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MEMBRANE-PROTEIN INTERACTIONS IN CELL SIGNALING AND MEMBRANE TRAFFICKING

Wonhwa Cho and Robert V. Stahelin
Department of Chemistry, University of Illinois at Chicago, Chicago, Illinois 60607-7061; email: wcho@uic.edu, rstahe1@uic.edu

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■ **Abstract** Research in the past decade has revealed that many cytosolic proteins are recruited to different cellular membranes to form protein-protein and lipid-protein interactions during cell signaling and membrane trafficking. Membrane recruitment of these peripheral proteins is mediated by a growing number of modular membrane-targeting domains, including C1, C2, PH, FYVE, PX, ENTH, ANTH, BAR, FERM, and tubby domains, that recognize specific lipid molecules in the membranes. Structural studies of these membrane-targeting domains demonstrate how they specifically recognize their cognate lipid ligands. However, the mechanisms by which these domains and their host proteins are recruited to and interact with various cell membranes are only beginning to unravel with recent computational studies, in vitro membrane binding studies using model membranes, and cellular translocation studies using fluorescent protein-tagged proteins. This review summarizes the recent progress in our understanding of how the kinetics and energetics of membrane-protein interactions are regulated during the cellular membrane targeting and activation of peripheral proteins.

CONTENTS

INTRODUCTION

Many cellular processes including cell signaling and membrane trafficking involve complex arrays of protein-protein and lipid-protein interactions. Research in the past decade has revealed that a large number of cellular proteins reversibly translocate to specific subcellular locations to form these macromolecular interactions (170). In particular, it has been recognized that many cytoplasmic proteins are recruited to different cellular membranes during cell signaling and membrane trafficking. These proteins are collectively known as peripheral proteins (as opposed to integral membrane proteins) or amphitrophic proteins. Peripheral proteins use different strategies for reversible membrane interactions. A large number of peripheral proteins contain one or more modular domains specialized in lipid binding. These lipid-binding structural modules, also known as membrane-targeting domains, include protein kinase C (PKC) conserved 1 (C1) (30, 189); PKC conserved 2 (C2) (30, 114, 138); pleckstrin homology (PH) (53, 92); Fab1, YOTB, Vac1, and EEA1 (FYVE) (163); Phox (PX) (187); epsin amino-terminal homology (ENTH) (39); AP180 amino-terminal homology (ANTH) (39); Bin amphiphysin Rvs (BAR) (64); band 4.1, ezrin, radixin, moesin (FERM) (21); and tubby domains (24). Also, there are peripheral proteins that do not have separate membrane-targeting domains but utilize a part of their molecular surface [e.g., secretory phospholipase A_2 (PLA_2)] or an amphipathic secondary structure [e.g., ADP-ribosylation factor (ARF)] to interact with the membrane. Another class of peripheral proteins (e.g., Src and Ras proteins) has covalently attached lipid anchors that embed in the lipid bilayer. This review summarizes the recent progress in our understanding of the mechanisms by which reversible binding of the nonlipidated peripheral proteins to different cell membranes is mediated and regulated, with an emphasis on how kinetics and energetics of their membrane-protein interactions are modulated during their membrane targeting and activation. The review describes recent biophysical studies for membrane-targeting domains and their host proteins using model membranes that have revealed how membrane-protein interactions are regulated by different factors, such as Ca^{2+}, phosphoinositides [phosphorylated derivatives of phosphatidylinositol (PI)] or protein phosphorylation, and cellular translocation studies with fluorescence protein-tagged domains that provide new insight into the correlation between the in vitro and cellular membrane targeting. Particular attention is given to the importance of partial membrane penetration of peripheral proteins in their membrane binding and biological activities, since this mode of

interaction is common across most peripheral proteins. For more exhaustive surveys on the membrane-targeting domains and lipidated peripheral proteins, readers are referred to several recent review articles (39, 41, 53, 136, 163, 187).

ENERGETICS AND KINETICS OF MEMBRANE-PROTEIN INTERACTIONS

Structure of Lipid Bilayers and Interfacial Preference of Amino Acids

Liquid-crystallographic determination of the structure of a 1,2-dioleoyl-*sn*-glycero-3-phosphocholine bilayer showed that the lipid bilayer has a highly polarized structure that consists of a central hydrocarbon core region and two flanking interfacial regions (180). The hydrocarbon region and the combined interfacial regions have comparable width (~30 Å each), and the interfaces thus account for roughly 50% of the total thickness of the bilayer (Figure 1, see color insert). The interfaces are heterogeneous in terms of chemical composition and polarity. The interfacial regions consist of a complex mixture of water, lipid backbone phosphate groups, headgroups, and the polar portion of the acyl chains, and the polarity profile changes dramatically over the 15 Å span of an interface, from the hydrocarbon region to the aqueous solution. Owing to this complex nature of the lipid bilayer, the location of the protein in the bilayer is a critical factor that governs the kinetics and energetics of its membrane interactions. In particular, the polarity profile in the interfacial region allows proteins with different characteristics to experience a range of membrane interactions. On the basis of their membrane location, peripheral proteins can be arbitrarily categorized into three classes (see Figure 1): (*a*) S-type proteins that are localized at the membrane surface and in the shallow interfacial region (i.e., outside of the level of the backbone phosphate group) and interact predominantly with the polar headgroups, (*b*) I-type proteins that penetrate significantly into the interfacial region (i.e., inside the level of the phosphate), and (*c*) H-type proteins that penetrate into the hydrocarbon core region of the lipid bilayer. Both I- and H-type peripheral proteins interact with both the polar headgroups and the hydrocarbon of the bilayer.

The propensity of 20 amino acids for the hydrocarbon core (hydrophobicity scale) and the interfacial region (interfacial hydrophobicity scale) has been both experimentally determined and computationally derived to understand the membrane localization and folding of surface-active peptides and integral membrane proteins (182). Consistent with their hydrophobic nature, aliphatic side chains score high in various hydrophobicity scales. Unexpectedly, aromatic residues Trp and Tyr have the highest affinity for the interfacial region despite their hydrophobic nature. Although the origin of the interfacial preference of these aromatic residues is not fully understood, this property has a profound implication in the membrane targeting of peripheral proteins. In particular, the aromatic residues play a critical role in the binding of peripheral proteins to membranes composed mainly of

zwitterionic phospholipids [i.e., phosphatidylcholine (PC) vesicles or the outer plasma membrane of mammalian cells] (67) because electrostatic attraction, which normally brings cationic proteins to anionic membrane surfaces, does not significantly contribute to this type of binding.

Energetics of Membrane-Protein Interactions

In protein-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 (34, 117). Recent studies using green fluorescent proteins (GFP) and GFP-fusion proteins showed that these proteins can freely diffuse in the cytosol despite molecular crowding (170). Because all intracellular membranes contain a varying degree of anionic lipids and most peripheral proteins contain cationic surfaces, at least locally, the membrane-protein binding, whether in vitro or in vivo, should be also initiated by diffusion and nonspecific electrostatic forces. In fact, both theoretical and experimental work has shown that although nonspecific electrostatic interactions may not be sufficient to anchor peripheral proteins at membrane surfaces, they are necessary for the membrane targeting and biological function of these proteins (111). The initial membrane adsorption of peripheral proteins facilitates specific interactions with lipids (and/or membrane proteins) by effectively reducing the dimensionality of the space through which the protein interacts with its lipid ligand (102). That is, the enhanced effective concentration of the protein at the membrane increases the probability that the protein is able to interact with both effectors and substrates. The initial membrane attachment can also facilitate the penetration of hydrophobic and aromatic residues on the surfaces of peripheral proteins (mainly H- and I-types) into the interfacial and hydrocarbon core regions of the lipid bilayer. These two processes are not mutually exclusive, as specific lipid binding (facilitated by nonspecific membrane recruitment) of some phosphoinositide-binding domains, such as FYVE (160), PX (156), and ENTH domains (161), induces their membrane penetration. Resulting specific interactions and/or hydrophobic interactions provide the proteins with extra binding energy that is necessary for their membrane recruitment and activity.

