DYNAMIC SHAPING OF CELLULAR MEMBRANES BY PHOSPHOLIPIDS AND MEMBRANE-DEFORMING PROTEINS

Shiro Suetsugu, Shusaku Kurisu, and Tadaomi Takenawa

Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara, Japan; Biosignal Research Center, Kobe University, Kobe, Hyogo, Japan; and Graduate School of Medicine, Kobe University, Kobe, Hyogo, Japan

Suetsugu S, Kurisu S, Takenawa T. Dynamic Shaping of Cellular Membranes by Phospholipids and Membrane-Deforming Proteins. Physiol Rev 94: 1219–1248, 2014; doi:10.1152/physrev.00040.2013.—All cellular compartments are separated from the external environment by a membrane, which consists of a lipid bilayer. Subcellular structures, including clathrin-coated pits, caveolae, filopodia, lamellipodia, podosomes, and other intracellular membrane systems, are molded into their specific submicron-scale shapes through various mechanisms. Cells construct their micro-structures on plasma membrane and execute vital functions for life, such as cell migration, cell division, endocytosis, exocytosis, and cytoskeletal regulation. The plasma membrane, rich in anionic phospholipids, utilizes the electrostatic nature of the lipids, specifically the phosphoinositides, to form interactions with cytosolic proteins. These cytosolic proteins have three modes of interaction: 1) electrostatic interaction through unstructured polycationic regions, 2) through structured phosphoinositide-specific binding domains, and 3) through structured domains that bind the membrane without specificity for particular phospholipid. Among the structured domains, there are several that have membrane-deforming activity, which is essential for the formation of concave or convex membrane curvature. These domains include the amphipathic helix, which deforms the membrane by hemi-insertion of the helix with both hydrophobic and electrostatic interactions, and/or the BAR domain superfamily, known to use their positively charged, curved structural surface to deform membranes. Below the membrane, actin filaments support the micro-structures through interactions with several BAR proteins as well as other scaffold proteins, resulting in outward and inward membrane micro-structure formation. Here, we describe the characteristics of phospholipids, and the mechanisms utilized by phosphoinositides to regulate cellular events. We then summarize the precise mechanisms underlying the construction of membrane micro-structures and their involvements in physiological and pathological processes.

I. INTRODUCTION 1219

II. MEMBRANE PHOSPHOLIPIDS 1220

III. PHOSPHOINOSITIDE-BINDING PROTEINS 1224

IV. GENERAL PRINCIPLES FOR... 1227

V. THE BAR DOMAIN SUPERFAMILY 1231

VI. BAR PROTEINS AND DISEASE 1237

VII. CONCLUSIONS AND OUTSTANDING... 1239

I. INTRODUCTION

Life on earth emerged through the appearance of self-sealing, curved, semipermeable membranes, which separate the inside of a structure from the outside environment. Cells are separated from the exterior by membrane envelopes, and thus need to uptake nutrients and excrete wastes through these barriers to sustain the dynamic activities necessary for life. Furthermore, cells have to recognize changes in the surrounding environment and communicate signals across their membranes, which is especially important for multicellular organisms. To consistently execute these vital functions under variable conditions, cells regulate the shape of their membranes in a way that reflects the specific needs of the cell in that environment and/or cellular event (e.g., cell migration, cell division, endocytosis, exocytosis, and cytoskeletal regulation). Our current knowledge of these cellular structures has been pioneered by using electron microscopy, which enables the visualization of the membrane micro-structures of individual cells. Recently, evidence concerning the molecular mechanisms underlying the formation of these fine micro-membrane structures has been revealed.

Eukaryotic cells contain intracellular membrane systems, whereas most noneukaryotic cell types only have a plasma membrane and no intracellular membranes. The internal membranes of eukaryotic cells generally contain numerous bends, resulting in high levels of membrane curvature. On
the other hand, the plasma membrane (PM) by itself is a large, relatively flat structure but contains multiple fine micro-membrane structures, that add high levels of curvature to the flat PM. These PM structures include outward protrusions, such as filopodia and lamellipodia, as well as invaginations, such as clathrin-coated pits and caveolae, all of which occur through the deformation of a limited area of the PM. The process of shaping the membrane is generally thought to be initialized by the generation of concave (positive) or convex (negative) membrane curvature, resulting in invaginations and protrusions, respectively. It is increasingly recognized that the formation of these areas of membrane curvature is induced by a variety of protein-driven mechanisms (3, 140, 174, 184, 255, 258, 274). Most PM curvature studies have focused on the cytosolic proteins that utilize electrostatic and hydrophobic interactions to interface with the negatively charged phospholipids (3, 17).

The phospholipids that form cellular membranes consist of a hydrophobic tail and a hydrophilic head group. Major phospholipids possessing the negatively charged head group include phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylinositol (PI), and its phosphorylated derivatives, phosphoinositides. PS and PA are thought to be constitutive components of membranes. PI is also present in high amounts at the cellular membrane. However, the head group of PI contains electrically neutral myo-inositol, and thus PI is not generally considered to contribute to the modulation of cellular functions. In contrast, phosphoinositides are generated enzymatically by the phosphorylation of PI through various PI kinases such as PI 3-kinases, and down-regulated by PI phosphatases such as PTEN, processes dependent on intracellular signaling in response to extracellular stimuli. The inositol ring of PI can be phosphorylated on the hydroxyl groups at C-3, C-4, and C-5 positions in different combinations, generating seven different phosphoinositides. The presence of multiple phosphate groups in the inositol ring results in much stronger negative charges, \(-2\) per one phosphate, than that for PS and PA, both of which contain net charge of \(-1\) at neutral pH (Figure 14). With these characteristics and dynamic production dependent on stimuli, phosphoinositides are considered to be fundamental lipid species for regulation of cellular processes.

These phosphate modifications of inositol allow specific recognition by phosphoinositide-binding proteins. The structural folds of these proteins provide pockets for various patterns of inositol ring phosphorylation, which allow them to recognize specific phosphoinositides (Figure 18). These domains that recognize specific phosphoinositides include pleckstrin homology (PH), Phox (PX), and epsin NH2-terminal homology (ENTH) and are found in proteins that act for signal transduction cascades (10, 23, 50, 292). In contrast, there are proteins with structural fold, but without specific recognition of phosphoinositides. The Bin-Amphiphysin-Rvs (BAR) domain superfamily, which include proteins containing BAR, N-BAR, FCH-BAR (F-BAR), and inverse-BAR (I-BAR) domains, bind phosphoinositides without strict recognition of the phosphate at the inositol ring. The structural fold of BAR domains is rather used to sense and/or to generate tubule-like structures of phosphoinositide-containing liposomes and cellular membranes (3, 174, 255). However, the structural fold of the proteins is not always necessary for the binding of proteins to phosphoinositide or anionic lipids. Some proteins have the clusters of basic amino acid residues lacking a structural fold (poly-cationic motif) that are responsible for lipid binding through nonspecific electrostatic interactions (155, 291).

When we observed the cellular membrane, the PM is supported and stabilized by the cortical actin cytoskeleton, which is formed and recycled through regulated polymerization and depolymerization processes. Interestingly, the lipid-binding proteins provide the connection between membrane curvature and reorganization of the actin cytoskeleton. For example, the BAR domain superfamily proteins, which sense and/or induce the curvature of the membrane, often interact with proteins that regulate actin reorganization (280, 293). Thus the formation of fine micro-membrane structures is thought to be organized by the binding of membrane-deforming proteins to negatively charged lipids, which leads to the interaction and concentration of actin polymerization regulators.

In this review, we describe the characteristics of membrane phospholipids, with an emphasis on phosphoinositides, and then the mechanisms utilized by phosphoinositides to regulate a variety of cellular events through their binding motifs and domains. We also discuss the relationship between phospholipids and the mechanisms underlying the organization of membrane curvature and micro-membrane structure formation, mainly focusing on the PM.

II. MEMBRANE PHOSPHOLIPIDS

A. Localization of Various Phospholipids in the PM

The PM is mainly constructed of phospholipids. The phospholipids form a bilayer (~5 nm in thickness) by utilizing their amphipathic characteristics, with the polar head groups facing the water and the hydrophobic fatty acyl chains facing each other on the interior of the bilayer. Many different types of phospholipids can be accommodated into a single bilayer, and the lipid composition is expected to influence the physiological functions of the membrane (Figure 1). The four primary phospholipids found in eukaryotic membranes are phosphatidylcholine (PC), phosphatidylethanolamine (PE), PS, and sphingomyelin (SM). In addition, two less abundant species, PI and PA, can also exist in the membranes. We still cannot exclude the existence of trace amount of other phospholipids, of which function is less
characterized (246). In addition, PI is used as a source of various trace amounts of “phosphorylated phosphatidylinositol,” known as phosphoinositide (10).

The various types of lipids show asymmetric distribution in the inner and outer leaflets of the PM lipid bilayer. PC and SM are predominantly localized in the outer membrane, whereas PE is primarily found in the inner membrane (64, 136). Furthermore, PS is exclusively found in the inner leaflet of the PM along with PI and several phosphoinositides, including PI 4-phosphate [PI(4)P], PI 4,5-bisphosphate [PI(4,5)P2], and PI 3,4,5-trisphosphate [PI(3,4,5)P3] (23, 50). The phospholipid composition also varies in each cell type and membrane (155, 308). In eukaryotic cells, PC, PE, and SM comprise 45–55, 15–25, and 5–10% of the total quantity of lipids, respectively (308), while PS and PA only account for 2–10 and 1–2%, respectively. On the other hand, PI represents ~10–15%, while phosphoinositides are only present as trace amounts and are generally less abundant by one order of magnitude. PI(4,5)P2 is the most abundant phosphoinositide and comprises ~0.5–1% of the total quantity of lipids, while the amounts of other phosphoinositides are even lower.

Although the number of phosphoinositides present in the membrane is very low, they nonetheless play important roles in receptor-activated signal transduction as they are generated (phosphorylated) and degraded (dephosphorylated) in a signal-dependent manner. It is well known that PI(4,5)P2 is hydrolyzed by phospholipase C to generate two second messengers, inositol 3,4,5-trisphosphate (IP3) and

\[
\text{IP}_3 = \text{IP}_4 - \text{IP}_5
\]

FIGURE 1. Phospholipid shape and the associated mechanism of membrane recognition and deformation. A: the shape of a lipid molecule is classified into three categories: cylindrical, conical, and inverted conical. Alone, each type of lipid will form a unique membrane structure; however, actual membranes, such as lipid bilayers, are composed of a mixture of these three types of lipids. Even taking into account the different lipid shapes, biological membranes are, overall, relatively flat when compared with the submicron scale structures, such as clathrin-coated pits. B: the protein domains that recognize and bind to lipids are currently classified into four categories: 1) simple, charge-driven interactions without recognition of specific lipid species; 2) lipid species recognition by the protein structure; 3) membrane recognition and deformation with the insertion of an amphipathic helix or a hydrophobic loop; and 4) protein structure-based deformation of the membrane (e.g., BAR domains).
The distribution of PS and phosphoinositides causes the electrostatic properties of the membrane to vary (17). Membranes of the early secretory pathway [e.g., endoplasmic reticulum (ER) and cis-Golgi] have a weakly charged cytosolic leaflet due to the scarcity of PS and phosphoinositides. In contrast, membranes of the late secretory pathway (e.g., endosome and PM) have a highly charged cytosolic leaflet because of their abundance of PS and phosphoinositides (17). The electrostatic nature of PS allows it to act as a partial substitute for phosphoinositides in membranes, especially during the interaction with cationic motifs. Membrane-deforming domains, such as BAR, F-BAR, and I-BAR, are one such example of cationic motifs that adapt to curved anionic membrane surfaces using electrostatic interactions (3). However, these domains seem to prefer PI(4,5)P2 to PS (173, 174). Therefore, phosphoinositides, rather than PS, are the preferred phospholipids for many proteins, likely owing to their stronger charge. This preferential interaction has made phosphoinositide a major player in controlling the construction of fine membrane structures utilized for a variety of cellular functions, including cell migration, membrane trafficking, and cell proliferation (50, 173).

