

# Invariant Leu Preceding Turn Motif Phosphorylation Site Controls the Interaction of Protein Kinase C with Hsp70\*

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Heat shock proteins play important roles in regulating signal transduction in cells by associating with, and stabilizing, diverse signaling molecules, including protein kinases. Previously, we have shown that heat shock protein Hsp70 associates with protein kinase C (PKC) via an interaction that is triggered by dephosphorylation at the turn phosphorylation motif. Here we have identified an invariant residue in the carboxyl terminus of PKC that mediates the binding to Hsp70. Specifically, we show that Hsp70 binds to Leu (Leu-640) immediately preceding the conserved turn motif autophosphorylation site (Thr-641) in PKC  $\beta$ II. Co-immunoprecipitation experiments reveal that mutation of Leu-640 to Gly decreases the interaction of Hsp70 with PKC  $\beta$ II. This weakened interaction between Hsp70 and the mutant PKCs results in accumulation of dephosphorylated PKC in the detergent-insoluble fraction of cells. In addition, the Hsp70-binding mutant is considerably more sensitive to down-regulation compared with WT PKC: disruption of Hsp70 binding leads to accelerated dephosphorylation and enhanced ubiquitination of mutant PKC upon phorbol ester treatment. Last, pulse-chase experiments demonstrate that Hsp70 preferentially binds the species of mature PKC that has become dephosphorylated compared with the newly synthesized protein that has yet to be phosphorylated. Thus, Hsp70 binds a hydrophobic residue preceding the turn motif, protecting PKC from down-regulation and sustaining the signaling lifetime of the kinase.

Hsp70<sup>3</sup> proteins are members of a large protein family of molecular chaperones. The role of heat shock proteins in facilitating protein folding and providing protection against cellular stress has been studied extensively (1). Hsp70 contains an N-terminal ATPase domain for binding co-chaperones and a C-terminal peptide-binding domain for substrate association. Hsp70 is known to participate in *de novo* folding of newly synthesized polypeptides by binding to the unfolded substrates to

prevent protein aggregation and assist protein folding in an ATP- and co-chaperone-dependent manner (2). The substrates of Hsp70 generally contain a hydrophobic region enriched particularly in the residues Leu, Ile, Val, and Phe (3). The Hsp70-binding sites are normally concealed inside the fully folded proteins but are exposed in nascent polypeptides or in proteins that have become denatured upon stress. Unmasking of hydrophobic regions in unfolded proteins signals Hsp70 to bind its substrates and begin the chaperone-assisted folding and refolding process.

An increasing number of studies have been focused on elucidating the role of Hsp70 in regulating signaling molecules in many physiological and pathological processes. For example, it has been shown that Hsp70 interacts with Raf-1 and hormone receptors and regulates their activity or cellular localization (2). It is known that levels of Hsp70 and other heat shock proteins are highly elevated in many human tumors and tumor cell lines (4). Overexpression of Hsp70 in tumor cells renders them resistant to TNF-induced apoptosis, and the level of Hsp70 expression correlates with tumorigenic potency of the tumor cells in mouse models (5). On the other hand, knock down of endogenous Hsp70 expression in human tumor cells, using either adenovirus-mediated delivery of antisense Hsp70 cDNA or transfection of siRNAs against Hsp70, results in cell death and inhibition of tumor formation (6–8). Although considerable evidence supports a role for Hsp70 in promoting cell survival and tumor progression, the molecular mechanisms underlying the tumor-promoting function of Hsp70 are not well understood. It is generally proposed that the increased expression of heat shock proteins may provide normal cells with a growth advantage to cope with increased amounts of denatured proteins resulting from chronic stress and the mutations accumulated during the tumorigenic process. Thus, Hsp70 may provide a general rescue mechanism to protect denatured or misfolded signaling molecules from aggregating by binding to them and shunting them into a refolding rather than degradation pathway.

The life cycle of PKC exemplifies how conformational changes are tightly coupled to kinase activity and protein stability. Specifically, newly synthesized PKC is associated with the membrane in an open conformation with the pseudosubstrate sequence out of the catalytic site and an exposed C terminus that allows docking of the upstream kinase PDK-1 (9, 10). Following phosphorylation of the activation loop site by PDK-1, the conventional PKCs are rapidly autophosphorylated at two sites, the turn motif site and the hydrophobic site, in the C terminus via an intramolecular mechanism (11). Fully phosphorylated PKC is now catalytically competent. It is released into the cytosol and adopts a closed conformation with the pseudosubstrate sequence folded into

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<sup>3</sup> The abbreviations used are: Hsp, heat shock protein; PKC, protein kinase C; DMEM, Dulbecco's modified Eagle's medium; GST, glutathione S-transferase; PDBu, phorbol-12,13-dibutyrate; PDK-1, phosphoinositide-dependent kinase 1; HA, hemagglutinin; WT, wild-type; PBS, phosphate-buffered saline; CT, C-terminus.

the active site of the enzyme, thus maintaining the matured PKC in an auto-inhibited state. Upon binding to the second messengers  $\text{Ca}^{2+}$  and diacylglycerol via its membrane targeting modules in the regulatory domain, conventional PKC is recruited to the membrane. This event expels the pseudosubstrate sequence from the active site, thus allowing substrate phosphorylation and downstream signaling. Activated PKC adopts an open confirmation in which the enzyme is sensitive to dephosphorylation. Prolonged activation of PKC, notably following treatment of cells with tumor promoting phorbol esters, triggers dephosphorylation and subsequent association of the dephosphorylated species with the detergent-insoluble fraction of cells and eventual degradation (12). It has been suggested that down-regulation of PKC is controlled by a proteasome-dependent pathway initiated by ubiquitination of the enzyme (13). However, the molecular events leading to ubiquitination of PKC have yet to be unveiled.

Previously we reported that Hsp70 associates with the C terminus of PKC, with the phosphorylation status of the turn motif site (Thr-641) providing the on and off switch for the interaction. We showed that disruption of the interaction between Hsp70 and PKC  $\beta$ II results in accumulation of dephosphorylated PKC in the detergent-insoluble fraction of cells and proposed that Hsp70 sustains the signaling lifetime of PKC by protecting PKC from activation-induced dephosphorylation and degradation. Here, we identify a primary determinant that mediates the binding of Hsp70 to dephosphorylated PKC. Specifically, we identify a conserved hydrophobic residue Leu-640 preceding the turn motif phosphorylation site that is responsible for binding Hsp70. Mutation of this hydrophobic residue accelerates dephosphorylation and down-regulation of PKC. We also show that Hsp70 preferentially binds to mature PKC that has been dephosphorylated compared with newly synthesized protein that has never been phosphorylated, revealing a molecular memory in the C terminus of PKC. These data reveal that Hsp70 recognizes conformationally distinct species, a mechanism that allows selective rescue and recycling of PKC that have been activated.

