Review

Phosphoinositide-binding interface proteins involved in shaping cell membranes

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Abstract: The mechanism by which cell and cell membrane shapes are created has long been a subject of great interest. Among the phosphoinositide-binding proteins, a group of proteins that can change the shape of membranes, in addition to the phosphoinositide-binding ability, has been found. These proteins, which contain membrane-deforming domains such as the BAR, EFC/F-BAR, and the IMD/I-BAR domains, led to inward-invaginated tubes or outward protrusions of the membrane, resulting in a variety of membrane shapes. Furthermore, these proteins not only bind to phosphoinositide, but also to the N-WASP/WAVE complex and the actin polymerization machinery, which generates a driving force to shape the membranes.

Keywords: phosphoinositide, membrane-deforming proteins, N-WASP, WAVE, BAR domain, F-BAR domain

1. Introduction

The mechanisms of cell polarity, patterning, and movement remain a compelling mystery, but reorganization of the cortical actin cytoskeleton and membrane deformation in response to extracellular stimuli are thought to be key events in these phenomena. Nevertheless, it is still unclear how these characteristic structures are formed by the dynamic collaboration between the actin cytoskeleton and the plasma membrane. We found a variety of membrane-deforming proteins,[1)–3)] connecting the actin cytoskeleton and the plasma membrane. These proteins contain the BAR, EFC/F-BAR, and IMD/I-BAR domains. We also found neural Wiskott-Aldrich syndrome protein (N-WASP) and WASP-family verprolin-homologous (WAVE) proteins, which are involved in cortical actin polymerization and cell movement.[4)–7)] Recently, it has been proved that these proteins dynamically collaborate to shape various membrane microstructures[7)] such as clathrin-coated pits and caveolae, which are involved in endocytosis, and protrusions of actin filaments such as filopodia and lamellipodia that lead to cell movement.

To understand this review more easily, molecular interactions of phosphoinositide-binding proteins are summarized in Fig. 1.

2. Phosphoinositide-binding proteins

Until the end of the 1980s, the basic concepts related to inositol lipid signaling were established mainly by 2 scientists Dr. Mike Berridge[8)] and Dr. Yasutomi Nishizuka.[9)] They proposed that phospholipase C (PLC) is activated through the receptors of a variety of hormones and growth factors, leading to the generation of 2 second messengers—diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (Ins(1,4,5)P3)—from phosphatidylinositol 4,5-bisphosphate(PI(4,5)P2), resulting in various cellular responses.

Besides their role as second messenger-generating lipids, phosphoinositides have been found to regulate actin reorganization by modulating the activity of actin regulatory proteins. We developed a monoclonal antibody against PI(4,5)P2[10),11)] and stained the culture cells with immunofluorescence. Surprisingly, we found that PI(4,5)P2 was present on cortical actin fibers in addition to plasma membranes. Subsequently, we found that vinculin and α-actinin are PI(4,5)P2-binding proteins[12)] and that their functions are regulated by PI(4,5)P2. We also demonstrated that PI(4,5)P2 binds to basic amino acid clusters in α-actinin, which are essential for

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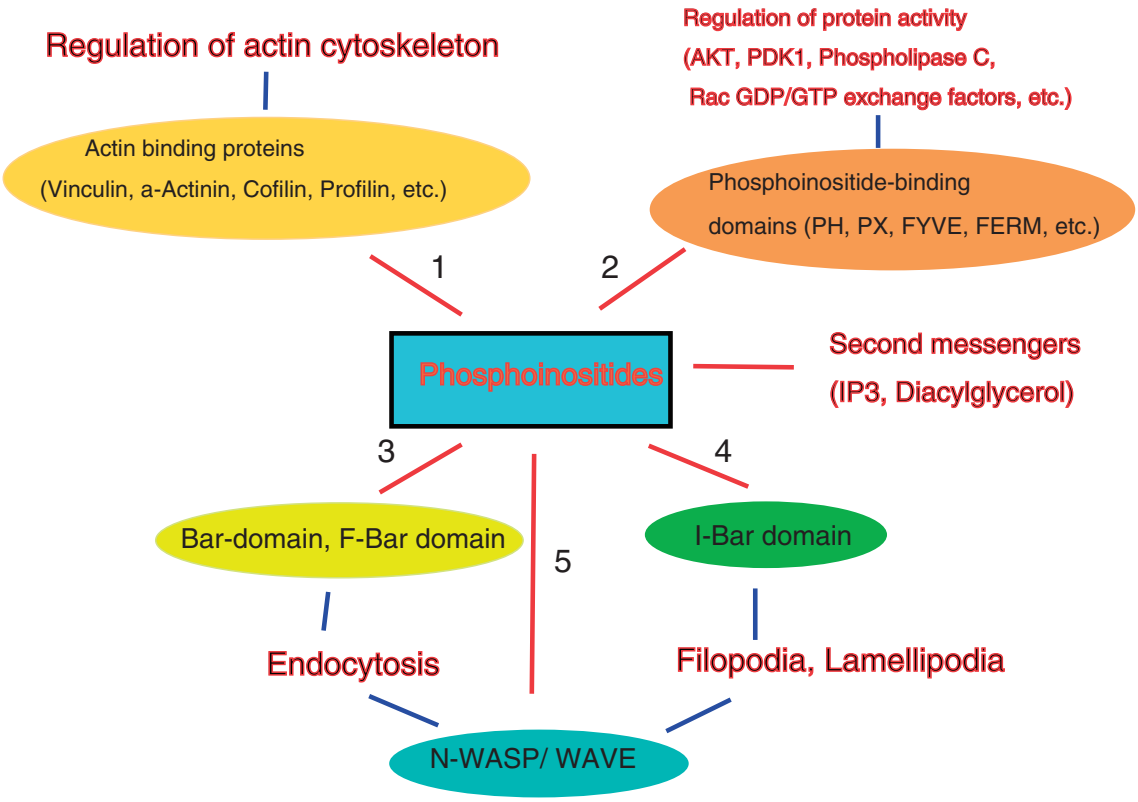

Fig. 1. A variety of physiological functions of phosphoinositide-binding proteins. Phosphatidylinositol (4, 5) bis-phosphate generates two second messengers, inositol (1, 4, 5) tris-phosphate(IP3) and diacylglycerol. In addition, phosphoinositides are involved in the regulation of actin binding proteins (1), various protein activities (2), endocytosis (3), membrane protrusions such as filopodia and lamellipodia (4) and the activities of N-WASP/WAVE proteins (5).

