that overexpressed Myc-PDK1 (Fig. 5). The phosphorylation of H_6-(H89)-C at Thr-197 by PDK1 was rapid and reached maximal in $\approx$10 min. In contrast, when extracts from nontransfected 293 cells were used, little phosphorylation was observed (data not shown).

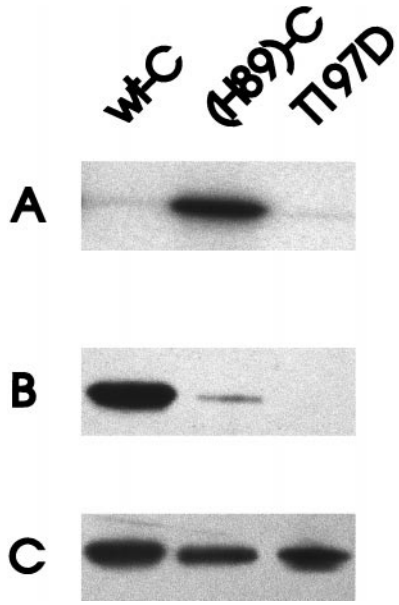

FIG. 4. Phosphorylation of (H89)-C by PDK1 at Thr-197. (*A*) Phosphorylation of wild-type C, H89-C, and mutant T197D by PDK1. Phosphorylation was analyzed by SDS/PAGE followed by autoradiography. (*B*) Western blot analysis using antibodies specific for the phosphorylated PKC activation loop. (*C*) Immunoblot using anti-C subunit antibodies to show equivalent amounts of wild-type C, (H89)-C, and T197D were used in *A* and *B*.

Activation of PKA by PDK1 Phosphorylation. To test the effect of PDK1 phosphorylation on the activity of H_6-(H89)-C, phosphorylation of H1, a known PKA substrate, was used to assay the activity of H_6-(H89)-C before and after incubation with PDK1. As shown in Fig. 6, once phosphorylated by PDK1, (H89)-C showed significant activation as demonstrated by an increase in phosphorylation of H1. Consistent with its inability

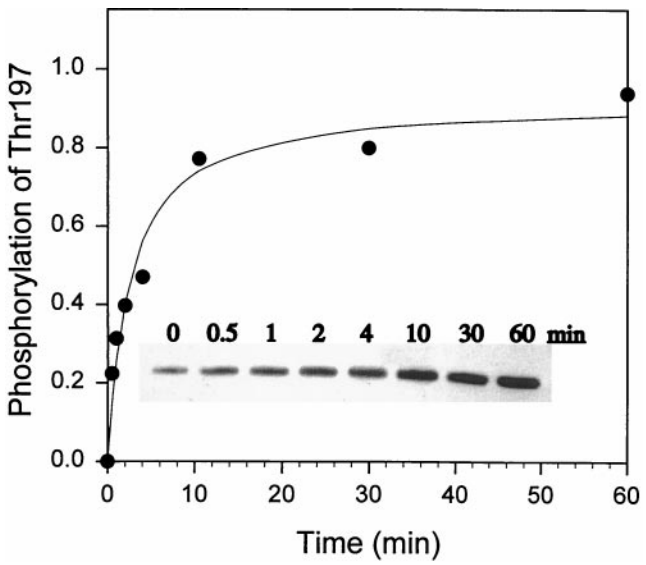

FIG. 5. Phosphorylation of (H89)-C by PDK1 as a function of time. H89-C (0.37 μg) was incubated with total cell extracts of 293 cells expressing Myc-PDK1 (7.5 μl) in the presence of 200 μM MgATP at room temperature. The total volume of the reaction mixture was 60 μl. At different time intervals, 5 μl were withdrawn, mixed with 5 μl 2X SDS sample buffer, and heated at 100°C for 2 min to stop the reaction. The phosphorylation of Thr-197 by PDK1 was probed by Western blot by using PKC antibodies that are also specific for C subunit that is phosphorylated at Thr-197. Control experiments using nontransfected 293 cell extract showed negligible phosphorylation of (H89)-C (data not shown). Phosphorylation units are arbitrary.

