Peptidyl-prolyl Isomerase Pin1 Controls Down-regulation of Conventional Protein Kinase C Isozymes

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Background: Conventional PKC isozymes have a putative Pin1 isomerization sequence at their turn motif phosphorylation site.

Results: Pin1 binds conventional PKCs and promotes their activation-induced down-regulation.

Conclusion: Pin1 isomerizes the phosphorylated turn motif of conventional PKC isozymes, priming them for subsequent down-regulation.

Significance: Pin1 provides a switch regulating the lifetime of conventional PKCs.

The down-regulation or cellular depletion of protein kinase C (PKC) attendant to prolonged activation by phorbol esters is a widely described property of this key family of signaling enzymes. However, neither the mechanism of down-regulation nor whether this mechanism occurs following stimulation by physiological agonists is known. Here we show that the peptidyl-prolyl isomerase Pin1 provides a timer for the lifetime of conventional PKC isozymes, converting the enzymes into a species that can be dephosphorylated and ubiquitinated following activation induced by either phorbol esters or natural agonists. The regulation by Pin1 requires both the catalytic activity of the isomerase and the presence of a Pro immediately following the phosphorylated Thr of the turn motif phosphorylation site, one of two C-terminal sites that is phosphorylated during the maturation of PKC isozymes. Furthermore, the second C-terminal phosphorylation site, the hydrophobic motif, docks Pin1 to PKC. Our data are consistent with a model in which Pin1 binds the hydrophobic motif of conventional PKC isozymes to catalyze the isomerization of the phospho-Thr-Pro peptide bond at the turn motif, thus converting these PKC isozymes into species that can be efficiently down-regulated following activation.

The peptidyl-prolyl cis-trans isomerase Pin1 is emerging as an important regulator of signal transduction pathways (1).

Pin1-catalyzed isomerization plays a key role in the control of normal cellular functions, most notably proliferation where Pin1 is essential for cell cycle progression (2). Pin1 belongs to the Parvulin family of peptidyl-prolyl cis-trans isomerases and is the only member that specifically isomerizes phospho-(Ser/Thr)-Pro ((Ser(P)/Thr(P))-Pro) motifs (3): the enzyme displays an ~1000-fold selectivity for peptides phosphorylated on the Ser/Thr preceding the Pro compared with unphosphorylated peptides (3).

Pin1-induced conformational changes in target proteins affect a variety of protein properties from folding to regulation of activity and stability. As a consequence, deregulation of phosphorylation steps and their attendant conformational changes often lead to disease (4). For example, Pin1 is down-regulated in degenerating neurons from Alzheimer disease patients, correlating with age-dependent neurodegeneration (5). Pin1 has also been implicated in cancer progression: levels of this protein are increased in many cancers, including those of the breast, prostate, brain, lung, and colon (6–9). Thus, Pin1 has been proposed to function as a catalyst for oncogenic pathways (10). The molecular mechanisms that lead to disease progression most likely involve postphosphorylation conformational changes catalyzed by Pin1 that are required for downstream effects.

Members of the protein kinase C (PKC) family of Ser/Thr kinases transduce an abundance of diverse signals that mediate processes such as cell cycle progression (11, 12), apoptosis (13), and immune responses (14). The PKC family consists of 10 isozymes that all possess an N-terminal regulatory domain, a conserved C-terminal catalytic core, and an autoinhibitory pseudosubstrate sequence (for reviews, see Refs. 15 and 16). The PKC family is subdivided into three subclasses based on the cofactor dependence of their regulatory domains: conventional (α, β, and γ; activated by diacylglycerol and Ca^{2+}) novel (ε, δ, θ, and η; activated by diacylglycerol), and atypical (ζ and i; insensitive to diacylglycerol or Ca^{2+}) isozymes. Before conventional PKC isozymes can be activated by second messengers, they undergo a series of ordered phosphorylations (17, 18) and conformational transitions. Newly synthesized, unphosphorylated
conventional PKC isozymes are loosely tethered at the membrane (19) with an exposed pseudosubstrate and an accessible C-terminal tail (20). The upstream kinase, phosphoinositide-dependent kinase 1 (PDK-1), docks onto the C-terminal tail of this newly synthesized conventional PKC (21), allowing efficient phosphorylation of the activation loop site (Thr641; numbering according to rat PKCβII) (17, 18, 22). This initial phosphorylation triggers two sequential phosphorylation events on the C-terminal tail that have recently been shown to depend on the mammalian target of rapamycin complex 2 (mTORC2) protein complex (23, 24). These sites are the turn motif (Thr641; numbering according to rat PKCβII) and the hydrophobic motif (Ser640; numbering according to rat PKCβII). The role of mTORC2 in these phosphorylations on PKC remains to be clarified. In the case of Akt, mTORC2 phosphorylates the turn motif site co-translationally (25). This is not the case with PKC because phosphorylation at the turn motif occurs after biosynthesis; the half-time of phosphorylation of newly synthesized PKC is on the order of 15 min (20). Once phosphorylated on the turn motif, PKC becomes phosphorylated at the hydrophobic motif via an intramolecular autophosphorylation (26). The fully phosphorylated conventional PKC then localizes to the cytosol where it is maintained in an inactive and phosphatase-resistant conformation (27, 28). This form is the major species of conventional PKC found in unstimulated cells. The phosphorylations at the PDK-1 site (activation loop) and at the turn and hydrophobic motifs are essential for PKC function; however, once PKC is matured by phosphorylation, phosphate on the activation loop (but not turn motif) becomes dispensable (19, 27).

Natural agonist-induced acute signaling by conventional PKC is terminated following removal of the second messengers (diacylglycerol and Ca2+), relocalizing conventional PKC to the cytosol in the closed, autoinhibited conformation. Chronic activation of conventional PKC, however, eventually results in the complete dephosphorylation and degradation of the enzyme by a ubiquitin/proteasome-dependent mechanism referred to as down-regulation (29–32). The classic trigger for chronic activation and subsequent down-regulation of conventional and novel PKC isozymes is phorbol ester treatment of cells (33). These potent analogues of diacylglycerol are not metabolized and thus cause sustained recruitment of PKC to membranes. Here, PKC is maintained in an open conformation that has a 2-orders of magnitude increased sensitivity to phosphatases (34). The recently discovered protein phosphatase PHLPP (pleckstrin homology (PH) domain leucine-rich repeat protein phosphatase (35)) catalyzes the first dephosphorylation event of PKC, which occurs on the hydrophobic motif and shunts PKC to the detergent-insoluble fraction of cells (36). Protein phosphatase 2A, which can dephosphorylate the activation loop and the hydrophobic motif in vitro (37), also contributes to the dephosphorylation of PKC in cells (38). Dephosphorylation has traditionally been considered to be the first step in phorbol ester-mediated down-regulation (39), although fully phosphorylated PKCα has been reported to be degraded in one study (40). D Dephosphorylated PKC can also be rescued by rephosphorylation in a manner that depends on the chaperone protein heat shock protein 70 (Hsp70), which specifically binds the dephosphorylated turn motif (41, 42).