Partial Membrane Penetration and Hydrophobic Interactions

Many secreted proteins and integral membrane proteins containing signal sequences are transported across membranes before they reach their final destination. In fact, the terms membrane translocation and protein targeting have been primarily used to describe these processes. (To circumvent confusion, these terms are avoided in this article. Membrane targeting used herein refers to reversible membrane recruitment.) Also, some transmembrane pore-forming peptides, such as gramicidin (36, 95) and alamethicin (25), fully insert themselves into the lipid bilayer. On the other hand, there are many surface-active peptides, including antibacterial magainin (96) and dermaseptin (110), that partially penetrate into the interfacial

and hydrocarbon core regions and thereby interact with both the polar headgroups and the hydrocarbon region of the bilayer. Many membrane-targeting domains and peripheral proteins also partly penetrate the lipid bilayer in the course of their membrane interaction (54, 156, 160, 161). Unlike peptides whose membrane insertion relies upon the induced amphiphilic secondary structures, the membrane penetration of these proteins may utilize more diverse local structures, including surface loops. Because hydrophobic side chains of proteins are not normally exposed to the molecular surface, membrane penetration of membrane-targeting domains and peripheral proteins often involves the conformational change of proteins at the membrane interface that exposes the buried hydrophobic side chains. As in the case of amphiphilic antibiotic peptides, biological activities of some peripheral proteins depend heavily on their partial membrane insertion (54, 156, 160, 161).

Membrane penetration of proteins has been measured by several semiquantitative and quantitative methods. Lipid monolayers have been used for measuring the ability of peripheral proteins to penetrate into the membrane (31, 174). The penetration is measured by monitoring either changes in surface tension of water (in terms of surface pressure) at constant surface area or changes in surface area at constant surface pressure. The former measurement allows the determination of the maximal surface pressure (i.e., lipid packing density) of the monolayer into which a protein can penetrate (31, 174). In conjunction with the estimated surface pressure of cell membranes, this value can be used to predict whether a peripheral protein can penetrate cell membranes (17, 40, 143). The latter method provides information about the surface area occupied by the membrane-incorporated protein. The monolayer technique has been successfully applied to many membrane-targeting domains and peripheral proteins, including phospholipases (10, 18, 94, 134) and PKCs (9, 11, 104–106, 153). However, the method provides relatively qualitative information about the membrane insertion, whereas quantitative data as to the depth of membrane penetration by the protein can only be derived indirectly in some cases. Three independent methods have been developed for more detailed depth measurements. The first method, termed Parallax analysis (1, 2, 6), is based on the quenching of an intrinsic fluorescent probe, Trp, by quenchers, such as dibromo and nitroxide groups, attached to either the polar headgroup or a specific acyl carbon of a phospholipid. The second method utilizes the quenching of the electron paramagnetic resonance (EPR) signal of a spin probe site specifically incorporated into the protein by a second paramagnetic species (70). This technique has also been applied to the H-/I-type C2 domains of group IVA PLA_2 ($cPLA_2\alpha$) (57), PKCα (86), and synaptotagmin (56) as well as the MARCKS protein (131). The H-type $cPLA_2\alpha$ C2 domain penetrates deeply into the interfacial and hydrocarbon core regions [i.e., 10 Å below (or above) the lipid phosphate], whereas the I-type C2 domains of synaptotagmin and PKCα exhibited a lesser degree of interfacial penetration (i.e., $\approx$5 Å below the phosphate). Finally, to circumvent the potentially perturbing effects of a fluorescent or spin probe, an X-ray reflectivity analysis of an unmodified peripheral protein interacting with a lipid monolayer has been recently developed (98). This method allows determination of the

penetration depth of the protein into the lipid monolayer and the angular orientation of the bound protein. The analysis shows that the C2 domain of cPLA$_2\alpha$ penetrates 11 Å into the monolayer with respect to the phospholipid headgroups.

Kinetics of Membrane-Protein Interactions

For protein-protein interactions that follow a two-step mechanism (see above), attractive electrostatic forces enhance association rate constant (k_a) and thereby promote the formation of an ensemble of weak, relatively nonspecific collisional complexes, whereas specific interactions, including electrostatic and hydrophobic interactions and hydrogen bonds, which stabilize tightly bound complexes, primarily lower the dissociation rate constant (k_d) (34, 179). Recent kinetic studies for a large number of peripheral proteins and their mutants by the fluorescence-based stopped-flow analysis (116), surface plasmon resonance (SPR) analysis (4, 5, 37, 59, 107, 125, 156–162), and fluorescence correlation spectroscopy (101) have supported a similar two-step mechanism for membrane-protein interactions, in which 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 and/or membrane penetration (Figure 2, see color insert). In general, protein residues (e.g., cationic membrane-binding residues) and other factors (e.g., Ca^{2+} or phosphorylation) that enhance the nonspecific, long-range electrostatic interactions primarily accelerate the association of proteins to anionic membranes (i.e., k_a effect), whereas protein residues and other factors that increase short-range specific interactions and/or membrane penetration mainly slow the membrane dissociation (i.e., k_d effect) (157). Aromatic residues, particularly Trp, play a unique and crucial role in binding to zwitterionic PC membranes (60, 67) by affecting both membrane association and dissociation steps (157).

IN VITRO VERSUS CELLULAR MEMBRANE-PROTEIN INTERACTIONS

Chemical Composition of Cell Membranes and Monitoring Lipid Dynamics

Membranes of cellular compartments have different lipid compositions, which are tightly regulated by local lipid metabolism and regulated lipid transport (154). Furthermore, plasma membrane, endosomes, and *trans*-Golgi network membranes maintain transbilayer asymmetry by actively transporting particular types of lipids from one opposing leaflet to the other (154). Cell membranes consist of two types of lipids. Bulk lipids, including PC, phosphatidylethanolamine (PE), phosphatidylserine (PS), PI, sphingomyelin, and cholesterol, mainly serve structural roles and their concentrations are relatively constant in each membrane. On the other hand, low-abundance lipids, such as diacylglycerol (DAG), phosphatidic acid

(PA), ceramide, or phosphoinositides, function as signaling molecules that recruit peripheral proteins in a spatiotemporally specific manner. The local concentration of these signaling lipids, which determines their activities, can fluctuate to a large degree in response to various cellular stimuli.

Because accumulating evidence has indicated that not only signaling lipids but also bulk lipids play important roles in the subcellular localization of peripheral proteins (see below), quantitative determination of the average lipid composition (both headgroup and acyl group composition) of cell membranes as well as spatiotemporal dynamics of signaling lipids is prerequisite for understanding the membrane targeting and activation of peripheral proteins under physiological conditions. Currently, however, only incomplete information is available for the lipid composition of cell membranes and spatiotemporal dynamics of signaling lipids (103). It is expected that recent mass spectrometry–based lipidomics studies (68) will yield more accurate and comprehensive information about the lipid composition of different membranes of various cells under different physiological conditions and the temporal dynamics of signaling lipids. Independently, the spatiotemporal dynamics of signaling lipids, including DAG and phosphoinositides, has been monitored in living cells using fluorescent protein-fused membrane-targeting domains as sensors (7, 73). With some technical improvement in sensitivity and quantification, this promising approach should provide valuable information about the spatiotemporal dynamics of signaling lipids and their effector proteins.

Correlation Between In Vitro and Cellular Membrane Binding

It was generally thought that specific subcellular localization of peripheral proteins would require protein-protein interactions, because lipid-protein interactions cannot confer exquisite specificity. Recent studies have indicated, however, that high specificity and affinity can be achieved through lipid-protein interactions. This in turn suggests that subcellular localization behaviors of peripheral proteins can be accounted for and, better yet, predicted by analyzing the kinetics and energetics of their binding to model membranes, provided that model membranes properly represent the cell membranes to which they are targeted. Although earlier studies on model peptides representing lipidated peripheral proteins supported this notion, direct evidence for the correlation between in vitro and cellular membrane binding of nonlipidated peripheral proteins was obtained only recently with the advance of analytical methodologies, i.e., SPR analysis (31, 113, 141) for in vitro kinetic and thermodynamic studies, fluorescence technology (119) for direct visualization, and, in some cases, quantitative analysis of membrane recruitment of peripheral proteins in living cells. In general, structural modification of proteins, including the deletion of a functional membrane-targeting domain, that greatly reduces their affinity for the model membranes also caused the cytosolic distribution of the proteins in the cell, supporting a correlation between the in vitro

and cellular membrane binding of peripheral proteins. One of the most convincing pieces of evidence for such a correlation has been provided by a series of recent studies on the C2 domains of 5-lipoxygenase (88), PLCδ (4), cPLA$_2\alpha$ and PKCα (162). Systematic comparison of in vitro affinity of these domains for vesicles of various compositions determined by SPR analysis and their subcellular translocation in HEK293 cells demonstrates that the subcellular localization of these C2 domains is determined primarily by their phospholipid headgroup selectivity and can be tailored by altering their phospholipid headgroup selectivity through point mutations (4, 88, 162). Furthermore, when k_a, k_d, and K_d (or K_a) for the in vitro vesicle binding of these C2 domains and their host proteins were compared with their cellular membrane translocation rates and cellular activities, excellent quantitative correlation was observed (R.V. Stahelin & W. Cho, unpublished results). Interestingly, results suggested that the k_d for a given C2 domain of its host protein should be smaller than a threshold value (approximately 0.01 s^{-1}) for the domain to show detectable subcellular translocation and cellular activities. In the case of FYVE domains, good quantitative correlation was observed between the K_d for in vitro binding and subcellular localization to early endosomes (14). Also, a threshold K_d necessary for the endosomal localization was found to be in the range of 0.1 to 0.2 μM. The existence of these narrow threshold values suggests that the membrane recruitment of peripheral proteins can be readily turned on and off by slight changes in kinetic and equilibrium parameters for membrane binding, which can be induced by many different factors, including a conformational change of protein and a change in the local concentration of a specific lipid.

