Molecular mechanism of membrane targeting by the GRP1 PH domain[S]

Ju He,* Rachel M. Haney,[†,§] Mohsin Vora,[†] Vladislav V. Verkhusha, Robert V. Stahelin,[†,§] and Tatiana G. Kutateladze[1,*]**

Department of Pharmacology,* University of Colorado Health Sciences Center, Aurora, CO; Department of Biochemistry and Molecular Biology,[†] Indiana University School of Medicine, South Bend, IN; Department of Chemistry and Biochemistry and The Walther Center for Cancer Research,[§] University of Notre Dame, South Bend, IN; and Department of Anatomy and Structural Biology,** Albert Einstein College of Medicine, Bronx, NY

Abstract **The general receptor for phosphoinositides isoform 1 (GRP1) is recruited to the plasma membrane in response to activation of phosphoinositide 3-kinases and accumulation of phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3]. GRP1's pleckstrin homology (PH) domain recognizes PtdIns(3,4,5)P_3 with high specificity and affinity, however, the precise mechanism of its association with membranes remains unclear. Here, we detail the molecular basis of membrane anchoring by the GRP1 PH domain. Our data reveal a multivalent membrane docking involving PtdIns(3,4,5)P_3 binding, regulated by pH and facilitated by electrostatic interactions with other anionic lipids. The specific recognition of PtdIns(3,4,5)P_3 triggers insertion of the GRP1 PH domain into membranes. An acidic environment enhances PtdIns(3,4,5)P_3 binding and increases membrane penetration as demonstrated by NMR and monolayer surface tension and surface plasmon resonance experiments. The GRP1 PH domain displays a 28 nM affinity for POPC/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine/PtdIns(3,4,5)P_3 vesicles at pH 6.0, but binds 22-fold weaker at pH 8.0. The pH sensitivity is attributed in part to the His355 residue, protonation of which is required for the robust interaction with PtdIns (3,4,5)P_3 and significant membrane penetration, as illustrated by mutagenesis data. The binding affinity of the GRP1 PH domain for PtdIns(3,4,5)P_3-containing vesicles is further amplified (by ~6-fold) by nonspecific electrostatic interactions with phosphatidylserine/phosphatidylinositol. Together, our results provide new insight into the multivalent mechanism of the membrane targeting and regulation of the GRP1 PH domain.**—He, J., R. M. Haney, M. Vora, V. V. Verkhusha, R. V. Stahelin, and T. G. Kutateladze. **Molecular mechanism of membrane targeting by the GRP1 PH domain. *J. Lipid Res.* 2008.** 49: **1807–1815.**

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The signaling lipid phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3] is produced in plasma membranes in response to stimulation of cell surface receptors by growth factors and hormones (1). Class I phosphoinositide (PI) 3-kinases phosphorylate the inositol headgroup of the relatively abundant phosphatidylinositol 4,5-bisphosphate [Ptdns(4,5)P_2], transiently elevating the level of PtdIns (3,4,5)P_3 from undetectable to nearly 10% of the PtdIns (4,5)P_2 level (2–4). The concentration of PtdIns(3,4,5)P_3 is tightly regulated by the activity of PI 5- and 3-phosphatases, such as SHIP1/2 and PTEN, which dephosphorylate the inositol ring generating PtdIns(3,4)P_2 and PtdIns (4,5)P_2 (5, 6). Despite the transitory accumulation and low concentrations in the plasma membrane, PtdIns (3,4,5)P_3 is implicated in fundamental biological processes including growth, proliferation, migration, and survival of cells (1, 7). The PtdIns(3,4,5)P_3-mediated signals are primarily recognized and transduced by pleckstrin homology (PH) domain-containing proteins that bind strongly and in some cases specifically to PtdIns(3,4,5)P_3. Mutations in the PH domains that disrupt or promote PtdIns (3,4,5)P_3 binding cause various signaling disarrays leading to severe immune system disorders and cancers

Abbreviations: GRP1, general receptor for phosphoinositides isoform 1; HSQC, heteronuclear single quantum coherence; IP4, inositol 1,3,4,5-tetrakisphosphate; PH, pleckstrin homology; PI, phosphoinositide; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; PS, phosphatidylserine; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; SPR, surface plasmon resonance.

[1] To whom correspondence should be addressed.
e-mail: Tatiana.Kutateladze@UCHSC.edu

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(8, 9). Among the best characterized PtdIns(3,4,5)P_3-recognizing protein effectors are Bruton's tyrosine kinase, ADP-ribosylation factor (Arf)-nucleotide binding site opener (ARNO), Cytohesin-1, and general receptor for phosphoinositides isoform 1 (GRP1) (10–12). GRP1 is a member of the GTP/GDP exchange factors family that catalyzes guanine nucleotide exchange of Arf proteins essential for actin rearrangement, endo- and exocytosis, membrane budding, and trafficking. Whereas the enzymatic activity of GRP1 is attributed to the central Sec7 homology domain, its membrane anchoring relies exclusively on the carboxy-terminal PH domain (13).

The PH domains comprise one of the largest families of signaling modules and are the most thoroughly characterized among PI binding domains (14, 15). The PH domain was identified within a set of human proteins in 1993 and named after the two homologous regions of pleckstrin, the major protein kinase C substrate of platelets (16, 17). Since then, it has been found in several hundred eukaryotic proteins involved in intracellular signaling, membrane trafficking, cytoskeletal dynamics, and lipid modifications. The PH domain contains ~120 residues that are folded in a highly conserved three-dimensional structure despite little sequence similarity between the family members. The fold consists of a seven-stranded β-barrel, capped by an amphipathic α helix at one edge, whereas the opposite edge is framed by three variable loops. The β1-β2, β3-β4, and β6-β7 loops form a PI binding pocket, and their length and primary sequence define the PH domain specificity. Like other PH modules, the GRP1 PH domain is electrostatically polarized and has a strong positive electrostatic potential surrounding the binding site, which may contribute to both the specific PtdIns(3,4,5)P_3 binding and the weak nonspecific electrostatic interactions with other acidic lipids in membranes (14, 18, 19). Although the majority of PH domains exhibit broad selectivity and associate with several PIs, recruiting their host proteins to different intracellular membranes, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 remain the most common targets for this family of lipid binding effectors.