actin-bundling activity.[13] PI(4,5)P2 also binds to a variety of actin-binding proteins including profilin,[14] vinculin,[12),15] gelsolin,[16] cofilin,[17] ERM (Ezrin/Radixin/Moesin),[18] N-WASP,[19),20] and WAVEs.[21),22] These proteins contain basic amino acids that stretch to interact with phosphoinositides. These results suggest that inositol lipid signaling influences the remodeling of the actin cytoskeleton through changes in the levels of PI(4,5)P2. Thus, the expression of PI(4)P 5-kinase α induces massive actin filament formation.[23] In contrast, expression of synaptojanin, a phosphoinositide phosphatase,[24] causes disruption of the actin filaments. In general, an increase in PI(4,5)P2 levels tends to promote actin filament formation, whereas a decrease in PI(4,5)P2 levels leads to actin depolymerization.[25]

3. The epsin N-terminal homology domain not only binds to PI(4,5)P2 but also deforms membranes

Actin-binding proteins have motifs consisting of basic amino acids to which phosphoinositides bind, thus regulating their functions.[26] On the other hand, a variety of membrane-associated proteins have phosphoinositide-binding domains that form three-dimensional structures and bind rather strongly to phosphoinositide.[27] The pleckstrin homology (PH) domain was the first phosphoinositide-binding domain to be reported, and it is conserved among a large number of proteins (more than 250) in the human proteome.[28),29] Additional phosphoinositide-binding domains have been identified, such as PX (Phox),[30)–32] FYVE (Fab1, YOTB, Vac1p, and EEA1),[33),34] and FERM (band 4.1, Ezrin, Radixin, and Moesin)[18),35] domains.

We also found that the epsin N-terminal homology (ENTH) domain was a new PI(4,5)P2 binding domain.[1] This domain has a unique characteristic that distinguishes it from the other phosphoinositide-binding proteins already reported. Surprisingly, the epsin ENTH domain exhibits membrane-deforming activity. Epsin is a component of the huge

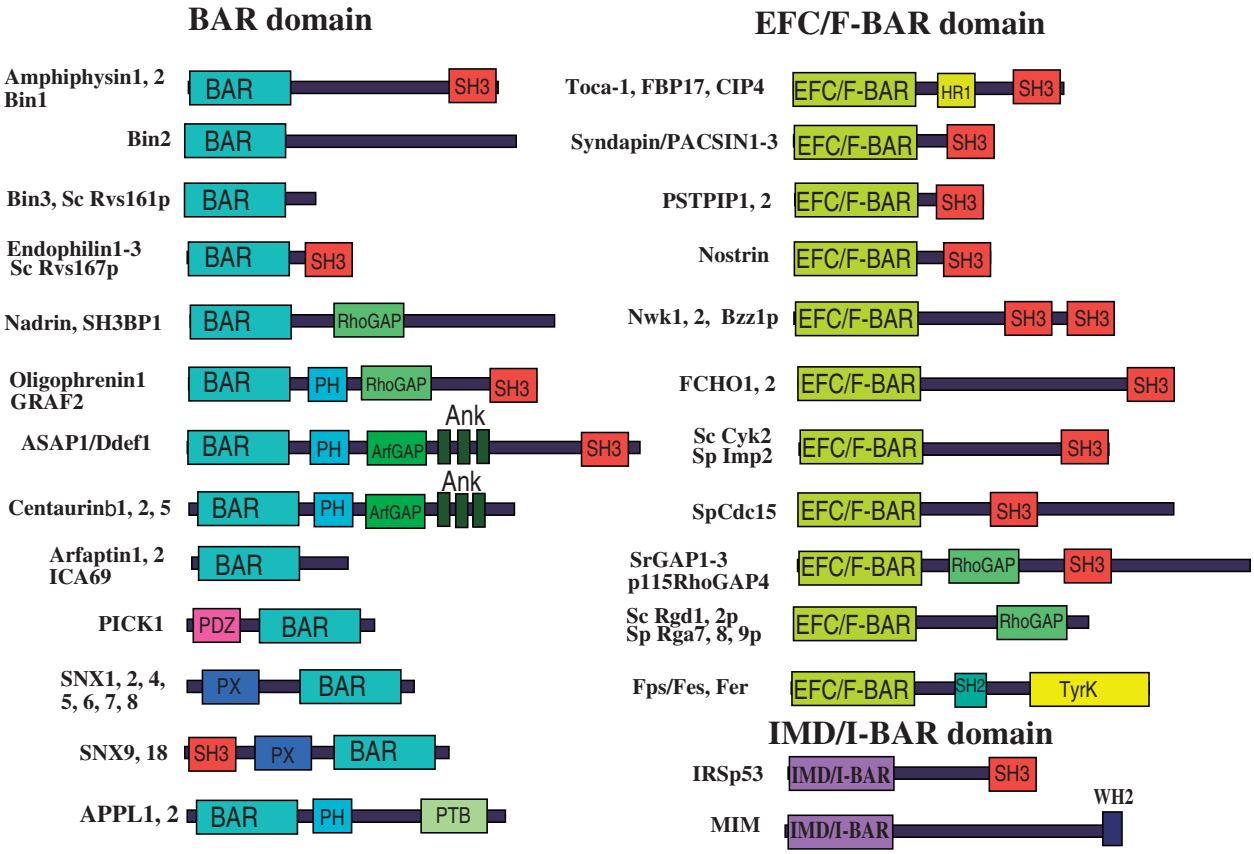

Fig. 2. Proteins that contain the BAR, EFC/F-BAR, and IMD/I-BAR domains.

clathrin complex and is involved in endocytosis. Structural study showed that the ENTH domain of epsin consists of an α-helix and an N-terminal unstructural region. Furthermore, nuclear magnetic resonance (NMR) analysis of the ENTH domain in the presence of Ins(1,4,5)P3 revealed that an unstructural region is strongly associated with Ins(1,4,5)P3. Comparison of chemical shift values in the presence or absence of Ins(1,4,5)P3, the head group of PI(4,5)P2, showed a large structural change at the N-terminal region upon PI(4,5)P2 binding. In fact, the complete deletion of N-terminal 18 residues or the alanine substitution of Arg8 abolished phosphoinositide binding. These results clearly indicate the key role of the N-terminal region in lipid binding. Subsequent elucidation of the crystal structure of the Ins(1,4,5)P3-bound epsin ENTH domain[36] showed that the N-terminal region forms an α-helix after binding to Ins(1,4,5)P3, thus retaining an amphipathic property. In addition, it has been demonstrated that the epsin ENTH domain can deform liposomes into tubules by inserting its N-terminal α-helix region into the liposome in a PI(4,5)P2-dependent manner. The tubulation activity of ENTH corresponds with its ability to deform a flat plasma membrane into an invaginated clathrin-coated pit. The key structural factor for membrane deformation is the presence of the amphipathic helix. In the epsin ENTH domain structure, an amphipathic helix is formed only when bound to PI(4,5)P2.[37),38)] This membrane deformation activity is essential for endocytosis of the epidermal growth factor (EGF).