TRIGGERING MECHANISMS FOR MEMBRANE TARGETING

In general, peripheral proteins that recognize specific (signaling) lipids have relatively high basal membrane affinity and thus respond spontaneously to the appearance of their cognate ligands. Conversely, the proteins that interact mainly with bulk lipids have low basal membrane affinity in the resting state, and their membrane binding must be triggered by specific signals, such as Ca^{2+} and protein (de)phosphorylation, that cause them to undergo either electrostatic switch or conformational changes. Even for the proteins that bind signaling lipids, their initial membrane interactions may induce conformational and/or electrostatic changes that lead to membrane insertion of hydrophobic and aromatic residues.

Electrostatic Switch Mechanism

Stimulus-induced nonspecific electrostatic interactions, or the electrostatic switch, provide a driving force for many peripheral proteins. The first example of the electrostatic switch in peripheral proteins was reported for the S-/I-type C2A domain of

synaptotagmin I (172). Ca^{2+} binding to this C2 domain increases the electrostatic potential at its membrane-binding surface and thereby increases its affinity for anionic membranes (172). Subsequent theoretical and experimental work showed that the same electrostatic switch mechanism is operative for the C2 domains of PKCβ (46), cPLA$_2\alpha$ (112), and PLCδ1 (4). In particular, for the H-/I-type C2 domain of cPLA$_2\alpha$,which prefers neutral PC membranes to anionic membranes and thus whose membrane binding relies mainly on hydrophobic interactions (115, 162), Ca^{2+}-induced electrostatic switch (neutralization in this case) was proposed to increase the membrane binding by lowering the desolvation penalty encountered in bringing charged residues to the membrane surface and thereby promoting the interfacial insertion of hydrophobic and aromatic residues (112). More recently, the importance of phosphoinositide-induced electrostatic switch in the membrane binding of FYVE (42, 160), PX (156), and ENTH domains (161) has come to light. Experimental and computational studies have shown that binding of a specific phosphoinositide to its binding pocket in these domains dramatically reduces the highly positive electrostatic potential on their membrane-binding surfaces, which in turn induces the membrane penetration of hydrophobic and aromatic residues (see below).

Conformational Switch Mechanism

Many peripheral proteins have been reported to undergo conformational changes in solution or at the membrane surface in response to ligand binding or protein (de)phosphorylation. The conformational changes can range from a minor local variation (e.g., rotation of an amino acid side chain) to a global transformation (e.g., exposure of buried secondary structure). In most cases, however, evidence for conformational changes has been obtained indirectly, e.g., through measuring the change in proteolytic patterns, and direct structural evidence is available only for limited cases. Ca^{2+} can induce conformational changes of its binding proteins. An NMR study showed that Ca^{2+} binding induces the rotation of side chains in the Ca^{2+}-binding loop of the synaptotagmin C2A domain (145). Also, specific lipid binding can lead to conformational changes. For instance, a recent crystallographic study showed that binding of PtdIns(4,5)P_2 to the epsin ENTH domain causes the formation of its N-terminal helix that is essential for its membrane penetration (54). Also, Ca^{2+}- and C2 domain–mediated binding of PKCα to the PS-containing membrane unlocks the interdomain tethering between its C1 and C2 domains and thereby triggers the membrane penetration of the C1 domain (11, 106). In addition, protein phosphorylation mediates varying degrees of conformational changes. In cPLA$_2\alpha$, phosphorylation of a serine (Ser-505) residue in the molecular hinge region causes the conformational change of the protein, which helps the interfacial penetration of the catalytic domain (38). For $p47^{phox}$, five-site phosphorylation of its C-terminal region releases the intramolecular tethering between its PX and SH3 domains, thereby allowing the PX domain to interact with the membrane (79). Conversely, dephosphorylation of multiple sites in the C-terminal tail of

PTEN abrogates the intramolecular electrostatic interactions and exposes surface cationic residues, which then interact with anionic phospholipids (37).

MEMBRANE-TARGETING DOMAINS AND THEIR HOST PERIPHERAL PROTEINS

C1 Domains and Host Proteins

The C1 domain (approximately 50 amino acids) is a cysteine-rich compact structure that contains five short β-strands, one short α-helix, and two zinc ions (22, 146, 189). The C1 domain was first identified as the interaction site for DAG and phorbol ester in PKCs (120, 121). DAG/phorbol ester-binding C1 domains have been subsequently found in other proteins with diverse functions. In general, C1 domains show a high degree of amino acid sequence homology. However, minor sequence variations in the ligand-binding region dramatically affect affinity for DAG and phorbol ester. More than 50 different mammalian proteins in the current protein database contain the C1 domain; among these C1 domains, those from protein kinase D (PKD/PKCμ), chimaerins, Ras-GRPs, Unc-13/Munc13 isoforms, and DAG kinases (β- and γ-isoforms) bind DAG and phorbol ester (22, 189).

MEMBRANE-BINDING MECHANISM The C1 domain represents a typical H-type protein that follows a two-step membrane-binding mechanism in which the initial membrane adsorption by nonspecific electrostatic interactions is followed by the membrane penetration (see Figure 2). The X-ray crystal structure of the PKCδ C1B domain shows that it has unique structural features that are consistent with this membrane-binding mechanism (192). The domain has a polar binding pocket for DAG/phorbol ester located at the tip of the molecule. This pocket is surrounded by hydrophobic and aromatic residues, which are adjoined by a ring of cationic residues in the middle part of the molecule (Figure 3, see color insert). Mutational studies of PKCα showed that clustered cationic residues in the C1A domain are involved in nonspecific electrostatic interactions with anionic phospholipids, which accelerate the initial membrane adsorption of the C1 domain (k_a effect) and also properly position the C1 domain at the membrane surface (11). An NMR study of the PKCγ C1B domain (185), a monolayer penetration study of PKCα (106), as well as vesicle binding studies of the PKCδ C1B domain (177), showed that the hydrophobic and aromatic residues surrounding the DAG-binding pocket penetrate the membrane. SPR measurements indicate that DAG binding increases the vesicle affinity (K_a) of the PKC C1 domains by more than two orders of magnitude mainly by reducing the k_d (5, 159), as is characteristic of a protein that interacts with membranes through specific or hydrophobic interactions. A monolayer penetration study of PKCα C1A domain indicated that membrane penetration of the C1 domain is necessary for DAG binding (104, 106), because the glycerol moiety of DAG is expected to be located deep within the interfacial region. The depth of membrane

penetration by the C1 domain has not been quantitatively measured yet; however, from the NMR spectra of the PKCγ C1B domain interacting with lipid micelles (185), it is estimated to be at least 10 Å below the level of the lipid phosphate, hence the classification as an H-type protein (see Figure 3). Because hydrophobic and aromatic residues surrounding the DAG-binding pocket are exposed, isolated C1 domains typically have a high tendency to aggregate in solution. Thus, the C1 domain in the full-length protein is expected to be buried in the inactive form of the enzyme and to become accessible to DAG or phorbol esters only after an interdomain conformational change (11, 106, 118, 159); in the case of PKCα, C1 domains are exposed upon Ca^{2+}-dependent, C2 domain–mediated membrane binding of the proteins (11, 106).

