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The GRP1 PH Domain, Like the AKT1 PH Domain, Possesses a Sentry Glutamate Residue Essential for Specific Targeting to Plasma Membrane PI(3,4,5)P_3

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ABSTRACT: During the appearance of the signaling lipid PI(3,4,5)P_3, an important subset of pleckstrin homology (PH) domains target signaling proteins to the plasma membrane. To ensure proper pathway regulation, such PI(3,4,5)P_3-specific PH domains must exclude the more prevalant, constitutive plasma membrane lipid PI(4,5)P_2 and bind the rare PI(3,4,5)P_3 target lipid with sufficiently high affinity. Our previous study of the E17K mutant of the protein kinase B (AKT1) PH domain, together with evidence from Carpten et al. [Carpten, J. D., et al. (2007) *Nature 448*, 439−444], revealed that the native AKT1 E17 residue serves as a sentry glutamate that excludes PI(4,5)P_2, thereby playing an essential role in specific PI(3,4,5)P_3 targeting [Landgraf, K. E., et al. (2008) *Biochemistry 47*, 12260−12269]. The sentry glutamate hypothesis proposes that an analogous sentry glutamate residue is a widespread feature of PI(3,4,5)P_3-specific PH domains, and that charge reversal mutation at the sentry glutamate position will yield both increased PI(4,5)P_2 affinity and constitutive plasma membrane targeting. To test this hypothesis, we investigated the E345 residue, a putative sentry glutamate, of the general receptor for phosphoinositides 1 (GRP1) PH domain. The results show that incorporation of the E345K charge reversal mutation into the GRP1 PH domain enhances PI(4,5)P_2 affinity 8-fold and yields constitutive plasma membrane targeting in cells, reminiscent of the effects of the E17K mutation in the AKT1 PH domain. Hydrolysis of plasma membrane PI(4,5)P_2 releases the E345K GRP1 PH domain into the cytoplasm, and the efficiency of this release increases when Arf6 binding is disrupted. Overall, the findings provide strong support for the sentry glutamate hypothesis and suggest that the GRP1 E345K mutation will be linked to changes in cell physiology and human pathologies, as demonstrated for AKT1 E17K [Carpten, J. D., et al. (2007) *Nature 448*, 439−444; Lindhurst, M. J., et al. (2011) *N. Engl. J. Med. 365*, 611−619]. Analysis of available PH domain structures suggests that a lone glutamate residue (or, in some cases, an aspartate) is a common, perhaps ubiquitous, feature of PI(3,4,5)P_3-specific binding pockets that functions to lower PI(4,5)P_2 affinity.

Signaling events at the cytoplasmic surface of the plasma membrane play a central role in a wide array of signaling cascades, and the signaling lipid PI(3,4,5)P_3 serves as an important second messenger at that membrane surface. Upon activation by upstream signals, the regulatory enzyme phosphoinositide 3-kinase (PI3K) phosphorylates the constitutive plasma membrane lipid PI(4,5)P_2 to generate PI(3,4,5)P_3.[4−6] The resulting appearance of a PI(3,4,5)P_3 signal in the plasma membrane recruits an array of signaling proteins to the membrane surface. Typically, these proteins possess a pleckstrin homology (PH) domain that specifically targets PI(3,4,5)P_3. Upon recruitment to the plasma membrane, such proteins are activated and regulate various essential cell processes, including growth, chemotaxis, DNA synthesis, cytoskeletal rearrangements, vesicle trafficking, and apoptosis.[4,6−16]

More than 561 human proteins contain PH domains, and a significant fraction of these PH domains are believed to bind PIP lipids with high affinity.[17] PH domains that are recruited to the plasma membrane specifically during a PI(3,4,5)P_3 signaling event face a significant target recognition challenge: they must selectively bind PI(3,4,5)P_3, which is a rare membrane component, while excluding the constitutive plasma membrane PI(4,5)P_2 that is at least 100-fold more prevalent even during a peak PI(3,4,5)P_3 signal.[4,13]

