Minireview

Pleckstrin homology domains and the cytoskeleton

Mark A. Lemmon[a,*], Kathryn M. Ferguson[a], Charles S. Abrams[b,*]

[a] *Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, 809C Stellar-Chance Laboratories, 422 Curie Boulevard, Philadelphia, PA 19104-6059, USA*

[b] *Department of Medicine, University of Pennsylvania School of Medicine, 912 BRB II/III, 421 Curie Boulevard, Philadelphia, PA 19104-6160, USA*

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Abstract Pleckstrin homology (PH) domains are 100–120 amino acid protein modules best known for their ability to bind phosphoinositides. All possess an identical core β-sandwich fold and display marked electrostatic sidedness. The binding site for phosphoinositides lies in the center of the positively charged face. In some cases this binding site is well defined, allowing highly specific and strong ligand binding. In several of these cases the PH domains specifically recognize 3-phosphorylated phosphoinositides, allowing them to drive membrane recruitment in response to phosphatidylinositol 3-kinase activation. Examples of these PH domain-containing proteins include certain Dbl family guanine nucleotide exchange factors, protein kinase B, PhdA, and pleckstrin-2. PH domain-mediated membrane recruitment of these proteins contributes to regulated actin assembly and cell polarization. Many other PH domain-containing cytoskeletal proteins, such as spectrin, have PH domains that bind weakly, and to all phosphoinositides. In these cases, the individual phosphoinositide interactions may not be sufficient for membrane association, but appear to require self-assembly of their host protein and/or cooperation with other anchoring motifs within the same molecule to drive membrane attachment.

Key words: PH domain; Cytoskeleton; Phosphoinositide; Actin; Membrane targeting

1. Introduction

Three reports in 1993 [1–3] pointed out the existence of a 100–120 residue stretch of amino acid sequence similarity in many proteins involved in cellular signaling, cytoskeletal organization, and other processes. This region of homology was proposed to define a protein module or domain, and was named the pleckstrin homology (or PH) domain since it had first been described by Haslam and colleagues as an internally repeated motif in the hematopoietic protein pleckstrin [4]. PH domains are now known to occur in a very large number of proteins, from yeast to mammals. The first draft of the human genome sequence [5] indicates that 252 different human proteins contain at least one PH domain, making it the 11th most common domain in humans. In *Saccharomyces cerevisiae*, some 27 different proteins contain a total of 36 PH domains, making the PH domain the 17th most common yeast domain [6]. The sequence characteristics used to identify PH domains appear to define a particular protein fold that has now been seen in the X-ray crystal structures and/or nuclear magnetic resonance (NMR) structures of some 13 different PH domains [7–12]. Each of these PH domains possesses an almost identical core β-sandwich structure (described below), despite pairwise sequence identities between PH domains that range from only around 10% to 30% in the best cases.

Although it is often assumed that all PH domains share a common function, they may instead share only the PH domain fold, and form subclasses with quite different functions. Recent studies have demonstrated that some PH domains can drive protein association with membranes through direct and specific recognition of polyphosphoinositides [7]. However, this is now realized to be a property of only a small fraction of PH domains [7,13] – perhaps just 10%. This raises the question as to how the other 90% function. Most of the these PH domains do appear to bind phosphoinositides, but do so very weakly and with little discernible specificity. This argues either that most PH domains require assistance from other domains in order to achieve their function, or that the physiological ligands of PH domains are not limited to polyphosphoinositides. We will discuss example of these possibilities in proteins involved in signaling and cytoskeletal function.

2. PH domain structure

As mentioned above, 13 different PH domain structures are known, and all share the same β-sandwich fold first observed in NMR structures of the N-terminal pleckstrin PH domain [14] and the β-spectrin PH domain [15]. The fold is illustrated in Fig. 1 for the β-spectrin PH domain. The amino-terminal half of the protein forms a four-stranded β-sheet, with an additional short α-helix in the β3/β4 loop (specific to the β-spectrin PH domain). The second half of the protein forms a β-sheet meander (strands β5–β7) that is near-orthogonal to the first sheet. The two sheets form a 'sandwich' that is filled with the hydrophobic core of the domain. To the left and right of the domain as seen in Fig. 1A are the 'closed' corners of the β-sandwich [16], at which the two sheets approach closely and are linked by tight turns or the continuation of β-strands. At the top and bottom of the structure in Fig. 1A are the two 'splayed' corners of the sandwich, which are capped off (at the top) by the C-terminal α-helix characteristic of all PH domains and (at the bottom) by the β1/β2, β6/β7