### EXPERIMENTAL PROCEDURES

**Materials**—Easy Tag [ $^{35}\text{S}$ ]methionine/cysteine ( $1175 \text{ Ci mmol}^{-1}$ ) was purchased from PerkinElmer Life Sciences. Methionine/cysteine-deficient DMEM was purchased from Invitrogen. PDBu was obtained from EMD/CalBiochem. A polyclonal antibody against PKC  $\beta$ II was purchased from Santa Cruz Biotechnology. A monoclonal antibody against Hsp70 and a monoclonal antibody against PKC  $\alpha$ , which recognizes PKC  $\beta$ II as well, were obtained from BD Biosciences. An anti-Myc monoclonal antibody (9B11) was purchased from Cell Signaling. An anti-HA high affinity rat monoclonal antibody was purchased from Roche Applied Science.

**Construction of Expression Plasmids**—Mutant PKC  $\beta$ II constructs including PKC  $\beta$ II-L640G and PKC  $\beta$ II-L640A were generated using a QuikChange site-directed mutagenesis kit (Stratagene). Construction of mammalian expression plasmids encoding glutathione *S*-transferase (GST)-fusion protein of the C terminus of WT PKC  $\beta$ II (GST-CT/WT) was described previously (10). The C-terminal fragment encoding amino acid residues 627–673 of the PKC  $\beta$ II mutant was amplified using PCR. The PCR

products were subcloned into pEBG vector (10) to yield the mutant GST-fusion protein expression construct GST-CT/L640G. The Myc-tagged Hsp70 was generated by PCR amplification of full-length Hsp 70 cDNA followed by subcloning of the products into pcDNA3.1Myc/His(–) vector (Invitrogen).

**Cell Transfection**—tsA201 or COS7 cells were maintained in DMEM (Cellgro) containing 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . Transient transfection of all cell types was carried out using Effectene transfection reagents (Qiagen).

**Cell Fractionation**—To examine cellular localization of WT and mutant PKC, transfected tsA201 cells were resuspended in Buffer A (50 mM  $\text{Na}_2\text{HPO}_4$ , 1 mM sodium pyrophosphate, 20 mM NaF, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 1 mM dithiothreitol, 200  $\mu\text{M}$  benzamidin, 40  $\mu\text{g ml}^{-1}$  leupeptin, 200  $\mu\text{M}$  phenylmethylsulfonyl fluoride). The cells were lysed by brief sonication, and the cell lysates were subjected to centrifugation at  $16,000 \times g$  for 5 min at  $4^\circ\text{C}$ . The resulting supernatant is defined as detergent-solubilized lysate. The pellet resulting from the centrifugation was defined as detergent-insoluble pellet.

**Immunoprecipitation**—To examine the interaction between PKC  $\beta$ II and Hsp70, tsA201 cells were transfected with either WT or mutant PKC  $\beta$ II and Myc-Hsp 70. The transfected cells were lysed in Buffer A, and the detergent-solubilized cell lysate was incubated with the anti-Myc monoclonal antibody and Ultra-link protein A/G beads (Pierce) at  $4^\circ\text{C}$  overnight. The immunoprecipitates were washed twice in Buffer A and twice in Buffer B (Buffer A plus 250 mM NaCl). Bound proteins were analyzed by SDS-PAGE and immunoblotting.

**GST Fusion Protein Pulldown Assay**—To examine the direct interaction between the C terminus of PKC  $\beta$ II and Hsp70, two separate transfections were carried out using tsA201 cells. One set of cells were transiently transfected with GST-CTs of PKC  $\beta$ II. The transfected cells were lysed in Buffer A, and the detergent-solubilized cell lysates were incubated with glutathione-Sepharose at  $4^\circ\text{C}$  for 2 h. Beads were washed twice in Buffer A and twice in Buffer B, and GST-CT bound beads were used in the subsequent pulldown experiments. The other set of cells were transfected with Myc-Hsp70 (tagged with a His<sub>6</sub>-tag as well), and the transfected cells were lysed in Buffer C (PBS plus 1% Triton X-100, 10 mM imidazole, 1 mM dithiothreitol, 200  $\mu\text{M}$  benzamidin, 40  $\mu\text{g ml}^{-1}$  leupeptin, and 200  $\mu\text{M}$  phenylmethylsulfonyl fluoride). The detergent-solubilized cell lysates were incubated with nickel-nitrilotriacetic acid (Qiagen) beads at  $4^\circ\text{C}$  for 1 h. The beads were washed in Buffer C and Myc-Hsp70 was eluted in PBS plus 250 mM imidazole. The GST-CT bound beads from above were incubated with eluted Myc-Hsp70 in PBS at  $4^\circ\text{C}$  for 4 h. The beads were washed with PBS and bound proteins were analyzed by SDS-PAGE and immunoblotting.

**Pulse-Chase Analysis of Protein Kinase  $\beta$ II Processing**—COS7 cells were transfected with WT and mutant PKC  $\beta$ II. 24–30 h post-transfection, cells were incubated with methionine/cysteine-deficient DMEM for 30 min at  $37^\circ\text{C}$ , as described previously (14). The cells were then pulse-labeled with  $1 \text{ mCi ml}^{-1}$  [ $^{35}\text{S}$ ]methionine/cysteine in methionine/cysteine-deficient DMEM for 7 min at  $37^\circ\text{C}$  and chased in chase medium (DMEM containing 5 mM unlabeled methionine and 5 mM unlabeled cysteine and 10% fetal bovine serum) for 0, 2, and

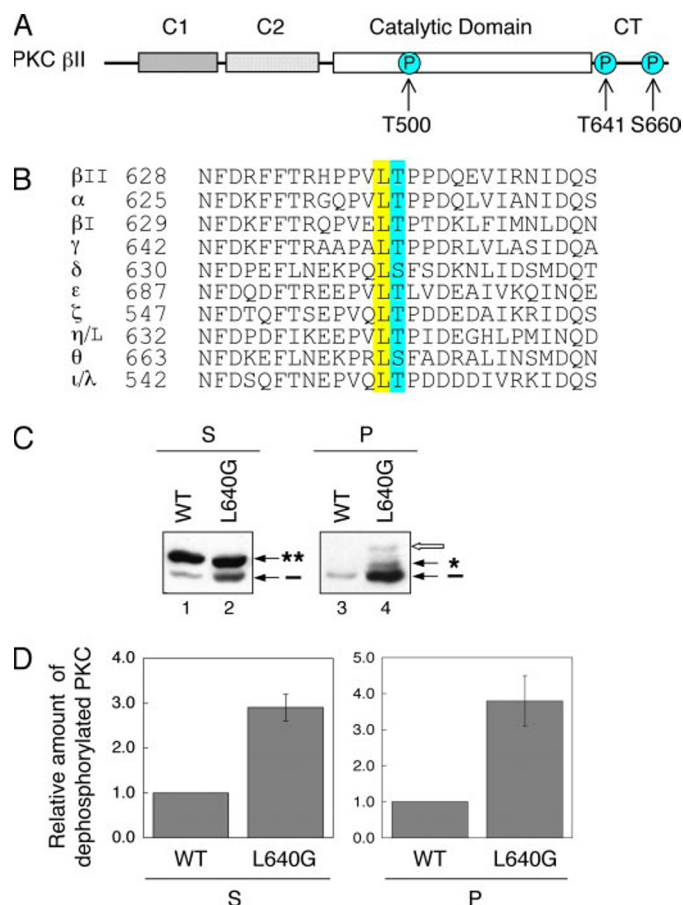
5.5 h. At the indicated times, cells were lysed in Buffer A, and PKC was immunoprecipitated with the anti-PKC  $\alpha$  monoclonal antibody (cross-reactive with PKC  $\beta$ II) coupled to Ultra-link protein A/G beads. The immunoprecipitates were washed three times with Buffer B and once with Buffer A. The immunoprecipitated proteins were separated by SDS-PAGE and transferred to PVDF membrane. The  $^{35}$ S signals were detected by autoradiography.

