Partitioning-defective Protein 6 (Par-6) Activates Atypical Protein Kinase C (aPKC) by Pseudosubstrate Displacement

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Background: Many cell polarities are controlled by the Par complex, which includes Par-6 and atypical protein kinase C (aPKC). How is aPKC activity controlled?

Results: A pseudosubstrate motif within aPKC inhibits its activity. Par-6 displaces the pseudosubstrate and increases aPKC activity.

Conclusion: Par-6 activates aPKC.

Significance: Par-6 couples aPKC localization and activation to precisely control cell polarity.

Atypical protein kinase C (aPKC) controls cell polarity by modulating substrate cortical localization. Aberrant aPKC activity disrupts polarity, yet the mechanisms that control aPKC remain poorly understood. We used a reconstituted system with purified components and a cultured cell cortical displacement assay to investigate aPKC regulation. We find that aPKC is auto-inhibited by two domains within its NH2-terminal regulatory half, a pseudosubstrate motif that occupies the kinase active site, and a C1 domain that assists in this process. The Par complex member Par-6, previously thought to inhibit aPKC, is a potent activator of aPKC in our assays. Par-6 and aPKC interact via PB1 domain heterodimerization, and this interaction activates aPKC by displacing the pseudosubstrate, although full activity requires the Par-6 CRIB-PDZ domains. We propose that, along with its previously described roles in controlling aPKC localization, Par-6 allosterically activates aPKC to allow for high spatial and temporal control of substrate phosphorylation and polarization.

The Par complex functions to create distinct, polarized cortical domains by coupling phosphorylation to cortical release (7–9). Through mechanisms that are still being elucidated, the Par complex becomes polarized to one cortical domain and keeps other, cell type-specific factors, localized to an opposite cortical domain (10–12). The activity of aPKC is a key output of the Par complex because the association of substrates with the cortex can be modulated by phosphorylation (7–9). For example, in Drosophila neuroblasts the protein Miranda localizes to a cortical domain opposite the Par complex, and its polarization requires aPKC activity (8). Miranda associates with the cortex via its cortical localization domain, but once this domain is phosphorylated by aPKC it is released into the cytoplasm leading to their mutually exclusive localization. Cortical association of the protein Numb is also modulated by aPKC phosphorylation, both in Drosophila and polarized mammalian cells (9), suggesting that coupling of aPKC-mediated phosphorylation to cortical displacement may be a general mechanism for Par-mediated polarity.

That activity of aPKC must be maintained within a certain range. In humans, inappropriate aPKC activity is associated with epithelial tumors (5, 6). Ectopic aPKC activity in neuroblasts leads to massive overproliferation and concomitant loss of differentiated cells (4). In current models, aPKC activity is controlled by a complex set of protein-protein “scaffolding” interactions (11, 13). In particular, Par-6 is thought to repress aPKC (7, 14, 15), suggesting that aPKC may have a high level of constitutive activity. Par-6 repression of aPKC is thought to be important in Drosophila sensory organ precursor (SOP) cells where aPKC activity is highly dynamic during mitosis (7). Early during SOP division, aPKC is held in an inactive complex along with Par-6 and the tumor suppressor Lethal giant larvae (Lgl). Activation is proposed to occur by phosphorylation of the Par-6 PB1 domain by the mitotic kinase Aurora A. Because the PB1 domain is the interaction site with aPKC (16–18), Par-6 dissociates from aPKC allowing Lgl to be phosphorylated and released from the complex. Finally, the Par complex member Baz becomes engaged and aPKC becomes fully activated. The mechanism by which aPKC activity is maintained within appropriate levels by dynamic scaffolding interactions has been unclear.
Par-6 Activation of aPKC

Although protein-protein interactions are thought to regulate aPKC, the precise mechanisms that control catalytic activity are unknown. All PKC isoforms contain NH2-terminal domains that are potentially important for controlling the activity of the COOH-terminal kinase domain (19). These domains include the aPKC-specific PB1 that binds Par-6 (18) and the C1 domain that binds lipid cofactors such as diacylglycerol in other PKC family members (20, 21), but whose function in aPKCs is unknown (22). PKCs also contain a short pseudosubstrate motif that resembles a true substrate but lacks a phosphorylatable residue, making it capable of acting as a competitive inhibitor. In other PKC isoforms the pseudosubstrate autoinhibits catalytic activity, but its role in regulating aPKC is unclear. In this work, we explore the interplay between internal aPKC regulatory elements and the protein-protein interactions that are thought to control aPKC activity during cell polarization.