*Corresponding authors. Fax: (1)-215-573 4764; (1)-215-573 7400.
E-mail addresses: mlemmon@mail.med.upenn.edu (M.A. Lemmon), abrams@mail.med.upenn.edu (C.S. Abrams).

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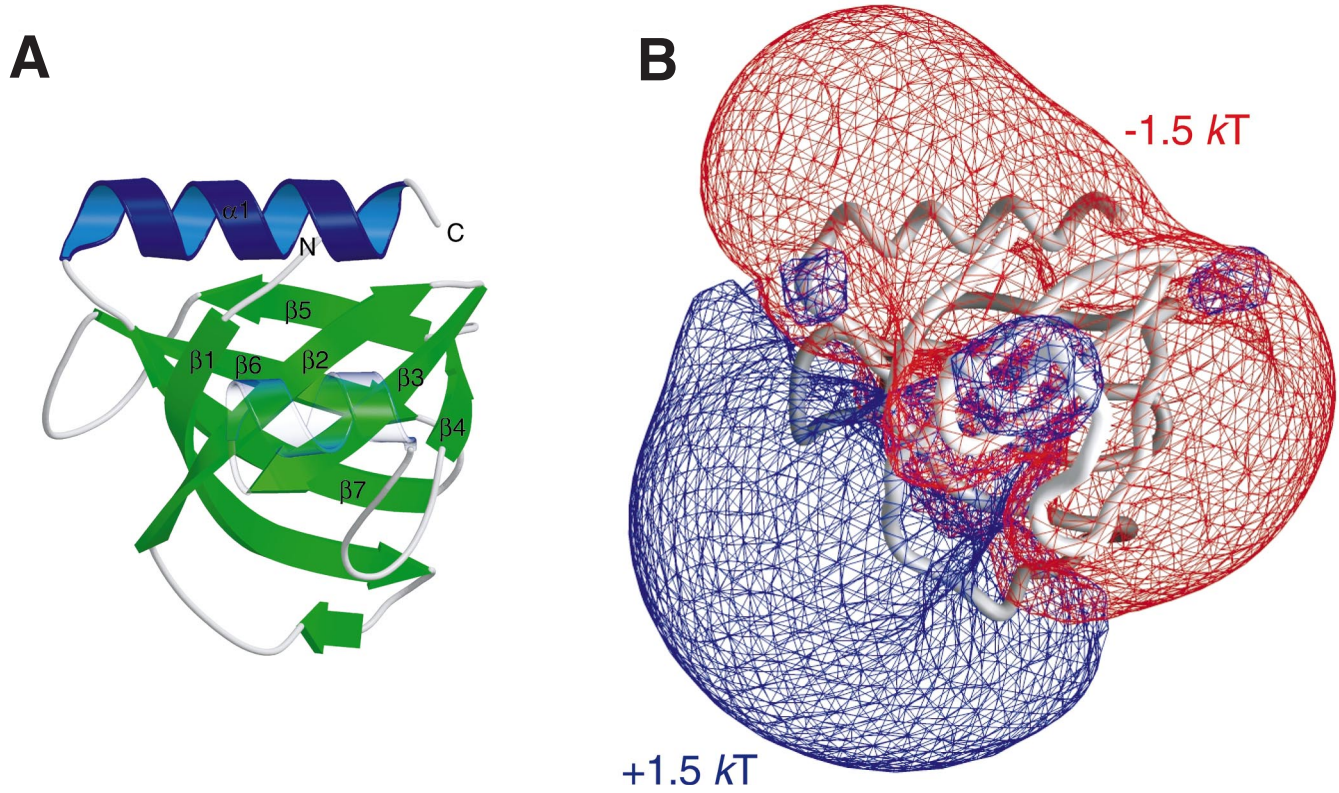