PH domains share a common core structure consisting of two antiparallel β-sheets forming a β-sandwich.[7,18] At one edge of the β-sandwich, up to three connecting loops provide basic side chains and other contacts that form the binding pocket for the negatively charged target lipid headgroup. The length and sequence of these loops provide each PH domain with its appropriate headgroup specificity,[19] and in principle, loop mutations could alter membrane recognition, yielding dramatic cellular consequences. For example, Carpten et al.[1] found that the E17K mutation in the PH domain of regulatory kinase AKT1 causes constitutive plasma membrane targeting, hyperactivation of AKT1 kinase, and transformation of tissue culture cells. This mutation is linked to multiple human pathologies, including cancers and Proteus syndrome.[1,3]

Our previous study of the E17K AKT1 PH domain revealed the mechanistic basis of its constitutive plasma membrane targeting, thereby uncovering a new molecular mechanism of carcinogenesis and leading to the sentry glutamate hypothesis of PI(3,4,5)P_3 specificity.[2] For proper silencing of AKT1 kinase (also termed protein kinase B or PKB), it is crucial that the PH domain remain docked to its inhibitory site on the kinase

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domain until a $PI(3,4,5)P_3$ signal recruits the PH domain and relieves autoinhibition, thereby activating the kinase.[20−23] Consequently, it is crucial that the PH domain exclude constitutive plasma membrane $PI(4,5)P_2$ to prevent kinase activation in the absence of a $PI(3,4,5)P_3$ signal. We found that the E17K mutation greatly enhances the affinity of the PH domain for $PI(4,5)P_2$, thereby explaining its known biological and pathological effects (including constitutive plasma membrane targeting, kinase hyperactivation, cell transformation, carcinogenicity, and tissue overgrowth[2]). In addition, we proposed that other $PI(3,4,5)P_3$-specific PH domains may also possess sentry glutamate residues.

The sentry glutamate hypothesis predicts that a glutamate-to-lysine charge reversal mutation at the sentry glutamate position of an arbitrary $PI(3,4,5)P_3$-specific PH domain will, like the AKT1 E17K mutation, both enhance $PI(4,5)P_2$ affinity and drive constitutive plasma membrane targeting.[2] The present study tests this hypothesis by introducing the E345K charge reversal mutation at the putative sentry glutamate residue of general receptor for phosphoinositides 1 (GRP1). GRP1 is an Arf6 guanidine nucleotide exchange factor (GEF) that catalyzes the activation of Arf6-GDP to Arf6-GTP at the plasma membrane surface. The GRP1 PH domain possesses a $PI(3,4,5)P_3$-specific binding pocket as well as an Arf6 binding site that includes residues I307 and K340 (Figure 1).[24,25]

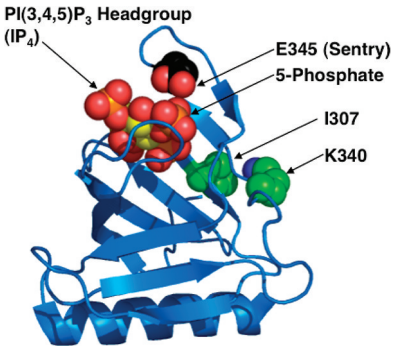

Figure 1. Locations of relevant residues in the crystal structure of the GRP1 PH domain.[18,26] The proposed sentry glutamate E345 is located adjacent to the binding pocket for the $PI(3,4,5)P_3$ headgroup (IP_4), near the 5-phosphate. The Arf6 docking surface includes residues I307 and K340.[24,25] This study employs the E345K charge reversal mutation to test the sentry glutamate hypothesis and the I307A mutation to weaken the interaction between the GRP1 PH domain and Arf6.

Previous studies have concluded that Arf6 and $PI(3,4,5)P_3$ binding both contribute to plasma membrane affinity.[25] The putative sentry glutamate residue of the GRP1 PH domain, E345 (Figure 1), is located adjacent to the $PI(3,4,5)P_3$ headgroup binding pocket. Notably, the GRP1 E345 residue is found on a different loop and on the opposite side of the $PI(3,4,5)P_3$ inositol ring relative to the AKT1 E17 residue,[18,26,27] but both glutamates are close enough to directly contact the bound headgroup. The diverse locations of putative sentry glutamates (see Discussion) are not surprising given the structural diversity of PH domain $PI(3,4,5)P_3$ binding pockets.

