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The substrates and binding partners of protein kinase C ϵ

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Abstract

The epsilon isoform of protein kinase C (PKC ϵ) has important roles in the function of the cardiac, immune and nervous systems. As a result of its diverse actions, PKC ϵ is the target of active drug discovery programs. A major research focus is to identify signaling cascades that include PKC ϵ and the substrates that PKC ϵ regulates. In this review we will identify and discuss those proteins that have been conclusively shown to be direct substrates of PKC ϵ by the best currently available means. We will also describe binding partners that anchor PKC ϵ near its substrates. We review the consequences of substrate phosphorylation and discuss cellular mechanisms by which target specificity is achieved. We begin with a brief overview of the biology of PKC ϵ and methods for substrate identification, and proceed with a discussion of substrate categories to identify common themes that emerge and how these may be used to guide future studies.

INTRODUCTION

The human kinome contains over 500 protein kinases [1]. Protein kinase C (PKC) (EC 2.7.11.13; <http://www.chem.qmul.ac.uk/iubmb/enzyme/>) belongs to the AGC group of serine-threonine kinases and transduces signals derived from lipid second messengers. PKC is a family of kinases encoded by nine genes with distinct biological functions. Conventional cPKC's (α , β , γ) are activated by calcium and diacylglycerol, novel nPKCs (β , ϵ , η , θ) are activated by diacylglycerol but not by calcium, and atypical aPKCs (ζ and τ/γ) are insensitive to calcium and diacylglycerol but are activated by other lipids and by phosphorylation. The ϵ isoform (PKC ϵ) is widely expressed throughout the body and has important roles in the function of the nervous [2,3], cardiac [4] and immune [5] systems. It is an attractive drug target for the treatment of several conditions such as inflammation [5], ischemia [6], addiction [7,8], pain [9], anxiety [10,11], and cancer [12,13]. Therefore, there is interest in developing PKC ϵ inhibitors and in discovering additional drug targets through identification of PKC ϵ substrates and the signaling pathways in which they participate.

In this review we discuss substrates of PKC ϵ and anchoring proteins that localize PKC ϵ near its substrates. We have focused on substrates that have been identified by multiple methods particularly those for which the phosphorylated residues have been determined. It is currently difficult to prove that any kinase directly phosphorylates a specific substrate *in vivo* and many surrogate measures are used to infer phosphorylation. These include *in vitro* phosphorylation assays with purified enzyme and substrate, radioactive labeling with ³²P or use of anti-phospho-specific antibodies to detect phosphorylation in intact tissues, and mutation of candidate phosphorylation sites to alter substrate function *in vivo*. It is only through a combination of these approaches that one can conclude with reasonable confidence that a particular substrate is phosphorylated by a specific kinase. Therefore, in this review, we have limited our attention to PKC ϵ substrates for which direct *in vitro* phosphorylation has been demonstrated in conjunction with one or more indirect approaches using intact tissues.

ACTIVATION AND PHOSPHORYLATION OF PKC ϵ

PKC ϵ shares many structural features (Figure 1) with other members of the PKC family including a C1 domain containing two cysteine-rich motifs that bind diacylglycerols, a C2-like phospholipid binding domain, a pseudosubstrate domain, C3 and C4 catalytic domains that contain a purine binding site for ATP, an activation loop, and a substrate recognition site [8]. A unique feature of PKC ϵ is a 6 amino acid actin-binding motif between the C1a and C1b subdomains [14] (see also below). Like other PKC isozymes, PKC ϵ is regulated by four key mechanisms: co-factor binding, phosphorylation, protein:protein interaction, and regulated degradation. A major endogenous activator is *sn*-1,2-diacylglycerol (DAG) generated by phospholipase C (PLC)-mediated hydrolysis of phosphoinositides. PKC activation by DAG can be mimicked by the addition of other compounds that bind the C1 domain, such as tumor promoting phorbol esters. PKC ϵ may also be activated by other lipids, such as arachidonic acid, or phosphoinositide bisphosphate (PIP2) [15,16].