B. Roles of the Phospholipid Head Group and Fatty Acid Tail

Phospholipids constituting the majority of lipid bilayers have a variety of polar head groups, differing in size and charge. For example, the polar heads of PE are smaller than those of PS, PC, and SM, whereas phosphoinositides have very large polar heads because of the presence of the phosphate-bearing inositol ring (FIGURE 1A). The size of the polar head group causes each of these lipid types to form different structures (127). PE is defined as a conical-shaped lipid and by itself forms a structure with negative curvature, similar to the inverted hexagonal phase of tubes, with the head groups on the inside and the hydrophobic tails on the outside (FIGURE 1A). In contrast, PI and phosphoinositides have an inverted conical shape and form structures with positive curvatures. PC, PS, and SM are cylindrical-shaped lipids, which preferentially form flat bilayer structures. In the eukaryotic PM, PC and PS are the most common constituents of the outer and inner leaflets, respectively. PC and PS alone form flat or gently curved planar membranes in vitro, but PE, cholesterol, and phosphoinositides cannot form bilayers without other lipids. Furthermore, the presence of these lipids causes destabilization of the planar membranes formed by PC and PS, which seems to be required for vesicle budding, fusion, and other shape changes involving biological membranes (127). Therefore, local accumulation of these lipids in specialized areas of the PM can modulate the membrane shape with their physical properties, electrostatic nature, and through the recruitment of membrane-binding proteins.

Lipid geometry also depends on the acyl chains, particularly the presence of double bonds that produce a kink in the middle of the chain (17). For example, the oleoyl chain consists of 18 carbons with one double bond (C18:1) and occupies a larger volume than a palmitoyl chain (C16:0) that consists of 16 carbons without any double bond. Thus the size of the polar heads and species of acyl chain influence the lipid packing of membranes. Furthermore, cholesterol, while not a phospholipid, is also abundant in the PM and has a particularly unique component because it is amphipathic, with a polar hydroxyl group and a hydrophobic planar steroid ring. These properties allow it to intercalate between the phospholipids, with its hydroxyl group near the polar head groups and its steroid ring parallel to the acyl chains of the phospholipids, thus improving lipid packing. The PM contains phospholipids with relatively high-saturated fatty acids as well as high levels of cholesterol allowing for tight lipid packing. In contrast, the ER is characterized by loose lipid packing due to the abundance of unsaturated phospholipids and scarcity of cholesterol.

PI and phosphoinositides contain high levels of polyunsaturated fatty acids, predominantly consisting of 1-stearyl and 2-arachidonyl acyl chains. An arachidonyl chain (C20:4) has four double bonds, and therefore, its acyl chain bends sharply. The large polar head group and abundance of arachidonic acid in phosphoinositides cause them to disturb lipid membrane packing and, in regions of the membrane where phosphoinositides are concentrated, larger spots of lipid-packing defects may be formed. Indeed, brain PI(4,5)P2 [dominantly 1-stearyl-2-arachidonyl PI(4,5)P2] was found to be a potent modifier of lipid bilayer when gramicidin A channels’ sensitivity to changes in lipid bilayer properties was measured (233). Furthermore, cytosolic proteins seem to interact electrostatically with loosely packed lipids more easily than closely packed lipids (16, 18, 170, 178). In contrast to the loose-packing nature of individual phosphoinositide molecule, phosphoinositides mixed with other phospholipids in a global cellular membrane are known to form phosphoinositide-enriched microdomains (156, 226). These microdomains are thought to be further stabilized or reinforced in the presence of phosphoinositide-clustering proteins (173, 234). Thus cytosolic proteins are probably more apt to access and interact with phosphoinositides that are concentrated at these “hot spots.”
C. Specific Localization of Each Phosphoinositide

There are seven phosphoinositides found in mammalian cells (FIGURE 2A): PI 3-phosphate [PI(3)P], PI(4)P, PI 5-phosphate [PI(5)P], PI 3,4-bisphosphate [PI(3,4)P2], PI 3,5-bisphosphate [PI(3,5)P2], PI(4,5)P2, and PI(3,4,5)P3. The type of phosphoinositide can be identified by determining the combination of phosphate groups at three sites (positions 3, 4, and 5) in the inositol ring. These phosphoinositides are synthesized from PI and localize at the restricted membranes (50).

Main phosphoinositides found at the PM are PI(4,5)P2, and less abundant PI(3,4)P2 and PI(3,4,5)P3, but each has its own specific localization pattern within the PM (FIGURE 2B). PI(4)P is enriched at the Golgi apparatus where it critically regulates Golgi integrity, while PI(3)P and PI(3,5)P2

FIGURE 2. Localization of phosphoinositides. A: the kinases and phosphatases important for the metabolism of a variety of phosphoinositides are shown. PI(4,5)P2, PI(3,4)P2, and PI(3,4,5)P3 are exclusively localized at the PM. The most abundant phosphoinositide, PI(4,5)P2, is mainly synthesized by PI(4)K, which is synthesized from PI by PI 4-kinase. The formed PI(4,5)P2 is further synthesized to PI(3,4,5)P3 by PI 3-kinase or degraded to two second messengers, DAG and IP3, by phospholipase C in response to extracellular stimuli. PI(3,4)P2 is formed from PI(4)P by PI(4)P 3-kinases or from PI(3,4,5)P3 by PI(3,4,5)P3 5-phosphatases, such as SHIP1, SHIP2, and SKIP.

B: localization of PI(3)P, PI(4)P, PI(4,5)P2, PI(3,4)P2, and PI(3,4,5)P3 determined using GFP-tagged domains that recognize each specific phosphoinositide. PI(3)P, PI(4)P, PI(4,5)P2, PI(3,4)P2, and PI(3,4,5)P3 were detected with the Fyve domain of Hrs and the PH domains of Fapp1, PLC6, Tapp1, and Grp1, respectively. With the use of the Akt PH domain, PI(3,4)P2 and PI(3,4,5)P3 were detected simultaneously. In the figure, "2 x" indicates two domains linked in tandem. Src-transformed cells and mouse fibroblasts were stained with rhodamine-phalloidin (red) to visualize the F-actin and with anti-HA or anti-myc antibody (green). PI(3)P is present at the endosome, while PI(4)P is present at the Golgi (arrows). PI(4,5)P2 is localized at the PM, and both PI(3,4)P2 and PI(3,4,5)P3 are localized at podosome/invadopodia (arrowheads). Bar: 20 μm. [Adapted from Oikawa et al. (193). Copyright 2008 Oikawa et al. Journal of Cell Biology. 182: 157–169. doi:10.1083/jcb.200901042.]
are enriched at the endosome and the multivesicular body, respectively. Recently, the role of PI(4)P at the plasma membrane as a pool of anionic phospholipid was discovered (99). The least characterized PI(5)P is found on the PM, in endosomes, and in the nucleus (263). It is noteworthy that each phosphoinositide can be found outside of its well-known subcellular location. For example, PI(4,5)P2 is found in the nucleus and is thought to regulate gene expression (177). The different localization of phosphoinositides in each organelle assists in the targeting of particular proteins to distinct membranes through the specific binding domains unique to each type of phosphoinositide. This subcellular localization of each phosphoinositide is mostly determined by its lipid kinases and phosphatases that are spatially restricted to specific membranes (119, 243).

PI(4,5)P2 is mainly synthesized from PI(4)P by PI(4)P 5-kinase (PIPSK), which consists of three isoforms, PI(4)P5Kα, PI(4)P5Kβ, and PI(4)P5Kγ (243). Although there is a synthetic pathway to form PI(4,5)P2 from PI(5)P, PI(5)P is very rare. Therefore, most PI(4,5)P2 is generally considered to be generated from PI(4)P. Degradation of PI(4,5)P2 is known to produce two second messengers, IP3 and DAG, when cells are activated by extracellular stimuli (13) and is an important step in the inositol lipid signaling system. In this reaction, receptor-activated phospholipase C is used to hydrolyze PI(4,5)P2 to IP3 and DAG. On the other hand, there are two types of phosphatases, 5-phosphatase and 4-phosphatase, that dephosphorylate PI(4,5)P2 to mainly control steady-state levels. PI(4,5)P2 is the most abundant phosphoinositol and presumably plays a pivotal role in cytoskeletal remodeling, endocytosis, and membrane deformation, which are induced by the interaction between the PM and certain cytosolic proteins (23, 50, 52, 83, 234).

PI(3,4,5)P3 is formed from PI(4)P by class I and II PI 3-kinases or through the dephosphorylation of PI(3,4,5)P3 by class I PI 3-kinases, such as SHIP1, SHIP2, and SKIP. PI(3,4,5)P3 is hydrolyzed to PI(3)P or PI(4)P by 4-phosphatases, such as INPP4A and INPP4B, or the 3-phosphatase PTEN (FIGURE 2A). PI(3,4,5)P3 serves as a localization signal for the construction of podosomes/invadopodia and as a regulator for the circular dorsal ruffle formation on the membrane (105, 193, 195).

PI(3,4,5)P3 is present in negligible levels under resting conditions, but in response to various extracellular stimuli, it is synthesized at the PM from PI(4,5)P2 by class I PI 3-kinases (72, 221, 309). Interestingly, PI(3,4,5)P3 is selectively present at the leading edge of the cells. In polarized epithelial cells, it is abundant in the basolateral membrane, but absent in the apical membrane (167). Furthermore, PI(3,4,5)P3 recruits cytosolic proteins to the PM through interaction with specific PI(3,4,5)P3-binding domains, particularly those containing PH domains. The aberrant accumulation of PI(3,4,5)P3, which can be caused by loss of PI(3,4,5)P3 3-phosphatase activity or an elevation in PI 3-kinase activity, causes a variety of diseases. For example, PTEN mutant mice develop several types of cancer (49), and disruption of the SHIP2 gene causes these mice to develop modified insulin signaling (268). The precise mechanisms causing these diseases are not yet clear, but based on these data, it is hypothesized that the development of each disease occurs through the altered functions of PI(3,4,5)P3 in different cellular compartments.

III. PHOSPHOINOSITIDE-BINDING PROTEINS

A. Electrostatic Interactions Between Phosphoinositides and Cytoskeletal Proteins

Phosphoinositide binding at cationic-charged regions rich in basic amino acid residues has been observed for a variety of cytoskeletal proteins (253). Importantly, a lot of actin regulatory proteins contain basic amino acid residues that can in fact interact with phosphoinositides, mainly PI(4,5)P2. Although the net charge of PI(4,5)P2 is less than that of PI(3,4,5)P3, the contribution of PI(3,4,5)P3 to the surface charge of the membrane is presumably low because of the lower amount of PI(3,4,5)P3 found in the membrane compared with PI(4,5)P2. PI(4,5)P2 has been shown to interact with multiple actin regulatory proteins, including vinculin (79, 89), talin (106, 296), α-actinin (79, 80), coflin (327), gelsolin (126), Ezrin-Radixin-Moesin (ERM) (111), and other actin regulatory proteins. The binding of phosphoinositides to the actin regulatory proteins regulates the rearrangement of cortical actin filaments, which support the membrane deformation to construct filopodia and lamellipodia. Furthermore, this binding also activates G-actin nucleation-promoting factors (NPFs), including neural Wiskott-Aldrich syndrome protein (N-WASP) and WASP-homologous verprolin homology protein 2 (WAVE2), both of which are known to be ubiquitously expressed in the cell (293). These data suggest that inositol lipid signaling influences the remodeling of the actin cytoskeleton and membrane morphology through changes in the levels of PI(4,5)P2 at the PM. Indeed, increased expression of PI(4)P5Kα induces massive actin filament formation (257), while expression of synaptojanin, a phosphoinositide phosphatase, causes disruption of actin filaments (240). Taken together, it appears that an increase in the level of PI(4,5)P2 tends to promote actin filament formation, whereas a decrease in the level of PI(4,5)P2 leads to actin depolymerization (125).

