Membrane Targeting by C1 and C2 Domains*

Wonhwa Cho†

From the Department of Chemistry, University of Illinois, Chicago, Illinois 60607-7061

Many peripheral proteins involved in cell signaling translocate to different cell membranes in response to specific cell stimuli. Because cellular functions and regulation of these proteins depend on their specific subcellular localization (1), understanding the mechanisms of membrane targeting is of great importance. The membrane targeting of diverse peripheral proteins is mediated by a limited number of membrane-targeting domains, including protein kinase C (PKC) conserved 1 (C1), PKC conserved 2 (C2), and pleckstrin homology domains. Recent structural and functional studies of individual membrane targeting domains as well as the peripheral proteins harboring these domains have provided new insights into the molecular mechanisms underlying the specific subcellular targeting and activation of peripheral proteins. This review summarizes the recent progress in our understanding of the mechanisms of membrane targeting of diverse peripheral proteins is mediated by the C1 and C2 domains and the peripheral proteins containing these domains and their subcellular targeting behaviors. There are several excellent reviews (2–6) that contain more exhaustive surveys on the membrane targeting domains.

Membrane-Protein Interactions

The membrane binding of peripheral proteins involves different types of interactions (Fig. 1) that depend upon the physicochemical properties of both membrane and protein. Membranes of different cellular compartments have different compositions of bulk lipids that can modulate membrane targeting of proteins either by providing unique microenvironments or by producing specific lipid metabolites, such as diacylglycerol (DAG) and phosphoinositides, that function as second messengers. Extensive structural and mutational studies of phospholipases A2 (PLA2) have shown that their membrane binding surfaces are composed of cationic, aliphatic, and aromatic residues (7). A recent study by surface plasmon resonance analysis indicated that cationic residues primarily accelerate the association of protein to anionic membrane surfaces, whereas aliphatic residues mainly slow the membrane dissociation by penetrating into the hydrophobic core of the membrane (8). Aromatic residues, particularly Trp, which has a preference for the water-lipid interface (9), play a pivotal role in binding to zwitterionic PC membranes (7, 10) by affecting both membrane association and dissociation steps (8). A priori, the physicochemical principles learned from these in vitro membrane binding studies should allow the prediction of the subcellular targeting behaviors of peripheral proteins, provided that the subcellular targeting is driven mainly by membrane-protein interactions.

Structure, Function, and Occurrence of C1 Domains

The C1 domain (~50 amino acids) is a cysteine-rich compact structure that contains five short β strands, a short α-helix, and two zinc ions (Fig. 2) (11, 12). The C1 domain was first identified as the interaction site for DAG and phorbol ester in PKCs (13). In conventional (α, β₁, β₂, and γ) and novel (δ, ε, θ, and η) PKCs, the C1 domain occurs in a tandem repeat (C1A and C1B). C1 domains have been subsequently found in other proteins with diverse functions, including protein kinase D (PKD/PKCμ), chimaerin, Ras-GRP, Unc-13, Munc13 isoforms, DAG kinases, and Raf (4). In general, C1 domains show a high degree of amino acid sequence homology. Some C1 domains, including those found in atypical PKCs (ζ and ι/λ), however, do not bind lipids due to minor sequence variations and might be involved in protein-protein interactions (4). This review will focus mainly on the C1 domains involved in DAG and phorbol ester binding.