Like other PKC isozymes, PKC ϵ must be primed through phosphorylation to display full enzymatic activity and respond to allosteric regulators. In common with other AGC kinase superfamily members, PKC isozymes are phosphorylated at three conserved priming sites in the catalytic domain: the activation loop (Thr-566), the Thr-Pro turn motif (Thr-710), and the hydrophobic Phe-Ser-Tyr motif (Ser-729). Phospholipid-dependent kinase 1 (PDK1) phosphorylates the activation loop, whereas the turn and hydrophobic motifs are autophosphorylated in conventional PKCs, and possibly in PKC ϵ [17], although recent evidence indicates that mammalian target of rapamycin complex 2 may transphosphorylate these sites [18,19]. In cPKC isozymes, these phosphorylations appear to be constitutive, whereas they are regulated in PKC ϵ . Thus, there exists a pool of unphosphorylated PKC ϵ that can be recruited into active signaling through stimulation of receptors coupled to PI3-kinases, which in turn activate PDK1 [17,20]. Immunoprecipitation and co-localization studies have demonstrated that unphosphorylated PKC ϵ is associated with the anchoring protein CG-NAP (centrosome and Golgi localized PKN-associated protein). This association occurs through the catalytic domain of PKC ϵ and localizes immature PKC ϵ to the Golgi apparatus [21].

Three additional sites in PKC ϵ have been identified that can be autophosphorylated *in vitro*: Ser-234, Ser-316, and Ser-368 [22]. However, in intact cells, it appears that these sites are trans-phosphorylated by conventional PKC isozymes. Phosphorylation at Ser-368 is required for binding of PKC ϵ to 14-3-3 proteins (see below). The functional consequences of phosphorylation at Ser-234 and Ser-316 are not yet known.

ANCHORING PROTEINS AND CHAPERONES

Receptors for Activated C-Kinase (RACKs)

Following activation, PKC ϵ translocates to specific subcellular compartments. This process involves interactions with anchoring proteins such as the “receptors for activated C-kinase” (RACKs), which serve to anchor PKC isozymes in close proximity to their substrates. Two RACKS have been cloned: RACK1, which preferentially binds activated PKC β II [23], and RACK2 (β' COP), a coatamer protein that binds activated PKC ϵ [24]. Disrupting the interaction between RACK2 and PKC ϵ is a goal of some drug discovery programs aimed at reducing PKC ϵ signaling [25]. Although the V1 region of PKC ϵ and the C-terminus of β' COP mediate a significant portion of this interaction, full length PKC ϵ binds also to the N terminus of β' COP, indicating that interactions are formed through other regions of these molecules. The PKC ϵ -RACK2 interaction is responsible for localizing active PKC ϵ to the Golgi apparatus [24], a localization that requires phosphorylation of PKC ϵ at S729 in the hydrophobic motif [26]. In cardiac myocytes RACK2 also localizes PKC ϵ to myofilaments

[27]. RACK1 can also interact with activated PKC ϵ . For example, in human glioma cells, PKC ϵ interacts with RACK1 to regulate integrin-mediated cell adhesion and motility [28].

PDZ and LIM domain protein 5 (PDLIM5)

PDLIM5 is a 63 kDa cytoplasmic protein also known as enigma homolog protein-1 (ENH1). It is a PKC ϵ binding protein that has been reported to bind N-type voltage-gated calcium channels [29]. The function of N-type calcium channels is increased by application of phorbol esters [30,31] and in transfected *Xenopus* oocytes PDLIM5 facilitates this potentiation through formation of a PKC ϵ -PDLIM5-N-type calcium channel complex [29]. PKC phosphorylates the pore-forming α subunit $\text{Ca}_v2.2\alpha$ of N-type calcium channels although it is not clear if PKC ϵ is the PKC isozyme responsible [30,31]. The zinc finger LIM domain of PDLIM5 is essential for the formation of the PKC ϵ -PDLIM5-N-type calcium channel complex [32]. However, a recent study was unable to find an interaction between PDLIM5 and $\text{Ca}_v2.2\alpha$, and questioned the role of PDLIM5 as an N-type channel adapter protein [33]. Interestingly, PDLIM5 may also perform a scaffolding role for PKD and L-type calcium channels in rat neonatal cardiomyocytes, to facilitate positive modulation of L-type calcium channel function by PKD [34]. Most notably, this positive modulation can be inhibited by treatment with the general PKC inhibitors chelerythrine and GF109203X. There is *in vitro* evidence that PKC ϵ can phosphorylate L-type calcium channels [35]. Given that PKC ϵ also binds PDLIM5 and can phosphorylate the activation loop of PKD [36] it seems reasonable to hypothesize that PKC ϵ is involved in the PKD-dependent regulation of L-type calcium channels and that PKD might participate in PKC ϵ regulation of N-type calcium channels.

