New insights into the regulation of protein kinase C and novel phorbol ester receptors

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ABSTRACT **Protein kinase C (PKC), a family of related serine-threonine kinases, is a key player in the cellular responses mediated by the second messenger diacylglycerol (DAG) and the phorbol ester tumor promoters. The traditional view of PKCs as DAG/phospholipid-regulated proteins has expanded in the last few years by three seminal discoveries. First, PKC activity and maturation is controlled by autophosphorylation and transphosphorylation mechanisms, which includes phosphorylation of PKC isozymes by phosphoinositide-dependent protein kinases (PDKs) and tyrosine kinases. Second, PKC activity and localization are regulated by direct interaction with different types of interacting proteins. Protein–protein interactions are now recognized as important mechanisms that target individual PKCs to different intracellular compartments and confer selectivity by associating individual isozymes with specific substrates. Last, the discovery of novel phorbol ester receptors lacking kinase activity allows us to speculate that some of the biological responses elicited by phorbol esters or by activation of receptors coupled to elevation in DAG levels could be mediated by PKC-independent pathways.—Ron, D., Kazanietz, M. G. New insights into the regulation of protein kinase C and novel phorbol ester receptors. *FASEB J.* 13, 1658–1676 (1999)**

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The discovery of protein kinase C (PKC) in 1977 by Nishizuka and co-workers represented a major breakthrough in the signal transduction field (1). Over the last two decades many laboratories have contributed to the elucidation of the molecular basis of PKC activation and regulation and, in an attempt to elucidate its function, have provided a wealth of information about PKC. PKC has been identified as the cellular receptor for the lipid second messenger diacylglycerol (DAG), and is therefore a key enzyme in the signaling mechanisms by activation of receptors coupled to phospholipase C, which leads to a transient elevation in DAG levels. The discovery that PKC is a high-affinity receptor for the phorbol ester tumor promoters established the basis of its involvement in multistage carcinogenesis and has provided us with powerful pharmacological tools with which to manipulate PKC both *in vitro* and in cellular systems (2). Initial purification experiments and subsequent molecular cloning studies revealed that PKC is a multifamily of lipid-regulated serine-threonine kinases that phosphorylates a variety of cellular proteins and plays an essential role in signal transduction mechanisms. The identification of specific substrates for PKC isozymes and the characterization of its functional role form an active area of research.

The early dogma for the regulation of PKC activity and function has been challenged in the last few years. The initial concepts were as follows: *1)* Ca^{2+} and/or lipids are the only regulators of PKC; *2)* PKC isozymes translocate upon activation from the cytosol to the plasma membrane; and *3)* PKC isozymes are the only cellular receptors for the phorbol esters and the second messenger DAG. These concepts were insufficient to explain the differences observed in cellular localization and function of individual PKC isozymes, nor did they explain the heterogeneity observed in the cellular responses of the phorbol esters.

This review will summarize the current status of the regulation of PKC activity, including structural aspects and the classical model of activation, and emphasize recent findings that have increased our knowledge of PKC regulation and phorbol ester actions. First, we will focus on the regulation of PKC activity by serine/threonine and tyrosine phosphorylation. Second, the regulation of PKC activity and function by subcellular localization via protein–pro-

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tein interactions, and the newly identified domains that mediate these interactions, will be discussed. Finally, we will focus on the novel phorbol ester/DAG receptors lacking kinase activity (α- and β-chimaerin isoforms, Unc-13/Munc13 isoforms, RasGRP) as potential mediators of phorbol ester/DAG actions.

STRUCTURE OF PKC ISOZYMES

Classification of PKC isozymes based on structure and cofactor/activator requirement

PKC comprises at least 10 structurally related phospholipid-dependent protein kinases (for reviews, see refs 3–5). PKC isozymes have been grouped into three subclasses according to their regulatory properties, which are conferred by specific domains in the proteins (**Fig. 1**). The 'conventional' or 'classical' PKCs (cPKCs) include PKC α, βI, βII, and γ. These isoforms can be activated by Ca^{2+} and/or by DAG and phorbol esters. The 'novel' PKCs (nPKCs) δ, ε, θ, and η can also be activated by DAG and phorbol esters but are Ca^{2+} independent. Finally, the atypical PKCs, which include PKCζ and PKCι (its mouse homologue has been named PKCλ), are unresponsive to Ca^{2+} and DAG/phorbol esters. A related enzyme, PKCμ or PKD, displays multiple unique features that makes it a distant relative of the PKC isozymes (6, 7). Each PKC isozyme is the product of a separate gene with the exception of PKCβI and βII, which are alternative spliced variants of the same gene.

Each PKC isozyme consists of a single polypeptide chain having two structurally well-defined domains: the amino-terminal regulatory domain and the carboxyl-terminal catalytic domain. The regulatory region possesses the motifs involved in the binding of the phospholipid cofactors and Ca^{2+} and participates in protein–protein interactions that regulate PKC activity and localization. The carboxyl-terminal region is the kinase domain and includes motifs involved in ATP and substrate binding. The regulatory and the catalytic domains are connected by a

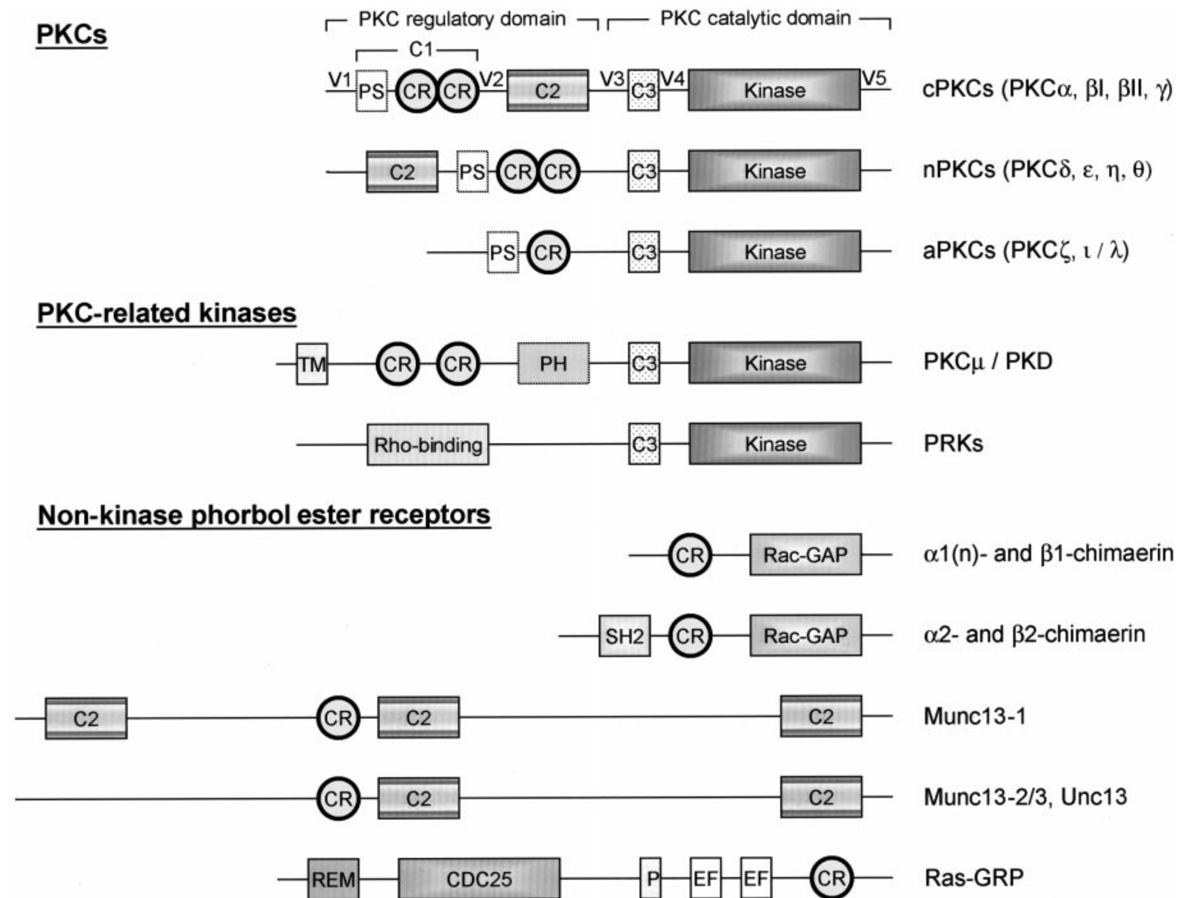

Figure 1. Structure of PKC, PKC-related kinases, and novel 'nonkinase' phorbol ester receptors. The different domains of PKC isozymes and related proteins are shown. The phorbol esters and the second messenger DAG bind to the cysteine-rich motif present in cPKCs and nPKCs. PKCμ/PKD, chimaerins, and C. *elegans* Unc-13 also bind phorbol esters and DAG with high affinity. PS, pseudosubstrate; CR, cysteine-rich domain; TM, transmembrane domain; PH, PH domain; SH2, SH2 domain; Rac-GAP, RacGTPase-activating protein domain; REM, Ras exchange motif; CDC25, region with homology to *Saccharomyces cerevisiae* CDC25 and guanine nucleotide exchange factor domain of Sos; P, proline-rich motif; EF, EF hand motif.