Notably, PI(4,5)P2 is unexpectedly concentrated in lipid rafts (90, 114, 210), where Rho- and ADP-ribosylation factor (Arf)-GTPases facilitate PI(4,5)P2-synthesizing PI 5-ki-
nases by inducing the recruitment of these kinases to these sites in the PM. Thus actin assembly can be initiated with remarkable efficiency at these PI(4,5)P2-rich rafts through the enhanced recruitment and activation of WASP and ERM proteins, leading to the promotion of the actin polymerization and actin filament assembly (92). Thus PI(4,5)P2-regulated rearrangement of actin filaments in compartmentally restricted areas of the PM seems to concentrate all of the factors necessary for micro-structure formation.

B. NPFs, N-WASP and WAVEs, Are Activated by Phosphoinositides in Combination With Other Factors

When cells start to migrate in response to chemoattractants, rapid actin polymerization is induced at the leading edge to form filopodia and lamellipodia. Such rapid actin polymerization is also necessary for forming other micro-membrane structures, such as endocytic pits. In resting cells, the barbed ends of actin filaments are capped by large redundant proteins to prevent spontaneous elongation of the filaments. Therefore, to trigger rapid new actin polymerization during the rearrangement of the cortical cytoskeleton in response to extracellular stimuli, cells must form actin nuclei from which new filaments can be polymerized. Thus actin polymerization takes place through two steps: actin nucleation and polymerization. First, the actin monomer (G-actin) forms an actin trimer, which serves as a seed or nucleation site for the polymerization. This step is time-consuming and is the rate-limiting step in de novo actin polymerization. Once a trimer is formed, the actin filament elongates from the barbed end depending on the concentration of G-actin. However, to induce quick actin polymerization at the leading edge of migrating cells in response to a stimulus, the time required for the nucleation step has to be shortened (200, 212, 283). NPFs that shorten the time required for a trimer formation have been discovered (231, 293) and include two WASP family proteins, WASP (286) and N-WASP (179, 180), and three WAVE family proteins, WAVE1, -2, and -3 (181, 282). Importantly, these proteins are also phosphoinositide-binding proteins.

N-WASP is anchored and activated by PI(4,5)P2 at the PM in the presence of Cdc42, inducing quick actin polymerization using the Arp2/3 complex (228). N-WASP also has several multifunctional domains, including a WASP homology 1 (WH1) domain, a basic region, a GDB/CRIB motif, a proline-rich region, and a verprolin-homology, coflin-like, and acidic domain (VCA) region (293). The WH1 region is essential for association with WIP/CR16/WICH (or WIRE) proteins, which are important for stabilization of N-WASP or WASP (134, 168, 222, 279). The basic region of N-WASP is the domain used to bind to PI(4,5)P2, which serves as an anchor point on the membrane. The VCA region, in which the V region binds to actin monomers and the CA region binds to the Arp2/3 complex, is the domain that plays an important role in shortening nucleation time. The Arp2/3 complex includes actin-like proteins, Arp2 and Arp3 (212). Therefore, when two Arps of the Arp2/3 complex and one actin monomer are gathered on the platform of VCA in N-WASP, the time-consuming nucleation process is skipped. However, it has been shown that full-length N-WASP does not activate the Arp2/3 complex as strongly as the VCA domain does alone, suggesting that the VCA region is masked in inactive N-WASP. Interestingly, the addition of PI(4,5)P2 and Cdc42 appears to unmask the VCA region, which consequently leads to the binding of the Arp2/3 complex to N-WASP, resulting in fast actin nucleation (137, 180, 228). The Arp2/3 complex further assembles actin filaments from the sides of already existing filaments, thus forming branched, mesh-like actin filaments (22, 77).

However, N-WASP forms straight actin filaments, such as filopodia and actin rods, in cells. Therefore, it is thought that actin bundling proteins probably reform the branched filaments into straight filaments after the branched-actin filaments are generated by the Arp2/3 complex (21, 310). It is interesting that filopodia-like structures can be reconstituted with N-WASP and Toca-1 or other BAR proteins, which have membrane-deforming abilities (152). It is likely that Toca-1 is recruited to the PI(4,5)P2-enriched area and then activates N-WASP by its membrane-deforming activity (287). In muscle, N-WASP can form unbranched filaments by using the multiple actin monomer-binding sites of Nebulin, the SH3-containing protein that binds to N-WASP. In this case, trimer assembly is thought to be mediated by the V region and Nebulin in forming the straight filaments (288).

Unlike N-WASP, WAVE is anchored and activated by PI(3,4,5)P3 in the presence of Rac, forming lamellipodia-like actin filaments (196, 281). There are three isoforms of WAVE: WAVE1, -2, and -3. All WAVEs also have a VCA region through which they activate the Arp2/3 complex (293). However, WAVEs are activated differently than N-WASP. Activation occurs by PI(3,4,5)P3 and Rac binding, inducing lamellipodia and membrane ruffle formation. WAVE1–3 can form a pentameric protein complex with HSPC300/BRICK, Abi1, Nap1, and Sra1/PIR121 (34, 120). When cells are activated by chemoattractants or growth factors, they form lamellipodia at the leading edge where PI(3,4,5)P3 is concentrated (112), indicating that the WAVE proteins, particularly the abundantly and ubiquitously expressed WAVE2 isoform, are essential for lamellipodia formation in this context (325).

WASP and WAVE family proteins play crucial roles in membrane deformation during endocytosis and formation of outward protrusions by supporting the structures and generating force by de novo actin polymerization. In addition to these NPFs, other NPFs have been recently discov-
C. Phosphoinositide-Binding Domains

A variety of membrane-associated proteins have phosphoinositide-binding domains with binding pockets for particular phospholipids. These domains recognize phosphoinositides with structural specificity (9, 291). Among them, the PH domain was the first phosphoinositide-binding domain reported and is conserved among a large number of proteins in the human proteome (103). The other phosphoinositide-binding domains are PX (31, 133, 323), Fab1, YOTB, Vac1p, and EE1 FYVE (FYVE) (84, 202), ENTH (71, 125), AP180 NH2-terminal homology (ANTH) (154, 275), some of PDZ such as those found in synaptotagmin and PLAs (37, 38, 45), band 4.1, Ezrin, Radixin, and Moesin (FERM) (111, 191), and glucosyltransferase, Rab-like GTPase activator, and myotubularin (GRAM) domains (12, 301).

Some of these phosphoinositide-binding domains show specific and high-affinity binding to a particular phosphoinositide. For example, PLCζ1 PH domain specifically binds PI(4,5)P2 as well as its head group IP3 (103). The PH domain of Grp1 binds exclusively to PI(3,4,5)P3, while that of Akt/PKB binds both PI(3,4,5)P3 and PI(3,4)P2 (73, 143). Tapp1 and Fapp1 PH domains specifically bind to PI(3,4)P2 and PI(4)P, respectively (54). The PH domain specificity has enabled the construction of probes used to detect the localization of these phosphoinositides (Figure 2B) (122, 193). Other than PH domain, GOLPH3, which consists of only one domain, specifically binds to PI(4)P (51, 321). However, the lipid recognition of the PH domains is generally less strict compared with that of other domains discussed here.

The PH domains that recognize PI(4,5)P2, PI(3,4,5)P3, or PI(3,4)P2 are involved in functions important during a variety of events in the PM. For example, once the PI(3,4,5)P3 hot spots are formed in the PM by the activation of PI3-kinase, Akt/PKB is recruited to the PM through the PH domain and is activated. Akt/PKB is a serine/threonine kinase that binds to PI(3,4,5)P3 through its NH2-terminal PH domain (73, 298) and is activated by phosphorylation at Thr-308 and Ser-473. Furthermore, activated Akt/PKB modulates a wide range of cellular functions, such as cell survival, proliferation, polarity formation, cortical actin regulation, and glucose metabolism. PI(3,4,5)P3 also modulates the activities of Cdc42 and Rac through the PH domains of their guanine nucleotide exchange factors (GEFs), such as Tiam1 (241) and Vav (100), leading to membrane ruffle formation.

Lipid specificity is also observed for the other phosphoinositide-binding domains. The ENTH domain specifically binds to PI(4,5)P2 (125). PI(3)P is enriched in early endosomes and acts as a recruiter of PI(3)P-binding proteins to this site, and the FYVE domains of early endosome antigen 1 (EEA1) and hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) show exclusive, high-affinity binding to PI(3)P. However, the FYVE domain of protrudin localizes to endosome and binds to PI(4,5)P2, PI(3,4)P2, and PI(3,4,5)P3 (87, 262). The PX domains of SNX3, NADPH oxidase component, and p40phox also bind to PI(3)P rather than PI(3,4)P2 (31, 56, 133, 319, 323). On the other hand, the p47phox PX domain binds both lipids, but binds more strongly to PI(3,4)P2.

A new lipid-binding domain, SH3YL1, Ysc84p/Lsb4p, Lsb3p, and plant FYVE protein (SYLF) domain, was recently identified in SH3YL1 protein (104). This domain preferentially binds to PI(3,4,5)P3. Furthermore, this domain contains an amphipathic α-helix in the NH2 terminus, which has the capability to deform liposomes into vesicles (104). The role of amphipathic helices in membrane deformation is described in further detail in a later section. The SYLF domain has been found to play an important role in forming circular dorsal ruffles (CDRs), which are characterized by short-lived, dynamic, ring-shaped F-actin-enriched structures thought to be involved in motile cell polarity formation, macropinocytosis, and the internalization of cell surface receptors. Interestingly, the formation of micro-membrane structures, such as CDRs and podosomes/invadopodia, has been shown to require PI(3,4)P2 in common (104, 193). In CDR formation, SH3YL1 is recruited to the dorsal PM region where PI(3,4,5)P3 is newly generated in response to receptor stimulation. Then, Arf-GTPase activating protein (GAP) with a Rho-GAP domain, an ankyrin repeat, and a PH domain 1 (ARAP1) is gathered to PI(3,4,5)P3 -enriched sites through its PH domain, and its Arf GAP activity is activated. Since SH3YL1 forms a protein complex with the PI(3,4,5)P3 5-phosphatase SHIP2, PI(3,4,5)P3 at CDRs is hydrolyzed to PI(3,4)P2. These data indicate that ARAP1 is first activated by the increase in PI(3,4,5)P3, and then becomes inactivated by its hydrolysis to PI(3,4)P2. Thus this phosphoinositide conversion leads to the transition of the nucleotide-binding state of Arfs from a GDP-bound (CDR expansion) to a GTP-bound form (CDR closure), which controls the size of the CDRs through the regulation of ARAP1 substrates, Arf1 and Arf5 (105). Therefore, conversion of PI(3,4,5)P3 to PI(3,4)P2 is not only
required for CDR formation but also regulates the dynamic movement of CDRs. Furthermore, Rac is suggested to be activated by Tiam1 (Rac GEF), which is known to be activated by PI(3,4,5)P3, at the early endosome and is then delivered to the CDRs (198) where it is involved in the rearrangement of actin filaments during CDR formation.