**EXPERIMENTAL PROCEDURES**

**Purification of aPKCs and aPKC/Par-6 Complex**—HEK293 F cells were transfected with pCMV His6-aPKC constructs for expression of individual aPKC variants or co-transfected with pCMV His6-Par-6 and pCMV aPKC (no His tag) for expression of the aPKC/Par-6 complex using the 293fectin transfection reagent (Life Technology). The cells were incubated at 37 °C for 72 h followed by sonication and centrifugation at 15,000 rpm for 30 min at 4 °C. Ammonium sulfate was added to the supernatant to a final concentration of 45% (w/v) and incubated at 4 °C for 30 min. The resulting precipitate was collected by centrifugation at 15,000 rpm for 30 min at 4 °C and resuspended in 50 mM NaH3PO4, 300 mM NaCl, 10 mM imidazole, adjusted to pH 8.0 with NaOH. The resuspended precipitate were incubated with Ni2+-nitrioltriacetic acid resin at 18 °C overnight. The bacterial lysate was incubated with GST-agarose (Sigma-Aldrich) at 4 °C for 15 min. For the complex with Par-6, the proteins were further purified by ion exchange.

**Expression and Purification of Lgl and Baz**—Full-length and residues 647–673 of Drosophila Lgl isoform A and Baz residues 905–1221 of isoform A (constituting the aPKC binding region) were cloned into pMAL-C2 vector (New England BioLabs), in which a tobacco etch virus protease recognition site was added following the MBP coding sequence. The residues Ser656 and Ser660 in Lgl 647–673 were mutated to alanines. The constructs were transformed into BL21 Escherichia coli cells. The expressions were induced by 0.4 mM isopropyl 1-thio-β-D-galactopyranoside at 18 °C overnight. The bacterial lysates were incubated with amylose resins (New England BioLabs). The resins were washed with MBP lysis buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM DTT). The MBP fusion proteins were eluted with MBP elution buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 5 mM maltose) and dialyzed at 4 °C overnight in 20 mM Tris-HCl, pH 7.5, 50 mM NaCl. The affinity-purified proteins were further purified by ion exchange.

**Affinity Chromatography “Pulldown” Binding Assays**—GST pulldowns were as described previously (23). Briefly, Drosophila aPKC 120–141 was cloned into pGEX-4T1 (GE Healthcare) which was transformed into E. coli strain BL21. Protein expression was induced by 0.4 mM isopropyl 1-thio-β-D-galactopyranoside at 18 °C overnight. The bacterial lysate was incubated with GST-agarose (Sigma-Aldrich) at 4 °C for 15 min. The GST resins were washed three times with 1× GST pulldown buffer (20 mM Hepes, 100 mM NaCl, 5 mM MgCl2, 1 mM DTT, 0.5% Triton X-100). For the experiment in Fig. 1E, 30 μg of His-aPKC A134D was added along with 10, 20, 40, 80, or 100 μM MBP-Lgl 647–673 S656A/S660A peptide and incubated at room temperature for 20 min in a reaction volume of 100 μl. The supernatant was removed and added to amylose resin (New England Biolabs). Both resins were washed three times with 1× GST pulldown buffer, 30 μl of 6× SDS loading buffer was added to each sample followed by separation on SDS-PAGE and transfer to nitrocellulose. The membrane was probed for aPKC with rabbit anti-aPKCα (1:5000) (Santa Cruz Biotechnology). The membrane was further incubated with goat anti-rabbit peroxidase-conjugated secondary antibody and visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

**In Vitro Kinase Activity Assay**—aPKC kinase activity was measured as described previously (24). Briefly, the purified aPKC variants and aPKC/Par-6 complex were diluted to concentrations at which the incorporation of radiolabeled phosphate from [γ-32P]ATP into MBP-Lgl peptide was linear with respect to time and the enzyme concentrations. The diluted enzymes were preincubated in the assay buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl2) with a wide range of MBP-Lgl peptide concentrations at 30 °C for 5 min. The reactions were initiated by adding 1 mM ATP spiked with [γ-32P]ATP (8.0 × 107/μmol of ATP). The reactions were incubated at 30 °C for 10 min. The reaction mixtures were blotted on Grade P81 phosphoroblotter (Whatman). The reactions were quenched by immediately submerging the blotted P81 paper in 75 mM H3PO4. 5 ml of scintillation fluid were added to measure the radioactive decays by liquid scintillation counter. For phosphorylation of Lgl full-length, the reactions were quenched by mixing with 6× SDS loading buffer. The quenched samples were analyzed by 12.5% SDS-PAGE and phosphorimaging. The intensities were analyzed by ImageQuant.