hinge region that is highly sensitive to proteolytic cleavage by cellular proteases.

The enzymes possess regions that are highly conserved between different PKC isozymes (regions C1 to C4) and variable regions (regions V1 to V5). The C1 region is present in all PKC isozymes. It contains an autoinhibitory domain or pseudosubstrate that binds to the substrate binding site in the catalytic domain and keeps the enzyme in an inactive state in the absence of cofactors and activators (8). The amino acid sequence of the pseudosubstrate resembles that of the phosphorylation motifs in PKC substrates, but possesses a nonphosphorylated amino acid (i.e., alanine) instead of serine or threonine (9). A distinct feature of the C1 region is the presence of the cysteine-rich domains, which are involved in binding of the second messenger DAG and phorbol esters in cPKCs and nPKCs (10–13).

While the cPKCs and nPKCs have two copies of these motifs in tandem, only a single copy is found in the aPKCs. A single cysteine-rich motif is also present in other proteins, including α- and β-chimaerins, Unc-13, Munc13 isoforms, Ras-GRP, Vav, and Raf (14–19) (Fig. 1).

The cPKCs possess a C2 region involved in Ca^{2+} binding immediately carboxyl-terminal to the cysteine-rich domains. A C2-like domain is present close to the amino-terminal end in nPKCs (20), although this domain is unable to bind Ca^{2+} (see below).

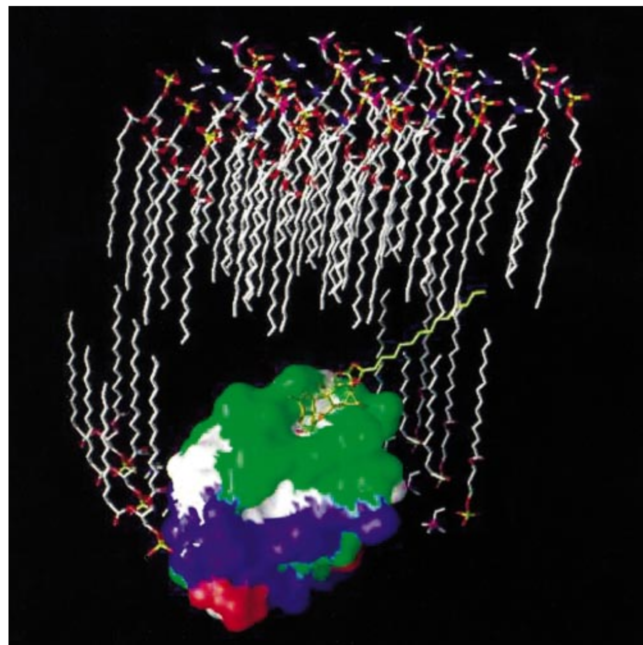

Figure 2. Model for the second cysteine-rich domain of PKCs complexed with PMA. Positively charged residues are shown in blue, negatively charged residues are red, hydrophobic residues are green, and neutral polar residues are white. The phorbol ester binds at the tip of the domain; rather than inducing a conformational change, it forms a hydrophobic cap over polar groups. By forming this continuous hydrophobic surface, PMA helps PKC insert into the lipid bilayer (see ref 42 for details).

PKCμ/PKD and PRKs: kinases related to PKC

A novel serine-threonine kinase regulated by DAG and phorbol esters was simultaneously identified by two groups and named PKCμ (human) or PKD (mouse) (6, 7). Although originally considered a new member of the PKC family, PKCμ/PKD differs from PKC isozymes in its regulation and substrate selectivity. PKCμ/PKD contains an amino-terminal putative transmembrane domain, a C1 region with two cysteine-rich domains that bind phorbol esters and DAGs (6, 7, 21), and a Pleckstrin homology (PH) domain (Fig. 1). No pseudosubstrate domain has been identified in PKCμ/PKD. The catalytic domain exhibits some degree of similarity with members of the PKC family, but is related more to the kinase domains of myosin light-chain kinase of *Dictyostelium* and Ca^{2+}/calmodulin-dependent kinase II (7). PKCμ/PKD does not catalyze significant phosphorylation of PKC substrates, including PKC pseudosubstrate-based peptides, histone, myelin basic protein, or protamine. The synthetic peptide syntide 2, a substrate of calmodulin-dependent kinases, is efficiently phosphorylated by PKCμ/PKD (22). This unique pattern of substrate recognition and its Golgi localization (23) suggest important functional differences between this novel kinase and the PKCs.

PKCμ/PKD is activated in cells through PKC-mediated phosphorylation and can therefore function downstream of PKCs (24). An additional form of regulation is through its PH domain; its deletion results in a marked increase in the basal activity of the enzyme, suggesting that the PH domain of PKCμ/PKD plays an inhibitory role in the regulation of its enzymatic activity (22). PKCμ/PKD may act as a scaffold protein for enzymes engaged in phosphoinositide synthesis, an effect that requires an intact amino-terminal region, including its transmembrane domain (25).

A second group of kinases with homology to PKC, named PRKs (PKC-related kinases), was recently described (3, 26, 27). PRK isoforms possess a kinase domain with homology to the kinase domain in PKCs (Fig. 1). Although these distant relatives of PKC isoforms, which lack a C1 domain, do not respond to phorbol esters or Ca^{2+}, they can be activated by the acidic phospholipids phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-triphosphate (3, 27). A distinct feature of PRKs is that their kinase activity is directly regulated by small GTP binding proteins. PRKs possess a binding site for the Rho small GTP binding proteins and belong to the family of Rho-activated kinases that includes rhotekin and rhophilin (3, 28, 29).

PROTEIN KINASE C DOMAINS

The cysteine-rich domains: binding sites for DAG and phorbol esters

Work performed by several groups led to the identification of PKC as a receptor for phorbol ester tumor promoters (30–34). The work pioneered by Blumberg and colleagues has provided important insights into the phorbol ester binding site in PKC isozymes and its interaction with ligands. Early experiments using [^{3}H]PDBu as a radioligand revealed that PKC isozymes bind phorbol esters with high affinity in the presence of phosphatidylserine as a cofactor (35) and that DAG and phorbol esters bind to the same site (36). Recombinant cPKC and nPKC isoforms expressed in Sf9 insect cells show similar low nanomolar affinities for [^{3}H]PDBu, suggesting a high degree of conservation in the binding site between the different PKC isozymes (37).

The first implication that cysteine-rich domains act as phorbol ester binding sites originated from experiments performed in Nishizuka's laboratory, in which either deletion of those motifs in PKCγ or mutation of conserved cysteines resulted in loss of phorbol ester binding (13). A deletion analysis by Quest et al. revealed a minimum domain of 43 amino acids required for phorbol ester binding (38). Each of these cysteine-rich domains possesses the motif $HX_{12}CX_2CX_{13/14}CX_2CX_4HX_2CX_7C$, where H is histidine, C is cysteine, and X is any other amino acid. Each domain tightly binds two atoms of Zn^{2+}, resulting in a stoichiometry of 4 Zn^{2+} per molecule of cPKC or nPKC (39).