DIFFERENTIAL LIGAND AFFINITIES Several laboratories have reported that the two C1 domains of conventional and novel PKCs (C1A and C1B) have disparate ligand affinities and distinct functional roles (11, 81, 106, 149, 167). Irie et al. (72) reported that C1B domains of PKCs have much higher intrinsic affinities for phorbol 12,13-dibutyrate (PDBu) than do C1A domains with an exception of PKCγ whose C1A domain has only modestly lower PDBu affinity than does its C1B domain. More recently, the intrinsic DAG affinities of PKC C1 domains have been determined by isothermal titration calorimetry using soluble DAG (5, 159). These studies showed that the C1A and C1B domains of PKCα and PKCδ have opposite affinities for DAG and phorbol ester, i.e., the C1A domain has high affinity for DAG and the C1B domain has high affinity for phorbol ester. In contrast, the C1A and C1B domains of PKCγ and PKCε (5) have comparably high affinities for DAG and PDBu. Consistent with these results, mutational studies of full-length proteins showed that the C1A domain is critical for the DAG-induced activation of PKCα and PKCδ (5, 159), whereas both C1A and C1B domains are involved in the DAG-induced activation of PKCγ and PKCε (5). Currently, the structural basis of differential DAG and phorbol ester affinities of PKC C1 domains is not fully understood. Nevertheless, these studies indicate that PKC isoforms may be activated by different mechanisms when activated by DAG and phorbol esters, thereby cautioning the exclusive use of phorbol esters for mechanistic studies of C1 domains and their host proteins. It was recently suggested that the C1 domain of RasGRP1 has selectivity for a form of DAG with less unsaturated acyl groups (found mainly in the Golgi apparatus) over DAG with polyunsaturated acyl groups (e.g., arachidonyl, abundant in the plasma membrane) (97) to account for specific Golgi targeting of this C1 domain. The C1B domains of PKCδ and PKCε also have been reported to interact with ceramide and arachidonic acid (81, 142), but their binding sites have not been located. The PKC C1 domains bind alcohols with high stereospecificity (148), presumably via a non-DAG-binding site (35).

SUBCELLULAR LOCALIZATION The cellular membrane recruitment of isolated C1 domains and their host proteins, mostly PKCs, in response to DAG and phorbol esters has been measured in various mammalian cells transfected with GFP-tagged

proteins. Normally, C1 domains and host proteins migrate to the membrane that contains DAG or phorbol ester. For instance, C1 domain (119) and PKC isoforms (84, 118, 132, 159, 168) are recruited to the plasma membrane in response to exogenous phorbol 12-myristate 13-acetate (PMA) or stimuli that induce the endogenous DAG formation because PMA and DAG are localized primarily at the plasma membrane. When less hydrophobic phorbol ester or DAG is fed into the cell or when DAG is formed at other intracellular membranes, the spatiotemporal dynamics of C1 domains and host proteins follow that of their C1 ligands (8, 13, 176). In general, residues essential for the in vitro membrane binding of C1 domains, particularly membrane-penetrating hydrophobic and aromatic residues, also play an important role in the subcellular targeting of PKC isoforms (5, 159), indicating good correlation between in vitro and cellular membrane-binding properties of C1 domains. Further discussion on the subcellular localization of C1 domain–containing proteins can be found in recent reviews (22, 146, 189).

C2 Domains and Host Proteins

The C2 domain (approximately 130 residues) was first discovered as the Ca^{2+}-binding site in conventional PKCs (114). More than 200 mammalian proteins (225 in the Pfam database) containing a C2 domain have been identified since, and most of them are involved in signal transduction [e.g., PKC, cPLA$_2\alpha$, PLC, plant phospholipase D (PLD), and phosphoinositide 3-kinase] or membrane trafficking (e.g., synaptotagmins, rabphilin-3A, and Munc-13) (114, 138). Structural analyses of multiple C2 domains have indicated that all C2 domains share a common fold, with eight-stranded antiparallel β-sandwich connected by variable loops (49, 126, 165, 166). The Ca^{2+}-binding sites are composed of three variable loops that contain ligands for multiple Ca^{2+} ions, and most Ca^{2+}-dependent C2 domains bind two to three Ca^{2+} ions (49, 126, 165, 166). When compared with C1 domains, C2 domains show much lower sequence homology, particularly in the loop regions, which is consistent with their greater functional diversity. Some of the Ca^{2+}-independent C2 domains, such as that of PTEN (37, 91), still bind the membrane, whereas others, such as the C2 domains of PLC β1 and β2, are involved in protein-protein interactions (178).

MEMBRANE-BINDING MECHANISMS C2 domains are unique among membrane-targeting domains in that they do not have a well-defined lipid-binding pocket and thus show relatively weak lipid specificity. Most Ca^{2+}-dependent membrane-binding C2 domains, including the C2 domains of conventional PKCs (87, 162, 173), PLCδ1 (49), and synaptotagmins (27), prefer anionic membranes to zwitterionic ones. In particular, the PKCα (162) and PLCδ1 C2 domains (49) exhibit PS selectivity. In contrast, the C2 domains of cPLA$_2\alpha$ (115, 162) and 5-lipoxygenase strongly favor PC membranes (88).

In general, anionic lipid-selective C2 domains are I-type and PC-preferring C2 domains are H-type. Three different roles have been proposed for Ca^{2+} ions in

the membrane targeting of the C2 domain: (*a*) inducing an electrostatic switch (112, 138), (*b*) inducing conformational changes in the calcium-binding regions (10, 88, 145), and (*c*) directly coordinating a lipid through calcium bridging or chelation (173). For the I-type C2 domains, Ca^{2+} ions primarily function as an electrostatic switch and/or bridge. Ca^{2+} binding dramatically enhances the positive electrostatic potential surrounding the Ca^{2+}-binding loops, which accelerates the association to the anionic membrane. Ca^{2+} can also mediate partial (i.e., ≈5 Å below the phosphate) membrane penetration (86) and/or PS coordination (173), both of which slow the membrane dissociation of the domain (k_d effect).

In the case of the H-type cPLA$_2\alpha$-C2 and the 5-lipoxygenase-C2, Ca^{2+}-induced electrostatic neutralization in the Ca^{2+}-binding loops has been proposed to promote the interfacial insertion of hydrophobic and aromatic residues (see above) (10, 88, 112). Thus, the main role of Ca^{2+} in this case is to reduce k_d through hydrophobic interactions. Mutational studies of the C2 domains of cPLA$_2\alpha$ and 5-lipoxygenase showed that aromatic and hydrophobic residues located in their Ca^{2+}-binding loops are important for their PC selectivity and membrane penetration (i.e., k_d effect) (88, 162). EPR analysis has shown that PKCα-C2 binds to the membrane in an orientation that optimizes its electrostatic interactions with the anionic membranes (86), whereas cPLA$_2\alpha$-C2 binds to the membrane in an orientation that optimizes the membrane penetration of its hydrophobic and aromatic residues (57) (see Figure 3).

Several C2 domains, including synaptotagmin-C2B (107), JFC1-C2A (26), Rsp5-C2 (45), and PKCα-C2 (32), have been reported to bind phosphoinositides in a Ca^{2+}-independent manner, presumably via the cationic cluster in the β-sandwich region (see Figure 3). For synaptotagmin-C2B, its Ca^{2+}-independent binding to PtdIns(4,5)P_2 prelocalizes the protein to PtdIns(4,5)P_2-rich membranes (140). Then, this Ca^{2+}-independent binding mode is rapidly switched to an energetically more favorable membrane-binding mode in response to Ca^{2+}. Because the cationic patch is conserved in virtually all C2 domains, this Ca^{2+}-independent phosphoinositide (or other anionic lipids) binding may represent a common membrane-binding mechanism of C2 domains at low Ca^{2+} concentrations, which may in turn prime C2 domains for stronger Ca^{2+}-dependent membrane binding. However, the cationic cluster of the C2 domain is buried in some C2 domain–containing peripheral proteins and may not be accessible for anionic lipid binding. For instance, mutation of the cationic patch in the full-length PKCβ_{II} (77) does not significantly affect their membrane binding.