It is clear that several of the phosphoinositide-binding domains have the capability to deform liposomes into tubules and small vesicles. For example, the ENTH domain deforms PI(4,5)P2-containing liposomes to small vesicles by inserting its NH2-terminal amphipathic helix into the membrane. Furthermore, the BAR, F-BAR, and I-BAR domains preferentially bind to PI(4,5)P2 and PI(3,4,5)P3, allowing them to deform PI(4,5)P2/PI(3,4,5)P3-enriched membranes into vesicles and tubular-like structures, although they can generally bind negative-charged phospholipids, such as PS. As discussed later, some BAR and F-BAR domain-containing proteins are accompanied by an extra lipid-binding domain, such as PH and PX domains. The presence of neighboring PH and PX domains may help to target the protein to sites where a particular phosphoinositide exists. The membrane deforming activity of BAR, F-BAR, and I-BAR domains is highly dependent on lipid-binding ability, and lipid-binding deficient mutants lose membrane-deforming activity.

**IV. GENERAL PRINCIPLES FOR MEMBRANE CURVATURE FORMATION**

The mechanisms of membrane deformation can be classified into three categories: lipid metabolism, protein scaffolding, and binding protein insertion/interaction (174). The representative proteins that are involved in membrane deformation are summarized in **TABLE 1**.

**A. Lipid Metabolism**

The shape of each lipid molecule, cylindrical, conical, or inverted conical, is different ([FIGURE 1A](#)). Enrichment of inverted conical or conical lipids is thought to drive membrane deformation. This enrichment can be achieved by increased lipid metabolism, which is mediated by the synthesis and/or transport of new lipid molecules to the membrane. The metabolism-mediated mechanisms include the enzymatic conversion of precursor lipids to usable forms as well as the addition of the head group onto the lipid backbone. The production of phosphoinositides with relatively large head groups might by itself be enough to induce membrane curvature, but the low concentration of phosphoinositides in the PM indicates that this is not the primary method used to alter global membrane curvature. However, phosphoinositides do appear to afford such effects locally in concentrated areas of the PM, such as clathrin-coated pits, caveolae, and near membrane receptors. Another metabolic mechanism employed to alter membrane curvature utilizes the hydrolysis of the acyl chain, which removes one of the two acyl chains in the lipid molecule. For example, PA is normally a conical lipid, but lysophosphatidic acid (LPA) is an inverted conical lipid. Therefore, conversion of PA to LPA is assumed to induce curvature formation. It was proposed that PA-hydrolyzing activity of endophilin was one of such example. However, the activity was the contaminant from the source of the protein purification, and indeed the membrane deformation was mediated by the BAR domain of endophilin described later (82).

The transport of lipid molecules to induce membrane curvature can be mediated by flippases, which induce lipid asymmetry between hemi-layers during cytokinesis and cell migration (57, 58). During apoptosis, type IV P-type ATPases (P4-ATPases) and CDC50 family proteins form a putative phospholipid flippase complex used for the translocation of PS and PE (135), exposing PS to the outer leaflet of the PM, which normally does not contain PS. Furthermore, the lipid scramblase, transmembrane protein 16F (TMEM16F), can also perform Ca2+-dependent scrambling of membrane phospholipids, resulting in the exposure of PS to the cell surface leading to apoptosis (285). However, the full mechanism of such enzymatic regulation and its precise effects on membrane curvature are still unclear.

**B. Indirect Protein Scaffolding**

Membrane deformation using the scaffold-mediated mechanism was first found for caged vehicles, such as clathrin-coated pits, which can be pinched off to form clathrin-coated vesicles. Clathrin coats are involved in clathrin-mediated endocytosis from the PM to form endosomes. Clathrin coats are not membrane-binding proteins, but they are connected to the membrane through adaptor proteins (APs) or cargo proteins (3, 50). On the other hand, COPI/II coats are involved in vesicle transport between the ER and the Golgi apparatus. Some components of COPI/II coats directly bind to the membrane, but GTases such as Arf play important roles for COPI/II assembly on the membrane (3, 50). In both cases, the formation of rigid lattice scaffolds, involving both the APs/cargo proteins as well as the secondary binding proteins, is thought to be the structural determinant of such caged vesicles.

The membrane curvature formation can also be just triggered by the assembly of proteins on the membrane without specific membrane binding properties. This mechanism is recently proposed as protein “crowding” for curvature formation (140, 246, 273). In this case, the assembly of proteins on the membrane with some anchoring to the membrane can bend the membrane. However, the existence of crowding mechanism in vivo is still unclear. The protein assembly of, for example, AP protein complex at the clathrin-coated pit might be considered to be such example (273).
<table>
<thead>
<tr>
<th>Classification</th>
<th>Name of Protein</th>
<th>Preferred Lipid</th>
<th>Amphipathic helix</th>
<th>Loop insertion</th>
<th>Structural fold</th>
<th>Multimer formation</th>
<th>Subcellular Structures</th>
<th>Remarks</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALPS motif</td>
<td>ArfGAP1</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>COP1 coat assembly</td>
<td></td>
<td>19, 55</td>
</tr>
<tr>
<td></td>
<td>GM1PI-21D</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cia-Golgi</td>
<td></td>
<td>205</td>
</tr>
<tr>
<td></td>
<td>Atg14L</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ER</td>
<td></td>
<td>204</td>
</tr>
<tr>
<td></td>
<td>Nup133</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>nuclear envelope</td>
<td></td>
<td>203</td>
</tr>
<tr>
<td>BAR domain</td>
<td>Arfaptin1</td>
<td>N</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td></td>
<td>Golgi</td>
<td></td>
<td>86, 294</td>
</tr>
<tr>
<td></td>
<td>Arfaptin2</td>
<td>N</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td></td>
<td>Golgi</td>
<td></td>
<td>189</td>
</tr>
<tr>
<td></td>
<td>PICK1</td>
<td>N</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td></td>
<td>Spine</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>APPL1, 2</td>
<td>P(3,4,5)P3</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td></td>
<td>Endosome With PH domain</td>
<td>157, 334</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ASAP1</td>
<td>N</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td></td>
<td>Invadopodia With Rab5</td>
<td>15, 129, 256</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SNX4,8,9,18</td>
<td>N</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td></td>
<td>Endosome With PX domain</td>
<td>98, 306, 307, 314</td>
<td></td>
</tr>
<tr>
<td>N-BAR domain</td>
<td>Amphiphysin1</td>
<td>P(4,5)P2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>Clathrin-coated pit</td>
<td></td>
<td>131, 208, 225, 271</td>
</tr>
<tr>
<td></td>
<td>Amphiphysin2</td>
<td>P(4,5)P2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>Clathrin-coated pit</td>
<td>Membrane insertion</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N-BAR domain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T-tubule helix HO</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Endophilin</td>
<td>P(4,5)P2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Clathrin-coated pit</td>
<td>Membrane insertion</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bin3</td>
<td>N</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>Lamellipodia</td>
<td></td>
<td>265</td>
</tr>
<tr>
<td>F-BAR domain</td>
<td>Toca-1</td>
<td>P(4,5)P2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>Clathrin-coated pit</td>
<td></td>
<td>26, 63, 123, 287</td>
</tr>
<tr>
<td></td>
<td>CIP4</td>
<td>P(4,5)P2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Clathrin-coated pit</td>
<td>Lamellipodia</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EB1P17</td>
<td>P(4,5)P2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Clathrin-coated pit</td>
<td></td>
<td>75, 132, 260, 303</td>
</tr>
<tr>
<td></td>
<td>FCho1,2</td>
<td>P(4,5)P2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>Clathrin-coated pit</td>
<td></td>
<td>107, 109</td>
</tr>
<tr>
<td></td>
<td>PSTPP1, 2</td>
<td>P(4,5)P2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>Podosome?</td>
<td></td>
<td>36, 302</td>
</tr>
<tr>
<td></td>
<td>Nostrin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lamellipodia</td>
<td>Caveolae?</td>
<td></td>
</tr>
<tr>
<td>IF-BAR domain?</td>
<td>srGAP1-4</td>
<td>N</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Filopodia</td>
<td>Outward protrusions</td>
<td>42, 59, 97, 159</td>
</tr>
<tr>
<td></td>
<td>FES</td>
<td>N</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Lamellipodia</td>
<td>Outward protrusions</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FER</td>
<td>N</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Lamellipodia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-BAR domain</td>
<td>IRSip53</td>
<td>P(3,4,5)P3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>Filopodia, lamellipodia</td>
<td>Outward Protrusions</td>
<td>1, 172, 234, 284, 332</td>
</tr>
<tr>
<td></td>
<td>MIM</td>
<td>N</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>Filopodia, lamellipodia</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IRETKS</td>
<td>N</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>Filopodia, lamellipodia</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PinkBAR</td>
<td>P(4,5)P2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>Planar membrane sheets close to adherence junction</td>
<td></td>
<td>213</td>
</tr>
<tr>
<td>Others</td>
<td>Epsin</td>
<td>P(4,5)P2</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Clathrin-coated pit</td>
<td>Synaptic vesicle</td>
<td></td>
</tr>
<tr>
<td></td>
<td>α-Synuclein</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Synaptic vesicle</td>
<td>Small GTPase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Arf1</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Circular dorsal ruffle</td>
<td>Caveolae</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Arf6</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Circular dorsal ruffle</td>
<td>Caveolae</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SH3YL1</td>
<td>P(3,4,5)P3</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Circular dorsal ruffle</td>
<td>Caveolae</td>
<td>Membrane scission molecule</td>
</tr>
<tr>
<td></td>
<td>EHD2</td>
<td>P(4,5)P2</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>Clathrin-coated pit</td>
<td>Caveolae</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dynamin</td>
<td>P(4,5)P2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Clathrin-coated pit</td>
<td>Caveolae</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Synaptotagmin</td>
<td>P(4,5)P2</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Synaptic vesicle protein</td>
<td>Defomation by G2 domain</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Flotillin</td>
<td>P(4,5)P2</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Clathrin-independent endocytic pathway</td>
<td>Hemin-insertion loops</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Caveolin</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Caveolae</td>
<td>Late endosome</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reticulin</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Caveolae</td>
<td>ER</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ESCRT complex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cytokinetic abscission</td>
<td>Autophagosome</td>
<td></td>
</tr>
</tbody>
</table>

N, negatively charged lipids.
Classically, the actin polymerization is thought to be a major source of force generation for membrane curvature formation, especially for protrusive structures such as lamellipodia and filopodia. The actin filaments exist in high density beneath the plasma membrane, and thus can be thought to be a mechanism for indirect protein scaffolding for membrane curvature formation. The elongating barbed ends of polymerizing actin filaments are thought to push membrane by their filling of the space between the filaments and fluctuating membrane, where the filling by actin filaments functions as “ratchet” for membrane protrusion (68, 174, 273).

C. Binding Protein Insertion/Interaction

There are two mechanisms of membrane deformation that occur through the direct binding of the proteins to the membrane. One utilizes the electrostatic interactions between the lipid-binding domains on the protein surface and the negatively charged lipids at the membrane. Two well-known classes of proteins with structural folds conducive to this type of electrostatic interaction are the BAR domain-containing proteins (BAR proteins) and the endosomal sorting complexes required for transport (ESCRT) proteins. The recently identified BAR proteins are thought to deform membranes primarily by electrostatic interaction followed by the recruitment of other membrane-modifying proteins. The ESCRT proteins utilize their electrostatic interactions with the membrane to function in membrane budding, which is essential for releasing virus vesicles to the outside of the cells as well as the biogenesis of membrane organelles containing vesicles, otherwise known as the multivesicular body (8, 108, 188). The details of ESCRT-mediated membrane changes are described in depth in other reviews (118, 219).