Here we report that Pin1 is required for the efficient down-regulation of conventional PKC isozymes that is triggered by either phorbol ester or natural agonist stimulation. Experiments using cells lacking Pin1, pharmacological inhibition of Pin1, or constructs of PKC unable to bind or be isomerized by Pin1, and peptide binding arrays revealed that Pin1 binds the C termini of the conventional PKC isozymes PKCα and PKCβII, converting them into a species that can be readily dephosphorylated and ubiquitinated. Our data are consistent with a model in which Pin1 catalyzes a cis/trans isomerization of the phospho-Thr-Pro peptide bond of the turn motif, thus converting PKC into a species that is down-regulation-sensitive. Thus, Pin1-mediated isomerization provides a molecular signal that primes conventional PKC isozymes for agonist-evoked down-regulation.

EXPERIMENTAL PROCEDURES

Plasmids—Rat PKCβII in pcDNA3, PKCβII-T641AAA, PKCβII-K371R (27), PKCβII-T660A (43), PKCe (44), and Myc-PDK-1 (22), have been described previously. Myc-tagged rat Xpress-tagged PKCζ and PKCθ were gifts from Alex Toker, PKCδ was from Peter Blumberg, and PKCα was a gift from Yusuf Hannun. GST-tagged PKCβII C-terminal constructs were generated as described (21). GST-Pin1 for bacterial expression was a gift from Joseph P. Noel, and GST-Pin1 and HA-Pin1 mammalian expression vectors were generated by PCR and subsequent cloning into the BamHI and NotI sites of pEBG vector (a gift from Bruce Mayer) or into the EcoRI and NotI sites 3’ to the HA epitope in pcDNA3-HA, respectively. PKCβII mutants were generated using QuikChange (Stratagene). 3HA-ubiquitin constructs were a kind gift from Vishva M. Dixit.

Antibodies and Materials—Antibodies for immunoblotting PKCα (sc-208), PKCβI (sc-209), PKCβII (sc-210), PKCδ (sc-937), PKCe (sc-214), and PKCζ (sc-216) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. PKCθ was detected using Omni-probe (Xpress tag) (Santa Cruz Biotechnology, sc-7270). HA-tagged ubiquitin was detected using a high affinity rat HA antibody (Roche Applied Science, 1 867 423). Endogenous ubiquitination was detected using a ubiquitin antibody (Covance, MMIs-258R), and Myc-tagged PDK-1 was detected with a Myc antibody (Covance, PRB-150P). Immunoprecipitation of PKCα and PKCβII was performed using an antibody generated against a region common to PKCα and PKCβII (BD Transduction Laboratories, 610108). A monoclonal antibody directed toward Hsp70 was also obtained from BD Transduction Laboratories (610607). The monoclonal Pin1 antibody was a gift from Kun Ping Lu. The polyclonal Pin1 antibody (3722) and the antibody detecting phosphorylated PKCβII/α (Ser660/663; 9371) were from Cell Signaling Technology, Inc. The γ-tubulin (T6074) and β-actin (A2066) antibodies were used.
from Sigma. Phorbol 12,13-dibutyrate (PDBu; 524390), MG-132 (474970), and diethyl-1,3,6,8-tetrahydro-1,3,6,8-tetraoxabenzo[imn][3,8]phenanthroline-2,7-diacetate (PiB; 529627) were obtained from Calbiochem. Protein A/G beads were purchased from Pierce (53133), and glutathione-Sepharose beads were from Amersham Biosciences (17-0756-01). EasyTag Expre<sup>35S</sup>S<sup>35</sup>S (1000 Ci/mmole) protein labeling mixture was purchased from PerkinElmer Life Sciences. N-Ethylmaleimide (E1271) and bombesin (B-126) were purchased from Sigma. 1-Oleoyl lysophosphatidic acid (LPA) was obtained from Cayman Chemical (62215).

**Cell Culture and Transfection**—COS7 cells, HeLa cells, 293T cells, Pin1+/-, and Pin1−/− mouse embryonic fibroblasts (MEFs; a gift from Kun-Ping Lu) were cultured in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin (MEFs; a gift from Kun-Ping Lu) were cultured in DMEM containing each time by centrifugation at 3000 × g for 10 min, thereafter washed three times in 1 ml of lysis buffer, pelleted by incubating with rotation overnight at 4 °C. Complexes were then allowed to form by incubating with rotation overnight at 4 °C. Complexes were thereafter washed three times in 1 ml of lysis buffer, pelleting each time by centrifugation at 3000 × g for 10 min (except for the PDBu-stimulated down-regulation experiments for which whole cell lysates were used), proteins were resolved by SDS-PAGE, transferred to PVDF, and visualized by immunoblotting. For immunoprecipitation, 1 μg of antibody and 30 μl of protein A/G beads were added to the cleared lysates. Immune complexes were then allowed to form by incubating with rotation overnight at 4 °C. Complexes were thereafter washed three times in 1 ml of lysis buffer, pelleting each time by centrifugation at 3000 × g for 3 min at 4 °C. GST pulldowns were performed in a similar way. However, instead of adding antibodies, 30 μl of washed GST-Sepharose beads (50:50 slurry) were added to the lysates followed by incubation overnight. Complexes were washed as described above.

**Cell Stimulation and in Vivo Ubiquitination Assay**—Cells were stimulated using 200 nM PDBu for the indicated times to induce ubiquitination or down-regulation and lysed in lysis buffer as described above. For ubiquitination assays using exogenous ubiquitin, COS7 cells in 6-well plates were transfected with 0.5 μg of cDNA encoding the indicated PKC isoform and 1.5 μg of cDNA 3HA-ubiquitin (Lys<sup>48</sup>-ubiquitin or Lys<sup>63</sup>-ubiquitin). For detection of endogenous ubiquitination, COS7 cells were transfected with the PKC isoform of interest. Sixteen to 24 h post-transfection, cells were stimulated with bombesin (10 nM) or LPA (10 μM) in the presence of the proteasome inhibitor MG-132 (10 μM). Cells were washed in ice-cold PBS and lysed on ice for 15 min in lysis buffer supplemented with N-ethylmaleimide (10 mM). Relevant proteins were immunoprecipitated from cleared lysates as described above. For ubiquitin experiments in the presence of the Pin1 inhibitor, cells were pretreated with PiB (10 μM) for 2 h and thereafter treated with bombesin (10 nM) or LPA (10 μM) and MG-132 (10 μM) as described above. For detection of endogenous ubiquitination in MEFS, confluent 10-cm dishes containing either Pin1−/− MEFS or control MEFS were used. Cells were stimulated with PDBu (200 nM) or LPA (10 μM) for 1 h in the presence of MG-132 (10 μM) and lysed in lysis buffer supplemented with N-ethylmaleimide (10 μM). Thereafter, PKC was immunoprecipitated as described above.