SUBCELLULAR LOCALIZATION The subcellular targeting of GFP-tagged C2 domains and host proteins has been studied in different mammalian cells. As described above, the subcellular localization behaviors of C2 domains are consistent with their in vitro membrane-binding properties. For instance, PS-selective C2 domains translocate to the PS-rich plasma membrane (4, 33, 162), whereas PC-preferring C2 domains migrate to the PC-abundant perinuclear region in response to Ca^{2+} (51, 88, 162). PtdIns(4,5)P_2-binding C2 domains are found in the

PtdIns(4,5)P_2-rich region of the plasma membrane (51). The C2A domain of JFC1 is localized to the plasma membrane via 3 phosphoinositides, PtdIns(3,4,5)P_3 in particular (26). Also, the C2 domain of yeast ubiquitin ligase Rsp5, which binds PtdIns(3)P and PtdIns(3,5)P_2 but not PS, is localized at late endosomes and multivesicular bodies (45). In general, this subcellular localization pattern of isolated C2 domains correlates with that of their host proteins if the C2 domain is the only membrane-targeting domain in the molecule (e.g., cPL$A_2\alpha$) (61). Even those with multiple membrane-targeting domains, such as conventional PKCs with both C1 and C2 domains, the C2 domain plays an important role in both their subcellular location and the kinetics and energetics of their membrane binding (106).

PH Domains and Host Proteins

PH domains are composed of 100 to 120 amino acids and found in more than 250 mammalian proteins, making them one of the most common domains. About 15% of these PH domains bind phosphoinositides with relative high affinity; however, their specificity varies widely. The PH domain of PLCδ1 binds PtdIns(4,5)P_2 (135), whereas the Btk and Grp1 PH domains have specificity for PtdIns(3,4,5)P_3. Also, the PH domain of Akt/PKB binds both PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (82). Moreover, PH domains that bind PtdIns(3)P, PtdIns(4)P, and PtdIns(3,5)P_2 have been identified (43). Many PH domains have low phosphoinositide affinities and do not yet have known functions. PH domains generally show low (i.e., less than 30%) sequence homology (53, 93), but all PH domains of known structure have a similar fold containing a β-sandwich and a C-terminal α-helix. The same structural fold has been observed in several other domains, such as the phosphotyrosine-binding (PTB) domain (194), the Enabled/VASP homology domain-1 (EVH1) (130), and the Ran-binding domain (175). All phosphoinositide-binding PH domains show pronounced electrostatic polarization with the strong positive electrostatic potential located on the phosphoinositide-binding surface (15, 16, 92, 133, 190).

MEMBRANE-BINDING MECHANISMS In general, PH domains are S-type proteins whose membrane binding does not involve significant interfacial penetration. Unlike other membrane-penetrating domains, such as FYVE, PX, and ENTH domains, PH domains do not have a deep ligand-binding pocket or hydrophobic and aromatic residues in the vicinity of the pocket (53, 92). Thus, the membrane binding of the PH domain is initially driven by nonspecific electrostatic interactions, which are followed by specific phosphoinositide binding (147). However, a recent solid-state NMR study suggests that hydrophobic residues of the PLCδ1 PH domain might penetrate the interfacial region to achieve hydrophobic interactions (171).

SUBCELLULAR LOCALIZATION The PH domains with high phosphoinositide affinity recruit their host proteins in a phosphoinositide-dependent manner. For instance, the PH domain of PLCδ1 is required for the plasma membrane localization

of the enzyme (188). Similarly, the PH domain of Akt/PKB recruits the protein to the PtdIns(3,4,5)P_3 site in the plasma membrane in response to phosphoinositide 3-kinase activation (82, 100). The cellular function of a large majority of PH domains with weak phosphoinositide affinity has not been well defined. Lemmon and colleagues (85, 92) have proposed that some of these PH domains, such as those from dynamins, increase their phosphoinositide affinity through oligomerization. Some PH domains may interact with both phosphoinositide and membrane proteins. For instance, the PH domain of the β-adrenergic receptor kinase, which has low PtdIns(4,5)P_2 affinity, drives the membrane targeting of the host protein by simultaneously interacting with the G$\beta\gamma$ subunit of heterotrimeric G proteins (128). Additional information on PH domains and their host proteins can be found in several recent reviews (41, 92).

FYVE Domains and Host Proteins

FYVE domains are small (70 to 80 amino acid) cysteine-rich domains that bind two zinc ions (163). FYVE domains are highly homologous, and most FYVE domains specifically bind PtdIns(3)P (123). PtdIns(3)P plays a key role in membrane trafficking and is found in specific subcellular locales, including the cytoplasmic face of early endosomes and internal vesicles of multivesicular bodies (164). Consistent with its in vitro PtdIns(3)P specificity, many FYVE domain–containing proteins are localized to these membranes and are involved in endocytic membrane trafficking (137, 144). In addition to their role in membrane trafficking, FYVE domain–containing proteins have been implicated in receptor signaling and actin cytoskeleton regulation. Recently, the plant homeodomain (PHD), a domain common to many chromatin-regulatory proteins, was shown to have FYVE domain–like structure (122) and bind PtdIns(5)P both in vitro and in vivo (63).

MEMBRANE-BINDING MECHANISM FYVE domains are H- or I-type domains (i.e., the depth of interfacial penetration is unknown) whose membrane binding involves initial membrane adsorption by nonspecific electrostatic interactions (42, 90, 160), specific lipid binding (123), interfacial penetration (160), and perhaps protein dimerization (44, 69, 99) (see Figure 2). The first X-ray structure of a FYVE domain from yeast protein Vps27p revealed that it has a shallow pocket that putatively binds PtdIns(3)P (109). On the basis of the similarity between the structures of the Vps27p FYVE and the C1B domain of PKCδ, Misra & Hurley (109) proposed that the FYVE domain binds to the membrane in a perpendicular "side-on" orientation, which would enable the binding of PtdIns(3)P to the pocket and simultaneous interfacial penetration of two Leu residues (Leu^{185} and Leu^{186}) in an exposed loop (termed the turret loop) (Figure 4, see color insert). Subsequent NMR studies of the FYVE domain of EEA1 in the presence and absence of a soluble PtdIns(3)P and/or lipid micelles suggest a membrane-binding mechanism in which nonspecific interfacial penetration precedes the binding of PtdIns(3)P to its pocket (89, 90). The recent X-ray structure of EEA1-FYVE-inositol 1,3-bisphosphate complex demonstrates how the FYVE domain stereospecifically recognizes the

PtdIns(3)P headgroup (44). This structure, in which the protein forms a homodimer stabilized by a coiled-coil conformation of the N-terminal extension, also suggests that the PtdIns(3)P-bound FYVE might have an "angled" orientation with respect to the membrane surface, which also allows hydrophobic residues in the turret loop to penetrate the membrane.

Recent biophysical and computational studies of the FYVE domains have shed new light on the membrane-binding mechanism and orientation of FYVE domains. The electrostatic potential calculation of Vps27p and Hrs FYVE domains showed that because of the presence of cationic residues, the turret loop region is surrounded by highly positive electrostatic potential, which drives the initial membrane association (k_a effect) (160). This membrane adsorption is followed by specific PtdIns(3)P binding, which then induces the interfacial penetration of hydrophobic and aromatic residues in the turret loop (160). SPR vesicle binding and monolayer penetration measurements indicated that PtdIns(3)P binding is not a consequence of but a prerequisite for the interfacial penetration of turret loop residues. PtdIns(3)P binding serves as an electrostatic switch that greatly reduces the positive potential surrounding the turret loop and thereby promotes the interfacial penetration of hydrophobic and aromatic turret loop residues (42, 160). Local conformational changes in the turret loop region caused by PtdIns(3)P binding have also been observed (89, 90). Both specific PtdIns(3)P binding and interfacial penetration slow the membrane dissociation (k_d effect) and thus stabilize the membrane-protein complex. This mechanism is further supported by a recent computational study of FYVE domain–phospholipids interactions using the finite difference Poisson-Boltzmann (FDPB) method (42). Furthermore, this calculation predicted that Vps27p FYVE and EEA1 FYVE would have the side-on and angled membrane orientation, respectively, owing to their distinct electrostatic potential distributions and different minimal free energy orientations at the membrane surface.