The details of how the PH domain recognizes the pattern of sequential 3-, 4-, and 5-phosphate groups in PtdIns(3,4,5)P_3 is provided by the crystal structure of the GRP1 PH domain in complex with inositol 1,3,4,5-tetrakisphosphate (IP4), an isolated headgroup of PtdIns(3,4,5)P_3 (20, 21). The tetrakisphosphate molecule lies in the center of a deep, positively charged pocket formed by the β1-β2, β3-β4, and β6-β7 loops and the strands they connect. The distal phosphates are the most buried in the pocket, whereas the 1-phosphate group is positioned near the tips of the loops. A network of hydrogen bonds, formed between conserved lysine and arginine residues of β2, β3, β4, and β7 and all four phosphate groups of IP4, efficiently restrains the inositol molecule. A unique β-hairpin in the long β6-β7 loop of GRP1 is involved in additional hydrogen bonding contacts with the 5-phosphate group of IP4. These additional interactions with the 5-phosphate account for the high specificity of the GRP1 PH domain toward PtdIns(3,4,5)P_3.

Despite the critical role in GRP1 function, the precise mechanism by which the PH domain associates with PtdIns(3,4,5)P_3-enriched membranes remains unclear. Because PtdIns(3,4,5)P_3 is present at a very low level even in stimulated cells, elucidation of the overall membrane targeting is essential for our understanding of how the GRP1 PH domain distinguishes PtdIns(3,4,5)P_3 and selectively binds this relatively rare lipid but does not recognize other, more-abundant PIs. Here we present the molecular basis of membrane docking, penetration, and pH regulation of the GRP1 PH domain based on structural and quantitative analysis of its interactions with lipids and membrane-mimicking monolayers and bilayers. Our results, derived from lipid binding measurements using NMR, surface plasmon resonance (SPR), liposome binding assays, and monolayer surface tension combined with mutagenesis data, provide novel insights into the mechanism of membrane recruitment and anchoring by GRP1.

MATERIALS AND METHODS

Subcloning, expression, and purification of GRP1 PH domain

The DNA fragment encoding residues 261–385 of the PH domain of human GRP1 was cloned into the pRSET A vector (Invitrogen). The unlabeled, ^{15}N-labeled, and ^{15}N/^{13}C-labeled proteins were expressed in *Escherichia coli* Rosetta in Luria-Bertani or minimal media supplemented with $^{15}NH_4Cl$ and $^{13}C_6$-glucose (Cambridge Isotope). Bacteria were harvested by centrifugation after induction with isopropyl-1-thio-β-D-galactopyranoside (0.1 mM) and lysed by French Press. The 6× His-fusion proteins were purified on a Talon-resin column (Clontech Laboratories, Inc.). The His tag was cleaved with EKMax (Invitrogen). The proteins were further purified by ion exchange chromatography on a HiTrap SP HP column (Amersham) in Bis-Tris buffer, pH 6.5, and concentrated in Millipore concentrators.

PCR mutagenesis of GRP1 PH

Site-directed mutagenesis of the GRP1 PH domain was performed using a QuikChange kit (Stratagene). The sequence of the H355A construct was confirmed by DNA sequencing.

NMR spectroscopy and sequence-specific assignments

Multidimensional homo- and heteronuclear NMR spectra were recorded at 25°C on Varian INOVA 500, 600, and 800 MHz spectrometers. The amino acid spin system and sequential assignments were made using three-dimensional ^{15}N-edited NOESY-heteronuclear single quantum coherence (HSQC) (22, 23), ^{15}N-edited TOCSY-HSQC (24) and triple-resonance experiments, such as HNCACB (25), CBCA(CO)NH (26), HNCO (26), C(CO)NH (27), and H(CCO)NH (27).

NMR titrations of lipids

The ^{1}H,^{15}N HSQC spectra of 0.2 mM uniformly ^{15}N-labeled PH domain were collected using 1,024 t_1 increments of 2,048 data points, 96 number of increments, and spectral widths of 7,500 and 1,367 Hz in the ^{1}H and ^{15}N dimensions, respectively. Lipid binding was characterized by monitoring chemical shift changes in the ^{1}H,^{15}N HSQC spectra of the PH domain as C_4-PtdIns(3,4,5)P_3, C_4-PtdIns(4,5)P_2, C_4-PtdIns(4)P (Echelon Biosciences, Inc.), and inositol hexakisphosphate (IP6) (Aldrich) were added step-

wise up to 4 mM. Significant changes in the resonances were judged to be greater than the average plus one standard deviation.

Monolayer measurements

The penetration of the wild-type and mutant GRP1 PH domains into the phospholipid monolayer was investigated by measuring the change in surface pressure (π) of invariable surface area during addition of the proteins. The experiments were performed using a 1 ml circular Teflon trough and wire probe connected to a Kibron MicroTrough X (Kibron, Inc., Helsinki). A lipid monolayer containing various combinations of phospholipids was spread onto the subphase composed of either 10 mM KH_2PO_4/0.16 M KCl (pH 6.0), 10 mM HEPES/0.16 M KCl (pH 7.4), or 10 mM HEPES/0.16 M KCl (pH 8.0) until the desired initial surface pressure (π_0) was reached. After stabilization of the signal (~5 min), 10 μg of protein was injected into the subphase through a hole in the wall of the trough. The surface pressure change ($\Delta\pi$) was monitored for 45 min. The $\Delta\pi$ value reached a maximum after 30 min in all experiments.