Through a combination of structural analysis and site-directed mutagenesis, the molecular basis of phorbol ester binding to PKC has been established (12, 40–42). Each cysteine-rich domain in PKC folds into a globular structure, and phorbol esters bind in a groove formed by pulling apart two β-sheets. Ligand binding does not induce significant changes in the conformation of the cysteine-rich domain, but rather 'caps' a hydrophilic site at the top of the structure forming a contiguous hydrophobic region that promotes insertion of the domain into the lipid bilayer (**Fig. 2**) (42). Essential residues for phorbol ester binding are missing in PKCζ and other cysteine-rich domain-containing proteins such as Raf or Vav, which compromise ligand binding (17, 43). Although initial reports have suggested that Vav is a phorbol ester receptor (44), it is now clear that Vav, although it preserves the Zn^{2+} binding characteristics, is unable to bind phorbol esters or related ligands even with low affinity (17).

The issue of equivalence between the cysteine-rich motifs in PKC is a subject of controversy and deserves careful attention. Several laboratories have reported that the stoichiometry of phorbol ester binding is ~1 mol of phorbol ester/mol of PKC (35, 45). These results contrast with experiments that show that each individual cysteine-rich domain is able to bind phorbol esters (10–13). Whether each cysteine-rich domain binds phorbol esters with similar affinity remains controversial.

In a series of papers, Stubbs and co-workers have proposed that PKCα contains two distinct binding sites with low and high affinity for the fluorescent ligand sapintoxin D and that distinct ligands differentially compete for each site (46–48). The level of PKC activation that can be achieved by DAG together with phorbol ester is greater than that observed with each individual activator at maximally stimulating concentrations. The nonequivalency of the cysteine-rich domains was also observed in yeast proliferation assays using deletion mutants of PKC (49). Studies with PKCδ, where each individual cysteine-rich domain has been mutated (Pro to Gly 11 in the motif), have revealed a differential pattern of translocation from cytosol to particulate fraction for each mutant (50). Moreover, these mutants show striking differences in their ability to translocate in response to different PKC ligands (51). Each cysteine-rich domain may have a different contribution to the activation of the enzyme. The dissociation between the affinity for binding and the potency for translocation suggests a complex mechanism for regulating membrane association and requires further analysis.

The C2 domain: binding site for Ca^{2+}, lipids, and proteins

The C2 domain in cPKCs is found immediately carboxyl-terminal to the cysteine-rich domains and is a Ca^{2+} binding site. Although first described for the cPKCs, the C2 domain has been recognized as a widespread domain. A great number of proteins containing C2 domains have been identified to date, most of them related to signal transduction mechanisms or membrane trafficking. Representative proteins containing C2 domains include synaptotagmins, phospholipase C isozymes, cytosolic phospholipase A_2, rabphilin-3A, Unc-13, and Munc13 isoforms. Despite a marked variation in primary sequences, structural analysis has revealed that all C2 domains fold similarly into a structure consisting of two four-stranded antiparallel β-sheets connected by variable loops at the end of each strand, with the Ca^{2+} binding site located at one end of the domain (52–54). It has been shown for PKCβ and synaptotagmin that five conserved Asp residues are involved in the coordination of two Ca^{2+} ions (53). The C2 domain acts as a membrane docking module, where the Ca^{2+} ions and basic residues contribute to electrostatic membrane binding.

Although not initially recognized, it has now been

shown that nPKCs possess a C2-like domain that is unable to bind Ca^{2+} (20). Important Asp residues required for Ca^{2+} binding are not present in the C2-like domain of nPKCs. It is believed that this domain in nPKCs may be involved in phospholipid binding and regulates lipid activation of nPKCs. A related lipid binding domain has also been reported in PRKs (3, 27).

The C2 domain of PKCs has a dual role in the regulation of PKC activity. In addition to its proposed lipid or Ca^{2+}/lipid binding sites, this domain regulates protein–protein interactions, as described later.

The kinase domain

The catalytic domain of PKC isozymes includes the C3 and C4 domains. The C3 domain possesses the binding site for ATP, the phosphate donor for phosphotransferase activity; the C4 domain possesses the binding site for substrates. When PKC is maintained in an inactive state, the pseudosubstrate occupies this site, blocking the binding of substrates. Structural information is not available for the PKC kinase domain. However, a modeled structure of the catalytic domain based on the 3-dimensional structure of cyclic AMP-dependent kinase (PKA) has been postulated (4, 55).

Although many substrates for PKC have been identified, synthetic peptides based on the pseudosubstrate region are efficiently phosphorylated by PKCs (37, 56). In elegant studies using peptide libraries, Cantley and co-workers have provided important information on the substrate selectivity for individual PKC isozymes. For most PKC isozymes, the predicted optimal sequences from position -3 to $+2$ relative to the phosphoserine were shown to be similar to the corresponding endogenous pseudosubstrate sequences, with some variations. In the library screening, all PKC isozymes (except PKCμ) show high selectivity for peptides with basic amino acids at position -6, -4, and -2 and variable residues in other positions (56).

REGULATION OF PKC ACTIVITY BY COFACTORS

Regulation by lipids

Biochemical and biophysical studies have provided a wealth of information about the lipid regulation of PKC activity by cofactors. The regulation of PKC activity by lipid cofactors has been extensively reviewed (see refs 5, 57–59). Experiments using mixed micelles or lipid bilayers have determined that acidic phospholipids are efficient cofactors for PKC activation, with a remarkable selectivity for phosphatidylserine. In addition to serving as an anchoring molecule through its binding to the cysteine-rich domains, DAG causes a dramatic increase in the affinity of cPKCs and nPKCs for phosphatidylserine (5, 58). In addition, binding of DAG to PKC confers selectivity for the phosphatidylserine headgroup among the different acidic phospholipids (60–62).

The accepted model of activation of PKC by lipids is that on binding of DAG (or phorbol esters) in the presence of the phospholipid cofactor, a conformational change in PKC results in the removal of the pseudosubstrate from its binding site and in the activation of the enzyme. It is believed that the cysteine-rich and C2 domains are not the only regions involved in phospholipid binding: the pseudosubstrate domain, once removed from its binding site, may also contribute to membrane binding through its basic residues (8, 57, 61, 62). Membrane association is reflected as a shift in subcellular localization or 'translocation' of cytosolic PKC to membrane compartments in cellular systems, a process that is also tightly controlled by protein–protein interactions (see below).

In addition to phospholipids, other lipids can stimulate PKC activity. Free fatty acids synergize with DAG for PKC activation (63), and lipids such as short-chain phosphatidylcholine derivatives, lysophosphatidic acid and phosphatidylinositol 3,4,5-triphosphate can activate PKC isozymes. It is likely that differential lipid requirements for individual PKC isoforms represent a potential mechanism to control isozyme specific functions in cells. One exciting finding supporting this concept is that the acidic phospholipid phosphatidylglycerol stimulates PKCβII in nuclear membranes, leading to phosphorylation of nuclear substrates by this isozyme. Identification of the phosphatidylglycerol binding site at the carboxyl-terminal region of PKCβII provides the first evidence of a phospholipid binding site in the catalytic domain, and suggests a high degree of complexity in the regulation of PKC activity by lipids (64).

Regulation of PKC activity by Ca^{2+}

Although the molecular events mediating Ca^{2+}-induced activation of cPKCs are not fully understood, experimental evidence supports a model of allosteric interaction between Ca^{2+} and phospholipids. Ca^{2+} increases the affinity of cPKCs for anionic phospholipids (65). Association to membranes and activation of kinase activity are differentially regulated by the cation, and the concentration of Ca^{2+} required for membrane binding is substantially lower than that required for activation (66). The model postulated by Newton suggests that low concentrations of the

cation promote a weak membrane interaction, which is accompanied by conformational changes that are not sufficient to promote activation of the enzyme. Higher Ca^{2+} concentrations, however, produce a conformational change in PKC that results in the release of the pseudosubstrate from its binding site in the catalytic domain, leading to enzyme activation (66). In support of this hypothesis, a mutation analysis of the C2 domain of PKCα shows that each individual Ca^{2+} ion and the Asp residues involved in their binding may have different roles in membrane binding and activation (67).

