ACTIVITY OF ATYPICAL PROTEIN KINASE C:

FROM REGULATION TO SUBSTRATE LOCALIZATION

by

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A DISSERTATION

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DISSERTATION ABSTRACT

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The phosphorylation activity of protein kinases is involved in virtually all biological processes of living organisms. As uncontrolled kinase cascades cause devastating defects such as cancer, cells employ complex regulatory networks to precisely control their activity. Atypical Protein Kinase C (aPKC) is a well-conserved protein kinase that plays a central role in the establishment of the Par complex-mediated cell polarity. The goal of my research is to understand how aPKC activity is regulated and how aPKC phosphorylates its substrates. The first part of my study focused on the mechanism by which intra- and intermolecular interactions regulate aPKC activity. aPKC contains a pseudosubstrate domain that acts as an internal inhibitor. Despite the presence of the cis-acting inhibitor, another Par complex member, Par-6, is thought to repress aPKC activity. To examine the precise mechanism by which the pseudosubstrate domain and Par-6 regulate aPKC activity, I reconstituted the system *in vitro* and performed a detailed kinetic analysis. We confirmed that the pseudosubstrate domain is responsible for the autoinhibition. Surprisingly, rather than acting as an inhibitor, Par-6 activates aPKC by displacing the pseudosubstrate from the kinase domain. Par-6 activation of aPKC is consistent with our observation that the Par-6/aPKC complex, but not aPKC alone, releases its substrate from the cell membrane in *Drosophila* S2 cells. The data

support a model in which aPKC activity is coupled to localization via Par-6. In the second part, I investigated how the phosphorylation activity of aPKC is coupled to cortical displacement of fate determinants, which often contain multiple phospho-accepting residues. Using Lgl as a model substrate in S2 localization assays, I examined the role of multiple phosphorylations and found that multi-site phosphorylation is required for cortical release. Also, I examined how aPKC phosphorylates Lgl in an *in vitro* kinase assay and found that aPKC cooperatively phosphorylates Lgl in an ordered manner. These results provide new insights into how multiple phosphorylation and phosphorylation rates could regulate localization behaviors of fate determinants at the cortex.

This dissertation contains previously published coauthored materials as well as unpublished materials.

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For Dad and Dan

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INTRODUCTION

PHOSPHORYLATION BY PROTEIN KINASES AS A KEY REGULATORY ELEMENT IN SIGNAL TRANSDUCTION

The explosion of scientific interest in protein kinase-mediated signal transduction dates back to the 1950's when phosphorylase kinase was originally discovered (1,2). At that time, it was known that phosphorylase, a metabolic enzyme, existed in an active form and an inactive form (3). In a search for the molecular mechanism responsible for converting phosphorylase from the inactive state to the active state, Krebs, Graves, and Fischer discovered that phosphorylase kinase *b* was responsible for the catalysis of phosphoryl-transfer reactions (4). It was later shown that phosphorylase kinase *b* itself was subject to phosphorylation to regulate its activity (5). These two breakthroughs introduced the idea that phosphorylation by protein kinases is a key regulatory element. Since then, a myriad of protein kinases have been identified and phosphorylation has been firmly established to be the most widespread form of post-translational modification.

The significance of phosphorylation as a signal is represented by the fact that ~2% of the human genome is dedicated to encode protein kinases, corresponding to more than 500 protein kinases. It has been estimated that about 30 % of all cellular proteins undergo phosphorylation at least once (2,6,7). It is not surprising that protein kinases are involved in every fundamental physiological process, ranging from metabolism, cell

division, differentiation, motility, trafficking, immunity, learning, memory, and more. Furthermore, protein kinases have been the focus of intense research efforts because malfunctioned and unregulated kinases have been linked to devastating diseases such as cancer. To develop therapeutics, scientists around the globe have been working to decipher the molecular mechanisms of the complex phosphorylation-mediated signaling networks. This extensive endeavor ranges from atomic level structural studies to more systemic cataloging like Kinome, which is a project to identify physiological kinase-substrate pairs in human genome and map the phosphorylation-based signaling networks (8). Though the scientific community has uncovered many of the mysteries, there are more questions to be answered to understand the entirety of kinase signaling.

In this study, I intend to discuss the molecular mechanisms by which atypical Protein Kinase C (aPKC) is regulated via both intra- and intermolecular interactions. Also, I will discuss how substrate specificity and preference leading to differences in aPKC activity. I hope that my research results would provide insights to general understanding of kinase regulation.

THE DAWN OF ATYPICAL PROTEIN KINASE C

In 1977, Nishizuka and colleagues published the first paper detailing the properties of the partially purified protein kinase from bovine cerebellum (9-11). In this paper, they showed that their enzyme phosphorylated histone and protamine in the presence of Mg^{2+}, and that their kinase seemed to be converted from a proenzyme to a highly active enzyme by limited proteolysis. In the following years, Nishizuka and his colleagues investigated the protease responsible for activating proteolysis and found that

the activator was, in fact, not a protein, but membrane-associate phospholipids (12). Interestingly, the purified phospholipids were not capable of activation unless an unusually high concentration of Ca^{2+} was present, which led to another discovery that diacylglycerol (DAG) was the main activator of PKC. Furthermore, they identified that PKC was the long-sought target of phorbol esters that function as potent tumor-promoting analogs of DAG (13). Several PKC isoforms were identified, and each described in detail (11,14-20). The findings provided the information that the isoforms were expressed in specific tissues and cells, and that cell signaling pathways involve the cell membrane, temporal and spatial regulation of the target proteins via multiple pathways, precise controls for activity of signaling molecules, and redundancy of the signaling systems. These early studies established the PKC family as a model for signal transduction (11,21).

It was not until the beginning of the 1990's that PKCζ and PKCλ/ι were first cloned and the subfamily of atypical protein kinase C emerged (22,23). They were originally discovered based on amino acid sequences similar to the other PKC family members, but aPKCs deeply puzzled the researchers because Ca^{2+}, DAG, and phorbol esters all failed to activate aPKC activity (24). It has been suggested that aPKCs are activated by other lipid cofactors such as phosphatitylinositols (PIs) (25), phosphatidic acid (26), phosphatidylinositol- (3,4,5)-triphosphate (27), phosphatidylserine (24), and ceraminde (27,28), but the exact activation mechanisms remain largely unknown for aPKCs. Nevertheless, there is a large body of evidence suggesting that aPKCs play a crucial role in cell growth and survival (22,23). Intense research efforts have been put forth develop therapeutic agents for aPKCs, as aberrant aPKC expression is associated

with poor prognosis in certain cancers (29), further emphasizing the importance of aPKCs.

aPKC AS A MEMBER OF THE PAR COMPLEX INVOLVED IN ESTABLISHING CELL POLARITY

Cell polarity is a dynamic process for living cells to create molecular distinction within the cell. In the event of polarization, a cell is required to establish physical segregation of proteins, lipids, and even organelles to the two opposing sides of the cells. These differentiated cellular poles play a vital role in the development of tissue structures and the maintenance of tissue homeostasis because the molecular polarity can define cell orientations, functions, and cell fates. For example, in epithelial cells, the apical domain faces the external environments to function as a barrier while the basolateral domain is attached to the underlying basal membrane through adhesion molecules to extracellular matrix (Fig. 1A). This apical-basolateral polarity contributes to the tissue shape and the direction of the transport. It has been suggested that a loss of the cell polarity in epithelial cells results in the tissue disorganization. When combined with the loss of cell-cell adhesion, unregulated polarity causes EMT and is associated with metastasis (30). Another type of cell polarity is observed in mammalian primary-cultured astrocytes (Fig. 1B). When a confluent monolayer of primary rat astrocytes are wounded by scratching, cells initiate reorganization of the microtubule organizing center, microtubule cytoskeleton, and the Golgi to establish cell polarity at the leading edge. This polarity axis allows the cells to direct cell protrusion and migration perpendicular to the scratch (31,32). In the case of *Caenorhabditis elegans*, just after the fertilization a zygote

undergoes cytoplasmic reorganization to establish anterior-posterior polarity, which cues the cell to divide asymmetrically and produce daughter cells with different behaviors and fates (33) (Fig. 1C). This process is called asymmetric cell division (ACD). Similar to *C. elegans,* in *Drosophila* neuroblasts, they set up molecular polarity during mitosis and utilize it to generate two different daughter cells with distinct fates; one remains as a neuroblast while the other becomes a ganglion mother cell (GMC), which further divides into neurons or glia cells (Fig. 1D). If ACD fails to progress properly, neuroblast would either be depleted or over-proliferate at the cost of neurons depending on the polarity defects in the fly brains. Thus, theses examples show that the establishment and maintenance of cell polarity have important implications to development and tissue homeostasis of metazoan cells. Then, how is cell polarity established?

The hint for the cellular function of aPKC emerged when it was identified as a core component involved in cell polarity. The six *par* genes and *PKC-3* (orthologue of aPKC) were first identified in *C. elegans* during a screen to find mutants that failed to establish anterior-posterior axis in the zygote. Normally, when *C. elegans* zygotes undergo the first mitosis, aPKC localizes to the anterior side of the zygote and excludes posterior components, such as Par-1 and Par-2, from the anterior side. However, *C. elegans* embryos lacking any of the Par complex members—consisting of Par-3, PKC-3 (*C. elegans* aPKC), and Par-6—failed to induce asymmetric contraction of the myosin II meshwork (34-36). In *Drosophila* neuroblasts, the null mutant of aPKC results in not only mislocalized Par-6, but also uniformly cortical localization of basal determinant Miranda. Similarly, aPKC fails to localize at the apical cortex when Par-6 is absent from neuroblasts (37). aPKC also phosphorylates Bazooka (Par-3 homologue) and controls its

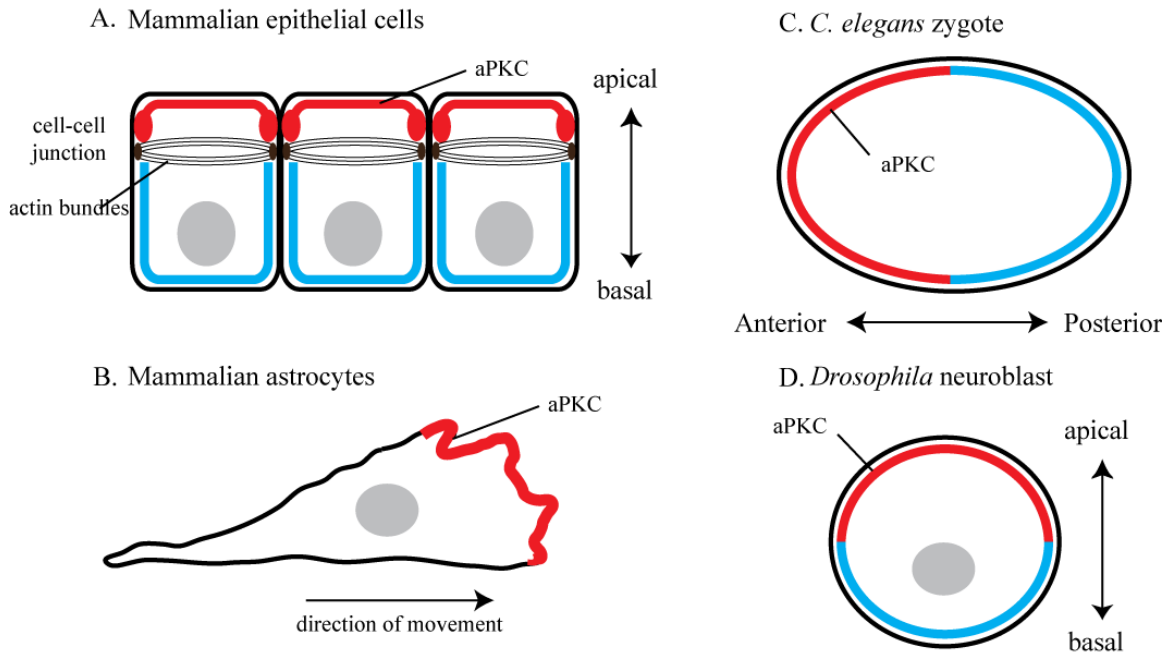

Figure 1. The different contexts of cell polarity and the localization of aPKC.

A. Mammalian epithelial cells with apicobasal polarity, which allows them to function as cellular barrier. aPKC is localized to the apical membrane.
B. Mammalian astrocytes establish polarity with respect to the direction of movement. aPKC is localized at the leading edge.
C. aPKC is localized at the anterior of *C.elegans* zygotes which divides asymmetrically along the anterior-posterior polarity axis.
D. Drosophila neuroblasts divide asymmetrically based on apical-basal polarity axis. aPKC is localized at the apical cortex. The figure is modified from [31].

function as the mutation of phosphorylation site impairs the Par-complex function (38,39). However, Bazooka shows slightly different localization from aPKC in *Drosophila*. In *apkc* null neuroblasts, Bazooka is still able to localize at the apical cortex, which suggests that it is upstream of aPKC (37). In mammalian epithelial cells, the Par complex is coupled to junction formations. Upon cell-cell contact, the Par complex is recruited to the spot-like adherens junctions (AJs) (40), which mature and differentiate into belt-like AJs and tight junctions (TJs). These junctional structures are located at the apex of the cells. The Par-complex plays an essential role in epithelial polarity, as

overexpression of a dominant-negative aPKC or Par-3 prevent the transition of AJs from spot-like to belt-like forms (31,40). The overwhelming evidence suggests that the Par-aPKC system is evolutionarily conserved proteins in function and plays an essential role in many different types of metazoan cell polarity. Also, these data suggest that localization of the Par complex is codependent and that the Par complex establishes polarity by somehow excluding proteins from an aPKC-containing domain.

A more mechanistic understanding of aPKC comes from the findings that expression of aPKC kinase-inactive mutant disrupts the assembly of TJs in mammalian and *Drosophila* epithelial cells (40,41). Also, it has been shown that aPKC directly interacts with Par-6 to form a stable dimer, and immunoprecipitation-coupled kinase assays show that the interaction with Par-6 represses aPKC activity (42). Furthermore, some of the differentiation factors that are excluded from aPKC-containing side of the membrane are aPKC substrates. Consistent with the kinase-dead mutant phenotypes, non-phosphorylatable mutant of Mira and Lgl in neuroblasts fail to be excluded from the apical cortex of the cell and remain uniformly cortical (43,44). Similarly, a non-phosphorylatable Numb mutant colocalizes with aPKC in SOP cells while wild type protein does not colocalize with aPKC (45). Taken together, it is clear that phosphorylation of several differentiation factors by aPKC is indispensible for the establishment of polarity, and aPKC activity is regulated by Par-6. However, little is known about the mechanism by which aPKC's catalytic activity is regulated.

STRUCTURAL DIVERGENCE OF ATYPICAL SUBCLASS RESPONSIBLE FOR THE DIFFERENCE IN ACTIVATION MECHANISMS

The activation mechanism of aPKC is not well understood, partially due to the fact that its domain structure differs from the other PKC family members. All PKC isoforms contain regulatory domains located in the N-terminus and highly conserved catalytic domains in the C-terminus. These isoforms are divided into three classes based on the regulatory domains: classical or conventional PKC (cPKC), novel PKC (nPKC), and atypical PKC (aPKC) (Fig. 2). Here, I discuss each domain of aPKC in detail to clarify the basis of the activation mechanism differences compared to the members of PKC family.

