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The Turn Motif Is a Phosphorylation Switch That Regulates the Binding of Hsp70 to Protein Kinase C*

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Heat shock proteins play central roles in ensuring the correct folding and maturation of cellular proteins. Here we show that the heat shock protein Hsp70 has a novel role in prolonging the lifetime of activated protein kinase C. We identified Hsp70 in a screen for binding partners for the carboxyl terminus of protein kinase C. Co-immunoprecipitation experiments revealed that Hsp70 specifically binds the unphosphorylated turn motif (Thr^{641} in protein kinase C βII), one of three priming sites phosphorylated during the maturation of protein kinase C family members. The interaction of Hsp70 with protein kinase C can be abolished *in vivo* by co-expression of fusion proteins encoding the carboxyl terminus of protein kinase C or the carboxyl terminus of Hsp70. Pulse-chase experiments reveal that Hsp70 does not regulate the maturation of protein kinase C: the rate of processing by phosphorylation is the same in the presence or absence of disrupting constructs. Rather, Hsp70 prolongs the lifetime of mature protein kinase C; disruption of the interaction promotes the accumulation of matured and then dephosphorylated protein kinase C in the detergent-insoluble fraction of cells. Furthermore, studies with K562 cells reveal that disruption of the interaction with Hsp70 slows the protein kinase C βII-mediated recovery of cells from PMA-induced growth arrest. Last, we show that other members of the AGC superfamily (Akt/protein kinase B and protein kinase A) also bind Hsp70 via their unphosphorylated turn motifs. Our data are consistent with a model in which Hsp70 binds the dephosphorylated carboxyl terminus of mature protein kinase C, thus stabilizing the protein and allowing re-phosphorylation of the enzyme. Disruption of this interaction prevents re-phosphorylation and targets the enzyme for down-regulation.

The function of signaling proteins is under acute regulation by macromolecular interactions that reversibly modulate their conformation and spatial distribution. These intra- and intermolecular rearrangements can be taxing to the stability of the proteins. Whereas the involvement of heat shock proteins in stabilizing the conformational transitions of newly synthesized proteins is well documented (1), mechanisms for stabilizing the conformational and structural transitions of mature proteins are less well understood.

Protein kinase C (PKC)[1] serves as a paradigm for a protein under acute structural and spatial regulation: its conformation and its binding partners change continuously during its lifetime in the cell. Specifically, newly synthesized protein associates with the membrane in an open conformation in which its autoinhibitory pseudosubstrate sequence is expelled from the active site (2), the upstream kinase PDK-1 is docked to the exposed carboxyl terminus (3), and the PDK-1 site on the activation loop sequence is accessible for phosphorylation (2). Following phosphorylation of the activation loop sequence, PDK-1 is released from the carboxyl terminus of PKC, thus exposing the carboxyl terminus for two rapid phosphorylations. In the case of conventional PKCs, this phosphorylation occurs by an intramolecular mechanism, underscoring the conformational flexibility of the carboxyl terminus (4). The mature, phosphorylated species is released to the cytosol and the pseudosubstrate gains access to the active site, thus maintaining the enzyme in an autoinhibited state. The enzyme is then activated following receptor-mediated generation of Ca^{2+} and diacylglycerol. These ligands recruit PKC to membranes by engaging its two membrane-targeting modules on the membrane, an event that provides the energy to expel the pseudosubstrate from the substrate-binding cavity and allow substrate phosphorylation. The membrane-bound, active species adopts an open conformation in which the enzyme is very sensitive to dephosphorylation. Prolonged activation results in significant dephosphorylation, association of the dephosphorylated species with the detergent-insoluble fraction of cells, and eventual down-regulation (5). Specific protein partners bind to each of the conformational/phosphorylation states of PKC (*i.e.* unphosphorylated, phosphorylated but inactive, phosphorylated and active, and dephosphorylated). What mechanisms stabilize PKC to the continual structural and spatial re-arrangements?

An increasing number of studies suggest that heat shock proteins may play a role not only in protein folding, but also in sustaining the activated state of protein kinases. Most notable is the interaction of Hsp90 with a number of protein kinases, including Src, Raf, and mitogen-activated protein kinase/extracellular signal-regulated kinase (6, 7). In the case of Src, binding to Hsp90 stabilizes the activity of Src, with studies in yeast indicating that the growth arrest resulting from expression of v-Src is overcome by lowering the level of Hsp90 expressed (8). Similarly, Hsp90 has recently been shown to protect Akt/protein kinase B from dephosphorylation, hence sustaining the active state of Akt and protecting cells from apoptosis (9).

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[1] The abbreviations used are: PKC, protein kinase C; Hsp, heat shock protein; PDK-1, phosphoinositide-dependent kinase 1; PKA, protein kinase A; DMEM, Dulbecco's modified Eagle's medium; PP1, protein phosphatase 1; HA, hemagglutinin; GST, glutathione *S*-transferase; PBD, peptide-binding domain; PBCT, combined peptide-binding domain and carboxyl terminus.

Hsp90 has also been shown to bind directly and stabilize PDK-1, thus prolonging the signaling lifetime of this pivotal kinase (10). In fact, an increasing number of chaperones are being identified as binding partners for particular protein kinases, including chaperone p32 for protein kinase D (11) and Hsp90 for the mitogen-activated protein kinase MOP (12). Thus, mounting evidence supports a common mechanism for increasing the lifetime of signaling molecules by direct interaction with molecular chaperones.

In this study we identified Hsp70 in a screen for binding partners for the carboxyl terminus of PKC. We show that Hsp70 binding is regulated by the phosphorylation state of the turn motif, one of the two conserved carboxyl-terminal phosphorylation sites, and that Hsp70 binds mature PKC that has become dephosphorylated, rather than newly synthesized PKC that has yet to be phosphorylated. Functional studies reveal that the interaction of Hsp70 with PKC prolongs the lifetime of active PKC and sustains its function. Hsp70 may generally regulate the function of AGC kinases: we show that the conserved turn motifs of protein kinase A (PKA) and Akt supports Hsp70 binding, apparently by the same phosphoregulated mechanism as PKC.

EXPERIMENTAL PROCEDURES

Materials—Easy Tag [^{35}S]methionine/cysteine (1175 Ci mmol^{-1}) was purchased from PerkinElmer Life Sciences. Methionine/cysteine-deficient DMEM was purchased from Invitrogen. PMA was obtained from Sigma. Biomax MR film for autoradiography was from Kodak. Protein phosphatase 1 (PP1) was obtained from Calbiochem. The K562 cell line was a gift of Dr. Alan P. Fields (University of Texas Medical Branch). The cDNA of rat Hsp70 was a gift of Dr. Marina Gelman (Stanford University). Expression constructs encoding HA-tagged wild-type PKA and the point mutant S338A were a gift of Dr. Susan Taylor (University of California, San Diego); expression constructs encoding Myc-tagged rat PKCζ and HA-tagged murine Aktα (wild-type and T450A) were a gift of Dr. Alex Toker (Harvard Medical School). A polyclonal antibody against the carboxyl terminus of PKCβII was purchased from Santa Cruz Biotechnology. A monoclonal antibody against Hsp70 and a monoclonal antibody against PKCα, which recognizes PKCβII as well, were obtained from Transduction Laboratories. All other materials were reagent grade.