As predicted by the sentry glutamate hypothesis, these findings indicate that the GRP1 E345K mutation increases the affinity of the PH domain for $PI(4,5)P_2$ and triggers constitutive plasma membrane targeting. The effects of this mutation on $PI(3,4,5)P_3$ and $PI(4,5)P_2$ binding, both in vitro and in live cells, provide strong evidence that the native E345 side chain is a sentry glutamate ensuring minimal plasma membrane association until the appearance of a $PI(3,4,5)P_3$ signal. More broadly, analysis of the available crystal structures of other $PI(3,4,5)P_3$-specific PH domains reveals analogous acidic side chains adjacent to the headgroup binding pocket that likely serve as sentry glutamate (or aspartate) residues to decrease $PI(4,5)P_2$ affinity, thereby ensuring plasma membrane targeting and pathway activation occurs only during a $PI(3,4,5)P_3$ signal.

MATERIALS AND METHODS

Reagents. All lipids were synthetic unless otherwise indicated. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (phosphatidylcholine, POPC, or PC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (phosphatidylethanolamine, POPE, or PE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (phosphatidylserine, POPS, or PS), natural L-α-phosphatidylinositol from bovine liver, natural L-α-phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$ or PIP_2] from porcine brain, and natural sphingomyelin from porcine brain were all purchased from Avanti Polar Lipids. 1,2-Dipalmitoylphosphatidylinositol 3,4,5-trisphosphate [phosphatidylinositol 3,4,5-trisphosphate or $PI(3,4,5)P_3$] was from Cayman Chemical. *N*-[5-(Dimethylamino)naphthalene-1-sulfonyl]-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (dansyl-PE or dPE) was from Molecular Probes. Cholesterol (CH) and inositol 1,2,3,4,5,6-hexakisphosphate (IP_6) were from Sigma. Lipofectamine 2000 transfection reagent was from Invitrogen. Dithiothreitol (DTT) was purchased from Research Products International. Human recombinant PDGF-BB was purchased from PeproTech.

Preparation of Purified PH Domains. For in vitro binding studies, the PH domains of human GRP1 (residues 255−392) and mouse PLCδ1 (residues 12−142) were cloned by polymerase chain reaction as previously described into the EcoRI/XbaI site of a glutathione *S*-transferase (GST) fusion vector.[13] Mutagenesis of GRP1 PH was conducted using a QuickChangeXLII site-directed mutagenesis kit (Stratagene). The GST fusion constructs were overexpressed in *Escherichia coli* BL21 cells and isolated on a glutathione affinity column as previously described.[13]

Preparation of Lipid Mixtures and Phospholipid Vesicles. Phospholipid bilayer vesicles were created using three lipid compositions (see Table 1) to mimic the plasma membrane inner leaflet either with or without a PIP lipid: PM(−)PIP, PM(+)$PI(4,5)P_2$, and PM(+)$PI(3,4,5)P_3$. All lipids were dissolved in chloroform except $PI(3,4,5)P_3$, which was dissolved in a chloroform/methanol/water mixture (1/2/0.8).

Table 1. Lipid Composition of Plasma Membrane Mimics

name	lipid mixture	lipid mol %
PM(+)PIP_n	PE/PC/PS/PI/SM/CH/dPE/PIP_n	27.5/10.5/21/4.5/4.5/25/5/2
PM	PE/PC/PS/PI/SM/CH/dPE	29.5/10.5/21/4.5/4.5/25/5

Subsequently, each given lipid mixture was created at the indicated mole ratios and dried under vacuum until all solvent was removed, and then hydrated by being vortexed with binding buffer [25 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.4 with KOH), 140 mM KCl, 15 mM NaCl, and 0.5 mM $MgCl_2$]. Finally, sonicated unilamellar phospholipid vesicles (SUVs) were prepared using a Misonix XL2020 probe sonicator as previously described,[2,13] yielding a total lipid concentration of 3 mM.

Fluorescence Spectroscopy. Equilibrium binding experiments were conducted on a Photon Technology International QM-2000-6SE steady state fluorescence spectrometer at 25 °C in binding buffer (described above) with 10 mM dithiothreitol (DTT) as previously described.[2,13] Excitation and emission slit widths were 1 and 8 nm, respectively. Wavelengths used for specific experiments are indicated below.