SUBCELLULAR LOCALIZATION Among the many FYVE domains characterized so far, few FYVE domains (e.g., those of endofin, FENS-1, and SARA) are autonomously targeted to endosomal membranes when ectopically expressed in the cell (74, 137, 144). Thus, questions remain whether lipid-protein interactions alone provide enough driving force for the subcellular targeting of the FYVE domains and whether other factors, such as domain oligomerization or other protein-protein interactions, are required for their membrane targeting. Dimerization-mediated endosomal targeting of several FYVE domains has been proposed (44, 69, 99). FYVE domains differ in their hydrophobicity in the putative dimer interface, which is proposed from the dimeric crystal structures of EEA1 and Hrs, and this difference could contribute to the differential dimerization and subcellular localization of FYVE domains. In support of this notion, a tandem fusion construct of the FYVE domains of Hrs readily translocated to endosomes, whereas a monomeric construct could not (62). Recently, it was reported (69) that the homodimerization of the SARA FYVE domain plays an important role in its endosomal localization, on the basis of the findings that the SARA FYVE domain has a high tendency to

dimerize in solution as well as in the cell, and that the induced dimerization of the frabin FYVE domain causes its endosomal localization. Our recent study has shown that minor structural variations in the turret loop of the FYVE domain cause large changes in in vitro membrane-binding properties and subcellular localization behaviors of the endofin and FENS-1 FYVE domains (14). Specifically, the endofin FYVE has an extra number of cationic residues and the FENS-1 FYVE has an 11-amino-acid insertion next to the turret loop. These unique structural variations dramatically increase their in vitro membrane affinity as well as interfacial penetration, and they are also responsible for their endosomal localization in the cell. Interestingly, the analytical ultracentrifugation analysis shows that FENS-1 and endofin FYVE domains are monomeric even with the protein concentration up to 30 μM. Thus, it would seem that homodimerization of FYVE domains is not a universal mechanism for endosomal targeting of all FYVE domains but that it may be necessary for the endosomal localization of only a subset of FYVE domains, such as EEA1 and SARA FYVE domains, whose host proteins form natural homodimers (23, 76).

PX Domains and Host Proteins

The PX domain is a structural module composed of 100 to 140 amino acids that was first identified in the $p40^{phox}$ and $p47^{phox}$ subunits of NADPH oxidase (129) and has since been found in a variety of other proteins involved in membrane trafficking (e.g., Mvp1p, Vps5p, Bem1p and Grd19p, and the sorting nexin family of proteins) and cell signaling [e.g., PLD, PI 3-kinases, cytokine-independent survival kinase (CISK), and FISH] (183). Recently, PX domains have been shown to interact with different phosphoinositides via conserved basic residues and target the host proteins to specific subcellular locations (78, 186). PX domains are similar to the PH domain in that they exhibit broad phosphoinositide specificity. Many PX domains, including those of Vam7p (28), sorting nexin 3 (186), and $p40^{phox}$ (78), specifically interact with PtdIns(3)P in vitro and also target the host proteins to early endosomes in the cell. In addition, most yeast PX domains bind PtdIns(3)P, albeit with varying affinities (191). In contrast, the PX domain of class II phosphoinositide 3-kinase-C2α (CPK) interacts with PtdIns(4,5)P_2 (152), and the $p47^{phox}$ PX domain preferentially interacts with PtdIns(3,4)P_2 (78). Also, the PX domains of the yeast protein Bem1p (3) and PLD1 (155) have specificity for PtdIns(4)P and PtdIns(3,4,5)P_3, respectively. The PX domain of Nox organizing protein 1 was reported to bind PtdIns(4)P, PtdIns(5)P, and PtdIns(3,5)P_2 (29).

The crystal structure of the $p40^{phox}$-PtdIns(3)P complex illustrated how the domain achieves the stereospecific recognition of PtdIns(3)P (20). The crystal structure of CISK-PX showed that this domain also has all the basic residues necessary for binding the 3-phosphate of PtdIns(3)P. The X-ray structure of the PX domain of $p47^{phox}$ revealed that this PX domain has two phospholipid-binding sites for PtdIns(3,4)P_2, respectively, and PA (or PS), respectively (79). The recent crystal structures of free and PtdIns(3)P-bound PX domain of yeast Grd19p

protein showed the lipid-induced local conformational changes involving putative membrane-penetrating hydrophobic residues (193).

MEMBRANE-BINDING MECHANISM In vitro membrane binding studies of the PX domains of p40^{phox} and p47^{phox} indicated that these PX domains are H- or I-type proteins (i.e., the depth of their interfacial penetration is unknown) whose membrane-binding mechanism is similar to that of FYVE domains. That is, initial membrane adsorption driven by nonspecific electrostatic interactions between the cationic molecular surface of the PX domain and the anionic membrane is followed by the specific phosphoinositide binding and subsequent interfacial penetration of hydrophobic and aromatic residues (79, 156). For the p47^{phox} PX domain with two separate phospholipid-binding pockets, PtdIns(3,4)P_2 and PA (or PS) induce the interfacial penetration of hydrophobic residues located near their respective binding sites by modulating local electrostatic potential changes (79) and possibly conformational changes (193). Simultaneous occupation of the binding pockets by ligands dramatically reduces the positive electrostatic potential, which in turn promotes more effective interfacial penetration of the domain, hence the synergistic effect of PtdIns(3,4)P_2 and PA (or PS) (79). The molecular modeling of the PLD1 PX domain (155) suggested the presence of a second lipid-binding pocket that is similar to that of p47^{phox} PX; however, this putative binding pocket nonspecifically interacts with anionic lipids. Furthermore, neither specific PtdIns(3,4,5)P_3 binding nor nonspecific anionic lipid binding significantly induces the interfacial penetration of the domain, partly because neither can significantly reduce the strongly positive electrostatic potential of this protein. Thus, it would seem that the PLD1 PX domain is a S-type protein whose initial membrane attachment by nonspecific electrostatic interactions is strengthened by specific phosphoinositide binding. More recently, it was suggested that the membrane binding of the CISK-PX domain might involve the domain homodimerization in addition to the phosphoinositide-induced interfacial penetration, on the basis of the finding that this PX domain tends to dimerize in the presence of the linker between the PX domain and the catalytic domain (184).

SUBCELLULAR LOCALIZATION Many PtdIns(3)P-binding PX domains, including the p40^{phox} PX domain, are localized at early endosomes when ectopically expressed in the cell (20, 78, 186). This suggests that these PX domains have higher affinity for PtdIns(3)P-containing membranes than most FYVE domains that are not targeted to endosomes. In fact, the affinity of p40^{phox} PX for PtdIns(3)P-containing vesicles is more than 10 times higher than that of the FYVE domains of Vps27p and Hrs, and it is above the threshold value for endosomal localization determined for FYVE domains (see above) (156). Cell studies with GFP-tagged p40^{phox} PX mutants in HEK293 cells showed good correlation between in vitro affinity for PtdIns(3)P-containing vesicles and the endosomal localization (156). In particular, hydrophobic and aromatic residues involved in interfacial penetration were crucial for the endosomal targeting of p40^{phox} PX. Among 15 PtdIns(3)

P-binding PX domains found in *Saccharomyces cerevisiae*, only four PX domains strongly bind PtdIns(3)P-containing vesicles and are likely to be capable of driving endosomal localization (191). Notably, many of the yeast proteins containing low-affinity PX domains have been found in multiprotein complexes. One example is the sorting nexin family, in which some family members have coiled-coil regions that can drive the homo- and/or hetero-oligomerization and endosomal targeting (187). The GFP-tagged $p47^{phox}$ PX was found in the cytosol in HEK293 cells presumably because of the low cellular concentration of PtdIns(3,4)P_2 (156). However, when PtdIns(3,4)P_2 was fed into cells and distributed among intracellular membranes, the $p47^{phox}$ PX was selectively targeted to the PS-rich plasma membrane, indicating that the secondary lipid-binding site might play a key role in the membrane targeting of $p47^{phox}$ PX (156).

Sequence comparisons of PX domains have shown that the domain contains several conserved regions, including a proline-rich motif (R(K)XXPXXP) that is characteristic of SH3 domain-binding motifs. Thus, it was speculated that some PX domains might be interacting partners of SH3 domain proteins (129). Recently, it was demonstrated that the PX domain of $p47^{phox}$ interacts with its C-terminal SH3 domain and inhibits binding of the PX domain to PtdIns(3,4)P_2-containing membranes. When five C-terminal serine residues are phosphorylated, this interdomain interaction is disrupted and the PX domain is allowed to interact with the membrane (79). Additionally, the PX domain of Scd2 interacts with its SH3 domain, preventing its binding to Cdc42 until Scd1 unleashes this interaction (48). PX domains may also be involved in interactions with other proteins, e.g., the PX domains of $p40^{phox}$ and $p47^{phox}$ bind moesin (181). However, the structural basis and the physiological role of this interaction have not been clarified.