Plasmid Constructs—Construction of the mammalian expression vectors encoding wild-type, kinase-inactive (K371R), and phosphorylation site mutants of PKCβII (PKCβII-T447A/T448A/T500A(T500A3), PKCβII-T634A/T638A/T641A (T641A3), PKCβII-S660A, and PKCβII-S660E) were described previously (2, 3, 13, 14). Similarly, construction of mammalian expression vectors encoding glutathione *S*-transferase (GST) fusion proteins of domains of PKCβII (GST-C1, GST-C2, and GST-CD (the catalytic domain), GST-CT (the wild type carboxyl terminus), GST-CT/S660A, GST-CT/S660E, and GST-CT/T641E/S660E (GST-CT/E2)) were described previously (3). cDNAs encoding the peptide-binding domain (PBD), the carboxyl terminus (CT70), or the combined peptide-binding domain and the carboxyl terminus (PBCT) of Hsp70 were amplified from full-length Hsp70 by PCR. The PCR products were subcloned into pEBG vector (3) to yield in-frame fusions of the various domains to GST (tag at NH_2 terminus). Carboxyl-terminal Myc-tagged constructs of Hsp70, the ATPase domain, or the PBCT region of Hsp70 were generated from full-length Hsp70 by PCR 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 and 1% penicillin/streptomycin at 37 °C in 5% CO_2. K562 cells were maintained in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in 5% CO_2. Transient transfection of all cell types was carried out using Effectene transfection reagents (Qiagen).

GST Fusion Protein Pull-down Assay—TsA201 cells were transiently transfected with GST-tagged domains of PKCβII or GST-tagged domains of Hsp70. In some experiments, cells were co-transfected with GST-tagged domains and wild-type or specified mutants of PKCβII, Akt, or PKA. The transfected cells were lysed in buffer A (50 mM Na_2HPO_4, 1 mM sodium pyrophosphate, 20 mM NaF, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 1 mM dithiothreitol, 200 μM benzamidine, 40 μg ml^{-1} leupeptin, 300 μM phenylmethylsulfonyl fluoride). The detergent-solublized cell lysates were incubated with glutathione-Sepharose overnight at 4 °C. Beads were washed twice in buffer A and twice in buffer B (buffer A plus 300 mM NaCl), and proteins bound to the glutathione-Sepharose beads were analyzed by SDS-PAGE and Western blot analysis using chemiluminescence. In some experiments, detergent-solublized lysates from cells expressing PKC, PKA, or Akt were treated with 2 units ml^{-1} PP1 for 30 min at room temperature to dephosphorylate the turn motif of these kinases (15).

Immunoprecipitation—The interaction between endogenous Hsp70 and endogenous PKCβII was examined in K562 cells. Cells were lysed in buffer A and the detergent-solublized cell lysate was incubated with an anti-PKCα monoclonal antibody and Ultra-link protein A/G beads (Pierce) overnight at 4 °C. The immunoprecipitates were washed twice in buffer A and twice in buffer B. Bound proteins were analyzed by SDS-PAGE and Western blot analysis using chemiluminescence.

Pulse-Chase Analysis of Protein Kinase βII Processing—COS7 cells were co-transfected with PKCβII and GST vector, GST-CT/S660A, or GST-PBCT. 24–30 h post-transfection, cells were incubated with methionine/cysteine-deficient DMEM for 30 min at 37 °C, as described previously (16). The cells were then pulse-labeled with 1 mCi ml^{-1} [^{35}S]methionine/cysteine in methionine/cysteine-deficient DMEM for 7 min at 37 °C, and chased in chase media (DMEM containing 5 mM unlabeled methionine and 5 mM unlabeled cysteine) for 0, 30, or 60 min. At the indicated times, cells were lysed in buffer A and PKC was immunoprecipitated with an anti-PKCα monoclonal antibody (cross-reactive with PKCβII, Transduction Laboratories) coupled to Ultra-link protein A/G beads. The immunoprecipitates were washed 3 times with buffer B and once with buffer A. The immunoprecipitated proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and ^{35}S incorporation detected by autoradiography.

K562 Cell Proliferation Assay—K562 cells were seeded at 2–3 × 10^5 ml^{-1} and treated with PMA (20 nM) for 2 days. PMA was removed by washing cells three times in growth media, as described (17), and cells were counted using a hemocytometer to establish the baseline number. Cells were then divided into 5 equal groups and plated in 6-well (25 mm) plates. One group of cells was allowed to grow in growth media (control). A second group of cells was grown in the continued presence of PMA. The other three groups of cells were transfected with PKCβII, GST-CT/S660A, or Myc-PBCT. Cell numbers were counted every 24 h for 3 days following transfection.

RESULTS

The Carboxyl Terminus of Protein Kinase C βII Binds Hsp70—The carboxyl terminus of PKC is required for the function of the enzyme: in addition to stabilizing the mature conformation of PKC by intramolecular interactions, it serves as the docking site for PDK-1 (3). To address whether this regulatory sequence mediates other key protein interactions, we initiated a study to identify binding partners of the carboxyl terminus. A construct of GST fused to the carboxyl-terminal 46 residues of PKCβII (GST-CT) was expressed in tsA2101 cells. Interacting proteins were identified by Coomassie Blue staining of gels from GST pull-down analysis of detergent-solublized cell lysates. A major band migrating at 70 kDa was present in pull-down analysis from cells expressing GST-CT but not GST alone (data not shown). This band was excised from the gel, analyzed by matrix-assisted laser desorption ionization-mass spectrometry, and identified as heat shock protein 70.

The interaction and specificity of the interaction of Hsp70 with the carboxyl terminus of PKC was confirmed by probing GST pull-downs of various domains of PKC (shown in Fig. 1*A*) expressed in tsA2101 cells with antibodies specific for Hsp70. Fig. 1*B* (*upper panel*) shows that endogenous Hsp70 bound constructs containing the carboxyl terminus (*GST-CT*; *lane 2*) and the entire catalytic domain (*GST-CD*; *lane 5*) of PKC. In contrast, no significant binding was detected to GST alone (*lane 1*), or fusion proteins of the C1 (*GST-C1*; *lane 3*) or C2 domains (*GST-C2*; *lane 4*). The level of Hsp70 expression was similar in all samples (*lower panel*). These results indicate that endogenous Hsp70 interacts with PKCβII *in vivo*, and that the interaction is mediated by the carboxyl terminus of PKC.