Fig. 1. A: Ribbon representation of the PH domain from β-spectrin, using coordinates from the X-ray crystal of Hyvönen et al. [33] (from which the bound Ins(1,4,5)P_3 has been removed). Conserved elements of secondary structure are labeled. This figure was generated using MOLSCRIPT [59]. B: The β-spectrin PH domain is shown in the same orientation as in A, with the calculated electrostatic potential shown, contoured at −1.5 kT (red) and +1.5 kT (blue). The backbone of the PH domain is represented by a gray worm. This figure was generated with GRASP [60].

loops and part of the β3/β4 loops of the domain. The same β-sandwich fold, with identical size and topology plus a C-terminal α-helix, has also been seen in several other domains that share no significant sequence similarity with PH domains [17]. Included in these examples are the phosphotyrosine binding (PTB) domain [18], the Ran binding domain (from Ran binding protein-2) [19], and the Enabled/VASP homology domain-1 (EVH1) [20]. Each of these domains recognizes a protein or peptide ligand in a distinct manner, providing suggestions of how PH domains might recognize putative protein targets. Like PH domains, PTB domains have also been reported to bind (albeit weakly) to phosphoinositides [18].

3. The PH domain as a polyphosphoinositide binding module

The determination of PH domain structures preceded understanding of their function. One of the first physical characteristics to be noted was that PH domains are strongly electrostatically polarized [15]. When the calculated electrostatic potential around the β-spectrin PH domain is plotted with a contour level of ±1.5 kT, a clearly defined region of positive potential is seen, centered on the β1/β2 loop [15] (Fig. 1B). The remainder of the protein is surrounded by negative electrostatic potential. Such electrostatic sidedness is a characteristic seen for all PH domains of known structure [17] and is highly reminiscent of the polarization seen in secretory phospholipase A_2 isozymes [21], which interact with acid phospholipids in cell membrane surfaces via their positively charged faces. Fesik and coworkers also noted a similarity between the PH domain fold and that of retinol binding protein [14], and this prompted them to propose that PH domains bind to membrane lipids, specifically to phosphatidylinositol (4,5)bisphosphate (PtdIns(4,5)P_2) [22]. They demonstrated that several PH domains can bind this relatively abundant component of the cytoplasmic face of cell membranes [22], and many subsequent studies have indicated that polyphosphoinositide binding is a property shared by most, if not all, PH domains [7,13,23].

However, in only very few cases is phosphoinositide binding by PH domains of high affinity and specificity. By far the majority of described PH domain/phosphoinositide interactions (90% or so) are of low affinity (K_D = 10 μM) and display little to no stereospecificity. Most known PH domain interactions with high affinity and specificity have now been studied extensively, and all appear to represent cases where phosphoinositide binding by the PH domain is both sufficient and necessary to drive membrane targeting of the PH domain-containing protein. One example of this is seen with phospholipase C-δ_1 (PLC-δ_1), the N-terminal PH domain of which is required for plasma membrane association of PLC-δ_1 in vivo [24]. The PLC-δ_1 PH domain specifically recognizes PtdIns(4,5)P_2 with high affinity ($K_D \approx 1.7$ μM) [7,13], and localizes strongly to the plasma membrane when expressed alone in yeast or mammalian cells [24–26]. Along similar lines, the PH domain at the amino-terminus of protein kinase B (PKB), also known as Akt, specifically recognizes both PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 [27,28], lipid second messengers that occur at significant levels in the plasma mem-