Liposome binding

The liposome binding assays were performed as described in (28). Briefly, solutions of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) (Avanti), and C_{16}- PtdIns(3,4,5)P_3 (Echelon Biosciences, Inc.) dissolved in $CHCl_3$:MeOH:H_2O (65:25:4) were mixed and dried down under vacuum. The lipids were resuspended in 50 mM MOPS, 150 mM KCl, 1 mM DTT, pH 7.0, and incubated at 65°C for 1 h. The liposomes were then frozen in liquid nitrogen and thawed at 37°C for three cycles. The liposome solution was passed through an Avanti extruder to make 1.0 μm liposomes. Liposomes were collected by centrifugation at 25,000 *g* for 20 min and resuspended to a final concentration of 4 mM total lipids in 100 μl of binding buffers, containing 150 mM NaCl, 1 mM DTT, and 50 mM either NaAc/HAc (pH 5.0), MES (pH 6.0), MOPS (pH 7.0), or Tris (pH 8.0). Liposomes were incubated with the 6× His-fusion GRP1 PH domain (100–250 μg/ml final protein concentration) for 30 min at room temperature and then collected again by centrifugation. The liposome pellets were resuspended in 100 μl of buffer and analyzed using SDS-PAGE and Coomassie brilliant blue staining.

SPR measurements

All SPR experiments were performed at 25°C as described previously (29). Equilibrium SPR measurements were carried out at a flow rate of 5 μl/min. After injecting 85 μl of GRP1 PH domain, the protein-lipid association and dissociation were measured for 1,020 s and 500 s, respectively. The sensorgrams were obtained using five or more different concentrations of the protein (within a 10-fold range of K_d) for each condition tested. The sensorgrams were corrected for refractive index change by subtracting the control surface response. A total of three data sets were generated to obtain a standard deviation. The maximal response (R_{eq}, saturation value) was determined from the plots and plotted versus protein concentrations (C). The K_d value was determined by a nonlinear least-squares analysis of the binding isotherm using the equation $R_{eq} = R_{max}/(1 + K_d/C)$ (30).

PtdIns(3,4,5)P_3 beads pull-down

The 2–5 μg of 6× His-GRP1 PH domain was incubated with 100 μl of PtdIns(3,4,5)P_3 beads or control beads (Echelon Biosciences, Inc.) suspended in 150 mM NaCl, 2 mM DTT, 0.5% NP40, and 50 mM either MES (pH 6.0), MOPS (pH 7.0), Tris (pH 8.0), or CHES (pH 9.0) buffers at room temperature for 1 h. The beads fraction and supernatant were separated by centrifugation and analyzed by SDS-PAGE.

RESULTS AND DISCUSSION

The PtdIns(3,4,5)P_3 lipid and its isolated headgroup occupy similar binding sites of the GRP1 PH domain

To establish the molecular basis of the lipid recognition, the interaction of the GRP1 PH domain with PtdIns(3,4,5)P_3 was investigated by NMR. The ^1H,^{15}N HSQC spectra of uniformly ^{15}N-labeled protein were collected while a soluble di-butanoyl (C_4) form of PtdIns(3,4,5)P_3 was gradually added into the NMR sample (**Fig. 1A, B**). As PtdIns(3,4,5)P_3 was titrated in, the resonances of the ligand-free PH domain decreased in intensity and disappeared completely once the protein-lipid ratio had reached 1:1 (Fig. 1B). Concomitantly, another set of resonances corresponding to the lipid-bound state of the protein gradually appeared and replaced the initial set of resonances at equal molar concentrations of the PH domain and PtdIns(3,4,5)P_3. This pattern of chemical shift changes is characteristic of slow exchange on the NMR time scale and indicates a robust interaction between the PH domain and PtdIns(3,4,5)P_3. Almost identical resonance perturbations in the PH domain spectra were induced by IP4, an isolated headgroup of PtdIns(3,4,5)P_3, suggesting that both molecules are bound in the same binding pocket, with the inositol ring involved in the most critical contacts. Only small differences were detected for three residues (K279 of the β1-β2 loop and T344 and A346 of the β6-β7 loop) (see supplementary Fig. I). These residues are located near the 1-phosphate group of IP4 and most likely are affected by the glycerol moiety, attached to this phosphate in the PtdIns(3,4,5)P_3 lipid.

PtdIns(3,4,5)P_3 binding triggers large resonance perturbations in the binding site of the GRP1 PH domain but does not induce global conformational changes

To identify the most-perturbed residues, the resonances of the lipid-bound and apo-states of the PH domain were assigned using a standard set of three-dimensional NMR experiments, and compared. The normalized ^1H and ^{15}N chemical shift differences are shown as a histogram in Fig. 1C. The most-pronounced resonance changes upon PtdIns(3,4,5)P_3 binding were observed for the K273, L274, G275, G276, V278, R284, and W285 residues of the β1 and β2 strands and the β1-β2 loop, I308 of β4, and G336, K343, N347, and E352 residues of the extended β6-β7 loop (Fig. 1C). All of these residues either directly interact with PtdIns(3,4,5)P_3, creating a dense net of hydrogen bonding contacts, or are located near the interacting residues, as seen in the crystal structure of the IP4-bound GRP1 PH domain (20, 21). The amino group of K273 forms hydrogen bonds with the 3- and 4-phosphate groups of the inositol ring. The backbone amides of G276 and V278, in concert with the side chain of K343, restrain the 5-phosphate moiety, whereas R284 is critical for coordinating the 3-