We next examined the interaction between endogenous (full-length) PKCβII and endogenous Hsp70. We used K562 cells

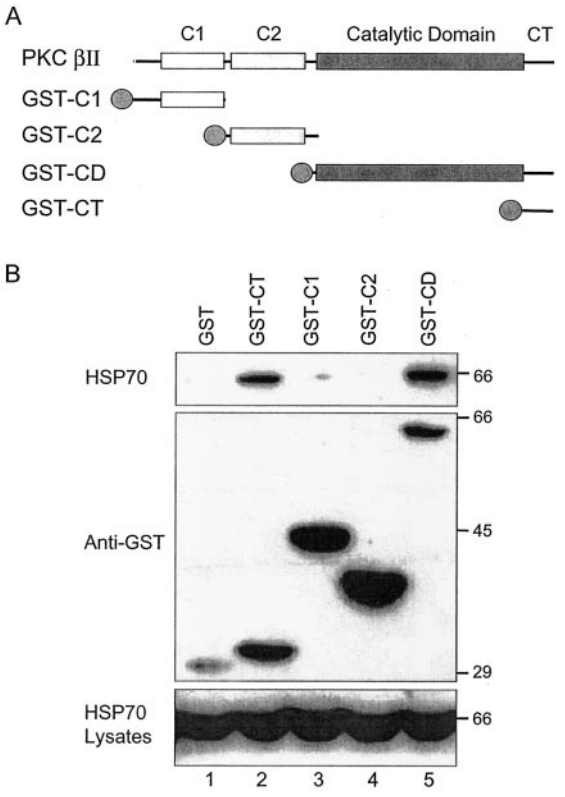

FIG. 1. **The carboxyl terminus of PKCβII interacts with Hsp70.** *A*, schematic representation of GST fusion proteins encoding different regions of PKCβII: the amino terminus and C1 domain (*GST-C1*), the C2 domain (*GST-C2*), the catalytic domain containing the carboxyl terminus (*GST-CD*), and the carboxyl-terminal 46 residues (*GST-CT*). *B*, Western blot showing endogenous Hsp70 in the detergent-solublized lysates from tsA201 transfected with the indicated GST-tagged fusion protein constructs (*lower panel*), GST constructs bound to glutathione-Sepharose beads following GST pull-down analysis from the detergent-solublized lysates (*middle panel*), and Hsp70 associated with glutathione-Sepharose beads (*upper panel*). Hsp70 was detected using an anti-Hsp70 antibody. Constructs were detected with an anti-GST antibody.

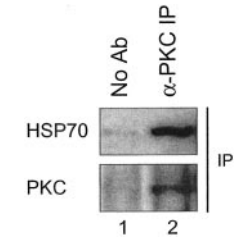

FIG. 2. **Endogenous PKC interacts with endogenous heat shock protein 70 *in vivo*.** PKC was immunoprecipitated (*IP*) from the detergent-soluble lysate of K562 cells with an anti-PKCα monoclonal antibody coupled to protein A/G-agarose (*lane 2*). As a negative control, the cell lysates were incubated with protein A/G-agarose alone (*lane 1*). Immune complexes were probed with an anti-PKCβII polyclonal antibody (*lane 2*, *lower panel*) or anti-Hsp70 antibody (*lane 2*, *upper panel*).

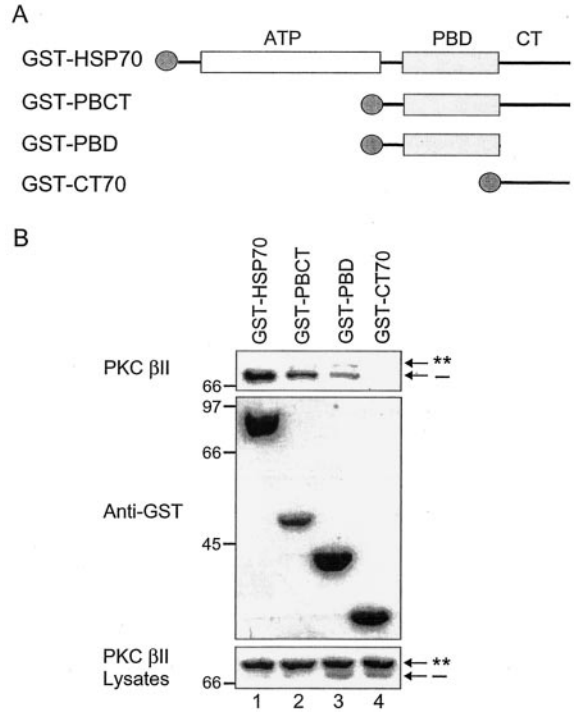

FIG. 3. **Mapping the binding domain for PKCβII in heat shock protein 70.** *A*, schematic representation of GST fusion proteins encoding different domains of Hsp70: the combined peptide-binding domain and the carboxyl terminus (*GST-PBCT*), the peptide-binding domain (*GST-PBD*), and the carboxyl terminus (*GST-CT70*). *B*, different GST-tagged fusion protein constructs of Hsp70 including GST-Hsp70, GST-PBCT, GST-PBD, and GST-CT70 (*lanes 1–4*, respectively) were co-transfected with PKCβII into tsA201 cells. Detergent-solublized cell lysates were incubated with glutathione-Sepharose. The proteins bound to the glutathione beads were separated on a SDS-PAGE gel and analyzed using immunoblotting. The *upper panel* shows the presence of PKCβII in the complexes with GST-Hsp70, GST-PBCT, and GST-PBD (*lanes 1–3*, respectively) but not GST-CT70 (*lane 4*), whereas the *lower panel* shows the total expression of PKCβII in the cell lysates. The PKCβII was detected on the blot using an anti-PKCβII antibody. The precursor PKC is labeled with a *dash* and the mature species (phosphates on all three *in vivo* sites) is labeled with *two asterisks* (**). Different GST fusion proteins of Hsp70 that bound to the glutathione beads were detected by an anti-GST antibody on the blot (*middle panel*).

because of their relatively high levels of endogenous PKCβII (17). PKC was immunoprecipitated from the Triton X-100-solublized cell lysate using an anti-PKCα monoclonal antibody that cross-reacts with PKCα and -βII. The *upper panel i*n Fig. 2 shows that endogenous Hsp70 was present in immune complexes with PKC (*lane 2*) but was not present in control samples in which antibody was omitted from precipitations (*lane 1*). PKCβII in the immunoprecipitates was detected by an anti-PKCβII antibody (*lane 2*, *lower panel*). These results show that endogenous PKCβII interacts with endogenous Hsp70 in intact cells.