**Arg-C Proteinase Sensitivity Assay**—Arg-C proteolysis was described previously (25). 30 μg of aPKC variants or aPKC/Par-6 complex was incubated with 1 μg of Arg-C proteinase (Sigma-Aldrich) at 37 °C for 120 min. Aliquots were removed at 0 and 120 min in equal volume of 6× SDS loading buffer. As negative control, aPKC variants were incubated at the same conditions without Arg-C proteinase. The samples were separated by 8% SDS-PAGE and transferred to nitrocellulose membrane. Western blots were performed to probe for aPKC proteolysis as described above.
S2 Lgl Cortical Localization Assay—Immunofluorescence was as described previously (26). Briefly, for S2 cell expression, aPKC was expressed using transient transfection with a modified pMT vector containing the Drosophila tubulin promoter in place of the metallothionein promoter. Myc:Par-6 and HA:Lgl coding sequences were cloned into the regular pMT vector using 5’-BglII and 3’-XhoI sites. Drosophila Schneider (S2) cells were maintained in Schneider’s medium with 10% FBS at room temperature. ~2 × 10^6 cells were seeded/well in a 6-well plate and transfected with 0.5 μg of each construct using Effectene transfection reagent according to the manufacturer’s protocol. After cells were incubated overnight and induced with 0.5 mM CuSO_4 for 24 h, 200 μl of cells were seeded on 12-mm diameter glass coverslips in a 24-well plate and allowed to adhere for 1 h. Cells were fixed for 20 min with 4% formaldehyde in PBS followed by three rinses of wash buffer (0.1% saponin in PBS) and two rinses of block buffer (0.1% saponin and 1% BSA in PBS). Coverslips were incubated overnight at 4 °C with the following primary antibodies: rabbit anti-aPKC (1:1000; Santa Cruz Biotechnology), mouse anti-HA (1:1000; Covance), rat anti-Par6 (1:1000; in house). Coverslips were then rinsed three times with blocking buffer, incubated at room temperature for 2 h with species-specific secondary antibodies (1:200; Jackson Immunoresearch), rinsed three times in washing buffer, and mounted in Vectashield Hardset Mounting Medium (Vector Laboratories). Images were acquired on a confocal microscope (Radiance; Bio-Rad Laboratories) using an oil immersion 60×1.4 NA objective, processed with ImageJ, and assembled in Adobe Illustrator.

Phosphorylation-coupled MBP Pulldown Assay—100 μg of the purified MBP-Lgl full-length and MBP-Lgl 647–673 S656A were incubated with amyllose resins (New England BioLabs) for 20 min at room temperature. The resins were washed three times with 1× MBP pulldown buffer (20 mM HEPES, 100 mM NaCl, 5 mM MgCl_2, 1 mM DTT, 0.5% Tween 20) and once with kinase assay buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl_2). 30 μg of aPKC WT/Par-6 complex was added to the washed resins with or without 0.5 mM ATP to allow phosphorylation. The reaction mixtures were incubated at 30 °C for 1 h. The reaction volume was 50 μl. The resins were washed three times with 1× MBP pulldown buffer. 6× SDS loading buffer was added to each sample. The samples were separated by 12.5% SDS-PAGE, and binding was analyzed by Western blotting probing for aPKC as described above.

RESULTS

aPKC Is Autoinhibited by Its Pseudosubstrate—We first identified internal elements within aPKC that regulate its kinase activity (aPKC domain structure is shown in Fig. 1A). In current models for aPKC regulation, Par-6 represses kinase activity (7, 11, 14, 15), suggesting that aPKC might be constitutively active. This model originates from experiments using immunoprecipitated aPKC and/or bacterially expressed Par-6 (14, 15), in some cases with a nonspecific substrate. To test aPKC regulation rigorously, we used high level expression in HEK293 cells followed by a three-step purification scheme (see “Experimental Procedures”). This method led to variable degrees of purity of the final products, depending on the aPKC variant (a schematic of aPKC variants used in this study is shown in Fig. 1B and supplemental Fig. S1A). Additionally, there was little difference in the degree of activation loop phosphorylation among the variants, with the exception that the K293W ATP binding pocket mutant (27) showed severely reduced modification (Supplemental Fig. S1B). We measured the activity of these preparations in in vitro kinase assays using a peptide from Lgl, a known substrate (28–30). Lgl contains three phosphorylatable serines, but we mutated two of them to alanine so that only a single site is available for phosphorylation to simplify the analysis (Fig. 1C). We measured initial rates of catalysis by following the transfer of a radiolabeled phosphorus from ATP to the Lgl peptide over a range of substrate concentrations (i.e. a Michaelis–Menten analysis).

We first compared the activities of full-length aPKC with that of its isolated kinase domain. We observed significant levels of activity for the kinase domain, but the activity of full-length aPKC was approximately equal to background activity, as assessed by measuring the activity of the ATP binding pocket mutant K293W (Fig. 1D), which results in an inactive kinase (27). The low activity of full-length aPKC precluded measurement of accurate K_m and k_cat values for this protein, but analysis of initial rates of the kinase domain yielded values of 4 μM and 1.8 s^{-1}, respectively. These values are similar to those from other catalytic domains from the AGC family of kinases (31, 32). The dramatic difference between the activity of full-length aPKC and its isolated kinase domain suggests that aPKC is autoinhibited and does not require other elements for regulation.