Quantitative Measurement of PIP Lipid Affinity and Specificity. Our previously described competitive displacement assay was used to quantitate the high-affinity binding of each PH domain to the selected PIP lipids.[2,13] In this approach, soluble inositol 1,2,3,4,5,6-hexakisphosphate (IP_6) was employed as a competitive inhibitor to drive equilibrium displacement of the PH domain from PIP lipid-containing membranes, while monitoring displacement by protein-to-membrane FRET. To determine the equilibrium dissociation constant for PIP lipid binding, $K_D(PIP_n)$, it was necessary to measure both the affinity of the free PH domain for the competitive inhibitor IP_6 and the competitive inhibition constant for IP_6 displacement of the PH domain from the target membrane.

The equilibrium dissociation constant of the free PH domain for the competitive inhibitor, $K_D(IP_6)$, was measured using an increase in the intrinsic Trp fluorescence to monitor binding of IP_6 to the protein. Increasing concentrations of IP_6 were titrated into a sample containing free PH domain protein (0.5 μM), 10 mM DTT, and binding buffer. The intrinsic tryptophan fluorescence was measured using the following excitation and emission wavelengths: λ_{ex} = 284 nm and λ_{em} = 322 nm (for GRP1 PH) or λ_{em} = 340 nm (for PLCδ1 PH). Sodium phosphate, at 6 times the corresponding IP_6 concentration, was also added to a control sample that was run parallel to the sample experiments to determine the effect of a highly anionic molecule on the Trp emission. The raw data were then corrected for dilution, and the control cuvette data were subsequently subtracted, yielding the final titration data. Finally, a nonlinear least-squares curve fit employing eq 1 determined the equilibrium dissociation constant for IP_6 [$K_D(IP_6)$]:

$$F = \Delta F_{max}\{[IP_6]/[K_D(IP_6) + [IP_6]]\} + C \quad (1)$$

where F is the observed fluorescence at a given IP_6 concentration ($[IP_6]$) and ΔF_{max} is the total magnitude of the fluorescence change at saturation with IP_6.

The equilibrium apparent inhibition constant, $K_I(IP_6)_{app}$, was measured by titrating IP_6 into a sample containing the PH domain bound to a membrane-associated PIP lipid, using a protein-to-membrane FRET assay to monitor the membrane-bound PH domain. The solution contained 0.5 μM PH domain, 10 mM DTT, 3 μM accessible PIP lipid, 300 μM total lipid, and binding buffer. The IP_6 competitively displaces the PH domain from the membrane, yielding a decrease in FRET. IP_6 was also added to a control sample that was run parallel to the sample experiments to determine the effect of free IP_6 on the FRET emission of free membranes. The control sample contained 10 mM DTT, 3 μM accessible PIP lipid, 300 μM total lipid, and binding buffer. The raw data were corrected for dilution, and then the control was subtracted from each sample. Finally, a nonlinear least-squares curve fit utilizing eq 2 determined the equilibrium apparent inhibition constant for IP_6 [$K_I(IP_6)_{app}$]:

$$F = \Delta F_{max}\{1 - [IP_6]/[K_I(IP_6)_{app} + [IP_6]]\} + C \quad (2)$$

where F is the observed fluorescence and ΔF_{max} is the total magnitude of the fluorescence change at saturation with IP_6. Using $K_D(IP_6)$ and $K_I(IP_6)_{app}$ and employing eq 3, the desired equilibrium dissociation constant for the binding of the PH domain to the membrane-associated PIP lipid [$K_D(PIP_n)$] was deduced:

$$K_I(IP_6)_{app} = K_D(IP_6)[1 + [PIP_n]_{free}/K_D(PIP_n)] \quad (3)$$

For additional details, see our previous publications.[2,13]

Quantitative Measurement of Association and Dissociation Kinetics. All kinetic experiments were conducted using an Applied Photophysics SX.17 stopped-flow fluorescence instrument to monitor changes in protein-to-membrane FRET induced by membrane association or dissociation, always in binding buffer with 10 mM DTT, at 25 °C as previously described.[2,13] Association kinetics were measured by rapidly mixing PH domain (0.5 μM) with SUVs (total lipid concentration of 300 μM) containing excess PIP_n target lipid (3 μM accessible). Under these conditions, the forward binding reaction is virtually irreversible because of the high affinity of the PH domain for PIP lipid, and best fit analysis of the binding time course with a double exponential yielded a predominant, fast component used to calculate the on rate constant (k_{on}). Dissociation kinetics were measured by rapidly mixing the preformed, PH domain−membrane complex (0.5 μM protein and 300 μM total lipid) with excess IP_6 (10 mM for the GRP1 WT PH domain with PIP_3, 20 mM for the GRP1 E345K or E345K/I307A PH domain with PIP_3, and 8 mM for the GRP1 E345K or E345K/I307A or PLCδ1 PH domain with PIP_2). Best fitting of a double-exponential function to the dissociation time course typically yielded fast and predominant slow components, and the latter was used to calculate the off rate constant (k_{off}) as previously described.[13]