**Autosport Peptide Array**—Peptide arrays were synthesized on nitrocellulose membranes using a MultiPep automated peptide synthesizer (INTAVIS Bioanalytical Instruments AG) as described (45). The peptide-containing membranes were activated in methanol and washed in distilled water and then in PBS with 0.05% Triton. Membranes were blocked in 5% milk and overlaid with purified His-Pin1 (1 μM). Bound Pin1 was subsequently detected using a horseradish peroxidase-conjugated anti-His antibody.

**siRNA**—Nineteen-nucleotide siRNAs targeting Pin1 (NCBI Reference Sequence NM_006221.3) were designed based on the algorithm developed by Amazrguoi and Prydz (46). The two 19-nucleotide sequences scoring best (siRNAs 1 and 2) for each sequence were ordered as preannealed duplexes from Dharmacon and carried 3’-dTdT overhangs. Duplex 3 was ordered from Integrated DNA Technologies and contained a 3’ overhang similar to the RNA region it was targeted against. The sequences for the sense strand of the siRNAs are as follows: 1, 5’-GGCCUAACAGAAAGUAAC-3’; 2, 5’-G GCCACAA-UCACGGACUU-3’; 3, 5’-UCAGCCGGAGUGUACAU-3’. The non-targeting control duplex was from Dharmacon (D-001210-01-20).

**Pulse-Chase and Kinase Assay**—For pulse-chase assays, cells were first incubated in Met/Cys-free DMEM for 30 min and then labeled with [35S]Met/Cys (0.1 mCi/ml) for 7 min. Cells were then chased in unlabeled Met/Cys for the times indicated in the figure legends. PKC was immunoprecipitated overnight and analyzed by SDS-PAGE and autoradiography as described (41). PKC activity assays were performed as described previously (43). Briefly, whole cell lysates (containing endogenous PKC) were diluted in buffer containing 20 mM Hepes, 0.1% Triton, 2 mM DTT, and 1 mM PMSF and incubated with 500 μM [γ-<sup>32</sup>P]ATP (0.1 mCi/mol), 25 mM MgCl<sub>2</sub>, and 50 μg/ml peptide substrate (Ac-FKKSFL-NH<sub>2</sub>) in the presence or absence of phosphatidylycerine/diacylglycerol vesicles (140 μM/3.8 μM) and 0.1 mM CaCl<sub>2</sub> at 30 °C for 5 min. The Ca<sup>2+</sup>/lipid-dependent activity was normalized to PKCα levels determined by Western blot analysis of the lysates.

**RT-PCR**—Total RNA was extracted from Pin1+/- and Pin1−/− MEFS stimulated for 24 h with PDBu using a Qiagen RNEasy kit according to the manufacturer’s instructions. The RNA concentration was measured on a Nanodrop ND-1000 spectrophotometer (Thermo), and equal amounts of RNA were used in RT-PCRs using a Qiagen OneStep RT-PCR kit (according to the manufacturer’s instructions), an annealing temperature of 55 °C, 25 cycles, and the following primers: mouse PKCα 1 forward, 5’-TGAAGACCCAAATCATTCCGC-3’; mouse PKCα 1 reverse, 5’-ACGAACCTATGCGACCTTCTTTA-3’; mouse PKCα 2 forward, 5’-AGAGGTGCACTTAGTT-
CGTTA-3'; mouse PKCa 2 reverse, 5'-GGCTTCCGTATGTGTGGATTTT-3'; mouse hypoxanthine-guanine phosphoribosyltransferase forward, 5'-GATTAGCGATGATGAACCA-3'; mouse hypoxanthine-guanine phosphoribosyltransferase reverse, 5'-GGCTTCCGTATGTGTGGATTTT-3'. PCR products were run on 2% agarose gels.

Prolines are indicated in yellow.

RESULTS

C Terminus of Conventional and Atypical PKCs Contain Conserved Pin1 Isomerization Site—We have shown previously that the phosphorylation state of the C-terminal phosphorylation sites in PKCβII, the turn motif (Thr641) and the hydrophobic motif (Ser660), function as determinants allowing specific protein interactions that regulate the maturation, stability, and function of PKCβII (21, 41). Curiously, we found that C-terminal binding partners discriminated between PKC that had never been phosphorylated (newly synthesized) and PKC that had matured and subsequently been dephosphorylated. Specifically, PDK-1 preferentially binds the C terminus of a PKC species that has never been phosphorylated (21), whereas Hsp70 preferentially binds the C terminus of a PKC species that has been dephosphorylated (41). Therefore, we reasoned that the conformation of the C terminus changes following phosphorylation/dephosphorylation. We noted that the conserved turn phosphorylation motif of all PKC isoforms except the novel isoforms ε, δ, and θ contains a Thr followed by a Pro (Fig. 1, residues marked in light blue and yellow, respectively). Given the selectivity of the peptidyl-prolyl cis-trans isomerase Pin1 for phosphorylated (Ser/Thr)-Pro motifs (3, 47), we hypothesized that Pin1 could regulate the transition between the various conformations that PKCβII is known to adopt during its life cycle.

Pin1 Binds C Terminus of Conventional PKC Isozymes—To test our hypothesis that Pin1 could account for the finding that the C termini of never-phosphorylated and dephosphorylated PKC differ, we explored whether Pin1 could recognize the C termini of PKC isoforms that have a Pro immediately following the phosphoacceptor Thr at the turn motif (Fig. 1). We designed and synthesized a peptide array containing overlapping 18-residue peptides covering the entire C-terminal domain of conventional PKCβII or novel PKCδ. One set of peptides had a non-phosphorylated residue at the turn motif (TM-Thr strip) and a second set had a phosphorylated residue (TMM-P-Thr strip). Overlay of the peptide array with bacterially expressed His-Pin1 revealed strong interactions with peptides covering a region between the turn motif and hydrophobic motif of PKCβII (residues 640–663; peptides marked in red), but no detectable interaction with corresponding peptides from the C-terminal tail of PKCβII was observed (Fig. 2, A and B). Interestingly, these results indicate that Pin1 might bind to an unconventional site in PKC rather than the typical (Ser(P)/Thr(P))-Pro motifs (3, 47), as expected that Pin1 could regulate the transition between the various conformations that PKCβII is known to adopt during its life cycle.