ENTH and ANTH Domains and Host Proteins

The ENTH domain is about 140 amino acids in length and has a compact globular structure of 8 α-helices connected by loops of varying length (39). This domain was first identified in the 90-kDa epsin protein that binds the clathrin adaptor AP-2 (83). Subsequently, the ENTH domain was identified in a number of proteins, such as CALM and AP180 (a brain-specific analog of CALM), involved in the early stages of the endocytic pathway by using homology searches. X-ray crystallographic and NMR studies have shown that these domains have similar structures despite the low sequence homology (54, 55, 75). Recent reports have indicated that ENTH domains can bind PtdIns(4,5)P_2 (55, 75). The X-ray structure of the ENTH domain of AP180/CALM revealed that a cluster of basic residues bind phosphate groups of PtdIns(4,5)P_2 (55). Surprisingly, the ENTH domain of epsin 1, which lacks this basic region, also binds PtdIns(4,5)P_2 (75). More recently, the X-ray structure of the epsin 1 ENTH domain complexed with inositol-1,4,5-trisphosphate [Ins(1,4,5)P_3] has been solved (54). This structure revealed the formation of a cationic phosphoinositide-binding pocket induced by Ins(1,4,5)P_3 and also suggested that hydrophobic residues on the same face of an α-helix, the formation of

which is induced by Ins(1,4,5)P_3 binding, may penetrate the membrane. On the basis of these results, it was suggested that the membrane penetration by the epsin 1 ENTH domain is essential for membrane deformation and vesicle budding during clathrin-mediated endocytosis (54). In contrast with the epsin 1 ENTH domain, the CALM/AP180 ENTH domain does not induce membrane deformation. Accordingly, ENTH domains are subdivided into two classes: ENTH for epsin 1 and its homologs and ANTH for AP180/CALM. Recently, the ENTH domains with different phosphoinositide specificity have been reported. The ENTH domain of epsin-related protein (epsinR) binds PtdIns(4)P (108), and the ENTH domains of yeast proteins Ent3p and Ent5p have specificity for PtdIns(3,5)P_2 (50, 58). Also, the ENTH domains of Huntingtin-interacting protein1 (HIP1) and HIP1-related protein bind PtdIns(3,4)P_2 and PtdIns(3,5)P_2 (71).

MEMBRANE-BINDING MECHANISMS Our recent biophysical analysis of the epsin 1 ENTH and AP180 ANTH domains showed that these two domains have distinctly different membrane-binding mechanisms (161) (Figure 4). The epsin ENTH domain is a H-type protein that initially binds anionic membranes by nonspecific electrostatic interactions, which is followed by the facilitated PtdIns(4,5)P_2 binding on the membrane surface. The initial membrane binding and/or PtdIns(4,5)P_2 binding induces dramatic conformational changes of the domain, leading to the formation of the amphipathic N-terminal α-helix (54). As in the case with FYVE (160) and PX domains (156), PtdIns(4,5)P_2 binding also serves as an electrostatic switch to neutralize the strong positive electrostatic potential on the membrane-binding surface and thereby promotes the interfacial penetration of hydrophobic and aromatic residues of the N-terminal α-helix. The depth of membrane penetration by the ENTH domain has not been determined; however, it is expected to penetrate into the hydrocarbon core region from the putative membrane binding orientation of the domain and the length the N-terminal α-helix (54). This PtdIns(4,5)P_2-induced penetration may account for the vesicle tubulation activity of the epsin ENTH domain.

In contrast, the AP180 ANTH domain is a S-type protein that binds the membrane by nonspecific electrostatic forces and subsequent specific interaction with PtdIns(4,5)P_2 that act as an electrostatic bridge between the protein and the membrane (see Figures 2 and 4). This ANTH domain has smaller k_a and larger k_d than the epsin ENTH domain because of weaker positive electrostatic potential and lack of interfacial penetration (161), respectively. The latter property is also consistent with its inability to tubulate liposomes (54). Intriguingly, there are many peripheral proteins, such as FYVE (160), PX (156), and C1 domains (106), that penetrate lipid monolayers as well as or more effectively than the epsin ENTH domain without inducing liposome tubulation. Although further studies are required to understand the molecular basis of this special activity of the epsin ENTH domain, it would seem that the insertion of a rigid α-helix distorts the membrane and causes curvature changes much more efficiently than a flexible loop, as was seen with many surface-active amphiphilic peptides.

Other Phosphoinositide-Binding Domains and Proteins

BAR DOMAINS BAR domains are composed of approximately 250 amino acids and are found in a number of proteins, including amphiphysin, endophilin, arfaptin2, nadrin2, centaurinβ2, and oligophrenin1, that are involved in synaptic vesicle endocytosis, muscle T cell tubule formation, and membrane remodeling (64, 127, 195). The BAR domain or some BAR domain–containing proteins bind, bend, and tubulate vesicles in vitro (52, 169). The recently determined structure of the *Drosophila* amphiphysin BAR domain provides the basis of its unique function (127). This domain forms an elongated banana-shaped dimer, with each monomer made of a coiled-coil of three long kinked α-helices. The concave surface of the dimer harbors several cationic clusters that interact with anionic membranes primarily through nonspecific electrostatic interactions. Consequently, amphiphysin and other BAR domains show weak lipid selectivity. Owing to the rigid concave shape of their membrane-binding surfaces, BAR domains can either bend membranes if the membrane-binding energy is high enough to drive the bending or simply sense curved membranes when membrane-binding energy is insufficient. As in the case of the epsin ENTH domain (54), BAR domains have an N-terminal extension that seems to form an extra amphiphilic α-helix upon lipid binding, at least in the case of the *Drosophila* BAR domain. However, unlike its counterpart in the epsin ENTH domain, this amphiphilic α-helix is important but not essential for vesicle tubulation. This suggests that, despite similar vesicle tubulation capabilities, the epsin ENTH domain and the amphiphysin BAR domain may have different membrane-binding mechanisms. Further studies are needed to understand the role of membrane penetration of the amphiphilic α-helix in the membrane-binding and vesicle-tabulating activity of the BAR domain.

FERM DOMAINS The FERM domain is composed of about 300 amino acids and is found in the N-terminal region of the ezrin/radixin/moesin (ERM) family of proteins, which function as cross-linkers between plasma membranes and actin filaments (21, 65, 124, 151). The FERM or FERM-like domain has also been identified in various other proteins, including a subset of protein tyrosine phosphatases. The FERM domain binds PtdIns(4,5)P_2 in vitro (65), while PtdIns(4,5)P_2 binding by ezrin is important for its cellular function. The interdomain interaction between the FERM domain and the C-terminal domain keeps ERM proteins in a dormant conformation in the cytoplasm. Phosphorylation of the C-terminal tail weakens this intramolecular interaction, allowing the FERM domain to interact with PtdIns(4,5)P_2 and membrane proteins in the plasma membrane (47). The crystal structure of the radixin FERM domain shows that the domain is composed of three subdomains (A, B, and C) that are arranged like a cloverleaf (65, 66, 124, 150, 151) and that the subdomain C is structurally similar to the PH domain. However, the PtdIns(4,5)P_2-binding site is formed in a basic cleft between subdomains A and C. Although the membrane binding of the FERM domain has not been fully characterized, the FERM domain is not expected to significantly

penetrate the PtdIns(4,5)P_2-containing membranes because its binding pocket is not surrounded by hydrophobic or aromatic residues. FERM domains may have multiple nonspecific PtdIns(4,5)P_2-binding motifs (19).

TUBBY DOMAINS Tubby domains are a highly conserved domain composed of about 260 amino acids and are located in the C terminus of all TULP (tubby-like protein) family proteins (24, 139). The Tub protein, a founding member of TULPs, shows dual subcellular localization at the plasma membrane and in the nucleus, which is achieved through the presence of competing localization signals in the N- and C-terminal domains. The N-terminal domain has a nuclear localization sequence and adopts completely nuclear localization, whereas the C-terminal tubby domain is localized to the plasma membrane on its own. The Tub tubby domain binds PtdIns(4,5)P_2 as well as PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, but not PtdIns(3,5)P_2 and singly phosphorylated phosphoinositides (139). The X-ray structure of the Tub tubby domain shows that the domain adopts a 12-stranded antiparallel β-barrel conformation that is filled with a central α-helix (139). The PtdIns(4,5)P_2-binding site is formed between three β-strands and an external helix, and its PtdIns(4,5)P_2-binding mode is similar to that of the CALM ANTH domain (55). Mutation of cationic residues involved in PtdIns(4,5)P_2 binding abrogates the plasma membrane localization of the tubby domain (139). Although further studies are needed to elucidate the membrane-binding mechanism of the tubby domain, it would seem that the domain is a S-type domain whose membrane-binding mechanism is similar to that of the ANTH domain. Interestingly, it has been proposed that PtdIns(4,5)P_2-mediated plasma membrane binding of the tubby domain sequesters the TULP away from its effectors that are located in the nucleus (139). Thus, membrane binding of tubby domains serves to keep their host proteins dormant in contrast to other membrane-targeting domains whose membrane binding leads to the activation of their host proteins.