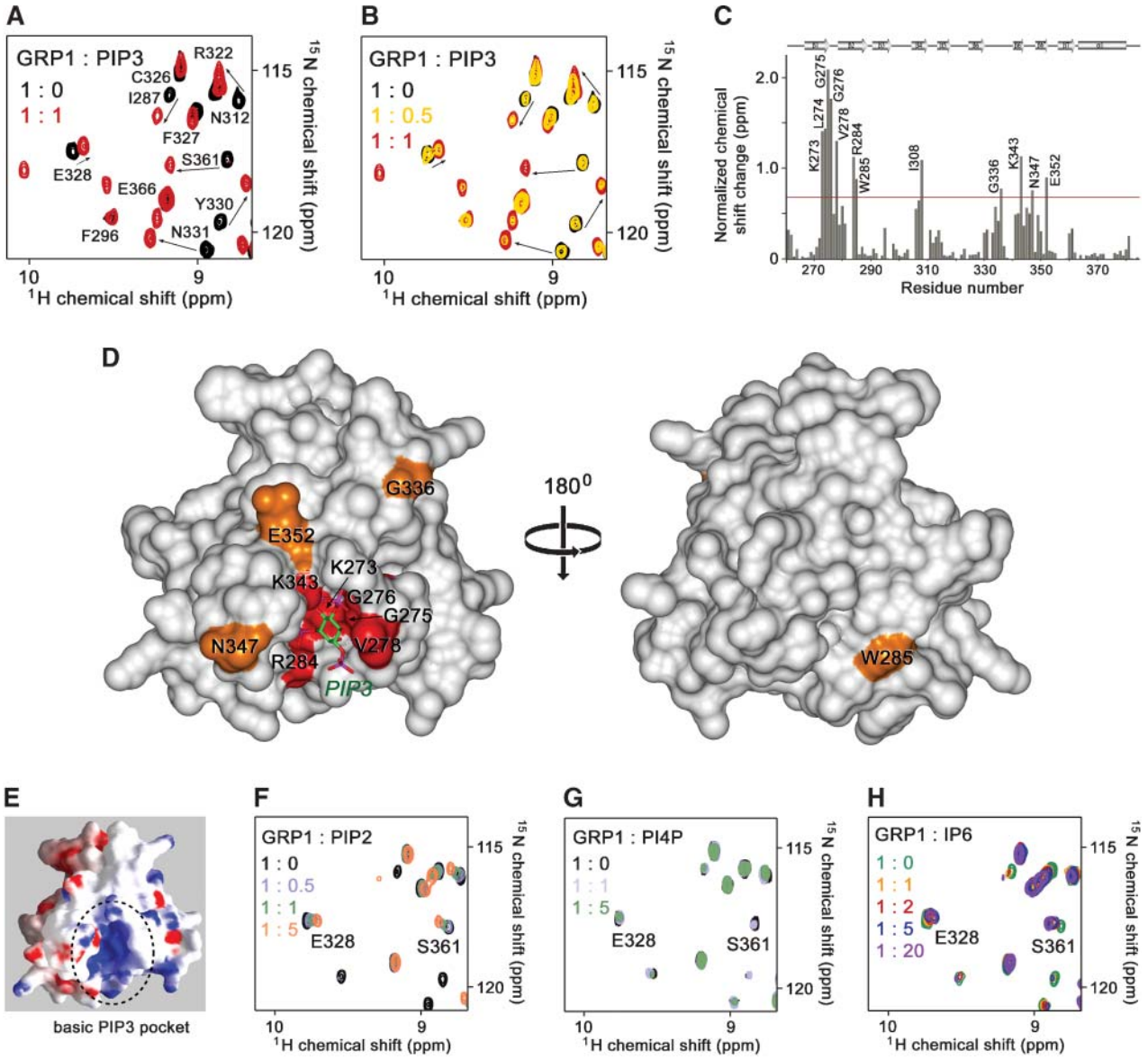

Fig. 1. The phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3 or PIP3] binding site of the general receptor for phosphoinositides isoform 1 (GRP1) pleckstrin homology (PH) domain. A: The superimposed ^{1}H,^{15}N heteronuclear single quantum coherence (HSQC) spectra of the PtdIns(3,4,5)P_3-bound (red) and the ligand-free (black) PH domain (0.2 mM). B: The ^{1}H,^{15}N HSQC spectra of the PH domain collected during titration with PtdIns(3,4,5)P_3. The spectra are color-coded according to the concentration of the lipid. C: The histogram shows normalized (42) chemical shift changes induced in the backbone amides of the PH domain by addition of PtdIns(3,4,5)P_3 at a 1:1 protein-to-lipid ratio. The horizontal line indicates the average change plus one standard deviation. The secondary structure is shown above the histogram. D: Residues that display significant chemical shift change in *c* are labeled on the GRP1 PH domain (Protein Data Bank ID: 1FGY) surface and colored in red and orange for large and medium changes, respectively. E: The electrostatic surface potential of the GRP1 PH domain, colored blue and red for positive and negative charges, respectively, reveals the basic pocket where the inositol headgroup is bound. F–H: The superimposed ^{1}H,^{15}N HSQC spectra of the ^{15}N-labeled GRP1 PH domain collected during gradual addition of (F) PtdIns(4,5)P_2 (or PIP2), (G) PtdIns(4)P (or PI4P), and (H) inositol hexakisphosphate. The spectra are color-coded according to the concentration of the ligands, as shown in the insets.

phosphate group. In addition, several amino acids were conformationally perturbed, being in close proximity to the residues involved in the hydrogen bonding contacts. Thus, L274 and G275 of the β1-β2 loop are flanked by the key residues, K273 and G276. The W285 residue is adjacent to R284, and I308 and E352 are near the R305 and N354 residues, which donate hydrogen bonds to the 3-phosphate and 5-phosphate, respectively.