4. Phosphoinositide binding and membrane deformation by the BAR and EFC/F-BAR domains

The Bin-Amphiphysin-Rvs167 (BAR) domain was originally found in the N-terminal region of highly conserved mammalian and yeast amphiphysins. Increasing numbers of BAR domain-containing proteins including amphiphysin, endophilin, nadrin, oligophrenin, arfaptin, sorting nexin, ASAP1, PICK1, APPL, and proteins of the centaurin subfamily have

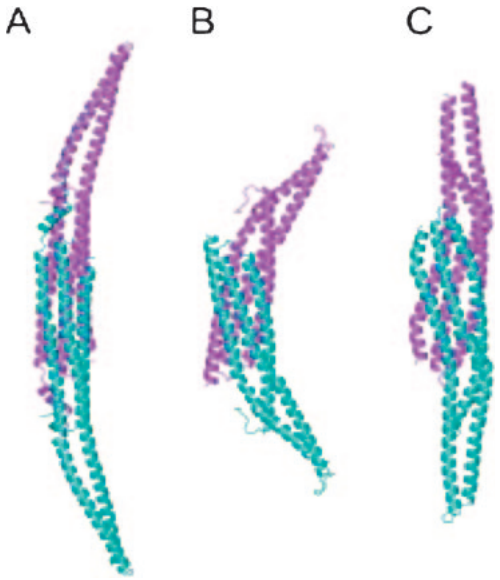

Fig. 3. Three-dimensional structures of BAR, EFC/F-BAR, and IMD/I-BAR domains. Ribbon representation of the dimmers of BAR (Amphiphysin), EFC/F-BAR (FBP17), and IMD/I-BAR (IRSp53).

been found (Fig. 2). All BAR domains form homodimers and deform PI(4,5)P2-containing liposomes to membrane tubules *in vitro*.[39] The structure of the BAR domain of amphiphysin was the first to be solved, and the protein was demonstrated to be important for membrane deformation. The BAR domain structure is a banana-shaped dimer in which the concave surface is positively charged (Fig. 3).[39] The positively charged concave surface of the domain attaches to the negatively charged inner surface of the plasma membrane, mostly through PI(4,5)P2 and phosphatidylserine. Furthermore, BAR domains from endophilin and amphiphysin have hydrophobic amino acids on their concave surfaces or dimer ends.[39)–41] The hydrophobic amino acids on the concave surface are inserted into the membrane, thereby strengthening the interaction between the membrane and the BAR domain. The BAR domain of amphiphysin deforms the membrane into narrow tubular invaginations, which are considered to correspond to the location of amphiphysin function at the late stage of endocytic vesicle formation. The BAR domain of SNX9 is closely connected to the PX domain, but the BAR and PX domain units of SNX9 have broad phosphoinositide specificity.[42]

The Fer-CIP homology (FCH) domain is found extensively in the pombe Cdc15 homology (PCH) protein family and is highly conserved from yeast to mammals. Most PCH proteins have the SH3 domain at the C-terminus. PCH family members are known to be involved in actin cytoskeleton organization and endocytic events. All PCH proteins possess an evolutionarily conserved region, which was predicted to be a coiled-coil structure extending to the C-terminal region from the FCH domain. These 2 regions, the FCH domain and the extended coiled-coil region, were found to be a single functional module. Therefore, we named this new domain the extended FCH (EFC) domain (also called the F-BAR domain).[2),43] EFC/F-BAR-containing proteins can be divided into 7 subfamilies: the CIP4 subfamily, Fes/Fer tyrosine kinases, srGAP subfamily, PACSIN/Syndapin subfamily, PSTPIP subfamily, Nostrin subfamily, and the FCH-domain only (FCHO) subfamily (Fig. 2). The EFC/F-BAR domain shows a weak homology to BAR domains, with well-conserved residues important for the helix packing. As is the case with the BAR domain, the EFC/F-BAR domain of FBP17 also induces robust membrane tubulation both *in vitro*[43),44] and *in vivo*.[2),43),45] The EFC/F-BAR domain of FBP17 show preferential binding to PI(4,5)P2, but it also binds to phosphatidylserine.[2),43] Positively charged amino acids are consistently conserved in the EFC/F-BAR domain and are required for lipid binding and for tubulation. Expression of CIP4, Toca-1, PSTPIP1, and PSTPIP2 also induced membrane tubulation in cells. However, expression of Fer resulted in little tubulation in cells, though the protein could efficiently induce tubulation in liposomes.[2] Expressions of srGAP1 and 2 did not induce tubulation, but rather, induced filopodia-like protrusions *in vivo*; srGAP2, in particular, formed remarkable filopodia-like protrusions.[46] These results suggest that EFC/F-BAR-containing proteins induce a variety of membrane shapes that are different from BAR domain-containing proteins.