PKC PHOSPHORYLATION

In recent years, accumulating evidence has indicated that phosphorylation is essential for the regulation of PKC. PKC is autophosphorylated on serine/threonine residues and is transphosphorylated on tyrosine and/or serine/threonine residues by upstream kinases. PKC phosphorylation is a processing event that regulates the maturation of the enzyme in addition to PKC activation or inactivation.

Serine/threonine phosphorylation

An emerging new theme in the regulation of PKC function by phosphorylation involves phosphoinositide-dependent protein kinase-1 (PDK1). Recently, studies by the laboratories of Parker (68) and Toker (69), have identified PKC isozymes as substrates for PDK1. Both groups have elegantly demonstrated that PDK1 phosphorylates PKCζ within the activation loop, which leads to the direct activation of this isozyme (68, 69). Furthermore, recent reports by these two groups, as well as from Newton and co-workers, suggest that PDK1 may be a universal PKC kinase, since PDK1 can phosphorylate cPKCs, nPKCs, and aPKCs. PKCδ (68), PKCζ (68, 69), PKCα, and PKCβII (70) are phosphorylated by PDK1 within the activation loop of the PKC kinase domain. This phosphorylation is an important regulatory step for PKC activation, since coexpression of PKCβII and an inactive mutant of PDK1 results in an inactive PKC (70). Furthermore, Thr to Ala mutation on the PDK1 phosphorylation site in PKCε, PKCδ, and PKCζ results in kinase-deficient, dominant negative mutants (71). Phosphorylation by PDK1 is followed by autophosphorylation of two additional sites within the carboxyl terminus of the sequence, namely, Thr-638 and Ser-657 for PKCα (72, 73) and Thr-641 and Ser-660 for PKCβII (74, 75). Mutagenesis of the autophosphorylation sites to Ala or Glu and dephosphorylation of these residues have revealed different functions for each site. The first autophosphorylation occurs on Thr-641 for PKCβII (75). This site is fundamental to the catalytic activity of PKCβII (75), PKCβI (76) and PKCδ (77). The importance of Thr-641 in the release of PKCβ from detergent-insoluble fraction to the cytosol has been demonstrated by Newton and co-workers (78). Phosphorylation on the same site in PKCα (Thr-638) is not required for its catalytic activity, but is important for the duration of activation and the rate of dephosphorylation of the enzyme (73). The second autophosphorylation site in PKCβII (Ser-660) is important for the correct folding of the enzyme and plays a regulatory role in substrate, ATP, and Ca^{2+} binding (74). Autophosphorylation on the same residue in PKCα (Ser-657), however, is important to lock the fully phosphorylated enzyme in a close conformation that is resistant to phosphatases (72). In summary, the phosphorylation of the activation loop by PDK1, followed by autophosphorylation on two additional sites are necessary for the localization of PKC isozymes and to obtain a catalytically competent conformation. Recently, Hannun and co-workers reported another function for PKC autophosphorylation. Using GFP-tagged PKCβII and mutants, they found that autophosphorylation of PKCβII on residues Thr-641 and Ser-660 is important for membrane dissociation of activated PKC from the plasma membrane and return of PKC to the cytoplasm after its activation (79).

Tyrosine phosphorylation

PKCδ is phosphorylated on tyrosine residues within the regulatory domain in response to a variety of stimuli, including the engagement of the FcεRI receptors in mast cells (80) and PDGF in 32D cells (81). Src (82, 83) and Lyn (82) have been identified as the tyrosine kinases responsible for this phosphorylation. The role of tyrosine phosphorylation for PKC activation, however, remains controversial. Whereas some reports show an absence of an effect on PKC activity (81), others have demonstrated an increase (84) as well as a decrease in activity (85). Nishizuka and co-workers have recently reported that PKCδ, as well as other PKC isozymes, were phosphorylated on tyrosine residues in response to stress response such as H_2O_2. The H_2O_2-induced tyrosine phosphorylation was mapped to the catalytic domain of PKC. Tyrosine phosphorylation induced by H_2O_2 was sufficient to induce prolonged PKC activation. (86).

PKC BINDING PROTEINS

Since the early 1990s it has become clear that in addition to binding to lipids, PKC can also interact with proteins via protein–protein interactions. These

TABLE 1. *PKC binding proteins*

Name	Type of PKC binding protein	Other functions	PKC substrate	Isozyme specificity	Reference
RACK1	RACK		No	PKCβII	(97)
RACK2	RACK	(β′COP), Golgi budding Vesicle trafficing	No	PKCε	(90)
P59fyn	Unknown	Tyrosine kinase	No	PKCθ	(132)
Clone72	STICK		Yes		(102)
Caveolin	Scaffolding protein	Lipid modification and metabolism	ND	PKCζ, PKCα	(106)
PICK1	Unknown		Yes	PKCα	(148–149)
AKAP79	Scaffolding protein	PKA anchoring protein	Yes		(108), (109)
ZIP/p62	Scaffolding protein	atypical PKCs			(112–113)
14-3-3τ	Scaffolding protein			PKCθ	(121)
Btk (Bruton tyrosine kinase)	Unknown	Tyrosine kinase	Yes	PKCβ, PKCε	(146), (179)
Nef	PKC inhibitor	HIV protein, down regulation of CD4 T cell surface receptors	No	PKCθ	(131)
Sdr (serum deprivation response) protein	STICK		Yes	PKCα	(103)
SRBC (sdr-related gen product that binds C kinase)	STICK		Yes	PKCδ	(180)
ASIP (atypical PKC isotype specific interacting protein)	Unknown			PKCζ, PKCλ/ι	(150)
RBCK1	Unknown	Transcription factor		PKCβ, PKCζ	(155)
XAP3	RACK (?)	Hepatitis B virus X-associated protein	No	PKCη	(150)
ENH (Enigma Homolog)	Unknown	Yes		PKCε, PKCβ	(152)
GAP43	Unknown		Yes	PKCδ	(130)
Syndecan-4		Transmembrane heparan sulfate proteoglycan	Yes	PKCα	(134–138)

interactions play an important role in the localization and function of PKC isozymes. In this review, we define PKC binding proteins as proteins that bind PKC directly via a nonsubstrate binding site, which may or may not be PKC substrates. The two methods most commonly used to identify PKC binding proteins are the overlay assay (87) and the yeast two-hybrid system. **Table 1** summarizes the current information on PKC binding proteins. These proteins may be grouped together on the basis of the conformation of the PKC required for binding, cofactor requirement, and whether or not the proteins are PKC substrates. PKC in an active conformation will bind to receptors for activated C kinase (RACKs) or substrates that interact with C kinase (STICKs), whereas inactive PKCs will interact with A kinase anchoring protein (AKAPs) and 14–3-3 (**Fig. 3*A, B***). PKC binding proteins may or may not be PKC substrates (for example STICKs and RACKs, respectively) and may require cofactors for binding to PKC (STICKS require phosphatidylserine; GAP-43 requires Ca^{2+}). Such proteins serve many functions (Fig. 3), which include localizing inactive (AKAPs) or active (RACKs) PKC isozymes to specific intracellular sites or serving as substrates (STICKs), shuttling proteins (RACK1), PKC activators (syndecan-4, F-actin), or PKC inhibitors (14–3-3, Nef).