PB1 domain

Modular domains often facilitate specific protein-protein interactions and play important roles in cellular functions. PB1 domains are dimerization domains present in scaffold proteins, conserved among amoebas, fungi, plants, and animals (46). They are about 80 amino acid long and form a ubiquitin-like compact globular fold, consisting of six-stranded β-sheets and two α-helices. Previous structural studies show that the PB1-PB1 interaction involves specific electrostatic contacts between a cluster of acidic amino acids of type I PB1 and the few basic residues including, invariant Lys of type II PB1 (46-48). A PB1 domain resides in the N-terminus of the aPKC regulatory domain, and is classified as type I/II because both the acidic cluster and the conserved Lys residue are present. It has been shown that the acidic patch of aPKC PB1 interacts with the basic

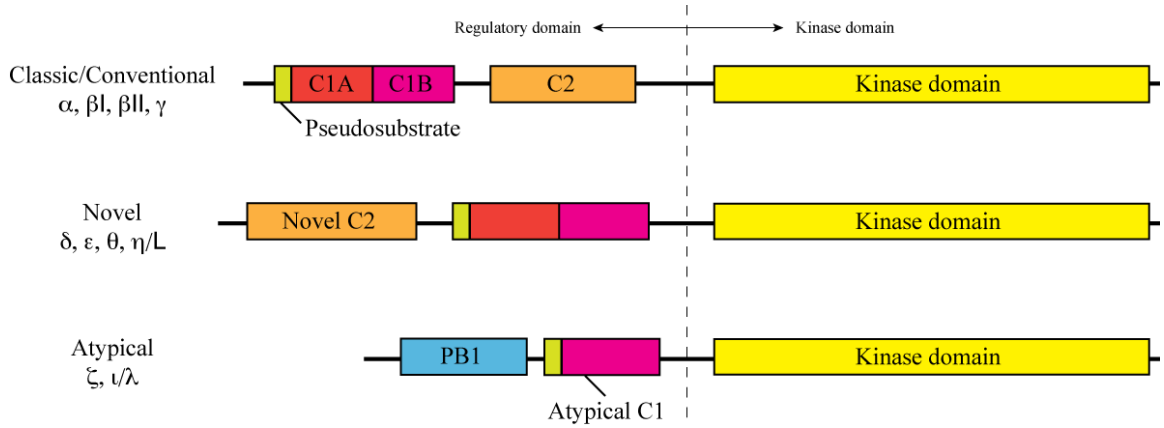

Figure 2. The domain structures of PKCs.

The regulatory domain of the cPKC class, which includes PKCα, βI, βII, and γ, consists of a pseudosubstrate motif in the N-terminus (a short stretch of amino acids that resembles the substrate binding motif except the phosphate-accepting Ser/Thr residue is replaced to Ala), twin C1 domains (tandem 50 residue long domains, C1A and C1B, containing 6 Cys and 2 His residues that coordinate 2 Zn^{2+} ions. They are responsible for DAG/phorbol esters sensitivity), and a C2 domain (Ca^{2+}-dependent, membrane-targeting). The nPKC class includes PKCδ, ε, θ, and η, and contains a novel C2 domain in the N-terminus (C2 domain in nPKC differ from cPKC because it lacks the Ca^{2+}-coordinating acidic residues. For this reason, nPKC isoforms are unresponsive to Ca^{2+}), a pseudosubstrate motif, and a pair of C1 domains. Finally, the regulatory domain of the aPKC class contains a PB1 domain (a dimerization domain to interact with other PB1-containing proteins), a pseudosubstrate motif, and a C1 domain (unlike the other PKCs, aPKCs only have one copy of C1 domain. Also, it has only one Zn^{2+} ion and lacks crucial basic residues for the interaction with DAG/phorbol esters). The figure is modified from [41].

residues in PB1 of Par-6, as yeast two-hybrid data and localization study in MDCK cells show that the mutations of either a critical acidic reside in aPKC PB1 or the conserved Lys in Par-6 PB1 disrupted the interaction (49). Furthermore, the X-ray crystallography data show that the two PB1 domains interact with each other via the charged regions in a front-to-back manner (50) (Fig. 3). The interaction with Par-6 is important for the localization of aPKC in polarized cells as loss of Par-6 is shown to correlated with mislocalized aPKC (37); however, it is not known how this interaction leads to polarization as opposed to the membrane localization of aPKC.

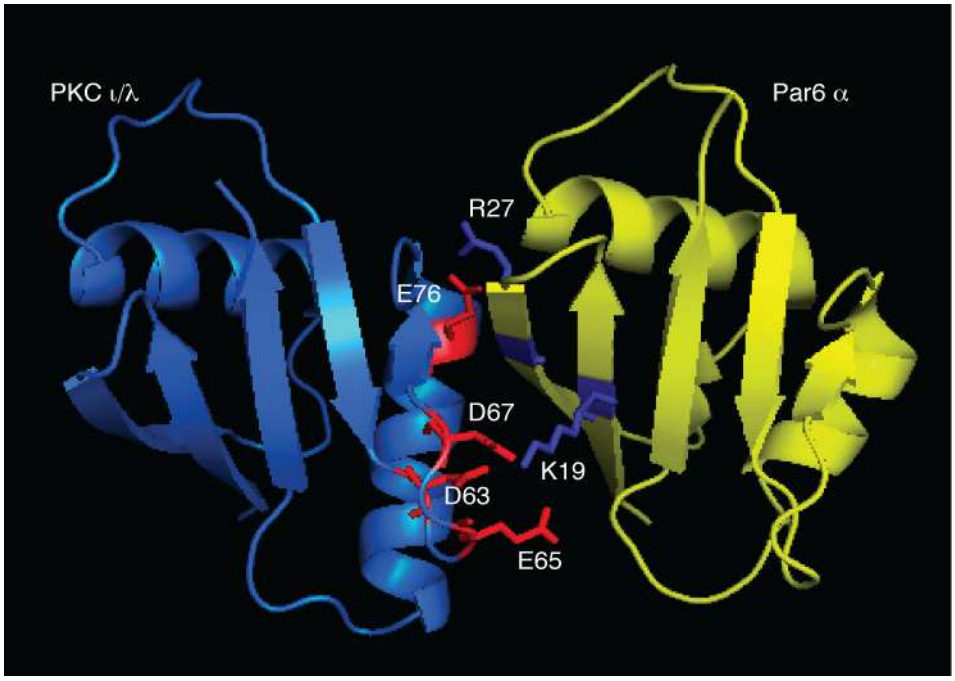

Figure 3. The crystal structure of PB1-PB1 dimer between PKCι/λ (blue) and Par-6α (yellow).

The complementary electrostatic charges allow the two domains to interact. The acidic residues of aPKC PB1 (red) and the basic residues of Par-6 (blue) are shown at the binding interface. PDB 1WMH

Pseudosubstrate motif

The presence of a *cis*-acting autoinhibitory motif is a common regulatory feature of protein kinases. Generally, a pseudosubstrate motif is a short stretch of amino acid that acts as a competitive inhibitor of the kinase activity since it resembles the substrate recognition sequence except that the phospho-accepting Ser/Thr residue is substituted to Ala and blocks the substrate binding (51). It was originally shown that the addition of synthesized PKC peptide that resembled the substrate phosphorylation site *in trans* reduced the catalytic activity of PKC in a dose-dependent manner. Kinetic analyses showed that the peptide inhibited PKC in a competitive manner, suggesting that it acted as a substrate antagonist by directly occupying the substrate-binding site in the catalytic

domain (52). Consistent with these data, while the PKC pseudosubstrate is protected from proteolysis when it is in an inactive state, it is highly sensitive to proteolysis upon activation (53). Extensive biochemical studies have indicated that binding to the activating lipid cofactors at the membrane supplies the energy necessary to release the PKC pseudosubstrate motif (54). However, since the lipid cofactors that activate PKCs do not activate aPKC, it is unclear whether aPKC pseudosubstrate motif autoinhibits the catalytic activity and is released in the same mechanism observed in PKCs.

C1 domain

In cPKC and nPKC, a pair of C1 domains, C1A and C1B, serves as membrane-targeting modules that bind DAG and phorbol esters for activation (55-59). They are characterized by the presence of six Cys and two His motif that coordinate two Zn^{2+} ions (each Zn^{2+} ion is coordinated by three Cys and one His). In contrast, there is only one C1 domain in aPKC. Although there are six Cys and two His residues similar to the other PKCs, aPKC C1 domain only contains one Zn^{2+}. Also, it is insensitive to both DAG and phorbol esters. The unresponsiveness to the lipid activators is attributed to a cluster of basic residues present in the domain (KRFNRRAFC). When four Arg residues are substituted to corresponding (uncharged) residues from PKCδ C1B domain, aPKCζ recovers the sensitivity to phorbol esters. Similarly, substitution of the residues from PKCδ C1B at the same position to Arg abolishes the sensitivity to phorbol esters (60).

Even though aPKC C1 domain does not interact with the known lipid activator, it may still function as membrane translocation module (unpublished data from Prehoda lab). Overexpression of aPKC C1 domain alone in *Drosophila* neuroblast results in

cortical localization (unpublished data). Additionally, the bacterially purified aPKC C1 domain interacts with certain phospholipids *in vitro*. Many questions remain as to the function of aPKC C1 domain, and it is of particular interest how aPKC localization via C1 domain is coupled to activation of its catalytic activity.

Catalytic domain

In general, catalytic domains of eukaryotic Ser/Thr protein kinases including aPKC are very well conserved and have similar tertiary structures (Fig. 4A). They are composed of an amino-terminal small subdomain (N-lobe) and a carboxyl-terminal large subdomain (C-lobe). The bilobal fold of protein kinases enables dynamic movement to assume different conformations and allows kinases to regulate their catalytic activity in response to cellular signals. There are several key features within the catalytic domain that regulate the coordinated movements for optimal phosphotransfer.

1. ATP binding site

One of the most conserved motifs within catalytic domains among the different kinases is a glycine-rich loop (consensus sequence of the loop is GXGXXG), which provides necessary flexibility for anchoring ATP. The loop is also important for excluding water to avoid unwanted hydrolysis of an ATP molecule. However, the third glycine of aPKC is replaced with alanine (61). This unique loop structure is reflected by the interaction between ATP and the "invariant lysine"— the most conserved and best characterized residue in protein kinases. The invariant lysine directly interacts with α-and

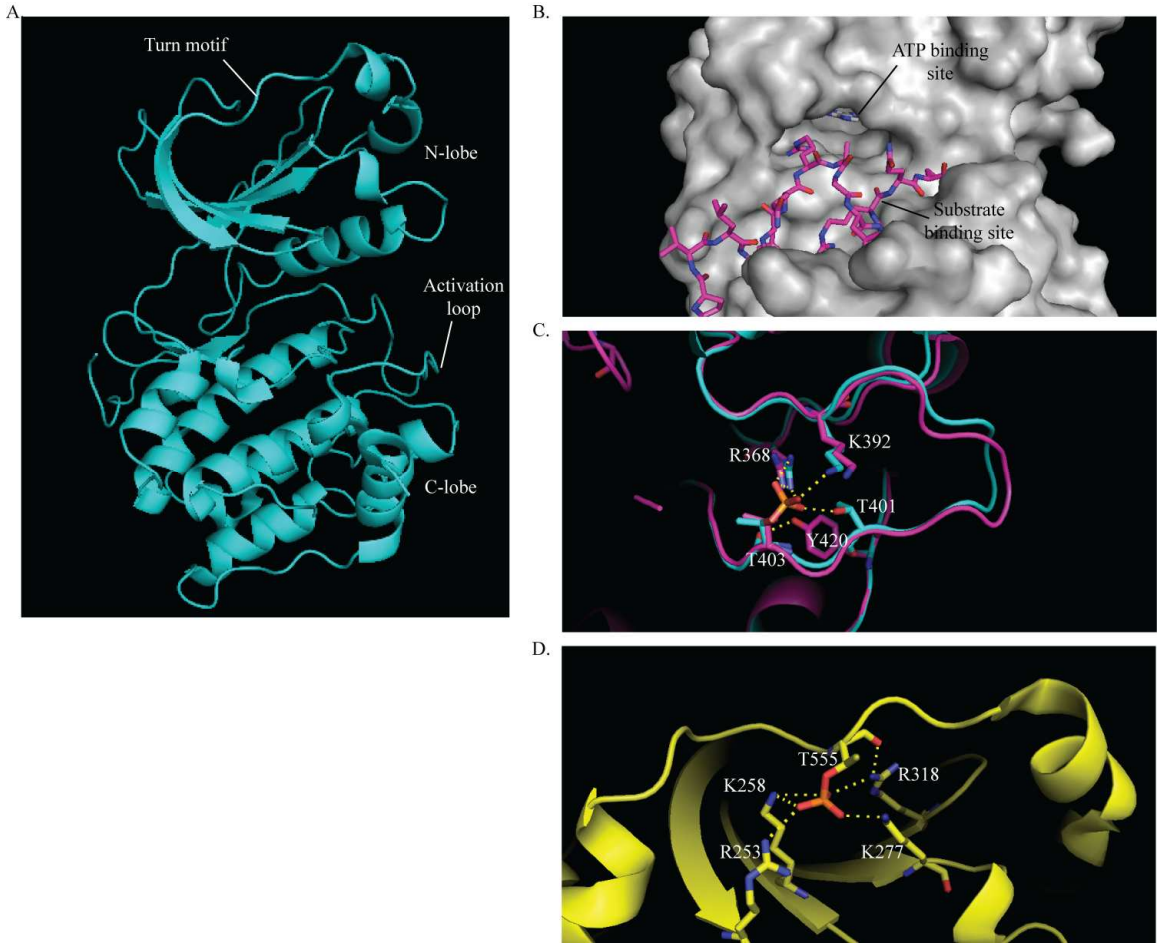

Figure 4. The structural features of aPKC catalytic domain.

A. The overall structure of human PKCι (PDB: 1ZRZ) showing the bilobal architecture with the activation loop and the turn motif.
B. ATP binding pocket and the substrate binding site. In a deep cleft between the two lobes is a binding site for one ATP molecule, and substrates bind across the front end of the ATP-binding pocket close to the γ-phosphate. In this figure, AMPPCP occupies the ATP binding site and Par-3 peptide substrate is bound at the substrate binding site (PDB: 4DC2).
C. Both activation loop with the phosphorylated T403 (cyan, PDB: 1ZRZ) and non-phosphorylated T403 (magenta, PDB: 4DC2) assume the active conformation. Phosphorylated T403 interacts with R368, K392, T401, and Y420 to order the loop. Non-phosphorylated T403 only interacts with Y420.
D. Phosphorylated T555 in the turn motif coordinates the N-lobe in a stable active form.

β-phosphates to anchor and orient the ATP molecule. Also, it forms a salt bridge with the carboxyl group of one of the glycine residues in the loop and stabilizes the three-dimensional structure of the catalytic domain (6,7). Typically, when this Lys residue is mutated to other amino acids including chemically similar Arg results in inactivation. In contrast, substitution of Lys to Arg in aPKC exhibits normal catalytic activity (61). These data indicates that the ATP-binding pocket of aPKC may differ from other kinases, including other PKC family members.

2. The activation loop

Many kinases require one or more phosphorylation event on the activation segment, which is defined as a stretch of 25-30 residues between two conserved tripeptide motifs (DFG and APE) within the C-lobe. The activation segment consists of the Mg^{2+} binding loop, the activation loop, and P+1 loop. The segment could adopt a variety of conformations when it is not phosphorylated and kinases are in inactive states. Once phosphorylated and active, however, the loop undergoes a dramatic conformational change to correctly align the residues involved in catalysis and provides a platform for the substrates (Fig. 4B) (7,62,63). The crystal structures of cyclic AMP-dependent protein kinase (PKA) (64), a cell cycle-dependent protein kinase (Cdk2) (65), and mitogen-activated protein kinase (66) have showed that the phosphorylated residue in the activation segment interacts with a pocket of positively charged patches on the surface of the kinase domain. These data reveal a common mechanism by which phosphorylation of the activation segment regulates the conformation of the substrate binding site.

In the case of aPKC, the requirement for the negative charge on the activation loop has been controversial (Fig. 4C). The crystal structure of human aPKCι has shown that the loop was phosphorylated and the catalytic domain was in an active form (67,68). Other studies have shown that aPKC and other PKC family members require a negative charge at the activation loop and that 3-phosphotidylinositide-dependent kinase -1 (PDK-1) is responsible for the phosphorylation (69-72). Either mutation of the phospho-accepting residue or deletion of PDK-1 results in reduced aPKC catalytic activity (70). However, another crystal structure of human PKCι with a physiologically relevant peptide substrate from Par-3 shows that the kinase domain can assume the same active conformation in the absence of the activating phosphorylation at the activation segment (73). The discrepancy may originate from that the kinase domain used for the crystal structure was a mutant whose invariant lysine was mutated to arginine, which is reported to be enzymatically active as mentioned above. It is of interest whether phosphorylation of the activation segment is required for aPKC or if the invariant lysine mutant does not require the negative charge on the loop to coordinate the substrate platform. It is also interesting to examine if the excess amount of peptide substrate present in the crystallization condition could order the substrate binding platform in the absence of the phosphate group on the activation segment.