**Analysis of PDBu-induced Dephosphorylation of PKC and Altered Association with Hsp70**—tsA201 cells were transiently transfected with WT and mutant PKC  $\beta$ II. Approximately 40 h post-transfection, the cells were treated with 400 nM PDBu for 0, 30, and 60 min. At each time point, the cells were lysed in Buffer A, and detergent-solubilized lysates were analyzed using SDS-PAGE and immunoblotting. Dephosphorylation of PKC  $\beta$ II is visualized by the appearance of the faster migrating band detected by the anti-PKC  $\beta$ II antibody. To examine whether newly synthesized or dephosphorylated PKC  $\beta$ II preferentially binds Hsp70, co-immunoprecipitation of PKC  $\beta$ II and Hsp70 were performed in the context of the pulse-chase experiments. Briefly, COS7 cells were transiently transfected with GFP-tagged PKC  $\beta$ II (the GFP-tagged construct was used to allow enough separation between PKC  $\beta$ II and endogenous PKC  $\alpha$  on the gel) and Myc-Hsp70. Approximately 24 h post-transfection, the cells were pulse-labeled with [ $^{35}$ S]methionine/cysteine as described above. The cells were either lysed immediately in Buffer A or chased for 6 h and treated with PDBu (400 nM) for 2 h and then lysed in Buffer A. Ten percent of the detergent-solubilized lysates were immunoprecipitated with the anti-PKC  $\alpha$  monoclonal antibody, and the rest of the lysates were immunoprecipitated with the anti-Myc monoclonal antibody coupled to Ultra-link protein A/G beads. The immunoprecipitates were washed three times with Buffer B and once with Buffer A. The immunoprecipitated proteins were analyzed using SDS-PAGE and autoradiography.

**Ubiquitination of PKC**—To examine the ubiquitination status of PKC  $\beta$ II *in vivo*, COS7 cells were co-transfected with a HA-tagged ubiquitin expression construct and WT or mutant PKC  $\beta$ II. Approximately 40 h post-transfection, the cells were treated with 1  $\mu$ M MG-132 for 30 min prior to addition of Me<sub>2</sub>SO or 200 nM PDBu for 30 min. The cells were then lysed in Buffer D (50 mM Tris, pH 7.4, 2 mM EDTA, 2 mM EGTA, 10% Triton X-100, 1 mM dithiothreitol, 200  $\mu$ M benzamidin, 40  $\mu$ g ml<sup>-1</sup> leupeptin, 200  $\mu$ M phenylmethylsulfonyl fluoride, and 10 mM *N*-ethylmaleimide). The detergent-solubilized cell lysate was incubated with the anti-PKC  $\alpha$  monoclonal antibody and Ultra-link protein A/G beads at 4 °C for 4 h. The immunoprecipitates were washed three times in Buffer E (Buffer D plus 250 mM NaCl) and once in Buffer D. Bound proteins were analyzed by SDS-PAGE and immunoblotting.

## RESULTS

**The C Terminus of PKC  $\beta$ II Contains a Putative Hsp70-binding Motif**—The two phosphorylation sites on the C terminus of PKC, the turn motif and the hydrophobic motif (Fig. 1A, Thr-641 and Ser-660 in PKC  $\beta$ II, respectively) serve as switches to control the interaction of the kinase with binding partners that control its maturation, signaling lifetime, and degradation (10,



**FIGURE 1. Mutation of a putative Hsp70-binding residue in the C terminus of PKC  $\beta$ II results in accumulation of dephosphorylated PKC.** A, schematic representation of the domain composition of PKC  $\beta$ II showing the C1 and C2 domains in the regulatory moiety, the catalytic domain, and C terminus (CT). The three *in vivo* phosphorylation sites (Thr-500, Thr-641, and Ser-660) in PKC  $\beta$ II are indicated. B, sequence alignment of PKC isozymes surrounding the turn motif phosphorylation site in the C terminus. The turn motif phosphorylation site (Thr-641 in PKC  $\beta$ II) is highlighted in blue; the highly conserved hydrophobic Leu residue (Leu-640 in PKC  $\beta$ II) is highlighted in yellow. C, Western blots showing the expression of WT PKC  $\beta$ II (lanes 1 and 3) and L640G mutant (lanes 2 and 4) in 10% of the detergent-solubilized lysate (S, lanes 1 and 2) and in 30% of the detergent-insoluble pellet (P, lanes 3 and 4). The expression of WT and mutant PKC  $\beta$ II was detected using an anti-PKC  $\beta$ II antibody. The unphosphorylated PKC is labeled with a dash, the partially phosphorylated species (phosphate on one of the two C-terminal phosphorylation sites) is labeled with one asterisk, and the fully phosphorylated species (phosphates on all three *in vivo* sites) is labeled with two asterisks. D, graphs showing quantified results of Western blots shown in C. The relative amount of dephosphorylated species was obtained by normalizing the amount of lower band to the total PKC expression, and this number for PKC  $\beta$ II WT was expressed as 1. Data represent the mean  $\pm$  S.E. of four independent experiments.

