Mechanistic Basis of Differential Cellular Responses of Phosphatidylinositol 3,4-Bisphosphate- and Phosphatidylinositol 3,4,5-Trisphosphate-binding Pleckstrin Homology Domains*

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Phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P2) and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) are lipid second messengers that regulate various cellular processes by recruiting a wide range of downstream effector proteins to membranes. Several pleckstrin homology (PH) domains have been reported to interact with PtdIns(3,4)P2 and PtdIns(3,4,5)P3. To understand how these PH domains differentially respond to PtdIns(3,4)P2 and PtdIns(3,4,5)P3 signals, we quantitatively determined the PtdIns(3,4)P2 and PtdIns(3,4,5)P3 binding properties of several PH domains, including Akt, ARNO, Btk, DAPP1, Grp1, and C-terminal TAPP1 PH domains by surface plasmon resonance and monolayer penetration analyses. The measurements revealed that these PH domains have significant different phosphoinositide specificities and affinities. Btk-PH and TAPP1-PH showed genuine PtdIns(3,4,5)P3 and PtdIns(3,4)P2 specificities, respectively, whereas other PH domains exhibited less pronounced specificities. Also, the PH domains showed different degrees of membrane penetration, which greatly affected the kinetics of their membrane dissociation. Mutational studies showed that the presence of two proximal hydrophobic residues on the membrane-binding surface of the PH domain is important for membrane penetration and sustained membrane residence. When NIH 3T3 cells were stimulated with platelet-derived growth factor to generate PtdIns(3,4,5)P3, reversible translocation of Btk-PH, Grp1-PH, ARNO-PH, DAPP1-PH, and its L177A mutant to the plasma membrane was consistent with their in vitro membrane binding properties. Collectively, these studies provide new insight into how various PH domains would differentially respond to cellular PtdIns(3,4)P2 and PtdIns(3,4,5)P3 signals.

Phosphoinositides (PIs)2 are mono- and polyphosphorylated derivatives of phosphatidylinositol (PtdIns) (1–3). Although PIs are minor components of membrane lipids, they regulate a wide range of biological processes, including cell proliferation, cell survival, differentiation, signal transduction, cytoskeleton organization, and membrane trafficking (1–3). PIs regulate these cellular processes primarily by serving as site-specific membrane signals that mediate the membrane recruitment and regulation of effector proteins. The PI-mediated cellular processes allow exceptional spatiotemporal specificity because the spatial and temporal distribution of various PIs is dynamically and tightly regulated by a series of PI-modifying enzymes, such as phospholipases, lipid kinases, and lipid phosphatases, located in different cell membranes (1–3). For instance, phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) are mainly found in the plasma membrane, whereas phosphatidylinositol 3-phosphate (PtdIns3P), phosphatidylinositol 4-phosphate (PtdIns4P), phosphatidylinositol 5-phosphate (PtdIns5P), and phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P2) are primarily present in endosomes, Golgi, nucleus, and multivesicular bodies, respectively (1–3). Also, some PIs, such as PtdIns(4,5)P2, are present at significant steady-state levels in quiescent cells, whereas phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P2) and PtdIns(3,4,5)P3 accumulate transiently in stimulated cells (4).

PtdIns(3,4)P2 and PtdIns(3,4,5)P3 have received much attention recently because of their critical role in cell signaling (4, 5). PtdIns(3,4,5)P3 is primarily generated from PtdIns(4,5)P2 by PI 3-kinases in response to specific receptor activation. PtdIns(3,4)P2 is formed either by hydrolysis of PtdIns(3,4,5)P3 by 5-phosphatases, such as Src homology 2 domain-containing inositol phosphatases (SHIP1 and SHIP2), or by phosphorylation of PtdIns(3)P and PtdIns(4)P by PI 4-kinase and PI 3-kinases, respectively (5). It has been reported that PtdIns(3,4,5)P3 accumulation after cell stimulation is immediate and transient, whereas PtdIns(3,4)P2 accumulation is slightly delayed and significantly more sustained in platelets (6, 7). Also, unlike PtdIns(3,4,5)P3 that is primarily present in the plasma membrane, PtdIns(3,4)P2 may be localized at endosomes, intralumi-
PH Domain Membrane Penetration

A

M^{59} \quad \beta_{6-\beta_{7}} \text{loop} \quad \beta_{3-\beta_{4}} \text{loop} \quad \beta_{1-\beta_{2}} \text{loop} \quad \text{Ins}(1,4,5)P_{3}

\begin{align*}
\text{N} & \quad \text{C} \\
\text{DAPP1-PH} & \quad \text{TAPP1c-PH} & \quad \text{Btk-PH} \\
\text{ARNO-PH} & \quad \text{Grp1-PH} & \quad \text{Akt-PH}
\end{align*}
nal vesicles, endoplasmic reticulum, as well as the plasma membrane (8).

A large majority of PI effector proteins contain one or more domains that specifically recognize individual PI species (9–12). PI binding domains include pleckstrin homology (PH) (13–15), Fab1, YOTB, Vac1, and EE1 (FYVE) (16, 17), Phox (PX) (18, 19), Epsin N-Terminal Homology (ENTH) (20–22), AP180 N-Terminal Homology (ANTH) (20–22), Bin Amphiphysin Rvs (BAR) (22–25), Band 4.1, Ezrin, Radixin, Moesin (FERM) (26), Tubby (27), and protein kinase C Conserved 2 (C2) (28–31) domains.

The PH domain is the first discovered PI binding domain (13–15), and this domain of 100–120 amino acids is present in a wide range of signaling and membrane trafficking proteins. Although PH domains show low amino acid sequence homology, they have a conserved structural fold (see Fig. 1A) that consists of a 7-stranded β-sandwich (β1 to β7), one end of which is capped by a C-terminal α-helix, and the open end is connected by three variable loops (β1–β2, β3–β4, and β6–β7 loops). PH domains also show characteristic electrostatic polarization with a strong positive electrostatic potential surrounding the variable loops (see Fig. 1B) (32). Structural and biochemical studies have indicated that the variable loops form both a specific lipid binding pocket and a nonspecific membrane-binding surface (13–15). PH domains exhibit a wide range of PI affinities and specificities, which originate from high variability in the length and sequence of the variable loops, the β1–β2 loop in particular (13, 15).