Membrane Binding of C1 Domains

Structural (12) and mutation (14) studies of PKCζ-C1B have defined the phorbol ester/DAG binding pocket. The polar binding pocket is located at the tip of the molecule and is surrounded by aliphatic and aromatic residues, which are adjoined by a ring of cationic residues in the middle part of the molecule (Fig. 2). A NMR study of PKCγ-C1B (15) and a monolayer penetration study of PKCa (16) showed that the hydrophobic residues of the C1 domain penetrate the membrane for DAG/phorbol ester binding. The phorbol ester binding seals the polar surface in the binding pocket and thereby generates a contiguous hydrophobic surface (12), which in turn greatly enhances the stability of the C1-membrane complex (17, 18). Further mutational studies of PKCs showed that clustered cationic residues in the C1A domain are involved in non-specific electrostatic interactions with anionic phospholipids (19). In agreement with this finding, the isolated C1 domain repeat (i.e. C1A + C1B) of PKCa exhibits little head group specificity among anionic phospholipids while discriminating against PC (16). Together these studies on PKC C1 domains have led to a model for C1 domain-membrane interactions illustrated in Fig. 2. In this model, cationic residues accelerate the initial adsorption of the C1 domain to the anionic membrane surfaces and properly position the C1 domain at the membrane surface. Then, the hydrophobic tip of the domain penetrates the membrane to bind DAG that is partially buried in the membrane because of its hydrophobic nature. Because C1 domains with the exposed hydrophobic patch are subject to protein aggregation in solution, a tightly controlled triggering mechanism would be necessary for the C1 domain-mediated membrane targeting. In the case of conventional PKCs, it has been shown that C1 domains are buried at the resting state and become accessible to DAG or phorbol esters only after Ca²⁺-dependent membrane binding (19, 20). The C1 domains of PKD/PKCμ (21, 22), Ras-GRP (23), and β₂-chimaerin (24) have also been shown to drive the cellular membrane targeting of the corresponding peripheral proteins in response to DAG and phorbol esters. Because no detailed analysis of their interactions with membranes has been reported, it is not known whether the mechanisms of C1 domain-mediated membrane binding of these peripheral proteins are similar to that of PKCs.

Differential Roles of Multiple C1 Domains in PKC

Conventional and novel PKCs contain two copies of C1 domains. Earlier studies on conventional PKC reported the one-to-one stoichiometry of PKC-DAG (25) and PKC-phorbol ester binding (26, 27), suggesting that only one C1 domain is directly involved in DAG/phorbol ester binding and PKC activation. A recent study of isolated C1A and C1B domains of PKCs revealed that C1B domains have much higher affinities for phorbol 12,13-dibutyrate than C1A.
domains (28). The only exception was PKCy, the C1A and C1B domains of which show comparably high affinities. For PKCα, good correlation was observed between the intrinsic phospholipid ester affinities of C1A and C1B domains and their relative importance in phospholipid ester-induced activation (29), supporting the notion that the disparate roles of C1 domains mainly derive from their different intrinsic affinities for phospholipid esters. More recently, however, it was shown that C1A and C1B domains of PKCα play an equivalent role in cellular membrane translocation in response to phorbol 12-myristate 13-acetate (PMA) despite their distinct phospholipid ester affinities (30). To date, correlation between the intrinsic DAG affinities of C1 domains and their relative importance in DAG-dependent PKC activation has not been documented. A series of spectrosopic vesicle binding studies of PKCα indicated that PKCα contains two phospholipid ester binding sites with high and low affinities and that DAG and phospholipid esters bind to the two sites with opposite affinity (31, 32). The notion that DAG and phospholipid esters might have different C1A versus C1B selectivity was further supported by the finding that the C1A domain is exclusively involved in the DAG-induced vesicle binding and activation of PKCα (16). Clearly, more studies are needed to fully understand these complex interactions of the C1 domains of PKC with their ligands.