15). Previously we demonstrated that dephosphorylation at the turn motif Thr-641 site promotes the interaction of Hsp70 with PKC  $\beta$ II. Here we set out to explore the molecular determinants driving the interaction. Examination of the sequence surrounding Thr-641 revealed that a hydrophobic residue, Leu-640, immediately precedes the turn motif phosphorylation site. Since Hsp70 prefers binding to hydrophobic residues (3), we explored whether Leu-640 mediates the binding of Hsp70 to PKC. Notably, a sequence alignment of all 10 PKC isozymes in the turn motif region showed that the hydrophobic residue Leu-640 is invariant among all PKC isozymes (Fig. 1B). To examine the role of this hydrophobic residue, we mutated Leu-640 to Gly (L640G) to disrupt the hydrophobicity and asked



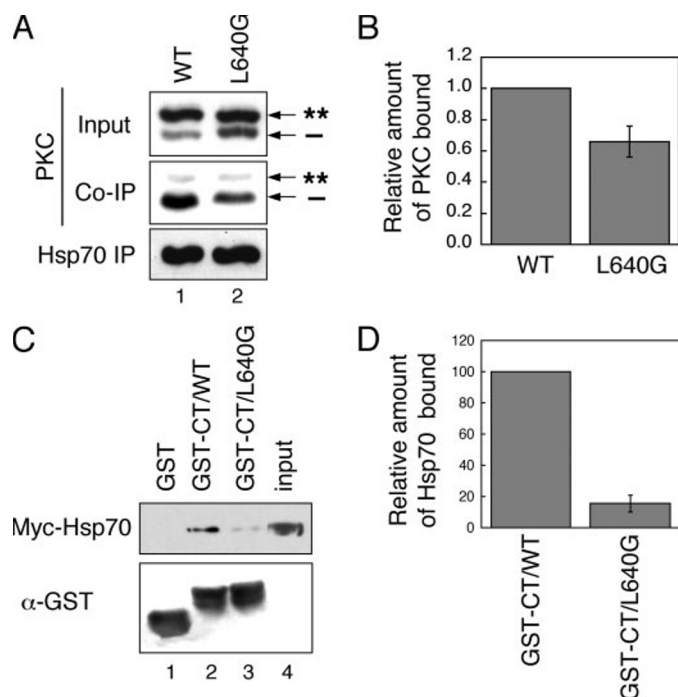
## Interaction of Hsp70 with PKC

how this mutation affected the maturation, stability, and down-regulation of PKC. We chose Gly over Ala because unlike Ala, Gly does not have an aliphatic side chain and, furthermore, results from peptide library screens indicate that Gly is slightly disfavored for binding Hsp70 (3).

First, we examined the subcellular distribution of WT PKC and the PKC L640G mutant in tsA201 cells. The Western blots in Fig. 1C show the partitioning of these constructs in the Triton-soluble and insoluble fractions of cells. As reported previously, the majority of WT PKC  $\beta$ II in the detergent-soluble supernatant migrated as an upper band (*double asterisk*) corresponding to a species quantitatively phosphorylated at both C-terminal positions (16) with a minor fraction of the protein migrating with a faster mobility (*dash*) corresponding to unphosphorylated PKC (Fig. 1C, *lane 1*). Only the unphosphorylated species was present in the detergent-insoluble pellet (Fig. 1C, *lane 3*). Mutation of Leu-640 to Gly in PKC  $\beta$ II resulted in a 3-fold increase in the amount of unphosphorylated PKC in the detergent-soluble fraction of cells (*lane 2*) and a 4-fold increase in the amount present in the detergent-insoluble pellet (*lane 4*). Note that a migration further retarded species is detected in the detergent-insoluble pellet for L640G mutant (*lane 4*, as indicated by an *open arrow*). This may reflect an ubiquitinated PKC species that is more easily observed for L640G mutant (detailed in Fig. 5). Mutation of Leu-640 to Ala also resulted in increased partitioning in the detergent-insoluble fraction, with the level intermediate between that of the WT and L640G mutant (data not shown), indicating that the dominant driving force altering the properties of these PKCs was the loss of the large aliphatic side chain of Leu rather than increased backbone flexibility. Additional mutation of the preceding residue, Val-639 to Ala, did not cause a further increase in the solubility of the L640G mutant, revealing that Leu-640 but not Val-639 affects the solubility of PKC (data not shown). These results reveal that Leu-640 plays important roles in stabilizing PKC, with mutation of this residue promoting the accumulation of unphosphorylated PKC in the detergent-insoluble fraction of cells.

**Mutation of Leu-640 in the Turn Motif of PKC  $\beta$ II Decreases Binding to Hsp70**—To further examine whether mutation of L640 reduces the binding of PKC to Hsp70, co-immunoprecipitation experiments were performed using tsA201 cells co-transfected with WT or mutant PKC together with a Myc-tagged Hsp70 expression construct. Consistent with our previous report, the faster migrating unphosphorylated species of PKC (*dash*) was the major species of PKC that co-immunoprecipitated with Myc-Hsp70 (Fig. 2A, *lane 1, middle panel*) (15). This unphosphorylated species was also specifically bound to Myc-Hsp70 in immunoprecipitations from cells expressing the L640G mutant construct (*lane 2*). Normalizing the amount of unphosphorylated PKC (*dash*) bound to Hsp70 (*middle panel*) to the amount of unphosphorylated PKC in the lysate (*upper panel*) revealed that the amount of Hsp70 bound to L640G mutant decreased to ~60% of the amount bound to WT PKC (Fig. 4B, *n* = 4).

We next addressed the effect of Leu-640 on the direct interaction with Hsp70 using GST pulldown experiments *in vitro*. GST alone or GST-tagged C-terminal constructs of WT or



**FIGURE 2. Mutation of Leu-640 of PKC  $\beta$ II decreases the binding of PKC to Hsp70 *in vivo*.** A, tsA201 cells were co-transfected with PKC  $\beta$ II WT or L640G (*lanes 1 and 2*, respectively) together with Myc-tagged Hsp70. The detergent-solubilized lysates were immunoprecipitated with the anti-Myc monoclonal antibody. Ten percent of the detergent-soluble lysates was analyzed to show the amount of WT and mutant PKC input (*upper panel*). The amount of PKC bound to Hsp70 in the immunoprecipitates is shown in the *middle panel*, and the amount of Hsp70 in the immunoprecipitates is shown in the *bottom panel*. The WT and mutant PKCs were detected using the anti-PKC  $\beta$ II antibody, and Hsp70 was detected using the anti-Hsp70 monoclonal antibody. The unphosphorylated PKC is labeled with a *dash* and the fully phosphorylated species is labeled with *two asterisks*. B, graph showing the quantified results from A. Western blots were scanned and quantified using a Bio-Rad GS-800 densitometer. The relative amount of PKC bound to Hsp70 was obtained by normalizing the amount of non-phosphorylated species of PKC in the immunoprecipitates to the amount in the lysate, and this number for WT PKC was expressed as 1. Data represent the mean  $\pm$  S.E. of four independent experiments. C, purified Myc-Hsp70 was incubated with glutathione beads pre-bound with GST or the GST-tagged C terminus of WT PKC  $\beta$ II (GST-CT/WT) or of L640G (GST-CT/L640G) (*lanes 1–3*, respectively), and proteins that bound to the beads were separated on a SDS-PAGE gel and analyzed using immunoblotting. The presence of Myc-Hsp70 in the beads (100%) or in the input (10%) was detected using the anti-Myc polyclonal antibody (*upper panel*), and the amount of GST and GST-CTs was visualized using the anti-GST antibody (*lower panel*). D, quantitative representations of Western blots shown in C. Graph showing relative amount of Myc-Hsp70 bound to the GST-tagged C terminus of PKC  $\beta$ II. The amount of Myc-Hsp70 bound to the GST-CT/WT was expressed as 100% binding. Data represent the mean  $\pm$  S.E. of three independent experiments.