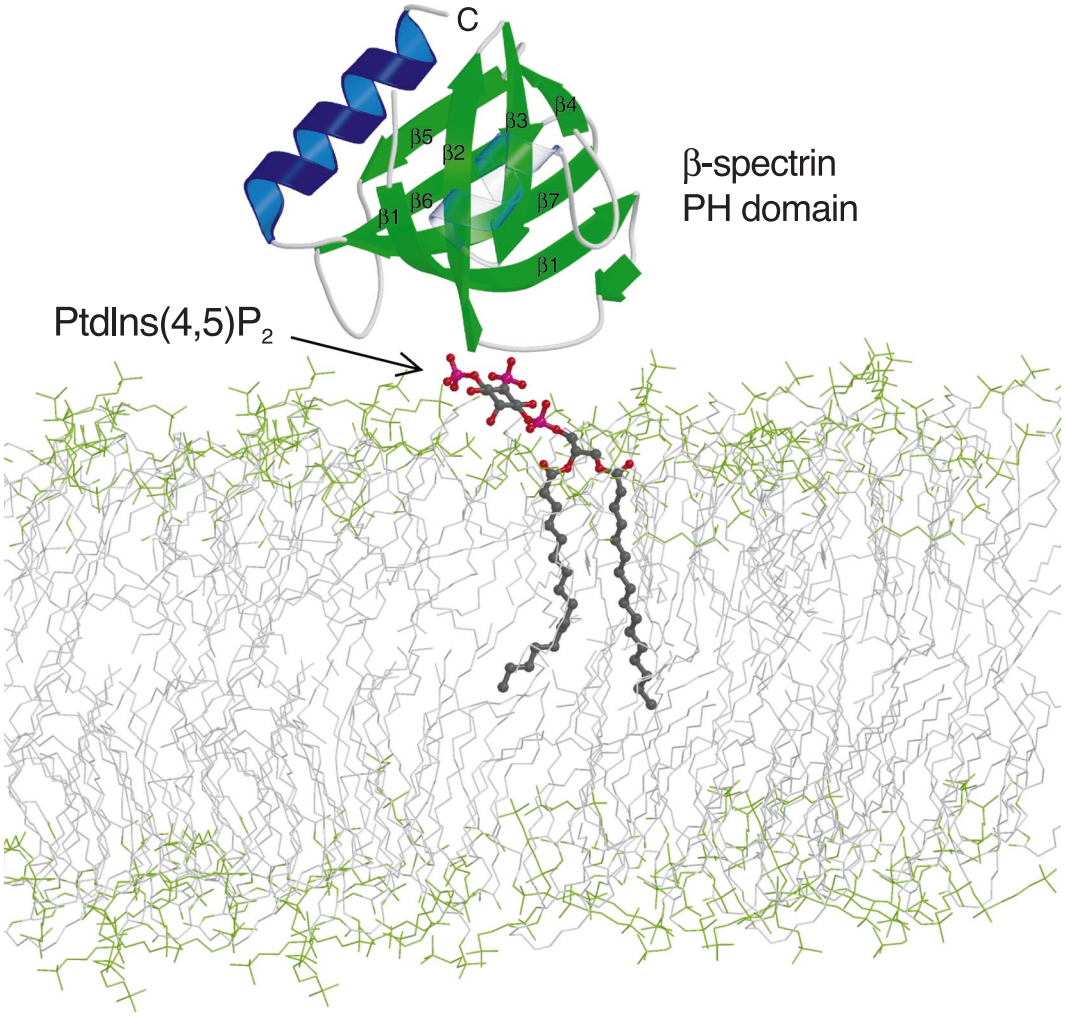

Fig. 2. Hypothetical view of how the PH domain from β-spectrin [33] binds to a membrane containing PtdIns(4,5)P_2. The X-ray crystal structure of the β-spectrin PH domain, with bound Ins(1,4,5)P_3 [33], is shown in a ribbon representation. The glycerol backbone and fatty acyl moiety from an idealized phospholipid have been added artificially to the Ins(1,4,5)P_3 headgroup to generate a hypothetical PtdIns(4,5)P_2 structure. The PtdIns(4,5)P_2 is embedded in a model for a dimyristoylphosphatidylcholine bilayer kindly provided by Dr. Herbert Treutlein. β-strands 1–7 are labeled, as is the C-terminus of the PH domain. This figure was generated using MOLSCRIPT [59].

brane only after phosphatidylinositol 3-kinase (PI 3-kinase) activation by cell surface receptor agonists [29]. PKB is recruited to the plasma membrane by binding of its PH domain to these transiently occurring lipid second messengers [30], providing a mechanism for direct regulation of PKB by agonist-stimulated PI 3-kinases [31,32].