Trif-related Adapter Molecule (TRAM)

Unlike RACK1, RACK2, or ENH1, some proteins that act as PKC ϵ scaffolds or chaperones are also PKC ϵ substrates. One such protein is the Trif-related adapter molecule (TRAM). Mice lacking PKC ϵ exhibit impaired immune function, most notably in the clearance of bacterial infections [37]. Further work on this phenotype has revealed that the response to lipopolysaccharide (LPS) is impaired in these mice. LPS is produced by gram-negative bacteria and promotes a robust immune response via Toll-like receptor 4 (TLR-4). TRAM is an adapter protein that is activated downstream of TLR4 and activates the transcription factor Interferon (IFN) Regulatory Factor 3. This activation leads to the production of cytokines including IFN-beta and RANTES. McGettrick and co-workers [38] showed that PKC ϵ phosphorylation of TRAM on serine 16 is key for signaling downstream of TLR4. This phosphorylation was demonstrated *in vitro* and was absent in lysates of LPS-treated fibroblasts from PKC ϵ knockout mice, but was restored by addition of PKC ϵ . Antibody depletion of PKC ϵ from lysates of LPS-treated THP1 cells also substantially reduced TRAM phosphorylation. Dephosphorylated TRAM is targeted to the cell membrane *via* myristoylation at its N-terminus. Following phosphorylation by PKC ϵ , TRAM is depleted from the membrane, although it is not clear where it goes. Mutating Ser-16 to alanine abolishes this effect as well as LPS-stimulated cytokine production. Recent studies have demonstrated a physical interaction between TLR4 and PKC ϵ that is mediated by another adaptor protein, MyD88 [39].

14-3-3 Proteins

The 14-3-3 family of proteins are encoded by seven different mammalian genes (α/β , ϵ , η , γ , τ/θ , ζ/δ and σ) and bind to sequence-specific, phosphopeptide binding motifs on several proteins, thereby regulating their function, stability, or localization [40]. Two optimal binding motifs have been identified, mode-I (RSXpS/TXP) and mode-II (RXXXpS/TXP). The V3 hinge region of PKC ϵ (Figure 1) contains a mode-I sequence surrounding Ser 346 and a weak mode II site surrounding Ser-368 [41]. GSK3- β phosphorylates PKC ϵ at Ser-346

whereas Ser-368 is autophosphorylated [22,41]. When bound to 14-3-3, PKC ϵ is active in the absence of lipids.

During the late stages of mitosis, 14-3-3 proteins associate with the V3 region of PKC ϵ and formation of this 14-3-3/PKC ϵ complex is required for the separation of two daughter cells (cytokinesis) during abscission, the final stage of the cell cycle [41]. Mutation of either V3 phosphorylation site prevents this association and impairs completion of cytokinesis. Likewise, knockout, depletion or inhibition of PKC ϵ impairs abscission. The mechanism by which PKC ϵ facilitates abscission is not known, but it may involve RhoA. PKC ϵ inhibition is associated with prolonged activation of RhoA and its persistent localization at the actomyosin ring that contracts to create a furrow before abscission. Presumably, inhibition and removal of RhoA is required for cytokinesis to be completed. The substrates that PKC ϵ phosphorylates to regulate RhoA are not yet known but likely candidates are Rho A guanine nucleotide exchange factors or GTPase activating proteins.

Histone H1

Histones are important components of chromatin that are essential for the chromosomal organization of DNA. Histone H1 was identified as a PKC ϵ binding protein using an overlay assay [42]. Subsequent *in vitro* phosphorylation analysis revealed that PKC ϵ can phosphorylate histone H1 but not H2A. The individual phosphorylated residues and the functional consequences are as yet unknown, but current evidence suggests that histone H1 anchors activated PKC ϵ in the nucleus where it can play a role in regulation of gene expression.

PKC ϵ REGULATION OF INTRACELLULAR SIGNALING

A common theme in cell signaling is the existence of kinase cascades that culminate in gene transcription. The activity of these cascades is under tight control and failures in regulation, resulting in aberrant gene transcription, may result in pathological conditions such as cancer. Roles for PKC ϵ have been demonstrated in many different systems, regulating diverse functions such as cell death and nociceptor sensitization [2,3] [12,13] [14]. However, within these intracellular signaling cascades only three subproteins have been positively identified as PKC ϵ substrates: the kinases Akt/PKB and PKD, and the transcription factor Stat3.