The Peptide-binding Domain of Hsp70 Interacts with Protein Kinase C—Hsp70 contains three defined functional domains (Fig. 3*A*): a 44-kDa ATPase domain (ATP), an 18-kDa peptide-binding domain (PBD), and a 10-kDa carboxyl-terminal sequence (CT70) (18). To map the region in Hsp70 that binds PKC, GST fusion constructs of different domains were co-expressed with recombinant PKCβII in tsA201 cells and their interaction with PKC determined by GST pull-down experiments from Triton X-100-solubilized cell lysates. Fig. 3*B* shows that PKCβII was expressed to similar levels in all samples (*lower panel*). Western blot analysis of the GST pull-down experiments confirmed the presence of the constructs in the pull-down experiments (*middle panel*). PKCβII associated with full-length Hsp70 (*GST-Hsp70*; *lane 1*), a construct of the peptide-binding and carboxyl-terminal domains (*GST-PBCT*; *lane 2*), and a construct of just the peptide-binding domain (*GST-PBD*; *lane 3*). No significant binding was detected to a construct of just the carboxyl-terminal sequence (*GST-CT70*; *lane 4*). PKCβII did not bind a Myc-tagged construct of the ATPase domain (data not shown). Thus, the peptide-binding domain of Hsp70 contains the primary determinants that mediate the binding of Hsp70 to PKC. The more robust binding to the combined PBCT construct compared with the peptide-binding domain alone revealed some contribution of the carboxyl ter-

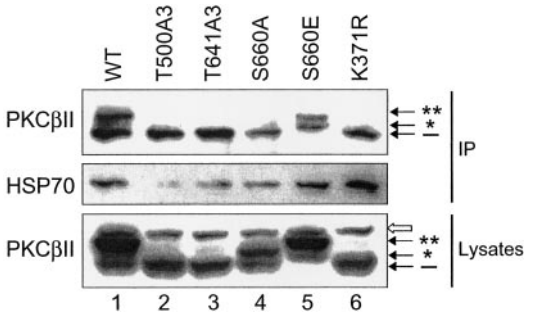

FIG. 4. **Hsp70 preferentially interacts with unphosphorylated PKC.** TsA201 cells were transiently co-transfected with Myc-tagged Hsp70 and either wild-type PKCβII (*lane 1*) or one of the following mutants of PKCβII: T500A3 (*lane 2*), T641A3 (*lane 3*), S660A (*lane 4*), S660E (*lane 5*), or K371R (*lane 6*). Cells were lysed and Hsp70 was immunoprecipitated (*IP*) with an anti-Myc antibody and protein A/G-agarose. Immunoprecipitated proteins were separated on a SDS-PAGE gel and analyzed by immunoblotting. PKC in the immune complexes was detected by an anti-PKCβII antibody (*upper panel*); Myc-Hsp70 was detected using the anti-Hsp70 antibody (*middle panel*). Wild-type and mutant PKCs in 10% of the detergent-solublized cell lysates were detected using the anti-PKCβII antibody (*lower panel*). Unphosphorylated PKC is labeled with a *dash* (-), the partially processed species (phosphate on one of the two carboxyl-terminal phosphorylation sites) is labeled with *one asterisk* (*), and the mature species (phosphate on both carboxyl-terminal sites) is labeled with *two asterisks* (**). (Note that phosphate on the PDK-1 site does not affect the electrophoretic mobility of PKC.) Endogenous PKCα in cell lysates was also labeled by the anti-PKCβII antibody and is indicated with the *open arrow* (*lower panel*).

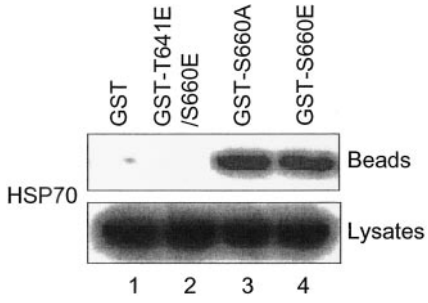

FIG. 5**. Negative charge at the turn motif abolishes the binding of Hsp70 to protein kinase C.** TsA201 cells were transiently transfected with GST vector (*lane 1*) or one of the following GST-tagged carboxyl-terminal constructs of PKCβII: GST-CT/T641E/S660E (*lane 2*), GST-S660A/CT (*lane 3*), or GST-S660E/CT (*lane 4*). The detergent-solublized cell lysates were incubated with glutathione-Sepharose beads and, following washes, bound proteins were separated by SDS-PAGE and analyzed using immunoblotting. Bound Hsp70 was detected using an anti-Hsp70 antibody (*upper panel*). Hsp70 expression in 10% of the lysates is shown in the *lower panel*.

minus, possibly by structuring the peptide-binding domain for optimal interaction with PKC, or by directly contributing binding determinants. The PBCT construct was used in subsequent experiments because it binds PKC better than the PBD construct.

PKCβII typically migrates as a doublet: the major slower migrating band represents phosphorylated PKC (Fig. 3, *double asterisk*) and the minor faster migrating band represents unphosphorylated PKC (*dash*). The PKC that associated with full-length Hsp70 was the unphosphorylated species (*upper panel*, *lane 1*); this species represented less than 10% of the total PKC in the lysate (*lower panel*). These data suggest that the phosphorylation state of PKC is important in the Hsp70 interaction.

Heat Shock 70 Preferentially Binds to Unphosphorylated Protein Kinase C—To further test whether Hsp70 specifically binds unphosphorylated PKCβII, wild-type enzyme and several phosphorylation deficient mutants were co-expressed with a Myc-tagged Hsp70 in tsA201 cells (Fig. 4). The *lower panel* in Fig. 4 shows the expression of these constructs and their relative electrophoretic mobilities. Wild-type PKCβII migrated primarily as the mature, phosphorylated protein (*double asterisk*) with a minor amount of unphosphorylated protein (*dash*) (*lane 1*), as in Fig. 3. Mutation of the activation loop (T500A3) or turn motif (T641A3) phosphorylation sites, or mutation to render PKC catalytically inactive (K371R), prevents phosphorylation of PKC and these mutants co-migrate with unphosphorylated PKC (*lanes 2*, *3*, and *6*) (2, 14). Mutation of the hydrophobic site to Ala (*lane 4*) or Glu (*lane 5*) still allows processing of PKC by phosphorylation (13), so that these constructs migrate as a major slower migrating species representing fully phosphorylated enzyme and a minor slower migrating species representing unphosphorylated protein. Note that the mobility of the full-phosphorylated S660A (*single asterisk*) was intermediate between that of full-phosphorylated (*double asterisk*) and completely unphosphorylated (*dash*) wild-type enzyme because one of the phosphorylation sites is occupied by Ala; conversely, the mobility of the unphosphorylated form of S660E is slower (*single asterisk*) than that of unphosphorylated wild-type enzyme (*dash*) because a negative charge occupies one of the phosphorylation sites. Note that the minor band migrating above full-phosphorylated PKBβII represents endogenous PKCα (indicated with *open arrow*).