Several PH domains have been reported to bind PtdIns(3,4,5)P3 and/or PtdIns(3,4)P2. For example, it has been reported that the PH domains of Bruton tyrosine kinase (Btk) (33, 34), general receptor for phosphoinositides-1 (Grp1) (35–38), and ADP-riboseylation factor nucleotide-binding site opener (ARNO) (35–38) are specific for PtdIns(3,4,5)P3, whereas the C-terminal PH domains of tandem-PH domain containing protein-1 (TAPP1) and -2 (TAPP2) specifically bind PtdIns(3,4)P2 (39). Also, the PH domains of protein kinase B (Akt) (40, 41), PI-dependent kinase 1 (PKD1) (42, 43), and dual adapter for phosphotyrosine and 3-phosphoinositides (DAPP1) (44) are known to bind both PtdIns(3,4,5)P3 and PtdIns(3,4)P2. However, PI specificities of these PH domains have been determined by a wide variety of methods under different conditions. This makes it difficult to quantitatively compare their PI specificities and affinities and predict which PH domain will preferentially bind to PtdIns(3,4)P2 and PtdIns(3,4,5)P3 formation under a given condition.

In general, membrane binding of lipid binding domains is initiated by nonspecific electrostatic interactions between anionic lipids and cationic protein residues, which is followed by specific ligand binding and/or partial membrane penetration (9). It has been shown that the partial membrane penetration is important for the membrane recruitment and activation of lipid binding domains and their host proteins (9). For this reason, the depth of membrane penetration has been determined for various lipid binding domains by several biophysical techniques, including electron spin resonance (45) and x-ray reflectivity (46, 47) measurements, and lipid binding domains have been classified into three groups, S-, I-, and H-types, depending on the degree of their membrane penetration (9). For FYVE (48), PX (49), and ENTH (50) domains, which belong to a membrane-penetrating H- or I-type, PI binding has been shown to trigger membrane penetration of the domains by inducing an electrostatic potential switch and/or local conformational changes. Although the depth of membrane penetration has not been reported for any PH domain, PH domains are generally thought to be S-type proteins whose membrane binding does not involve a significant degree of membrane penetration (9, 10, 13). This is largely because of the presence of a large number of cationic residues and the apparent lack of hydrophobic residues on their membrane-binding surfaces (32, 51). However, recent studies have indicated that the PH domain of phospholipase C (PLC) δ1 significantly penetrates the membrane (52, 53).

To systematically study the mechanism by which PtdIns(3,4)P2 and PtdIns(3,4,5)P3 induce the membrane recruitment of their effector proteins, we quantitatively determined by surface plasmon resonance (SPR) and monolayer penetration analyses the PI affinities, specificities, and membrane binding properties of several PH domains that have been reported to bind PtdIns(3,4,5)P2 and/or PtdIns(3,4,5)P3. The results reveal new PI specificities for these PH domains and also show that they have distinctively different membrane binding mechanisms, different degrees of membrane penetration in particular, due in part to variable distribution of hydrophobic residues in their membrane binding loops.

**EXPERIMENTAL PROCEDURES**

**Materials**—1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P2), phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P3), and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) were purchased from Cayman. The concentrations of the phospholipids were determined by a modified Bartlett analysis. Octyl glucoside was purchased from Fisher. The Pioneer L1 sensor chip was
purchased from Biacore AB (Piscataway, NJ). Fatty acid-free bovine serum albumin was from Bayer, Inc. (Kankakee, IL).

Vector Construction and Mutagenesis—The cDNAs of murine Grp1, human Btk, murine ARNO, and human DAPP1 PH domains were subcloned into the vector pKTM, between the restriction sites BamH1 and EcoRI. pKTM is a modified vector of pET-21a (+), with an optional N-terminal His6 tag and a Gly-Ser linker. DAPP1 PH domain mutants, L117A, V178A, and R184A, were generated by the overlap extension PCR and subcloned into the pKTM vector. The cDNAs of the human Akt PH domain and the human TAPP1 C-terminal (TAPP1c) PH domain were subcloned into the pGEX-4T-1 (Novagen, Madison, WI) vector, containing an N-terminal glutathione S-transferase fusion. PH domain mutants were generated by the overlap extension PCR and subcloned into corresponding vectors. All of the above constructs were transformed into DH5α cells for plasmid isolation, and their DNA sequences were verified. Those constructs subcloned into the pGEX-4T-1 vector were transformed into Escherichia coli BL21 cells, whereas those subcloned into the pKTM vector were transformed into E. coli BL21(DE3) cells for protein expression. For the cellular translocation work in NIH 3T3 cells, the PH domains of Grp1, Btk, ARNO, and DAPP1, and its L177A mutant, respectively, were subcloned between the HindIII and PstI sites of the mCerulean-C1 vector (54) and pEGFP-C2 vector, respectively, to yield the PH domains with cyan fluorescence protein (CFP) (54) and enhanced fluorescence green protein (EGFP), respectively, at the N terminus. The mCerulean-C1 vector was a generous gift of Dr. David Piston.

Protein Expression and Purification—For the expression of the Akt PH domain and the wild type and mutants of the TAPP1c PH domain, 1 liter of Luria broth containing 100 μg/ml ampicillin was inoculated with BL21 cells harboring the PH domain construct and grown at 37 °C until the absorbance at 600 nm reached 0.6. Protein expression was induced by the addition of 250 μM isopropyl 1-thio-β-D-galactopyranoside (Research Products, Mount Prospect, IL), and cells were harvested by centrifugation for 10 min at 5000 g, and the resulting pellet was resuspended in 20 ml of 50 mM NaH2PO4, pH 8.0, containing 0.3 M NaCl, 10 mM imidazole, 50 μM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, and 0.1% Triton X-100. The solution was then sonicated for 10 min (30 s sonication followed by 30 s cooling on ice). This was followed by centrifugation for 30 min (48,000 g × 4 °C). The supernatant was filtered into a 50-ml tube, and 1 ml of nickel-nitrilotriacetic acid resin (Qiagen, Valencia, CA) was added. The mixture was incubated on ice with gentle stirring (80 rpm) for 1 h. After this time, the mixture was poured onto a column filled with 20 ml of 50 mM NaH2PO4, pH 8.0, containing 300 mM NaCl and 10 mM imidazole. After sequentially washing the nonspecifically bound protein with 50 ml of 50 mM NaH2PO4, pH 8.0, 300 mM NaCl containing 10, 15, 20, and 50 mM imidazole, respectively, the protein was eluted in five fractions using 1 ml of 50 mM NaH2PO4, pH 8.0, containing 300 mM NaCl and 300 mM imidazole. All the fractions were pooled and diluted with deionized water to 50 ml and purified using either a Q-Sepharose column for Akt-PH or a S-Sepharose column for all other PH domains, which was connected to the ÄKTA FPLC system. The column was equilibrated with 20 mM MES buffer, pH 6.5 (Grp1-PH and ARNO-PH), 20 mM Tris buffer, pH 7.4 (Btk-PH and TAPP1c-PH), or 20 mM Tris buffer, pH 8.0 (Akt-PH), depending on the isoelectric point of the PH domain, and the elution was performed with the linear increase of KCl from 0 to 2 M in the same buffer. Protein purity was checked on an 18% polyacrylamide gel, and the protein concentration was determined by the bicinchoninic acid method (Pierce). The purified proteins were stored in 20 mM Tris-HCl buffer, pH 7.4, with 0.16 M KCl.