We first solved the structure of the EFC from CIP4 and FBP17 among the EFC/F-BAR domains (Fig. 3).[47] The EFC/F-BAR domains of Toca-1 are almost identical to those of CIP4 and FBP17.[2),43),47] The EFC/F-BAR domain forms a banana-shaped dimer in which the positively charged concave surface binds to the negatively charged membrane.[47] Besides the interface for domain dimerization, there are additional interfaces between neighboring EFC/F-BAR dimers. The dimers are arranged to form filaments. These interfaces involve several hydrogen-bonding interactions between neighboring dimers. Phase-contrast cryo-transmission electron microscopy (cryo-TEM) has shown striated structures, which

represent the tight EFC/F-BAR spirals covering the tubular membranes. These striations seemed to be perpendicular to the main axis of the tubular membranes. The average spacing between neighboring striations was 42 Å, and the diameters of EFC/F-BAR-induced tubular membranes were about 600 Å. As for the BAR domain, overexpression of the EFC/F-BAR domain protein fragment alone induces tubular membrane invaginations in cells. Overexpression of full-length FBP17 also induces tubulation. The curvature generated by the EFC/F-BAR domain of CIP4 and FBP17 was much larger than that induced by the BAR domains from amphiphysin or endophilin, and the curvature appeared to correspond to the curvature of the initial stages of clathrin-coated pits. FBP17 and CIP4 function in the endocytosis of clathrin-coated pits, presumably by recruiting dynamin and actin polymerization machinery to the membrane curvature for clathrin-coated pits.[2),43),47),48)] In a reconstitution assay, FBP17 and Toca-1 induced N-WASP and the Arp2/3 complex-mediated actin polymerization, which is dependent on membrane curvature.[49)]

PACSIN/Syndapin forms 1 subfamily of the F-BAR domain protein family.[7),43)] The crystal structure of the PACSIN F-BAR domain shows a shallower concave surface, and the membrane tubules induced by the PACSIN F-BAR are narrower than those induced by the F-BAR domains of FBP17, CIP4, and Toca-1. The narrower diameter of the tubules induced by PACSIN2 suggests that PACSIN2 is recruited to the clathrin-coated pit at the late stage of clathrin-coated vesicle fission, during which narrower membrane tubules connect the plasma membrane to the coated vesicles. However, PACSIN did not colocalize with clathrin.[50)] Therefore, the F-BAR domain of PACSIN2 forms a clearly distinct subfamily in the F-BAR family, and PACSIN2 appears to be involved in different biogenesis of cellular organelles than the BAR or F-BAR domains. The F-BAR domains of PACSIN1 and PACSIN2[51)] have a hydrophobic insertion loop, as found in the endophilin BAR domain and in the MIM IMD/I-BAR domain. More importantly, in contrast to the canonical F-BAR proteins, such as FBP17 and Toca-1, PACSIN2 overexpression did not cause membrane tubule formation in cells. However, the treatment of cells with latrunculin B induced membrane tubulation, suggesting that the actin filament suppresses tubulation to maintain the plasma membrane and to prevent the plasma membrane structures from undergoing excess tubulation or invagination. Although the mechanism of suppression of tubulation by actin polymerization is unknown, the SH3 domain of PACSIN/Syndapin also binds to N-WASP and dynamin.[52)]

The EFC/F-BAR domains bind to phosphatidylserine and PI(4,5)P_2 and deform artificial liposomes and cell membranes into tubules. The binding of the EFC/F-BAR domains to phosphatidylserine has been demonstrated by many laboratories and is supported by its crystal structure.[2),43),47),51)] However, on the basis of its strong negative charge and its involvement in endocytosis, the binding of the EFC/F-BAR domain to PI(4,5)P_2 is also plausible; moreover, many proteins involved in endocytosis bind to PI(4,5)P_2.[44)] The F-BAR domain of FCHo2 also forms a banana-shaped dimer, but the curvature of its membrane-binding concave surface is larger than that of EFC/F-BAR, CIP4, and FBP17.[47),53)] In contrast to the EFC/F-BAR of CIP4 and Toca-1, the lateral surface of the F-BAR domain of FCHo2 is curved.[53)] However, the location of the FCHo2 function in cells is still unclear, and except the F-BAR domain, no functional domains have been assigned to FCHo1 or FCHo2.

5. Tyrosine kinase activity of Fer is regulated through the binding of phosphatidic acid to EFC/F-Bar and FX domains

In the case of Fer, the N-terminal EFC/F-BAR domain could not strongly bind to liposomes consisting of acidic phospholipids; but, that of CIP4 could bind to liposomes remarkably. The Fer protein binds to liposomes with high affinity through an unknown FX domain that is near the EFC/F-BAR domain.[54)] This domain preferentially binds to phosphatidic acid (PA) among a variety of acidic phospholipids. Interestingly, the addition of PA-containing liposome remarkably increases tyrosine kinase activity of Fer and promotes the phosphorylation of cortactin. This, in turn, activates Arp2/3 complex-mediated actin polymerization and Vav, which is an activator of Rac. The knockdown of Fer expression suppresses migration and expression of wild-type Fer could rescue this migration. In contrast, the expression of a PA-binding-deficient mutant in which simultaneous substitutions of positively charged residues in the FX domain (Arg417, Arg425, and His426) with alanine (FX AAA) were made, could not rescue migration. Further, the overexpression of Fer enhanced lamellipodia formation and cell migration. More importantly, the simultaneous expression of both GFP-Fer and HA-phospholipase D2 (PLD2) resulted in a

marked increase in the number of migrated cells, whereas this synergistic activation was abolished when wild-type PLD2 was replaced with a lipase-dead form of PLD2 (PLD2KR). Thus, it is clear that the PLD-PA pathway promotes cell migration via Fer-induced actin polymerization, beneath the plasma membrane.[54]

6. N-WASP and WAVE assemble cortical actin filaments, which generates a motile force

Rapid actin polymerization is induced to form filopodia and lamellipodia when cells move in response to extracellular signals. In general, the barbed ends of actin filaments are capped by large redundant proteins to prevent spontaneous elongation of the filaments in resting cells. Therefore, to trigger rapid actin polymerization for rearrangement of the cortical cytoskeleton and movement in response to extracellular stimuli, cells must expose these barbed ends or form actin nuclei to produce new filaments. We found 2 new proteins—N-WASP[19),20)] and WAVEs[21),55)]—that activate actin nucleation proteins, i.e., the Arp2/3 complex,[56)] and then elongate actin filaments from the sides of the already existing actin filaments (Fig. 4). This results in the mesh-like actin filaments that are typically observed in the leading edges of moving cells.[5),6)] Thus, this system can bypass the step involving the exposure of barbed ends in order to quickly form mesh-like actin filaments when cells move.