Akt (Protein Kinase B)

Akt is a serine/threonine kinase important for cell survival and proliferation, angiogenesis, metabolism and protein translation [43–45]. Activation of Akt requires phosphorylation at both Thr-308 and Ser-473. Phosphorylation of Thr-308 is mediated by PDK1 [46,47]. Several other kinases phosphorylate Akt at Ser-473, including integrin-linked kinase (ILK), mitogen-activated protein kinase-2 (MAPKAPK-2), DNA-dependent protein kinase (DNA-PK) and the rictor-mTOR complex (reviewed in [12]). Cell culture studies have also implicated PKC ϵ in the regulation of Akt [32,48,49]. In the heart, activated PKC ϵ forms a complex with Akt and eNOS. Active Akt binds to PKC ϵ via its pleckstrin homology domain, and formation of this three-member complex is enhanced by activation of PKC ϵ . *In vitro*, PKC ϵ phosphorylates Akt at Ser-473 and increases its activity. PKC ϵ also increases the phosphorylation of eNOS at Ser-1171 and increases eNOS activity. These PKC ϵ mediated events appear to contribute to the cardioprotective effects of moderate ethanol consumption [50].

Protein Kinase D

PKD1, formerly known as PKC μ , is a protein kinase that, like most PKC isozymes, is activated by phorbol esters, but contains a kinase domain that more closely resembles that of

calcium/calmodulin dependent protein kinases. PKD1 is activated in response to a variety of regulatory peptides and growth factors [51]. Like other kinases, PKD1 must be phosphorylated in its activation loop to be active. Two residues have been identified, Ser-744 and Ser-748, and PKC ϵ phosphorylates both *in vitro* [36].

Stat 3

The Stat3 transcription factor is constitutively active in a variety of human cancers. Stat3 requires phosphorylation at two sites for activation. Tyr-705 is phosphorylated by a Janus kinase, usually JAK1 [52]. Ser-727, which resides in the transactivation domain, can be phosphorylated by several kinases including PKC ϵ . In prostate adenocarcinoma cells PKC ϵ forms a complex with Stat3 and phosphorylates Stat3 at Ser-727 [53]. Knockdown of PKC ϵ with RNA interference inhibits Stat3 Ser727 phosphorylation and reduces the activity of Stat3, measured by DNA-binding and activation of transcription. These results identify a potential mechanism by which PKC ϵ may promote cancer. They also provide, to our knowledge, the only known example of a transcription factor that is a substrate of PKC ϵ .

PKC ϵ REGULATION OF ION CHANNELS

Type A γ -aminobutyric acid (GABA $_A$) Receptors

GABA $_A$ receptors mediate most inhibitory neurotransmission in the nervous system and our studies with PKC ϵ knockout mice indicate that PKC ϵ phosphorylates and regulates the function of these receptors. PKC ϵ knockout mice show increased behavioral responses to ataxic and hypnotic doses of ethanol and benzodiazepines [54]. The positive allosteric effects of these drugs on GABA $_A$ receptor function are also increased in crude synaptoneurosomes (microsacs) prepared from the frontal cortex or cerebellum of PKC ϵ knockout mice. *In vitro* phosphorylation studies and electrophysiological studies of receptors with mutations at known PKC phosphorylation sites, revealed that PKC ϵ phosphorylates Ser-327 on $\gamma 2$ subunits, and that phosphorylation at this site reduces the response of GABA $_A$ receptors to ethanol and benzodiazepines. A striking feature of ethanol intoxication is the development of acute tolerance to ethanol during a single drinking session despite the persistence of elevated blood ethanol levels [55]. PKC ϵ phosphorylation of $\gamma 2$ S327 appears to participate in the development of this acute tolerance. Treatment *in vivo* with ethanol increases phosphorylation of PKC ϵ at Ser-729 in the cerebellum measured 1 hour later, suggesting that ethanol increases the activity of cerebellar PKC ϵ [56]. *In vivo* exposure to ethanol also increases phosphorylation of $\gamma 2$ S327 and reduces the sensitivity of cerebellar GABA $_A$ receptors to ethanol *in vitro* at this time [56,57]. These findings suggest an important role for PKC ϵ in mediating acute tolerance to the intoxicating effects of ethanol.