SPR Measurements—All SPR measurements were performed at 23 °C using a lipid-coated L1 chip in the BIACore X system as described previously (55). Briefly, after washing the sensor chip surface with the running buffer (20 mM HEPES, pH 7.4, containing 0.16 M KCl), POPC/POPE/PI (77:20:3), and POPC/POPE/POPS (80:20), vesicles were injected at 5 μl/min to the active surface and the control surface, respectively, to give the same resonance unit values. The level of lipid coating for both surfaces was kept at the minimum that is necessary for preventing the nonspecific adsorption to the sensor chips. This low surface coverage minimized the mass transport effect and kept the total protein concentration (P0) above the total concentration of protein-binding sites on vesicles (M0) (56). The control surface was also coated with 40 μl of BSA (0.1 mg/ml in the running buffer) at a flow rate of 5 μl/min before the injection of PH domains to minimize nonspecific adsorption of PH domains to the control surface. Equilibrium SPR measurements were done at the flow rate of 5 μl/min to allow sufficient time for the R values of the association phase to reach near-equilibrium values (Req) (57). For the expression of the Grp1, Btk, ARNO, and DAPP1 PH domains, 1 liter of Luria broth containing 100 μg/ml ampicillin was inoculated with BL21(DE3) cells harboring each construct, and the culture was grown at 37 °C until absorbance at 600 nm reached 0.5. At this time, 1 mM of isopropyl 1-thio-β-D-galactopyranoside was added, and cells were then incubated at 25 °C for 14 h. Cells were harvested for 10 min at 5000 g, and the resulting pellet was resuspended in 20 ml of 50 mM NaH2PO4, pH 8.0, containing 0.3 M NaCl, 10 mM imidazole, 50 μM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, and 0.1% Triton X-100. The solution was then sonicated for 10 min (30 s sonication followed by 30 s cooling on ice). This was followed by centrifugation for 30 min (48,000 g × 4 °C). The supernatant was filtered into a 50-ml tube, and 1 ml of nickel-nitrilotriacetic acid resin (Qiagen, Valencia, CA) was added. The mixture was incubated on ice with gentle stirring (80 rpm) for 1 h. After this time, the mixture was poured onto a column filled with 20 ml of 50 mM NaH2PO4, pH 8.0, containing 300 mM NaCl and 10 mM imidazole. After sequentially washing the nonspecifically bound protein with 50 ml of 50 mM NaH2PO4, pH 8.0, 300 mM NaCl containing 10, 15, 20, and 50 mM imidazole, respectively, the protein was eluted in five fractions using 1 ml of 50 mM NaH2PO4, pH 8.0, containing 300 mM NaCl and 300 mM imidazole. All the fractions were pooled and diluted with deionized water to 50 ml and purified using either a Q-Sepharose column for Akt-PH or a S-Sepharose column for all other PH domains, which was connected to the ÄKTA FPLC system. The column was equilibrated with 20 mM MES buffer, pH 6.5 (Grp1-PH and ARNO-PH), 20 mM Tris buffer, pH 7.4 (Btk-PH and TAPP1c-PH), or 20 mM Tris buffer, pH 8.0 (Akt-PH), depending on the isoelectric point of the PH domain, and the elution was performed with the linear increase of KCl from 0 to 2 M in the same buffer. Protein purity was checked on an 18% polyacrylamide gel, and the protein concentration was determined by the bicinchoninic acid method (Pierce). The purified proteins were stored in 20 mM Tris-HCl buffer, pH 7.4, with 0.16 M KCl.
index change by subtracting the control surface response from it. Assuming a Langmuir-type binding between the protein (P) and protein-binding sites (M) on vesicles (i.e., P + M ⇌ PM) (56), $R_{eq}$ values were then plotted versus $P_0$, and the $K_d$ value was determined by a nonlinear least squares analysis of the binding isotherm using an equation, $R_{eq} = R_{max} / (1 + K_d/P_0)$ (56). Each data set was repeated three or more times to calculate average and S.D. values. For kinetic SPR measurements, the flow rate was maintained at 15 µl/min for both association and dissociation phases. Some kinetic data were analyzed with BiAevaluation 3.0 software (Biacore) to determine the rate constants of association ($k_a$) and dissociation ($k_d$), using a one-step (i.e., P + M ⇌ PM) or two-step 1:1 (i.e., P + M ⇌ PM ⇌ P'M) protein-membrane binding model.

**Monolayer Measurements**—The penetration of PH domains into the phospholipid monolayers of different lipid compositions was measured in terms of the change in surface pressure ($\pi$) using a 10-ml circular Teflon trough and a Wilhelmy plate connected to a Cahn microbalance as described previously (58).

**Cellular Translocation Measurements**—NIH 3T3 cells were seeded into 8 wells of a sterile Nunc Lak-TeKII™ chambered cover glass plate, which was filled with 400 µl of Dulbecco’s modified Eagle’s medium (DMEM) and 10% (v/v) fetal bovine serum and incubated at 37 °C with 5% CO2 for 24 h. For transfection NIH 3T3 cells were incubated for 4 h with the mCerulean-C1 vector harboring each PH domain (1 µg/ml) in the presence of the Lipofectamine 2000 reagent in OPTI-MEM (Invitrogen). Co-transfection of cells with CFPltk-PH and EGFP-DAPP1-PH (or EGFP-Btk-PH and CFPI-DAPP1-PH) was performed with corresponding two plasmids in a 2:1 ratio to balance the emission intensities of two fluorophores. Cells were then incubated in DMEM with 10% fetal bovine serum overnight, washed with DMEM twice, and incubated in DMEM without serum for another 8–10 h. For imaging, cells were washed twice with the Hanks’ balanced salt solutions.

The membrane translocation of PH domains was monitored at fixed intervals (every 7 s) using a custom-built combination laser-scanning multiphoton microscope (59) after stimulation with 50 ng/ml of human platelet-derived growth factor-BB (PDGF-BB; Invitrogen) in 200 µl of the same medium. For two-channel dual imaging of CFP- and EGFP-tagged proteins, fluorophores were excited at 840 nm, and 480/40 and 525/50 bandpass filters and a Q505 long pass beam splitter were used to separate the emission signals. Images were analyzed using SimFCS (a kind gift of Dr. Enrico Gratton) as described previously (60). Specifically, regions of interest in the cytosol were defined, and the average intensity in a square (1 × 1 µm) was obtained with respect to time. Membrane intensities were determined for each frame in individual cells by extending a line from the cytosol to the outside of the cell and reading off the intensity with distance along the line. Intensity values corresponding to the place on the line indicating the edge of the cell were averaged. Lines were drawn in at least three places in each cell, and membrane intensity was determined. These values were averaged, and the resultant cytosolic intensity values were converted to a ratio for each frame: membrane/(membrane + cytosol). Each experiment was repeated at least three times on a given day and was repeated at least 2 different days with different transfected cells.