WASP has been identified as the causative gene product of Wiskott-Aldrich syndrome, which is characterized by eczema, bleeding, and recurrent infections.[57)] This protein is expressed exclusively in hematopoietic cells. Lymphocytes from WAS patients show cytoskeletal abnormalities with a reduction in the number of cell surface microvilli.[58),59)] WASP binds Cdc42 through its GBD/CRIB (GTPase-binding domain/Cdc42 and Rac interactive binding) domain, and overexpression of WASP induces formation of actin clusters.[60)] Although these data suggest that WASP is related to the organization of actin filaments, the physiological function was unclear until the isolation of N-WASP. N-WASP was first purified as a protein that bound the Ash/Grb2 Src-homology 3 (SH3) domain,[61),62)] and the corresponding cDNA was isolated in 1996.[19)] N-WASP shows approximately 50% amino acid homology with WASP, and it contains several multifunctional domains (Fig. 4), including the WH1/EVH1 (WASP homology/Ena VASP homology) domain, a basic region, the GBD/CRIB domain, a proline-rich region, the verprolin-homology region (V), a cofilin-like region (C), and an acidic region (A).

The basic region of N-WASP binds PI(4,5)P2,[19),20)] and N-WASP is thought to be anchored to the membrane through this region. Full-length N-WASP does not activate the Arp2/3 complex as strongly as it activates the VCA region, suggesting that the VCA region is masked in the inactive N-WASP. In the presence of Cdc42 and PI(4,5)P2, however, N-WASP activates the Arp2/3 complex to a level comparable to that of VCA protein.[56)] Although we had previously predicted that the basic region interacts with the VCA region, leading to the masking of the VCA region, these data also support the idea that the folded N-WASP molecule is unfolded by the binding of Cdc42 and PI(4,5)P2, thus exposing the VCA region. Rohatgi *et al.*[63)] showed that the interaction between the GBD/CRIB domain and C-terminal region must be intramolecular because N-WASP is present as a monomer in solution. They further showed that PI(4,5)P2 influences the activity of N-WASP through a conserved basic sequence located near the Cdc42 binding site. As with Cdc42, PI(4,5)P2 reduces the interaction between the N-terminal and C-terminal regions.

In addition to N-WASP, we have also identified WAVE as a novel protein with a VCA domain.[21),55)] This protein has a WAVE homology domain (WHD), a basic region (B), a proline-rich region (P), and a VCA region (Fig. 4). Therefore, the C-terminal region of this protein is highly homologous to that of N-WASP. WAVE, however, does not have the GBD/CRIB domain, and therefore, it is not likely to be activated downstream of Cdc42 in the signaling cascade. Furthermore, 2 additional homologous but unique genes were isolated. They were named WAVE2 and WAVE3, and the original WAVE was renamed WAVE1.[55)]

To date, 3 isoforms of WAVE proteins have been isolated.[55)] Endogenous WAVE localizes at the area of membrane ruffling that is induced by treatment with platelet-derived growth factor (PDGF) and expression of dominant active Rac.[21)] A mutant WAVE protein with the V domain deleted, which acts as a dominant-negative, blocks the formation of membrane ruffles induced by Rac. These data suggest that WAVE regulates membrane ruffle formation through activation of the Arp2/3 complex downstream of Rac. WAVE has previously been shown to form complexes with Rac when both molecules were