Direct protein insertion into the membrane is another mechanism used to alter membrane curvature. This mechanism of membrane deformation was first characterized for proteins containing an amphipathic helix, whereby the cylindrical helix is laid down on the membrane, exposing the hydrophilic surface to the cytosol (3) (FIGURE 1A). Although the exposure of this surface prevents the penetration of the helix into the membrane, like that of a transmembrane protein, the hydrophobic surface alone is still inserted into the hemilayer of the membrane, thereby enlarging the area of the inner leaflet of the bilayer and causing membrane deformation. If the hydrophilic surface is positively charged, the helix interacts with anionic phospholipids in the cytosolic layer of the membrane, while inserting the other side into the hydrophobic interior, which is the case for the ENTH domain of epsin and the N-BAR domain of amphiphsin and endophilin (FIGURE 3). The positively charged, unstructured amino acids in the NH2-terminal region of the ENTH domain, in combination with the structure region of the domain, interact specifically with the head group of PI(4,5)P2. The interaction of ENTH domain and PI(4,5)P2-containing membrane then turns the last 17 residues into an amphipathic helix, which is then inserted into the membrane (FIGURE 3) (32, 71, 125, 328).

Furthermore, if the hydrophilic surface of the helix does not have a strong charge, then the helix has a tendency to function as a sensor for membrane curvature, capturing the vesicles for intracellular vesicle transport. Such a sensor function has been found in the amphipathic lipid packing sensor (ALPS) motif of the ArfGAP1, which is involved in COPI coat disassembly (19, 55). ALPS motifs have been found in proteins associated with the nuclear envelope (Nup133), the ER (Arg14L), and the cis-Golgi (ArfGAP1, GMAP-210) and thus seem to be involved in the events of the early secretory pathway (3). The ALPS motif of ArfGAP1 is unstructured in solution, but inserts its bulky hydrophobic residues between loosely packed lipids, and forms an amphipathic helix on highly curved membranes. This helix appears to favor insertion at sites of lipid packing defects that arise in the external leaflet of a liposome when its curvature increases. Furthermore, the ALPS/ArfGAP1 helix differs from classical amphipathic helices, as there is an abundance of serine and threonine residues on its polar face. Thus ALPS motifs favor curvature and lipid-packaging defects rather than curvature and electrostatic interactions. These ALPS motifs are characterized by their secondary amphipathic helix structures and are not related to a specific amino acid sequence (19, 55).

In addition to amphipathic helices, proteins with stretches of hydrophobic residues are also known to form loop/wedge-shaped insertions into the membrane, inducing deformation. Caveolins and reticulons are examples of such membrane-embedded proteins that, after interacting with the membrane, undergo oligomerization to generate curvature. Furthermore, self-assembly of caveolin and reticulin can induce membrane remodeling to create plasma membrane invaginations and tubulate the ER (52, 255, 259, 273, 312). A similar protein structure is also found in flotilin, which is a scaffold protein involved in a less-characterized clathrin-independent endocytic pathway (52, 255, 312).

There are two additional important examples for membrane deformation mediated by membrane insertion. One is the eps15 homology (EH) domain-containing proteins 2 (EHD2) protein. The EHD2 protein is known to oligomerize, and this oligomerization and the insertion appear to be essential for membrane deformation (43). The C2 domain from several proteins, such as synaptotagmin, Doc2a/b, and group IVA cytosolic phospholipase A2 (cPLA2α), induces and/or senses membrane curvature likely through membrane insertion, which occurs in Ca2+-dependent manner (117, 166, 317, 331). The roles and mechanisms of C2 domain-mediated membrane deformation are discussed in other reviews (155, 175).
FIGURE 3. Diagrams of the membrane-deforming proteins during their interaction with the membrane. The representative structure of the different membrane-deforming proteins and their mode of action are illustrated. The BAR domains, actin regulatory proteins, and dynamin are shown with their structures in contact with the membrane. Proteins with amphipathic helices are shown with a wheel diagram at the helix. The wheel diagram was generated by HELIQUEST (85).
V. THE BAR DOMAIN SUPERFAMILY

A. Identification of BAR Proteins

BAR proteins are classified as having a BAR (N-BAR), F-BAR, or I-BAR domain depending on their sequence (Table 1). However, all BAR proteins are composed of a helix bundle, where three helices of one monomer form into a dimer, producing a six-helix bundle that displays various degrees of intrinsic curvature (Figure 3). Thus the structural classification of these three BAR domain subfamilies is less important. However, the important distinction between BAR proteins is in the degree of curvature innate to each BAR domain, which allows a cell to generate a large range of varying curvature when utilizing a scaffolding mechanism. In all BAR domains, the positively charged residues are enriched at a particular surface of the dimer, identifying it as the membrane contact site (280). With the exception of the I-BAR proteins, the membrane contact surface is found on the concave side (or the inside of the arc-shaped protein dimer). Considering only the dimer of the BAR domains, most BAR domains are thought to fit with negatively charged lipid membranes through their positive concave face (Figures 1B and 3). Furthermore, they induce membrane tubulation in vitro. Importantly, the membrane tubules formed by the BAR proteins in these experiments are topologically the same as those found in the membrane invaginations of in vivo plasma membranes.

It is still unclear whether BAR domains actually deform the membrane in cells directly or rather sense the membrane and then recruit other membrane-deforming proteins. However, membrane deformation and membrane sensing cannot be distinguished in cells. The sensing and deformation would be inherently linked processes, and one may be the other under different conditions. It has been experimentally shown that purified BAR proteins can deform the membrane in vitro, but it seems that the concentration of the BAR proteins appears to distinguish its deforming and sensing activities (271). Furthermore, in a model experiment where a nanofabricated pattern was applied onto the plasma membrane, the BAR proteins appeared to be recruited upon membrane deformation, thus supporting the curvature sensing ability of the BAR proteins (81).

Interestingly, the BAR domains from amphiphysin and Rvs161/167 have putative amphipathic helices at both ends of the dimer units of the BAR domain and are thus called N-BAR domains (208). The amphipathic helix, in conjugation with the concave structure, is important for its ability to sense and induce membrane curvature. Hence, the N-BAR module plays a dual role in regulating membrane curvature. Insertion of NH2-terminal amphipathic helices into membranes causes changes in lipid packing and effectively creates local membrane curvature. Thus the NH2-terminal amphipathic stretch plays pivotal roles in membrane tubulation and shallow helical fold insertion into the membrane. The scaffolding and loop/wedge insertion mechanism are independent of each other; however, both are thought to work simultaneously to induce membrane curvature. In addition, the amphipathic helix can also function as a sensor for membrane curvature as it acts as an anchor for the membrane and can sense high levels of positive curvature as in case of ALPS motif. Thus N-BAR proteins also could sense curved membrane domains by probing the surface for lipid packing defects. Other than that found in amphiphysin, there are only a small number of other BAR domains that have such insertions. For example, the endophilin BAR domain has a similar amphipathic helix at the end of its dimer, but also has a helix at the middle of its dimer (Figure 3) (169). The PACSIN1/Syndapin I and PACSIN2/Syndapin II F-BAR domains also have the membrane insertion loops at the membrane interacting surface (Figure 4A) (261, 315). The missing-in-metastasis (MIM) I-BAR domain also causes the membrane insertion (234). However, the other BAR domain has not been reported to have such a membrane insertion mechanism.

Epsin ENTH domain, which has a hydrophobic insertion, is apt to form small vesicles and extremely narrow tubules. In contrast, BAR domain scaffolds generally induce membrane tubulation. However, N-BAR domains, which have both insertions and BAR domain scaffolds, can generate a mixture of vesicles and tubules. In fact, an increased number of amphipathic helices on a BAR domain correlates with increased vesiculation and smaller vesicle size, driving membrane scission (24). Moreover, a high concentration of BAR domains with amphipathic insertions has been shown to induce vesiculation of liposomes (24, 91). In terms of physical chemistry, each lipid molecule is thought to adapt itself to an energetically lower state. It might be reasonable to assume that the lower state is achieved by the vesiculation of the tubulated membrane. It is likely that the high degree of membrane curvature generated by a hydrophobic insertion can make phase separation of membrane lipids by clustering specific lipids, which probably leads to the formation of boundaries between lipid clusters on the membrane (232, 267). This boundary might function as cutting line for vesiculation or scission of the membrane (4, 11, 158, 232). It is proposed that shallow hydrophobic insertions are sufficient for vesicle formation, driving membrane fission, whereas crescent-like protein scaffolds, such as BAR domains without amphipathic helix or loop insertion support formation of membrane tubules, hence disfavoring fission.

B. Clathrin-Mediated Endocytosis

The most extensively studied function of BAR domains involves clathrin-mediated endocytosis (CME) (23, 161).
CME is initialized by clathrin-coated pit formation, which is the proposed site of sequential recruitment and detachment of the BAR proteins (295). It is hypothesized that first, an F-BAR protein with shallow curvature, FCHo1/2, is recruited, which initiates the assembly of the clathrin-coated pits (FIGURE 4A) (107, 305). Then, F-BAR proteins, such as FBP17 and PACSINs, are recruited, followed by BAR domains with significant curvature, such as endophilin and amphiphysin (23, 142, 295). The FCHo1/2 proteins are unique among the BAR proteins because they do not have the SH3 domain found in other BAR proteins, but instead, FHCo1/2 has a motif for binding to epsin and other mole-

FIGURE 4. The interplay between membrane deforming proteins and the cytoskeleton. A: possible sequential recruitment of membrane deforming proteins and the organization of the actin filaments (40, 107, 295). The actin filaments induced by F-BAR protein were estimated based on Suetsugu (277). [Part of this schematic is modified from Suetsugu and Gautreau (280).] B: the I-BAR protein domain and the actin cytoskeleton assembly at the protruding filopodia and lamellipodia. C: transverse section of the clathrin-coated pit shown in A, highlighting the direction of actin filament organization. D: actin filaments utilized for protrusion (filopodia and lamellipodia) formation as well as clathrin-mediated endocytosis are illustrated. The abbreviations B and P indicate the barbed-end (fast-growing end) and the pointed-end (slow-growing end), respectively. The B ends always face the membrane, presumably providing the force for membrane shape formation. [Adapted from Suetsugu (278), by permission of Oxford University Press.]
Clathrin-coated pit formation is initiated in PI(4,5)P2-enriched membranes, and PI(4,5)P2 is essential for the early steps in CME, namely, nucleation, cargo selection, and coat assembly, whereas the latter steps, such as scission and uncoating, depend on PI(4,5)P2 elimination. A lot of proteins associated with clathrin-coated pits bind directly to PI(4,5)P2, but clathrin does not. The clathrin-binding proteins, such as epsin and AP180, have ENTH and ANTH domains, respectively, that interact with PI(4,5)P2. Classical BAR domain-containing proteins, such as endophilin and amphiphysin, also bind to PI(4,5)P2 and can deform PI(4,5)P2-containing membranes (329). However, it should be noted that BAR domains generally do not recognize PI(4,5)P2 specifically, rather bind to membrane with electrostatic interactions. Furthermore, dynamin also binds to PI(4,5)P2 through its PH domain. After the early steps of CME are complete, endophilin is thought to recruit the PI(4,5)P2 phosphatase synaptojanin, which depletes PI(4,5)P2 from the clathrin-coated pits, thereby inducing clathrin uncoating and scission (207, 249). Thus the CME process is closely regulated by PI(4,5)P2.

Furthermore, the BAR proteins appear to finely regulate actin polymerization during CME, depending on the membrane size and structure. The first example, demonstrating the dependency of actin polymerization on the size of the membrane, indicated that actin polymerization by Toca-1 and FBP17 in combination with N-WASP was affected by the diameter of the liposome (287). The curvature-dependent formation of actin filaments was also observed when actin polymerization was induced by the BAR-PX domain of SNX 9 on phosphoinositide-coated silica beads of a defined curvature (83). In this case, the actin filaments were found to be elongated toward the neck of constricting clathrin-coated pits (FIGURE 4D) (40, 277). Therefore, actin polymerization was found to push the membrane in analogous ways during both filopodia, lamellipodia, and clathrin-coated pit formation, presumably under the regulation of a diverse set of BAR domain proteins (FIGURE 4, B AND D) (280).