**RESULTS**

**PI Specificities of PH Domains**—It has been reported that the C-terminal PH domain of TAPP1 (TAPP1c) (39) and the PH domains of DAPP1 (44), Akt (40, 41), Btk (33, 34), ARNO (35–38), PDK1 (42, 43), and Grp1 (35–38) have high affinity and specificity for PtdIns(3,4,5)P3 and/or PtdIns(3,4,5)P2. Some of these PH domains, Akt-PH and Grp1-PH in particular, have been used as sensors for cellular PtdIns(3,4,5)P3 (61). However, PI specificities and affinities of these PH domains have not been quantitatively determined under the same conditions, making it difficult to directly compare their membrane binding properties and casting doubt on the use of these domains as specific and sensitive PI probes that can compete with endogenous PI effector proteins. We therefore quantitatively characterized the binding of these PH domains to mixed vesicles (POPC/POPE/PI (77:20:3)) containing various D3-PIs, including PtdIns3P, PtdIns(3,4)P2, PtdIns(3,5)P2, and PtdIns(3,4,5)P3, by equilibrium SPR analysis. Fig. 2 illustrates representative sensograms for the binding of Btk-PH to POPC/POPE/PtdIns(3,4,5)P3 vesicles and the binding isotherm generated from the sensograms. $K_d$ values determined for various PH domains and vesicles are listed in Table 1.

When compared among the PH domains, only Btk-PH showed genuine specificity for PtdIns(3,4,5)P3. It exhibited little to no affinity for other PIs, including PtdIns(3,4)P2, Grp1-PH also exhibited selectivity (i.e. 4.3-fold) for PtdIns(3,4,5)P3 over PtdIns(3,4)P2, but it had significant affinity for PtdIns(3,4)P2-containing vesicles. Btk-PH had ∼2-fold
higher affinity for POPC/POPE/PtdIns(3,4,5)P3 (77:20:3) vesicles than Grp1-PH, and together these two PH domains had higher PtdIns(3,4,5)P3 affinity than any other PH domain tested, indicating that these PH domains should be able to respond to PtdIns(3,4,5)P3 formation more effectively than POPC/POPE/PtdIns(3,4,5)P3 (77:20:3) vesicles. TAPP1c-PH showed similar PtdIns(3,4)P2-dependent monolayer penetration, but its monolayer penetration was weaker than DAPP1-PH (Fig. 3C). Thus, this PH domain might or might not be able to penetrate cell membranes depending on the local concentration of PtdIns(3,4)P2 and the local membrane structure. The notion that PtdIns(3,4,5)P2 (and PtdIns(3,4,5)P3 to a lesser extent) specifically induces the membrane penetration of DAPP1-PH and TAPP1c-PH is further supported by the finding that the mutation of essential PI ligands of DAPP1-PH (i.e. R184A) (see Fig. 3B) and TAPP1c-PH (i.e. R211A) (data not shown) abrogated the PtdIns(3,4,5)P2-enhanced monolayer penetration of these PH domains. Furthermore, PtdIns(4,5)P2, had a negligible effect on the monolayer penetration of the DAPP1-PH (Fig. 3A) and TAPP1c-PH (data not shown).

When compared with DAPP1-PH and TAPP1c-PH, Akt-PH (data not shown), ARNO-PH (Fig. 3D), and Grp1-PH (Fig. 3E) showed smaller but definite increases in monolayer penetration with $\pi_r$ ranging from 27 to 29 dynes/cm, in the presence of PtdIns(3,4,5)P2 or PtdIns(3,4,5)P3. Thus, membrane interactions of these domains are not likely to involve extensive membrane penetration but their modest membrane penetration activities may still play some roles under certain conditions (see below).

Among all PH domains tested, Btk-PH had the highest affinity for POPC/POPE/PtdIns(3,4,5)P3 (77:20:3) vesicles, indicating that the membrane penetration of the above PH domains into monolayers composed of various phospholipids. The lipid monolayer of a given surface pressure ($\pi_0$) was spread at a constant area, and the change in surface pressure ($\Delta \pi$) was monitored after the injection of proteins into the subphase. In general, $\Delta \pi$ is inversely proportional to $\pi_0$ of the phospholipid monolayer, and an extrapolation of $\Delta \pi$ versus $\pi_0$ yields $\pi_c$, which specifies an upper limit of $\pi_0$, that a protein can penetrate (56). The surface pressure of cell membranes and large unilamellar vesicles has been estimated to be 31–35 dynes/cm (63–65). Thus, for a protein to effectively penetrate a particular cell membrane (or large vesicles), it should have the $\pi_c$ value above this range for the monolayer whose lipid composition mimics that of the cell membrane.

As shown in Fig. 3, all the six PH domains penetrated a POPC/POPE (80:20 in mol %) monolayer with $\pi_c = 25–26$ dynes/cm. This indicates that they have some degree of intrinsic PI-independent membrane penetrating activities; however, these activities are not strong enough to allow penetration into densely packed bilayers, including large unilamellar vesicles and cell membranes. When 3 mol % of PtdIns(3,4)P2 or PtdIns(3,4,5)P3 was added to the monolayer (i.e. POPC/POPE/PI (77:20:3)), most of them showed enhanced monolayer penetration, but the degree of increase widely varied among the PH domains. The most dramatic effect was seen with DAPP1-PH (Fig. 3A). This domain was able to effectively penetrate the POPC/POPE/PtdIns(3,4,5)P3 (77:20:3) monolayer with $\pi_0 > 30$ dynes/cm (i.e. $\pi_c \approx 34$ dynes/cm), suggesting that DAPP1-PH could penetrate cell membranes in a PtdIns(3,4,5)P3-dependent manner. Consistent with PtdIns(3,4,5)P3 selectivity of DAPP1-PH (see Table 1), PtdIns(3,4,5)P3 had a smaller, albeit still significant, effect on the monolayer penetration of DAPP1-PH. TAPP1c-PH showed similar PtdIns(3,4,5)P3-dependent monolayer penetration, but its monolayer penetration was weaker than DAPP1-PH ($\pi_c \approx 30$ dynes/cm; Fig. 3C). Thus, this PH domain might or might not be able to penetrate cell membranes depending on the local concentration of PtdIns(3,4,5)P3 and the local membrane structure.