Subcellular Targeting by C1 Domains

The subcellular targeting of isolated C1 domains and PKC holoenzymes in response to DAG and phospholipid esters has been measured in various mammalian cells transfected with green fluorescent protein (GFP)-tagged proteins (20, 33–39). PKCy translocated to plasma membrane in response to PMA in COS-7 cells (33). In RBL cells, PKCy and its C1A, C1B, and C1A-C1B domains all showed the translocation to plasma membrane in response to PMA or DAG (35). As is the case with PKCy, PKCβ1 (40, 41), PKCδ (34), PKCε (39), and PKCδ-C1A-C1B (38) were shown to translocate to plasma membrane in response to PMA or DAG. In the case of PKC holoenzymes, Ca2+-enhanced the rate of translocation of proteins but did not affect their localization. Later studies showed that whereas PMA and other hydrophobic phospholipid esters induced the initial translocation of PKCα to the plasma membrane, less hydrophobic DAG and PDB caused PKCα to translocate to the perinuclear region (36, 37). Our cell study2 of GFP-PKCα with a fluorescent phospholipid ester analog, sapintoxin D, supported the notion that the differential subcellular localization of C1 ligands is a main determinant of the differential subcellular targeting of PKCs. This in turn suggests that the in vitro membrane binding and the subcellular targeting of C1 domains are driven by the same forces. Binding to neither adapter proteins nor cytoskeleton appears to contribute significantly to the subcellular targeting of PKC-C1 domains because the putative adapter binding site is not located in the C1 domains (42) and the cytoskeleton inhibitors show little effect on C1 domain translocation (35, 39).

Structure, Function, and Occurrence of C2 Domains

The C2 domain (~130 residues) was first discovered as the Ca2+-binding domain in conventional PKCs (15). A great number of proteins containing the C2 domain have been identified since, and most of them are involved in signal transduction (e.g., PKC, cytosolic PLA2 (cPLA2), phospholipases C, plant phospholipase D, and phosphatidylinositol 3-kinase) or membrane trafficking (e.g., syntaptogamins, rabphilin-3A, and Unc-13) (2, 3). Structural analyses of multiple C2 domains have indicated that all C2 domains share a common fold of eight-stranded antiparallel β-sandwich connected by variable loops, with the Ca2+-binding sites located at one side of the domain (Fig. 2) (43–47). When compared with C1 domains, C2 domains show a larger degree of variation in amino acid sequence, particularly in the loop regions (2, 3). Consistent with this finding, C2 domains show greater functional diversities. Most Ca2+-dependent membrane-binding C2 domains prefer anionic membranes to zwitterionic ones; however, cPLA2-C2 strongly favors PC membranes (48). Among anionic lipid-selective C2 domains, PKCo-C2 (16) and PLCδ1-C2 (49) exhibit PS selectivity. Furthermore, there are many non-Ca2+-binding C2 domains; some of them, such as PTEN-C2 (50), still bind the membrane, and others might be involved in protein-protein interactions (2, 3). This review will mainly deal with the C2 domains that bind phospholipids in a Ca2+-dependent manner.

Ca2+-dependent Membrane Binding of C2 Domains

The Ca2+-binding sites of C2 domains are composed of three variable loops that contain ligands for multiple Ca2+ ions. Structural (43–47) and binding analyses (51–53) have determined the calcium binding stoichiometry, geometry, and affinity for several C2 domains. Two major roles of Ca2+ ions in the membrane targeting of the C2 domain have been experimentally demonstrated. The first role of Ca2+ ions is to provide a bridge between the C2 domain and anionic phospholipids. This Ca2+-bridge model is supported by an x-ray structure of the PKCα-C2 (C2α)–PS complex (46), showing that a short-chain PS molecule is specifically coordinated to a Ca2+-ion and other residues in the Ca2+-binding loops. This structure also accounts for the PS selectivity of PKCα-C2 (16). The second role of Ca2+ ions is to induce intra- or interdomain conformational changes, which in turn trigger membrane-protein interactions. Despite earlier negative reports (43), accumulating evidence has supported the occurrence of Ca2+-induced conformational changes in the C2 domain (44, 52–55). Recent structure-function studies of the C2 domains of PKCα (18) and cPLA2α (56), both of which bind two Ca2+-ions, showed that two Ca2+-ions play distinct roles, with one primarily involved in inducing the conformational changes and the other in Ca2+-bridging. The main difference is that Ca2+-induced conformational changes are critical for the membrane binding of cPLA2-C2, whereas Ca2+-bridging is a relatively more important role of PKCα-C2.