3. Turn motif and hydrophobic motif

For PKC family members, the phosphorylation of the activation segment triggers phosphorylation of two additional sites (51,74). This series of phosphorylation events is called maturation, for the lack of this process leads to inactive kinases. One of the

phosphorylation occurs at the turn motif, which is a motif located in a proline-rich domain of the kinase domain and positioned at the apex of the N-lobe in the three dimensional structure (Fig. 4D). The inactive kinase expressed in cultured mammalian cells lacks the phosphate group at the turn motif (75). The selective dephosphorylation of the turn motif from classic PKCβII results in inactivation of the kinase activity (76). However, the turn motif is not phosphorylated unless the activation loop is phosphorylated by PDK-1. Biochemical evidence shows that the negative charge at the turn motif locks the kinase in the catalytically competent conformation (51). It is unclear whether the turn motif plays a role in aPKCs. Considering the similarity of the primary sequence between the other PKC family members and aPKCs, it is likely that the turn motif phosphorylation plays an important role in the maturation of aPKC catalytic domain. Also, all the crystal structures of PKCι which are in the active conformation show the presence of a phosphate group at this position (67,68,73). Further investigation is required to clarify if aPKC follows the trend of the other PKCs.

The second phosphorylation site is at the hydrophobic motif, which is C-terminal of the turn motif. It has been shown that this site is autophosphorylated in PKC isozymes with the exception to PKCδ (77), which an upstream kinase is shown to phosphorylate at this position. It has been suggested that the negative charge at the hydrophobic motif may not be critical to the catalytic activity of the enzymes because mutation of the phospho-accepting serine to alanine still generates activatable PKCα and PKCβII (51). In the case of aPKC, the phospho-accepting residue is substituted to a phospho-mimetic glutamic acid (68). Balendran and colleagues showed that the hydrophobic motif of aPKC serves as the docking site for PDK-1 because mutation in the motif significantly reduced the

ability of aPKC to interact with PDK-1 (78). However, the mutation of the acidic residue did not affect the interaction. Thus, more investigation is needed to decipher the exact role of the phospho-mimetic residue in the aPKC hydrophobic motif.

4. PDZ ligand

The C-terminal tails are the most variable part of the otherwise well-conserved PKC family members. Recent studies indicate that this region is a critical determinant for isoform -pecific targeting and cellular functions (55). In the case of PKCε and PKCδ, the tail regions play important roles in determining the isoform-specific localization and biological functions (79). Also, they are critical for the catalytic competence of PKCε, for deletion of the tail residues reduces the phosphorylation activity in a length-dependent manner (80). Similarly, truncation of the last 10 residues from PKCα leads to acatalytically inactive kinase, due to the lack of activation loop phosphorylation (81). Interestingly, modeling studies for both PKCε and PKCα showed that the tail regions intramolecularly interacted with the N-lobe. The interaction influenced the structure of the ATP binding site and ultimately their enzymatic activity (55). These data suggest that the very distal portion of the PKC kinase domain significantly impact the catalytic competence.

Additionally, PKCα and aPKC contain a PDZ domain consensus binding motif (PDZ ligand) at the C-terminal tail and have been reported to interact with PDZ domain-containing proteins. For example, PDZ ligand of PKCα interacts with PICK1, which serves as a scaffold to localize the kinase by associating with itself and other PDZ domain-containing proteins at the membrane (82). Also, PDZ domain of Discs Large Homologue 1 (DLG1) interacts with PKCα via PDZ ligand to regulate the cell migration

(83). In *C. elegans* embryos, a PDZ-PDZ ligand-mediated interaction between Par-6 and aPKC has been shown to be dispensable for the localization of Par-6 because mutations that abolish the interaction did not affect Par-6 functionality (84). It is unclear whether PDZ ligand of aPKC plays regulatory roles with Par-6. However, the kinetic analyses of the complex between Par-6 and an aPKC PDZ ligand-deleted mutant show reduced activity (unpublished data). Also, PDZ ligand deletion mutant localizes in the cytoplasm of neuroblasts (unpublished data). Our data suggest that aPKC PDZ ligand is involved in both regulating the catalytic activity and localization. It is of interest to clarify this discrepancy as well as to examine how PDZ ligand-mediated regulation of aPKC would tie into the rest of the complex network of aPKC activation mechanism.

BRIDGE TO CHAPTER II

As described above, there are many components that could regulate the catalytic activity of aPKC. However, it is not well understood how all these elements participate in controlling aPKC in a concerted manner. In the following chapter, I discuss how the intramolecular interaction within aPKC regulates its catalytic activity. Also, I challenge the standing model that Par-6 represses aPKC activity. Previous study indicated that aPKC on its own is constitutively active, though it contains autoinhibitory pseudosubstrate domain. The inhibitory mechanism of Par-6 is also unclear. To understand the complex regulatory network of aPKC, I realized that aPKC must be purified, not immunoprecipitaed. Also, I needed a method that directly measured the phosphorylation rates using physiologically relevant substrates instead of the traditional immunoprecipitation-coupled kinase assay where aPKC, along with other cellular

constituents, were immunoprecipitated from cellular lysate and mixed with a generic substrate to test the kinase activity. This method inherently presents three issues: 1) other proteins that directly and indirectly interact with the target kinase will be present in the assay, and 2) the concentration of the kinase will be unknown, and 3) the readout of the kinase activity is qualitative not quantitative. In the following chapter, I describe the intricate regulatory mechanism of aPKC using both a reconstituted system as well as cellular context.

Chapter II and III includes previously published and unpublished co-authored materials, respectively.

CHAPTER II

PARTITIONING-DEFECTIVE PROTEIN (PAR-6) ACTIVATES ATYPICAL PROTEIN KINASE C BY PSEUDOSUBSTRATE DISPLACEMENT

This chapter contains previously published co-authored material taken with permission from:

Graybill, C., Wee, B., Atwood, S. X., and Prehoda, K. E. (2012) Partitioning-defective protein (Par-6) activates atypical Protein Kinase C by pseudosubstrate displacement. Journal of Biological Chemistry. 287(25):21003-11.

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INTRODUCTION

The Par complex functions to create distinct, polarized cortical domains by coupling phosphorylation to cortical release (43,45,85). 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 (86-88). The activity of aPKC is a key output of the Par complex as the association of substrates with the cortex can be modulated by phosphorylation (43,45,85). For example, in

Drosophila neuroblasts the protein Miranda localizes to a cortical domain opposite the Par complex and its polarization requires aPKC activity (43). 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 (45), 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 (89,90). Ectopic aPKC activity in neuroblasts leads to massive overproliferation and concomitant loss of differentiated cells (91). In current models, aPKC activity is controlled by a complex set of protein-protein "scaffolding" interactions (87,92). In particular, Par-6 is thought to repress aPKC (42,85,93), 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 (85). 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. As the PB1 domain is the interaction site with aPKC (48,50,94), 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.

Although protein-protein interactions are thought to regulate aPKC, the precise mechanisms that control catalytic activity have been unclear. All PKC isoforms contain NH2-terminal domains that are potentially important for controlling the activity of the COOH-terminal kinase domain (74). These domains include the aPKC-specific PB1 that binds Par-6 (50), and the C1 domain that binds lipid cofactors such as diacylglycerol in other PKC family members (95,96), but whose function in aPKCs is unknown (60). 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.

MATERIALS AND METHODS

Purification of aPKCs and aPKC/Par-6 complex

HEK293 F cells were transfected with pCMV His_6-aPKC constructs for expression of individual aPKC variants or co-transfected with pCMV His_6-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 hours 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 with Ni^{2+} lysis buffer (50 mM NaH_3PO_4, 300 mM NaCl, 10 mM Imidazole, adjusted to pH 8.0 with NaOH). The resuspended precipitate were incubated with Ni^{2+} NTA resins for 45 min at 4 °C. The resins were washed with the lysis buffer. The proteins were eluted using Ni^{2+} elution buffer (50 mM NaH_3PO_4, 300 mM NaCl, 250 mM Imidazole, adjusted to pH8.0 with NaOH). The eluted proteins were dialyzed at 4 °C for 4 hours. For the complex with Par-6, the proteins were further purified with a size exclusion column (S200 10/30; GE Health). The concentration of aPKC was determined by comparing its reactivity with an anti-aPKC antibody (Santa Cruz Biotech) with that of a standard of known concentration (bacterially expressed aPKC kinase domain purified and quantified using a Bradford dye binding assay) on a western blot.

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 Inc.), in which a TEV protease recognition site was added following the MBP coding sequence. The residues S656 and S660 in Lgl 647-673 were mutated to alanines. The constructs were transformed into BL21 *E. coli* cells. The expressions were induced by 0.4 mM IPTG at 18 °C overnight. The bacterial lysates were incubated with amylose resins (New England BioLabs Inc.). 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 20mM Tris·HCl pH7.5, 50mM NaCl. The affinity purified proteins were further purified by ion exchange.

Affinity chromatography "pulldown" binding assays

GST pulldowns were as previously described (97). Briefly, *Drosophila* aPKC 120-141 was cloned into pGEX-4T1 (GE Healthcare Life Sciences) which was transformed into *E. coli* strain BL21. Protein expression was induced by 0.4 mM IPTG 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 with 1x GST pulldown buffer (20 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.5 % Triton X-100) for 3 times. For the experiment in Figure 1*E*, 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 with 1x GST pulldown buffer 3 times. 30 µL of 6x 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-aPKCzeta (1:2000) (Santa Cruz). 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 previously described (98). Briefly, the purified aPKC variants and aPKC/Par-6 complex were diluted to concentrations at which the incorporation of radiolabeled phosphate from [γ^{-32}P]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 $MgCl_2$) with a wide range of MBP-Lgl peptide concentrations at 30 °C for 5 min. The reactions were initiated by adding 1mM ATP spiked with [γ^{-32}P]ATP (~1.0 x 10^5/nmol ATP). The reactions were incubated at 30 °C for 10 min. The reaction mixtures were blotted on Grade P81 phosphopaper (Whatman). The reactions were quenched by immediately submerging the blotted P81 paper in 75 mM H_3PO_4. 5mL 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 6x SDS loading buffer. The quenched samples were analyzed by 12.5 % SDS-PAGE and phosphorimager. The intensities were analyzed by ImageQuant.

Arg-C proteinase sensitivity assay

Arg-C proteolysis was described previously (99). 30 µg of aPKC variants or aPKC/Par-6 complex were incubated with 1 µg of endoproteinase Arg-C proteinase (Sigma-Aldrich) at 37 °C for 120 min. Aliquots were removed at 0 and 120 min into equal volume of 6x 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 previously described (100). 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 metallothionen 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. ~$2x10^6$ cells were seeded per 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 24h, 200μL of cells were seeded on 12 mm diameter glass coverslips in a 24-well plate and allowed to adhere for 1h. Cells were fixed for 20 minutes with 4% formaldehyde in PBS followed by 3 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^oC 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 3x with blocking buffer, incubated at room temperature for 2h with species-specific secondary antibodies (1:200; Jackson Immunoresearch), rinsed 3x in washing buffer, and mounted in Vectashield Hardset Mounting Medium (Vector Laboratories). Images were acquired on a confocal microscope (Radiance; Biorad laboratories) using an oil-immersion 60x 1.4NA 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 S660A peptide were incubated with amylose resins (New England BioLabs Inc.) for 20 min at room temperature. The resins were washed with 1x MBP pulldown buffer (20 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.5 % Tween 20) for 3 times 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 hour. The reaction volume was 500 µL. The resins were washed with 1x MBP pulldown buffer for 3 times. 6x SDS loading buffer was added to each sample. The samples were separated by 12.5% SDS-PAGE and binding was analyzed by western blots 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. 5*A*). In current models for aPKC regulation, Par-6 represses kinase activity (42,85,87,93), suggesting that aPKC might be constitutively active. This model originates from experiments using immunoprecipitated aPKC and/or bacterially expressed Par-6 (42,93), in some cases with a non-specific substrate. To rigorously test aPKC regulation, we used high level expression in HEK293 cells followed by a three step purification scheme (see methods). 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 are shown in Fig. 1*B*; Supplemental Fig. S1*A*). 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 (101) showed severely reduced modification (Supplemental Fig. S1*B*). We measured the activity of these preparations in *in vitro* kinase assays using a peptide from Lethal giant larvae (Lgl), a known substrate (44,102,103). 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. 5*C*). We measured initial rates of catalysis by following the transfer of a radiolabeled-phosphorous from ATP to the Lgl peptide over a range of substrate concentrations (i.e. a Michealis-Menten analysis).

We first compared the activities of full length aPKC to that of its isolated kinase domain. We observed significant levels of activity for the kinase domain but full-length aPKC's activity was approximately equal to background activity, as assessed by measuring the activity of the ATP binding pocket mutant K293W (Fig. 5*D*), which results in an inactive kinase (101). 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 (104,105). 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.

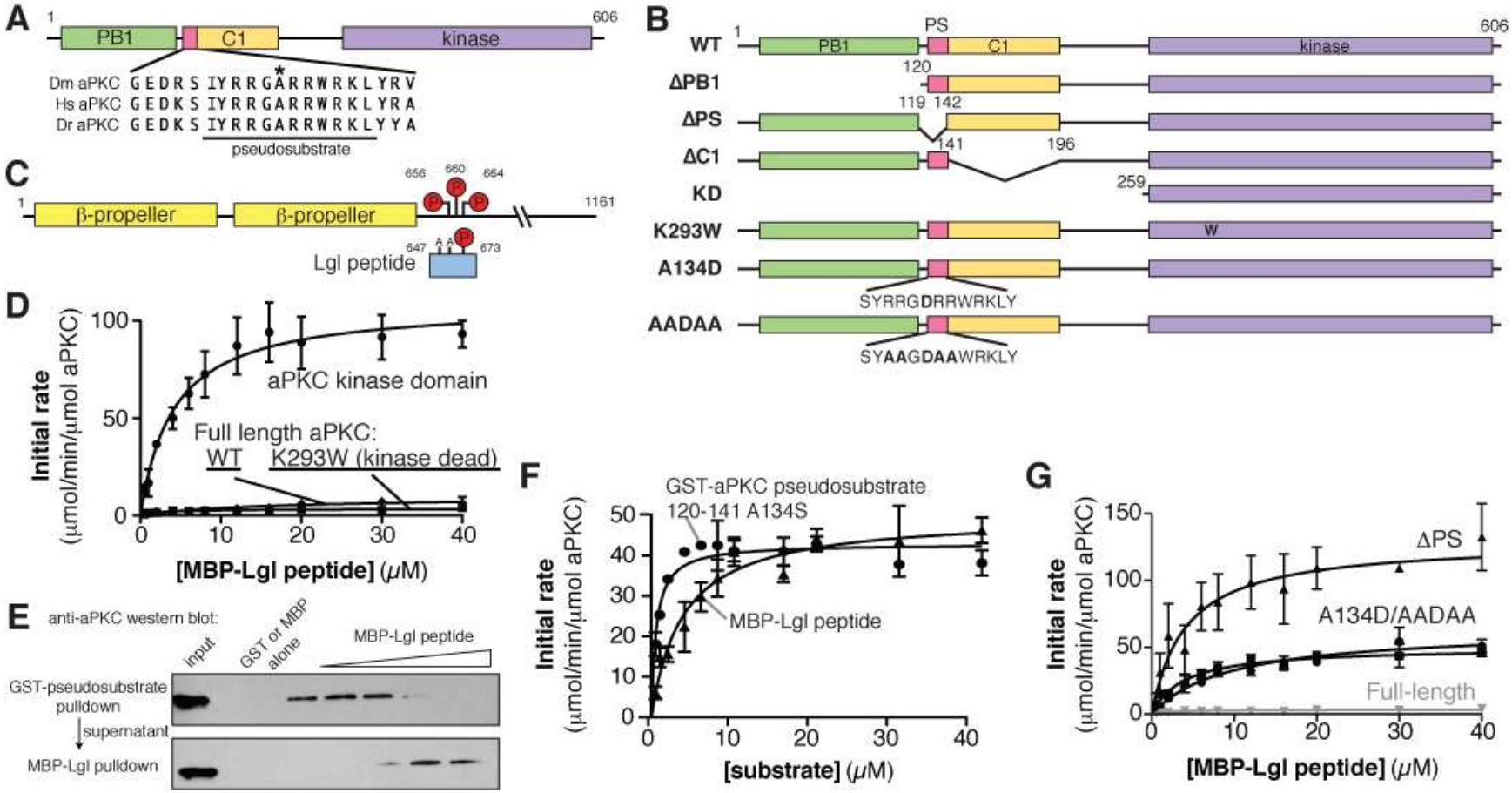
To identify domain(s) that inhibit aPKC's activity, we measured the rates of substrate phosphorylation by variants that lacked individual domains within the NH_2-terminal regulatory region. Like other PKC family members, aPKC contains a pseudosubstrate motif that can act as an internal competitive inhibitor. A peptide containing the pseudosubstrate sequence competes with binding of true substrates (Fig. 5*E*) 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. 5*F*). Consistent with these observations, deletion of pseudosubstrate domain dramatically increased aPKC activity indicating that it is required for aPKC autoinhibition (Fig. 5*G*). To further characterize the pseudosubstrate's role 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 increase aPKC activity (Fig. 5*G*). 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. 6*A*). The aPKC sequence also contains a C1 domain directly COOH-terminal to the pseudosubstrate. In other PKC isoforms, the C1 binds diacylglycerol but C1 function in aPKC is unknown. We found that deletion of C1

increased aPKC activity to a level similar to pseudosubstrate mutants (Fig. 6*A*) demonstrating it plays a critical role in synergizing with the pseudosubstrate to maintain the autoinhibited state.