Effects of PI on Membrane Penetration of PH Domains—Our studies on FYVE (48), PX (49), and ENTH (50) domains have shown that PI binding triggers membrane penetration of the domains by inducing an electrostatic potential switch and/or local conformational changes. However, most PH domains are thought not to significantly penetrate the membrane. To date, only the PLCc1 PH domain has been reported to have a significant membrane penetrating activity (52, 53). To see if other PH domains have membrane-penetrating activity and how PIs may affect their membrane penetration, we measured the penetration of the above PH domains into monolayers composed of various phospholipids. The lipid monolayer of a given surface pressure ($\pi_0$) was spread at a constant area, and the change in surface pressure ($\Delta \pi$) was monitored after the injection of proteins into the subphase. In general, $\Delta \pi$ is inversely proportional to $\pi_0$ of the phospholipid monolayer, and an extrapolation of $\Delta \pi$ versus $\pi_0$ yields $\pi_c$, which specifies an upper limit of $\pi_0$, that a protein can penetrate (56). The surface pressure of cell membranes and large unilamellar vesicles has been estimated to be 31–35 dynes/cm (63–65). Thus, for a protein to effectively penetrate a particular cell membrane (or large vesicles), it should have the $\pi_c$ value above this range for the monolayer whose lipid composition mimics that of the cell membrane.

As shown in Fig. 3, all the six PH domains penetrated a POPC/POPE (80:20 in mol %) monolayer with $\pi_c = 25–26$ dynes/cm. This indicates that they have some degree of intrinsic PI-independent membrane penetrating activities; however, these activities are not strong enough to allow penetration into densely packed bilayers, including large unilamellar vesicles and cell membranes. When 3 mol % of PtdIns(3,4)P2 or PtdIns(3,4,5)P3 was added to the monolayer (i.e. POPC/POPE/PI (77:20:3)), most of them showed enhanced monolayer penetration, but the degree of increase widely varied among the PH domains. The most dramatic effect was seen with DAPP1-PH (Fig. 3A). This domain was able to effectively penetrate the POPC/POPE/PtdIns(3,4,5)P3 (77:20:3) monolayer with $\pi_0 > 30$ dynes/cm (i.e. $\pi_c \approx 34$ dynes/cm), suggesting that DAPP1-PH could penetrate cell membranes in a PtdIns(3,4,5)P3-dependent manner. Consistent with PtdIns(3,4,5)P3 selectivity of DAPP1-PH (see Table 1), PtdIns(3,4,5)P3 had a smaller, albeit still significant, effect on the monolayer penetration of DAPP1-PH. TAPP1c-PH showed similar PtdIns(3,4,5)P3-dependent monolayer penetration, but its monolayer penetration was weaker than DAPP1-PH ($\pi_c \approx 30$ dynes/cm; Fig. 3C). Thus, this PH domain might or might not be able to penetrate cell membranes depending on the local concentration of PtdIns(3,4,5)P3 and the local membrane structure.

The not-measurable values were not determined for the SPR experiments. The SPR determination quantitatively re-assesses the PI specificities of the above PH domains into monolayers composed of various phospholipids. The lipid monolayer of a given surface pressure ($\pi_0$) was spread at a constant area, and the change in surface pressure ($\Delta \pi$) was monitored after the injection of proteins into the subphase. In general, $\Delta \pi$ is inversely proportional to $\pi_0$ of the phospholipid monolayer, and an extrapolation of $\Delta \pi$ versus $\pi_0$ yields $\pi_c$, which specifies an upper limit of $\pi_0$, that a protein can penetrate (56). The surface pressure of cell membranes and large unilamellar vesicles has been estimated to be 31–35 dynes/cm (63–65). Thus, for a protein to effectively penetrate a particular cell membrane (or large vesicles), it should have the $\pi_c$ value above this range for the monolayer whose lipid composition mimics that of the cell membrane.

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Effects of PI-induced Membrane Penetration on Membrane Dissociation of PH Domains—To investigate how differential membrane penetrating activities of the PH domains may affect their membrane binding activities, we measured the kinetics of membrane association and dissociation of the PH domains by the SPR analysis. Our previous studies on various lipid binding domains and membrane-binding proteins have shown that nonspecific electrostatic interactions accelerate their membrane association, whereas short range specific interactions and membrane penetration slow the membrane dissociation (9, 55). It was therefore expected that membrane-penetrating PH domains, DAPP1-PH in particular, show slower membrane dissociation than weak penetrating PH domains, such as Btk-PH.

We injected the same concentration (final concentration of 200 nM) of PH domains to the sensor chip coated with POPC/POPE/PtdIns(3,4,5)P_3 (77:20:3) (Fig. 4A) or POPC/POPE/PtdIns(3,4)P_2 (77:20:3) (Fig. 4B) vesicles and monitored the kinetics of vesicle binding. Clearly, PH domains displayed diverse kinetic patterns. Rate constants (k_a and k_d) could not be robustly determined and directly compared in this study because the observed kinetics for the PH domains followed complex patterns and did not uniformly conform to either a one-step (i.e. P + M → PM) or a two-step (i.e. P + M → PM → P*M) 1:1 binding model. Kinetics of membrane association for some PH domains, including TAPP1c-PH, could be fit with the one-step 1:1 binding model, but their membrane dissociation kinetics did not follow the same model. For this reason, only the qualitative comparison of kinetic patterns was made. As for the membrane association, different maximal resonance unit values caused by these PH domains reflect their different affinities for the vesicles (see Table 1) and different degrees of membrane penetration (see Fig. 3). As far as the rate of association is concerned, Btk-PH (Fig. 4A) and TAPP1c-PH (Fig. 4B) apparently were the fastest for PtdIns(3,4,5)P_3- and PtdIns(3,4)P_2-containing vesicles, respectively. This is consistent with highly positive electrostatic potentials on the membrane-binding surfaces of these PH domains (15).

When the kinetics of membrane dissociation was compared, noticeable different patterns were seen among PH domains. In the presence of POPC/POPE/PtdIns(3,4,5)P_3 (77:20:3) vesicles (Fig. 4A), Btk-PH dissociated faster than Grp1-PH and ARNO-PH, which is consistent with their monolayer penetration capabilities. In particular, ARNO-PH showed extremely slow dissociation after a rapid initial phase that accounted for ~20% of total dissociation. As a result, even after exhaustive elution (i.e. >20-min), about 75% of ARNO-PH still remained on the membrane surface (data not shown). When POPC/POPE/PtdIns(3,4)P_2 (77:20:3) vesicles were used (Fig. 4B), the DAPP1
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PH domain showed much slower dissociation than any other PH domain. This again shows that the ability of this PH domain to effectively penetrate densely packed lipid monolayers allowed this PH domain to reside on the membrane longer. Similarly to ARNO-PH, Akt-PH showed a rapid initial dissociation, followed by a much slower phase. Thus, it appears that the partial membrane penetration of PH domains plays a significant role in the kinetics of their membrane interactions, membrane dissociation in particular.