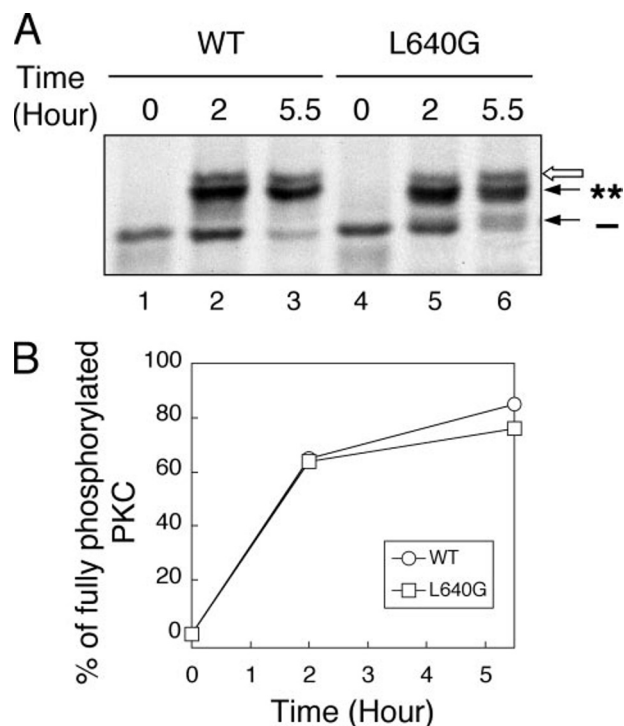
mutant PKC  $\beta$ II were expressed in tsA201 cells and fusion proteins purified using glutathione-Sepharose; Myc-Hsp70 (additionally tagged with His<sub>6</sub>) was purified from transfected cells using nickel-nitrilotriacetic acid affinity beads and eluted into solution for the subsequent binding assay. GST pulldown experiments were then performed using GST- or GST-CT (WT and mutants)-bound beads and purified Myc-Hsp70. The Western blot in Fig. 2C shows the C-terminal construct effectively bound Hsp70 (Fig. 2C, *lane 2*), and that this interaction was compromised upon mutation of Leu-640 (*lane 3*). As a negative control, GST alone did not bind Hsp70 (*lane 1*). The amount of Myc-Hsp70 added to the assays is shown as input (*lane 4*). Quantitation of the amount of Hsp70 present in GST

pulldowns from three independent experiments revealed mutation of Leu-640 in the C-terminal construct of PKC  $\beta$ II decreased binding of Hsp70 to about 10% of the binding observed with the WT construct. Taken together, these results indicate that Leu-640 in the turn motif of PKC  $\beta$ II is the primary determinant that, along with dephosphorylated Thr-641, drives the binding of Hsp70 to PKC.

**Mutation of the Hsp70-binding Site of PKC  $\beta$ II Does Not Affect the Maturation of PKC**—We showed in Fig. 1C that mutation of Leu-640 in the C terminus of PKC  $\beta$ II resulted in accumulation of unphosphorylated PKC in cells. There are two species of unphosphorylated PKC in cells: 1) newly synthesized PKC that has yet to be phosphorylated and 2) mature PKC that has been fully phosphorylated and then dephosphorylated. Thus, we set out to ask whether the accumulation of unphosphorylated PKC reflected a decrease in the rate of phosphorylation of newly synthesized PKC or whether it reflected acceleration in the rate of dephosphorylation of mature PKC following activation. Specifically, we analyzed whether mutation in the Hsp70-binding site alters the maturation process or the activation-induced dephosphorylation of PKC. We first performed pulse-chase experiments to monitor the maturation process of WT PKC  $\beta$ II and L640G mutant (Fig. 3). COS7 cells transfected with WT or L640G mutant were pulse-labeled with [ $^{35}$ S]Met/Cys and chased for 0, 2, and 5.5 h. PKC was immunoprecipitated from cell lysates at each time point. The autoradiogram in Fig. 3 shows that the newly synthesized PKC  $\beta$ II migrated as a single faster-migrating band (*dash*; lane 1) and then shifted to a slower migrating band (*double asterisk*) during the time of chase (lanes 2 and 3). As reported previously, this shift in electrophoretic mobility results from autophosphorylation of the turn motif and hydrophobic motif sites in the C terminus of PKC  $\beta$ II during its maturation process (14). Importantly, mutation of the Hsp70-binding site in PKC  $\beta$ II had no significant effect on the rate of processing of PKC (Fig. 3A, lanes 4–6). The percentage of fully phosphorylated PKC at each time point was similar for L640G mutant and WT PKC  $\beta$ II (Fig. 3B). Note that disruption of Hsp70 binding by mutating the binding site in PKC  $\beta$ II thus had little effect on the processing rate of PKC, revealing that newly synthesized PKC was not the species of unphosphorylated PKC that accumulates following disruption of the interaction with Hsp70.

**Disruption of Hsp70 Binding Accelerates PDBu-induced Dephosphorylation and Promotes Ubiquitination of PKC  $\beta$ II**—It is well known that prolonged activation of PKC triggered by long term phorbol ester exposure leads to dephosphorylation and degradation of PKC (17, 18). Ubiquitination of PKC has been observed upon receptor-mediated activation or phorbol ester stimulation, suggesting that down-regulation of PKC utilizes a proteasome-dependent pathway (13). Thus, we next addressed whether mutations of the Hsp70-binding site alter the dephosphorylation and down-regulation process of PKC.

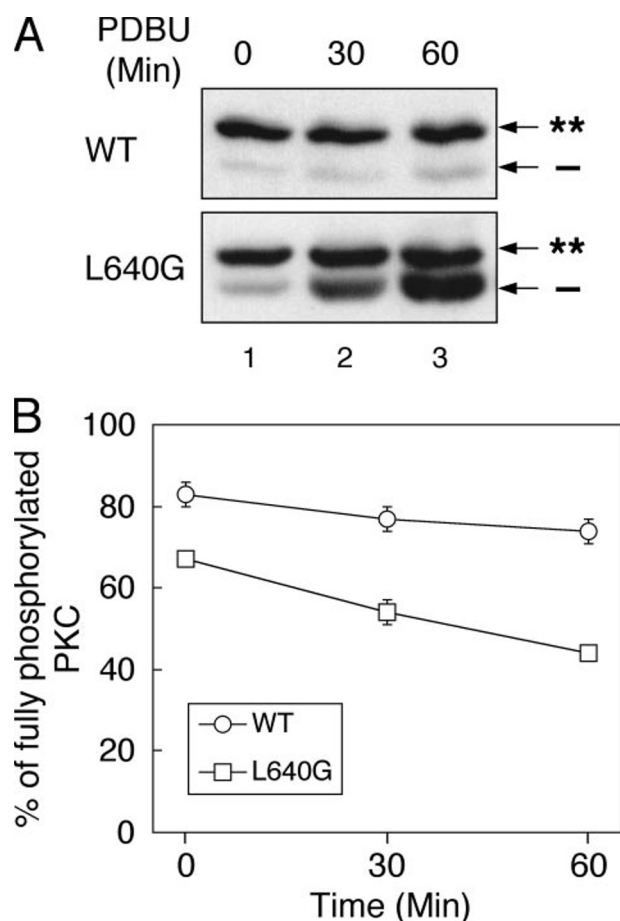
tsA201 cells were transfected with PKC  $\beta$ II-WT or L640G mutant, and the cells were treated with PDBu for 0, 30, or 60 min (Fig. 4, lanes 1–3). Similarly as reported previously for PKC  $\alpha$  (18, 19), the majority of WT PKC  $\beta$ II was fully phosphorylated (*double asterisk*) in unstimulated cells (lane 1) and became dephosphorylated (*dash*) slowly over the time course of 60 min