Fluorescent Fusion Proteins. The pEGFP-GRP1 PH construct was a kind gift to our lab from B. Tycko (Columbia University Medical Center, New York, NY),[28] and pRFP-PLCδ1 PH was a kind gift from M. Katan (Cancer Research UK Centre for Cell and Molecular Biology, London, U.K.). In each plasmid, the fluorescent protein was replaced with citrine fluorescent protein (Cit) by subcloning. Mutagenesis of Cit-GRP1 PH to generate the indicated mutations was conducted using a QuickChangeXLII site-directed mutagenesis kit (Stratagene).

Transfections into Live Cells. NIH 3T3 cells (American Type Culture Collection) were cultured in DMEM containing 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.292 mg/mL glutamine in 5% CO_2 at 37 °C. Cells were plated in 6 cm culture dishes at a density of 4.2 × 10^5 cells/dish and sat overnight. Transfections were conducted with 4 μg of total DNA using lipofectamine 2000 (Invitrogen) with OptiMEM (Invitrogen) according to the manufacturer's protocol. Prior to imaging, transfected cells (24−48 h after transfection) were plated in 35 mm glass

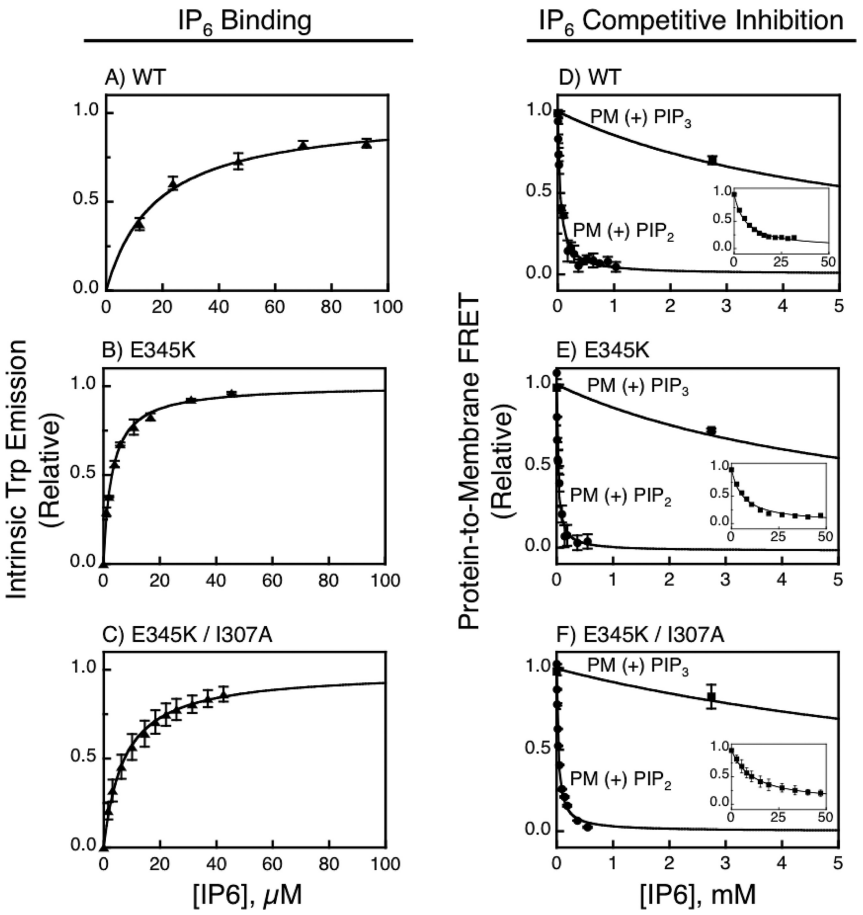

Figure 2. Quantitative equilibrium binding and specificity measurements. (A−C) Direct measurement of binding of IP_6 to the free PH domain in which each protein at 0.5 μM was titrated with IP_6 while the increase in tryptophan emission resulting from complex formation was quantitated (see Materials and Methods). (D−F) Competitive displacement assay for the indicated PH domain bound to either $PI(3,4,5)P_3$ (■) or $PI(4,5,)P_2$ (●) on plasma membrane-like sonicated unilamellar vesicles. To form the protein−membrane complex, protein (0.5 μM) was mixed with target vesicles (300 μM total lipid and 3 μM accessible PIP lipid). Subsequently, protein was displaced from target vesicles by titration with the competitive inhibitor IP_6 while the decrease in protein-to-membrane FRET was monitored (see Materials and Methods). In each panel, assays were conducted in triplicate at 25 °C in a physiological buffer, data points are averages (±one standard deviation), and best fit curves were defined by eq 1 (A−C) or 2 (D−F). Insets in panels D−F show full titration for proteins bound to $PI(3,4,5)P_3$. Table 2 presents parameters defined by averaging best fit values defined by three or more independent measurements.

bottom dishes (MatTek) at a density of 1×10^5 cells/dish and adhered for 1−2 h. Where indicated, serum starvation of cells was conducted in DMEM containing 0.2% BSA and 0.292 mg/mL glutamine and the cells were incubated for 6 h in 5% CO_2 at 37 °C.