Transient Receptor Potential Vanilloid 1 (TRPV1)

TRPV1 is a ligand-gated, non-selective, cation channel activated by capsaicin, heat, protons, leukotrienes and anandamide [58]. It is expressed by small-diameter nociceptive neurons and integrates thermal and chemical stimuli as a polymodal receptor [59–61]. TRPV1 knockout mice show reduced sensitivity to noxious thermal stimuli and reduced inflammatory thermal hyperalgesia [58,59]. PKC modulates TRPV1 function. Brief (<3 min) treatment with phorbol esters reduces the thermal threshold for activation and enhances activation by capsaicin, anandamide, ATP and bradykinin; this effect is inhibited by PKC inhibitors [62] [63,64]

In heterologous expression systems, phorbol esters also activate TRPV1 currents directly by a PKC-dependent mechanism [63,65,66], but this is evidently not commonly observed in rat DRG neurons [64]. Two different groups have identified Ser-502 and Ser-800 as important for phorbol ester enhancement of agonist and heat responses [67,68]. *In vitro*, PKC ϵ

phosphorylates Ser-502 and Ser-800, and in HEK-293 cells, intracellular application of the ϵ V1-2 peptide inhibitor of PKC ϵ , which inhibits PKC ϵ binding to RACK2, reduces bradykinin-mediated enhancement of TRPV1 function [62]. Studies using the ϵ V1-2 peptide also demonstrate that activation of neurokinin-1 receptors [57] or protease-activated-receptor 2 [69] enhances TRPV1 function *via* PKC ϵ . More recently, PKD1 has been found to associate with and enhance the activity of TRPV1 through phosphorylation of TRPV1 at S116 [70]. This could provide a mechanism for indirect activation of TRPV1 by PKC ϵ , since, as discussed above, PKC ϵ phosphorylates the activation loop of PKD1 thereby stimulating PKD1 activity [36,71,72].

PKC ϵ AND THE CYTOSKELETON

The cytoskeleton is composed of three structural protein elements; microtubules, intermediate filaments and actin microfilaments. PKC ϵ regulates the function of all three of these structural elements (reviewed in [73]) by interacting with, and in some cases phosphorylating, proteins that form these structures.

Peripherin

Peripherin is an intermediate filament protein that is exclusively neuronal, being expressed at particularly high levels in the peripheral nervous system [74]. The formation of peripherin-containing protein aggregates in motor neurons is a feature of amyotrophic lateral sclerosis (ALS). Aggregates of endogenous peripherin can also form in SK-N-BE(2)C neuroblastoma cells when these cells are treated with phorbol esters. Peripherin does not appear to be directly phosphorylated by PKC ϵ but immunoprecipitation studies show that peripherin binds to PKC ϵ in these cells [75]. Interestingly, the regulatory C1b domain of PKC ϵ mediates the formation of this complex; the C2 domain does not participate, nor does peripherin bind the C1b domain of another PKC isozyme, PKC α . Complex formation is not regulated by PKC ϵ activity. However, overexpression of peripherin in these cells causes peripherin aggregation, and this aggregate formation is reduced by knockdown or inhibition of PKC ϵ and enhanced by phorbol esters or overexpression PKC ϵ . Thus, these findings indicate an important role for PKC ϵ in peripherin aggregation [75].

Vimentin

Vimentin is an intermediate filament protein found predominantly in cells of mesenchymal origin [76]. Like peripherin, vimentin is not directly phosphorylated by PKC ϵ , but a number of PKC ϵ -dependent phosphorylation events regulate the function of both vimentin and beta-1 integrins. Integrins are transmembrane proteins that mediate cell attachment to the extracellular matrix and thus are important for cell motility [77]. In motile cells, beta-1 integrin-containing adhesive structures are continually cycled from the trailing to the leading edge of cells in a PKC ϵ -dependent manner [78]. The cycling of beta-1 integrins requires phosphorylation of vimentin; this occurs in a PKC ϵ -dependent manner, but PKC ϵ does not appear to be the kinase that is directly responsible [79]. In primary cultures of cardiac fibroblasts, treatment with angiotensin II promotes adhesion of cells to a collagen-1 matrix. Angiotensin II also promotes co-immunoprecipitation of beta-1 integrin with PKC ϵ and stimulates phosphorylation of PKC ϵ at Ser-729 in these cells. Angiotensin II does not stimulate adhesion of cells that lack PKC ϵ , suggesting that activation of PKC ϵ and association of PKC ϵ with beta-1 integrin are required for angiotensin II-stimulated adhesion of cardiac fibroblasts to collagen [80].