Along with the BAR proteins, N-WASP is thought to induce actin polymerization at the clathrin-coated pits, as indicated in the several examples described above (FIGURE 4, A, C, AND D) (40). However, N-WASP-mediated actin polymerization by itself is not required for endocytosis in cultured cells under normal conditions without membrane tension. Notably, this actin polymerization is required for endocytosis under membrane tension, presumably to support the membrane during scission (25, 67, 322), indicating a context-specific need for N-WASP-mediated polymerization. In addition to N-WASP, other actin regulatory proteins, such as WAVE, formin, VASP, and Cobl, can also function downstream of the BAR proteins (74, 78, 94, 150, 250, 326).

C. Other Invagination/Endocytotic Structures

The involvement of BAR domain proteins in the formation of other membrane structures is less clear. Caveolae are flask-shaped invaginations that are enriched in cholesterol, sphingolipids, and PI(4,5)P2 (76, 258). The major structural component of caveolae is the integral membrane protein caveolin, a membrane-embedded protein that undergoes oligomerization to generate curvature (229). Caveolins have transmembrane domains that contain an amphipathic helix inserted into the bilayer (201). This helix also binds cholesterol and could cause the raftlike lipid composition of the caveolae (61). Recent work has identified a protein family associated with caveolae, which includes cavin1–4. Cavin proteins interact with caveolin in mature caveolae, and the deletion of cavin results in the loss of caveolae (101, 110). Thus this cavin complex may function as a scaffold for caveolin and other components of caveolae.

With regard to F-BAR-domain proteins, PACSIN2 was shown to be present in caveolae and appears to be important for morphogenesis of the caveolae structure (102, 146, 254). Indeed, depletion of PACSIN2 results in the loss of morphologically defined caveolae. Similar to the BAR proteins in the clathrin-coated pits, PACSIN2 binds to dynamin and N-WASP through its SH3 domain (102, 254). There is an Asn-Pro-Phe (NPF) motif in PACSIN2 that can bind to EHD2 (43, 186), which is thought to stabilize the caveolae through the association with actin filaments (276), likely induced by PI(4,5)P2 (FIGURE 3) (76, 266). Thus it is clear that PACSIN2 plays an important role in the formation of caveolae. Another F-BAR protein, NOSTRIN, is also reported to be localized at caveolae, although it is not clear specifically how NOSTRIN functions in this membrane structure (244).
Among the variety of endocytic pathways that internalize numerous cargoes, several appear to proceed independently of the canonical protein clathrin. The clathrin-independent carriers (CLIC) and GPI-enriched endocytic compartments (GEEC) pathways play major roles in such uptake (141, 235). GTPase regulator associated with focal adhesion kinase-1 (GRAF1) protein, which contains an NH2-terminal BAR domain, a PH domain, a Rho-GAP domain, a proline-rich domain, and a COOH-terminal SH3 domain, is shown to be important in coordinating small G protein signaling and membrane remodeling to facilitate internalization of CLIC/GEEC pathway cargoes (162). In this pathway, GRAF1 localizes to P(4,5)P2-enriched sites in the membrane via its NH2-terminal BAR and PH domains, and deforms the membrane to facilitate endocytic intermediate formation.

**D. Intracellular Organelles**

The membranes of intracellular organelles have also emerged as a site of BAR domain function. For example, the PX-BAR domain of SNX18 is shown to function in autophagosome formation (144). Furthermore, the endosome contains SNX1, -4, -8, -9, and -18, all of which have BAR domains, and appear to be important during the formation of tubular networks and in endosome trafficking (98, 299, 306, 307). Endosomes also contain adaptor protein containing a PH domain, phosphotyrosine-binding domain, and leucine zipper motif 1 (APPL1), which contains an NH2-terminal BAR domain, a central PH domain, and a COOH-terminal phosphotyrosine-binding (PTB) domain (FIGURE 3). The crystal structure of the BAR domain dimer of APPL1 revealed that it contains two four-helical bundles (FIGURE 3), whereas other BAR domain dimers have only three helices in each bundle (157, 334). The PH domain is located at the ends of the BAR domain dimer, and all of the BAR, PH, and PTB domains have shown preferential binding to P(3,4,5)P3. The BAR-PH domain has also been shown to fold together, forming the binding site for the small GTP-binding protein Rab5.

The Golgi network also contains BAR proteins, such as Arfaptin, which is the BAR domain only protein and modulates small GTPases, such as Arl and Arf, for Golgi trafficking (86, 294). For example, Arfaptin 2 binds to Arl1 through its BAR domain (FIGURE 3) and is then recruited to the Golgi membrane, where it induces membrane tubules. Interestingly, the Arl1 in complex with the BAR domain of Arfaptin 2 resides at a similar position as the PX domain in the PX-BAR unit of SNX9 (FIGURE 3) (189), suggesting a similar mechanism underlying BAR domain targeting to specific organelle membranes.

**E. Membrane Protrusions**

The protrusive structures found on membrane surfaces had previously been believed to be organized solely by the power of actin polymerization (160). Therefore, it was surprising that membrane deformation was found to be, at least in part, mediated by BAR proteins. Currently, there are two classes of BAR proteins with protrusion forming ability, proteins containing an I-BAR, and some of the F-BAR domain-containing proteins, which comprise only a small population of the entire BAR protein superfamily. The I-BAR domain, which has a kink in the helix, was first found in IRSp53 and then later discovered in MIM (172, 284). The kink produces the surface of the inverted membrane, to the opposite direction than do classical BAR domains. The I-BARs can deform membranes in vitro into protrusion-like shapes, consequently clarifying the role of I-BAR domain in negative curvature formation (FIGURE 3).

However, it is still uncertain whether such membrane deformations are actually taking place in vivo, although several researchers have concluded that I-BAR proteins are localized at and involved in the formation of filopodia and lamellipodia (1, 20, 251) (FIGURE 4B). In other words, it is still unknown whether protrusions forming independently from actin filaments actually exist in the natural cellular environment. As Ahmed et al. (1) pointed out, the artificial filopodia induced by the I-BAR domain in cell culture and the "genuine" filopodia observed in vivo show distinctively different dynamics: the former is relatively stable and less motile with a lifetime of more than 10 min, compared with the latter with a lifetime of 79–142 s. Importantly, the full-length IRSp53 has the scaffolding ability to generate actin-mediated forces, which may explain the differences in the filopodia dynamics. The relative contribution of curvature-sensing, -generating, and scaffolding is currently unknown, and further studies are necessary to provide additional insight into the mechanism of IRSp53-mediated filopodium formation.

Recently, another I-BAR function has been proposed in Drosophila, where I-BAR proteins indirectly help protrusion formation by preventing invagination for endocytosis from occurring at these membrane sites (218). In addition, Pinkbar, a relative of IRSp53, has more flat surface for membrane interaction and functions in generating planar membrane sheets in intestinal cells (FIGURE 3) (213).

Another class of BAR domains with protrusion-forming activity was found in srGAP proteins that were classically called as Slit-Robo GTPase activating proteins. The srGAP family of proteins consists of four family members, srGAP1, 2, 3, and 4, which have from the NH2 terminus an F-BAR domain, a Rho-GAP, and an SH3 domain. Although each family member contains a GAP domain, there are differences in GAP activity between the proteins. The Rho-GAP domain of srGAP1 has been shown to promote GTP hydrolysis of Cdc42 and RhoA (320), whereas the GAP domains of srGAP2 and srGAP3 are both specific for Rac1 (97, 269), and srGAP4 can act on both Cdc42 and Rac1 (311). All four family members display spatially and tem-
porally distinct patterns of expression in the central nervous system (5, 70) and have been shown to regulate cell migration and neuronal morphology in mammalian cells. Surprisingly, the overexpression of the F-BAR of srGAP2 did not cause invagination, but instead led to outward protrusions along the cell surface (29, 42, 59, 97). As well, srGAP1 and srGAP3 induce filopodia formation, although they are less potent than srGAP2. And incubating this domain with liposomes caused deformations of the membrane similar to those created by the IRSp53 I-BAR domain in vitro. These data were unexpected because F-BAR domains, in general, had been believed to induce positive membrane curvature. However, it is plausible that the F-BAR domain of srGAP functions like I-BAR, although there is no reported structure for the F-BAR domain of those proteins to corroborate this hypothesis. Thus this family of Rho-GAP is now defined as inverse F-BAR (IF-BAR), which is functionally distinct from other F-BAR domains.

Recent studies show that the F-BAR-containing srGAP family of proteins can regulate dendritic spine morphogenesis. Dendritic spines, small bulbous protrusions found on neuronal dendrites, are postsynaptic structures that receive inputs from axons. Spine formation, remodeling, and shape change are associated with the brain’s ability to store and process information in response to an experience. With the use of primary cultures of neurons, both srGAP2 and srGAP3 were shown to promote dendritic spine maturation, presumably by facilitating dendritic filopodia formation at the site of developing spines (29, 30). The overexpression of the GAS7 F-BAR domain has also been reported to induce cellular protrusions, which are suggested to be related to neurite extension (330). However, the mechanisms of these relationships remain unclear because the structures have not been solved yet.

The Fes or Fer proteins had first been characterized as members of nonreceptor protein tyrosine kinases, which play important roles in intracellular signal transduction for cell adhesion and migration (95). The Fes or Fer proteins have the F-BAR domain, SH2 domain, and tyrosine kinase domain. The Fes or Fer has a recently characterized neighboring FX domain. Whereas the F-BAR domain of Fer alone is not sufficient for membrane binding, the FX domain is found to be required for efficient binding. Furthermore, the FX domain specifically binds to PA, but not PI(4,5)P2, and functions in concert with the F-BAR domain (124). Indeed, the addition of PA seems to enhance the tyrosine kinase activity of Fer in vitro, and phospholipase D (PLD)-generated PA appears to serve as an activator in response to extracellular stimuli, since tyrosine kinase activation was suppressed by the addition of PLD inhibitors. Furthermore, the FX domain may be important for targeting the F-BAR domain of Fer to the lamellipodia formation sites in the membrane as the F-BAR-FX domain was found to be necessary for lamellipodia formation by Fer.

F. How Is the BAR Domain Superfamily Regulated?

The BAR domain superfamily is unique in its structural characteristics and its structure-membrane curvature interactions. Therefore, because of their numerous biological functions, it is important to understand how the BAR domain superfamily is regulated. A large number of cytosolic proteins are known to be activated and transduce signals upon phosphorylation or the addition of a charge to the protein. Recently, the BAR domains of ACAP4 have been reported to be activated by phosphorylation during membrane association in a manner dependent on epidermal growth factor (EGF) stimulation (333). However, when we consider the increase of negative charge brought by phosphorylation, the consequence might be the opposite, because an addition of negative charge may enhance repulsion from anionic lipids of the membrane. Drosophila syndapin/PACSIN is also reported to be phosphorylated, but in this case, the phosphorylation inactivates the membrane tubulation ability (289). Mammalian PACSIN1 and PACSIN2 are also reported to be phosphorylated, which results in defective neuronal morphologies and/or functions (2, 217). Because phosphorylation introduces a strong negative charge, it is assumed that the electrostatic membrane interactions are destroyed, thereby leading to weaker membrane affinity.

Autoinhibition is a widely used mechanism for the regulation of multidomain proteins. For example, N-WASP is regulated by the intramolecular interaction between the VCA and the CRIB motif (228). This interaction is released by the binding of PI(4,5)P2 and Cdc42, thereby freeing the VCA to associate with the Arp2/3 complex. WAVE is also regulated by protein complex formation, where the VCA region is exposed through phosphorylation and binding of the small GTPase Rac (197). However, among the BAR proteins, such autoinhibition has not been reported. Only one curious example is PACSIN1, in which the SH3 domain is proposed to interact with the F-BAR domain intramurally; however, the lack of a 1:1 stoichiometry for the F-BAR and the SH3 domains in the crystal structure indicates that this is a weak interaction (224).