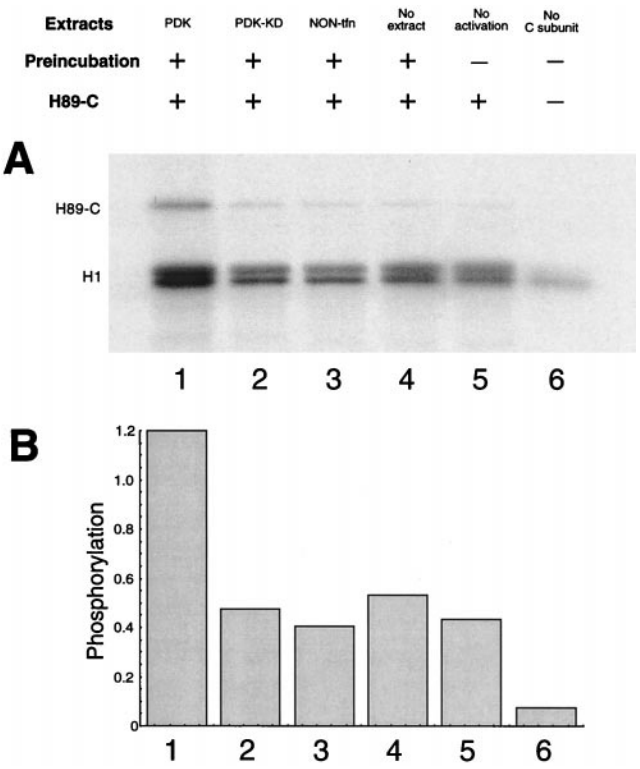

FIG. 6. Activation of PKA by PDK1. The S100 fractions of 293 cells (2.5 μl) independently transfected with Myc-PDK1 and Myc-PDK1-KD, as well as nontransfected 293 cells were immunoprecipitated by Myc antibodies and protein G-Sepharose as in Fig. 1. The immunoprecipitates were then mixed with histidine-tagged (H89)-C (0.125 μg) in the presence of [γ-^{32}P]ATP. After incubation at room temperature for 30 min, the protein G beads were removed by centrifugation and H1 (1 μg) was added. The reaction mixtures were further incubated at room temperature for additional 10 min. Phosphorylated H1 was resolved by SDS/PAGE and visualized by autoradiography. Phosphorylation of H1 was quantitated by phosphoimaging. Units are arbitrary.

to phosphorylate H_6-(H89)-C, Myc-PDK1-KD did not exhibit any effect on (H89)-C activity. These data indicate that phosphorylation of H_6-(H89)-C by PDK1 leads to the activation of PKA. The basal H1 phosphorylation activity of H_6-(H89)-C did not increase significantly if the protein was preincubated with only MgATP (Fig. 6, lane 5), indicating that the basal activity for phosphorylation of H_6-(H89)-C was not caused by activation by autophosphorylation. Earlier studies indicate that unphosphorylated C subunit has a k_{cat} value comparable with that of the wild-type enzyme whereas the K_m values for both substrates are raised significantly (13, 14). Therefore, it is not surprising to see that H_6-(H89)-C had a considerable amount of basal H1 phosphorylation activity under the experimental conditions that were used.

DISCUSSION

Although activation of many protein kinases is mediated by phosphorylation at a critical site on the activation loop by a heterologous protein kinase (4), phosphorylation at this site in the catalytic subunit of PKA until now has been poorly understood both in terms of the specific kinase that mediates this phosphorylation and in terms of the overall location and regulation of this process in cells. The results described here, demonstrating that PDK1 can serve as a PKA kinase (PKAK), open up new dimensions to our thinking about how the catalytic subunit is assembled and regulated.

Using recombinant catalytic subunit expressed in the presence of a specific PKA inhibitor, H89, to prevent autophos-

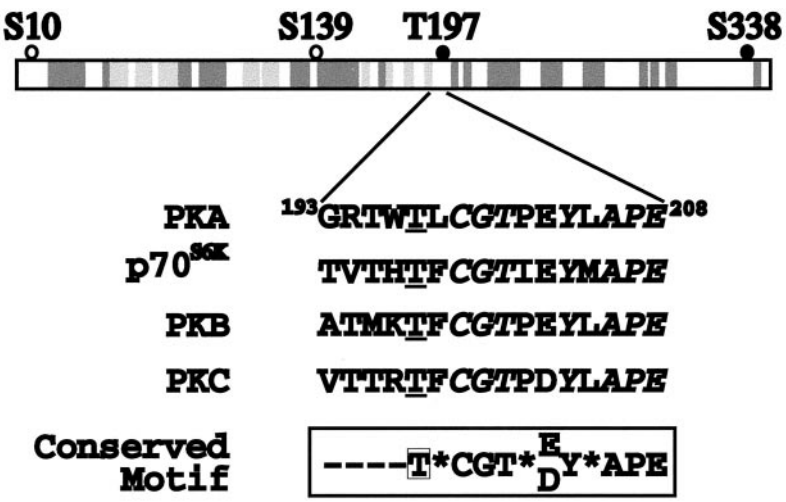

FIG. 7. Sequence alignment of the activation loop region of PKA, PKB, PKC, and $p70^{s6k}$. The specific Thr residue whose phosphorylation is important for PKA activity is underlined and identical amino acid residues are highlighted in italic. The consensus site is indicated at the bottom. The * corresponds to hydrophobic residues.