**FIGURE 1.** C termini of conventional and atypical PKCs contain conserved Pin1 isomerization site. A sequence alignment of the C-terminal segments of protein kinase C isozymes is shown. NCBI Reference Sequences for human PKC isoforms are from the University of California, Santa Cruz genome browser (α, NM_002737; β/βII, NP_002738; γ, NM_002739; δ, NM_002740; ζ, NM_002744; ε, NM_005400; δ, NM_006254; θ, NM_006257). Protein sequences were aligned using ClustalW. The conserved turn motif and hydrophobic motif phosphorylation sites are indicated in light blue, and the turn motif prolines are indicated in yellow.
consecutive position in the peptide ("Ala scan"; Fig. 2D). The top peptide in the scan is the wild-type sequence and reproduces the binding of Pin1 to the C-terminal sequences in the original array (Fig. 2A). The interaction with the wild-type peptide was abolished upon Ala replacement of a number of key residues (residues boxed in light gray; also marked with a red
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**Pin1 Affects Interactions between PKCβII and PDK-1 but Not Hsp70**—To determine whether Pin1 helps to control the conformational changes that occur as PKC matures, we assessed whether it affects the interactions of PKC with binding partners that discriminate based on its conformation. PDK-1 interacts with unphosphorylated, newly synthesized PKC, whereas Hsp70 prefers dephosphorylated PKC. Thus, we investigated whether Pin1-mediated isomerization could provide the “molecular memory” that controls the history-dependent association of PDK-1 and Hsp70 with PKC. To address this, we tested whether depletion of Pin1 affected the binding of these two proteins to PKC. We expressed PKCβII and Myc-PDK-1 together with control siRNA or Pin1-specific siRNA in HeLa cells, immunoprecipitated PKCβII from the lysate, and analyzed its interaction with PDK-1 or Hsp70 by Western blot. In control cells expressing endogenous Pin1, we observed basal interactions among PKCβII, PDK-1, and Hsp70 (Fig. 3A, lane 1). Knockdown of Pin1 caused an ~8-fold increase in the amount of PDK-1 bound to PKCβII (Fig. 3A, PDK-1 blot, lane 2). In contrast, knockdown of Pin1 had no significant effect on the interaction between Hsp70 and PKCβII (Fig. 3A, Hsp70 blot, lane 2). Because Hsp70 itself has Pro isomerase activity (48), it may not discriminate between cis and trans conformers of the turn motif. However, our data clearly establish that the binding of PDK-1 to PKC is dramatically increased in cells lacking Pin1, consistent with the model that isomerization of the turn motif by Pin1 provides the molecular memory that allows PDK-1 to discriminate between never-phosphorylated and dephosphorylated PKC.

**Pin1 Distinguishes between Mature and Immature PKCs**—Given that Pin1 controls interactions that are dependent on the conformational state of PKC, we asked whether the PKC-Pin1 interaction was itself conformation-dependent. To discriminate between phosphorylated, dephosphorylated, and unphosphorylated (i.e. never-phosphorylated) PKC, we used wild-type PKCβII (phosphorylated and dephosphorylated) and kinase-dead (K371R) PKCβII (unphosphorylated). Phorbol esters trigger the dephosphorylation of mature, fully phosphorylated PKCβII. On the other hand, kinase-dead PKCβII (K371R) is never phosphorylated and hence not dephosphorylated after PDbu treatment. COS7 cells were co-transfected with GST-Pin1 and either wild-type PKCβII or PKCβII-K371R, and the interaction between Pin1 and the kinase was monitored following phorbol ester treatment of cells (Fig. 3B). In the absence of PDbu, wild-type PKCβII migrated as a single band on SDS-PAGE corresponding to the migration position of kinase quantitatively phosphorylated at the two C-terminal sites (Fig. 3B, lower panel, lane 1; the fully phosphorylated species is indicated with a double asterisk). PDbu treatment resulted in the accumulation of a faster migrating species corresponding to PKC dephosphorylated at both C-terminal sites (Fig. 3B, lower panel, lanes 1–6) (37). GST pulldown experiments revealed a dra-

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**FIGURE 2. Conventional PKC and Pin1 interact via determinants in PKC C-terminal hydrophobic motif.** A and B, His-Pin1 was overlaid on an array of 18-mer peptides derived from the C-terminal tail of rat PKCβII (A) or PKCβI (B) with either a non-phosphorylated residue at the turn motif (Thr<sup>641</sup> or Ser<sup>643</sup>); indicated by an asterisk) or a phosphorylated residue at the turn motif (Thr(P)<sup>641</sup> or Ser(P)<sup>643</sup>); and binding was detected as described under “Experimental Procedures.” The peptide sequences used in the peptide array are indicated to the right of the blot; each peptide shares 16 amino acids in common with the next peptide in the array. Sequences in red indicate peptides that bound strongly to Pin1; sequences in green indicated peptides that only displayed significant binding to Pin1 when the turn motif Thr was phosphorylated. C, GST pulldowns and lysates from COS7 cells expressing GST alone (lane 1) or the GST-tagged C-terminal region of PKCβII (lane 2) in combination with HA-Pin1 were analyzed by Western blotting (WB) using GST and HA antibodies. D, an “Ala scan array” comprising the region in PKCβII found to interact with Pin1 (from A; residues 639–663 in rat βII with Ala substitutions at each position) was incubated with pure His-Pin1, and the amount of binding was determined using the chemiluminescent signal from an HRP-conjugated anti-His antibody. The residue in the original peptide (top) replaced with Ala in the scan is indicated in red. Residues required for the interaction with Pin1 are indicated in light gray boxes in the sequence on the right and with red asterisks on the left of the strip; those that influence the interaction but are not strictly required are boxed in yellow and indicated with a gray asterisk on the left of the strip. E, HeLa cells were transfected with wild-type PKCβII and GST (lane 1), wild-type PKCβII and GST-Pin1 (lane 1), the TP motif mutant PKCβII-P642A and GST-Pin1 (lane 3), or the hydrophobic motif mutant PKCβII-F66S/L-F659A and GST-Pin1 (lane 4). Cells were lysed 24 h post-transfection, and the resulting cleared lysates were subjected to PKCβII immunoprecipitation (IP). Pin1 that co-immunoprecipitated with PKCβII was detected using a GST antibody. F–H, COS7 cells were transfected with conventional (F), atypical (G), and novel (H) PKC isoforms in combination with GST or GST-Pin1 as indicated. Lysates and GST pulldowns were subsequently analyzed for the presence of GST and the various PKC isoforms as described under “Experimental Procedures.”
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**Figure 3.** PKC binding partners PDK-1, Hsp70, and Pin1 distinguish between newly synthesized (never-phosphorylated) and dephosphorylated forms of PKC. A, HeLa cells were transfected with control siRNA or Pin1 siRNA in combination with PKCβII and PDK-1. Twenty-four hours later, the cells were lysed, and PKCβII was immunoprecipitated (IP) from the lysates. Both immunoprecipitates and lysates were analyzed for PDK-1, Hsp70, and Pin1. PDBu-mediated dephosphorylation at Thr641 was sufficient to trigger the PDBu-mediated action of Pin1 with two phosphorylation mutants of PKCβII: PKCβII-S660A and PKCβII-T641AAA (this construct is mutated at Thr641, Thr643, and Ser645 in the turn motif region to prevent compensating autophosphorylations (27)). PKCβII-S660A is processed by phosphorylation, so a mature species phosphorylated only at the turn motif (Thr641) accumulates (Fig. 3D, lower panel, lane 1, double asterisk). Under the conditions of our experiments, ~70% of the S660A construct was phosphorylated at Thr641 (Fig. 3D, double asterisk), and ~30% was not phosphorylated (dash). Both species were weakly pulled down by GST-Pin1 (Fig. 3D, lane 1). Treatment with PDBu resulted in an accumulation of faster migrating, dephosphorylated PKC (Fig. 3D, Lysate, lane 2). This species bound with significantly higher affinity to Pin1 than either the phosphorylated PKC in the same sample or the unphosphorylated PKC present prior to PDBu treatment (Fig. 3D, pull-down). Thus, the S660A construct had the same sensitivity to phorbol esters as the wild-type enzyme, revealing that dephosphorylation at Thr641 was sufficient to trigger the PDBu-mediated high affinity interaction between Pin1 and PKCβII (Fig. 3D, pull-down).
FIGURE 4. Pin1 increases susceptibility of conventional but not novel PKC isozymes to ubiquitination. A, Pin1+/+ MEFs or Pin1−/− MEFs were left untreated or stimulated with PDBu in the presence of the proteasome inhibitor MG-132 for 1 h. PKCα was immunoprecipitated (IP), and endogenous ubiquitination was detected by Western blotting (WB). Immunoprecipitates were subsequently reprobed with a PKCα antibody. B, COS7 cells were transfected with PKCβII and HA-ubiquitin in combination with GST or with GST-Pin1. The day after transfection, cells were treated with PDBu in the presence of MG-132 for 1 h and lysed. PKCβII was immunoprecipitated and analyzed for ubiquitination by Western blotting using HA antibodies. Lysates were also analyzed for Pin1 (GST), PKCβII, and γ-tubulin (as a loading control). Pin1+/+ MEFs or Pin1−/− MEFs were left untreated or stimulated with LPA in the presence of the proteasome inhibitor MG-132 for 1 h. C, MEFs were treated as in A and examined for ubiquitination of endogenous PKCδ. D and E, PKCα (D) or PKCδ (E) was immunoprecipitated, and endogenous ubiquitination was detected by Western blotting. Immunoprecipitates were subsequently reprobed with antibodies to PKCα or PKCδ.