RACKs

RACKs were originally identified by Mochly-Rosen and co-workers, who in the early 1990s proposed that the intracellular localization of PKC isozymes upon activation is mediated via interaction with isozyme-specific anchoring proteins (88, 89). RACKs were first characterized as Triton-X-100 insoluble proteins that bind PKC isozymes only in the presence of PKC activators (88, 89). The PKC–RACK interaction is mediated, at least in part, by the C2 region in cPKCs and the C2-like region (within the V1 region) in nPKCs (90–96). Two RACKs have been identified to date by using overlay assays: RACK1, which specifi-

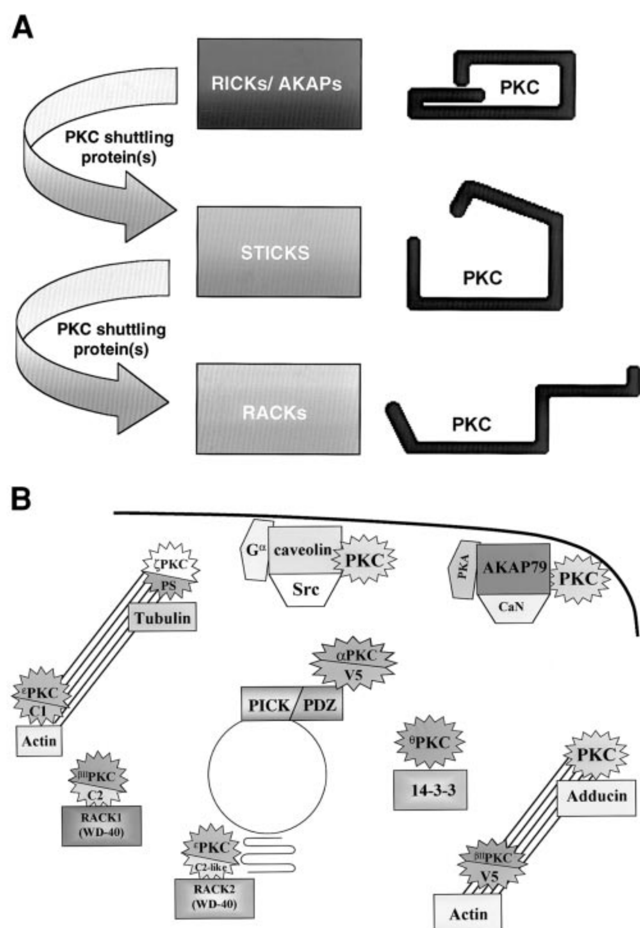

Figure 3. PKC binding proteins serve different functions in the regulation of function of PKC isozymes. *A)* Inactive PKC isozymes bind to AKAPs and/or the putative RICKs that inhibit PKC activity and localize the inactive isozymes to specific intracellular sites. In the presence of phosphatidylserine, PKCs bind and phosphorylate STICKs. Fully active PKCs bind to RACKs localized to specific sites, allowing the PKC isozymes to phosphorylate specific substrates. Shuttling proteins such as RACK1 may facilitate the distribution from a PKC binding protein to another *(B)*. PKC binding proteins include cytoskeletal proteins, scaffolding proteins, and organelle associated proteins. PKC binding proteins can localize to different intracellular sites; their localization can be fixed or mobile.

cally interacts with PKCβII (97, 98), and RACK2 (β'-COP), which specifically interacts with PKCε (90). Both RACKs bind PKC in its active conformation. Despite the differences in sequence between RACK1 and RACK2, the two proteins share common features. Although RACK1 and RACK2 are not PKC substrates, both increase PKC phosphorylation of substrates (90, 97). RACKs contain 'WD40 repeats', a motif known to mediate protein–protein interactions. The role of WD40 repeats in PKC binding proteins is discussed below. It was recently observed that RACK1 translocates in response to PKC activation. RACK1 translocates to the same site as activated PKCβII and specifically associates with this isozyme upon activation. These findings suggest a potential role for RACK1 as a PKC shuttling protein (93).

STICKs

STICKs are an important group of PKC binding proteins that were discovered by Jaken and co-workers. STICKs require phosphatydilserine for interaction and are PKC substrates. Phosphorylation of STICKs regulates their association to PKC. The identified STICKs include MARCKs, MacMARCKs, α-adducin, β-adducin, γ-adducin, and clone 72 (SseCKs) (99–103). STICKs are involved in a variety of functions. For example, adducins are cytoskeletal proteins involved in the interaction between actin and spectrin. Expression, localization, and phosphorylated states of α-adducin and γ-adducin have been correlated with renal tumor progression (99–101). Clone 72, a major PKC binding protein in REF52 fibroblasts, is involved in cytoskeleton remodeling and cell growth (102). Another STICK is the serum deprivation response protein (Sdr) that binds and localizes PKCα within the microdomain of caveolae (103).

Scaffolding proteins

Scaffolding proteins cluster signaling proteins, thus allowing a tight control of cellular pathways as well as cross talk between different cascades. Scaffolding proteins such as caveolin, AKAPs, and INAD cluster PKC to specific intracellular sites. PKC scaffolding proteins that have been identified to date bind to PKC in its inactive conformation.

Caveolin

Caveolin targets a variety of signaling proteins such as α-subunits of G-proteins, Src, and EGF receptor to the caveolae (104–106). PKCα localizes to the microdomain of the caveolae (103, 106). PKCα, in addition to PKCζ, also associates with caveolin, whereas PKCε was not found to associate with this protein (106). Caveolin interaction with PKC results in the inhibition of PKC activity. Moreover, a short peptide derived from caveolin interacts directly with PKC and inhibits its kinase activity (106).

AKAPs

Two members of the AKAP family of proteins that target PKA to a specific intracellular site are also scaffolding proteins for PKC. Elegant work by Scott and co-workers has demonstrated that AKAP79 assembles PKA, phosphatase 2B, and PKC at postsynaptic dendritic fractions (107–109). AKAP79 inhibits PKC activity (108). Ca^{2+}-calmodulin as well as DAG

release PKC from AKAP79 (109). In HEK-293 cells, AKAP79 is phosphorylated after PMA stimulation (110). Phosphorylation of AKAP79 by PKC regulates its subcellular compartmentalization (110). Thus, there is a reciprocal regulation of AKAP79 and PKC with regard to subcellular localization. Another AKAP identified by Scott and co-workers, AKAP250 (Gravin), assembles both PKA and PKC to the filopodia in human erythroleukemia cells (111).

p62/ZIP (PKCζz interacting protein)

p62/ZIP was isolated as a binding protein for the atypical PKCs (112, 113). p62/ZIP is a poor PKC substrate (112). This protein has been implicated in targeting atypical PKC isozymes to a lysosome-targeted endosomal compartment (113). Since p62/ZIP is also associated with other proteins such as the cytokine receptor EBI-3 and p56Lck, it is possible that p62/ZIP is yet another example of an isozyme-selective scaffolding protein.

INAD

Zucker and co-workers have identified the *Drosophila* INAD as a scaffolding protein that assembles signaling proteins that participate in phototransduction. These proteins include the light-sensitive TRP ion channel, calmodulin, rhodopsin, phospholipase C, and PKC (114, 115). INAD contains PDZ domains that mediate its interaction with PKC (see below).

14–3-3

14–3-3 is a family of highly homologous proteins that are ubiquitously expressed. The role of 14–3-3 proteins in PKC signal transduction remains controversial. 14–3-3 isozymes may either inhibit (116, 117) or enhance PKC activity (118, 119). The dimeric structure of 14–3-3 enables the protein to serve as an adaptor or scaffold for a variety of signaling proteins, including PKC (120). Meller et al. have reported a specific interaction between a PKC isozyme (PKCθ) and a 14–3-3 isozyme (14–3-3τ) in T cells that inhibits PKCθ translocation and function (121). This interaction only occurs with inactive PKCθ, which may help keep this PKC isozyme in its inactive conformation. 14–3-3τ may represent an example of a RICK (a receptor for inactive C kinase), which binds to PKC and targets the inactive isozyme to specific intracellular sites.

Direct interaction of PKC isozymes with cytoskeletal proteins

PKC isozymes associate with cytoskeletal proteins, and a review on the subject has recently been published elsewhere (122). The interaction between PKCs and cytoskeletal proteins is at least in part isozyme-selective. An example of this isozyme specificity is PKCζ, which associates with tubulin via the pseudosubstrate region (123). Terrian and co-workers have demonstrated that PKCε specifically binds F-actin via an actin binding site within the C1 region (124, 125). F-actin activates PKCε in the absence of phospholipids (124). PKCβII (but not PKCβI) also interacts with F-actin via its V5 domain and translocates to the actin cytoskeleton upon activation (126). PKCβII selectively phosphorylates actin, although actin is a poor substrate. The interaction of PKCβII with actin results in a significant enhancement in autophosphorylation and in an alteration in substrate specificity (126). Furthermore, the interaction between PKCβII and actin protects PKC from degradation and down-regulation (126). The interaction between PKC and F-actin was also observed in *Aplysia* (127).