To identify domain(s) that inhibit aPKC activity, we measured the rates of substrate phosphorylation by variants that lacked individual domains within the NH2-terminal regulatory region. Like other PKC family members, aPKC contains a pseudosubstrate motif that can act as an internal competitive inhibitor. A peptide containing the pseudosubstrate sequence competes with binding of true substrates (Fig. 1E), suggesting that the pseudosubstrate can interact with the kinase domain active site. This conclusion is further supported by phosphorylation of the pseudosubstrate when the alanine residue that would sit in the active site is mutated to a serine residue (Fig. 1F). Consistent with these observations, deletion of pseudosubstrate domain dramatically increased aPKC activity, indicating that it is required for aPKC autoinhibition (Fig. 1G). To characterize further the role of the pseudosubstrate in autoinhibition, we made versions of aPKC with point mutations in residues thought to be critical for its interaction with the kinase domain. Mutation of the key alanine residue to a phosphomimetic aspartic acid increases kinase activity presumably by causing “substrate release.” Additional mutation of basic residues near the phosphorylated residue that are important for kinase domain interaction also increases aPKC activity (Fig. 1G). Thus, the pseudosubstrate is a critical element of aPKC autoinhibition.

We also tested the role of other aPKC domains in regulating catalytic activity. In contrast to the pseudosubstrate, the PB1 domain, which mediates interaction with Par-6, does not directly regulate aPKC activity as its deletion had no detectable effect on catalytic activity (Fig. 2A). The aPKC sequence also contains a C1 domain directly COOH-terminal to the pseudo-
substrate. In other PKC isoforms, the C1 binds diacylglycerol, but the C1 function in aPKC is unknown. We found that deletion of C1 increased aPKC activity to a level similar to pseudosubstrate mutants (Fig. 2A), demonstrating that it plays a critical role in synergizing with the pseudosubstrate to maintain the autoinhibited state.

To dissect further the role of the pseudosubstrate in regulating aPKC, we took advantage of a protease sensitivity assay in which pseudosubstrate cleavage by Arg-C is inhibited by its interaction with the kinase domain (25). The aPKC sequence contains two arginine dipeptides that are located in its pseudosubstrate motif (Fig. 1A), demonstrating that it plays a critical role in synergizing with the pseudosubstrate to maintain the autoinhibited state.

Par-6 Activates aPKC—Our observation that aPKC is autoinhibited in vitro, we sought to determine whether it is also autoinhibited in a cellular context. The aPKC substrate Lgl localizes to the cortex of cultured Drosophila S2 cells, but phosphorylation by aPKC causes its displacement into the cytoplasm. When aPKC is expressed with Lgl, a significant fraction remains co-localized with its low catalytic activity, further supporting a model in which aPKC is autoinhibited by the synergistic activity of C1 and pseudosubstrate.

Although our data indicate that aPKC is autoinhibited in vitro, we sought to determine whether it is also autoinhibited in a cellular context. The aPKC substrate Lgl localizes to the cortex of cultured Drosophila S2 cells, but phosphorylation by aPKC causes its displacement into the cytoplasm. When aPKC is expressed with Lgl, a significant fraction remains co-localized with its low catalytic activity, further supporting a model in which aPKC is autoinhibited by the synergistic activity of C1 and pseudosubstrate.
we decided to revisit the role of Par-6 in aPKC regulation. Previous studies used bacterially expressed Par-6 to investigate its effect on aPKC activity. However, we noticed that bacterially prepared Par-6 is highly aggregated (Fig. 3A), presumably because of its PB1 domain as the CRIB-PDZ fragment is soluble and monomeric. Thus, previously observed aPKC repression may have been due to nonspecific effects of the aggregated protein. To overcome this problem, we co-expressed Par-6 with aPKC in HEK293 cells and purified them together as a complex. When prepared in this manner, Par-6 and aPKC form a discrete complex as assessed by gel filtration chromatography (Fig. 3B) and with high purity as shown by SDS-PAGE (Fig. 3C).

To determine the effect of Par-6 on aPKC activity, we first compared the activity of the co-purified aPKC/Par-6 complex with that of aPKC alone. Par-6 and aPKC interact via their PB1 domains (18), and Par-6 also contains semi-CRIB and PDZ domains (33) (Fig. 4A). Rather than lowering the activity of aPKC, we found that aPKC/Par-6 activity is significantly higher than aPKC alone, comparable with the activity of the aPKC pseudosubstrate mutants (Fig. 4B). This effect is due, in part, to the Par-6 PB1 domain because this domain alone is sufficient to activate aPKC (Fig. 4C). The PB1 does not activate to the same level as full-length Par-6, however, indicating that the CRIB-PDZ domain does participate in aPKC activation. Thus, we conclude that Par-6 activates aPKC through PB1-PB1 interactions and possibly additional interactions elsewhere among the two proteins.