pull-down, PKCβIII blot). In contrast, analysis of GST pull-downs from cells expressing PKCβIII-T641AAA showed no dramatic change in the interaction with Pin1 after PDBu treatment (Fig. 3D, lanes 3 and 4). This result is consistent with our previous observation that Pin1 selectively binds PKCβII that has been dephosphorylated compared with PKC that has never been phosphorylated (Fig. 3B). We also noted that overexposure of blots from such experiments revealed a clear laddering/smeared pattern for PKC following phorbol ester treatment (data not shown), suggesting that the PKC interacting with Pin1 after PDBu treatment may be ubiquitinated.

Pin1 Controls Ubiquitination of PKCα and PKCβII but not PKCδ—We next explored whether Pin1 regulates the ubiquitination of PKC in cells. First, we examined the PDBu-stimulated ubiquitination of endogenous PKCα and PKCδ in control MEFs compared with that in Pin1-deficient MEFs (49). We saw a robust increase in the ubiquitination of PKCα immunoprecipitated from Pin1+/+ but not Pin−/− MEFs following PDBu treatment (Fig. 4A). In contrast, PDBu-induced ubiquitination of the novel isozyme PKCδ was comparable in control MEFs and Pin1−/− MEFs (Fig. 4C). PDBu did not induce the ubiquitination of the atypical PKCζ, an isozyme that cannot bind phorbol esters (data not shown). To explore the physiological relevance of the Pin1-induced ubiquitination, we explored whether the natural agonist LPA induces the ubiquitination of conventional and novel PKC isozymes in a manner that depends on Pin1. PKCα or PKCδ was immunoprecipitated from control or Pin1-deficient MEFs that had been treated with vehicle or LPA and the proteasome inhibitor MG-132 for 1 h. Both PKCα and PKCδ became ubiquitinated following LPA treatment of control MEFs (Fig. 4, D and E, lanes 3 and 7). Interestingly, the LPA-triggered ubiquitination of PKCα, but not PKCδ, was markedly reduced in Pin1−/− cells compared with control cells (Fig. 4, D and E, compare lanes 3 and 4 and lanes 7 and 8). In addition, overexpression of Pin1 significantly increased the exogenous PDBu-mediated ubiquitination of PKCβIII compared with cells in which PKCβII was expressed with GST alone (Fig. 4B, lane 4 and lane 2, respectively). We also observed that increasing the amount of Pin1 expression reduced the expression of PKCβIII (Fig. 4B, βIII blot, lanses 1 and 3). Our results reveal that Pin1 is required for the efficient ubiquitination of the conventional PKCα, but not the novel PKCδ, following stimulation of cells with either phorbol esters or a natural G-protein-coupled receptor agonist.

Inhibition of Catalytic Activity of Pin1 Interferes with Bombesin-stimulated Ubiquitination of Conventional PKC Isozymes—To test whether the catalytic activity of Pin1 is required for the ubiquitination of conventional PKC isozymes, we explored whether agonist-induced ubiquitination was sensitive to the Pin1 inhibitor PiB (50). COS7 cells overexpressing conven-
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Inhibition of catalytic activity of Pin1 interferes with bombesin-stimulated ubiquitination of conventional but not novel PKC isozymes. A and B, COS7 cells transfected with PKC\(\alpha\) (A) or PKC\(\delta\) (B) were pretreated with vehicle or the Pin1 inhibitor PiB for 2 h and thereafter stimulated with bombesin in the presence of MG-132 for 0, 30, or 60 min. The overexpressed PKC isozyme was immunoprecipitated (IP), and the amount of the isozyme and degree of ubiquitination were analyzed by Western blot (WB). C–E, COS7 cells were transfected to express wild-type PKC\(\beta\)II (C), PKC\(\beta\)II-P642A (D), or PKC\(\beta\)II-F656A/F659A (E). Approximately 24 h after transfection, the cells were pretreated with vehicle or PiB for 2 h and then stimulated with bombesin in the presence of MG-132 for 0, 30, or 60 min (‘) to induce ubiquitination. Each PKC mutant was immunoprecipitated and analyzed for endogenous ubiquitination and total PKC\(\beta\)II levels. F, COS7 cells overexpressing PKC\(\alpha\) and either HA-Lys48-ubiquitin or HA-Lys63-ubiquitin were stimulated with bombesin for the indicated times, immunoprecipitated, and analyzed for ubiquitination by probing Western blots with an anti-HA antibody. G and H, COS7 cells were co-transfected with PKC\(\alpha\) and HA-Lys48- or HA-Lys63-ubiquitin (Ubiq) and analyzed as in G.

Note that bombesin treatment of MEFs did not induce ubiquitination of any PKC isoforms; it is not known whether fibroblasts express bombesin receptors.

These data reveal that the prolyl isomerase activity of Pin1 is required to convert the conventional PKC isoforms \(\alpha\) and \(\beta\)II into readily ubiquitinated species. In contrast, ubiquitination of PKC\(\delta\), which lacks Pin1 binding and the residues necessary for isomerization, occurs independently of Pin1.