Other PKC binding proteins

A number of PKC binding proteins do not share sequence homology or functional characteristics with the PKC binding proteins described previously. One such protein that interacts with PKCλ/ι and was cloned in a yeast two-hybrid screening is LIP (PKCλ/ι interacting protein). LIP binds to the cysteine-rich domain of PKCλ/ι but not to PKCζ, and serves as an activator of the enzyme (128). A second atypical PKC binding protein is PAR-4, which interacts with the cysteine-rich domains of atypical PKCs and is involved in apoptotic responses (129). Parker and co-workers characterized another protein, GAP43 (growth-associated protein, neuromodulin), which is a major PKC substrate in neurons. GAP43 is a PKCδ binding protein and interacts with the C2-like domain of this nPKC (130), suggesting that GAP43 may play a role in the subcellular localization of PKCδ.

The human immunodeficiency virus protein Nef interacts with PKCθ in T cells and inhibits PKCθ translocation and activity (131). Recently, the nonreceptor tyrosine kinase Fyn was found to be directly associated with PKCθ in T cells (132). Since PKCθ translocates to the T cell receptor after T cell receptor activation (133), it may be possible that Fyn, which is also known to associate with the T cell receptor, plays a role as an anchoring protein for activated PKCθ. The direct interaction between PKC isozymes and nonreceptor tyrosine kinases is not restricted to PKCθ and Fyn. PKCδ associates with the nonreceptor tyrosine kinase Src via tyrosine phosphorylation-dependent and -independent mechanisms (82, 83).

Another PKC interacting protein is syndecan-4, a member of the transmembrane matrix binding pro-

teoglycans. The cytoplasmic tail of syndecan-4 interacts with the kinase domain of PKCα, which results in the localization of PKCα to focal contacts and in the activation of the isozyme (134, 135). Furthermore, syndecan-4 is phosphorylated in response to PKC activation (136). The phosphorylation status of syndecan-4 does not affect its binding to PKCα, although it regulates its activity (137, 138).

Protein interacting domains in PKC binding proteins

Figure. 3*B* and **Fig. 4*A*** summarize the current knowledge on the protein interacting domains in PKC binding proteins.

WD40 repeats

WD40 repeats are sequences of approximately 40 amino acids having conserved amino acids at the amino-terminal (usually Trp or W, and Asp or D) and at the carboxyl-terminal of the repeat. WD40 repeats have been found in more than 100 proteins (139). The seven WD40 repeats of the β-subunit of G-proteins make a seven-bladed β-propeller, which explains the protein–protein interaction properties of this motif (140). WD40 repeats are found in RACKs (90, 97). The entire sequence of RACK1 consists of seven continuous WD40 repeats (97). WD40 repeats in RACK2 account for only 40% percent of its sequence (90). Another WD40-containing protein, the telomerase associate protein 1 (TEP1), associates specifically with the PKCα isozyme in human breast cancer cells (141). Since RACKs are isozyme-selective PKC anchoring proteins, it may be possible that TEP1 is a RACK for PKCα.

PH domain

Plecktrin homology domains consist of ~100 amino acids, which bind polyphosphoinositides as well as proteins and are present in a wide variety of signaling proteins (142). Several reports have shown an association of PKC isozymes with PH domain-containing proteins (143–146). For example, the PH domain of rac protein kinase associates with the regulatory domain of PKCζ (143, 144). PKCβI associates with the tyrosine kinase Btk in mast cells via its PH domain, which results in the phosphorylation of Btk and inhibition of its kinase activity (146).

PDZ domain

The PDZ (Psd-95, Dlg, and Zo-1) domain is found in a variety of scaffolding proteins that mediate the organization of signaling networks (147). Thus far, three PKC binding proteins that contain PDZ domains have been identified, namely, PICK1, INAD, and ASIP, which interact with the V5 region of PKC isozymes. PICK1 was isolated in a yeast two-hybrid screening, using the catalytic domain of PKCα as bait

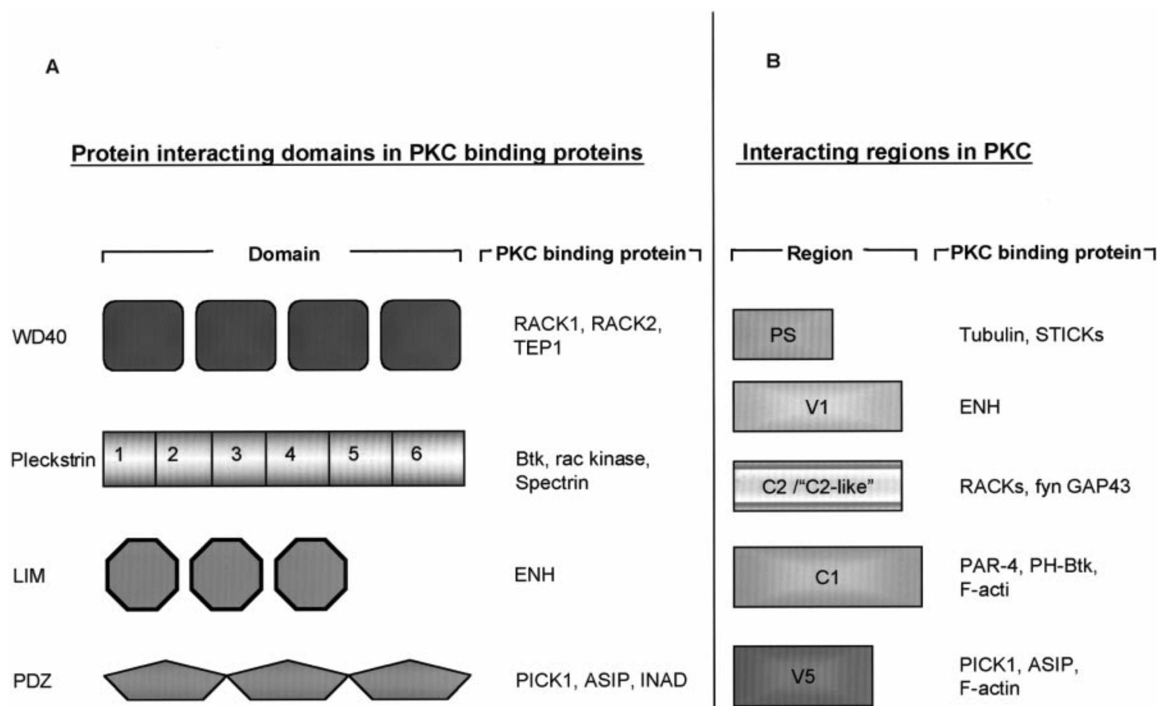

Figure 4. The interactions between PKC and PKC binding proteins are mediated via protein domains. *A)* Domains that mediate protein–protein interactions found in PKC binding proteins. *B)* Regions in PKC isozymes that interact with PKC binding proteins.

(148). PICK1 localizes PKCα to a perinuclear region (148, 149). The *Drosophila* scaffolding protein INAD contains five PDZ domains, two of which interact directly with *Drosophila* eye PKC (114, 115). This association occurs in the absence of PKC activation. The PKC binding site for INAD has been mapped to the carboxyl-terminal region of PKC, although sites outside the V5 region may also be involved (115). Finally, the third PDZ-containing protein ASIP, which was recently identified by Ohno and coworkers, is a novel atypical PKC-specific interacting protein. This protein contains three PDZ domains. ASIP localizes atypical PKCs to tight junctions and may play a role in the maintenance of epithelial cell polarity (150).

LIM domain

This domain is a cysteine-rich protein–protein interaction domain that has been found in more than 60 proteins (151). The LIM domain-containing protein ENH (Enigma homologue) was cloned using the yeast two-hybrid system and the regulatory domain of PKCβ as the bait (152). ENH contains three LIM domains, one of which interacts with the V1 region of PKCβ and PKCε (152). Although PKCβ phosphorylates ENH, the interaction is not dependent on the phosphorylation state of ENH (152).