Mapping the chemical shift changes onto the GRP1 PH domain surface revealed a well-defined, deep pocket where PtdIns(3,4,5)P_3 is bound (Fig. 1D). Whereas the outer walls of the pocket are formed by neutral or hydrophobic residues, the inner region displays a strong positive potential (Fig. 1E), implying that electrostatic and hydrogen-bonding interactions play a major role in restraining the inositol ring. A unique set of these contacts is respon-

sible for the strong preference of the GRP1 PH domain for PtdIns(3,4,5)P_3 over other negatively charged lipids. For example, PtdIns(4,5)P_2 was bound in the same binding pocket, but this interaction was much weaker, judging by significant line broadening and/or gradual movement of NMR resonances, indicative of an intermediate-to-fast exchange (Fig. 1F). This is in agreement with the reported affinities of the GRP1 PH domain for PtdIns(3,4,5)P_3 (50 nM) and PtdIns(4,5)P_2 ($\sim 10^4$-fold lower) (18), and for the headgroups of these lipids, IP4 [27 nM [31]; 35 nM (12)] and IP3 [>4.5 μM (31); 6 μM (12)]. Furthermore, no chemical shift changes were observed upon titration of PtdIns(4)P, demonstrating that this most-abundant in plasma membrane monophosphorylated PI does not bind at all (Fig. 1G). On the other hand, an increase of the net negative charge of the headgroup did not enhance the binding. A 20-fold excess of IP6 caused only minor changes in the NMR spectrum of the GRP1 PH domain (Fig. 1H), confirming a weak binding in the micromolar range (31). Taken together, these results demonstrate that the GRP1 PH domain specifically recognizes the pattern of sequential phosphate groups in the inositol ring of PtdIns (3,4,5)P_3. The lack of significant resonance perturbations in other regions of the PH domain suggests that the PtdIns (3,4,5)P_3 binding induces local but not global conformational changes in the protein.

PtdIns(3,4,5)P_3 binding facilitates the membrane insertion of the GRP1 PH domain

A number of PI binding modules, including FYVE, PX, and ENTH domains have been shown to penetrate membranes following the recognition of PIs (30, 32–34). The PI coordination partially reduces positive potential surrounding the binding pocket, facilitating insertion of the adjacent hydrophobic residues into the bilayer. In general, PH domains harbor fewer hydrophobic residues near the PI binding site, however, recent studies have demonstrated that the PH domains of PLCδ1 (35, 36) and DAPP1 (37) penetrate membranes significantly. Initially, the ability of the GRP1 PH domain to penetrate membranes was examined by monitoring changes in surface tension of lipid monolayers in a neutral (pH 7.4) buffer. Phospholipid monolayers at the air-water interface serve as a highly sensitive tool for measuring the membrane penetration by peripheral proteins (30, 32–34). A POPC/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) (80:20) or POPC/POPE/PtdIns(3,4,5)P_3 (77:20:3) monolayer of initial surface pressure (π_0) was spread at constant area, and the change in surface pressure ($\Delta\pi$) after the injection of the protein was monitored (**Fig. 2A**, open and filled circles). $\Delta\pi$ is inversely proportional to π_0, and an extrapolation of $\Delta\pi_0$ versus π_0 yields the critical surface pressure π_c, which specifies an upper limit of π_0 that a protein can penetrate into (38). As shown in Fig. 2A, the GRP1 PH domain has low intrinsic membrane-penetrating ability. The π_c value of a POPC/POPE monolayer was found to be ~22 dyne/cm. Thus, in the absence of PtdIns(3,4,5)P_3, the GRP1 PH domain does not significantly insert into membranes. However, incorporation of 3 mol% PtdIns (3,4,5)P_3 into the monolayer substantially enhanced penetration, raising the π_c value to ~28 dyne/cm.

Membrane insertion of the GRP1 PH domain is increased in the acidic pH

We next examined the extent of the membrane penetration of the GRP1 PH domain into PtdIns(3,4,5)P_3-containing monolayers by varying the pH of the subphase buffer (Fig. 2B). When the pH was increased from 7.4 to 8.0, the monolayer surface pressure, π_c, was reduced from 28 to 26 dyne/cm, indicating that basic conditions inhibit the membrane insertion of the GRP1 PH domain. Conversely, the π_c value rose to 31 dyne/cm at pH 6.0, revealing that the membrane-penetrating ability is enhanced by an acidic environment. These results demonstrate that in low pH media, the GRP1 PH domain can easily penetrate physiological bilayers, because the surface pressure of cell membranes and large unilamellar vesicles is estimated to be in the range of 30–35 dyne/cm (30, 34, 38). In the absence of PtdIns(3,4,5)P_3, the GRP1 PH domain did not insert, and the π_c value of POPC/POPE monolayers remained unchanged for all pH conditions tested (~22 dyne/cm). Thus, PtdIns(3,4,5)P_3 is required for the strong anchoring and insertion of the protein into membranes. Likewise, PtdIns(3)P and PtdIns(4,5)P_2 are necessary for the FYVE, PX, and ENTH domains to sufficiently penetrate phospholipid monolayers or micelles (30, 33, 34, 39).

Targeting of the GRP1 PH domain to PtdIns(3,4,5)P_3-containing bilayers is pH dependent

To test whether the pH dependence is preserved for PtdIns(3,4,5)P_3 embedded in bilayers, the protein-lipid interaction was investigated by liposome binding assays. The GRP1 PH domain was incubated with small unilamellar vesicles (SUVs) composed of PC, PE, and PtdIns(3,4,5)P_3 at pH 5.0, 6.0, 7.0, or 8.0. Following centrifugation, the partitioning of the protein between the supernatant and pelleted fraction was examined. As shown in Fig. 2B, C, ~95% of the GRP1 PH domain was retained in the pelleted liposome fraction at a low pH of 6.0. At each progressively higher pH value, the protein was increasingly redistributed to the supernatant. Densitometry analysis of the gel bands revealed that binding to PtdIns(3,4,5)P_3-enriched vesicles was reduced from ~95% to ~25% as a result of alkalization of the buffer from pH 6.0 to pH 8.0 (Fig. 2C). In the absence of PtdIns(3,4,5)P_3, the protein did not associate with SUVs, confirming that PtdIns(3,4,5)P_3 is necessary for the membrane localization of GRP1.