Next, we mutated residues in PKC\(\beta\)II identified as essential for Pin1 binding or isomerization and asked how mutation of these residues affected agonist-evoked ubiquitination of PKC. Specifically, we tested the effect of Pin1 inhibition on the bombesin-evoked ubiquitination of wild-type PKC\(\beta\)II, PKC\(\beta\)II-P642A (loss of isomerizable residue), and PKC\(\beta\)II-F656A/F659A (loss of Pin1 binding). PiB strongly inhibited the bombesin-triggered ubiquitination of wild-type PKC\(\beta\)II (Fig. 5C) but had little effect on the ubiquitination of the P642A mutant (Fig. 5D). Bombesin triggered significant ubiquitination of PKC\(\beta\)II-F656A/F659A as well, but importantly, this ubiquitination was relatively insensitive to Pin1 inhibition (Fig. 5E) compared with that of the wild-type enzyme. These data are...
consistent with Pin1 binding to the hydrophobic motif being essential for isomerization of the turn motif. Furthermore, disruption of the interaction between Pin1 and PKC abolishes the sensitivity of agonist-evoked ubiquitination to Pin1 inhibition. Note that the high agonist-evoked ubiquitination of the P642A and F656A/F659A mutants likely results from decreased protein stability of these constructs.

We next addressed the nature of the bombesin-induced ubiquitin linkage on conventional PKC isozymes. The functional outcome of polyubiquitination of proteins depends on the lysine utilized in the formation of the ubiquitin chain (51). Typically, substrates destined for degradation through the proteasomal pathway are targeted with a ubiquitin chain in which at least four successive ubiquitins are linked together through an isopeptide bond between Lys48 in the last ubiquitin in the chain and the C-terminal Gly (Gly76) in the new ubiquitin molecule (52). Ubiquitin chains can also be formed through conjugation to Lys63 in the ubiquitin molecule. It has been suggested that the latter type of ubiquitin chain is not a target for proteasomal degradation but rather acts as a signal in several non-degradative processes in a cell (53). It is currently not known what type(s) of ubiquitin chain becomes attached to non-degradative processes in a cell (53). It is currently not known whether Pin1 affects the rate of degradation of these isozymes.

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Specifically, we examined the effect of Pin1 knockdown on the basal protein stability of conventional PKC isozymes. Because Pin1 regulates the ubiquitination of PKCα and PKCβII in response to natural agonists, we investigated whether Pin1 affects the rate of degradation of these isozymes.

Specifically, we examined the effect of Pin1 knockdown on the levels of PKCα, PKCβII, PKCζ, and PKCδ following cycloheximide treatment. HeLa cells were treated with control siRNA or Pin1 siRNA for 72 h to induce efficient Pin1 knockdown. Thereafter, cells were treated with cycloheximide to prevent new protein synthesis and harvested at the time points indicated in Fig. 6A. Under conditions of efficient knockdown of Pin1 (Fig. 6A, top two panels), we observed the following effects on PKC isozymes: PKCα, PKCβII, and PKCζ were considerably less stable in Pin1-expressing cells compared with Pin1 knockdown cells, whereas PKCδ was equally stable in the presence or absence of Pin1. Quantitation of Western blots from Fig. 6A (see graphs) revealed that PKC degradation was ~3-fold slower in Pin1 knockdown cells compared with control cells: about 50% of both PKCα and PKCβII was degraded in 3 h in control cells compared with 9 h in Pin1 knockdown cells, and PKCζ was degraded with a half-life of ~7 h in control cells compared with 12 h in knockdown cells. In striking contrast, Pin1 knockdown had no detectable effect on the rate of degradation of PKCδ: the half-life of PKCδ was 6 h in control and knockdown cells. This result is consistent with the finding that Pin1 does not bind PKCδ (Fig. 2H) or alter its ubiquitination state (Fig. 4, A and B).

We next examined whether Pin1 affects the synthesis or maturation of conventional PKCs using cells depleted of Pin1. Newly synthesized conventional PKCs are processed by a series of ordered phosphorylations that can be visualized by a mobility shift in pulse-chase experiments. The first phosphorylation by PDK-1 does not cause a mobility shift, whereas the phosphorylations at the two C-terminal positions do (37). To address the effects of Pin1 on the maturation of PKCβII, cells were pulsed with 35S-labeled Met/Cys for 7 min to label the unphosphorylated, newly synthesized pool of PKC. After this, cells were chased in medium containing unlabeled Met/Cys for 10, 30, or 60 min to monitor the mobility shifts accompanying C-terminal autophosphorylation of the newly synthesized PKC. The autoradiogram showing immunoprecipitated PKC from such pulse-chase experiments revealed that endogenous PKCβII in HT1080 cells was processed at a comparable rate in control cells (Fig. 6C, lanes 1–4) and in cells where Pin1 was knocked down (24 h after transfection) (Fig. 6C, lanes 5–8): the ratio of phosphorylated (double asterisk) to unphosphorylated PKC (dash) was similar at all chase points (Fig. 6C, compare e.g. lanes 3 and 7) with a half-time of processing on the order of 30 min.

Given that Pin1 depletion decreases the rate of conventional PKC degradation without affecting synthesis, we hypothesized that the steady-state levels of conventional PKCs would be increased in these cells. Indeed, in HeLa cells depleted of Pin1, the levels of PKCα and PKCβII were increased by ~50% at 24 h after transfection (Fig. 6B). We validated the decrease in conventional PKC expression induced by Pin1 by overexpressing increasing amounts of GST-tagged Pin1 in COS7 cells and examining the effects on PKCβII levels. At the highest ratio of GST–Pin1 to PKCβII (Fig. 6D, lane 5), Pin1 reduced the protein levels of PKCβII 5-fold to 21 ± 15% (Fig. 6E; average ± S.D., n = 7) of the levels in control cells transfected with PKCβII and GST alone. Taken together, these data suggest that Pin1 decreases the steady-state levels of conventional PKC isozymes by increasing their rate of degradation.