The Myc-tagged Hsp70 was immunoprecipitated from the detergent-solublized lysates of cells expressing PKCβII constructs with an anti-Myc antibody and its presence detected with an anti-Hsp70 antibody (Fig. 4, *middle panel*). Fig. 4 (*upper panel*) shows that each construct of PKC was complexed with Hsp70. Importantly, the unphosphorylated species of PKC was enriched by over an order of magnitude in the immune complexes relative to phosphorylated PKC, whereas unphosphorylated protein represented about 10% of the wild-type (*lane 1*) and S660E (*lane 5*) PKC in the total cell lysate, it represented over 90% of the PKC in the complex with Hsp70. Unphosphorylated S660E (*lane 6*; fastest migrating band) was co-precipitated as efficiently as unphosphorylated wild-type enzyme (*lane 1*) and unphosphorylated S660A (*lane 5*). Thus, negative charge at the hydrophobic motif does not influence the interaction of Hsp70 with PKC. However, phosphorylated S660A (position indicated with *single asterisk* in the *lower panel* of Fig. 4) did not interact with Hsp70 (*lane 5*; only the slowest migrating band is present on the beads); this species of PKC has phosphate on the turn motif but no charge on the hydrophobic motif, indicating that phosphorylation of the turn motif, in contrast to that of the hydrophobic motif, abolishes the interaction with Hsp70. Thus, Hsp70 selectively binds unphosphorylated PKC and in a manner that is sensitive to the charge at the turn motif but not the hydrophobic motif.

Phosphorylation of the Carboxyl-terminal Turn Motif Abolishes the Interaction of Hsp70 with Protein Kinase C—The above data show that Hsp70 selectively binds unphosphorylated PKC, yet experiments with the S660E mutant showed that this interaction is not sensitive to the phosphorylation of the hydrophobic motif. This led us to explore whether phosphorylation of the turn motif regulates the interaction of PKC with Hsp70. TsA201 cells were transfected with GST alone (Fig. 5, *lane 1*) or the following fusion constructs of the carboxyl terminus: GST-T641E/S660E in which both turn and hydrophobic sites were mutated to Glu, GST-S660A (*lane 3*), and GST-S660E (*lane 4*). GST pull-down experiments showed that the carboxyl-terminal constructs with mutations at Ser^{660} effectively bound Hsp70, independently of the charge at this position (*upper panel*, *lanes 3* and *4*). In contrast, additional mutation of Thr^{641} to Glu to mimic the phosphate at the turn motif abolished the interaction of the carboxyl terminus with Hsp70

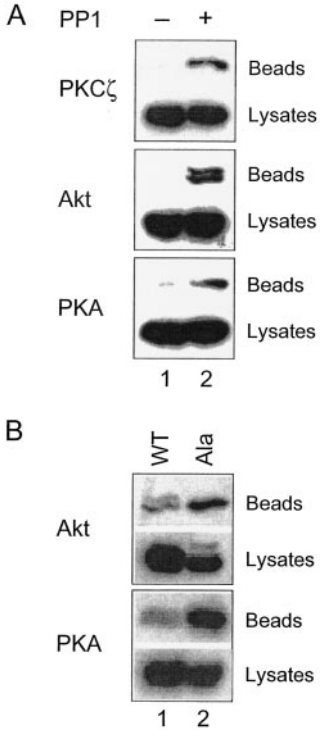

FIG. 6. **Phosphorylation of the turn motif of the AGC kinase family members controls their interaction with Hsp70.** *A*, tsA201 cells were transiently co-transfected with GST-Hsp70 and Myc-tagged PKCζ, HA-tagged Akt, or HA-tagged PKA. The detergent-solublized cell lysates from each transfection were divided into two equal aliquots and treated without (*lane 1*) or with (*lane 2*) PP1. Cell lysates were then incubated with glutathione-Sepharose beads to pull down GST-Hsp70. Bound PKCζ was detected by an anti-PKCζ antibody (*upper panel*, *beads*) and bound Akt and PKA were detected by an anti-HA antibody (*middle* and *lower panels*, respectively, *beads*). The total protein expression of each kinase in 5% of cell lysates was detected on the blot as well (*lysates*). *B*, tsA201 cells were transiently co-transfected with GST-Hsp70 and either HA-tagged wild-type or T450A mutant of Akt (*upper panel, lanes 1* and *2*, respectively), and HA-tagged wild-type or S338A mutant of PKA (*lower panel*, *lanes 1* and *2*, respectively). The detergent-solublized cell lysates were incubated with glutathione-Sepharose beads to pull-down GST-Hsp70. Bound Akt and PKA were detected by probing Western blots with an anti-HA antibody (*beads*). The total protein expression in 5% of cell lysates was detected by the anti-HA antibody as well (*lysates*).

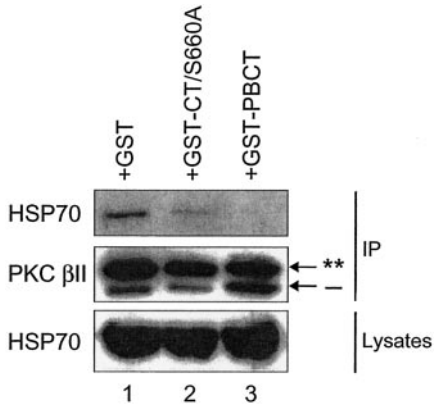

FIG. 7. **The interaction of Hsp70 with protein kinase C is disrupted by co-expression of fusion proteins encoding the interaction domain in either PKCβII or Hsp70.** TsA201 cells were co-transfected with PKCβII and one of the following GST-tagged constructs: GST vector (*lane 1*), GST-CT/S660A (*lane 2*), or GST-PBCT (*lane 3*). Cells were lysed in 1% Triton X-100 and PKC was immunoprecipitated with an anti-PKCα monoclonal antibody coupled to protein A/G-agarose (this antibody binds PKCα and -βII). Endogenous Hsp70 in the immune complex was detected by Western blot analysis using an anti-Hsp70 antibody (*upper panel*). PKCβII in the immune complexes was detected by an anti-PKCβII antibody. The *lower panel* shows the total expression of endogenous Hsp70 in 10% of cell lysates as detected by the anti-Hsp70 antibody.

(upper panel, *lane 2*). This result suggests that phosphorylation at the turn motif (but not hydrophobic) provides an on/off switch for the interaction of Hsp70 with PKC.

Hsp70 Interacts with Other AGC Kinase Family Members via Their Unphosphorylated Turn Motif—The turn motif is conserved throughout the AGC family of kinases (19), prompting us to explore whether phosphorylation-regulated binding of Hsp70 was conserved among the PKC family and even generally among AGC superfamily members. Specifically, we asked whether three other kinases bind Hsp70: PKCζ, a member of the atypical class of PKC isozymes that is most divergent from the conventional class of which PKCβII is a member; Akt (protein kinase B); and the archetypal AGC family member, PKA. Similar to the PKC family members, Akt and PKA are constitutively phosphorylated at their turn motifs (20, 21). Each of these kinases was co-expressed with GST-Hsp70 in tsA201 cells. Detergent-solublized cell lysates were then treated without or with PP1 to dephosphorylate the kinases. Control or phosphatase-treated lysates were then subjected to GST pull-down analysis and the beads were probed for relevant kinases with specific antibodies. Fig. 6*A* shows that no significant binding of PKCζ, Akt, or PKA was detected in nonphosphatase samples (*lane 1*). However, significant association of all three kinases was observed in phosphatase-treated samples (*lane 2*). Thus, Hsp70 selectively binds the dephosphorylated species of these AGC family members.