Affinity of the GRP1 PH domain for PtdIns(3,4,5)P_3-containing vesicles increases in an acidic environment

To quantitatively assess the effect of pH, equilibrium binding constants of the GRP1 PH domain's association with bilayers were measured by SPR (Fig. 2D and **Table 1**). Binding of the PH domain to POPC/POPE/PtdIns(3,4,5)P_3 (78:20:2) vesicles was monitored at pH 6.0, 7.4, and 8.0. POPC/POPE (80:20) vesicles were used as a control, because it has been shown that the GRP1 PH domain is

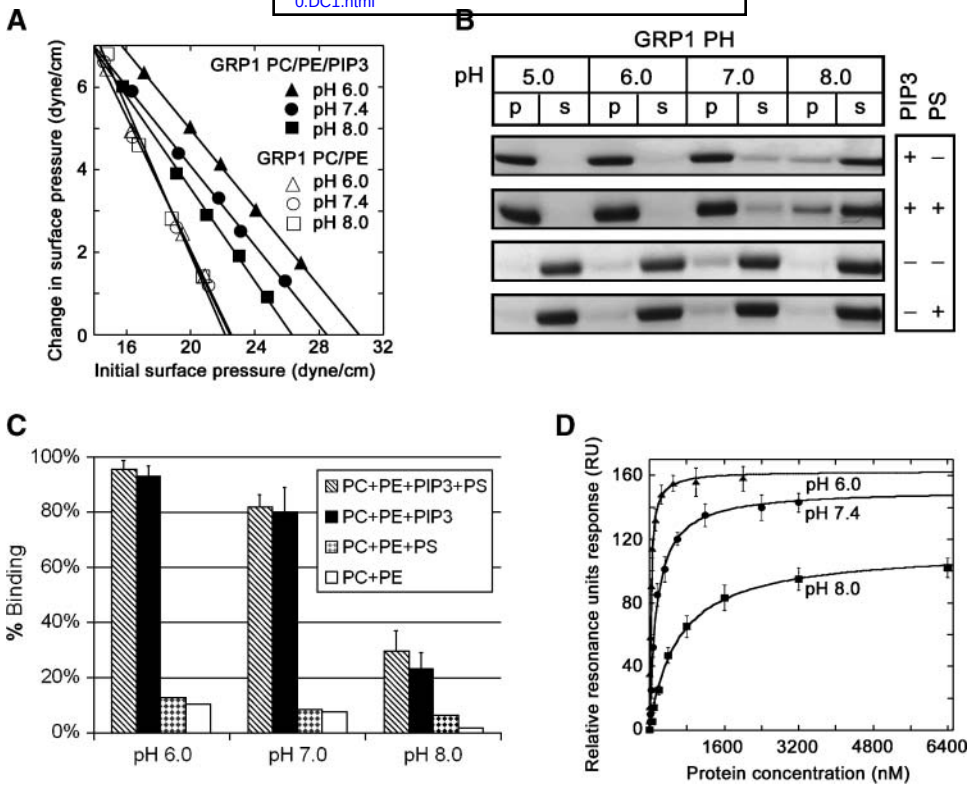

Fig. 2. Binding of the GRP1 PH domain to PtdIns(3,4,5)P_3-containing monolayers and bilayers is pH dependent. A: Insertion of the GRP1 PH domain into a POPC/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) (80:20) monolayer (open symbols) and a POPC/POPE/PtdIns(3,4,5)P_3 (77:20:3) monolayer (filled symbols) at pH 6.0 (triangle), 7.4 (circle), and 8.0 (square) monitored as a function of π. B,C: The SDS-PAGE gel and the histogram show partitioning of the PH domain between the supernatant (s) and liposome pellet (p) at indicated pHs in the liposome binding assays. The experimental points were averaged over at least three measurements. D: Affinity of the GRP1 PH domain for PtdIns(3,4,5)P_3-containing membranes increases in an acidic environment. Surface plasmon resonance measurements used to determine K_ds of the PH domain association with POPC/POPE/PtdIns(3,4,5)P_3 vesicles. The solid line represents a theoretical curve obtained based on R_{max} and K_d values determined by nonlinear least-squares analysis. Error bars indicate ± SD.

unable to localize with these vesicles even at high concentrations of protein. The obtained dissociation constants (K_ds) demonstrate that the GRP1 PH domain associated with POPC/POPE/PtdIns(3,4,5)P_3 vesicles, however, the binding affinity was significantly decreased when the pH of the buffer was raised from 6.0 to 8.0 (Table 1). At pH 6.0, the PH domain exhibited a 28 nM affinity for POPC/POPE/PtdIns(3,4,5)P_3 vesicles, whereas this interaction was 22-fold weaker at pH 8.0 (Table 1). Thus, our data indicate that pH can mediate association of the GRP1 PH domain with PtdIns(3,4,5)P_3-containing bilayers.

PtdIns(3,4,5)P_3 binding of the GRP1 PH domain is pH sensitive

To examine whether the pH dependence is attributable to the direct interaction between GRP1 PH and PtdIns(3,4,5)P_3, this binding was characterized in the absence of membrane mimetics by PtdIns(3,4,5)P_3 pull-down assays and NMR spectroscopy. The His-tag fusion GRP1 PH domain was incubated with PtdIns(3,4,5)P_3-immobilized beads at pH 6.0, 7.0, 8.0, and 9.0, and association with the lipid was examined by sedimentation and SDS-PAGE (**Fig. 3A**). The GRP1 PH domain showed a steady decrease in binding to PtdIns(3,4,5)P_3 beads when pH was raised from 6.0 to 9.0, indicating that the PtdIns(3,4,5)P_3 interaction is indeed pH sensitive and can be strengthened by the acidic media.