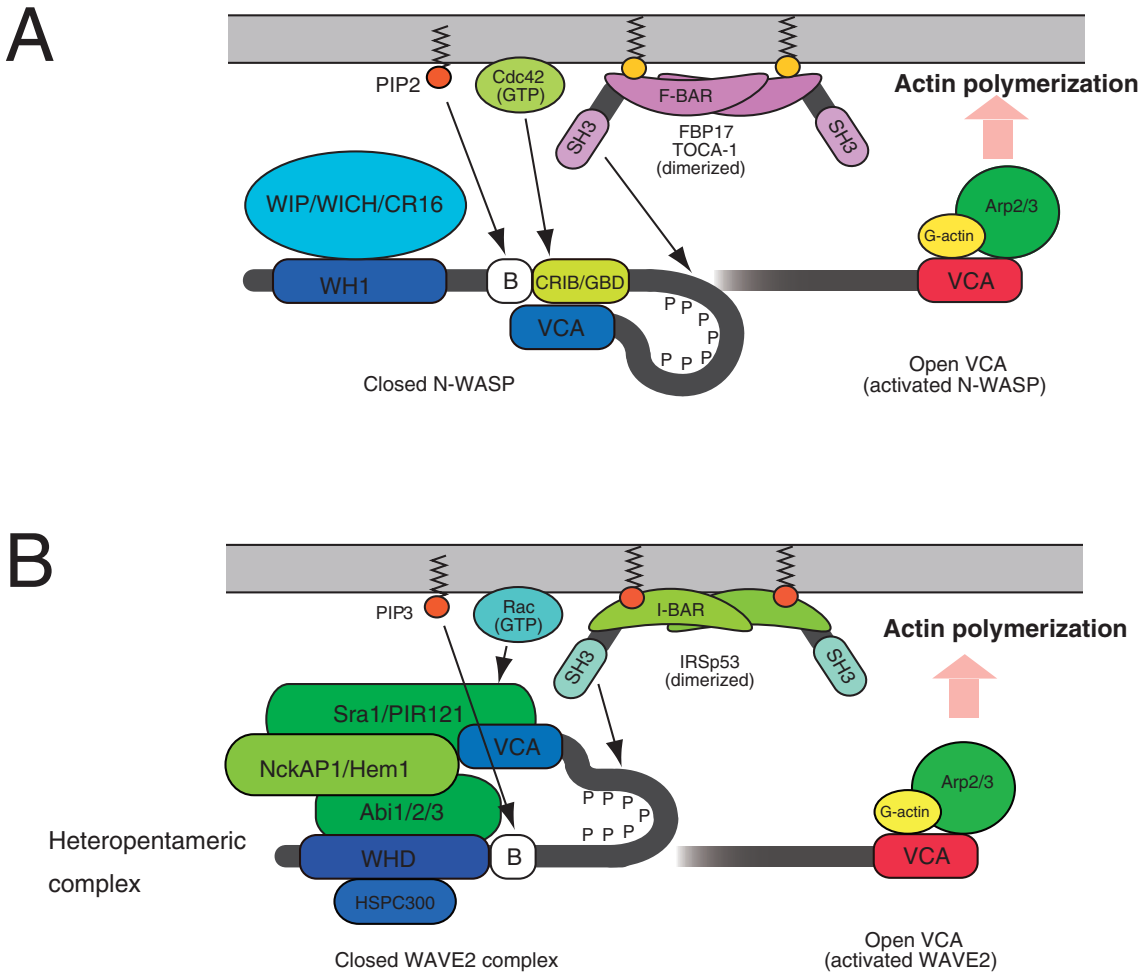

Fig. 4. Activation mechanism of N-WASP and WAVE. N-WASP has an N-terminal WASP-homology 1 (WH1) domain, where WIP, CR16, or WICH bind. WAVE2 has an N-terminal WAVE-/SCAR-homology domain (WHD/SHD) that mediates protein complex formation with HSPC300, Abi, Nap1, and PIR121/Sra1. The basic region (B) is common to both N-WASP and WAVE2, where phosphoinositides (PIP2 or PIP3) bind for protein localization or activation of the Arp2/3 complex. N-WASP has the Cdc42-Rac-interactive binding region (CRIB) for Cdc42 binding. WAVE2 binds to Rac through PIR121/Sra1 in the WAVE2 complex, and through IRSp53, which binds to the proline-rich (Pro-rich) region of WAVE2. F-BAR containing proteins such as Toca-1 and FBP-7 bind to proline-rich regions of N-WASP, providing the driving force for membrane deformation. On the other hand, IMD/I-BAR of IRSp53 binds to proline-rich regions of WAVE, which generates protrusive force.

expressed in Cos7 cells.[21),64)] However, this binding does not appear to be direct, suggesting that at least 1 additional factor is required to link Rac and WAVE. Because all WAVEs have the WHD in the N-terminal region, this domain was predicted to be the site that receives the signal from Rac. It has now become clear that WAVE proteins form a heteropentameric complex (the WAVE complex) with Sra1/CYFIP1, Nap1, Abelson-interactor (Abi) 1/2/3, and HSPC300/Brick1 through the WHD domain, whose components seem to be conserved among species ranging from plants to humans.[64),65)] Among these binding proteins, Sra/CYFIP1 has been found to bind to Rac. Lack of any of these components destabilizes the WAVE complex, leading to proteasomal degradation of all the components.[66),67)] Biochemical studies suggest that direct stoichiometric association of the WHD domain with Abi and HSPC300 appears to contribute to the formation of the WAVE complex.[68)] All known WHD domains contain conserved coiled-coil motifs spanning at least 36 amino acids. These motifs are thought to associate tightly with other coiled-coil motifs existing in Abi and HSPC300. WAVEs localize at the tips of

lamellipodia, which are flat protrusions of cells extending in the direction of cell movement.[69] Lamellipodia are filled with dense networks of branched actin filaments. This actin architecture is generated by the activity of the Rho family small GTPase Rac, and WAVE was originally identified as a downstream effector for Rac-mediated actin polymerization. Subsequently, WAVEs were found to activate the Arp2/3 complex, and now WAVEs are known to be key proteins acting downstream of Rac to trigger actin polymerization by the Arp2/3 complex.[6] WAVEs are essential for cell motility since motility is accomplished by lamellipodial extension-substrate adhesion cycles. The localization of WAVEs at the tips of lamellipodia is regulated by mechanisms similar, but not identical, to those of N-WASP. Through the basic domain, WAVE2 preferentially binds to and is recruited to phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_2) rather than PtdIns(4,5)P_2.[22] The Rho-family GTPase Rac seems to recruit WAVEs to the membrane and activate them. Recently, Lebensohn and Kirschner[70] reported the detailed activation mechanism of WAVE2 by Rac. According to them (Fig. 4), WAVE2 complexes are basally inactive, though previous reports claimed the presence of a constitutive activity. Activation of the WAVE2 complex requires simultaneous interaction with prenylated Rac-GTP and acidic phospholipids as well as a specific state of phosphorylation. The GTP form of prenylated Rac seems to bind to a WAVE complex component, Sra1.[71] This interaction presumably recruits WAVEs to the membrane where WAVE is activated by acidic phospholipids, prenylated Rac, and phosphorylation.

Regulation of WAVE2 function by IRSp53 has also been suggested.[4),72] The proline-rich domain of mammalian WAVEs binds to the SH3 domain of IRSp53, which belongs to the RCB (Rac binding)/IMD (IRSp53-MIM homology domain)-containing proteins (another class of membrane-associated protein families). The RCB/IMD simultaneously binds to activated Rac, which contributes to Rac-dependent localization of WAVEs.[4),72)–75] Interestingly, as compared to WAVE1 and WAVE3, WAVE2 has a much stronger affinity to IRSp53.[72] However, immunopurified WAVE2 complex containing IRSp53 remains inactive, suggesting that IRSp53 by itself does not activate the WAVE complex.[70] Therefore, interaction with IRSp53 is likely to contribute specifically to the tip localization of WAVE2.

7. BAR and EFC/F-BAR domain-containing proteins are connected to the actin cytoskeleton through N-WASP

Several kinds of invaginated structures are formed at the plasma membrane. The most representative structures are clathrin-coated pits and caveolae. The clathrin-coated pit is rapidly internalized into cells by forming clathrin-coated vesicles. In the scission of the vesicles, actin polymerization appears to play important roles. Actin polymerization is also suggested to be involved in subsequent vesicle movement inside the cell. The endocytosis machinery includes many BAR- and EFC/F-BAR-containing proteins, and most of them bind to N-WASP as well as to the membrane scissor dynamin.[2),7),39),43),76),77]

In the case of Syndapin II/PACSIN2, it was first reported that overexpression of the SH3 domain inhibits the endocytosis of transferrin.[52),78] This inhibition was apparently a consequence of titrating out dynamin or N-WASP from the invagination structures, because the C-terminal SH3 domain of PACSIN2/Syndapin II binds to N-WASP and dynamin.[79]

Among the proteins that have BAR and EFC/F-BAR domains, amphiphysin, endophilin, SNX9, FBP17, and Toca-1 bind to N-WASP through their SH3 domains and activate N-WASP in a membrane-dependent manner (Fig. 4). The other signaling cascades, such as those mediated by small GTPases, are integrated on N-WASP to regulate the Arp2/3 complex-mediated actin polymerization.[42),49),80] PICK1 does not have an SH3 domain, but contains a PDZ domain. Interestingly, the BAR domain of PICK1 has been shown to interact with the Arp2/3 complex to suppress the nucleation of actin filaments, thereby suppressing the endocytosis of neurotransmitter receptors.[81),82]

The yeast WASP homologue Las17p was identified in a screen of mutants defective in endocytosis.[78),83),84] Las17p and Vrp1p, a yeast homologue of WASP-interacting protein (WIP), are recruited to clathrin-coated pits in the early stages of endocytosis with Bzz1p and Rvs167p, the yeast F-BAR, and BAR-containing proteins.[83)–85] Toca-1 in mammalian cells also forms a protein complex with N-WASP and WIP.[74] The recruitment of N-WASP and the involvement of the actin cytoskeleton in endocytosis have also been found in mammalian cells.[84),86] Therefore, the roles of WASP and N-WASP in endocytosis are well conserved from yeast to mammals.