Structural Basis of Differential Membrane Penetration of PH Domains—To understand the basis of differential membrane penetration activities of the above PH domains, we examined their tertiary structures and performed mutational analysis. The reported crystal structures of PH domain-PI complexes (see Fig. 1) suggest that differential membrane penetrating abilities of the PH domains derive from the variable distribution of hydrophobic residues on their putative membrane-binding surfaces. Interestingly, DAPP1-PH has two prominently protruding hydrophobic residues, Leu177 and Val178, side-by-side on their putative membrane-binding surfaces. Interestingly, DAPP1-PH has two prominently protruding hydrophobic residues, Val204 and Met205 in its β3–β4 loop (68). Thus, this PH domain is expected to be as effective as TAPP1c-PH in terms of membrane penetration. On the other hand, Akt-PH (69), ARNO-PH (70), and Grp1-PH (71) have one hydrophobic residue on the putative membrane-binding surfaces, which may contribute to their modest membrane penetrating activities. Also, Btk-PH (72) has no fully exposed hydrophobic residue on the membrane-binding surface (Phe44 near the membrane-binding surface is not fully exposed), which accounts for its lower monolayer penetration. Thus, it appears that the presence of two neighboring hydrophobic residues in a protruding loop is important for a PH domain to penetrate densely packed monolayers and bilayers.

To test the notion that surface hydrophobic residues are important for the membrane penetration and sustained membrane residence of PH domains, we mutated two hydrophobic residues (Leu177 and Val178) of DAPP1-PH to Ala and measured the effects of mutations on its membrane binding. As shown in Fig. 3B, the mutation of a single hydrophobic residue to Ala was enough to essentially abrogate the PtdIns(3,4)P2-enhanced monolayer penetration of the PH domain, i.e. both mutants penetrated the POPC/POPE/PtdIns(3,4)P2 (77:20:3) monolayer only as effectively as the wild type DAPP1-PH penetrated the POPC/POPE (80:20) monolayer. Furthermore, the kinetic SPR analysis of these proteins (see Fig. 4C) revealed that L177A and V178A dissociated from the membrane much faster than the wild type, behaving similarly to other PH domains with weaker membrane penetrating activities. In particular, L177A showed >70% dissociation from the membrane. L177A and V178A also had significantly lower affinities for POPC/POPE/PtdIns(3,4)P2 (77:20:3) vesicles than wild type (see Table 1).

To see if surface hydrophobic residues play the same role for other PH domains, we mutated two hydrophobic residues (Val204 and Met205; see Fig. 1) of TAPP1c-PH and monitored the membrane dissociation kinetics of TAPP1c-PH after 5 min (see Fig. 4D) and less than 40% dissociation after 30 min (see Fig. 4D) and less than 40% dissociation after exhaustiv elution (data not shown). Under the same condition, however, V204A and M205A exhibited about 95 and 80% dissociation. Notice also that the membrane dissociation of M205A to V204A was much faster than that of the wild type TAPP1c-PH.
We also introduced two hydrophobic residues into the β1-β2 loop of Btk-PH by the K18F/K19L mutation to see if Btk-PH could be converted into one with DAPP1-PH-like properties. As shown in Fig. 4A, this mutant showed significantly slower membrane association than the wild type Btk-PH. This is presumably because of the removal of two lysines that are involved in nonspecific electrostatic interaction with the anionic membrane, which facilitates the membrane association. More importantly, the mutant exhibited extremely slow membrane dissociation; little dissociation was observed even after exhaustive elution. Collectively, these results support the notion that two proximal hydrophobic residues on the membrane-binding surface are essential for a PH domain to penetrate compactly packed lipid bilayers, including cell membranes, in a PI-dependent manner. The results also indicate that the partial membrane dissociation of PH domains is because of PI-induced membrane insertion of hydrophobic residues on their membrane-binding surfaces.

**Differential Membrane Cellular Translocation of PH Domains**—To see if cellular membrane targeting behaviors of PH domains are governed by their membrane binding properties, we monitored the PtdIns(3,4,5)P$_3$-mediated subcellular translocation of Btk-PH, Grp1-PH, ARNO-PH, and DAPP1-PH each tagged with the modified CFP at their N termini, in NIH 3T3 cells. The cell populations expressing similar levels of PH domains were selected by visual inspection of CFP fluorescence intensity and used for translocation measurements. A minimum of quadruple measurements was performed for each protein with at least 5 cells monitored for each measurement. Typically, >80% of cell population showed similar behaviors with respect to membrane translocation of PH domains.

Fig. 5 shows the time-lapse images of PH domains in representative cells. When expressed in NIH...
3T3 cells grown in serum-supplemented media, all PH domains showed some degree of pre-localization at the plasma membrane of quiescent cells (data not shown), which was largely suppressed by serum starvation. As reported previously (73), nuclear localization was observed for all PH domains, with Grp1-PH showing the most pronounced nuclear distribution (Fig. 5A). When NIH 3T3 cells were treated with 50 ng/ml PDGF, which was reported to produce PtdIns(3,4,5)P_3 in the plasma membrane (74, 75), cytosolic Btk-PH and Grp1-PH rapidly translocated to the plasma membrane (Fig. 5A and C). In particular, Btk-PH showed the fastest translocation to the plasma membrane, completing the translocation within 2 min (Fig. 5C). In contrast, ARNO-PH exhibited much slower plasma membrane translocation. Interestingly, Btk-PH moved back to the cytoplasm rather quickly after reaching the maximal plasma membrane translocation. Most important, DAPP1-PH and its L177A mutant from the plasma membrane membrane targeting, we then measured the dissociation of vesicle binding shown in Fig. 4A.

To further investigate the correlation between in vitro membrane binding properties of PH domains and their cellular membrane targeting, we then measured the dissociation of DAPP1-PH and its L177A mutant from the plasma membrane after PDGF stimulation (Fig. 5D). Although DAPP1-PH prefers PtdIns(3,4,5)P_3 to PtdIns(3,4,5)P_5, it still has higher affinity for PtdIns(3,4,5)P_3-containing vesicles than ARNO-PH (see Table 1). Accordingly, both DAPP1-PH and L177A translocated to the plasma membrane in response to PDGF stimulation faster than ARNO-PH, and slightly slower than Grp1-PH. Most important, DAPP1-PH stayed bound to the plasma membrane much longer than Btk-PH and Grp1-PH. It gradually dissociated from the membrane only after 8 min. In contrast, L177A started to dissociate from the plasma membrane as fast as Btk-PH even before it reached the full association.