4. Low-affinity binding of polyphosphoinositides to PH domains

While 'high-affinity' PH domains can function independently as signal-regulated membrane-targeting modules, the function of PH domains that bind only weakly and non-specifically to polyphosphoinositides is less clear. However, there is a great deal of evidence supporting the importance of these PH domains and a role for their weak phosphoinositide interactions. The β-spectrin PH domain and the N-terminal pleckstrin PH domain both bind PtdIns(4,5)P_2 with low affinity (K_D in the 30–50 μM range) and poor specificity [22,33]. NMR studies showed that the positively charged face of the N-terminal pleckstrin PH domain is responsible for PtdIns(4,5)P_2 binding to this domain [22]. Similarly, a crystal structure of the complex formed between the β-spectrin PH and inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3), published by Saraste and coworkers [33], showed that the binding site for the PtdIns(4,5)P_2 headgroup lies in the center of the positively charged lobe seen in Fig. 1B. The binding site for Ins(1,4,5)P_3 in this case is less well defined or buried than binding sites seen in structures of high-affinity PH domain complexes [34].

We suggest that the weak interactions seen with the pleckstrin and spectrin PH domains are driven by rather non-specific, or delocalized, electrostatic attraction between an anionic ligand and the positively charged face of the electrostatically polarized protein domain. In these and several other cases, inositol phosphates bind with affinities that reflect the number of phosphate groups that they contain, rather than the specific spatial arrangement of these groups [35]. A model for binding of the β-spectrin PH domain to PtdIns(4,5)P_2-containing membranes, based on studies by Saraste and colleagues [33], is presented in Fig. 2. Comparison with the electrostatic representation in Fig. 1B illustrates how docking of the PH domain on the membrane, guided by the crystallographically determined position of the bound headgroup,

abuts the positively charged surface of the domain against the negatively charged surface of the membrane.

Alone, weak interactions of the sort described here are unlikely to be sufficient to drive membrane association of PH domain-containing proteins. It remains possible that the phosphoinositide binding observed in vitro for these weakly binding domains has no relevance in vivo, and that protein or other ligands are more relevant. However, PH domains from this class tend to remain cytoplasmic when expressed in isolation, arguing against clear membrane targets that have simply not yet been identified. It therefore seems most likely that phosphoinositide binding by the PH domain cooperates with other interactions mediated by the same domain, or by other domains in the same protein (or complex of proteins) to drive multivalent membrane association. One piece of evidence for this was reported for the PH domain from the β-adrenergic receptor kinase (β-ARK), which binds PtdIns(4,5)P_2 with a K_D in the 100 μM range [36], but also appears to bind $G_{\beta\gamma}$ subunits of heterotrimeric G proteins through a C-terminal extension [37]. Neither one of these interactions alone is sufficient for β-ARK membrane association. However, simultaneous interaction of the PH domain with both ligands appears to be sufficient to drive efficient membrane targeting of β-ARK [38]. Similar situations may occur for PH domains that bind (in addition to phosphoinositides) actin [39], the receptor for activated C-kinase (RACK1) [40], or protein kinase C [41], which have all been described.

Weak phosphoinositide binding by a PH domain might instead cooperate with interactions mediated by an entirely separate domain within the same protein, as has been observed for the Rac1 guanine nucleotide exchange factor Tiam-1. The N-terminal PH domain and a separate protein binding domain must cooperate to drive appropriate subcellular targeting of Tiam-1 [42]. Other examples are provided by pleckstrin with its two PH domains (see below), and several other molecules in which more than one lipid binding modules cooperate to drive membrane association. The different domains that cooperate in driving membrane association may be in the same protein or in different proteins. If they are in separate proteins (in a homo- or heteromeric complex), then regulation of oligomer formation could control membrane targeting, as has been suggested for the large GTPase dynamin [43].

5. PH domains and the cytoskeleton

Many cytoskeletal proteins and proteins that regulate the cytoskeleton contain PH domains. Some have PH domains from the specific and high-affinity class discussed above. Some have PH domains that bind phosphoinositides with low affinity and specificity. Still others appear to show significant specificity for 3-phosphorylated phosphoinositides (if not always high affinity). Regulation of membrane association in each case is likely to be fundamentally different. For proteins with high-affinity and specific PH domains, simple alteration of lipid species in the membrane can directly alter membrane association. Regulated membrane association of proteins with low-affinity, non-specific PH domains need not involve alteration of lipids in the membrane, but may instead require modulation of protein–protein interactions. Proteins with low-affinity PH domains that show some phosphoinositide specificity may respond to simultaneous changes in phosphoinositide profile and protein–protein interactions.