Keratin

Keratins are intermediate filament proteins that are expressed primarily in epithelial cells. There are numerous forms of keratin. In rat pituitary GH₄C₁ cells, thyrotropin releasing

hormone (TRH) stimulates PKC ϵ phosphorylation of keratin 8. Treatment with TRH also causes PKC ϵ and keratin 8 to colocalize. Keratin 8 is hyperphosphorylated in PKC ϵ overexpressing cells and is directly phosphorylated by PKC ϵ *in vitro* at Ser-8 and Ser-23. The functional consequences of this phosphorylation are unclear but may involve regulation of cell-cell contacts in epithelial cells [73].

Actin

Actin exists in two conformational states, globular and filamentous (F-actin). PKC ϵ is unique among PKC isozymes in containing an F-actin-binding region. This six amino acid sequence (LKKQET) is located at amino acids 223-228 on PKC ϵ . When PKC ϵ is in the inactive state, this region is not exposed and thus actin is not bound. However, following treatment with PKC activators such as phorbol esters, diacylglycerol or arachidonic acid, PKC ϵ undergoes a conformational change and the actin-binding region is exposed. Interestingly, binding of PKC ϵ to filamentous actin stabilizes the active conformation of the enzyme [14,81]. The PKC ϵ -actin interaction has profound consequences for the actin cytoskeleton, most notably promoting the formation of F-actin through several mechanisms, including inhibiting the depolymerization of F-actin, increasing the rate of actin filament elongation and overturning the inhibition of actin nucleation by thymosin β 4 [82]. Functionally, the formation of a PKC ϵ -F-actin complex appears to be important for glutamate exocytosis from dentate gyrus granule cells of the hippocampus [14].

IQ motif-containing GTPase activating protein 1 (IQGAP1)

The Rho-family GTPases (Rho, Rac and Cdc42) are key regulators of cytoskeletal function. These GTPases cycle between an active state (GTP-bound) and an inactive state (GDP-bound). The switch between active and inactive is mediated by their intrinsic GTPase activity. The IQ motif-containing GTPase activating protein 1 (IQGAP1) is a calcium sensitive GTPase scaffolding protein that binds to Rac1 and Cdc42 and promotes their active state [83–85]. IQGAP1 and PKC ϵ can be co-immunoprecipitated and IQGAP1 contains a consensus PKC phosphorylation site (Ser-1443) at its C-terminus [86]. *In vitro* phosphorylation assays show that PKC ϵ can phosphorylate IQGAP1 at this residue. The consequences of Ser-1443 phosphorylation have not been conclusively demonstrated, but transfection of NIE-115 neuroblastoma cells with an IQGAP1 mutant that contains S1441E/S1443D mutations to mimic phosphorylation at these residues increases neurite outgrowth when cells are plated on laminin. A smaller increase in neurite outgrowth is seen when cells are transfected with native IQGAP1 whereas no increase is observed with an S1441A/S1443A mutant. Ser-1441 is not phosphorylated by PKC ϵ [87]. Interestingly, an emerging literature suggests that IQGAP1 exists in a complex containing beta-1 integrins [88–90], whose cycling is controlled in a PKC ϵ -vimentin dependent manner as described above.

Cardiac Myosin Binding Protein (CMyBPC)

CMyBPC is a myosin binding protein that has an essential role in cardiac contractility [91] and is a substrate for PKC ϵ [92,93]. Transgenic mice that overexpress PKC ϵ in the heart develop a dilated cardiomyopathy between the ages of 9 and 12 months. This pathology is associated with hyperphosphorylation of CMyBPC. *In silico* analysis of the CMyBPC protein sequence suggests Ser-302 as a potential PKC ϵ phosphorylation site and peptides containing this site and its surrounding sequence can be phosphorylated by PKC ϵ *in vitro*. These data suggest that increased PKC ϵ phosphorylation of CMyBPC at Ser-302 contributes to aberrant cardiac function in this model of cardiomyopathy.