It would not be surprising if the regulation of BAR proteins depended on molecule-molecule interactions (i.e., interactions with other proteins or lipids). This type of mechanism is known to be utilized in the regulation of SH3 binding proteins. In this process, if IRSp53 is phosphorylated by Par1 kinase or an unidentified kinase downstream of GSK3β, then the binding of the SH3 domain is hindered by the binding of 14–3–3 protein (39, 130, 227). However, to date, no regulatory mechanism controlling the membrane deforming ability of the BAR proteins has been discovered.

So far, it is not clear how BAR domain assembly, which is required for forming a stable membrane micro-structures, is
regulated. Membrane bending due to thermal fluctuation may be sufficient for triggering BAR domain assembly. This initial BAR assembly likely results in the assembly of several additional molecules, which may lock the membrane into the conformational structure formed by the protein domains. In this case, BAR proteins are pure sensors of membrane curvatures, of which no particular regulation might be required. However, this theory has not yet been tested directly. If BAR proteins are actively engaged in the formation of membrane curvature, then it is still unclear how the membrane deforming ability of the BAR proteins is regulated, even 9 years after the structural determination of the amphiphysin BAR domain.

G. Higher Order Structure Assembly

When we consider how proteins with positively charged surfaces and/or amphipathic helices deform membranes, it is not likely that a single molecule or single dimer could act alone to deform the membrane in such a specific and effective manner. Instead, the assembly of many molecules at the same place of the membrane is certainly required for membrane deformation. For such assembly, it is quite reasonable to expect that the enzymatic production of phosphoinositides, such as PI(4,5)P₂, would recruit membrane-deforming proteins. The assembly of several of these molecules may trigger the “polymerization” of membrane deforming proteins necessary for definite curvature generation (123, 260). In such a scenario, the concentration of the membrane-deforming protein will need to be fairly high, presumably in the micromolar range.

When the structures of BAR, F-BAR, and I-BAR proteins were discovered, it was obvious that a dimer structure of the BAR domains was responsible for binding the protein to the curved membranes. However, BAR domains not only bind to membranes of the appropriate curvature to stabilize them, but can also act to deform membranes into such a shape (75, 183). Therefore, it is reasonable to suggest that major curvature deformations (e.g., protrusions, invaginations, etc.) might be formed by the assembly of the BAR domains into chains, sheets, and/or spirals. Thus the shape of membrane contact surface is determined by not only the dimer unit structure, but also the assembly of higher order structures on the surface of the membrane.

The first example of higher order assembly formation found in nature was the putative filament formed by the F-BAR domains of CIP4 and FBP17, where tightly packed F-BAR spirals were observed on the surface of the tubules (FIGURE 3) (260). Subsequent research has identified spiral formation by endophilin and has further revealed some unique spacing patterns among the spirals of this endophilin assembly on the membrane tubules (FIGURE 3) (183). However, this filament formation was not clearly observed for other BAR domains. With regard to PACSIN1 and Pinkbar, the higher ordered structure is proposed to function for formation of membrane curvature (213, 224). Especially, Trp141 of Pinkbar is shown to be involved in lateral contact for formation of planar sheet of the I-BAR domain. It is important to note that such insights into the assembly of BAR proteins into higher order structures are typically based on the protein packing observed in the crystallized form. In a crystal, the proteins must interact with each other to form a stabilized crystal structure, and the contact sites between molecules can be determined from the spatial organization of the proteins in these structures. However, it is still unclear whether such higher order structures actually exist for the membrane-deforming proteins.

Furthermore, the localization of the BAR protein is also expected to affect its ability to form higher order structures, and, importantly, proteins with similar structures do not necessarily localize in the same areas of the cellular membrane. For example, the F-BAR protein FBP17 is reported to localize at clathrin-coated pits (260), while its homolog, CIP4, can localize to lamellipodia (163, 236, 297). In addition, although the three PACSIN isoforms have a similar structure, PACSIN2 and -3 were localized at caveolae, while PACSIN1 was not (254). PACSIN2 is also involved in EGF receptor internalization, presumably through clathrin-coated pits (46, 295). These data suggest that the BAR domain is not the only determinant of BAR protein localization. Rather, the formation of a protein complex might be more important than the BAR domain-membrane interaction for the localization of the protein.

The exploration of BAR protein localization and assembly formation is limited by the current methods used to visualize live cells (e.g., microscope resolution and membrane/protein visualization methods). Furthermore, the diffraction limit of a live cell also causes difficulties, as most of the membrane structures formed by BAR proteins are below this limit, thus preventing the use of conventional microscopy to examine BAR protein-mediated deformation. Recently, superresolution microscopy, such as stochastic optical reconstruction microscopy (STORM), photo-activated localization microscopy (PALM), and structured illumination microscopy (SIM), has been used to try to overcome such limitations, and preliminary studies have been initiated to reveal how membrane structure is built up by cytosolic proteins (14, 116). However, superresolution microscopy is not without its own limitations, and although the membrane micro-structure probes can be rapidly developed, the signal density still needs to be increased to effectively observe the shape of the membrane. However, this methodology represents a large step toward visualizing protein expression and localization, as accomplished with conventional microscopy, in an electron microscopy-grade image of the cellular structures. This type of detailed visualization of proteins and membranes will be indispensable to
increase understanding of the function and regulation of BAR proteins during membrane morphogenesis.

VI. BAR PROTEINS AND DISEASE

The members of the BAR protein superfamily play crucial roles in the formation of fine membrane micro-structures, such as membrane protrusions, filopodia, lamellipodia, and endocytic invaginations. Therefore, defects in the function of these proteins are likely related to a variety of diseases. Cell culture and animal model knockout studies are powerful tools used to determine the physiological role of a protein in vivo. Here, we describe several BAR protein mutations connected to disease pathology as well as the phenotypic consequences of lacking one or more BAR protein in a mouse model.

A. Cancer

Cancer cells have different morphology compared with the parental normal cells. Although the relationships between shape formation by BAR proteins and cancer formation is not clear, several reports showed the involvement of BAR proteins in cancer. The amphiphysin-like protein Bin1 has been reported to contribute to the Myc and Raf pathways which play a crucial role in apoptosis and senescence (214), and one isoform appears to act as a tumor suppressor by inhibiting c-Myc. Loss of Bin1 in mosaic mice was also associated with overall organ tissue inflammation and an increased occurrence of lung and liver carcinomas (33). Deletion of Bin3 has also been associated with increased lymphomas in aging animals as well as juvenile cataract formation, with a near total loss of F-actin in the lens fiber cells (220).

Numerous BAR proteins are known to be involved in podosome/invadopodia formation in cancer cells. Formation of these F-actin-rich fingerlike membrane protrusions drives cell migration and invasion into the extracellular matrix (ECM) (185). Podosomes/invadopodia were initially discovered in cells transformed with the Rous sarcoma virus as well as in monocyte-derived cells, such as osteoclasts and macrophages (44, 164, 165). The BAR protein CIP4 has been shown to be localized at the leading edge and podosomes and may be involved in ECM invasion (115, 209). In addition to CIP4, FBP17 is also localized and appears to function in the podosomes (300). Furthermore, both proteins have been shown to be involved in invadopodia formation in invasive breast and bladder cancer cells, respectively (209, 324), and CIP4 are expressed at high levels in the invasive breast cancer cell line MDA-MB 231, while lower levels are found in the less invasive cell line MCF-7. It has been suggested that CIP4 expression promotes breast cancer cell invasion and invadopodia formation through the activation of N-WASP (209).

CIP4 is also expressed at abnormally high levels in chronic lymphocytic leukemia (CLL) cells compared with normal B cells or other subtypes of B-cell malignancies. Experimental CIP4 knockdown in CLL cell lines causes decreased activation of WASP, but increased activation of PAK1 and p38 mitogen-activated protein kinase (MAPK), resulting in impaired lamellipodium assembly and loss of directional migration (163). In addition, overexpression of CIP4 in CLL patients seems to be associated with aggressiveness of the disease and a shorter survival. However, the inconsistent evidence is also reported. According to that report, CIP4 may function as a suppressor of Src-induced invadopodia and invasion in breast tumor cells by promoting endocytosis of MT1-MMP (115). Therefore, it is likely that CIP4 plays different roles dependent on the types of cancer cells. As well, CIP4 gene knockout mice only cause delayed and decreased endocytosis (65). Although such partial suppression of endocytosis probably implicates the redundancy of these proteins and limitation of knockout studies, CIP4-mediated endocytosis might be involved in some forms of cancer progression.

The BAR and GAP protein, ASAP1, as well as the I-BAR protein IRSp53 are also involved in cancer cell podosome/invadopodia formation (15, 194). ASAP1 is an Arf-GAP known to regulate membrane trafficking and actin cytoskeleton organization (121, 223). It contains an NH2-terminal BAR domain, followed by a PH domain, an Arf GAP domain, an ankyrin repeat, a proline-rich region, and an SH3 domain. On the membrane surface, ASAP1 catalyzes the hydrolysis of GTP bound to Arf family GTP-binding proteins (Arfs), preferably to Arf6. Arf6 is localized at the PM where it functions in a wide range of processes, such as endocytosis, cytokinesis, and the reorganization of the actin cytoskeleton, possibly through the action of increasing PI(4,5)P2 synthesis and Rho family G protein regulation (88). The BAR domain of ASAP1 is critical for in vivo function of ASAP1, and the PH domain selectively binds to PI(4,5)P2 (129). The PH domain is functionally integrated with the GAP domain, raising the possibility that the BAR domain affects GAP activity through the PH-BAR folding mechanism. It has also been reported that ASAP1 is a substrate of the tyrosine kinase Src and binds to a variety of actin regulatory proteins, including cortactin, which is a required component for podosome formation (15). Indeed, ASAP1, particularly the BAR domain was found to be required for podosome formation in breast cancer cells and NIH 3T3 expressing active c-Src. Furthermore, such podosome formation was inhibited by the overexpression of the BAR-PH domain (15). These results suggest that the recognition of PI(4,5)P2 by the BAR-PH domains trigger subsequent signaling events in podosome formation including the regulation of Rho family G proteins, the reorganization of actin filaments, and possibly the membrane curvature generation.
Another phosphoinositide, \( \text{Pl}(3,4)\text{P}_2 \), is required for podosome/invadopodia formation in a unique way. Tks5/FISH, a scaffold protein, has been shown to play an essential role in invadopodia formation and is upregulated in invasive forms of cancer (252). This protein consists of one PX domain and five SH3 domains and binds through PX domain to \( \text{Pl}(3,4)\text{P}_2 \) (193, 195). Tks5 is essential for podosome/invadopodia formation in many different cell types (38, 193, 252, 321). However, it is not clear yet how Tks5 plays a role in the podosome/invadopodia formation. Recently, a new sequential model of invadopodia assembly is presented (242) based on high-resolution spatiotemporal live cell imaging. According to that model, cortactin, N-WASP, coflin, and actin arrive together to form the invadopodia precursor, followed by Tks5 recruitment. Tks5 then forms a complex with N-WASP, cortactin, coflin, and actin. Next, \( \text{Pl}(3,4,5)\text{P}_3 \) gets enriched in a ring around the precursor core. SHIP2, a \( \text{Pl}(3,4,5)\text{P}_3 \) 5-phosphatase, also localizes at the invadopodia core and hydrolyzes \( \text{Pl}(3,4)\text{P}_2 \) to \( \text{Pl}(3,4)\text{P}_2 \) which anchors the Tks5 complex to the precursor core and stabilizes the invadopodia (FIGURE 2).