5.1. Dbl family GEFs

Dbl family proteins are guanine nucleotide exchange factors (GEFs) that mediate activation of Rac/Rho family small GTPases by catalyzing the exchange of bound GDP for GTP. GEF activation of Rac/Rho GTPases plays a critical role in actin assembly initiated by all cell surface receptors. Every Dbl family protein contains a Dbl homology (DH) domain responsible for its GEF activity [44], which is always immediately followed by a PH domain. Ligand binding to the PH domain is thought to regulate DH domain activity. In the case of Vav-1, it has been proposed that PI 3-kinase products bind to the PH domain and enhance the ability of the DH domain to activate Rac/Rho GTPases. Supporting this hypothesis, high concentrations of PtdIns(3,4,5)P_3 were reported to enhance Vav-1 exchange activity in vitro [45]. Interestingly, the same study indicated that PtdIns(4,5)P_2 inhibits the exchange activity of Vav-1 [45], suggesting that the PH domain may exert a negative influence on Vav activity, and that PtdIns(3,4,5)P_3 (but not PtdIns(4,5)P_2) can somehow relieve this inhibition. In line with this hypothesis, deletion of the PH domain from Vav-1 was recently shown to result in a mutant with constitutive in vivo exchange activity [46]. Similarly, the PH domain of Sos was reported to have an inhibitory effect upon its DH domain in vivo, and this could be relieved by deletion of the PH domain [47]. Based on these observations and on structural studies it has been suggested that the PH domain of a DH/PH pair may block access to the binding site for the Rac/Rho small G protein on the DH domain [11,48]. Phosphoinositide binding to the PH domain could alter the average position of the PH domain with respect to the DH domain and thus relieve this steric block, although precise details of how this might occur are far from clear. Most PH domains that follow DH domains appear to exhibit both low affinity and poor specificity for phosphoinositides [49], suggesting that the regulatory mechanism is more complicated than suggested in this simple view. Furthermore, binding of phosphoinositides to the PH domain does not alter in vitro DH domain activity under all conditions [49]. Clearly, a great deal more work is required in order to fully understand the role of the PH domain in controlling the adjacent DH domain in Dbl family members.

5.2. PH domains in cell polarization and chemotaxis

Several recent studies have indicated that PH domains play critical roles in the spatial regulation of actin assembly required for proper chemotaxis – providing a clear link between PH domains and coordination of cytoskeletal organization. This link appears to involve PI 3-kinase activation. Protein kinase B or its isolated PI 3-kinase product binding PH domain has been shown to localize specifically to the leading edge of chemotaxing cells [50]. So have the *Dictyostelium* proteins CRAC and PhdA, which both have PH domains likely to bind PI 3-kinase products. In mammalian neutrophils [51] and in *Dictyostelium* [52,53], it has been demonstrated dramatically with GFP fusion proteins that the PH domains of PKB, CRAC, and PhdA acquire a highly polarized localization upon application of a chemotactic stimulus gradient.

A requirement for PKB in directing cell polarity is suggested by the finding that PKB-deficient *Dictyostelium* do

not extend pseudopodia in a polarized manner following exposure to chemoattractant gradients [54]. PhdA is one of the PH domain-containing proteins that localize to the leading edge of *Dictyostelium* when exposed to a cAMP gradient [53]. Polarized PhdA localization is clearly PI 3-kinase-dependent, and it has been shown both that loss-of-function mutations in PhdA lead to defects in actin polymerization at the leading edge of chemotaxing cells, and that dominant negative PhdA mutations impair pseudopod formation at the leading edge [53]. A model has thus emerged in which localized production of PI 3-kinase products is thought to recruit PhdA via its PH domain, with PhdA then serving as a scaffolding protein to localize other proteins required for actin assembly at the leading edge.