Cytoskeletal interaction with other PKC ϵ substrates

PKC ϵ interactions with the cytoskeleton may be important for the regulation of other PKC ϵ substrates. For example, PKC ϵ -dependent cytoskeletal regulation appears to be important for PKC ϵ regulation of pain. Injection of epinephrine into the hindpaw of rodents produces a hyperalgesia that is dependent on PKC ϵ and can be mimicked by direct injection of PKC ϵ activators [9]. This hyperalgesia can be blocked by agents that disrupt each of the three major cytoskeletal elements; microtubules, intermediate filaments and actin microfilaments. Disrupting the cytoskeleton does not affect baseline analgesia suggesting that hyperalgesia mediated by PKC ϵ activation requires an intact cytoskeleton. In a separate study, the same authors observed that hyperalgesia induced by a PKC ϵ activator could be attenuated by blocking antibodies against beta-1 integrin [94], suggesting that PKC ϵ -dependent beta1 integrin cycling and regulation of beta1 integrin by the PKC ϵ substrate IQGAP1 are functionally important for PKC ϵ -mediated hyperalgesia.

PKC ϵ interactions with the cytoskeleton may promote oncogenesis. Cell culture studies have demonstrated that overexpression of PKC ϵ is associated with anchorage-independent cell growth and increased tumorigenicity, and some cancer cell lines display increased PKC ϵ activity (reviewed in [12]). These findings have *in vivo* significance since overexpression of PKC ϵ in the epidermis enhances development of squamous cell carcinoma after topical treatment with tumor promoters [95]. Several of the PKC ϵ substrates and binding partners described here may contribute to the pro-oncogenic properties of PKC ϵ . For example, the actin-binding domain of PKC ϵ is required for the metastatic potential of cells over expressing PKC ϵ , injection of NIH3T3 cell lines that over express PKC ϵ into nude mice causes tumors that metastasize. Injection of NIH 3T3 cell lines that overexpress a deletion mutant of PKC ϵ lacking the actin binding site still cause tumors but those tumors do not metastasize [96]. *In vitro* these deletion mutants do not show the invasive morphology and phenotype of cells that over express native PKC ϵ . It has been proposed that Rho GTPases and beta-1 integrins act downstream of PKC ϵ in these systems to mediate some of the pro-oncogenic effects of PKC ϵ [28,88,97].

CONCLUSION AND FUTURE DIRECTIONS

Using a combination of *in vitro* phosphorylation, regulation of kinase activity in intact cells and mutation of putative phosphorylation sites in candidate substrates, it is possible to identify kinase substrates with reasonable certainty. Substrates of PKC ϵ identified by these approaches are summarized in table 1. There exist however many examples where not all criteria have been met and therefore a candidate substrate has not been shown to be direct phosphorylated by PKC ϵ . For example, PKC ϵ plays a role in regulating gap junctions and has been postulated to phosphorylate connexin-43, but there are no direct *in vitro* phosphorylation data yet to support that conclusion [98,99]. Similarly, a role for PKC ϵ has been demonstrated in intracellular signaling cascades, such as the MAPK pathway, without direct substrates being identified [100]. A large volume of literature points to a critical role for PKC ϵ in ischemic preconditioning [4], the phenomenon whereby brief periods of ischemia and reperfusion confer protection from subsequent ischemic episodes. Again, the PKC ϵ substrates involved in this process are not yet known. One candidate is the mitochondrial permeability transition pore, which is a large conductance multiprotein complex involved in ischemic cell death. PKC ϵ is a component of the complex along with the voltage-dependent anion channel 1 (VDAC1), adenine nucleotide translocase (ANT1), hexokinase II, and cyclophilin D [101]. Functional studies demonstrate that activation of PKC ϵ attenuates pore opening, consistent with its role in ischemic preconditioning. *In vitro*, PKC ϵ can phosphorylate VDAC1, although the kinetics of this reaction and the phosphorylated residues are not known. Several other potential substrates have been proposed including the mitochondrial ATPase-sensitive K⁺ channel and cytochrome c

oxidase subunit IV (Reviewed in [6]). However, direct *in vitro* phosphorylation of these targets has not, to our knowledge, been demonstrated.

Even if *in vitro* studies demonstrate that a kinase can directly phosphorylate a protein, there is still uncertainty about whether the protein is an immediate substrate of that kinase *in vivo*. Recent developments in chemical genetics have led to a new approach that capitalizes on the fact that all kinases share a highly conserved purine binding pocket. Shokat and colleagues [102] have developed a strategy that utilizes the conserved features of the purine binding domain to engineer mutants specific for ATP analogs and modified inhibitors that bind competitively with ATP at this site. The mutation is made to replace a large “gatekeeper” residue with a small amino acid such as glycine or alanine, and is designed to be functionally silent with respect to kinase activity and substrate specificity. This generates an analog selective (AS) mutant that can utilize non-natural ATP analogs with bulky residues at the N⁶ position. Only the AS kinase, but not wild type kinases, can use N⁶-substituted analogs as phosphate donors. Therefore, only unique substrates of the AS-kinase are labeled by the ATP analogs. This approach has been successfully used to identify direct substrates of several kinases including JNK, v-Src, and cdk1 [103]. We have used an AS-kinase mutant of PKCε to study autophosphorylation of PKCε [22].