The I-BAR domain-containing protein IRSp53, which can produce negative curvature, may also act as a scaffold protein, rather than a membrane-deforming protein, during podosome formation, although its full function is still unclear (194). IRSp53 consists of an I-BAR domain, a CRIB motif, and an SH3 domain. The CRIB motif binds to Cdc42 and the SH3 domain binds to Eps8, N-WASP, WAVE2, MENA, and VASP (251), solidifying its potential as a scaffold protein, on which a variety of actin regulatory proteins can be assembled for inducing actin polymerization.

Some BAR family proteins act as tumor suppressors. F-BAR-only protein, PSTPIP2, antagonizes actin polymerization in the podosome by competing with FBP17 at the membrane (302). Furthermore, the I-BAR containing protein MIM is present in normal tissues, but is missing in several metastatic cell lines, and this downregulation is associated with the progression of bladder transitional carcinomas (316). Thus MIM has been proposed as a suppressor of metastasis genes in bladder cancer (153).

These accumulating evidences clearly indicate the involvement of BAR proteins in cancer. However, how the membrane morphology generated by the BAR protein relates to cancer formation remains unclear.

B. Immune System Disorders

Multiple F-BAR proteins affect immune cell function through their effects on membrane cytoskeleton remodeling. For example, an alternatively spliced form of CIP4 localizes in the phagocytic cup of RAW murine macrophage cells, indicating a potential function in phagocytosis (53). Furthermore, CIP4 is necessary for integrin-dependent T cell trafficking, and CIP4-deficient T lymphocytes cause impaired T-cell-dependent antibody response, impaired contact hypersensitivity, and defective adhesion to immobilized VCAM1 and ICAM1 in endothelial cells, leading to impaired transendothelial migration (148).

Two additional F-BAR proteins, PSTPIP1 and PSTPIP2, containing an F-BAR and SH3 domain and a sole F-BAR domain, respectively, are also involved in immune function. PSTPIP1 recruits PTP-PEST to immune synapses where it negatively regulates the antigen-dependent activation of T cells. Point mutations of PSTPIP1 are found in conjunction with pyogenic sterile arthritis, pyoderma gangrenosum, and acne (PAPA) syndrome (318), where the binding to PTP-PEST was markedly reduced. In contrast, the interaction of the mutated PSTPIP1 with pyrin protein, which mediates the inflammatory response and is responsible for the familial Mediterranean fever protein, was markedly increased (264). PSTPIP2 also appears to be a negative regulator of the immune system as it antagonizes FBP17 by competing for membrane binding sites, resulting in the suppression of immune system hyperactivation (302). PSTPIP2 point mutations and protein deficiency in mice in fact induce auto-inflammatory disease and exhibit a similar phenotype to chronic recurrent multifocal osteomyelitis (35, 66, 96).

C. Muscle Disorders

The membrane-deforming ability of the BAR proteins was pioneered by the study of amphiphysin with mutation defective in muscle formation. Amphiphysin is necessary for the organization of the excitation-contraction coupling machinery (t tubule), which is long invagination in muscles, but it is not necessary for synaptic vesicle endocytosis in Drosophila (225). In mammals, mutation of amphiphysin 2 (Bin1) causes autosomal recessive centronuclear myopathy by interfering with the remodeling of t tubules and/or endocytic membranes (152, 225). In this case, two missense mutations affecting the BAR domain disrupt the membrane tubulation properties in transfected cells (206).

Bin3, consisting only of an N-BAR domain, is known to be required for proper formation of multinucleated muscles both in vitro and in vivo. During early myogenesis, Bin3 promotes migration of differentiated muscle cells, where it colocalizes with F-actin in lamellipodia. Importantly, Bin3 forms a complex with Rho GTPases, such as Rac1 and Cdc42, which are essential for myotube formation (265). Bin3 knockout mice showed the delayed muscle regeneration (265). Interestingly, lack of Bin3 in differentiated muscle cells prior to myotube formation led to defects in lamellipodia formation and cell migration in addition to significantly decreased levels of active Rac1 and Cdc42. These data suggest a major role for a Bin3-dependent signaling pathway in regulating Rac1- and Cdc42-dependent processes during myotube formation (265).
D. Neuronal Diseases

The endocytosis and exocytosis of neurotransmitters and their receptors are crucial for a large number of neuronal functions. Dynamin, endophilin, and PACSIN1/Syndapin I are essentially involved in such processes, presumably through their functions in scission, subsequent uncoating of cargo, and/or recycling. PACSIN1 is crucially involved in the morphogenesis of neuronal cells in cooperation with N-WASP and dynamin (48). Furthermore, PACSIN1 knockout mice suffer from seizures, a phenotype consistent with excessive hippocampal network activity (143) as well as defects in presynaptic membrane trafficking processes. At the molecular level, it appears that PACSIN1 plays an important role in the recruitment of all dynamin isoforms and is a central player in vesicle-membrane fission reactions (145, 216). Thus PACSIN1 acts as a pivotal membrane-anchoring factor for the dynamins during regeneration of synaptic vesicles.

Unlike PACSIN1, triple knockout of all three mouse endophilin proteins did not cause defects in endocytotic vesicle scission, but led to defects in the uncoating of clathrin-coated vesicles, thereby reducing the dynamics of synapse transmission (182). This defect is likely caused by the loss of the ability of endophilin to bind to and recruit synaptotagmin, the PI(4,5)P2 phosphatase that functions to uncoat these vesicles (207, 249). However, further studies are needed to elucidate the precise mechanisms involved in this process (6, 145, 171, 182, 290).

Interestingly, CIP4 appears to function in the formation of neurite, although it is unclear how CIP4 functions in endocytosis or other processes in neurons. CIP4-null neurons are specifically precocious in forming neurites (stage 1–2 transition), but not in polarization (stage 2–3 transition), indicating that CIP4 expression inhibits neuritogenesis. If CIP4 is a negative regulator of neuritogenesis, then the CIP4-null neurons would be expected to form longer neurites than those of the wild-type controls, and this is indeed the case. The CIP4-null cortical neurons extend axons 1.5 times longer than the controls at 1 day in vitro (236). One additional link between CIP4 and neuronal health is through the huntingtin gene, which CIP4 can bind to, thus potentially affecting the symptoms and progression of Huntington’s disease (113).

As described above, srGAPs which have Rho-GAP activities are important multifunctional adaptor proteins involved in various aspects of neuronal development, including axon guidance, neuronal migration, spine maturation, and synaptic plasticity. Therefore, the defects of srGAP genes are thought to link to some neurodevelopmental disorders, such as mental retardation, schizophrenia, and seizure. The srGAP3 protein, alternative name of mental-disorder associated GAP protein (MEGAP), is reported to be disrupted and functionally inactivated by a translocation breakpoint in a patient who shares some characteristic clinical features, such as hypotonia and severe mental retardation, with the 3p– syndrome (caused by deletions affecting many genes at the terminal end of chromosome 3p) (60). Loss of srGAP3 was found to result in reduced density of spines and be linked to impaired learning and memory (29). Its knockout mice lead to mismigration of postnatal neural progenitors and blockage of the cerebral aqueduct, inducing lethal hydrocephalus (139) or schizophrenia-related behaviors (313). On the other hand, srGAP2 is implicated in a severe neurodevelopmental syndrome causing early infantile epileptic encephalopathy (239). Interestingly, there are human specific splicing isoforms of srGAP2, which might be involved in human specific brain development (30).

Intriguingly, the role of PACSIN1/Syndapin I also extends to cilia formation in the sensory hair cells of the inner ear in zebrafish, where it is required for formation of both microtubule-dependent kinocilia and F-actin-rich stereocilia (247). Furthermore, two less characterized F-BAR domain-containing proteins, FCHSD1 and FCHSD2, are also reported to be involved in hair cell stereocilia function through regulation of actin polymerization (28).

VII. CONCLUSIONS AND OUTSTANDING QUESTIONS

All cellular organelles have a membrane separating it from the rest of the cell, with the cell itself being surrounded by the PM. Although the development of the electron microscope has helped to reveal many of the micro-structures present on these membranes, these structures are often complex, difficult to visualize, and short-lived. Thus we only have a fragmented understanding of how these membrane structures are generated. Many of these structures are thought to be generated through protein-lipid interactions, and numerous factors in the genome appear to encode proteins for such functions. Recently, noncoding RNAs have been shown to function in various aspects of membrane biochemistry/morphology, mainly through manipulation of transcription. However, RNA and other nucleic acids do have a strong negative charge, making it possible, albeit at high concentrations, for them to affect the interactions between the anionic membrane lipids and the cationic residues of membrane deforming proteins. However, it is not clear whether RNA and other nucleic acids interact with membrane-deforming proteins.

Although we have a basic understanding of the membrane-binding proteins at present, there are no genome-wide studies identifying the whole list of proteins with lipid-binding abilities. Several attempts have been made to begin the membrane-binding domain screening process (138); however, few membrane-binding gene families have been found to date. When we consider the total number of human proteins, then the total number of known membrane curvature-
related proteins might be small in percentage. The occurrence of the proteins containing an ALPS motif in the whole genome is totally 385 among the 14,049 human proteins analyzed, suggesting 2.7% of the total number of human proteins according to the prediction program (55). Furthermore, the total number of BAR domain-containing proteins in the human genome, including BAR, F-BAR, and I-BAR, is ~70 (238), while the number of ENTH domain-containing proteins is only 21, according to the SMART database (248). With such a low percentage of proteins being able to interact with the membrane, the functional loss of just one can lead to any number of cellular defects or diseases. However, we know that the cluster of the basic-charged amino acid residues is sufficient for membrane binding, and there are thought to be a lot of proteins with such amino acid sequence. Therefore, we can assume that we only know a part of the whole list of the membrane binding protein. Thus it is increasingly important to determine the possible membrane-binding ability of all of the proteins to understand how proteins are regulating membrane morphology in a particular organism, such as humans.

Our ability to study membrane-protein interactions is entirely dependent on the methodology used to visualize this relationship. The structural determination of protein domains, combined with superresolution microscopy (which enables resolution as high as 10 nm, corresponding to the size of a single protein), will likely revolutionize the field, pushing our understanding of membrane structure formation forward. With these techniques, we may eventually be able to map individual proteins, molecule by molecule, to determine their specific location in the cell. With the ability to identify protein localization in such nanoscale structures, we are confident that the next generation of cell biology inquiry will begin. These new techniques should be applied to study the assembly of BAR proteins and their binding proteins, as well as the localization of phosphoinositides. At present, we do not have a lot of examples of ordered assembly of proteins in vivo. The new techniques might unravel unexpected ordered assembly of the BAR proteins as we have already observed in in vitro reconstitution experiments.

ACKNOWLEDGMENTS

S. Suetsugu and T. Takenawa contributed equally to this work.

Address for reprint requests and other correspondence: T. Takenawa, Graduate School of Medicine, Kobe University, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan (e-mail: takenawa@med.kobe-u.ac.jp).

GRANTS

This work was supported by the Japan Society for the Promotion of Science (JSPS) through the “Funding Program for Next Generation World-Leading Researchers (NEXT Program)” initiated by the Council for Science and Technology Policy (to S. Suetsugu), JSPS Grants-in-Aid for Scientific Research Grant 25860215 (to S. Kurisu), and JSPS Grants-in-Aid for Scientific Research Grant 23227005 (to T. Takenawa).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES

SHAPING OF CELLULAR MEMBRANES


146. Kochubey O, Lou X, Schneggenburger R. Regulation of transmitter release by Ca2+


149. Kochubey O, Lou X, Schneggenburger R. Regulation of transmitter release by Ca2+


159. Loisel TP, Boujemaa R, Pantaloni D, Carlier MF. Reconstitution of actin-based motility by 10.220.33.5 on April 20, 2017 http://physrev.physiology.org/ Downloaded from
SHAPING OF CELLULAR MEMBRANES


Physiol Rev • VOL 94 • OCTOBER 2014 • www.prv.org

Downloaded from http://physrev.physiology.org/ by 10.220.33.5 on April 20, 2017