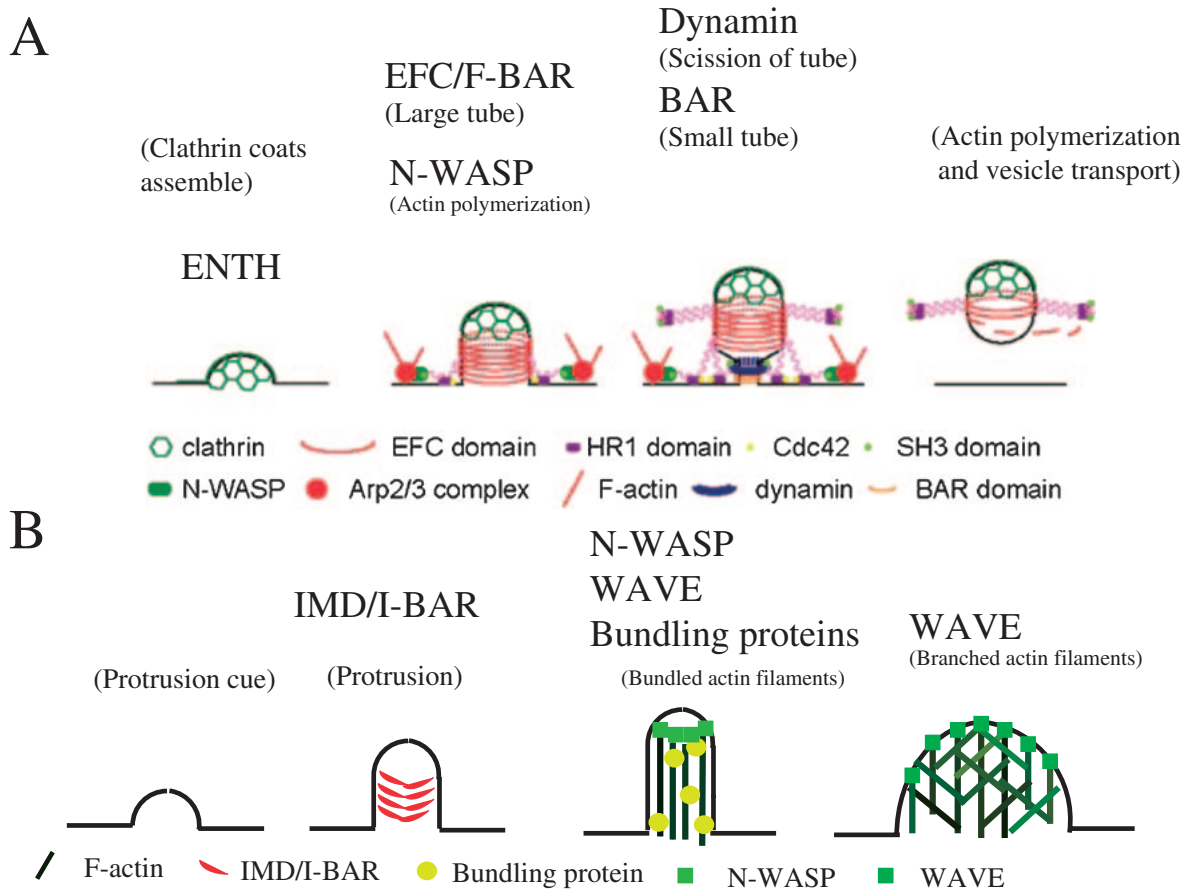

Fig. 5. Functional models of the BAR, EFC/F-BAR, or IMD/I-BAR-containing proteins and of WASP/WAVE proteins in inward or outward deformation of the membrane. A. Role of BAR, EFC/F-BAR, and N-WASP in endocytosis. B. Role of IRSp53 and WAVE in the formation of outward protrusion.

In mammalian cells, a detailed analysis of the time course of protein recruitment to the clathrin-coated pit and subsequent internalization into cells was performed.[47),86)] N-WASP, the Arp2/3 complex, and FBP17 were maximally recruited to the clathrin-coated pit when the clathrin disappeared or was endocytosed from the membrane (Fig. 5). Interestingly, dynamin recruitment to clathrin was faster than that of N-WASP or the Arp2/3 complex and was maximal when the amount of clathrin reached its maximum.[86)] Dynamin recruitment should be faster than that of FBP17 because N-WASP and FBP17 are recruited simultaneously to the clathrin-coated pit.[47)] Moreover, the tubules induced by FBP17 were antagonized by the expression of dynamin, but this dynamin-mediated antagonism of tubulation was dependent on actin filaments,[43)] suggesting that the actin filaments mediated by N-WASP are essential for the dynamin-mediated fission of tubulated membranes or clathrin-coated pits. The yeast homolog of dynamin Vps1p interacts with the actin filament binding protein Sla1p and functions in actin cytoskeletal organization.[87)]

8. IRSp53-containing IMD/I-BAR domain induces outward protrusions

There are several types of protrusive structures in cells. Most of these structures contain actin filaments, presumably for their stability, for the uptake of extracellular materials, and for the mechanical strength required for cell motility. The best-characterized cellular protrusive structures are filopodia and lamellipodia.[7),88)–90)]

Filopodia are cellular protrusions with spike- or needle-like morphology that contain bundled actin filaments. Lamellipodia are relatively flat cellular

structures that protrude in the direction of cell movement. Branched actin filaments fill the inside of the lamellipodia. Protrusive structures driven by actin polymerization are also observed at phagocytosis. During phagocytosis, a protruding lamellipodia-like structure surrounds the material that is incorporated into the cell.