Finally, we co-transfected the NIH 3T3 cells with EGFP-Btk-PH and CFP-DAPP1-PH (or vice versa) and simultaneously monitored their membrane translocation by two-channel imaging in response to PDGF activation to verify that these two PH domains show distinct membrane translocation patterns under the same conditions. As illustrated in Fig. 5, B and E, individual behaviors of EGFP-Btk-PH and CFP-DAPP1-PH in co-transfected cells were essentially the same as their behaviors in independently transfected cells, i.e. Btk-PH rapidly bound to and dissociated from the plasma membrane in response to PDGF activation, whereas DAPP1-PH showed much slower membrane dissociation. The same pattern was seen when NIH 3T3 cells were co-transfected with CFP-Btk-PH and EGFP-DAPP1-PH (data not shown). These co-transfection experiments thus rule out a possibility that differential responses of PH domains to PDGF activation are due to variable effects of their overexpression on PtdIns(3,4,5)P_3 signaling pathways in NIH 3T3 cells. Collectively, our results show that the kinetics of PtdIns(3,4,5)P_3-mediated subcellular localization of the PH domains is well correlated with their relative affinity for PtdIns(3,4,5)P_3-containing vesicles (Table 1) and, more importantly, the kinetics of binding to PtdIns(3,4,5)P_3-containing vesicles (Fig. 4A). This semi-quantitative correlation supports the notion that differential cellular membrane targeting behaviors of PH domains are largely attributed to their divergent membrane binding properties.

**DISCUSSION**

In cell signaling, a single signal mediator, such as Ca^{2+} and a lipid second messenger, can mediate divergent signaling pathways in a spatially and temporally specific manner. A majority of PI s serve as site-specific membrane signals that recruit and activate effector proteins, and generally, each PI is recognized by multiple effector molecules. This raises a question as to how a PI selectively recruits and activates a particular effector protein under a specific condition. A commonly proposed mechanism is the membrane recruitment by multiple interactions, involving a lipid and a protein adaptor or two different lipids, for example, which would confer an extra level of specificity and control (1, 76). It is also possible that the compartmentalization through the formation of signaling complexes at the membrane allows only a small set of proteins to see the locally confined PI molecules. Another possibility is that PI effectors have such divergent membrane binding properties that they can differentially respond to emerging PI molecules. The present study investigates the last aspect using several PtdIns(3,4)P_2- and PtdIns(3,4,5)P_3-binding PH domains as models.

Because the lipid selectivity of many PH domains has been determined under different conditions, we reexamined the lipid selectivity of representative PtdIns(3,4)P_2- and PtdIns(3,4,5)P_3-binding PH domains by SPR analysis. This study redefines PI selectivity for some PH domains (ARNO, DAPP1, Grp1, and Akt), and confirms previous reports on other PH domains (Btk and TAPP1c). In general, our PI selectivity data are in good agreement with the reported structural information on PH domain-PI complexes. For example, the pronounced specificity of Btk-PH for PtdIns(3,4,5)P_3-containing vesicles is consistent with its large binding pocket containing ligands for D3, D4, and D5 phosphoryl groups (72). Its extremely low affinity for other PIs indicates that partial occupancy of the binding pocket of Btk-PH is not conducive to productive PI binding. Also, high PtdIns(3,4)P_2 specificity of TAPP1c-PH has been attributed to the presence of an Ala residue in the β1–β2 loop that sterically discriminates against the D5 phosphate group (67). Furthermore, the modest preference of DAPP1-PH and Akt-PH for PtdIns(3,4)P_2 over PtdIns(3,4,5)P_3 agrees with the finding that they do not have specific binding sites for the D5 phosphate in the pockets (66, 69). Significant PtdIns(3,4,5)P_3 selectivity of Grp1-PH is also consistent with its structure showing a well defined binding site for the D5 phosphate. However, it is somewhat unexpected to find that a structurally similar ARNO-PH has modest selectivity for PtdIns(3,4)P_3 over PtdIns(3,4,5)P_3 because it also has a well defined binding site for the D5 phosphate. It should be noted that splice variants of ARNO/Grp1 that contain three Gly residues in the β1–β2 loop have different lipid selectivity than the major forms that have two glycines in the same loop (38, 70). Our sequencing analysis verified that...
ARNO-PH used herein has two glycines in the loop (data not shown) and thus our specificity data are not because of structural variation. It thus appears that binding to the D5 phosphate to ARNO-PH does not contribute much to the overall ligand binding energy. This notion is consistent with the finding that ARNO-PH has much lower affinity for PtdIns(3,4,5)P3-containing vesicles than Btk-PH or Grp1-PH.

Because PtdIns(3,4)P2 and PtdIns(3,4,5)P3 are lipid second messengers that accumulate only transiently in stimulated cells, their effector proteins must be able to rapidly and proficiently respond to these signals. This entails high affinity (i.e., low $K_0$) and rapid association kinetics (i.e., high $k_a$). Our results show that TAPP1c-PH and Btk-PH meet these requirements as specific PtdIns(3,4)P2 and PtdIns(3,4,5)P3 effector proteins. They have high PI specificities (i.e., 20-fold selectivity for their preferred PIs) and affinities ($K_0 < 100$ nM), and they also show rapid membrane association kinetics (Fig. 4, A and B). Although individual rate constants for PH domains could not be accurately measured in this study because of complex kinetic patterns, their $k_a$ values estimated from their association kinetic phases, assuming one-step 1:1 membrane-protein binding, are in the range of $10^4$--$10^5$ M$^{-1}$ s$^{-1}$, which compare favorably with those of other fast lipid/membrane binding domains (9). Grp1-PH also has high affinity for PtdIns(3,4,5)P3-containing vesicles, but it displays significant affinity for PtdIns(3,4)P2-containing vesicles, and its association rate constant appears to be smaller than that of Btk-PH (Fig. 4B).

On the basis of these membrane binding properties, one would expect that Btk-PH and TAPP1c-PH would respond to emerging cellular PtdIns(3,4,5)P3 and PtdIns(3,4)P2 signals, respectively, more effectively than other PH domains if lipid-protein interactions provide a primary driving force for membrane targeting of these proteins. Grp1-PH should serve as a proficient effector for PtdIns(3,4,5)P3, but it may also respond to the formation of PtdIns(3,4)P2. Likewise, the PH domains of Akt, ARNO, and DAPP1, which do not clearly distinguish between PtdIns(3,4)P2 and PtdIns(3,4,5)P3, may serve as dual effectors for PtdIns(3,4)P2 and PtdIns(3,4,5)P3, but their cellular responses would be slower than Btk-PH and Grp1-PH for PtdIns(3,4,5)P3 and TAPP1c-PH for PtdIns(3,4)P2. Our cell translocation results support this notion. When the formation of PtdIns(3,4,5)P3 is induced by PDGF in NIH 3T3 cells, Btk-PH shows the fastest translocation to the plasma membrane, which is followed by Grp1-PH and ARNO-PH.