**FIGURE 3. Mutation of Leu-640 in the Hsp70-binding site does not affect the maturation of PKC  $\beta$ II.** A, autoradiogram from pulse-chase analysis of COS7 cells transfected with PKC  $\beta$ II WT (lanes 1–3) and L640G (lanes 4–6). Transfected cells were labeled with [ $^{35}$ S]methionine/cysteine for 7 min and chased for 0 min (lanes 1 and 4), 2 h (lanes 2 and 5), and 5.5 h (lanes 3 and 6). The WT and mutant PKC  $\beta$ II were immunoprecipitated from detergent-solubilized cell lysates with the anti-PKC  $\alpha$  antibody and analyzed by SDS-PAGE and autoradiography. Position of newly synthesized protein that is not yet phosphorylated is indicated by a *dash*; the *double asterisk* denotes position of full-phosphorylated PKC. Note that a minor slower migrating band visible above the matured PKC  $\beta$ II (marked by an *open arrow*) represents endogenous PKC  $\alpha$  that was immunoprecipitated by the anti-PKC  $\alpha$  antibody used in the experiments. Data shown are representative of three independent pulse-chase experiments. B, quantitative representations of the autoradiogram shown in A. The graph shows relative amount of fully phosphorylated mature PKC as a percentage of the total PKC protein.

(Fig. 4A, upper panel). Specifically, the amount of fully phosphorylated PKC  $\beta$ II-WT decreased slightly from 83 to 74% following 60-min PDBu treatment (Fig. 4B). In contrast, the rate of dephosphorylation was significantly increased for the Hsp70-binding mutant (Fig. 4A, lower panel). Sixty minutes of PDBu treatment caused the amount of phosphorylated L640G to decrease from 67 to 44% of the total PKC. These data reveal that binding of Hsp70 to the C terminus of PKC protects the enzyme from activation-induced dephosphorylation.

To further examine the down-regulation process of PKC, we performed cellular ubiquitination experiments. COS7 cells were transfected with PKC  $\beta$ II-WT or L640G mutant together with a HA-tagged ubiquitin expression construct. The cells were treated with a proteasome inhibitor MG-132 for 30 min to transiently inhibit protein degradation. This treatment is used routinely to trap ubiquitinated proteins in cells. The cells were then treated with Me<sub>2</sub>SO or PDBu for 30 min. The WT and mutant PKCs were immunoprecipitated from detergent-solubilized lysates. Ubiquitination of PKC was detected on Western blot using an anti-HA antibody to probe for the covalently bound HA-ubiquitin (Fig. 5A). No significant ubiquitination was detected on WT PKC  $\beta$ II under basal conditions (Fig. 5A,



**FIGURE 4. Time course of PDBu-induced dephosphorylation of PKC  $\beta$ II.** A, tsA201 cells were transfected with PKC  $\beta$ II WT and L640G. The cells were treated with PDBu (300 nM) for 0, 30, and 60 min (lanes 1–3, respectively). The detergent-solubilized cell lysates were separated by SDS-PAGE and analyzed using Western blotting. The WT and mutant PKCs were detected on the blot using the anti-PKC  $\beta$ II antibody. The unphosphorylated PKC is labeled with a *dash*, and the fully phosphorylated species is labeled with *two asterisks*. B, graph showing quantified results from three independent experiments. Western blots as shown in A were scanned and quantified using a Bio-Rad GS-800 densitometer. The relative amount of fully phosphorylated PKC was expressed as the percentage of total PKC. Data represent the mean  $\pm$  S.E. of three independent experiments.

lane 1), and PDBu treatment caused a modest increase in the amount of ubiquitinated species (lane 2). The basal ubiquitination of L640G mutant was only slightly increased compared with that of WT enzyme (lane 3). However, PDBu caused a dramatic increase in the ubiquitination of the Hsp70-binding mutant (lane 4). The relative ubiquitination was quantified by normalizing the amount of ubiquitination to the amount of PKC in the immunoprecipitates, and the results from three independent experiments are summarized in Fig. 5B. The graph shows that PDBu caused an 11-fold increase in the ubiquitination of L640G mutant and only a 2-fold increase in the ubiquitination of WT PKC  $\beta$ II. Taken together, these data reveal that binding of Hsp70 to PKC protects PKC from dephosphorylation, ubiquitination, and degradation. Thus, the increased amount of unphosphorylated species observed with the L640G mutant results from accumulation of the dephosphorylated mature enzyme.

**Hsp70 Preferentially Binds to Dephosphorylated PKC**—The foregoing data reveal that Hsp70 controls signal termination by