PERSPECTIVES

Recent structural, computational, biophysical, and cell studies have provided a unifying view on how peripheral proteins interact with membranes and how differences in their membrane-binding mechanisms lead to distinct functions. With the availability of whole-genome sequence information for many different organisms, an increasing number of membrane-targeting domains and peripheral proteins will be identified in the near future. Elucidation of the kinetics and thermodynamics of membrane-protein interactions for all these peripheral proteins under physiological conditions is a challenging task that requires multidisciplinary experimental and theoretical approaches. Recent development in various fluorescence spectroscopic techniques, such as fluorescence correlation spectroscopy, offers new opportunities to directly determine the kinetic and thermodynamic parameters of membrane-protein interactions in living cells. This exciting future research, in

conjunction with other lines of investigation, will provide more direct and detailed mechanistic information about how cellular membrane binding and activation of a wide range of peripheral proteins is regulated.

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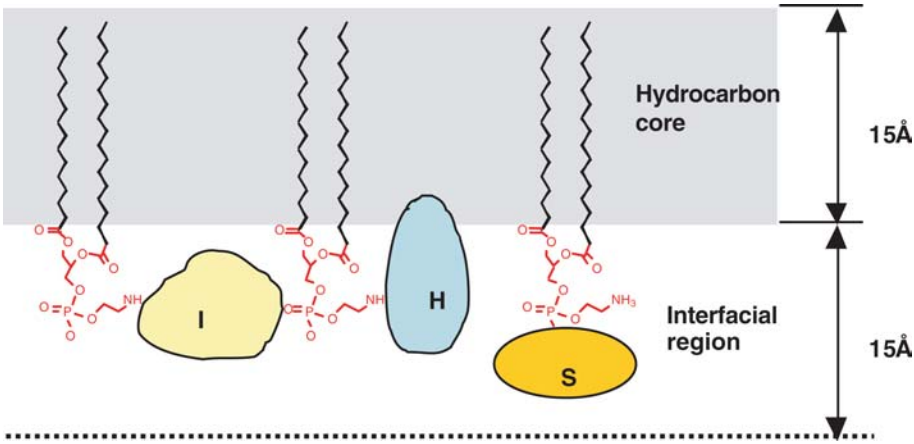

Figure 1 The structure of a phospholipid bilayer and the membrane location of three classes of peripheral proteins. The bilayer is composed of the hydrocarbon core region and the interfacial region with approximately equal thickness. I- and H-type proteins penetrate beyond the lipid phosphate group, whereas S-type proteins show little interfacial penetration. See the text for further description. Only a monolayer of a PC bilayer is shown here, with the polar groups of lipid in red.

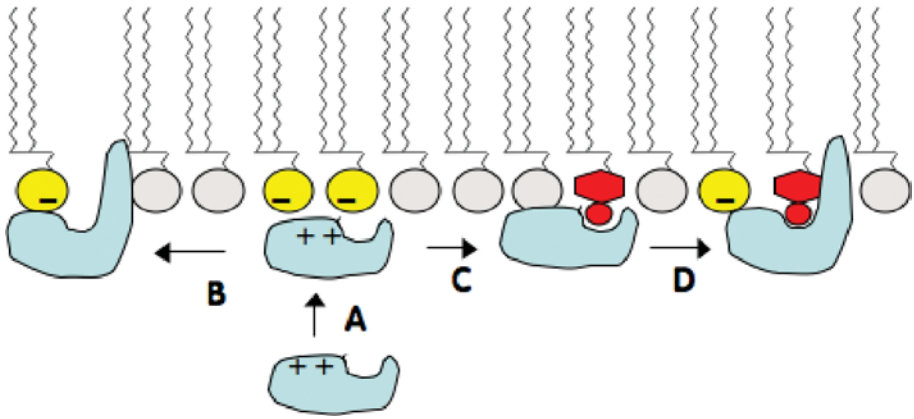

Figure 2 Membrane-binding mechanisms of peripheral proteins. Initial membrane adsorption of peripheral proteins (Step A) is driven by nonspecific electrostatic interactions and diffusion. Step A can be either autonomously performed or triggered by a Ca^{2+}-induced electrostatic switch. The membrane-attached protein can either undergo membrane penetration (Step B) or bind a specific lipid (Step C) to achieve a more stable membrane-protein complex. For many phosphoinositide-binding proteins, specific lipid binding also leads to the membrane penetration of the protein (Step D).

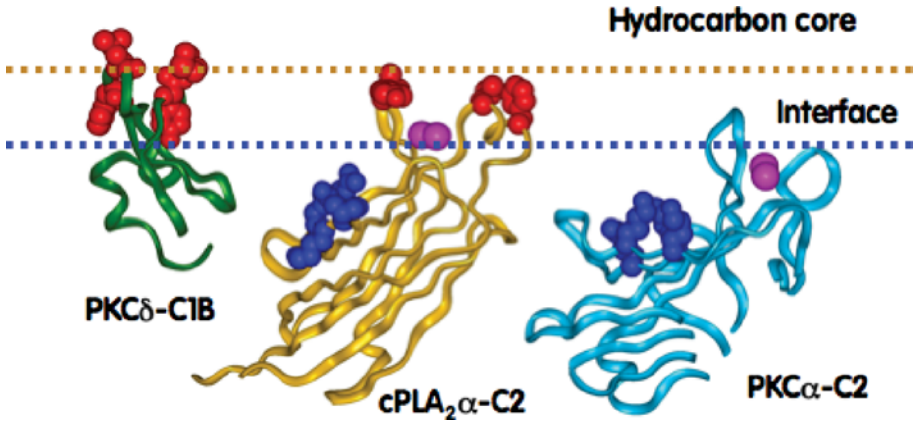

Figure 3 Proposed membrane-binding modes for PKCδ C1B, $cPLA_2\alpha$ C2, and PKCα C2 domains. The backbones of the three domains are shown in ribbon diagram, and membrane-penetrating aromatic and hydrophobic residues are shown in space-filling representation and colored red. Cationic clusters of C2 domains that have been implicated in phosphoinositide binding are shown in blue. For $cPLA_2\alpha$-C2 (57) and PKCα-C2 (86), the depth of membrane penetration and the orientation has been determined by EPR analysis.

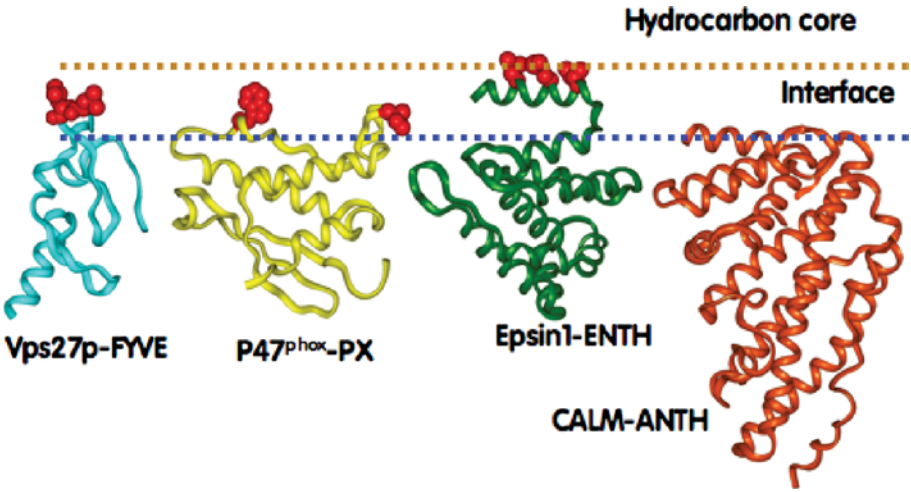

Figure 4 Proposed membrane-binding modes for Vps27p FYVE, $p47^{phox}$-PX, epsin1-ENTH, and CALM-ANTH domains. The backbones of the three domains are shown in ribbon diagram, and membrane-penetrating aromatic and hydrophobic residues are shown in space-filling representation and colored red. The depth of membrane penetration and the membrane-bound orientation have not been directly determined for these domains.