Figure 5 (next page)**.** aPKC is autoinhibited by its pseudosubstrate motif.

A. aPKC domain structure. An alignment of the the pseudosubstrate motifs from flies (Dm), humans (Hs), and fish (Dr) is shown with the alanine that is in the position of the phosphorylatable residue in a typical substrate starred.
B. Lgl domain structure. LCR, low complexity region. The three residues phosphorylated by aPKC are shown, as is the peptide that was used for the majority of the kinase activity assays showing the phosphorylation sites that were removed by mutation to alanine.
C. aPKC constructs used in this study.
D. Comparison of full-length aPKC catalytic activity to that of the isolated kinase domain. The initial rate of phosphorylation (µmol/minute) of a Maltose binding protein fusion of the Lgl peptide (MBP-Lgl peptide; see panel B) is shown for three aPKC variants: the isolated kinase domain, full-length, and K293W which disrupts the ATP binding site of the kinase domain.
E. The pseudosubstrate interacts with aPKC and the Lgl peptide competes with their interaction. Addition of MBP-Lgl peptide reduces the amount of aPKC retained on glutathione agarose beads adsorbed with a GST fusion of the aPKC pseudosubstrate (top western). The supernatant was adsorbed onto amylose resin (bottom western) to confirm that the MBP-Lgl fusion formed a complex with aPKC. The A134D aPKC pseudosubstrate variant (see panel B) was used for the pull-down so that the internal pseudosubstrate did not compete with the pull-down.
F. The pseudosubstrate containing an alanine to serine mutation is readily phosphorylated by aPKC. Comparison of the initial phosphorylation rate of a GST fusion of the pseudosubstrate containing mutation A134S (starred residue in panel A) to MBP-Lgl peptide. The “AADAA” aPKC pseudosubstrate variant (see panel B) was used for the kinetic assay.
G. Perturbation of the pseudosubstrate activates aPKC. ΔPS, aPKC that lacks the pseudosubstrate; A134D, aPKC containing an aspartic acid in place of the starred alanine in panel A; AADAA, aPKC that contains A134D along with mutations in adjacent basic residues that interact with the kinase domain. The activity of full-length aPKC is shown for comparison.


A
PB1
C1
kinase
Dm aPKC GEDRS IYRRGARRWRKLYRV
Hs aPKC GEDKS IYRRGARRWRKLYRA
Dr aPKC GEDKS IYRRGARRWRKLYYA
pseudosubstrate
B
WT
ΔPB1
ΔPS
ΔC1
KD
K293W
A134D
AADAA
PS
SYRRGDRRWRKLY
SYAAGDAAWRKLY
C
β-propeller
β-propeller
Lgl peptide
D
aPKC kinase domain
Full length aPKC:
WT
K293W (kinase dead)
Initial rate
(μmol/min/μmol aPKC)
[MBP-Lgl peptide] (μM)
E
anti-aPKC western blot:
input
GST or MBP alone
MBP-Lgl peptide
GST-pseudosubstrate pulldown
supernatant
MBP-Lgl pulldown
F
GST-aPKC pseudosubstrate 120-141 A134S
MBP-Lgl peptide
[substrate] (μM)
G
ΔPS
A134D/AADAA
Full-length

To further dissect 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 (99). The aPKC sequence contains two arginine dipeptides that are located in its pseudosubstrate and could potentially be cleaved by Arg-C (Fig. 5*A*). However, we observed no cleavage of full length wild-type aPKC with Arg-C over the course of 120 minutes (Fig. 6*B*), consistent with its low catalytic activity. In contrast, the version of aPKC with an aspartic acid mutation in pseudosubstrate domain (A134D), which we found to be highly active in the kinase assay, is Arg-C sensitive. Mutation of the pseudosubstrate basic residues to alanine in the context of the activating A134D mutation (AADAA) abolished protease sensitivity, confirming that the pseudosubstrate is the cleavage site. Interestingly, we found that removal of the C1 domain increases Arg-C sensitivity of the pseudosubstrate indicating that the C1 functions to regulate aPKC activity by assisting pseudosubstrate interaction with the kinase domain. Thus, deletion of the C1 likely causes a dramatic change in the conformation of aPKC. Taken together, these results indicate that pseudosubstrate protease sensitivity is correlated with catalytic activity, further supporting a model in which aPKC is autoinhibited by the synergistic activity of C1 and pseudosubstrate.

While our data indicates that aPKC is autoinhibited *in vitro,* we sought to determine if 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 at the cortex, consistent with aPKC autoinhibition (Figs. 6*C*,*D*). When autoinhibition is disruputed, such as when the

pseudosubstrate is mutated (leading to high activity *in vitro)*, Lgl is efficiently displaced from the cortex. Thus, both *in vitro* and in a cellular context, aPKC is autoinhibited.

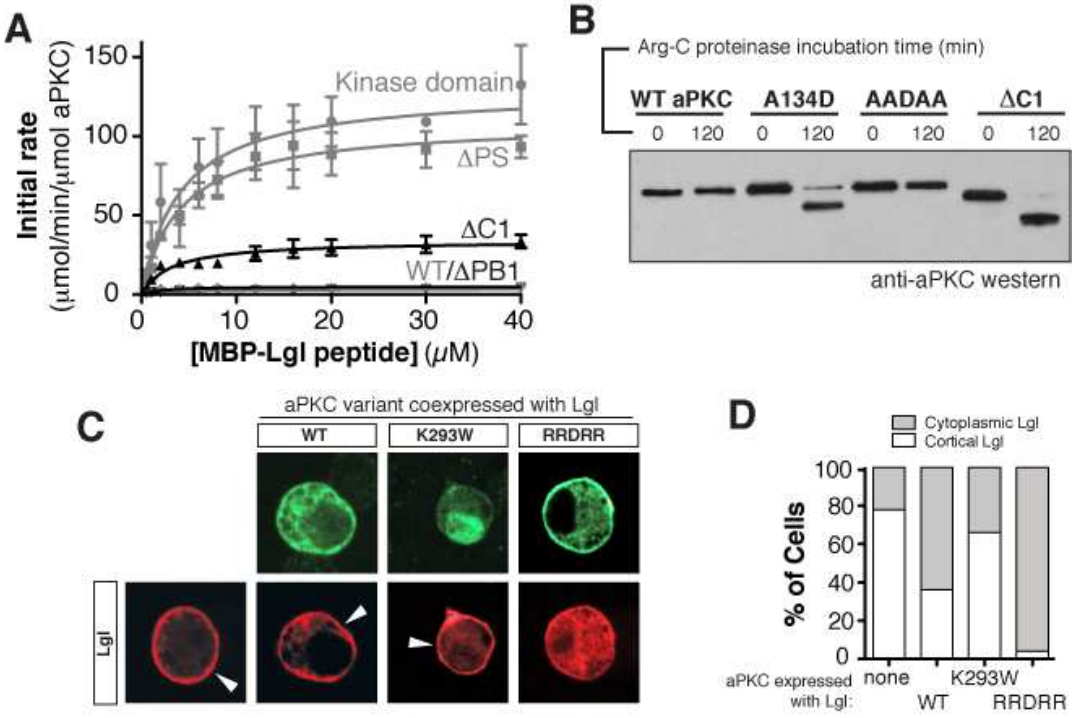


Figure 6. The C1 and pseudosubstrate synergistically repress aPKC activity.

A. Deletion of the PB1 domain has no effect on aPKC catalytic activity whereas loss of the C1 activates aPKC. The activity of the isolated kinase domain and full-length aPKC, both intact and lacking the pseudosubstrate, are shown for comparison.
B. Mutations that activate aPKC are sensitive to the Arg-C protease. Arg-C protease cleaves at the arginine dipeptides that are located solely in the aPKC pseudosubstrate. Wild-type aPKC is insensitive to Arg-C, presumably because the pseudosubstrate is bound to the kinase domain. The A134D aPKC is Arg-C sensitive, consistent with its increased activity. The AADAA mutation removes the protease cleavage site, confirming that cleavage does not occur at other sites under the conditions used. Deletion of the C1 exposes the pseudosubstrate indicating that the C1 functions, at least in part, to displace the pseudosubstrate.
C. Lgl cortical localization assay for aPKC activity. Lgl localizes to the cortex of cultured S2 cells (first column) even when co-expressed with aPKC (aPKC WT) or a kinase dead mutant (aPKC K293W), but becomes displaced into the cytoplasm with expressed with an aPKC pseudosubstrate mutant (aPKC RRDRR). Arrowheads indicate cortical Lgl signal.
D. Quantification of Lgl localization in S2 cells. The percentage of cells with cortical or cytoplasmic Lgl is shown when expressed by itself or with the aPKC variants shown in panel C. For each condition, 50 cells were examined.

Par-6 activates aPKC

Our observation that aPKC is autoinhibited is inconsistent with the current model in which Par-6 inhibits aPKC activity. The lack of detectable kinase activity for full-length aPKC under our assay conditions suggests that further inhibition is unlikely to be physiologically relevant. Thus, 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. 7*A*), 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 non-specific 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. 7*B*), and with high purity as shown by SDS-PAGE (Fig. 7*C*).

To determine the effect of Par-6 on aPKC activity, we first compared the activity of the co-purified aPKC/Par-6 complex to that of aPKC alone. Par-6 and aPKC interact via their PB1 domains (50), and Par-6 also contains semi-CRIB and PDZ domains (106) (Fig. 8*A*). Rather than lowering the activity of aPKC, we found that Par-6/aPKC activity is significantly higher than aPKC alone, comparable to the activity of the aPKC pseudosubstrate mutants (Fig. 8*B*). This effect is due, in part, to the Par-6 PB1 domain as this domain alone is sufficient to activate aPKC (Fig. 8*C*). 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.

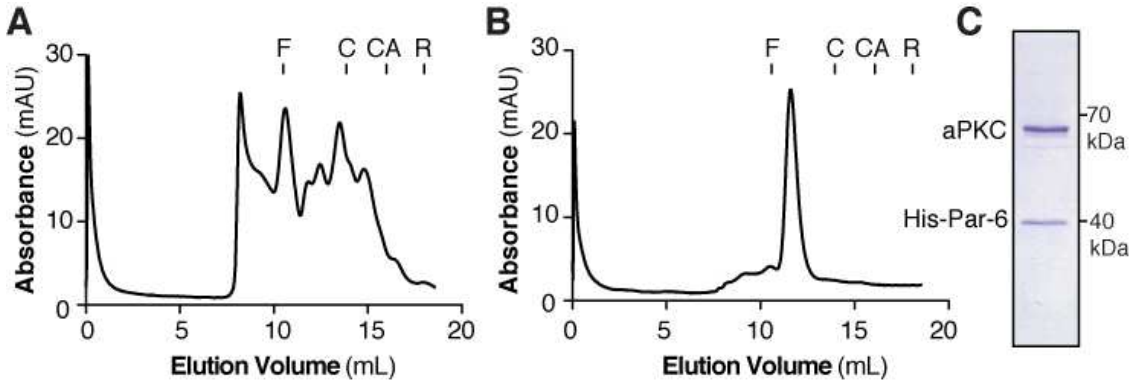


Figure 7. Purification of the Par-6/aPKC complex.

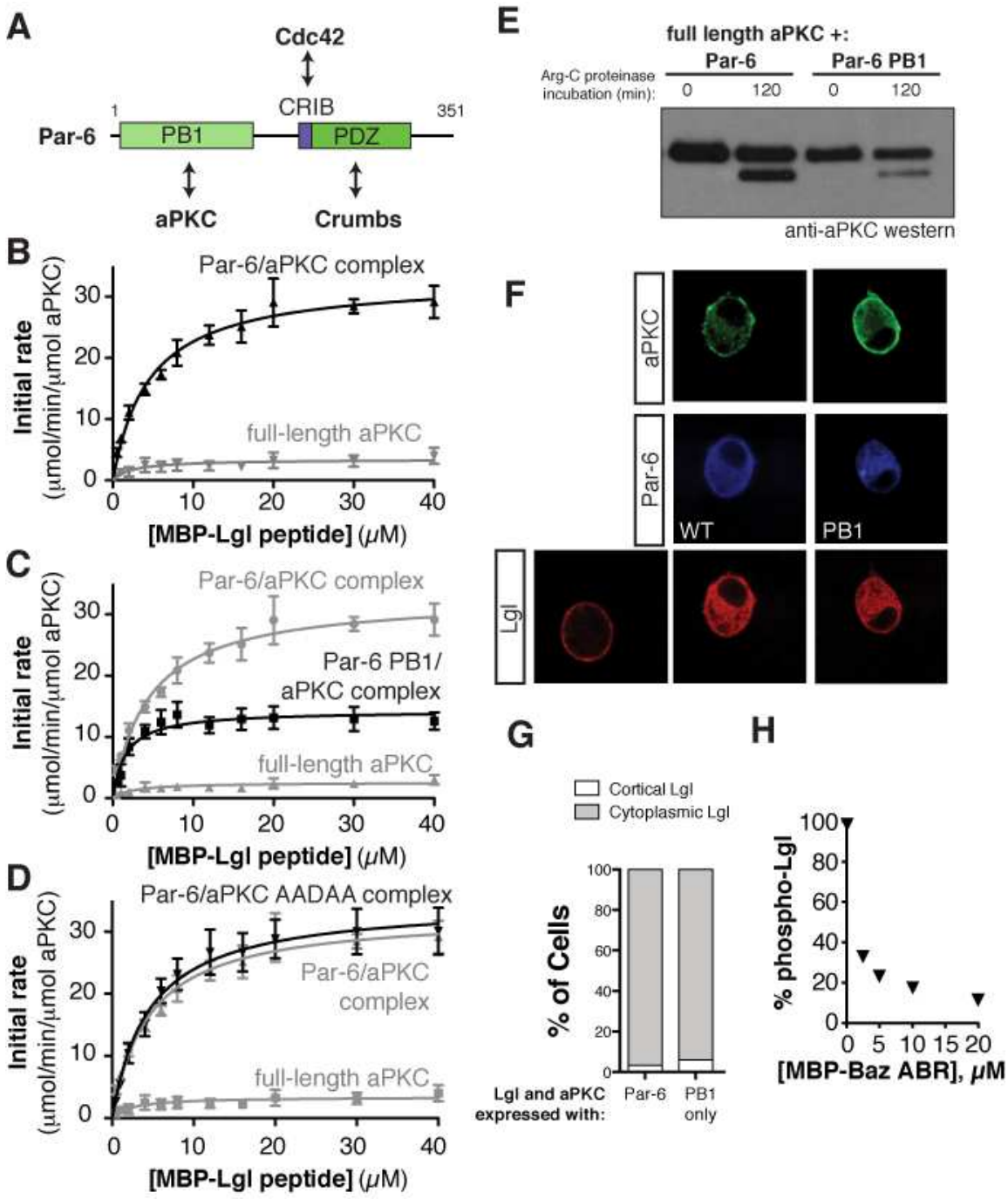
A. Bacterially expressed Par-6 is highly aggregated. Gel filtration chromatogram of purified bacterially expressed Par-6. Marks represent elution volume of standard proteins (F, ferritin–440 kDa; C, conalbumin–75 kDa; CA, carbonic anhydrase–29 kDa; R, RNAse A–13.7 kDa).
B. Par-6/aPKC form a discrete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

How does Par-6 activate aPKC? To determine if activation occurs through the pseudosubstrate, we measured the activity of Par-6 in complex with aPKC containing activating pseudosubstrate mutants (Fig. 8*D*). 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 Par-6/aPKC complex (Fig. 8*E*). As opposed to aPKC alone, the pseudosubstrate in the Par-6/aPKC 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 (Figs. 8*F,G*). While aPKC alone is unable to displace Lgl into the cortex, 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.

Figure 8 (next page)**.** Par-6 activates, and Baz represses aPKC.