TABLE 1. Lipid binding properties of the GRP1 PH domain and H355A mutant

Protein	POPC/POPE/PtdIns(3,4,5)P_3 (77:20:3)	POPC/POPE/POPS/PI/Cholesterol/PtdIns(3,4,5)P_3 (9:35:22:9:22:3)
	K_d (nM)	
GRP1 wt pH 6.0	28 ± 2	4.8 ± 2
H355A pH 6.0	120 ± 10	22 ± 5
GRP1 wt pH 7.4	150 ± 10	28 ± 6
H355A pH 7.4	480 ± 50	88 ± 9
GRP1 wt pH 8.0	620 ± 40	110 ± 20
H355A pH 8.0	970 ± 60	240 ± 30

POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; PI, phosphatidylinositol; GRP1, general receptor for phosphoinositides isoform 1.

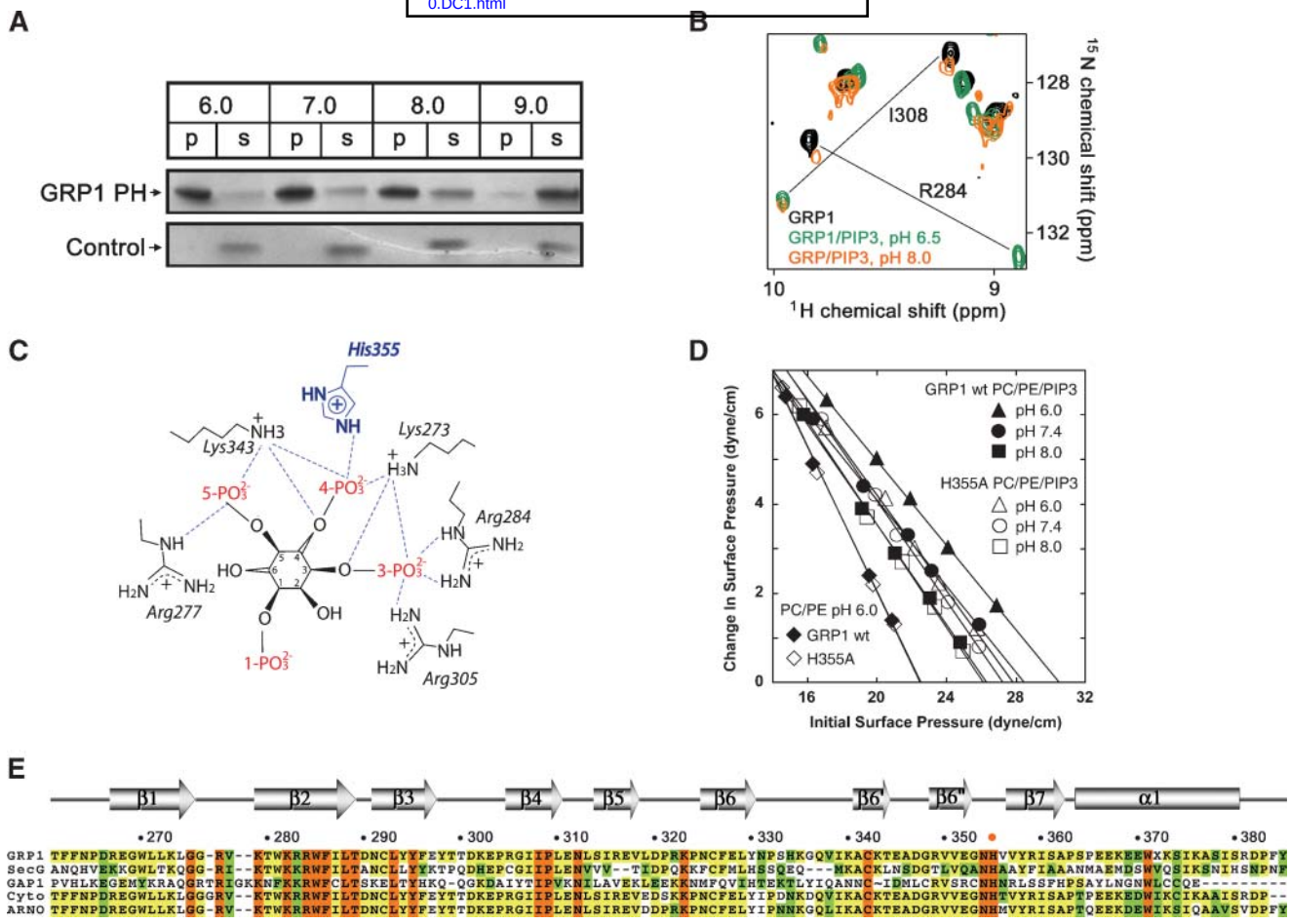

Fig. 3. The direct interaction of the GRP1 PH domain with PtdIns(3,4,5)P_3 is enhanced by lowering the pH. A: The SDS-PAGE gel displays the distribution of the His-tag fusion PH domain between the supernatant (s) and pelleted PtdIns(3,4,5)P_3 beads or control beads (p) at indicated pHs. B: Superimposed ^{1}H,^{15}N HSQC spectra of the ^{15}N-labeled GRP1 PH domain (0.2 mM) recorded in the absence (black) and presence of C_4-PtdIns(3,4,5)P_3 at pH 6.5 (green) and 8.0 (orange). C: His355 of the GRP1 PH domain mediates PtdIns(3,4,5)P_3 binding. A schematic diagram showing the PtdIns(3,4,5)P_3 headgroup coordination by the GRP1 PH domain (Protein Data Bank ID: 1FGY). Only charged residues of the proteins are depicted for clarity. D: Insertion of the wild-type GRP1 PH domain (filled symbols) and H355A mutant (open symbols) into a POPC/POPE (80:20) monolayer at pH 6.0 (diamond) and a POPC/POPE/PtdIns(3,4,5)P_3 (77:20:3) monolayer at pH 6.0 (triangle), 7.4 (circle), and 8.0 (square) monitored as a function of π. E: Alignment of the PH domain sequences: absolutely, moderately, and weakly conserved residues are colored orange, green, and yellow, respectively. The secondary structure is shown above the sequences. The conserved His355 residue of the GRP1 PH domain is indicated by the orange circle.