Ring finger-containing proteins

Two proteins possessing ring finger zinc binding domains have recently been identified as PKC binding proteins (153–155). RBCK1 (RBCC protein interacting with PKC) was cloned using the yeast two-hybrid system and the regulatory domain of PKCβ as a bait (153). RBCK1 interacts with PKCζ and PKCβ, and acts as a transcription factor (153, 154). XAP3 (X-associated protein 3), a protein that binds to the transcriptional transactivator hepatitis B virus X protein, is another candidate as an isozyme selective PKC anchoring protein. XAP3 shares 85% homology with RBCK1, and interacts specifically with PKCη, allowing it to come into close proximity with its substrate protein X. PKCη in turn phosphorylates protein X that results in activation of transcription (155).

Protein interacting regions in PKC

The identification of PKC interacting proteins suggests that specific regions within the structure of PKC should serve as binding motifs. These motifs, present in either the regulatory or catalytic domain of PKC isozymes, are depicted in Fig. 4*B*. Short sequences within the kinase domain of PKC have been identified as interacting motifs for the PDZ-containing proteins PICK1 (149) and ASIP (150), as well as for caveolin (106), F-actin (125), and syndecan-4 (134).

Using chimeras of the regulatory and catalytic domains of different PKC isozymes, the laboratories of Blumberg (156) and Fields (157) have provided evidence for a role of the catalytic domain in targeting PKC isozymes to specific cellular sites. However, most of the protein binding sequences are localized to the regulatory domain. The pseudosubstrate sequence has been reported to mediate, at least in part, the interaction with STICKs (158) and to mediate PKCζ binding to tubulin (123). Short sequences within the C1 region of PKCε were identified as F-actin binding motifs (125). The C1 region in atypical PKCs was identified as the PAR4 binding site (129). The C2 region contains the RACK binding site (97, 98) and the GAP43 binding site in PKCδ (130). This C2 region in PKCβ also possesses the pseudo-RACK binding site (159), which is a short sequence that shares high homology to RACK1. This motif may bind to the RACK binding site, thereby keeping PKC in an inactive conformation (159). Another autoregulatory sequence has recently been identified in the C1 domain of PKCα and PKCε. This C1-derived sequence interacts with the kinase domain of PKC in a lipid-independent manner (160). It is likely that this motif also contributes to maintaining PKC in an inactive conformation.

NOVEL 'NONKINASE' PHORBOL ESTER/DAG RECEPTORS

α- and β-chimaerins: phorbol ester receptors with Rac-GAP activity

For many years, the only receptors known for phorbol esters and DAG were the cPKCs and nPKCs. However, not all the phorbol ester responses can be attributed to PKC isozymes. A potential explanation for the heterogeneity in phorbol ester responses in cells is the presence of phorbol ester receptors distinct from those of the PKC family. Additional phorbol ester receptors have recently been identified: mammalian α- and β-chimaerins, Ras-GRP, and *Caenorhabditis elegans* Unc-13 (Fig. 1 and **Table 2**). Each of these novel phorbol ester receptors possess a single copy of the cysteine-rich domain, suggesting that, as previously observed with PKC deletion mutants and isolated domains, only one copy of the motif is sufficient for high-affinity binding of phorbol esters and DAG (11, 38, 161).

The first evidence for a nonkinase phorbol ester receptor was the cloning of α1-chimaerin (formerly known as n-chimaerin). This 38 kDa protein is highly expressed in the brain and resembles a 'chimera' between the regulatory domain of PKC and BCR, the

TABLE 2. *Novel nonkinase phorbol ester/DAG receptors*

Protein	Tissue distribution	Activity	Cellular functions described
α1-(n)-Chimaerin	Brain, others[a]	Rac-GAP	Alteration of adhesive properties in fibroblasts Induction of lamellipodia and filopodia (cooperation with Rac)
α2-Chimaerin	Brain, testis, others[b]	Rac-GAP	Unknown
β1-Chimaerin	Testis	Rac-GAP	Unknown
β2-Chimaerin	Cerebellum, others[c]	Rac-GAP	Unknown
C. elegans Unc-13	*C. elegans* nervous system	Unknown	Unknown
Munc13-1/2/3[d]	Brain	Unknown	Interaction with proteins of exocytotic machinary
Ras-GRP	Brain, thymus, spleen, bone marrow	Ras exchange factor	Transformation in fibroblasts

[a] α1-Chimaerin was detected in A549 lung carcinoma cells (L. B. Areces, M. G. Kazanietz, and P. M. Blumberg, unpublished data). [b] α2-Chimaerin was detected at low levels in non-neuronal cell lines, including HeLa cervical carcinoma and HepG2 hepatoma cells. [c] Using RT-PCR, we have observed that β2-chimaerin was expressed ubiquitously, although low levels of expression were found in most cases (161a). [d] Munc13 isoforms have not been analyzed for phorbol ester binding.

breakpoint cluster region protein involved in the translocation of Philadelphia chromosome in chronic myelogenous leukemia. Sequence alignment of α1-chimaerin with known phorbol ester receptors revealed ~40% homology at the cysteine-rich domain with PKC isozymes (16). The initial report using bacterially expressed α1-chimaerin showed a K_d of 30–50 nM for [^{3}H]PDBu in the presence of phosphatidylserine (162). The K_d for Sf9-expressed α1-chimaerin, however, is substantially lower (K_d=0.2 nM); it is within the same range of affinities for cPKCs and nPKCs under similar experimental conditions (163). As expected, deletion of the cysteine-rich domain of α1-chimaerin resulted in complete loss of phorbol ester binding (162), and mutation of cysteines in chimaerins (which were shown to be essential for phorbol ester binding in PKC isozymes) completely abolished [^{3}H]PDBu binding (M. J. Caloca and M. G. Kazanietz, unpublished results).

A comparison of phorbol ester binding properties of α1-chimaerin with PKCα shows that both receptors are virtually indistinguishable in ligand binding structure–activity and phospholipid requirement for phorbol ester binding. Like PKCs, acidic phospholipids reconstitute binding activity, with phosphatidylserine as the most efficient phospholipid. The PKC inhibitors calphostin C and sphingosine inhibit [^{3}H]PDBu binding to α1-chimaerin and PKCα with similar potencies, suggesting that PKC inhibitors acting on the cysteine-rich domain may not be suitable tools for selective PKC inhibition (163).

The chimaerin family has expanded with the cloning of novel isoforms: α2-, β1-, and β2-chimaerins. The different chimaerin isoforms come from alternative splicing of the α- and β-chimaerin genes (18, 164, 165). Using Sf9-expressed β2-chimaerin, Caloca et al. (14) have shown high-affinity [^{3}H]PDBu binding for this novel receptor. β2-Chimaerin is also a high-affinity receptor for the bryostatins, a unique class of PKC activators. Similar affinities for DAGs were observed for α1-chimaerin, β2-chimaerin, and PKCs (14, 163). The flexibility conferred by the rotatable bonds of the DAGs may allow a better 'accommodation' within the binding site of the different cysteine-rich domains and therefore allow a correspondingly positive interaction between the active residues of the pharmacophore and each cysteine-rich domain. An important observation is that thymeleatoxin, an analog of the second-stage tumor promoter mezerein, has a markedly lower affinity for β2-chimaerin than for PKCs, providing the first ligand capable of distinguishing between these two classes of phorbol ester receptors (14).

The biological roles for the chimaerins and their relation to DAG signaling are not yet defined. The chimaerins do not possess a kinase domain, but rather have a carboxyl-terminal GAP (GTPase-activating protein) domain. Both α- and β-chimaerins accelerate *in vitro* the hydrolysis of GTP from Rac, a member of the of the Rho family of small GTP binding proteins, with little or no effect on Cdc42 and Rho (164, 166, 167). Thus, chimaerins down-regulate Rac function.