We next addressed whether, as occurs for PKC, phosphorylation of the turn motif is the switch that abolishes Hsp70 binding to Akt and PKA. TsA201 cells were co-transfected with GST-Hsp70 and either wild-type Akt or PKA (which are quantitatively phosphorylated at the turn motif), or the turn motif mutants of each kinase, T450A or S338A, respectively. Hsp70 was pulled down with glutathione-Sepharose beads and pellets were analyzed for bound kinases using the antibodies for Akt or PKA. Fig. 6*B* shows modest association of both wild-type Akt and PKC (*lane 1*) with Hsp70. However, mutation of the turn motif to Ala resulted in a dramatic increase in association (*lane 2*). Thus, similar to its interaction with PKC, Hsp70 binds Akt and PKA via the unphosphorylated turn motif.

Co-expression of the Binding Domain of Either Protein Disrupts the Binding of Hsp70 to Protein Kinase C in Vivo—We next investigated whether the interaction between full-length PKCβII and Hsp70 could be disrupted by overexpression of fusion proteins encoding either the carboxyl terminus of PKC or the PBCT domain of Hsp70. Constructs of the carboxyl terminus of PKC bind PDK-1 and promote the autophosphorylation of PKC (3). To avoid disrupting the interaction with PDK-1, we focused on a construct of the carboxyl terminus that has a mutation that abolishes PDK-1 binding but does not affect Hsp70 binding: a carboxyl-terminal construct with Ser^{660} (a key PDK-1-docking site) mutated to Ala. TsA201 cells were co-transfected with PKCβII and GST (Fig. 7, *lane 1*), GST-CT/S660A (*lane 2*), or GST-PBCT (*lane 3*). PKC in the detergent-solublized cell lysates was immunoprecipitated and bound Hsp70 was detected with anti-Hsp70 antibodies. As in previous experiments (*e.g.* Fig. 4), Fig. 7 shows that Hsp70 was present in the PKC immune complex (*lane 1*). However, the amount of associated Hsp70 was significantly reduced in cells co-expressing the region of PKC that binds Hsp70 (GST-CT/S660A; *lane 2*) and abolished in cells co-expressing the region of Hsp70 that binds PKC (GST-PBCT; *lane 3*). Comparable amounts of PKCβII were in the immunoprecipitates (*middle panel*; note detecting antibody binds both unphosphorylated (*dash*) and

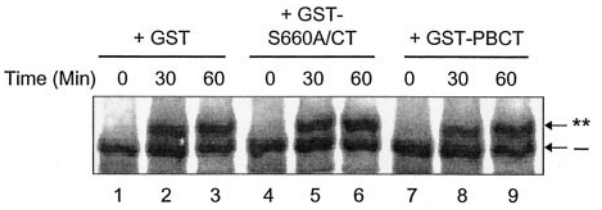

FIG. 8. **Hsp70 does not regulate the maturation of protein kinase C.** Autoradiogram from pulse-chase analysis of COS7 cells co-transfected with GFP-tagged PKCβII and GST vector (*lanes 1–3*), GST-CT/S660A (*lanes 4–6*), or GST-PBCT (*lanes 7–9*). Transfected cells were metabolically labeled with [^{35}S]methionine/cysteine for 7 min and chased for 0 min (*lanes 1*, *4*, and *7*), 30 min (*lanes 2*, *5*, and *8*), or 60 min (*lanes 3*, *6*, and *9*). GFP-PKCβII was immunoprecipitated from detergent-solublized cell lysates with the anti-PKCα antibody (see legend to Fig. 7) and analyzed by SDS-PAGE and autoradiography. Position of newly synthesized protein that is not yet phosphorylated is indicated by the *dash* (-); the *double asterisk* denotes position of phosphorylated (mature) PKC.

phosphorylated (*double asterisk*) PKC). Similarly, the expression of endogenous Hsp70 was similar in all samples (*lower panel*). These data reveal that binding domains from either protein can be used to disrupt the interaction of full-length PKC with Hsp70 *in vivo*.

Disrupting the Interaction of Hsp70 with Protein Kinase C Does Not Affect the Maturation of Protein Kinase C—The foregoing data reveal that Hsp70 preferentially binds unphosphorylated PKC. Two species of PKC are unphosphorylated in the cell: 1) newly synthesized PKC that has yet to be phosphorylated, and 2) mature PKC that has been processed by phosphorylation and then dephosphorylated. Given the role of Hsp70 in protein folding, we first asked whether Hsp70 has a role in the maturation process of PKCβII. Specifically, we asked whether disrupting the interaction with Hsp70 slows the maturation of PKC. This maturation can be detected by pulse-chase experiments: the processing phosphorylations of the carboxyl terminus result in readily detectable shifts in the electrophoretic mobility in the pool of ^{35}S-labeled PKC that is being chased (16). COS7 cells were co-transfected with GFP-tagged PKCβII (GFP-PKCβII was used to increase ^{35}S incorporation) and GST alone (*lanes 1–3*), GST-CT/S660A (*lanes 4–6*), or GST-PBCT (*lanes 7–9*). The cells were labeled with [^{35}S]Cys/Met for 7 min and the pool of labeled proteins was chased for 0 (*lanes 1*, *4*, and *7*), 30 (*lanes 2*, *5*, and *8*), or 60 min (*lanes 3*, *6*, and *9*) in unlabeled media. PKC was immunoprecipitated from cell lysates. The autoradiogram in Fig. 8 shows that newly synthesized PKCβII migrated as a single faster migrating band (*dash*) immediately following the chase (*lane 1*), and then shifted to a slower migrating species with time (*double asterisk*) (*lanes 2* and *3*). As previously published, PKCβII was processed with a half-time on the order of 60 min (16). Importantly, co-expression of neither disruptor had any effect on the rate of PKCβII processing. The ratio of matured to precursor PKCβII was the same in control cells and cells co-expressing GST-CT/S660A or GST-PBCT (compare *lanes 2*, *5*, and *8*). These data suggest that the interaction between Hsp70 and PKC is not likely to involve the maturation of newly synthesized PKCβII.