The enhancement of PtdIns(3,4,5)P_3 binding in a low pH environment was substantiated by NMR resonance perturbations observed in the PtdIns(3,4,5)P_3-bound GRP1 PH domain upon varying the solution pH (Fig. 3B). The ^{1}H,^{15}N HSQC spectra of the PH domain were recorded in the absence (black) and presence (green) of PtdIns(3,4,5)P_3 at a constant pH of 6.5. Binding of PtdIns(3,4,5)P_3 caused large chemical shift changes in the protein (Fig. 3B). As the pH of the sample was increased to 8.0, the crosspeaks corresponding to the PtdIns(3,4,5)P_3-bound PH domain (orange) shifted toward their positions in the ligand-free protein, thus indicating a weaker interaction under basic conditions. Consequently, the lipid-bound and -free states of the GRP1 PH domain appear to be stabilized by lowering and raising the pH, respectively. Taken together, our data demonstrate that the direct interaction of the GRP1 PH domain with PtdIns(3,4,5)P_3 is pH dependent and becomes stronger in acidic conditions.

His355 of the GRP1 PH domain mediates PtdIns(3,4,5)P_3 binding

The PtdIns(3,4,5)P_3 binding pocket of the GRP1 PH domain contains the His355 residue, which forms a hydrogen bond with the 4-phosphate group of PtdIns(3,4,5)P_3 (Fig. 3C). To determine whether His355 plays a role in the pH sensitivity, it was replaced with Ala, and binding of the H355A mutant to POPC/POPE/PtdIns(3,4,5)P_3 vesicles was tested at different pH values. We found that the H355A mutant associates with vesicles ~4-fold weaker than the wild-type protein at pH 6.0, 3-fold weaker at pH 7.4, and 1.6-fold weaker at pH 8.0 (Table 1). Overall, the binding affinity of H355A dropped by 8-fold when pH was changed from 6.0 to 8.0. In contrast, the wild-type GRP1 PH domain showed a 22-fold decrease in affinity. The H355A mutant did not entirely abolish this interaction, suggesting that lysine and arginine residues in the PtdIns(3,4,5)P_3 binding site play critical roles in coordinating the

lipid (Fig. 3C). Unlike the wild-type protein, the H355A mutant was unable to effectively insert into the POPC/POPE/PtdIns(3,4,5)P_3 monolayer at any pH (Fig. 3D). In fact, it had lower penetrating power at pH 6.0, than did the wild-type GRP1 PH domain at pH 7.4. Thus, His355 is necessary for the strong membrane anchoring and insertion, and its protonation increases the binding affinity. These results also support the idea that membrane penetration by the GPR1 PH domain requires efficient PtdIns(3,4,5)P_3 binding, which in turn can be promoted by acidic media.

Nonspecific electrostatic interactions with PS/PI enhance the GRP1 PH domain binding to PtdIns(3,4,5)P_3-containing vesicles

Recent studies have shown that nonspecific electrostatic interactions with anionic phospholipids often stimulate membrane association of peripheral proteins. This can be particularly important in the case of the inner leaflet of the plasma membrane, where the anionic lipid concentration is high (40). To determine whether the interactions with acidic lipids other than PtdIns(3,4,5)P_3 contribute to the membrane targeting by the GRP1 PH domain, we examined its association with plasma membrane mimetic liposomes containing PC, PE, PS, PI, cholesterol, and PtdIns(3,4,5)P_3 (Table 1). The wild-type GRP1 PH domain bound the membrane-mimicking vesicles with a K_d of 4.8 nM at pH 6.0. Thus, the binding affinity increased nearly 6-fold for the vesicles containing anionic PS/PI/PtdIns(3,4,5)P_3 lipids in comparison to the POPC/POPE/PtdIns(3,4,5)P_3 vesicles. A comparable ~6-fold amplification of the affinity was observed for the H355A mutant. This points to the significant contribution of the nonspecific electrostatic interactions to the membrane docking of the GRP1 PH domain and is in line with the recent report showing a 12-fold increase in the membrane affinity of GRP1 due to the presence of acidic lipids (18). Similarly, both the wild-type GRP1 PH domain and its H355A mutant exhibited an ~5- to 6-fold increase in the affinity for the plasma membrane mimetic at pH 7.4 and pH 8.0, indicating comparable contributions of nonspecific electrostatic interactions at any pH. As in other PI binding modules (19, 30, 32, 41), a strong positive potential surrounding the PtdIns(3,4,5)P_3 binding pocket of the GRP1 PH domain may facilitate the initial membrane association, reinforcing PtdIns(3,4,5)P_3 binding and alleviating penetration.

The pH sensitivity of the GRP1 PH domain was maintained in the interaction with the plasma membrane-mimicking liposomes. The binding affinity was reduced by ~23-fold upon alkalization of the buffer from pH 6.0 to pH 8.0 (Table 1). This decrease was similar to the 22-fold drop in binding affinity toward POPC/POPE/PtdIns(3,4,5)P_3 vesicles, suggesting that the pH dependence is primarily due to PtdIns(3,4,5)P_3 binding rather than to nonspecific electrostatic interactions with other acidic lipids.

In conclusion, our results reveal the multifaceted membrane docking by the GRP1 PH domain involving PtdIns(3,4,5)P_3 recognition that is *i*) facilitated by stabilizing electrostatic interactions with other acidic lipids, *ii*) regulated by pH, and *iii*) accompanied by insertion into the membrane. The His355 residue in the PI binding site may undergo reversible protonation/deprotonation as cytosolic pH fluctuations occur (28). This could modulate the affinity of the GRP1 PH domain for transiently accumulated PtdIns(3,4,5)P_3 in the plasma membrane. Future research is necessary to establish the physiological significance of pH sensing by the GRP1 PH domain. Alignment of the PH domain sequences shows conservation of His355 in ARNO, Cyto, GAP1, and SecG (Fig. 3E), suggesting a general regulatory mode for this subset of PH domains.

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