A. Par-6 domain structure and interactions.
B. Par-6 increases the catalytic activity of aPKC. The activity of full-length aPKC is shown for comparison.
C. The 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 pseudosubtrate ("AADAA"), thereby activating aPKC (see Fig. 1G), has no effect in the Par-6/aPKC complex. The activities of full-length aPKC and Par-6 activated aPKC are shown for comparison.
E. The aPKC pseudosubstrate is protease sensitive when aPKC is bound to full-length Par-6 or its PB1 domain (see Fig. 1G for comparison to 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. Quantification of the Lgl localization data shown in panel F 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.



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 (85). To determine if Baz influences aPKC activity,

we examined the effect of the Baz aPKC binding region (ABR; Baz residues 905-1221) on Lgl phosphorylation by Par-6/aPKC (Fig. 8*H*). Addition of Baz dramatically decreased the extent of Lgl peptide phosphorylation.

Lgl is efficiently phosphorylated and released from the Par-6/aPKC complex

In *Drosophila* SOP cells, Lgl is thought to remain in a complex with aPKC until Aurora A activity phosphorylates Par-6, activating the complex (85). 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 Par-6/aPKC. We examined if Par-6/aPKC phosphorylated full-length Lgl *in vitro* and observed significant phosphorylation, although somewhat less than with the Lgl peptide (Fig. 9*A*). We also tested whether Lgl is a stable part of the Par complex using a Maltose Binding Protein (MBP) fused Lgl adsorbed onto amylose resin. This protein efficiently pulls down Par-6/aPKC in the absence of ATP, but addition of ATP abolished the interaction (Fig. 9*B*). The result of this interaction assay indicates that MBP-Lgl is released from Par-6/aPKC once it is phosphorylated, as expected for an enzyme's substrate, suggesting that Lgl transiently associates with the Par complex.

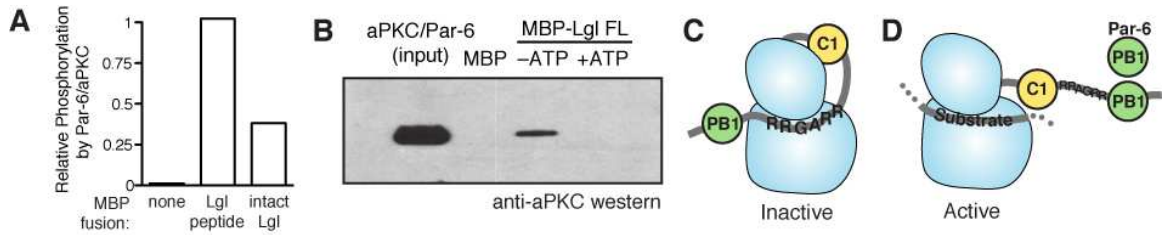


Figure 9. Lgl is a transient component of the Par complex.

A. Full-length Lgl is phosphorylated by the Par-6/aPKC complex. Comparison of the relative phosphorylation of Lgl full-length and the Lgl peptide (see Fig. 1A).
B. Phosphorylated Lgl is released from the complex. Although MBP-Lgl efficiently pulls down aPKC in the absence of ATP, the addition of ATP causes release of the enzyme, such that it is no longer pulled-down.
C. Model for autoinhibited aPKC. The C1 and pseudosubstrate synergistically repress the aPKC catalytic domain. D, Model for Par-6 activated aPKC. The interaction of the Par-6 PB1 with aPKC causes release of the pseudosubstrate from the aPKC catalytic domain.

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 the 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 of substrate, the kinase domain is approximately 50-fold more active than full length aPKC. We focused on three NH_2-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 (107), the C1 domain makes contacts 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 isn't required for autoinhibition, its interaction with the Par-6 PB1 leads to activation. Par-6 activation of aPKC doesn't lead to full activity, as the kinase domain alone is still significantly more active. However, the ability of Par-6/aPKC 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 (Figs. 9*C,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 (108), 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 (106,107). In aPKC, activation instead occurs by binding to the PB1 domain which lies on the opposite side of the pseudosubstrate (Fig. 5*A*).

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 aPKC 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.

BRIDGE TO CHAPTER III

In this chapter, I have demonstrated how intra- and intermolecular interactions work synergistically to regulate aPKC activity and localization. In a polarized cell, aPKC activity outputs as exclusion of aPKC substrates from the cortex. How does phosphorylation result in displacement of substrate molecules? One of the observations is that Mira, Lgl, and Numb all contain multiple phosphorylation sites. However, it is unclear if multi-site phosphorylation is involved in the regulation of displacement mechanisms. Also, little is know about how aPKC phosphorylates its substrates at

multiple locations. In the following chapter, I describe how multi-site phosphorylation plays a vital role in substrate release from the membrane using Lgl as a model substrate in cultured *Drosophila* S2 cells. Finally, I investigate how aPKC preferentially phosphorylates Lgl at three serine residues using detailed kinetic assays.

CHAPTER III

ORDERED MULTI-SITE PHOSPHORYLATION OF LETHAL GIANT LARVAE BY ATYPICAL PROTEIN KINASE C

This chapter contains unpublished material prepared for submission to *Journal of Biological Chemistry.*

Author contributions: K. E. Prehoda and I designed research; I performed experiments; K. E. Prehoda and I wrote the manuscript.

INTRODUCTION

Many cells organize their cortical regions into large, molecularly discrete domains. The resulting cell polarity is essential for a broad array of processes, including cytokinesis, movement, and asymmetric division (86,87). The atypical Protein Kinase C (aPKC), part of the Par polarity complex, has emerged as a key organizer of the cell cortex (31,37,109). In Par-mediated polarity, upstream components specify the location and activity of aPKC. Once at the cortex, aPKC phosphorylates substrates, displacing them into the cytoplasm, thereby ensuring that they only occupy cortical regions opposite aPKC (37,91,110). Because a key aspect of Par polarity is phosphorylation coupled cortical displacement, we have investigated the detailed kinetics of aPKC substrate phosphorylation and the requirements for release from the cortex.

The Par complex directs polarity in diverse systems using a mechanism that relies on aPKC-mediated phosphorylation. Besides aPKC, the Par complex consists of Par-3 (Bazooka in flies) and Par-6. These proteins, along with a large number of upstream regulators, control aPKC localization and kinase activity in cells ranging from epithelia to neural stem cells. When not bound to Par-6, aPKC exists in an autoinhibited form with an internal pseudosubstrate motif bound to its kinase domain (111). Recruitment to the cortex by Par-3 and Par-6 activates aPKC such that it can efficiently phosphorylate target proteins, such as the tumor suppressor Lethal giant larvae (Lgl) and the fate determinants Numb and Miranda. Once phosphorylated, these substrates become cytoplasmic, effectively displacing them from cortical regions containing aPKC. In the absence of aPKC catalytic activity, these substrates become depolarized with severe physiological consequences (43-45).

As substrate phosphorylation by aPKC appears to be a central element of polarity, we have investigated the detailed kinetics of the process. Many aPKC substrates contain numerous phosphorylation sites (e.g. 3 sites for Lgl (44), 5 sites for Mira (43), and 5 sites for Numb (45)) and non-phosphorylatable mutants are not polarized by aPKC. These studies suggest that multi-site phosphorylation is an important component of aPKC phosphorylation, although this hypothesis has not been tested. Is phosphorylation at all sites required for cortical displacement? Are the kinetic parameters for aPKC phosphorylation the same at each site? Is there synergy between phosphorylation at each site?

Here, we investigated the role of the multiple phosphorylation using Lgl as a model aPKC substrate. First, we tested the number of phosphorylation sites needed for

cortical displacement of Lgl using cultured *Drosophila* S2 cells. We find that Lgl requires at least two, possibly three, phosphorylations for membrane displacement. Also, we examined how aPKC phosphorylates Lgl at three Ser residues using *in vitro* kinase assays. We find that the three Ser sites are not kinetically equivalent, for aPKC shows a clear preference among them. Furthermore, we utilized phospho-mimetic mutants to test whether or not multi-phosphorylation is dependent. We find that it is dependent but the effects depend on the position of the phospho-mimetic mutation(s). We conclude that phosphorylation of Lgl at multiple sites by aPKC plays a vital role in regulation of Lgl localization. Our results may be extended to the other aPKC substrates and differentiation factors to understand the mechanism of polarization. Our findings could also provide new insight to the substrate recognition and preference of aPKC.

MATERIALS AND METHODS

S2 Lgl cortical localization assay

Immunofluorescence was as previously described (100). 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 metallothionen promoter. mCherry: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. ~$2x10^6$ cells were seeded per 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 24h, 200μL of cells were seeded on 12 mm diameter glass coverslips in a 24-well plate and allowed to adhere for 1h. Cells were fixed for 20 minutes with 4% formaldehyde in PBS followed by 3 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^oC with rabbit anti-aPKC antibody (1:1000; Santa Cruz Biotechnology). Coverslips were then rinsed 3x with blocking buffer, incubated at room temperature for 2h with species-specific secondary antibodies (1:200; Jackson Immunoresearch), rinsed 3x in washing buffer, and mounted in Vectashield Hardset Mounting Medium (Vector Laboratories). Images were acquired on a confocal microscope (Radiance; Biorad laboratories) using an oil-immersion 60x 1.4NA objective, processed with ImageJ, and assembled in Adobe Illustrator.

Purification of aPKC 259-606 (Kinase Domain)

HEK293 F cells ($1X10^6$ cells/mL) were transfected with pCMV His_6-aPKC 259-606 and pCMV dPDK-1 (without any tag) using the 293fectin transfection reagent (Life Technology). The cells were incubated at 37 °C for 48 hours. To harvest, the cells were resuspended with Ni^{2+} lysis buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1 mM MgCl, 10 mM β-ME, 10 mM Imidazole, adjusted to pH 7.5 with NaOH). Then, the cells were lysed open using syringes and 21-gauge needles and the whole cell lysate was centrifugation at 15,000 rpm for 30 min. at 4 °C. The supernatant was incubated with Ni^{2+} NTA resins for 45 min at 4 °C. The resins were washed with the lysis buffer. The proteins were eluted using Ni^{2+} elution buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1 mM MgCl, 10 mM B-ME, 250 mM Imidazole, adjusted to pH 7.5 with NaOH). The

eluted proteins were dialyzed at 4 °C for 4 hours. The concentration of aPKC was determined by comparing its reactivity with an anti-aPKC antibody (Santa Cruz Biotech) with that of a standard of known concentration (bacterially expressed aPKC kinase domain purified and quantified using a Bradford dye binding assay) on a western blot.

Expression and purification of Lgl peptides

The esidues 647-673 of *Drosophila* Lgl isoform A were cloned into pMAL-C2 vector (New England BioLabs Inc.), in which a TEV protease recognition site was added following the MBP coding sequence. The residues S656, S660, and S660 in Lgl 647-673 were mutated to alanines or aspartic acids. The constructs were transformed into BL21 *E. coli* cells. The expressions were induced by 0.4 mM IPTG at 18 °C overnight. The bacterial lysates were incubated with amylose resins (New England BioLabs Inc.). 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 20mM Tris□HCl pH7.5, 50mM NaCl, 1 mM DTT.

In vitro kinase activity assay

aPKC kinase activity was measured as previously described (98). Briefly, the purified aPKC kinase domain was diluted to concentrations at which the incorporation of radiolabeled phosphate from [γ^{-32}P]ATP into MBP-Lgl peptides were 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 $MgCl_2$) with a wide range

of MBP-Lgl peptide concentrations at 30 °C for 5 min. The reactions were initiated by adding 1mM ATP spiked with [γ^{-32}P]ATP (~1.0 x 10^6/nmol ATP). The reactions were incubated at 30 °C for 10 min. The reaction mixtures were blotted on Grade P81 phosphopaper (Whatman). The reactions were quenched by immediately submerging the blotted P81 paper in 75 mM H_3PO_4. 5mL of scintillation fluid were added to measure the radioactive decays by liquid scintillation counter.

Kinetic modeling

Copasi was used to model the phosphorylation reaction of wild type Lgl. To investigate if phosphorylations are cooperative, we used measured K_M and V_{max} values of SAA, ASA, and AAS for the first phosphorylation, SDA, SAD, ASD, DSA, ADS, and DAS for the second phosphorylation, and SDD, DSD, and DDS for the third phosphorylation. The phosphorylations were plotted over time.

Separation of single-, double-, and triple-phosphorylated Lgl peptides

A reaction containing 15 μM MBP-Lgl 647-673, aPKC kinase domain, 1 mM ATP in the reaction buffer described above was set up and incubated at 30 °C for 90 min. Small aliquots of the reaction mixture were quenched with SDS-PAGE loading buffer periodically during the incubation. The quenched samples were run on Mn^{2+}- Phos-tag SDS-PAGE (Wako USA). The phosphorylated Lgl was detected on western blot using mouse anti-MBP (1:1000; Santa Cruz Biotechnology) and bovine anti-mouse HRP (1:2000; Santa Cruz Biotechnology).

RESULTS

Lgl cortical displacement requires phosphorylation at multiple serines

aPKC phosphorylates Lgl at three serine residues *in vitro*, and non-phosphorylatable Lgl fails to be released from the plasma membrane when expressed in neuroblasts. Non-phosphorylatable Lgl also causes the fate determinant Miranda to become depolarized (44). These data indicate that phosphorylation plays a crucial role in Lgl localization and function. However, the role of Lgl's multiple phosphorylation sites is unknown.

To determine the role of individual Lgl phosphorylation events in its localization, we mutated the phosphorylatable serine residues to alanine in all possible combinations (Fig. 10A). The resulting proteins were tested for their localization in cultured *Drosophila* S2 cells, which have been utilized previously to investigate the membrane localization of aPKC substrates including Lgl. Wild type Lgl localizes at the plasma membrane when expressed alone, and in the cytoplasm when co-expressed with active aPKC. When there is only one Ser residue available for aPKC to phosphorylate, Lgl remained at the cortex regardless of the position of the phosphosite (fig. 10B). The comparison of cytoplasmic/cortical intensity ratios showed that the mutants are not significantly different from Lgl alone ($p < 0.01$) (Fig. 10C), suggesting that a single phosphorylation is not sufficient to release Lgl from the membrane.

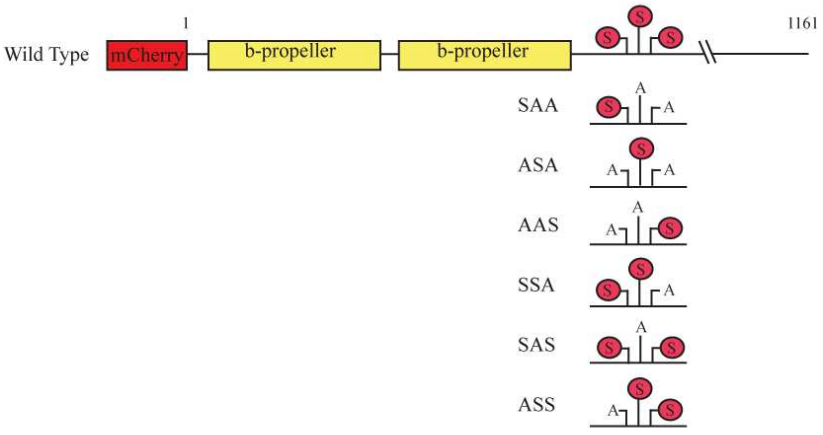
While one phosphorylation does not lead to a detectable effect on Lgl's localization, Lgl mostly localized in the cytoplasm when two of the three serine residues were available for aPKC to phosphorylate (fig. 10B). However, the degree of cytoplasmic

localization was still distinguishable from phosphorylated wild type Lgl as the cortical/cytoplasmic ratio for the two phosphorylation site Lgls are statistically different from both Lgl alone and Lgl with aPKC (Fig. 10C). Our data showed the general trend of increased cytoplasmic localization and reduced cortical/cytoplasmic intensity ratio, as the number of the available phosphorylation sites increased. These data suggest that Lgl needs to be phosphorylated at all three phosphorylation sites to be completely displaced from the membrane.