How does Par-6 activate aPKC? To determine whether activation occurs through the pseudosubstrate, we measured the activity of Par-6 in complex with aPKC containing activating pseudosubstrate mutants (Fig. 4D). We observed that this complex has activity similar to the Par-6 complex with wild-type aPKC, suggesting that Par-6 acts through the pseudosubstrate to increase aPKC activity. We verified the effect on the pseudosubstrate by assessing the Arg-C sensitivity of the aPKC/Par-6 complex (Fig. 4E). As opposed to aPKC alone, the pseudosubstrate in the aPKC/Par-6 complex is readily digested by Arg-C. The pseudosubstrate is also accessible to Arg-C in the Par-6 PB1 complex with aPKC, but to a lesser degree, consistent with the lower activity of this complex. We conclude that Par-6 activates aPKC by displacing the pseudosubstrate from the kinase domain.

We also tested whether Par-6 activates aPKC in a cellular context using the Lgl cortical localization assay in S2 cells (Fig. 4, F and G). Whereas aPKC alone is unable to displace Lgl into the cytoplasm, co-expression of Par-6 or just its PB1 domain led to cytoplasmic Lgl, consistent with our in vitro observations. Thus, measurements in vitro and in cells indicate that Par-6 activates aPKC catalytic activity rather than repressing it.

In addition to Par-6, the “Par complex” also includes Baz. As Baz is an aPKC substrate, it may compete with phosphorylation of other substrates like Lgl, although Baz has been proposed to activate aPKC (7). To determine whether Baz influences aPKC activity, we examined the effect of the Baz aPKC binding region (Baz residues 905–1221) on Lgl phosphorylation by Par-6/ aPKC (Fig. 4H). Addition of Baz dramatically decreased the extent of Lgl peptide phosphorylation.

Lgl Is Efficiently Phosphorylated and Released from aPKC/Par-6 Complex—In Drosophila SOP cells, Lgl is thought to remain in a complex with aPKC until Aurora A activity phos-
FIGURE 3. Purification of the Par-6/aPKC complex. 

A. Bacterially expressed Par-6 is highly aggregated as shown in the gel filtration chromatogram of purified bacterially expressed Par-6. Marks represent elution volume of standard proteins (F, ferritin 440 kDa; C, conalbumin 75 kDa; CA, carbonic anhydrase 29 kDa; R, RNase A 13.7 kDa). B. Par-6/aPKC form a discrete complex as shown in the gel filtration chromatogram of aPKC/Par-6 purified from HEK293 cells. Marks represent elution volume of standard proteins as in A. C. Purity of the aPKC/Par-6 complex was assessed by gel electrophoresis and Coomassie Brilliant Blue staining.

FIGURE 4. Par-6 activates and Baz represses aPKC. 

A. Par-6 domain structure and interactions are shown. B. Par-6 increases catalytic activity of aPKC. The activity of full-length aPKC is shown for comparison. C. Par-6 PB1 domain is sufficient for aPKC activation. The activities of full-length aPKC and Par-6 activated aPKC are shown for comparison. D. Mutation of the pseudosubstrate does not increase aPKC activity when bound to Par-6. A mutation that inactivates the pseudosubstrate (AADAA), thereby activating aPKC (see Fig. 1G), has no effect on the aPKC/Par-6 complex. The activities of full-length aPKC and Par-6-activated aPKC are shown for comparison. E. aPKC pseudosubstrate is protease-sensitive when aPKC is bound to full-length Par-6 or its PB1 domain (see Fig. 1G for comparison with wild-type aPKC). F. Co-expression of Par-6 or its PB1 domain with aPKC causes cortical displacement of Lgl in S2 cells. Lgl (red signal) associates with the S2 cell cortex, but phosphorylation by aPKC (green) causes displacement into the cytoplasm. Although aPKC itself has no effect on Lgl localization (Fig. 2C), co-expression with Par-6 (blue) leads to cytoplasmic Lgl. G. Lgl localization data shown in F is quantified for 50 cells. H. Bazooka (Baz) inhibits aPKC phosphorylation of the Lgl peptide. Addition of the Baz aPKC binding region (ABR) causes a decrease in the extent of Lgl that is phosphorylated by aPKC. Error bars, S.E.
phosphorylates Par-6, activating the complex (7). A key prediction of this model is that Lgl is a stable member of a ternary complex with Par-6 and aPKC, even though Lgl is an aPKC substrate. Our work above used a small peptide from Lgl that is phosphorylated by the complex, and it is possible that full-length Lgl represses aPKC activity and remains associated with aPKC/Par-6. We examined whether aPKC/Par-6 phosphorylated full-length Lgl in vitro and observed significant phosphorylation, although somewhat less than with the Lgl peptide (Fig. 5A). We also tested whether Lgl is a stable part of the Par complex using a MBP-fused Lgl adsorbed onto amylose resin. This protein efficiently pulls down aPKC/Par-6 in the absence of ATP, but addition of ATP abolished the interaction (Fig. 5B). The result of this interaction assay indicates that MBP-Lgl is released from aPKC/Par-6 once it is phosphorylated, as expected for the substrate of an enzyme, suggesting that Lgl transiently associates with the Par complex.