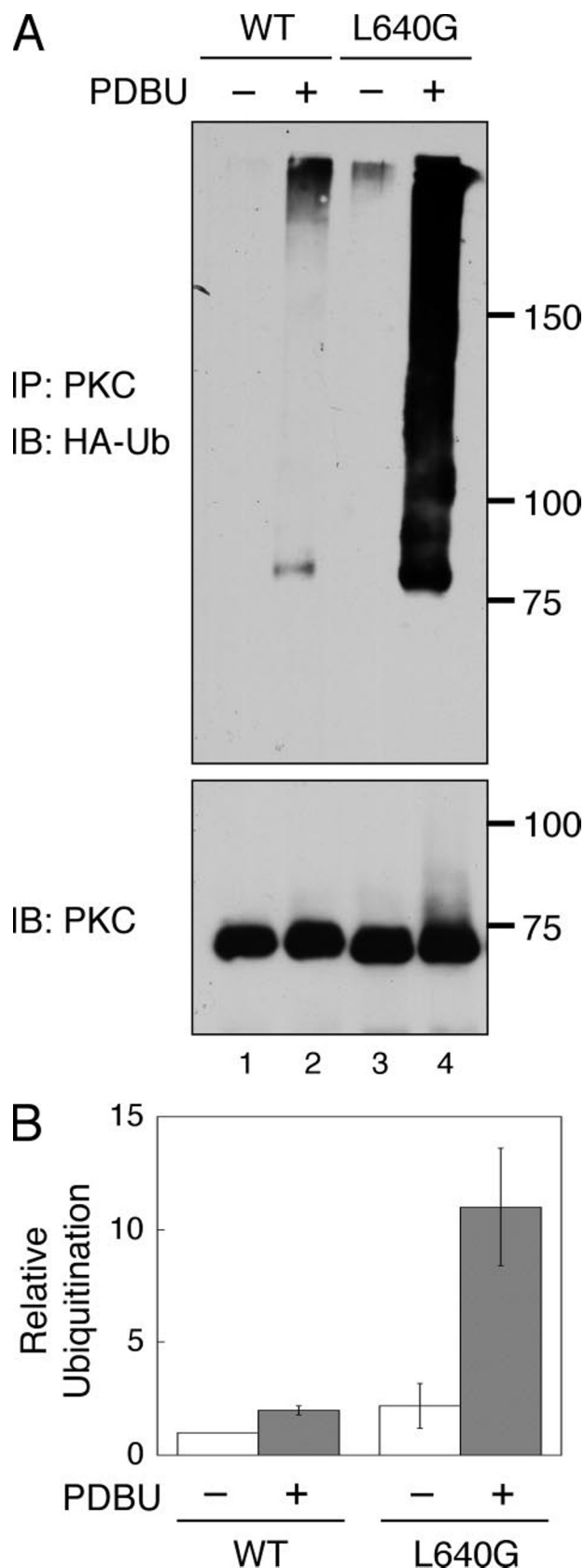
binding dephosphorylated PKC rather than controlling maturation of unphosphorylated PKC. Thus, we hypothesized that Hsp70 is able to distinguish between PKC that has never been phosphorylated and PKC that has been dephosphorylated. To test this hypothesis, we performed co-immunoprecipitation experiments in conjunction with pulse-chase experiments to allow us to visualize newly synthesized unphosphorylated PKC, mature phosphorylated PKC, and mature dephosphorylated PKC. COS7 cells co-transfected with GFP-tagged PKC  $\beta$ II and Myc-Hsp70 were pulse-labeled with [ $^{35}$ S]Met/Cys for 7 min. The autoradiogram in Fig. 6A shows that immediately after the pulse labeling, the majority of GFP-PKC  $\beta$ II proteins migrated with a faster mobility (*dash*) corresponding to the newly synthesized never-phosphorylated species (Fig. 6A, lane 1). In a companion experiment, cells were chased for 6 h to allow complete phosphorylation of PKC and then treated with PDBu to induce dephosphorylation. After 2-h PDBu treatment, both fully phosphorylated (*double asterisk*) and dephosphorylated (*dash*) species were observed on the autoradiogram (Fig. 6A, lane 2). Hsp70 was immunoprecipitated from both sets of lysates and the amount of bound PKC analyzed. As shown in the *middle panel* (Fig. 6A), more dephosphorylated PKC (lane 2) was co-immunoprecipitated with Hsp70 compared with the never-phosphorylated PKC (lane 1). Quantitation of the data from three independent experiments revealed that the amount of dephosphorylated PKC bound to Hsp70 was 3-fold higher than the amount of newly synthesized PKC (Fig. 6B). These results demonstrate that the two species of unphosphorylated PKC indeed are biochemically different proteins. Consistent with its role in stabilizing PKC after activation, Hsp70 preferentially recognizes the dephosphorylated C terminus of PKC.

## DISCUSSION

In this study we identify the Leu immediately preceding the turn motif phosphorylation site as the major determinant that mediates the binding of Hsp70 to dephosphorylated PKC  $\beta$ II. Mutation of this residue to Gly results in reduction in the binding of Hsp70 to PKC, acceleration of PDBu-induced dephosphorylation, and enhanced ubiquitination of PKC upon activation. In contrast, mutation of this residue has no significant effect on the maturation of PKC. Furthermore, our data reveal that the C terminus of PKC has a molecular memory: we show that Hsp70 discriminates between PKC that has never been phosphorylated and PKC that has matured and been dephosphorylated. Taken together, our data demonstrate that Hsp70 binds the conserved Leu preceding the turn motif phosphorylation site via an interaction that depends on the prior processing and then dephosphorylation of the turn motif. Binding of Hsp70 following activation-induced dephosphorylation protects PKC from down-regulation and prolongs the signaling lifetime of the enzyme, presumably by promoting rephosphorylation and re-entry into the signaling pool of enzyme.

Binding data with Hsp70 reveal that the C terminus of PKC has molecular memory: the molecular chaperone discriminates between the C terminus that has never been phosphorylated and the C terminus that has been dephosphorylated. We have previously demonstrated that another C-terminal binding partner, PDK-1, discriminates between these two species of PKC. In





**FIGURE 5. Mutation of Leu-640 promotes PDBu-induced ubiquitination of PKC  $\beta$ II.** A, COS7 cells were co-transfected with PKC  $\beta$ II WT (lanes 1 and 2) or L640G (lanes 3 and 4) together with a HA-tagged ubiquitin expression construct. The transfected cells were treated with Me<sub>2</sub>SO (lanes 1 and 3) or with 200 nM PDBu (lanes 2 and 4) for 30 min. The detergent-solubilized cell lysates

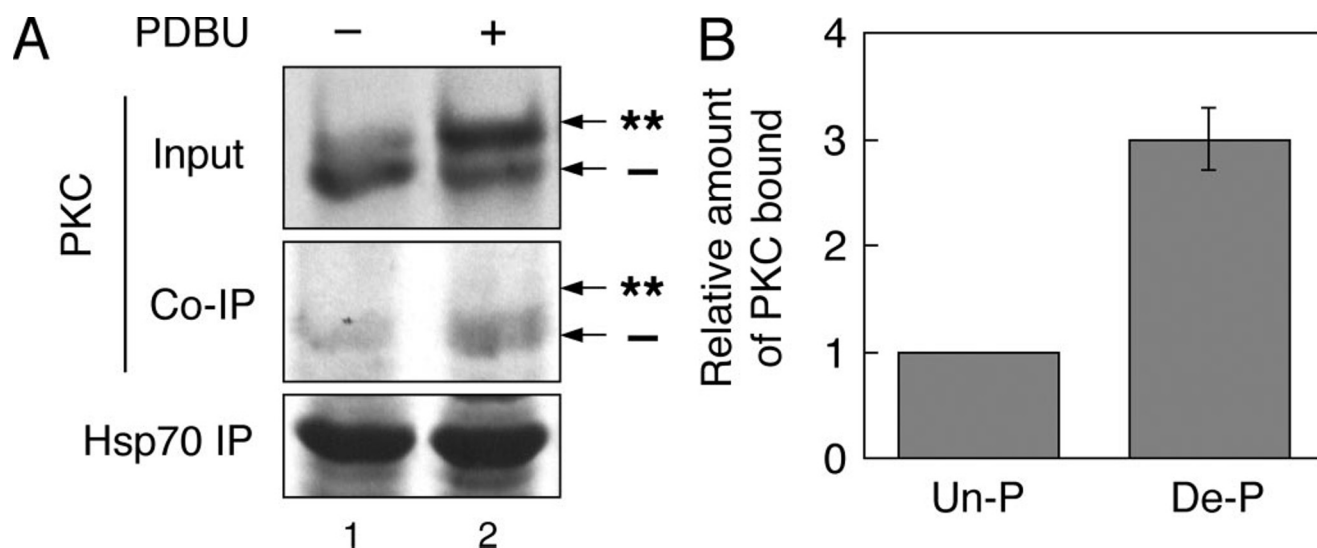
the case of PDK-1, however, the discrimination is reversed: PDK-1 preferentially binds PKC that has never been phosphorylated compared with PKC that has been phosphorylated. These findings reveal that PKC adopts different conformations at the beginning and the end of its life cycle. This allows otherwise indistinguishable un/de-phosphorylated proteins to be targeted to different pathways in cells by allowing association with distinct binding partners. Thus, PDK-1 preferentially binds unphosphorylated PKC and allows it to mature and Hsp70 binds dephosphorylated PKC and protects it from degradation.