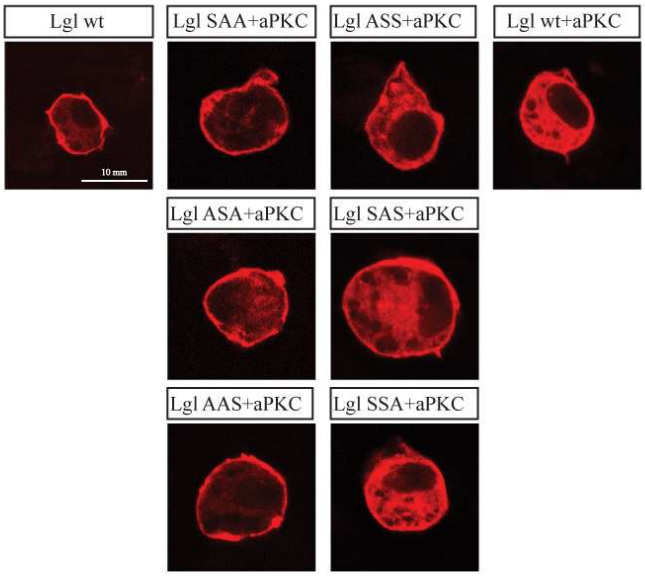
Figure 10 (next page)**.** Lgl requires multiple phosphorylation for cortical displacement.

A. Lgl domain structure and aPKC phosphosite positions. Localization assays used full length Lgl whereas the kinetic assays were performed with maltose-binding protein-fused Lgl residues 647-673.The localization of mCherry-Lgl mutants in cultured *Drsophila* S2 cells. The localization assays show the more phosphorylatable, the more cytoplasmic Lgl localizes.
B. The localization of mCherry-Lgl wild-type and aPKC phosphosite mutants in cultured *Drsophila* S2 cells.
C. Quantification of Lgl cortical localization (n = 50 for each condition). ANOVA statistical analysis reveals that cortical recruitment of wild-type Lgl is indistinguishable from singly-phosphorylated Lgl (SAA, ASA, and AAS), and Lgl variants with two phosphorylatable sites (ASS, SAS, and SSA) are distinguishable from wild-type.

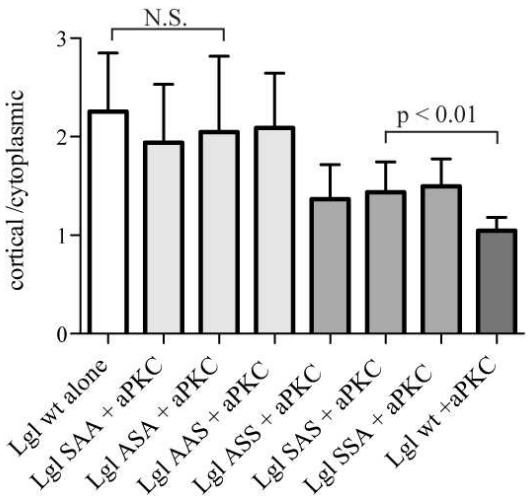
A.


1
1161
Wild Type
mCherry
b-propeller
b-propeller
S
S
S
SAA
A
S
A
A
ASA
S
A
A
AAS
A
A
S
SSA
S
S
A
SAS
A
S
S
ASS
S
A
S

B.


Lgl wt
Lgl SAA+aPKC
Lgl ASS+aPKC
Lgl wt+aPKC
10 mm
Lgl ASA+aPKC
Lgl SAS+aPKC
Lgl AAS+aPKC
Lgl SSA+aPKC

C.


N.S.
p < 0.01
cortical /cytoplasmic
3
2
1
0
Lgl wt alone
Lgl SAA + aPKC
Lgl ASA + aPKC
Lgl AAS + aPKC
Lgl ASS + aPKC
Lgl SAS + aPKC
Lgl SSA + aPKC
Lgl wt +aPKC

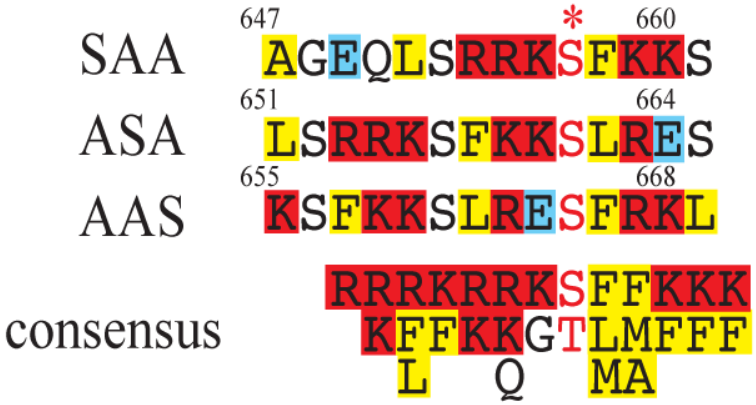
aPKC preferentially phosphorylates Lgl at Ser^{656} and Ser^{664}

The localization assays demonstrated the necessity of multiple phosphorylation for Lgl displacement. Thus, intrinsic kinetic differences between the three sites could be very important for Lgl localization and function. However, it was unclear whether aPKC would preferentially phosphorylate the serine residues or if each of the three sites are equivalent. Compared to the consensus sequence (Fig. 11A), the amino acid sequence alignment surrounding the phosphorylation sites for each Lgl peptide shows that all peptides satisfy the requirement at P-5, P-2, and P+1 positions while they all fail to match at P-4 and P+2 positions. Also, there are more variations at N-terminal residues away from each phosphorylation sites. Therefore, comparison of each of the Lgl subsite sequences with the consensus sequence suggests that aPKC could have different kinetic parameters for each of the three sites.

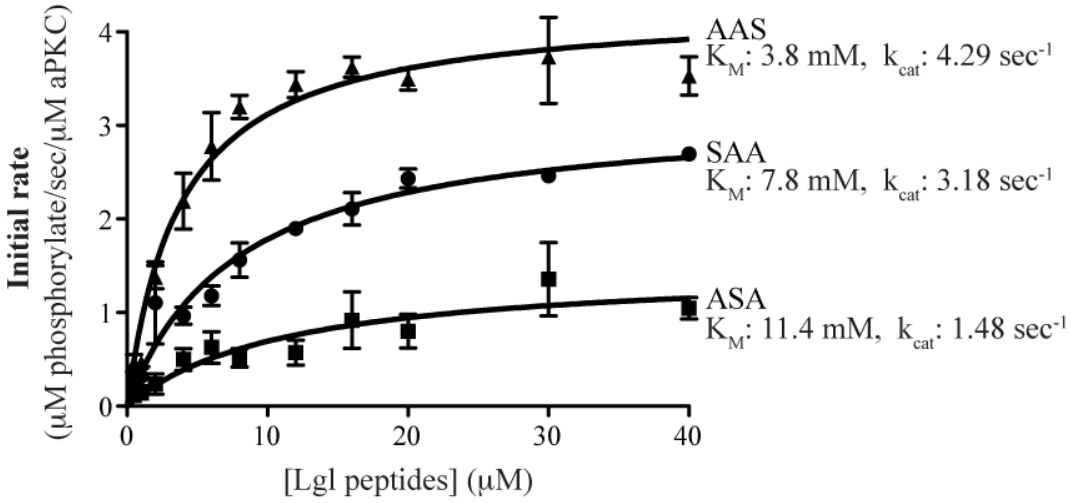
Figure 11 (next page)**.** Non-equivalent phosphorylation of the three Lgl phosphosites by aPKC.

A. . Amino acid sequence alignment of Lgl phosphorylation sites. The phopho-accepting serines (P_0) are labeled with red asterisks. The optimal consensus sequence is shown at the bottom (112). Note that multiple residues are accepted in some positions.
B. Kinetic analyses of each singly-phosphorylated Lgl peptide, showing differences in K_M and k_{cat}.
C. The possible pathways to phosphorylate all three phosphosites. Phosphorylated residues are highlighted with red. If the phosphorylation events are independent, based on the results from b, preferential phosphorylation occurs in the order of Ser^{664} $\rightarrow$ Ser^{656} $\rightarrow$ Ser^{660}.

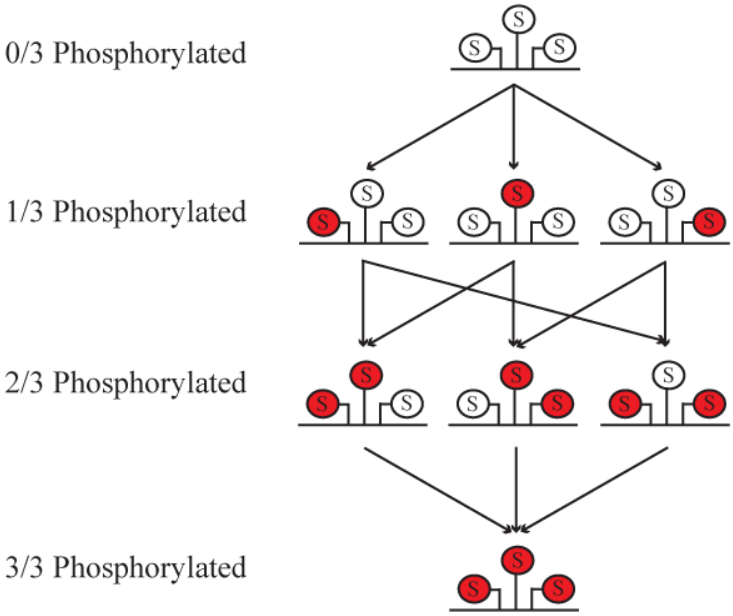
A.


647
660
SAA
AGEQLSRRKSFKKS
651
664
ASA
LSRRKSFKKSLRES
655
668
AAS
KSFKKSLRESFRKL
consensus
RRRKRRKSFFKKK
KFFKKGTLMFFF
L Q MA

B.


Initial rate
(μM phosphorylate/sec/μM aPKC)
[Lgl peptides] (μM)
AAS
K_M: 3.8 mM, k_cat: 4.29 sec-1
SAA
K_M: 7.8 mM, k_cat: 3.18 sec-1
ASA
K_M: 11.4 mM, k_cat: 1.48 sec-1

C.


0/3 Phosphorylated
1/3 Phosphorylated
2/3 Phosphorylated
3/3 Phosphorylated

To determine whether the three serine residues are equivalent or not, we generated singly-phoshorylatable Lgl peptides and performed the kinase assays. Using Michaelis-Menten analyses, we determined the catalytic efficiency (k_{cat}/K_M) for phosphorylation at each subsite. As Fig. 11B shows, K_M values for each serine were 7.8 µM, 11.4 µM, and 3.8 µM and k_{cat} values were 3.18 sec^{-1}, 1.48 sec^{-1}, and 4.29 sec^{-1} for SAA, ASA, and ASA, respectively. The catalytic efficiency values were 0.407 μM^{-1} sec^{-1}, 0.130 μM^{-1} sec^{-1}, and 1.141 μM^{-1} sec^{-1}, respectively. There are six possible pathways to phosphorylate all three Lgl serine residues (Fig. 11C) and, if the sites were independent, our data indicate a preferential order of Ser^{664} > Ser^{656} > Ser^{660}.

aPKC phosphorylation of Lgl is cooperative

As each serine residue in Lgl is only separated by three residues, it is likely that phosphorylation events interact with one another and phosphorylation at one site could influence the kinetic parameters of others (i.e. cooperativity). To investigate whether Lgl phosphorylation by aPKC is cooperative, we utilized phospho-mimetic mutants of Lgl peptides and tested them in kinase activity assays.

First, we tested how phosphorylation of a serine residue affects phosphorylation of directly neighboring sites (Fig. 12A). The values of K_M for SDA, DSA, ASD, and ADS were 14.4 µM, 5.9 µM, 7.9 µM, and 6.2 µM and k_{cat} were 3.31 sec^{-1}, 1.82 sec^{-1}, 0.69 sec^{-1}, and 4.82 sec^{-1}, respectively. The catalytic efficiencies were 0.23 μM^{-1} sec^{-1}, 0.31 μM^{-1} sec^{-1}, 0.09 μM^{-1} sec^{-1}, and 0.77 μM^{-1} sec^{-1}, respectively. Compared to SAA, SDA showed 50 % reduction in the catalytic efficiency. The reduction was attributed by K_M increase. Both DSA and ASD showed decrease in K_M values relative to ASA. However,

the replacement of Ser^{656} to an Asp residue increased k_{cat} and the catalytic efficiency while the replacement at Ser^{664} decreased both values. Finally, ADS showed an increase in K_M value, reducing phosphorylation rate compared to AAS. These data indicate that prior phosphorylations on the direct neighboring sites reduce the phosphorylation rates of Ser^{656} and Ser^{664}. In contrast, the presence of the Asp residue at the adjacent site had favorable effect for Ser^{660}, which may indicate that the negative charges may be needed for its efficient phosphorylation.

We next examined how a phosphate group influences the non-directly neighboring site using SAD and DAS peptides (Fig. 12B). Initial rate analyses showed that K_M values were 27.37 μM and 3.73 μM and the values for k_{cat} were 4.41 sec^{-1} and 3.38 sec^{-1}, respectively. The catalytic efficiencies were 0.16 μM^{-1} sec^{-1} and 0.91 μM^{-1} sec^{-1}. The effect of the Asp residue at P+8 was more pronounced than at P+4 for Ser^{656}, for K_M value SDA was 2 times higher than SAA and the effect on k_{cat} was minimal while K_M value of SAD was 3 times higher and k_{cat} was 40 % higher than SAA. For DAS, on the other hand, the effect was more subtle, in which k_{cat} was reduced from AAS by 20 % and almost no change in K_M. Combined together, the data show that phosphomimetic

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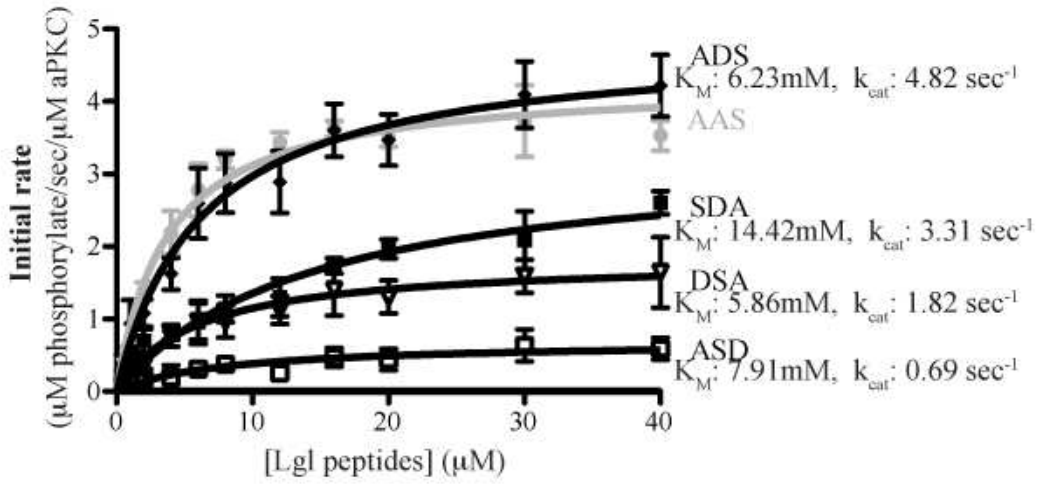
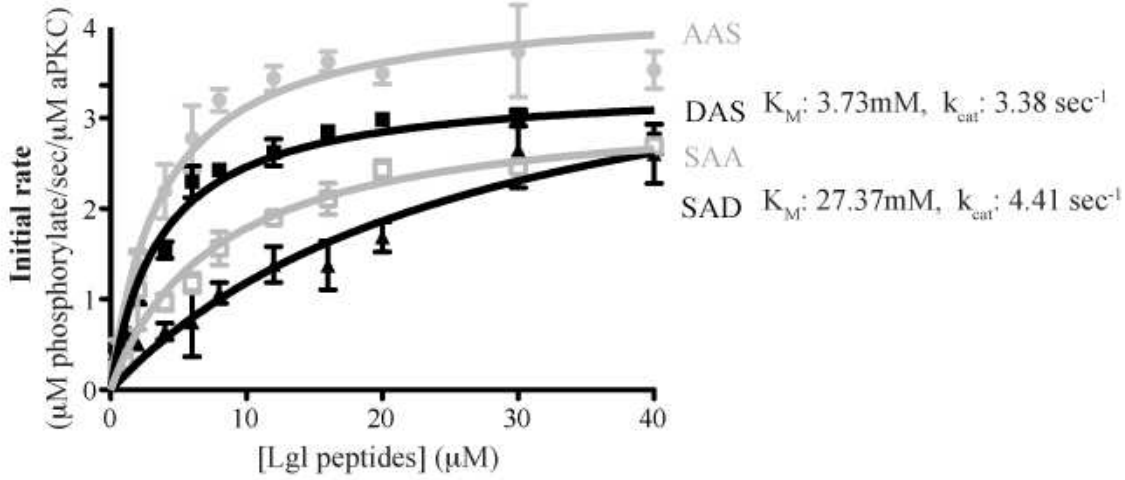
Figure 12 (next page). Phosphorylation events are cooperative.