**DISCUSSION**

Although aPKC activity is required for many cell polarities, excess activity can lead to tissue disorganization and overproliferation. Thus, a central question in cell polarity is how aPKC catalytic activity is kept in an appropriate range during dynamic processes such as asymmetric cell division. In the current study, we investigated the interplay between elements within aPKC and protein-protein interactions with Par-6 in regulating catalytic activity. We used both an in vitro reconstitution strategy along with a cultured cell cortical displacement assay to measure aPKC activity under a wide variety of contexts. Our observations indicate that aPKC is strongly autoinhibited and that interaction with Par-6 causes activation.

**Dual Domain Autoinhibition of aPKC Kinase Domain**—We observed that aPKC is strongly autoinhibited. Although the full-length protein has detectable activity, it is significantly less active than the isolated kinase domain. For example, at 10 μM substrate, the kinase domain is ~50-fold more active than full-length aPKC. We focused on three NH2-terminal domains as candidates for autoinhibition. The PB1 domain that binds the Par complex member Par-6 by PB1 heterodimerization is not required for autoinhibition. Surprisingly, however, aPKC autoinhibition is brought about by collaboration of the two other domains, the kinase-interacting pseudosubstrate and the C1 domain. The aPKC pseudosubstrate efficiently interacts with the kinase domain and is readily phosphorylated when the decoy alanine is replaced with a phosphorylatable residue. Activation of aPKC by mutation of key kinase-interacting residues leads to exposure of the pseudosubstrate, as detected by protease sensitivity. Pseudosubstrate exposure and kinase activation also occur when the C1 domain is deleted, indicating that it plays a previously unappreciated role in regulating kinase activity through the pseudosubstrate. In a recent structure of canonical PKC (34), the C1 domain makes contact with the kinase domain, suggesting that it could make contacts that provide additional stabilization of the pseudosubstrate interaction.

**Allosteric Activation of aPKC by Par-6**—Although the aPKC PB1 domain is not required for autoinhibition, its interaction with the Par-6 PB1 led to activation. Par-6 activation of aPKC does not lead to full activity, as the kinase domain alone is still significantly more active. However, the ability of aPKC/Par-6 to displace Lgl from the cortex of S2 cells suggests that this level of activity may be sufficient for physiological function. Par-6 activation appears to occur through an allosteric mechanism in which the key inhibitory pseudosubstrate interaction is displaced. How might Par-6 binding influence pseudosubstrate interaction with the kinase domain? Based on the close proximity of the PB1 and pseudosubstrate in the aPKC sequence, we propose a steric model in which PB1-PB1 interaction is incompatible with aPKC autoinhibition (Fig. 5, C and D). In this model the pseudosubstrate occupies the kinase active site and is assisted by C1 interactions with the kinase domain. Recent work on the mammalian form of aPKC also implicates the C1 in regulation (35), although to a greater extent than described here. In canonical PKCs the C1 domain couples diacylglycerol binding to activation by binding to the kinase N-lobe (34, 36). In aPKC, activation instead occurs by binding to the PB1 domain which lies on the opposite side of the pseudosubstrate (Fig. 1A).

**Par-6 Couples aPKC Localization and Activation**—How is aPKC regulated during complex polarization processes such as asymmetric cell division? Current models include Par-6 repression of aPKC as a core component, but our results suggest that these models should be reexamined. How might aPKC autoinhibition and activation by Par-6 regulate polarity? In Drosophila neuroblasts and SOP cells, aPKC is cytoplasmic early in the cell cycle but becomes polarized to the cortex by metaphase. Localization of aPKC occurs by interactions with Par-6, suggesting that this early, unlocalized pool of aPKC may not be...
bound to Par-6 and therefore, autoinhibited. By metaphase, Par-6 becomes polarized to the cortex where it can recruit and activate aPKC. The coupling of a PKC localization to its activation would ensure that substrate phosphorylation only occurs at the correct place and time.

How do other Par complex regulatory components influence activity? The substrates Lgl and Baz have been proposed to regulate aPKC-Lgl by inhibiting and Baz by activating catalytic activity. We have found that Lgl behaves as a typical kinase substrate, only transiently associating with the enzyme. Baz also acts as a substrate, competing for the active site with other substrates such as Lgl. In SOP cells, Lgl is localized uniformly to the cortex early in the cell cycle but ultimately becomes polarized to a domain opposite to aPKC before being completely displaced into the cytoplasm. This dynamic pattern of localization correlates well with low aPKC activity early in the cell cycle (due to autoinhibition) followed by polarized activation from interactions with Par-6. Baz, on the other hand, remains localized in the same cortical domain with Par-6 and aPKC (although it localizes to a separate region in epithelia cells). Further work, both in vitro and in vivo, is required to understand how the constellation of proteins that interact with Par-6 and aPKC regulate catalytic activity and cellular function.