Finally, we assessed the effects of Pin1 depletion on the intrinsic catalytic activity of PKC by analyzing the specific activity of PKC in Pin1-deficient cells. Lipid-dependent activity was measured in lysates from control cells or Pin1 knockdown cells. Pin1 knockdown by siRNA did not significantly affect the lipid-dependent specific activity of PKC (Fig. 6F). In all kinase assays, activity in the lysate was normalized to the expression of PKCα. The specific activity of PKC from control MEFs and Pin1−/− MEFs was also the same (data not shown). These data reveal that Pin1 regulates the amount of PKC in cells and that the specific activity of this PKC is unaffected by the presence or absence of Pin1.
Pin1 Controls Down-regulation of Conventional PKC Isozymes

**A**

Control siRNA

Pin1 siRNA

PKCα

Pin1 siRNA

PKCβ1

Control siRNA

Pin1 siRNA

PKCβ2

Control siRNA

Pin1 siRNA

PKCδ

Control siRNA

Pin1 siRNA

γ-Tubulin

**B**

PKCβ1 (% control)

Control

Cycloheximide (h)

Knockdown

Control

Knockdown

PKCβ1 (% control)

Control

Cycloheximide (h)

Knockdown

Control

Knockdown

**C**

PKCβ1(IP)

Control siRNA

Pin1 siRNA

γ-Tubulin

**D**

PKCβ1

Control siRNA

Pin1 siRNA

γ-Tubulin

**E**

PKCβ1 (% control)

Control

PKCβ1

Pin1

**F**

PKC activity (relative units)

Control

PKCβ1

Pin1

PKCβ1

Pin1

PKCβ1

Pin1

PKCβ1

Pin1

PKCβ1

Pin1

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PKCβ1

Pin1
**Pin1 Controls Down-regulation of Conventional PKC**

**Pin1 Promotes Degradation of PKCα in Response to Prolonged Agonist Stimulation**—To assess possible mechanisms underlying the down-regulation of mature, phosphorylated PKC, we examined phorbol ester-stimulated PKCα degradation in the presence or absence of Pin1. In Pin1+/+ MEFs, prolonged stimulation with PDBu induced a decrease (70% ± 5% [average ± S.E.] at 24 h post-PDBu) in the protein levels of endogenous PKCα (Fig. 7, A and B), resulting from degradation of the dephosphorylated form of the protein. In MEFs lacking Pin1, however, the PDBu-induced decrease in PKCα protein levels was much less pronounced (30% ± 10% at 24 h post-PDBu; Fig. 7, A and B), suggesting that the absence of Pin1 rendered PKCα resistant to degradation. The rate of PDBu-induced degradation, reflecting the decrease in protein levels over time, was calculated from the data in Fig. 7B and found to be significantly lower in the Pin1−/− MEFs than in the Pin1+/+ MEFs (Fig. 7C; n = 7). Correspondingly, PKCα had a much longer half-life in the Pin1−/− MEFs (50 ± 20 h, average ± S.E.) than in the Pin1+/+ MEFs (16 ± 2 h, average ± S.E.).

To verify that the increase in PKCα protein levels observed in the Pin1−/− MEFs after PDBu stimulation was caused by changes to the protein rather than the mRNA, we examined PKCα protein and mRNA levels at 24 h after PDBu stimulation by Western blot and RT-PCR, respectively (Fig. 7, D–F). Although the protein levels of PKCα were increased in the Pin1−/− MEFs relative to the Pin1+/+ MEFs (Fig. 7F, light gray bars), there was no change in the mRNA levels (as assessed using two different primer sets; Fig. 7F, dark gray bars). These data again point to a specific role for Pin1 in controlling the degradation of conventional PKC proteins. Altogether, our data support a model in which Pin1 catalyzes the isomerization of the Thr-Pro bond from cis to trans, an event that converts conventional PKC isozymes into a form that is more readily dephosphorylated, ubiquitinated, and thus down-regulated.

**DISCUSSION**

Phosphorylation at the C-terminal turn motif of PKC is a constitutive step in the maturation of the enzyme (15). Here we show that this motif, along with the hydrophobic motif, also controls the termination of signaling by conventional PKC isozymes. Specifically, we show that the peptidyl-prolyl cis-trans isomerase Pin1 converts conventional PKC isozymes into species that are efficiently ubiquitinated following phorbol ester or agonist stimulation. Peptide array analysis reveals that Pin1 binds C-terminal determinants in the hydrophobic motif segment and that the interaction with the C terminus is additionally strengthened when the turn motif is phosphorylated. Our data support a model in which this interaction results in a cis/trans isomerization of the turn motif Thr-Pro peptide bond that converts PKC into a species that is more readily down-regulated.
regulated by dephosphorylation and ubiquitination: locking the Thr-Pro peptidyl bond in trans by replacing the turn motif Pro with Ala locks PKC into a readily down-regulated species, whereas inhibition of Pin1 catalytic activity retains PKC in a conformation that is not readily down-regulated. Thus, our data unveil a new model in which Pin1 regulates the conventional PKC isoforms by controlling a conformation-dependent switch in the C-terminal tail that terminates the lifetime of the enzyme.

**Consensus Thr-Pro of Turn Motif Is Pin1-controlled Timing Switch**—Biochemical studies have revealed that phosphorylation at the turn motif locks the conventional PKCα and PKCβII into catalytically competent, thermally stable species that are relatively resistant to dephosphorylation at all three processing sites (27, 54). Lack of phosphate at the turn motif shunts conventional PKC to the detergent-insoluble fraction where it is eventually degraded. We have also shown that the molecular chaperone Hsp70 can rescue PKC from phorbol ester-directed degradation: it specifically binds the dephosphorylated turn motif with data supporting a model in which it allows PKC to become rephosphorylated and re-enter the pool of signaling-competent enzyme (41, 42). Thus, the turn motif both controls the processing of conventional PKC by phosphorylation and following dephosphorylation recruits Hsp70 to sustain the signaling lifetime of the enzyme.

Our data unveil an added level of complexity to regulation by turn motif phosphorylation: Pin1-catalyzed cis/trans isomerization converts conventional PKC into a species that is efficiently ubiquitinated and degraded following activation. Pin1 interacts with and isomerizes proteins phosphorylated at Ser or Thr residues preceding a Pro (55). Consistent with this, our data reveal that Pin1 controls the ubiquitination of wild-type PKCβII whose turn motif phosphorylation site comprises the consensus TP sequence but not the construct in which the Pro is mutated to Ala (P642A) nor the novel isoform PKCδ whose turn motif phosphoacceptor site is not followed by Pro. Although the consensus binding site for Pin1 has been shown to comprise a (Ser(P)/Thr(P))-Pro motif, we show here that the phosphorylated PKCβII TP motif is not the only determinant of the interaction between Pin1 and PKC. Additional interactions with the segment around the hydrophobic motif control binding, revealing an unconventional, novel consensus for Pin1 interaction. Peptide array analysis identified a specific segment surrounding the hydrophobic phosphorylation motif that mediates binding to Pin1. Note that although many of the key residues in this segment identified in the array are conserved among conventional PKC isoforms a few are not. It is possible that weakening of one interaction is compensated by strengthening in another elsewhere in the segment.

The conversion of conventional PKC into a species that can be ubiquitinated also requires the catalytic activity of Pin1. First, we show that the Pin1 inhibitor PBl abolishes the agonist-induced ubiquitination of the Pin1-dependent isoforms PKCα and PKCβII but has no effect on the agonist-induced ubiquitination of the novel isoform PKCδ or the Pin1-independent PKCβII mutants P642A and F656A/F659A (Fig. 5, A–E). Thus, the effects of Pin1 depend on the intrinsic isomerase activity of the enzyme and the integrity of both the hydrophobic motif and the phosho-Thr-Pro consensus sequence at the turn motif.