A-C. Phosphorylation rates of individual Lgl sites are affected by the phosphorylation state of neighboring sites (as assessed by phosphomimetic residues). Panel (a) shows the effect of modification at directly neighboring sites, while panel (b) shows the effect of more distant modifications. Finally, (c) demonstrates the effect of modification at both of the other sites. The data show - the phosphorylation rates are cooperative but the affects differ depending on the positions of the phospho-accepting residue and modified site(s). Based on the results from a-c, phosphorylation primarily occurs in the order of $Ser^{664} \rightarrow Ser^{660} \rightarrow Ser^{656}$.

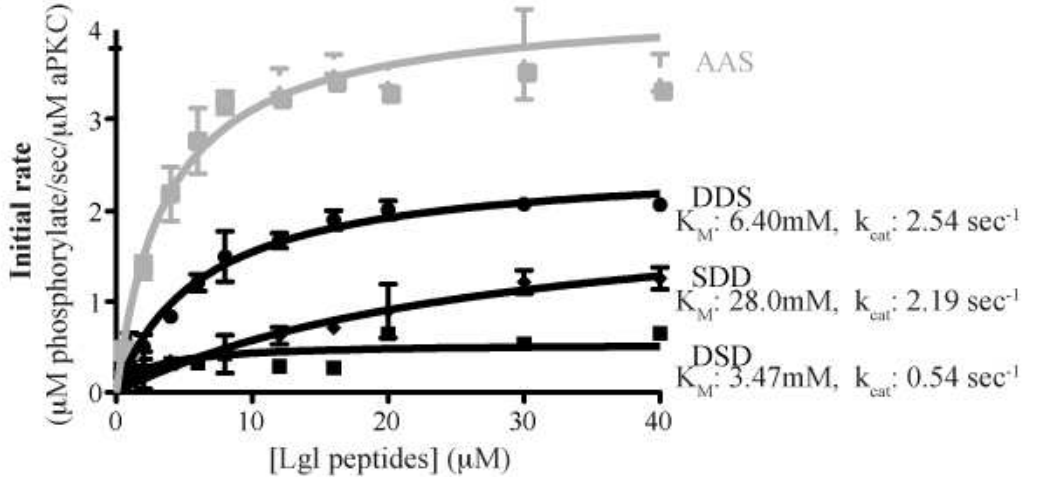
A.


Initial rate
(μM phosphorylate/sec/μM aPKC)
[Lgl peptides] (μM)
ADS
K_M: 6.23mM, k_cat: 4.82 sec^-1
AAS
SDA
K_M: 14.42mM, k_cat: 3.31 sec^-1
DSA
K_M: 5.86mM, k_cat: 1.82 sec^-1
ASD
K_M: 7.91mM, k_cat: 0.69 sec^-1

B.


Initial rate
(μM phosphorylate/sec/μM aPKC)
[Lgl peptides] (μM)
AAS
DAS K_M: 3.73mM, k_cat: 3.38 sec^-1
SAA
SAD K_M: 27.37mM, k_cat: 4.41 sec^-1

C.


Initial rate
(μM phosphorylate/sec/μM aPKC)
[Lgl peptides] (μM)
AAS
DDS
K_M: 6.40mM, k_cat: 2.54 sec^-1
SDD
K_M: 28.0mM, k_cat: 2.19 sec^-1
DSD
K_M: 3.47mM, k_cat: 0.54 sec^-1

residue at P+8 influenced k_{cat}, but it changed K_M at P+4. These data indicate that proximal phosphorylation at P-8 or P+8 reduces catalytic efficiency.

We next examined how a phosphate group influences the non-directly neighboring site using SAD and DAS peptides (Fig. 12B). Initial rate analyses showed that K_M values were 27.37 μM and 3.73 μM and the values for k_{cat} were 4.41 sec^{-1} and 3.38 sec^{-1}, respectively. The catalytic efficiencies were 0.16 μM^{-1} sec^{-1} and 0.91 μM^{-1} sec^{-1}. The effect of the Asp residue at P+8 was more pronounced than at P+4 for Ser^{656}, for K_M value SDA was 2 times higher than SAA and the effect on k_{cat} was minimal while K_M value of SAD was 3 times higher and k_{cat} was 40 % higher than SAA. For DAS, on the other hand, the effect was more subtle, in which k_{cat} was reduced from AAS by 20 % and almost no change in K_M. Combined together, the data show that phosphomimetic residue at P+8 influenced k_{cat}, but it changed K_M at P+4. These data indicate that proximal phosphorylation at P-8 or P+8 reduces catalytic efficiency.

Finally, we examined how two phosphate groups affect the phosphorylation of the remaining Ser residues using SDD, DSD, and DDS Lgl peptides (Fig. 12C). The values of K_M were 26.37 μM, 2.47 μM, and 6.40 μM and k_{cat} were 2.04 sec^{-1}, 0.54 sec^{-1}, and 2.54 sec^{-1}, respectively. The catalytic efficiencies were 0.08 μM^{-1} sec^{-1}, 0.22 μM^{-1} sec^{-1}, and 0.40 μM^{-1} sec^{-1}, respectively. These data show that the presence of two Asp residues decrease the phosphorylation rates of Ser^{656} and Ser^{664} while they increase that of Ser^{660}. These results indicate that Ser^{660} is more likely to be phosphorylated after Ser^{656} and Ser^{664} are phosphorylated.

All the kinetic parameters measured showed that the replacement of Ser^{660} to Asp residues resulted in increased K_M values for Ser^{656} and Ser^{664}. On the other hand, the

presence of phosphomimetic residue(s) were beneficial to Ser^{660}. Taken together, the primary order of phosphorylation is likely to be Ser^{664} -> Ser^{660} -> Ser^{656} while the secondary pathway may be Ser^{656} -> Ser^{664} -> Ser^{660}.

Ordered phosphorylations of aPKC

Considering how similar the sequences of the three Lgl phosphorylation subsites are, it was surprising how aPKC presented clear preferences among them. We reasoned that the residues which all three subsites failed to satisfy the consensus sequence requirement may not contribute much in the activity differences observed, and that the preferential phosphorylation of Ser^{664} originated from the differences in the residues surrounding Ser^{664} and Ser^{660}.

To investigate the basis for the subsites preference within Lgl, we examined series of mutations in the context of Lgl ASA (Fig. 13A). First, we noticed that ASA peptide had a Glu residue at P+3 position while SAA and AAS peptides had a Lys at the same position. The values of K_M and k_{cat} for ASA E663K mutant were 12.39 μM and 1.68 μM^{-1} sec^{-1}, respectively, indicating that the replacement of Glu to Lys at P+3 does not increase the rate of ASA phosphorylation. We also tested ASA L661F mutant. Although ASA mutant satisfies the hydrophobic residue requirement at P+1 position, both SAA and AAS peptides had a Phe residue. The values for K_M and k_{cat} for ASA L661F were 10.66 μM and 2.47 μM^{-1} sec^{-1}, respectively, suggesting that the substitution of Lue to Phe at P+1 position did not affect K_M but k_{cat} increased by 66%, compared to ASA. When ASA L661F E663K double-mutant was tested, k_{cat} value was 1.70 μM^{-1} sec^{-1} and comparable to ASA, but K_M value was reduced down to 1.88 μM. This was 6 times lower

than ASA and 2 times lower than AAS, implying that the those two residues may play roles in binding affinity.

We followed the concentration of singly, doubly, and triply phosphorylated Lgl to determine how well the kinetic parameters from each site could predict bulk phosphorylation behavior. Using SDS-PAGE gels containing Mn^{2+} and Phos-tag, we were able to resolve each of the phosphorylated species (Fig. 13B). We then quantified the degree of phosphorylation and compared to a simulation of wild type Lgl phosphorylation (Fig. 13C).

DISCUSSION

Phosphorylation coupled release of aPKC substrates from the plasma membrane is a central element of Par-mediated polarization. The mechanism by which substrates are released from cortex has been unclear, however. In the case of the tumor suppressor Lgl, phosphorylation by aPKC is crucial for not only asymmetric cell division but also epithelial cell polarity and cell migration (103). In the present work, we have found that

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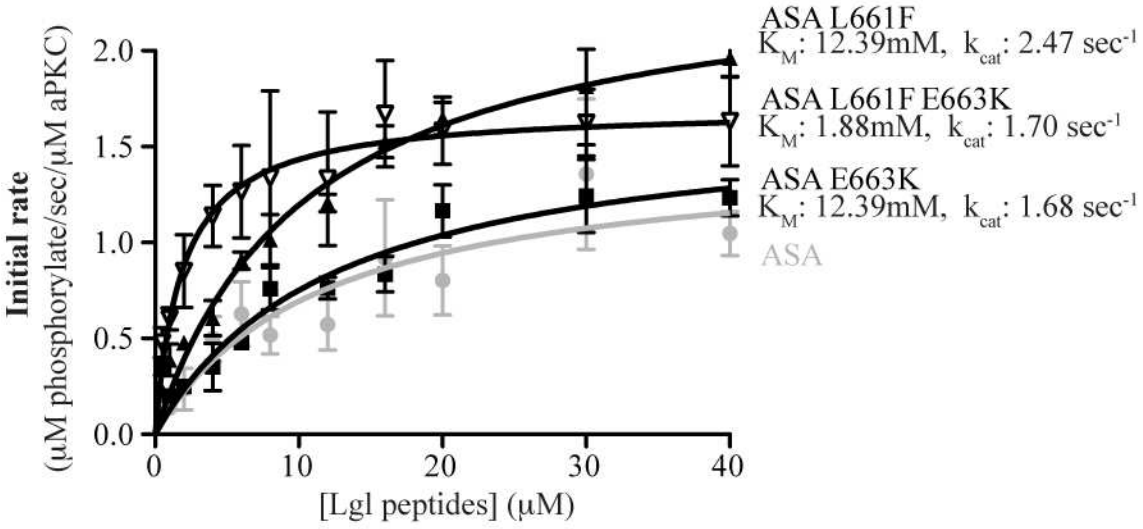
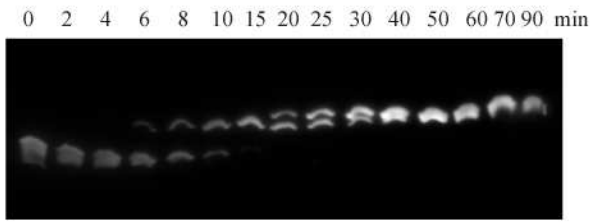
Figure 13 (next page). Ordered phosphorylations of aPKC

A. Critical recognition positions that determine the K_M for aPKC phosphorylation. Mutation of L661F and E663K together converted K_M of ASA peptide to that of AAS peptide. Phosphorylation of Lgl peptide with three serines. 15 mM of wild type Lgl peptide was phosphorylated for 90 min in the presence of aPKC kinase domain and 1 mM ATP. The phosphorylated Lgl was separated on Mn2+ Phos-tag SDS-PAGE.
B. Time course of wild-type Lgl phosphorylation as determined by phospho-resolving gel electrophoresis.
C. Modeling of Lgl phosphorylation by aPKC. The degree of phosphorylation was quantified using the data from panel (B) and overlaid with a simulation of wild type Lgl phosphorylation (solid lines) using the data measured in the previous sections.

A.

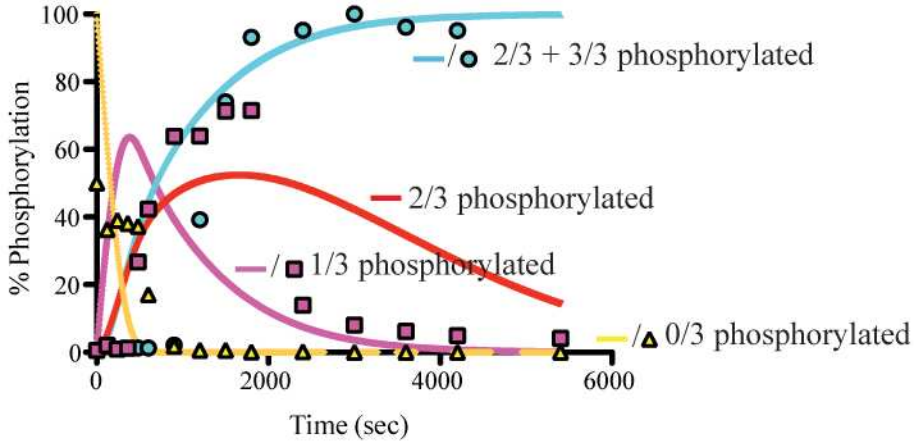


B.



WB: Mouse anti-MBP

C.



aPKC phosphorylation of Lgl at each of its three phosphosites is required for full displacement from the cortex in a cultured S2 cell model system. Because multi-site phosphorylation is essential for cortical release, and therefore polarization, we investigated the kinetics at each site, and how phosphorylation at one site influences others. A mathematical model for Lgl phosphorylation based on the empirical parameters we measured is in good agreement with the overall rates of Lgl phosphorylation that we observed.

Multi-site phosphorylation is coupled to cortical release of Lgl

We first addressed the relationship between the number of phosphorylations and cortical localization of Lgl by expressing various Lgl constructs in cultured *Drosophila* S2 cells. We observed a general trend of increasing cytoplasmic Lgl with increasing phosphorylation states, indicating an additive effect of multi-site phosphorylation on membrane interaction. Similar behavior has been observed with another aPKC substrate, myristoylated-alanine rich C-kinase substrate (MARCKS). Phosphorylation of three serine residues in MARCKS reduces its electrostatic attraction for the membrane, resulting in translocation of MARCKS into the cytoplasm (113-115). Multi-site phosphorylation of aPKC substrates could be a general mechanism for altering the electrostatic charges of the substrates to regulate their membrane interactions in cell. It is of interest to determine whether multi-site phosphorylation exhibits the same effects on other aPKC substrates such as Miranda and Numb.

Phosphorylation order as regulatory element for cortical displacement

We examined whether phosphorylation of each serine residues were equivalent and found that aPKC preferentially phosphorylates Lgl in the order of $Ser^{664} > Ser^{656} > Ser^{660}$, indicating that the phosphosites are not equivalent. We also tested if phosphosites interact with one another and found that the effects of phosphorylation (using phosphomimetic residues as a proxy) depend on their position relative to the target serine. For example, the presence of Asp at Ser^{656} increases K_M of adjacent sites. On the other hand, the phosphmimetic residue(s) decreased K_M for Ser^{660}. Our data indicate the primary pathway for Lgl phosphorylation is $Ser^{664} \rightarrow Ser^{660} \rightarrow Ser^{656}$ while the secondary pathway is $Ser^{656} \rightarrow Ser^{664} \rightarrow Ser^{660}$. The addition of phosphate groups on substrates in an ordered manner may serve as another layer of regulation in cortical displacement, as differences in kinetic parameters could contribute to how well fate determinants interact with the plasma membrane (i.e. the association and dissociation rates). Kinetic analyses of other aPKC substrates, combined with localization assays, will be useful for more generalized understanding of the relationship between the number of phosphorylation and cortical release behaviors.

Flexibility in aPKC substrate recognition sequence

Substrate recognition is an important aspect of substrate binding and specificity. Though the three serine residues and the surrounding amino acid sequences are similar to one another, aPKC showed a clear preference among them, suggesting that the subtle difference in sequence could have a large impact on kinetic parameters and substrate preference. Lgl phosphorylation sites showed some deviation from the known consensus

amino acid sequence of aPKC substrates (73,104,112). All Lgl peptides satisfied the requirement at P-5, P-2, and P+1 positions while they all failed to match at P-4 and P+2 positions. Our data demonstrate that the mutation of Leu to Phe at the P+1 position slightly increased V_{max}, suggesting that the larger hydrophobic residue interacts more favorably with the kinase interaction surface. In contrast, mutation of Glu to Lys at the P+3 site, thought to be critical for recognition, did not influence the phosphorylation rate at Ser^{660}. Taken together, our study suggests that the collective properties of the phospho-accepting residue regions plays a key role in preferential binding and efficient catalysis. This could explain the range of amino acid requirements seen in a single recognition site. Our study emphasizes the complexity of substrate specificity and the flexibility of aPKC's substrate recognition mechanism.

BRIDGE TO CHAPTER IV

The data presented in Chapter III demonstrated how Lgl phosphorylation at multiple serine residues plays a crucial role in cortical displacement. The kinetic analyses show that aPKC preferentially phosphorylates Lgl although the amino acid sequences surrounding the phospho-accepting serine are quite similar. Also, the phosphomimetic mutants indicate that phosphorylation events are cooperative. These findings could provide new insights to the role of phosphorylation in cortical release of aPKC substrates. In the next chapter, I summarize my thesis research and discuss some of the interesting questions that await the answers.