5.3. Pleckstrin

Pleckstrin is well known because it contains the two prototypic PH domains, which individually appear to bind phosphoinositides with relatively low affinity and poor specificity. Pleckstrin is expressed only in hematopoietic cells, where it is one of the most abundant proteins, accounting for approximately 1% of total cellular protein. Pleckstrin was initially described as a prominent substrate of PKC [4], and pleckstrin activity is regulated by PKC phosphorylation at three sites immediately adjacent to the N-terminal PH domain [23]. Once phosphorylated, pleckstrin is a negative regulator of 3- and 5-polyphosphoinositide production. When expressed in a variety of transformed and primary cell lines, PKC-phosphorylated pleckstrin causes a dissolution of stress fibers, enhanced cell spreading, and appearance of morphology suggesting increased F-actin levels [23]. Some of these effects may arise directly from the ability of pleckstrin to associate with phospholipids via its two PH domains, since phospholipids contribute to actin organization by regulating actin capping and GEFs. However, the effects were also shown to involve activation of Rho family GTPases and integrins [55,56].

In addition to the hematopoietic-restricted pleckstrin, a widely expressed paralog, named pleckstrin-2, exists [57]. Pleckstrin-2 has a similar domain structure to pleckstrin, but is not regulated by PKC. The PH domains of pleckstrin-2 appear to show a much higher specificity for PI 3-kinase products than do their pleckstrin counterparts. Consistent with this, it has been found that pleckstrin-2 induces ruffle formation and cell spreading in a PI 3-kinase-dependent manner [57]. As proposed for the regulators of cell polarity in chemotaxis, it is likely that pleckstrin-2 contributes to local actin organization once recruited to membranes by binding to PI 3-kinase products.

5.4. Cytoskeletal proteins with PH domains that do not bind PI 3-kinase products

Specific, regulated recruitment of PH domain-containing proteins to regions at which PI 3-kinase products are generated appears to participate in spatial organization of the cytoskeleton in several of the examples given above. For illustration, this process appears to result in nucleation of actin polymerization at the leading edge of chemotaxing cells. However, not all PH domains in cytoskeletal proteins bind PI 3-kinase products, suggesting that this mechanism does not always apply. As mentioned previously, the β-spectrin PH domain, for example, binds weakly to all phosphoinositides. It is likely that the physiological ligand of such PH domains is the most abundant polyphosphoinositide, PtdIns(4,5)P_2. The likely structural basis for PtdIns(4,5)P_2 binding by the β-spectrin PH domain is represented in Fig. 2. Note that the positively charged face of the domain seen in Fig. 1A directly faces the negatively charged membrane surface. Spectrin assemblies or networks contain many spectrin molecules, and therefore many PH domains. Such assemblies will be capable of high-avidity (multivalent) PH domain-mediated membrane association that will be very strong in spite of the fact that each single PH domain interaction with PtdIns(4,5)P_2 is weak. It is likely that much of the membrane attachment of the cytoskeleton is achieved through this type of multivalent interaction with membrane lipids. Such a mechanism allows for a situation in which only assembled cytoskeletal components, and not the individual proteins, will bind strongly to cellular membranes. Raucher et al. [58] have elegantly shown using laser tweezers that PtdIns(4,5)P_2 plays an important role in adhesion of the cytoskeleton to the plasma membrane. They specifically sequestered cellular PtdIns(4,5)P_2 by over-expression of the PH domain from PLC-δ_1, and found that the adhesion energy between the cytoskeleton and the plasma membrane was reduced significantly. Several cellular stimuli known to depress PtdIns(4,5)P_2 levels had similar effects.

6. Conclusion

Many cytoskeletal proteins contain domains that bind phosphoinositides, the PH domain being just one example of several classes. How membrane attachment of these proteins is regulated and remodeled in vivo is only just beginning to be elucidated. An appreciation is growing that cytoskeletal and associated proteins, when membrane-bound, will also directly affect the distribution and accessibility of phosphoinositides in the membrane. The consequences of this, and how it participates in cellular control, will be the focus of intensive investigation as tools are sharpened for analyzing phospholipids in living cells.

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