Analysis of the solved structure of the PKCβII kinase core suggests a structural basis for how Pin1-catalyzed isomerization of the Thr-Pro peptide bond of the turn motif can convert PKC into a species that is readily ubiquitinated (56). In the solved structure, the Thr-Pro bond is in trans, resulting in the phosho-Thr at position 641 being relatively solvent-exposed. Consistent with exposure, this site is sensitive to dephosphorylation when mature PKCβII is in the active, membrane-bound, and “open” conformation (34). Ubiquitination follows dephosphorylation of PKC that has been activated. Molecular modeling of the Thr-Pro bond in cis reveals that the phosphate now pivots into the core of the protein, potentially forming an electrostatic interaction with Arg415 (Fig. 8A). Importantly, Arg415 is invariant among all PKC isozymes that have the Thr-Pro motif: it is present in conventional PKCα and PKCβII but is not present in novel PKCδ, which lacks the Thr-Pro sequence at the turn motif. This residue forms part of a basic binding pocket recently identified by Frödin and co-workers (57) as promoting a “zipper-like” association of the C-terminal tail turn motif and hydrophobic motif of AGC kinases with the upper lobe of the kinase core. Our data reveal that this “zipper-like” association is loosened by Pin1 through isomerization, exposing the phosphorylated C-terminal sites to dephosphorylation.

**Functional Effects of Pin1 on PKC**—Pin1 does not affect the rate of processing of conventional PKC by phosphorylation nor does it affect the catalytic activity of conventional PKC. Rather, Pin1 accelerates the agonist-evoked down-regulation of conventional PKC isoforms.

The novel isoform PKCδ does not contain the TP consensus motif and lacks several key residues in the hydrophobic motif important for PKCβII-Pin1 interaction. Consistent with the model that the TP motif is required for Pin1 to control the turn motif, Pin1 did not interact with PKCδ nor was the agonist-induced ubiquitination of PKCδ sensitive to Pin1 inhibition. Because novel isoforms lack the TP molecular timer, we would predict that they are synthesized in a conformation that is already degradation-sensitive. Consistent with this, Blumberg and co-workers (58) have shown that the rate of phorbol ester-mediated down-regulation of the novel PKC isoforms δ and ε is ∼5 times faster than that of the conventional PKCα.

There is precedent for Pin1 destabilizing signaling proteins as we report here for conventional PKC isoforms. The stability of c-Myc is also increased in cells lacking Pin1: protein expression and the half-life of c-Myc are increased in a manner analogous to our finding for conventional PKC (59). In contrast, Pin1 stabilizes several other proteins. p65 (RelA) levels are up-regulated, and the protein has increased nuclear accumulation when Pin1 levels are elevated (60). In addition, genotoxin-mediated accumulation of p73 (61) and p53 (62, 63) is impaired in Pin1-deficient cells. Thus, Pin1 controls conformational switches that regulate the lifetime of signaling molecules by both positive and negative mechanisms.

Pin1 has also been proposed to control the mitochondrial accumulation of the adapter protein p66Shc following phosphorylation by PKCβII (64). Furthermore, it was recently shown that Pin1 interacts with PKCα and that PKCα can phos-
phorylate Pin1 (65). Thus, Pin1 is emerging as a novel regulator of PKC signaling function both through directly controlling the lifetime of conventional PKC isozymes and by controlling the function of downstream targets of PKC.

**Pin1 Is a Molecular Timer in Life Cycle of Conventional PKC**—Fig. 8B presents a model for how cis/trans isomerization catalyzed by Pin1 controls the lifetime of conventional PKC isozymes. Our data are consistent with a model in which the peptidyl-prolyl bond at the turn motif of newly synthesized conventional PKC isozymes is in a cis conformation (Fig. 8B, Species 1). PDK-1 docks onto the C-terminal tail of newly synthesized PKC and phosphorylates the activation loop (Thr<sup>500</sup>).
rendering PKC catalytically competent (17, 18, 22). Upon dis- engagement of PDK-1 from the C terminus, PKC becomes rapidly phosphorylated on the turn motif, an event that depends on mTORC2 (23) and on the intrinsic catalytic activity of PKC. Phosphorylation of the turn motif is required to process func- tional PKC, and it is also the rate-limiting step in the matura- tion of PKC (19). Phosphorylation on the turn motif is followed immediately by intramolecular autoprophosphorylation of the hydrophobic motif (26) to yield the mature, activatable form of PKC. We suggest that Pin1 binds this fully phosphorylated and catalytically competent species via determinants surrounding the hydrophobic motif and via the phosphorylated Thr of the turn motif (Fig. 8B, Species 2). Upon PKC activation, Pin1 catalyze a cis to trans isomerization of the phospho-Thr-Pro bond of the turn motif, unmasking the phospho-Thr (see the mod- eled structure in Fig. 8A and see Fig. 8B, Species 3). This unmasking renders PKC much more sensitive to dephosphory- lation (Fig. 8B, Species 4) following agonist activation, result- ing in the ubiquitination and degradation of PKC. PKC can, however, be rescued from degradation: binding of Hsp70 to the phosphorylated turn motif recycles PKC into the pool of sig- naling-competent enzyme presumably by permitting the rei- somerization and phosphorylation of PKC (41, 42). Thus, isomerization of conventional PKC isoforms by Pin1 provides a conformation-dependent degradation switch: in the absence of Pin1, conventional PKC isoforms are trapped in a conforma- tion that cannot be efficiently ubiquitinated and down-regu- dated following agonist stimulation. This switch can be bypassed by locking the peptide bond after the phosphorylated turn motif Thr in trans as occurs in novel PKC isoforms where the Pro is absent or by mutagenesis of the PKCβII Pro to Ala as shown in this report.

Peptide array data revealed that Pin1 has a higher affinity for C-terminal peptides with phospho-Thr versus unphospho- lated Thr at the turn motif, but co-immunoprecipitation stud- ies revealed that Pin1 preferentially binds dephosphorylated PKC in cells. One possible explanation is that the C-terminal tail of full-length PKC is in a more exposed conformation when the turn motif and hydrophobic motif are dephosphorylated, thus favoring Pin1 binding. Similar results occur with the bind- ing of PDK-1: although it binds constructs of the C terminus that are phosphorylated at the hydrophobic motif with much higher affinity than unphosphorylated constructs, in the con- text of the full-length protein, it preferentially binds dephos- phorylated enzyme (21).

The Pin1-controlled timing switch on the turn motif of PKC provides a new level of complexity to the life cycle of the enzyme where down-regulation of conventional (but not novel) PKC isoforms requires a priming step by Pin1. It remains to be established whether the Pin1 step is itself regulated or part of the constitutive processing of PKC. Interestingly, isomerization of the TP motif provides a molecular mechanism to increase the degradation of PKC that is unique to the conventional PKC isoforms. Precise control of the amplitude of the PKC signal is critical to normal physiology, and dysregulation of the levels of conventional PKC isoforms accompany many diseased states, most notably cancer (66). Whether dysregulation of the Pin1-mediated down-regulation switch accounts for the elevated lev- els of PKC in these disease states also remains to be explored.

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