Disrupting the Interaction of Hsp70 with Protein Kinase C Causes Accumulation of Dephosphorylated Protein Kinase C in the Detergent-insoluble Fraction of Cells—We next tested whether Hsp70 was involved in the down-regulation of PKC by binding mature PKC that has been dephosphorylated. PKC is dephosphorylated following prolonged activation as occurs following phorbol ester treatment (5). This dephosphorylated species accumulates in the Triton X-100-insoluble fraction of cells, and is eventually degraded as part of the down-regulation of the enzyme. To test whether Hsp70 is involved in the dephosphorylation and down-regulation of PKC, we examined whether disruption of the Hsp70/PKC interaction influenced the accumulation of dephosphorylated PKC in the detergent-insoluble fraction of cells. TsA201 cells were co-transfected with PKCβII and with GST alone (Fig. 9, *lanes 1* and *2*), GST-CT/S660A (*lanes 3* and *4*), or GST-PBCT (*lanes 5* and *6*). The cells were lysed in buffer containing 1% Triton X-100, and the Triton-soluble supernatant (S) was separated from the Triton-insoluble pellet (P) by centrifugation. Ten percent of each fraction was analyzed by SDS-PAGE and PKCβII associated with each fraction was detected using an anti-PKCβII antibody. Fig. 9*A* shows that the majority of wild-type PKCβII was expressed in the Triton-soluble fraction (*lane 1*) and was fully phosphorylated (*double asterisk*). Minor unphosphorylated species were present in the pellet (*dash*). Disruption of the Hsp70 interaction by co-expression of disrupting domains caused a modest (*GST-S660A/CT*; *lanes 3* and *4*) to dramatic (*GST-PBCT*; *lanes 5* and *6*) increase in the fraction of dephosphorylated PKC in the pellet. The much stronger effect of the GST-PBCT disruptor is consistent with quantitative disruption of the Hsp70/PKC interaction (Fig. 7). Data from three independent experiments are quantified in Fig. 9*B* and show that co-expression of the disruptor GST-PBCT caused an ~6-fold increase in the amount of unphosphorylated PKC in the pellet.

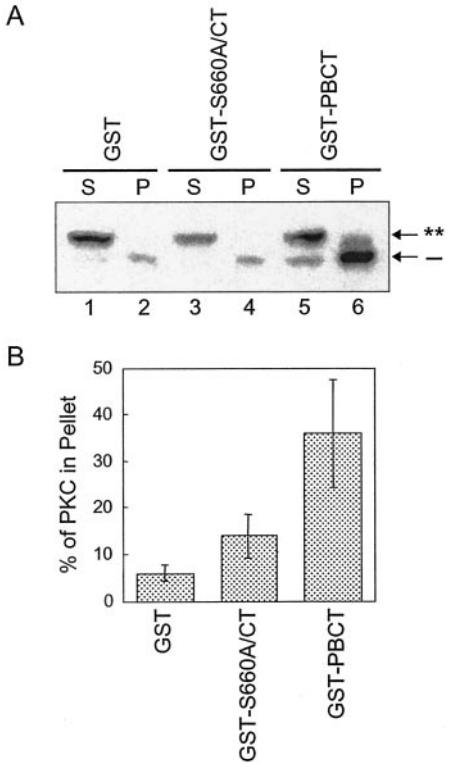

FIG. 9. **Hsp70 prevents the accumulation of dephosphorylated protein kinase C in the detergent-insoluble fraction of cells.** TsA201 cells were co-transfected with PKCβII and one of the following GST-tagged disrupting constructs: GST alone (*lanes 1* and *2*), GST-CT/S660A (*lanes 3* and *4*), or GST-PBCT (*lanes 5* and *6*). Cells were lysed in 1% Triton X-100 and fractionated into detergent-soluble and detergent-insoluble fractions. *A*, Western blot showing the expression of PKCβII in the detergent-soluble supernatant (*S*) and detergent-insoluble pellet (*P*) detected with an anti-PKCβII antibody. The *dash* indicates the position of unphosphorylated PKC; the *double asterisk* denotes the position of fully phosphorylated PKC. *B*, quantitation of the data in *A* showing the percent of PKC in the pellet. The ECL signals shown in *panel A* were scanned and quantified by a CCD camera using a GeneGnome bioimaging system (Syngene). The percentage of PKC in the detergent-insoluble pellet for cells expressing PKC + GST, PKC + GST-CT/S660A, or PKC + GST-PBCT was 6 ± 2, 14 ± 5, and 36 ± 12 (n = 3), respectively.

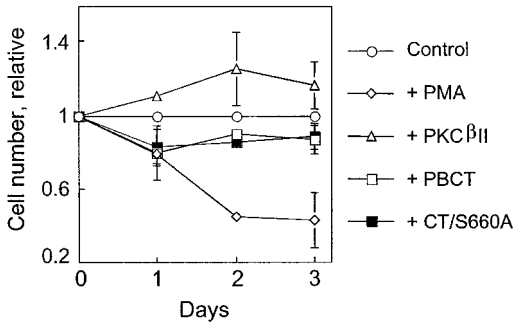

FIG. 10. **Disrupting the interaction of Hsp70 with protein kinase C inhibits the recovery of cells from PMA-induced growth arrest.** K562 cells were treated with PMA (20 nM) for 2 days to induce cell growth arrest. At this time point (day 0), the cells were washed three times with growth media to remove PMA, reseeded, and treated as follows: (*a*) cells were kept in growth media (*Control*, ○); (*b*) cells were returned to PMA (20 nM) containing media (+*PMA*, ◇); (*c*) cells were transfected with GFP-tagged PKCβII (+PKCβII, △); (*d*) cells were transfected with the GST-CT/S660A fusion protein construct (+CT/S660A, ■); and (*e*) cells were transfected with the Myc-PBCT fusion protein construct (+PBCT, □). The number of cells were counted at 1, 2, and 3 days after the treatment. The number of cells at each time point was normalized to the number of cells in the control sample (treatment a). Data represent the mean ± S.E. of three independent experiments.

Because pulse-chase analysis revealed that this disruptor has no effect on the amount of PKC that has not yet been phosphorylated, this unphosphorylated species reflects mature protein that has been dephosphorylated. The finding that disruption of the interaction of Hsp70 with PKC results in accumulation of dephosphorylated PKC in the detergent-insoluble pellet indicates that Hsp70 prevents the down-regulation of PKC.

Disrupting the Interaction with Hsp70 Inhibits Protein Kinase C Function in Vivo—To further examine the functional significance of the interaction of Hsp70 with PKC, we examined the effect of disrupting this interaction on the proliferation of K562 cells. Fields and co-workers (17) have elegantly shown that PKCβII plays a key role in proliferation of these cells. Specifically, PMA treatment of these cells causes a dose-dependent arrest of cell growth that results from down-regulation of endogenous PKCβII (17). Consistent with this, the rate of cell proliferation was correlated with the levels of PKCβII expression in K562 cells. Taking advantage of this PKCβII read-out, we tested whether disruption of the PKC/Hsp70 interaction affects cell growth. K562 cells were pretreated with PMA for 2 days to arrest cell growth, then subjected to various treatments outlined in the legend to Fig. 10. Control cells were returned to growth media to recover from the PMA treatment. The number of cells started to increase, and these numbers were normalized to 1 (Fig. 10, *open circles*). Cells that had continued exposure to PMA declined in numbers (*open diamonds*), as previously reported (17). The growth rate of cells transfected with either disruptor was consistently lower than that of the control cells (+*CT/S660A*, *closed squares*; +*PBCT*, *open squares*). For comparison, cells transfected with PKCβII recovered better from the growth arrest compared with the control cells (+*PKCβII*, *open triangles*). Although the changes in cell numbers resulting from the various transfections were highly reproducible ($n = 3$), the differences relative to the control were modest (about 5–10% reduction in growth rate for disruptors). However, given the poor transfection efficiency of K562 cells (around 5–10% (data not shown)), these changes suggest a significant slowing, if not complete arrest, in the growth rate of the cells successfully transfected with the disruptors. Since the functional PKCβII is required for K562 cell proliferation, our results suggested that interaction between PKCβII and Hsp70 is important for proper function of PKCβII in intact cells.