For a long time, the local cellular structural elements of filopodia and lamellipodia morphologies were considered to be generated solely as a consequence of actin polymerization. WAVE2-mediated activation of the Arp2/3 complex is essential for the formation of branched actin filaments in lamellipodia.[7] The actin bundle formation of filopodia appears to be generated by several pathways, which are classified as Arp2/3 dependent and Arp2/3 independent.[91] In the Arp2/3-dependent mechanisms, the branched filament is bundled by additional factors. In the Arp2/3-independent mechanisms, the unbranched filaments are bundled. These mechanisms appear to be sufficient for the generation of protrusions. Therefore, the discovery of membrane protrusions lacking actin filaments was surprising, and their existence *in vivo* is still in question.

We have already found that IRSp53 plays an important role in lamellipodia formation by WAVE2.[4),72] IRSp53 and MIM contain the IMD/inverse-BAR (I-BAR) domain (Fig. 2). The IMD/I-BAR domain binds to the membrane through its convex surface. Owing to the inverted geometry of the membrane-binding surface (Fig. 3), the IMD domain is involved in the plasma membrane protrusions of filopodia and lamellipodia (Fig. 5).[4),73),92),93] The binding of IMD to the membrane on the inner surface of the tubules was confirmed by cryo-electron microscopy.[91] Most of the interaction occurred through phosphatidylserine, but a preference for $PI(4,5)P_2$ and $PI(3,4,5)P_3$ was observed for the IMD of IRSp53.[91),94] The IMD from MIM also has an α helix for insertion into the membrane.[91]

The IMD of IRSp53 binds to the active form of Rac, whereas the IMD of MIM binds to the inactive form.[4),72),95] These differences in the affinities to the small GTPases may modulate the membrane binding ability of the IMDs.

It is clear that the overexpression of the IMD fragment alone induces highly dynamic membrane protrusions that persist even in the presence of an actin polymerization inhibitor.[4),93),96),97] When the full-length proteins are overexpressed, the induced protrusions contain actin filaments, presumably because the SH3 domain recruits proteins that induce the formation of actin filaments. However, several regions without actin filaments were observed in these protrusions.

Interestingly, the SH3 domain of IRSp53 binds to the Arp2/3 activator; WAVE2, which plays essential roles in lamellipodium formation; and also to N-WASP, which is considered to function in filopodium formation and endocytosis.[4),72),98] MIM has the V/WH2 domain and directly binds to actin.[88),99] The binding of IMD to the actin filament has been confirmed by several laboratories.[4),92),93] The IRSp53 SH3 domain also binds to dynamin,[98] but the significance of dynamin in membrane protrusions is still unclear. IRSp53 also has a PDZ binding motif, and it binds to several proteins with the PDZ domain, which may be important for the assembly of some cellular structures.[100)–103]

Currently, the IMDs and srGAP2 are reported to induce filopodia-like protrusions *in vivo*. However, it is not clear whether srGAP2, which has an EFC/F-BAR domain, induces filopodia-like protrusions using the same mechanism as that used by IMD. This problem cannot be solved until the three-dimensional structure of srGAP2 is determined. Interestingly, IRSp53 is involved in both filopodium and lamellipodium formation, as suggested from its localization and also from its binding to WAVE2 and N-WASP, VASP, and Mena.[72),98),104),105] An analysis with N-WASP knockout cells indicated that the IRSp53-mediated formation of filopodium-like protrusions requires N-WASP, but its Arp2/3 complex-activating ability was not involved in protrusion formation.[98] The small interfering RNA (siRNA)-mediated knockdown of IRSp53 has also revealed its role in lamellipodia formation.[4]

The lamellipodia-like structures induced by WAVE2 and IRSp53 are involved in phagocytosis.[75),106] Interestingly, the EFC/F-BAR protein FBP17 was involved in both phagocytosis and the formation of podosomes, which are invasive structures that degrade the extracellular matrix.[107]

9. Phosphoinositides and membrane shape

Membrane remodeling and curvature formation are observed in cells during formation of the complex shapes of not only the plasma membrane, but also of intracellular organelles such as Golgi bodies, endosomes, multivesicular bodies, and the endoplasmic reticulum. The expression of EFC/F-BAR and BAR domains induced membrane tubulation toward the intracellular space, whereas myotubularin and IMD of IRSp53 induced filopodia-like membrane deforma-

tion toward outer space.[104] Currently, it is not clear how such a difference has evolved between the formation of membrane curvature in vesicles budding toward the cytosol and the formation of structures protruding toward the exterior of cells. In any case, dimerization/oligomerization of protein domains and their lipid-binding capabilities seem to be necessary for membrane deformation. The shapes of each cell and its organelles are different and very complicated, and comprise different membrane curvatures and sizes. Production of these complicated shapes is most likely triggered by the interaction between membrane phosphoinositides and their binding domains.

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Profile

Tadaomi Takenawa was born in 1944 in Yamaguchi prefecture, Japan. He graduated from Kyoto University, Faculty of Pharmaceutical Science in 1966. He began research concerned with free inositol and inositollipids under Professor Toru Tsumita at Institute of Medical Science, University of Tokyo. In 1974, he was appointed to Lecturer, Institute of Basic Science, School of Medicine, Tsukuba University, and then promoted to associate professor of Department of Biochemistry (Professor, Yositaka Nagai) School of Medicine, University of Tokyo in 1980. In 1983, he was invited as a chief of Department of Biosignals, Tokyo Metropolitan Institute of Gerontology. He worked as Professor of Department of Biochemistry, Institute of Medical Science, University of Tokyo from 1992 to the retirement as a Professor Emeritus in 2007. He is currently working as a Professor of Laboratory of Lipid Biochemistry, School of Medicine, Kobe University. He has been working on inositollipid signalings from starting of research. He found new inositollipid binding proteins that can change the shape of cell membrane. These proteins, which contain membrane-deforming domains such as the BAR, EFC/F-BAR, and the IMD/I-BAR domains, led to inward-invaginated tubes or outward protrusions of the membrane, resulting in a variety of membrane shapes. Furthermore, these proteins not only bind to phosphoinositide, but also to the N-WASP/WAVE complex and the actin polymerization machinery, which generates a driving force to shape the membranes.