Non-Ca2+-coordinating protein residues in the Ca2+-binding loops also play important roles in the membrane binding and phospholipid selectivity of the C2 domain. A large degree of structural variations have been found in the Ca2+-binding loops of C2 domains in terms of both primary and tertiary structures (43–47). Mutational (56, 57) and labeling (58, 59) studies have identified the residues in the Ca2+-binding loops that play a key role in membrane binding. In general, cationic residues on the surface of Ca2+-binding loops are important for anionic lipid-selective C2 domains (57, 60), whereas aliphatic and aromatic residues are essential for PC-selective cPLA2-C2 (56, 58, 59). A predominant cationic cluster in the β-sandwich region has been implicated in inositol polyphosphate binding of syntaptogamin II (61) but does not significantly affect the membrane binding of conventional PKCs (62) and cPLA2α (56). Together, these in vitro studies have indicated that anionic lipid-selective C2 domains and cPLA2α-C2 have distinct membrane binding modes. As shown in Fig. 2, PKCo-C2 binds to the membrane in an orientation that optimizes its electrostatic interactions with the anionic membrane (46, 57, 60), whereas cPLA2α-C2 binds to the membrane in an orientation that optimizes the membrane penetration of its hydrophobic residues (56, 58, 59, 63). Interestingly, membrane binding properties of cPLA2 and its C2 domain are similar (56, 64), whereas those of PKCo and its C2 domain have noticeable differences (16, 18). This discrepancy implies that the relative contribution of a C2 domain to the membrane binding of a peripheral protein depends on the structural context of the protein, particularly on the presence of other membrane targeting domains in the same molecule.
exhibited good correlation with their altered membrane binding. These C2 domains is primarily driven by forces that govern their binding kinetics. Thus, it appears that the subcellular targeting of Ca\(^{2+}\)/H9251 (20), PKC\(\gamma\) (20) and PKC\(\alpha\) (41). The regulatory interactions between C1 and C2 domains have also been suggested for the targeting and regulation of different PKC isoforms. In the case of PKC\(\alpha\), a single anionic residue in the C1A domain is implicated in the tethering of C1A to the other part of the molecule, most likely the C2 domain (19). The putative tethering keeps the protein in an inactive conformation at the resting state and is disrupted specifically by the Ca\(^{2+}\)-dependent binding of the C2 domain to PS in the membrane, which in turn leads to the membrane penetration and DAG binding of the C1A domain and PKC activation. Similarly, it was suggested that the C2-like domain of novel PKC interacts with the C1 domain to regulate the enzyme activity (72). Further studies will reveal more examples of interdomain interactions in the membrane targeting of peripheral domains.

### Interactions of C1 and C2 Domains

The majority of peripheral proteins have two (or more) membrane targeting domains although the presence of extra domains is not absolutely required for their membrane translocation. This suggests that their membrane targeting and activation might involve a synergistic and/or regulatory interplay of the membrane targeting domains. A synergistic action of C1 and C2 domains in the prolonged membrane localization of PKC was demonstrated for PKC\(\gamma\) (20) and PKC\(\alpha\) (41). The regulatory interactions between C1 and C2 domains have also been suggested for the targeting and regulation of different PKC isoforms. In the case of PKC\(\alpha\), a single anionic residue in the C1A domain is implicated in the tethering of C1A to the other part of the molecule, most likely the C2 domain (19). The putative tethering keeps the protein in an inactive conformation at the resting state and is disrupted specifically by the Ca\(^{2+}\)-dependent binding of the C2 domain to PS in the membrane, which in turn leads to the membrane penetration and DAG binding of the C1A domain and PKC activation. Similarly, it was suggested that the C2-like domain of novel PKC interacts with the C1 domain to regulate the enzyme activity (72). Further studies will reveal more examples of interdomain interactions in the membrane targeting of peripheral domains.