Although the Rac-GAP activity of chimaerins *in vitro* has been well documented, the cellular effects of these phorbol ester receptors as regulators of Rac-mediated functions in cells remain almost unexplored. It may be possible that chimaerins inhibit Rac-mediated responses, such as cytoskeleton organization, activation of c-Jun amino-terminal kinase (JNK), regulation of cell growth and cell cycle, malignant transformation, and control of NADPH oxidase activity in neutrophils (168–171). The differential tissue expression of chimaerin isoforms suggests unique roles for each isozyme. The presence of a putative SH2 domain at the amino-terminal region of α2- and β2-chimaerin suggests potential interactions with phosphotyrosine proteins (18, 164). An attractive but still untested hypothesis is

that these phorbol ester receptors may integrate signals from GTP binding proteins, tyrosine kinase, and DAG generation.

An important question is whether phorbol esters and/or DAG can activate chimaerin Rac-GAP activity. Using *in vitro* GAP assays, Lim and co-workers have postulated an allosteric model for activation of α1-chimaerin by phorbol esters. However, the data reported by these authors show only a 10–15% activation by phorbol esters in the presence of phosphatidylserine (166). In another report with α2-chimaerin, the same authors did not detect any phorbol ester effect on GAP activity (164). α1-Chimaerin GAP activity can be regulated by phosphatidylserine and phosphatidic acid. In contrast to PKC, lysophosphatidic acid and fatty acids inhibit the catalytic activity of α1-chimaerin (164). The issue of a specific regulation of individual chimaerin isoforms by lipids and phorbol esters needs to be addressed.

A possible second model of regulation is that DAG and the phorbol esters target the chimaerins to a cellular compartment shared by Rac or other chimaerin targets. In support of this positional model is the observation that β2-chimaerin, like PKCs, is subjected to translocation by phorbol esters in cells (14). These data also support the finding that a single cysteine-rich domain is capable of supporting translocation, as determined with isolated cysteine-rich domains and mutated PKC isozymes (50, 51, 172). Translocation of β2-chimaerin requires higher concentrations of PMA and has slower kinetics compared to PKCα (14). A likely explanation for the difference between both types of receptors is that important structural motifs controlling translocation, such as phospholipid binding sites and/or sites of protein–protein interactions, are missing in chimaerin isoforms. β2-Chimaerin translocates to a perinuclear compartment after phorbol ester treatment (161a). A detailed analysis of the function of α- and β-chimaerins as mediators of phorbol ester actions is therefore of great importance and is currently under investigation.

Unc-13 and related proteins

The *Unc-13* gene from *C. elegans* encodes a large protein (1734 amino acids) that was identified as part of a group of mutants with defects in coordinated movement. Mutations in the *Unc-13* gene cause a severe phenotype characterized by its abnormal neuronal connections and impairment in cholinergic neurotransmission in the nematode. Molecular cloning of the *Unc-13* gene revealed a central domain with sequence similarity to the regulatory domain of PKC, which includes C1 and C2-related domains. The Unc-13 protein possesses a single cysteine-rich domain with a high degree of sequence homology to those in PKCs and chimaerins (19).

Although the initial characterization of Unc-13 protein showed phospholipid independence for phorbol ester binding (19), subsequent experiments demonstrated that Unc-13 binds phorbol esters and DAG with high affinity in a phospholipid-dependent manner (161, 173). Modest differences in binding affinities for phorbol ester analogs and phospholipid requirement for binding were observed between the recombinant cysteine-rich domains of Unc-13 and PKCδ expressed in *Escherichia coli*. As observed for α1-chimaerin, the PKC inhibitor calphostin C also inhibits [^{3}H]PDBu binding to Unc-13 (161).

Subsequent experiments identified three Unc-13 mammalian homologues: Munc13–1, Munc13–2, and Munc13–3 (174). Munc13s are also large proteins (195–225 kDa) and are highly expressed in the brain. The structural similarities between Unc-13 and Munc13 proteins predict a conservation of function in different organisms. Munc13 isoforms are enriched in synaptosomes and localized to the plasma membrane, raising the possibility that these proteins may have a role in neurotransmitter release (174, 175). To our knowledge, Munc-13 proteins have not been examined for phorbol ester/DAG binding, but structural similarities between cysteine-rich domains of Munc13 and other receptors predict that they are high-affinity receptors for phorbol esters.

The homology between Unc-13/Munc13 and PKCs not only extends to the C1 cysteine-rich domain, but also to the C2 domain. Two C2-related regions have been found in Unc-13 and Munc13 isoforms: one central, located immediately carboxyl-terminal to the cysteine-rich domain, and one at the carboxyl-terminal. A third, distantly related C2-like domain was also found in Munc13–1. These C2-like domains may be involved in phospholipid binding in a Ca^{2+}-independent manner (174).

The demonstration that Munc13 interacts with proteins of the exocytotic machinery, including Doc2, syntaxin, synaptobrevin, and synaptotagmin, suggests a potential role of these large proteins as scaffold-like structures (175, 176). It was proposed that Munc13s modulate or stabilize an intermediate form of the synaptic core complex (175). Whether lipids or phorbol esters regulate their function and/or localization in cells is not known.

RasGRP (Ras guanyl-releasing protein): a link between phorbol ester activation and the Ras pathway

This novel phorbol ester receptor has recently been isolated using a cloning approach to identify proteins that enhance Ras signaling (15). RasGRP is

highly expressed in brain (mainly hippocampus) and thymus, as well as in bone marrow and spleen. Sequence analysis shows a single cysteine-rich domain at the carboxyl-terminal region that resembles those in PKC isozymes. Phorbol ester binding studies confirmed that RasGRP is indeed a phorbol ester receptor (15).

RasGRP possesses a catalytic region, the CDC25 box, named for the prototypic Ras activator from *Saccharomyces cerevisiae*, and a Ras exchange motif (REM), which is conserved among the guanyl nucleotide-releasing factors interacting with members of the Ras family (177). The CDC25 box has ~50% similarity with the Ras activators SOS1 and RasGRF1. *In vitro*, RasGRP enhances the dissociation of the Ras-GDP complex and favors the association of Ras with GTP. In addition to the Ras activation domains, RasGRP possesses a structure resembling a pair of EF hands, which binds Ca^{2+}, and proline clusters, which could serve as binding sites for SH3 domains.

The phorbol ester binding site appears to be involved in the recruitment of RasGRP to the plasma membrane, where it can activate Ras. Indeed, PMA treatment results in translocation of RasGRP from soluble to particulate fractions. Rat2 fibroblasts expressing RasGRP assume a transformed morphology when exposed to phorbol esters. Recruitment of RasGRP to the plasma membrane by phorbol esters results in the activation of downstream effectors of Ras, i.e., MAPK. A mutated RasGRP lacking the cysteine-rich domain failed to translocate and activate MAPK after PMA treatment. Furthermore, it did not induce a malignant phenotype in fibroblasts. The DAG/phorbol ester binding site in RasGRP may therefore have a dominant role in RasGRP activation (15, 178). In light of these results, it would be essential to re-evaluate the participation of PKC in Ras signaling pathways.

CONCLUSION

Our initial view of the regulation of PKC activity by lipid cofactors and Ca^{2+} has expanded in recent years with the elucidation of novel regulatory mechanisms, namely, PKC phosphorylation and protein–protein interactions. Association to specific anchoring and scaffolding proteins may be a key mechanism for conferring substrate specificity to PKC isozymes. In addition, PKC binding proteins may regulate cross talk between PKC isozymes and other signaling molecules and serve as activators of inhibitors of PKC activity. Understanding the complexity of these protein–protein interactions will allow the elucidation of isozyme-specific signaling in different cellular systems and will help to design isozyme-specific pharmacological tools. Thus far, the interaction of PKC isozymes with each of their binding proteins has been studied separately. A challenge for the future will be to integrate and elucidate the dynamics of these tight connections.

The discovery of 'non-PKC' phorbol ester receptors adds an additional level of complexity. Our assumption that signaling by DAG proceeds through PKC to generate a biological response has been challenged by the discovery of chimaerins, Unc-13-related proteins, and Ras-GRP, novel high-affinity phorbol ester receptors lacking kinase activity. The use of phorbol esters as agents for assessing the involvement of PKC in cellular responses may lead in some cases to ambiguous conclusions. Selective pharmacological tools will be necessary to dissect the role of individual phorbol ester/DAG receptors in cellular functions. FJ

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