phorylation, PDK1 expressed in 293 cells was shown here to be a PKAK. Kinase activity was dependent on the expressed enzyme and was observed by using either extracts of cells that overexpress PDK1 or immunoprecipitates of PDK1 from 293 cells. Autophosphorylation of the catalytic subunit under these conditions was slow compared with the rapid phosphorylation by PDK1. The highly specific inhibitor of PKA, PKI, also had no significant effect on the PDK1-mediated phosphorylation of the catalytic subunit. Phosphorylation of the catalytic subunit by PDK1 also led to an increase in its catalytic activity by using histone as a substrate. Two strategies were used to demonstrate that the site of phosphorylation by PDK1 was Thr-197. A mutant form of the catalytic subunit where Thr-197 was replaced by Asp, was not a substrate for PDK1. In addition, the time-dependent phosphorylation could be monitored with antibodies that specifically recognize only catalytic subunit that is phosphorylated on Thr-197. Thr-197 is, therefore, the site that is recognized by PDK1.

So far all known PDK1 substrates belong to the AGC subfamily of Ser/Thr protein kinases and share a high degree of sequence similarity in the region that flanks the Thr phosphorylation site. Unlike many protein kinases consensus sites, amino acids important for PDK1 recognition appear to be localized carboxyl terminal to the P site; no apparent sequence similarity is observed in the region that is amino terminal to the P site. Based on sequence comparison of PKA, PKB, PKC, and $p70^{s6k}$ (Fig. 7), a tentative consensus phosphorylation motif for PDK1 is T*CGT*(E/D)Y*APE, which corresponds to the P through P+11 region. The * corresponds to hydrophobic residues. This sequence recognition motif may reflect a unique mechanism of substrate recognition for kinase activating protein kinases such as PDK1.

Although it is not clear that PDK1 is responsible for the *in vivo* phosphorylation of the catalytic subunit, it is very likely that PDK1 or one of its homologs is the *in vivo* PKAK. The properties of PKA phosphorylation by PDK1 described here are very consistent with the recent results of Cauthron *et al.* (19) who demonstrated a partially purified PKAK activity from a mutant form of S49 mouse lymphoma cells. If PDK1 is the *in vivo* kinase and has broad specificity *in vitro* as demonstrated here and in earlier studies with PKB (20, 21) and S6 kinase (22, 23), then it will be extremely important to determine whether specificity is achieved in part by recruitment of PDK1 to specific subsites. Colocalization may be of special importance for PDK1 not only because of its plextrin homolog domain but also because it appears to have a high level of constitutive activity (20, 21). We have not tested the effect of phospholipids on the PKAK activity of PDK1, but this clearly will have important implications for the *in vivo* function of PDK1.

The AGC family of protein kinases, especially PKA and PKC, are ubiquitous in mammalian cells. They regulate a myriad of processes that are critical for cell growth, development, death, and homeostasis. In contrast to many protein kinases such as mitogen-activated protein kinase and cell cycle-dependent protein kinase 2, which undergo dynamic, phosphorylation-dependent changes in their activation loops (5–8, 10, 13), the activation loop phosphorylation sites in enzymes such as PKA and PKC are relatively stable in the purified mammalian enzymes (10, 30). All of the PKA structures to date are of the mature phosphorylated enzyme, and in all of these structures, the activation loop has been very stable. This discovery of a PKAK opens up several new dimensions in our thinking about this ubiquitous enzyme. In addition to resolving the physiological role of PDK1 in the activation and phosphorylation of PKA, it will be of equal importance to characterize the structure of the dephosphorylated catalytic subunit to determine whether the activation loop and the carboxyl terminal tail have undergone significant conformational reorganization. Taken together with recent reports of the catalytic subunit involvement in a novel IκB:NFκB complex in which activation is mediated by a presumably cAMP-independent process (31), it will be very important to rigorously reexamine our thinking about PKA. The conventional view of a static holoenzyme complex that is activated in response to cAMP, a view that has predominated for many decades, may be too simplistic.

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