Several modifications to the use of AS-kinases have facilitated identification of direct substrates. Most AS-kinases can use ATP-γ-S analogs to label substrates, thereby producing thiophosphorylated instead of phosphorylated residues. Alkylation of the thiophosphate group with *p*-nitrobenzylmesylate yields a unique epitope tag that can be used to immunopurify the direct substrate and analyze the phosphorylation site by mass spectrometry [104]. Alternatively, thiophosphate residues can react with iodoacetamido-biotin at low pH (<4.0) allowing the substrate to be affinity purified using streptavidin beads [105]. In addition, some kinases can use ATP biotinylated at the γ position instead of ATP to transfer biotin directly to a substrate for affinity purification [106]. Finally, as an alternative to the AS-kinase method, it is also possible to identify direct substrates using tandem affinity purification tagging (Tap-tag kinase and cross-linking of substrate and kinase using reagents such as dithiobissulfosuccinimidyl) [107].

Once a direct substrate has been identified, specific sites of phosphorylation can be determined by mass spectrometry. For large substrate proteins or proteins with several sites of phosphorylation, one can use bioinformatics tools to predict phosphorylation sites, which can be validated by *in vitro* phosphorylation assays with substrate mutants that contain alanine substitutions at these sites. Tools that only scan for simple phosphorylation motifs suffer from a high rate of false positive hits. Other programs use a variety of techniques to reduce the false positive rate. For example, GPS (<http://gps.biocuckoo.org/>) classifies protein kinases into hierarchies and calculates the theoretically maximal false positive rate for each cluster of protein kinases. Linding et al [108] have developed a different computational tool, NetworKIN (<http://networkin.info/search.php>), that matches known phosphorylation motifs with information from several literature sources and databases to improve accuracy. Future developments in bioinformatics tools should greatly aid in the search for phosphorylation sites on substrates of all kinases, including substrates of PKCε.

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Figure 1.

Schematic diagram of PKC ϵ primary structure. Shown are common (C) regions conserved between members of the PKC family, including the pseudosubstrate (PS) domain that binds to the substrate recognition site when the enzyme is inactive, the neighboring C1a and C1b domains that bind diacylglycerols, phosphatidylserine and phorbol esters, and the C2-like domain which contains a site ($_{14}\text{EAVSLKPT}^{21}$) for binding to RACK2 and a “pseudoRACK sequence ($\text{yR}; _{85}\text{HDAPIGYD}^{92}$)” homologous to the corresponding binding site in RACK2. Also shown is the kinase domain, which is highly conserved between family members, and contains sites for binding of ATP and protein substrates. Regions unique to PKC ϵ include an actin binding domain that lies between the two C1 subdomains and the V3 hinge region which contains an autophosphorylation site (Ser-368) necessary for binding of 14-3-3 proteins. Like other kinases, PKC ϵ must be primed to exhibit full activity. Priming phosphorylation sites at the activation loop, turn motif and hydrophobic motif are shown in blue in the kinase and V5 domains. Two other sites that can be autophosphorylated *in vitro* are shown in green.

Table 1

Summary of the currently identified substrates and binding partners of PKC ϵ .

Protein	Type	Ref
RACK2/ β' -COP	Anchor/Binding Protein	[24,28]
RACK1		[28]
PDLIM5 (ENH-1)		[29,32]
TRAM		[38]
Histone H1		[42]
14-3-3		[41]
PKC ϵ	Kinase	[17,22]
Akt/PKB		[48,49]
PKD (PKC μ)		[36]
Stat 3	Transcription Factor	[53]
GABA $_A$ γ 2 subunit	Ion Channel	[57]
TRPV1		[62]
Peripherin	Cytoskeleton (intermediate filament)	[75]
Vimentin		[79]
Keratin		[109]
Actin	Cytoskeleton	[14,81,82]
IQGAP1		[86]
CMyBPC		[92,93]