### Roles of Bulk Lipids and Lipid Second Messengers

In both protein-protein and membrane-protein interactions, the initial formation of nonspecific collisional complexes, driven by diffusion and electrostatic forces, is followed by the formation of tightly bound complexes, which are stabilized by specific interactions (8, 73). The former interactions mainly enhance the association rate whereas the latter interactions primarily decrease the dissociation rate. For C1 domains, the initial binding is driven by nonspecific electrostatic interactions between cationic residues with bulk anionic lipids, but the tight complex formation is achieved by both hydrophobic interactions between hydrophobic C1 residues and the membrane core and hydrogen bonds between polar C1 residues and DAG (Fig. 2). Accordingly, the subcellular localization of the C1 domain is determined primarily by the location of DAG and phorbol ester, although the bulk lipid composition of the targeting membrane can affect the kinetics of translocation (20). For anionic lipid-selective C2 domains, the initial binding is driven by electrostatic interactions via C2-bound Ca\(^{2+}\), cationic residues, or a combination of both. However, the paucity of multiple specific interactions required for tight complex formation renders the binding relatively weak and transient (20). On the other hand, the membrane binding of PC-selective cPLA\(_2\)-C2 is slowly driven by interactions between aromatic residues and PC molecules (8), but the binding is tight due to membrane penetration and the resulting hydrophobic interactions. In accordance with these membrane

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3 R. Stahelin, J. Rafter, and W. Cho, manuscript in preparation.
4 S. Kulkarni and W. Cho, manuscript in preparation.
binding properties, cPLA₂-C2 alone can achieve the membrane localization of cPLA₂ in the cell, whereas both C1 and C2 domains are required for the prolonged membrane localization of conventional PKC (20, 41). Unlike the case of the C1 domain, the bulk lipid composition of the membrane is an important determinant of Ca²⁺-dependent localization of the C2 domains. For the reliable prediction of subcellular localization of C2 domains based on their lipid selectivity, the lipid compositions of various cellular membranes of different cells need to be fully characterized.

Conclusions and Future Direction

Results summarized in this review show that much of the spatiotemporal dynamics of the peripheral proteins harboring membrane-binding C1 and C2 domains can be accounted for by the physicochemical properties that govern their in vitro membrane binding properties. The subcellular targeting of peripheral proteins containing a single targeting domain (e.g. cPLA₂) usually reflects the membrane binding properties of the domain, whereas that of peripheral proteins with multiple C1 and C2 domains is determined by the delicate interplay of the domains and the synergistic actions of their agonists (e.g. Ca²⁺ and DAG). The principles learned from these studies should help in understanding the subcellular targeting of peripheral proteins containing other membrane targeting domains. Because C1 and C2 domains can also interact with other proteins (42), the subcellular targeting of some C1- and C2-containing proteins might involve both membrane-protein and protein-protein interactions. Moreover, protein phosphorylation might play an important regulatory role in the subcellular targeting of peripheral proteins (40, 41). Further systematic studies are required to comprehensively address these important issues. Finally, cellular translocation studies of peripheral proteins have been focused mainly on membrane translocation so far. To fully understand spatiotemporal dynamics and regulation of signaling peripheral proteins, however, it is necessary to simultaneously monitor the spatiotemporal dynamics and activities of peripheral proteins as well as the spatiotemporal dynamics of their stimuli in living cells. Recent technological advances in light microscopy and cell biology should greatly facilitate this advance today.

Acknowledgments—I thank M. Medkova, L. Bittova, R. Stahelin, J. Kafer, S. Das, M. Sumandea, S. Kulkarni, M. Digman, and B. Ananayaranan for their contributions. I apologize to the many authors whose work could not be cited directly because of page limitation.

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J. Biol. Chem. 2001, 276:32407-32410.
doi: 10.1074/jbc.R100007200 originally published online June 29, 2001

Access the most updated version of this article at doi: 10.1074/jbc.R100007200

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