It should be noted that there is some noticeable discrepancy between our membrane affinity data and published data. For example, Grp1-PH (35–38) and Akt-PH (40, 41) have been reported to have higher affinity for PtdIns(3,4,5)P3 than Btk-PH. However, our results show that Btk-PH has 2-10-fold higher affinity for PtdIns(3,4,5)P3-containing vesicles than Grp1-PH and Akt-PH, respectively. This type of discrepancy in membrane binding parameters, which is commonly found for lipid binding domains, arises mainly from the fact that affinity data were obtained by various methods (e.g., lipid overlay, vesicle pelletting, fluorometric, or SPR assay) with highly variable sensitivity and accuracy under different conditions (e.g., PIs in different physical states). This makes it difficult to directly compare reported membrane affinities. The semi-quantitative correlation between our in vitro membrane binding data and cell data not only validates our affinity data but also underscores the importance of rigorously and systematically determining membrane binding parameters of lipid binding domains under well-defined conditions.

Another key finding in this study is that all PH domains but Btk-PH show PI-induced membrane penetration, although the degree of penetration varies widely among them. PH domains have been generally considered as S-type proteins that primarily interact with the membrane surface because of the presence of a cluster of basic residues on their putative membrane-binding surfaces (9). So far, membrane penetration has been reported only for the PH domain of PLCδ1 (52, 53). In particular, Flesch et al. (53) reported that PLCδ1-PH has strong PI-independent monolayer penetration and partial dissociation from the vesicle-coated SPR chip. However, this study was performed with excessively high PI concentrations (i.e., 20 mol%) in monolayers and bilayers, which casts doubt on the validity and physiological relevance of their findings and interpretation. Our study employing physiologically more relevant PI concentrations (i.e., 1–5 mol%) clearly shows that PtdIns(3,4)P2, and/or PtdIns(3,4,5)P3 significantly enhance the monolayer penetration of PH domains. For DAPP1-PH and TAPP1c-PH with the highest monolayer penetrating activities, the monolayer penetration is greatly promoted by PtdIns(3,4)P2. In particular, 1–3 mol% of PtdIns(3,4)P2 allows DAPP1-PH to effectively insert its hydrophobic residues on the β1–β2 loop into densely packed monolayers and bilayers. Thus, PtdIns(3,4)P2 would seem to trigger the penetration of DAPP1-PH (and perhaps TAPP1c-PH) into the cell membrane. On the basis of our previous studies on FYVE (48), PX (49), and ENTH (50) domains, we postulate that PtdIns(3,4)P2 binding induces electrostatic attenuation of the membrane-binding surface of DAPP1-PH (i.e., reducing the positive electrostatic potential surrounding hydrophobic residues, thereby allowing them to penetrate the membrane without paying the significant dehydration penalty) and/or causes local conformational changes. Our results indicate that the presence of two neighboring hydrophobic residues (Leu$^{177}$ and Val$^{178}$ for DAPP1-PH) is critical for this strong membrane-penetrating activity as the substitution of either residue by a less hydrophobic one in DAPP1-PH mutants or in other PH domains greatly weakens the monolayer penetration and shortens the residence time at the lipid bilayers. Also, introduction of two neighboring hydrophobic residues into the membrane binding loop of Btk-PH converts it into a DAPP1-PH-like domain with strong membrane-penetrating activity.

It has been reported that sustained membrane residence, which is achieved through membrane insertion of hydrophobic residues, is essential for the activation and processive actions of membrane-binding proteins (9). Although we could not quantitatively fit the complex membrane binding kinetics of PH domains using a single model, monolayer and kinetic SPR data of wild type and mutant PH domains indicate that membrane penetration of PH domains, mediated by hydrophobic residues on their membrane-binding surfaces, plays a key role in regulating the dissociation of PH domains from model and cell membranes. DAPP1-PH with two membrane-penetrating hydrophobic residues shows extremely slow in vitro and cellular membrane dissociation, whereas removal of either...
of two hydrophobic residues dramatically accelerates the membrane dissociation. Even for those PH domains with modest monolayer penetrating activities, their membrane dissociation kinetics seem to be greatly influenced by the partial membrane penetration of a hydrophobic residue(s) on their membrane-binding surfaces. Btk-PH is unique among PtdIns(3,4,5)P$_3$-binding PH domains in that it shows rapid dissociation for the plasma membrane presumably in response to the removal of PtdIns(3,4,5)P$_3$. Thus, its rapid kinetics of membrane association and dissociation may enable Btk-PH to serve as a genuine sensor for cellular PtdIns(3,4,5)P$_3$.

It should be noted that there is some discrepancy between our monolayer penetration data and membrane dissociation data. For instance, ARNO-PH and Grp1-PH show similar monolayer penetration behaviors, but ARNO-PH dissociates from model and cellular membranes more slowly than Grp1-PH. Although it is beyond the scope of this investigation, determination of the membrane-bound topology of different PH domains and the depth of their membrane penetration should provide further insight into the basis of different membrane dissociation behaviors of PH domains. Also, protein-protein interactions, which may play a significant role in subcellular localization and cellular actions of PH domains (13–15), can slow the membrane dissociation of proteins (62). This may at least partially account for the different cellular membrane dissociation behaviors between Grp1-PH and ARNO-PH, for example.

Cellular membrane recruitment has been studied for many PH domains with different PI specificities (13–15). Yet it remains unclear how each PI can differentially regulates many PH domains. Due in part to a lack of kinetic and thermodynamic parameters of membrane binding determined under well-defined conditions, however, cellular membrane translocation properties of PH domains with similar PI specificities (e.g. PtdIns(3,4,5)P$_3$) have not been systematically compared. This study provides such information for the PH domains that interact with two important lipid second messengers, PtdIns(3,4)P$_2$ and/or PtdIns(3,4,5)P$_3$. A good overall correlation between the membrane binding properties of these PH domains and their cellular translocation indicates that the kinetics of PI-mediated cellular membrane recruitment of these PH domains is governed, to a large extent, by their membrane binding properties. This should also help account for different cellular functions and regulation of the proteins in which these PH domains reside. Furthermore, our results should form the foundation of systematic and quantitative assessment of different cellular membrane translocation properties of a large number of PH domains and their host proteins.

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