DISCUSSION

In this study we show that the molecular chaperone, Hsp70, binds the carboxyl terminus of at least three AGC family members: PKC isozymes, Akt, and PKA. The interaction is mediated primarily by the peptide-binding domain on Hsp70 and the dephosphorylated turn motif on the protein kinases. In the case of PKC, the functional consequence of this interaction is not to assist in the maturation of PKC, but, rather to prevent the down-regulation of the mature enzyme. These results underscore an emerging role of molecular chaperones in sustaining the lifetime of signal transduction molecules. In particular, our data reveal a novel mechanism for combating the inactivating phosphorylations that turn off kinase function.

The Carboxyl Terminus as a Multiprotein Docking Site—The carboxyl terminus has long been known to be important for the structural stability of PKC. Studies in the early 1990s showed that deletion of the carboxyl-terminal residues of PKCα resulted in the accumulation of inactive PKC in the detergent-insoluble fraction of cells (22). Much evidence also pointed to important roles in regulating the function of the enzyme. For example, studies with chimeric proteins revealed that the presence of just the carboxyl-terminal sequence of a particular isozyme was sufficient to confer functional characteristics to a chimera containing all but the carboxyl terminus of a second isozyme (23, 24). The discovery that the carboxyl terminus is constitutively phosphorylated at two highly conserved sites, the turn motif and the hydrophobic site, initiated a flurry of studies on the role of these phosphorylations (13, 14, 25–28). Although some analyses have been confounded by the presence of compensatory phosphorylation triggered by mutation of the turn motif, the general consensus is that both sites are important to lock PKC in a thermally stable, phosphatase-resistant conformation.

The finding that the hydrophobic motif in the carboxyl terminus provides a docking site for PDK-1 revealed that this region of the protein has an essential role in mediating protein/protein interactions (3). In addition to anchoring PDK-1, the carboxyl terminus may bind proteins that dictate the localization of PKC; a recent study by Mochly-Rosen and co-workers (29) showed that overexpression of a construct containing 6 residues from the carboxyl terminus of PKCβII selectively inhibited the phorbol ester-mediated translocation of this isozyme. Thus, the carboxyl terminus is important for the structural stability of PKC and for allowing the interaction with key regulatory proteins.

The Turn and Hydrophobic Motifs Regulate the Interaction with Their Docking Partner by Different Molecular and Temporal Mechanisms—Our data reveal that each of the phosphorylation sites on the carboxyl terminus of PKC serves as a phosphorylation-regulated binding site for a protein that directly regulates the function of PKC. The hydrophobic site provides the primary determinants for binding PDK-1 and the turn motif provides the primary determinants for docking Hsp70. Both proteins selectively bind unphosphorylated PKC, but the regulation by phosphorylation occurs by two very different mechanisms. In the case of PDK-1, the reduced affinity for phosphorylated PKC results from steric and conformational effects that mask the docking site to PDK-1 binding in the mature (but inactive) conformation of PKC. In fact, PDK-1 has a higher intrinsic affinity for phosphorylated hydrophobic motif compared with unphosphorylated motif as evidenced by its tighter binding to isolated constructs of the carboxyl terminus that are phosphorylated compared with constructs that are not phosphorylated (3). Thus, PDK-1 actually prefers to bind to the phosphorylated hydrophobic motif, but does not do so in the context of the full-length proteins because of limited accessibil-

ity. In contrast, Hsp70 does not bind mature PKC because phosphorylation of the turn motif directly abolishes binding.

Binding to PDK-1 and Hsp70 appear to occur at different points in the life cycle of PKC. PDK-1 binds newly synthesized enzyme that has yet to be phosphorylated, whereas Hsp70 binds mature PKC that has been dephosphorylated. This binding appears to be independent: disrupting the interaction of PKC with Hsp70 has no detectable effect on the processing of PKC by PDK-1. One possible explanation is that PKC that has not been phosphorylated and PKC that has matured and then been dephosphorylated are not conformationally equivalent species. Thus, the accessibility of the carboxyl-terminal-binding sites may be sensitive to the phosphorylation history of PKC.

PKC and Heat Shock—Our data reveal that Hsp70 prolongs the lifetime of PKC and, possibly, that of the other AGC kinases with which it interacts. Interestingly, many of these kinases are themselves activated following cellular stress such as heat shock. For example, Akt, which plays a key role in cell survival, becomes activated following stresses such as heat shock (30–33). In the case of PKC, phorbol esters have been shown to enhance Hsp70 expression and heat-induced stress responses (34). Fever-like hyperthermia, as well as phorbol esters, have also been reported to cause Hsp70 and PKC to associate with a cytoskeletal component containing spectrin (35). Some PKC isozymes have also been reported to directly phosphorylate heat shock proteins such as Hsp25/27 (36). Our finding that Hsp70 prolongs the lifetime of kinases whose function is required to combat heat shock provides an example of feed forward regulation of a protective response.

Model—Taken together with previous findings, our data are consistent with a model in which PDK-1 docks the unphosphorylated carboxyl terminus of newly synthesized PKC and promotes its maturation. Hsp70 does not appear to play a significant role in this step. Following activation of the mature enzyme by binding lipid second messengers, PKC adopts an open conformation that is highly phosphatase-sensitive (15, 37). The dephosphorylated enzyme has a greatly reduced thermal stability and, if maintained in this state for long enough, associates with the detergent-insoluble cell fraction and is targeted for down-regulation. Hsp70 binds this dephosphorylated species via the turn motif and prevents the association with the detergent-insoluble cell fraction. Based on the mechanism of binding of Hsp70 to nascent polypeptides (1), we hypothesize that Hsp70 binds a partly unfolded carboxyl terminus, preventing aggregation, and allowing re-autophosphorylation of the enzyme. If the interaction with Hsp70 is disrupted, the dephosphorylated PKC may aggregate and no longer be competent for re-autophosphorylation. This mechanism of protecting PKC by dephosphorylation triggered association with Hsp70 provides a highly effective mechanism to combat the voracious activity of cellular phosphatases. Thus, the turn motif of AGC kinase family members may serve as a protection switch to invite Hsp70 binding in the face of an onslaught of inactivating phosphatase activity. Our data with PKC show that this protective interaction with Hsp70 is required for effective PKC function.

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