REFERENCES

1. Nance, J., and Zallen, J. A. (2011) Elaborating polarity: PAR proteins and the cytoskeleton. Development 138, 799–809
2. Suzuki, A., and Ohno, S. (2006) The PAR-aPKC system: lessons in polarity. J. Cell Sci. 119, 979–987
3. Goldstein, B., and Macara, I. G. (2007) The PAR proteins: fundamental players in animal cell polarization. Dev. Cell 13, 609–622
4. Lee, C. Y., Robinson, K. J., and Doe, C. Q. (2006) Lgl, Pins and aPKC regulate neuroblast self-renewal versus differentiation. Nature 439, 594–598
5. Eder, A. M., Sui, X., Rosen, D. G., Nolden, L. K., Cheng, K. W., Lahab, J. P., Kango-Singh, M., Lu, K. H., Warneke, C. L., Atkinson, E. N., Bedrosian, I., Keyomarsi, K., Kuo, W. L., Gray, J. W., Yin, J. G., Liu, I., Halder, G., and Mills, G. B. (2005) Atypical PKCs contribute to poor prognosis through loss of apical-basal polarity and cyclin E overexpression in ovarian cancer. Proc. Natl. Acad. Sci. U.S.A. 102, 12519–12524
6. Regala, R. P., Weems, C., Jameson, L., Copland, J. A., Thompson, E. A., and Fields, A. P. (2005) Atypical protein kinase Cε plays a critical role in human lung cancer cell growth and tumorigenicity. J. Biol. Chem. 280, 31109–31115
7. Wirtz-Peitz, F., Nishimaga, T., and Knoblich, J. A. (2008) Linking cell cycle to asymmetric division: Aurora-A phosphorylates the Par complex to regulate Numb localization. Cell 135, 161–173
8. Atwood, S. X., and Prehoda, K. E. (2009) aPKC phosphorylates Miranda to polarize fate determinants during neuroblast asymmetric cell division. Curr. Biol. 19, 723–729
9. Smith, C. A., Lau, K. M., Rahmani, Z., Dho, S. E., Brothers, G., She, Y. M., Berry, D. M., Bonn, E., Thibault, P., Schweig, F., Le Borgne, R., and McGlade, C. J. (2007) aPKC-mediated phosphorylation regulates asymmetric membrane localization of the cell fate determinant Numb. EMBO J. 26, 468–480
10. Prehoda, K. E. (2009) Polarization of Drosophila neuroblasts during asymmetric division. Cold Spring Harbor Perspect. Biol. 1, a001388
11. Knoblich, J. A. (2010) Asymmetric cell division: recent developments and their implications for tumour biology. Nat. Rev. Mol. Cell. Biol. 11, 849–860
12. Betschinger, J., Eisenhaber, F., and Knoblich, J. A. (2005) Phosphorylation-induced autoinhibition regulates the cytoskeletal protein Lethal (2) giant larvae. Curr. Biol. 15, 276–282
13. Rosse, C., Linch, M., Kerrmorgant, S., Cameron, A. J., Boeckele, K., and Parker, P. J. (2010) PKC and the control of localized signal dynamics. Nat. Rev. Mol. Cell. Biol. 11, 103–112
14. Atwood, S. X., Chabu, C., Penkert, R. R., Doe, C. Q., and Prehoda, K. E. (2007) Cdc42 acts downstream of Bazooka to regulate neuroblast polarity through Par-6 aPKC. J. Cell Sci. 120, 3200–3206
15. Yamanaka, T., Horikoshi, Y., Suzuki, A., Sugiyama, Y., Kitamura, K., Maniwa, R., Nagai, Y., Yamashita, A., Hirose, T., Ishikawa, H., and Ohno, S. (2001) Par-6 regulates aPKC activity in a novel way and mediates cell-cell contact-induced formation of the epithelial junctional complex. Genes Cells 6, 721–731
16. Lin, D., Edwards, A. S., Fawcett, J. P., Mbanamu, G., Scott, J. D., and Pawson, T. (2000) A mammalian PAR-3-PAR-6-complex implicated in Cdc42/Rac1 and aPKC signalling and cell polarity. Nat. Cell Biol. 2, 540–547
17. Moscat, J., Diaz-Meco, M. T., Albert, A., and Campuzano, S. (2006) Cell signaling and function organized by P1B domain interactions. Mol. Cell 23, 631–643
18. Hirano, Y., Yoshinaga, S., Takeya, R., Suzuki, N. N., Horiuchi, M., Kohijima, M., Sumimoto, H., and Inagaki, F. (2005) Structure of a cell polarity regulator, a complex between atypical PKC and Par6 P1B domains. J. Biol. Chem. 280, 9653–9661