Hsp70 may protect PKC from down-regulation by simply binding and preventing aggregation of the dephosphorylated enzyme or by shielding the enzyme from the ubiquitination machinery. Alternatively, Hsp70 may serve as a molecular chaperone to refold dephosphorylated PKC upon binding and allow PKC to become rephosphorylated and recycled. Specifically, Hsp70 may convert PKC into a conformation that can be recognized by PDK-1, thus triggering the rephosphorylation process.

Our data also demonstrate directly that dephosphorylation and ubiquitination of PKC are tightly coupled in cells. Specifically, PDBu treatment is able to induce dephosphorylation and ubiquitination of PKC within a similar time frame, and increased dephosphorylation is linked with enhanced ubiquitination as evidenced by the Hsp70-binding mutant of PKC. Thus, dephosphorylation may serve as a trigger for ubiquitination of PKC.

Our finding that Leu-640 is important for Hsp70 binding is consistent with the previously reported substrate preference of Hsp70. Screens of peptide libraries reveal that Leu is the most favored residue for the binding of DnaK (a bacterial version of Hsp70) to peptides, with Val and Ile also favored. Interestingly, acidic residues are highly disfavored for binding Hsp70 (3). In the case of PKC, we show that the Leu immediately preceding the turn motif phosphorylation site is critical for Hsp70 binding. This Leu is conserved in all PKC isoforms (Fig. 1B). In addition, Ile or Val is found in the equivalent position in Akt and protein kinase A, respectively (20). Similarly, all PKC isoforms (including the atypical ones), Akt, and protein kinase A are regulated by phosphorylation at the turn motif. In the case of PKC, phosphorylation at the turn motif site (Thr-641 adjacent to the Hsp70-binding site) abolishes Hsp70 binding (15). The molecular switching function of the turn motif phosphorylation can be explained by the highly disfavored binding caused by negative charge in the Hsp70 recognition motif (3). Given the conservation of the turn motif phosphorylation site and preceding hydrophobic residue among AGC protein kinase family members, it is tempting to speculate that Hsp70 provides a common regulatory mechanism for all AGC family protein kinases. Although we have used phorbol esters as a tool

were immunoprecipitated with the anti-PKC  $\alpha$  monoclonal antibody. The ubiquitination of WT PKC  $\beta$ II and L640G was detected using the anti-HA monoclonal antibody (upper panel), and the same blot was stripped and re-probed with the anti-PKC  $\beta$ II antibody to detect the presence of PKC  $\beta$ II (lower panel). B, graph showing the quantified results as shown in A. Western blots were scanned and quantified using a Bio-Rad GS-800 densitometer. The relative ubiquitination was calculated by normalizing the amount of ubiquitination to the amount of PKC in the immunoprecipitates. The relative ubiquitination for WT PKC  $\beta$ II under the control condition was normalized to 1. Data represent the mean  $\pm$  S.E. ( $n = 3$ ).



**FIGURE 6. Hsp70 preferentially interacts with dephosphorylated PKC  $\beta$ II.** A, autoradiogram from pulse-chase analysis of COS7 cells co-transfected with Myc-Hsp70 and GFP-PKC  $\beta$ II. The transfected cells were labeled with [ $^{35}$ S]methionine/cysteine and chased for 0 min (lane 1) or 6 h and then treated with PDBu (400 nM) for 2 h (lane 2). Ten percent of the detergent-solubilized cell lysates was immunoprecipitated with the anti-PKC  $\alpha$  monoclonal antibody to show the phosphorylation profile of GFP-PKC as input (top panel). Myc-Hsp70 was immunoprecipitated with the anti-Myc monoclonal antibody (bottom panel), and GFP-PKC in the immune complex with Hsp70 was shown in the middle panel. The unphosphorylated PKC (newly synthesized or dephosphorylated) is labeled with a dash and the fully phosphorylated species is labeled with two asterisks. Note that only the unphosphorylated PKC co-immunoprecipitated with Hsp70. B, graph showing the quantified results of the data shown in A. The relative amount of binding of PKC for Hsp70 was determined by normalizing the amount of dephosphorylated PKC in the immune complex with Hsp70 to the amount of dephosphorylated PKC in the input and to the amount of Hsp70 in the immunoprecipitates. The relative binding of Hsp70 for the newly synthesized PKC was normalized to 1. Data represent the mean  $\pm$  S.E. of three independent experiments.

to promote the degradation of PKC, we would propose that Hsp70 controls the lifetime of these AGC kinases by controlling their stability following activation-induced dephosphorylation.

Expression levels of Hsp70 are highly elevated in many tumors (4), leading to its consideration as a tumor-promoting factor. Several potential roles have been proposed for Hsp70 in tumorigenesis. Experimental evidence suggests that Hsp70 serves as an anti-apoptosis factor by suppressing the activation of stress kinase JNK (21, 22) and by preventing the processing of procaspases 9 and 3 (21). Furthermore, Hsp70 has been shown to specifically interact with pro-apoptotic factors including apoptosis protease activating factor-1 and apoptosis-inducing factor, thus inhibiting their function (23, 24). Interestingly, elevation of PKC expression has also been linked to tumorigenesis and tumor progression (25, 26). However, the cause for increasing expression of PKC in cancer is unknown. In this study, we demonstrate that Hsp70 serves as a stabilizing factor for PKC by protecting it against activation-induced dephosphorylation and down-regulation. Thus, increased levels of Hsp70 in cancer cells may extend the lifetime of PKC proteins, resulting in higher PKC expression. Further studies of the interaction of Hsp70 with PKC in cancer are of potential in developing novel therapies in cancer treatment.

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