CHAPTER IV

CONCLUSIONS AND FUTURE CONSIDERATIONS

CONCLUSIONS

Complex signaling networks are employed in cells to temporally and spatially regulate the catalytic activity of protein kinases, as dysfunctional kinase cascades often lead to devastating diseases such as cancers. aPKC is a well-conserved regulator of cell polarity in metazoan systems. Unregulated phosphorylation activity of aPKC causes defects in development and tissue homeostasis, suggesting that aPKC plays crucial roles in the grand scheme of cell polarization. Despite its physiological significance, little is known about the regulatory mechanism of aPKC.

In Chapter II, I described both intra- and intermolecular regulation of aPKC activity. Previously, it was thought that aPKC was constitutively active though it contained a pseudosubstrate domain, whose function was reported to inhibit kinase activities in other kinases. To challenge the existing model, I developed a reconstituted system and more stringent kinetic method. With the new methods, I was able to determine that full length aPKC's catalytic activity was autoinhibited by the *cis*-acting pseudosubstrate domain. Consistent with the kinetic data, Arg-C proteinase sensitivity assay also showed that full length aPKC was in an inactive conformation. Additionally, as S2 localization assay showed that the pseudosubstrate mutant, but not full length aPKC, displaced Lgl from the cortex. The above findings led me to reinvestigate the role of Par-6, another component of the Par complex. It was previously shown that Par-6

repressed aPKC activity, but it seemed unnecessary to repress already inactive aPKC. To our surprise, the kinetic analyses and the Arg-C proteinase assays showed that Par-6 activated aPKC by displacing pseudosubstrate domain. Consistent with activation, Lgl was released from the membrane when both aPKC and Par-6 were co-expressed. Taken together, our data suggest a dual role of Par-6 in aPKC polarization and aPKC activity. In other words, aPKC activity is coupled to localization via Par-6.

In Chapter III, I discussed how the activity of aPKC translates to the localization patterns of its substrates. I realized that fate determinants such as Lgl, Mira, and Numb contained multiple aPKC phosphorylation sites. However, it has never been directly tested if multi-site phosphorylations are necessary for their localization behaviors. Using Lgl as a model substrate in S2 localization assay, I found that Lgl localized at the cortex in the absence of aPKC. As the number of phosphorylations of Lgl increased, the cytoplasmic localization of Lgl increased. As the singly phosphorylatable Lgl was not statistically different from non-phosphorylated Lgl, our data suggest that multiple phosphorylation is required for displacement of Lgl from the membrane. We also performed kinetic analyses to examine how aPKC phosphorylated three serine residues in Lgl. Despite similarities among the residues surrounding the phospho-accepting serine residues, aPKC exhibited clear preferences among them. Finally, I tested whether the presence of negative charge(s) on the neighboring site(s) affect the preceding phosphorylation events. The results of phosphomimetic mutant Lgls showed that the phosphorylation is cooperative although the effects depended on the position of the phospho-accepting serine residue relative to the acidic residue(s). These findings imply a

possible general mechanism of cortical displacement where rates of substrate phosphorylation could regulate how long it would associate with the membrane.

My thesis research depicts how both *cis*- and t*rans*-acting regulatory elements regulate aPKC activity and localization. My research also shows how aPKC activity is coupled to substrate localizations. These findings provide deeper understanding of how the Par complex mediates the establishment of cellular polarization.

FUTURE CONSIDERATIONS

The data presented in Chapter II show that Par-6 PB1 was sufficient to partially displace pseudosubstrate domain and activate aPKC activity. However, the kinetic data suggest that the C-terminal part of Par-6 (CRIB-PDZ) also participate in the regulation of aPKC, as Par-6 PB1cannot fully recover the activity level of aPKC/Par-6 wild type complex. To examine the involvement of Par-6 CRIB-PDZ in aPKC regulation, we first tested the direct interaction between the two proteins using GST pull down assays using bacterially expressed GST-fused Par-6 CRIB-PDZ. The binding assay showed that wild type aPKC interacted with both full length Par-6 wt and Par-6 CRIB-PDZ, though CRIB-PDZ binding was weaker than wt (Fig. 14A). Because aPKC contains PDZ ligand at the very distal end of the catalytic domain, it is likely that they interact each other in PDZ-PDZ ligand mediated manner. Next, we tested the kinetic effect of this interaction in the kinase assay. To do so, we first investigated the activity of aPKC mutant lacking PDZ ligand (aPKC ΔPDZL) was comparable to aPKC wild type. Then, we purified aPKC ΔPDZL in complex with ful length Par-6. The mutant complex showed higher activity than wild type aPKC alone, but it was only partially activated, suggesting that

PDZ/CRIB-PDZ interaction regulates aPKC activity (Fig. 14B). We also purified aPKC ΔPDZL/Par-6 PB1 complex and tested in the kinase assay. Interestingly, the activity of this complex was equivalent to full length aPKC, suggesting that displacement of pseudosubstrate domain by Par-6 PB1 requires the interaction between PDZ-PDZ ligand. The exact mechanism remains to be tested.

In the literature, it has been reported that a Rho family GTPase Cdc42 is an upstream of aPKC that apically localizes in neuroblasts when bound to GTP (94,116). It is also shown to interact with Par-6 via CRIB-PDZ when it is in GTP bound form (117). This interaction rescues aPKC activity from Par-6 repression *in vitro* though the recovery was marginal (93). Now that we think Par-6 is an activator of aPKC, the role of Cdc42 is unclear. One possibility is that Cdc42 may be another component that couples the localization to the activity of aPKC as Cdc42 localizes at the apical cortex prior to Par-6. It is also possible that Cdc42 further activates aPKC activity in the context of aPKC/Par-6, as the activity of the complex is about 50 % of aPKC kinase domain alone.

In Chapter III, I used Lgl as a model substrate to examine how multiple phosphorylation plays a role in its localization and how aPKC preferentially phosphorylates those sites. It is possible that the addition of the multiple phosphates may attenuate the interaction with the plasma membrane as the electrostatic charges of substrate change (113,114). To examine if this is a general mechanism that other aPKC substrate utilizes, this hypothesis needs to be tested with other proteins such as Mira and Numb. It would be also beneficial to compare if the phosphorylation rate differences among the substrates correlates to how long they remain bound to the membrane. It would be of interest to see if one could manipulate the cellular localization of an

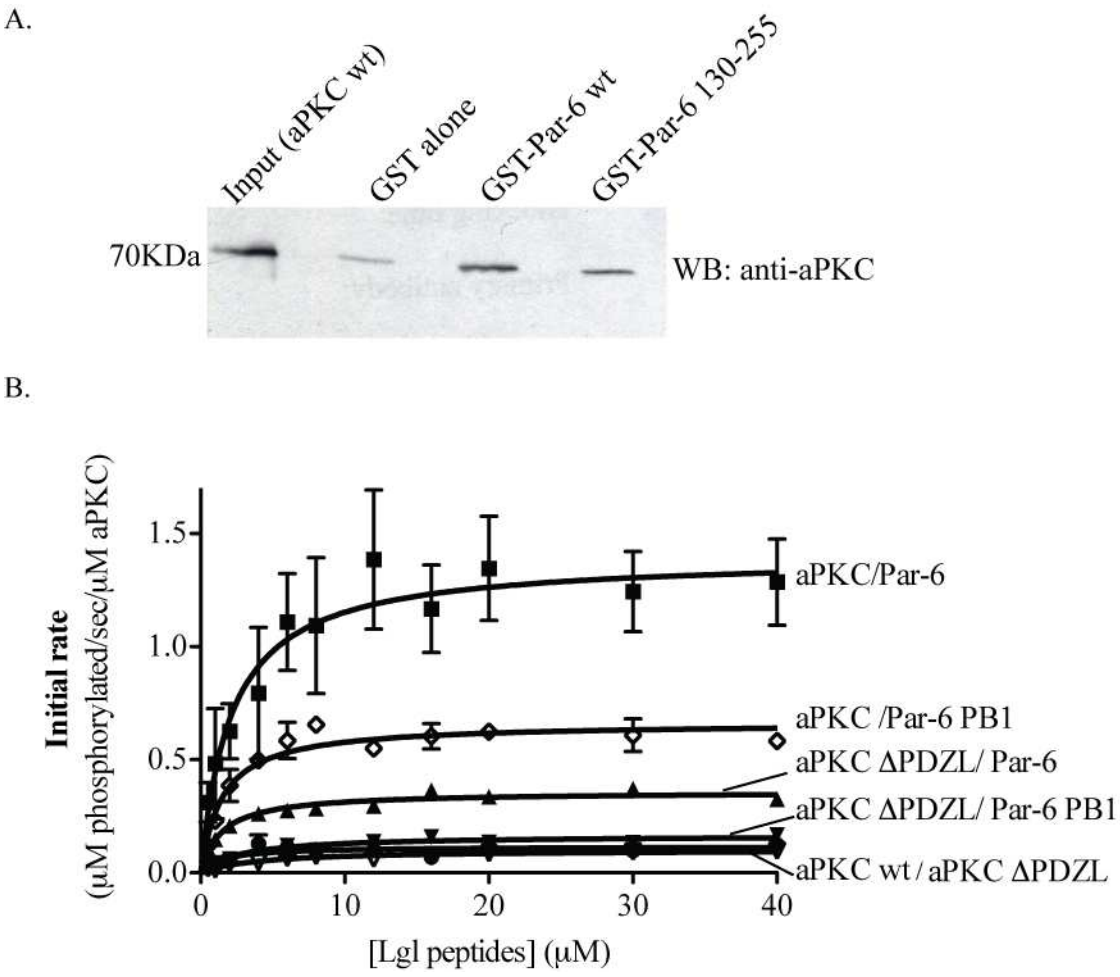


Figure 14. Par-6 CRIB-PDZ participates in regulation of aPKC activity**.**

A. GST pulldown assay to test the direct interaction between aPKC wt and Par-6 130-255 (CRIB-PDZ). Bacterially expressed GST-fusion Par-6 and GST-Par-6 CRIB-PDZ were immobilized onto glutathione-agarose. Then, aPKC wt was allowed to interact with GST-fusion proteins. The interaction was examined by a western blot probing against aPKC with rabbit anti-aPKC antibody.
B. The effects of aPKC PDZ ligand on its aPKC catalytic activity. Deletion of PDZ ligand from aPKC (aPKC ΔPDZL) did not affect the enzyme activity and the mutant showed the similar activity level with full length aPKC alone. aPKC ΔPDZL in complex with Par-6 wt exhibits partial activation compared to full length alone. But it was less than that of aPKC/Par-6 PB1 complex. aPKC ΔPDZL/ Par-6 PB1 complex show activity level comparative to aPKC wt.

artificially engineered substrate whose number of phospho-accepting serines and phosphorylation rates are modulated.

CONCLUDING REMARKS

As our understanding of signal transduction increases, it is clear that multiple layers of complex regulatory mechanisms precisely control localization and activity of protein kinases. My research shows that aPKC is no exception to this general phenomenon. Here, I have described the intricate but elegant intra- and intermolecular mechanisms responsible for the regulation aPKC. Additionally, I have also depicted that aPKC phosphorylation activity outputs as displacement of its substrate from the plasma membrane. These results demonstrate a seemingly simple strategy of cells where activity and localizations of the enzyme as well as its substrates are coupled one another. These regulatory mechanisms may be general methods that protein kinases utilize in cell signaling.

APPENDIX

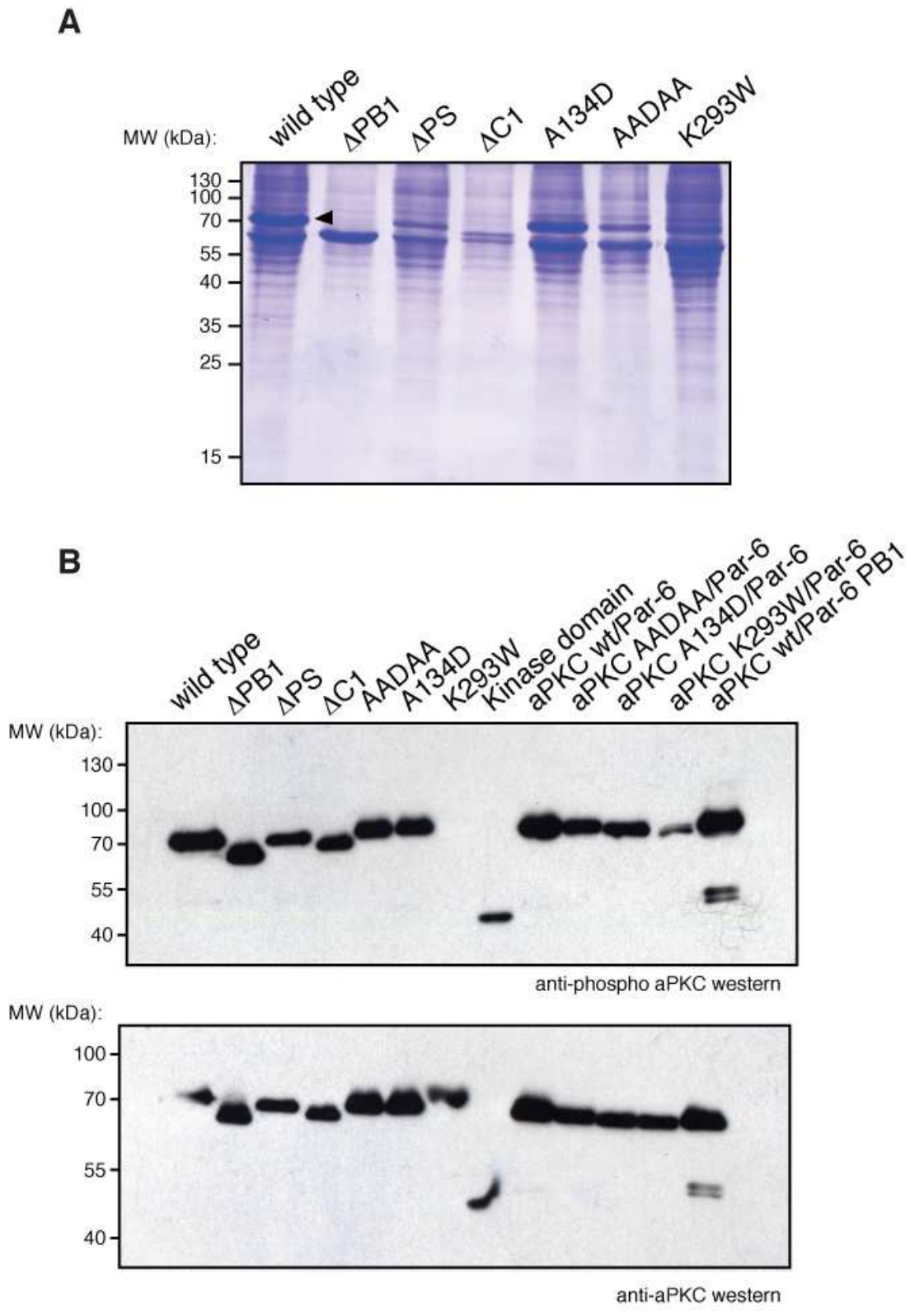
SUPPLEMENTAL MATERIALS FOR CHAPTER II

Abbreviations used in this chapter:

GST	Glutathione-S-transferase
MBP	Maltose-binding protein

Supplemental figure 1. Characterization of aPKC proteins used in this study.

A. Purity as assessed by SDS-PAGE and coomassie brilliant blue staining. The band corresponding to wild type aPKC (as identified by reactivity with the anti-aPKC antibody) is marked with an arrowhead.
B. Activation loop phosphorylation. Upper panel shows western signal using an antibody specific to aPKC containing a phosphorylated activation loop. The lower panel shows signal for total aPKC.


A
MW (kDa):
wild type
ΔPB1
ΔPS
ΔC1
A134D
AADAA
K293W
130
100
70
55
40
35
25
15
B
wild type
ΔPB1
ΔPS
ΔC1
AADAA
A134D
K293W
Kinase domain
aPKC wt/Par-6
aPKC AADAA/Par-6
aPKC A134D/Par-6
aPKC K293W/Par-6
aPKC wt/Par-6 PB1
MW (kDa):
130
100
70
55
40
anti-phospho aPKC western
MW (kDa):
100
70
55
40
anti-aPKC western

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