19. Newton, A. C. (2010) Protein kinase C: poised to signal. Am. J. Physiol. Endocrinol. Metab. 298, E395–402
20. Giorgione, J. R., Lin, J. H., McCammon, J. A., and Newton, A. C. (2006) Increased membrane affinity of the C1 domain of protein kinase Cδ, compensates for the lack of involvement of its C2 domain in membrane recruitment. J. Biol. Chem. 281, 1660–1669
21. Colón-González, F., and Kazanietz, M. G. (2006) C1 domains exposed: from diacylglycerol binding to protein-protein interactions. Biochim. Biophys. Acta 1761, 827–837
22. Pu, Y., Peach, M. L., Garfield, S. H., Wincovitch, S., Marquez, V. E., and Blumberg, P. M. (2006) Effects on ligand interaction and membrane translocation of the positively charged arginine residues situated along the C1 domain binding cleft in the atypical protein kinase C isoforms. J. Biol. Chem. 281, 33773–33788
23. Nishikawa, K., Toker, A., Johannes, F. J., Songyang, Z., and Cantley, L. C. (1997) Determination of the specific substrate sequence motifs of protein kinase C isozymes. J. Biol. Chem. 272, 952–960
24. House, C., Wettenhall, R. E., and Kemp, B. E. (1987) The influence of basic residues on the substrate specificity of protein kinase C. J. Biol. Chem. 262, 772–777
25. Dutil, E. M., and Newton, A. C. (2000) Dual role of pseudosubstrate in the coordinated regulation of protein kinase C by phosphorylation and diacylglycerol. J. Biol. Chem. 275, 10697–10701
26. Peterson, F. C., Penkert, R. R., Volkman, B. F., and Prehoda, K. E. (2004) Cdc42 regulates the Par-6 PDZ domain through an allosteric CRIB-PDZ transition. Mol. Cell 13, 665–676
27. Leonard, T. A., Różycki, B., Saidi, L. F., Hummer, G., and Hurley, J. H. (2011) Crystal structure and allosteric activation of protein kinase CβII. Cell 144, 55–66
28. Leonard, T. A., and Hurley, J. H. (2011) Regulation of protein kinases by lipids. Curr. Opin. Struct. Biol. 21, 785–791
29. Plant, P. J., Fawcett, J. P., Lin, D. C., Holdorf, A. D., Binns, K., Kulkarni, S., and Pawson, T. (2003) A polarity complex of mPar-6 and atypical PKC binds, phosphorylates and regulates mammalian Lgl. Nat. Cell Biol. 5, 301–308
30. Qian, Y., and Prehoda, K. E. (2006) Interdomain interactions in the tumor suppressor discs large regulate binding to the synaptic protein Guk. Holder. J. Biol. Chem. 281, 35757–35763
31. Hastie, C. J., McLachlan, H. J., and Cohen, P. (2006) Assay of protein kinases using radiolabeled ATP: a protocol. Nat. Protoc. 1, 968–971
32. Lopez-Garcia, L. A., Schulze, J. O., Fröhner, W., Zhang, H., Süss, E., Weber, N., Navratil, J., Amon, S., Hindle, V., Zeuzem, S., Jürgensen, T. I., Alzari, P. M., Neimanis, S., Engel, M., and Biondi, R. M. (2011) Allosteric regulation of protein kinase PKCγ by the N-terminal C1 domain and small compounds to the PIF-pocket. Chem. Biol. 18, 1463–1473
33. Berra, E., Diaz-Meco, M. T., Domínguez, I., Municio, M. M., Sanz, L,
Lozano, J., Chapkin, R. S., and Moscat, J. (1993) Protein kinase Cζ isoform is critical for mitogenic signal transduction. Cell 74, 555–563
34. Betschinger, J., Mechtler, K., and Knoblich, J. A. (2003) The Par complex directs asymmetric cell division by phosphorylating the cytoskeletal protein Lgl. Nature 422, 326–330
35. Yamanaka, T., Horikoshi, Y., Sugiyama, Y., Ishiyama, C., Suzuki, A., Hirose, T., Iwamatsu, A., Shinohara, A., and Ohno, S. (2003) Mammalian Lgl forms a protein complex with PAR-6 and aPKC independently of PAR-3 to regulate epithelial cell polarity. Curr. Biol. 13, 734–743
36. Wee, B., Johnston, C. A., Prehoda, K. E., and Doe, C. Q. (2011) Canoe binds RanGTP to promote Pins(TPR)/Mud-mediated spindle orientation. J. Cell Biol. 195, 369–376