Cell Imaging Studies. Transfected NIH 3T3 cells were rinsed and incubated in HBSS buffered with 25 mM HEPES (pH 7.4) (HHBSS) containing 0.01% endotoxin-free BSA. In experiments involving Ca^{2+}-stimulated $PI(4,5)P_2$ hydrolysis by phospholipase C, HBSS was purchased free of Ca^{2+} and Mg^{2+} and subsequently supplemented with 0.493 mM magnesium chloride and 0.407 mM magnesium sulfate to provide an initial Ca^{2+}-free extracellular environment. Immediately prior to imaging, ionomycin was added to a final concentration of 10 μM. Subsequently, at time zero, Ca^{2+} was added at the indicated extracellular concentration to trigger Ca^{2+} influx as previously described.[2,29,30] Citrine images were acquired on a Nikon inverted microscope equipped with a 60 × 1.4 NA oil immersion objective, a CFP/YFP-Cit/RFP dichroic mirror, single-band excitation and emission filters (Chroma Technology), a CoolSNAP ES camera (Photometrics), and a mercury lamp. For the kinetic time courses, time-lapse imaging involved 300 ms acquisitions with a total of 30 s between image sets analyzed in ImageJ (http://rsb.info.nih.gov/ij/).

RESULTS

Strategy and PH Domains Employed. This study of the putative sentry glutamate E345 of GRP1 began with the construction of a set of PH domains possessing side chains of varying charge at position 345. Our previous study[2] of AKT1 sentry glutamate E17K employed three sentry position variants: E17(WT), E17Q, and E17K. To facilitate comparisons with this previous study, we constructed and isolated the three corresponding GRP1 PH domains: E345(WT), E345Q, and E345K. The relative affinities of these three GRP1 PH domains for membrane-bound $PI(3,4,5)P_3$ and $PI(4,5)P_2$, each embedded in bilayers mimicking the lipid composition of the plasma membrane, were initially measured in a qualitative assay.[2] The resulting relative affinities were as follows: E345K > E345Q > E345(WT) for $PI(3,4,5)P_3$ and the same order for $PI(4,5)P_2$. These trends matched those previously observed for the AKT1 PH domain: E17K > E17Q > E17(WT) for both $PI(3,4,5)P_3$ and $PI(4,5)P_2$.[2] Thus, as predicted by the sentry glutamate hypothesis and previously noted for the AKT1 PH

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Table 2. Equilibrium and Kinetic Parameters for PH Domains Binding to Plasma Membrane Mimics Containing PIP Lipids

			equilibrium		kinetics		
PH domain	$K_D(IP_6)$ (μM)	plasma membrane mimic[a]	$K_I(IP_6)$ (mM)	$K_D(PIP_n)$ (nM)	k_{on} ($\times 10^6$ M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (k_{off}/k_{on}) (nM)
WT	20 ± 3	PM(+)PI(3,4,5)P_3	5 ± 2	14 ± 8	14 ± 1	0.79 ± 0.01	55 ± 1
		PM(+)PI(4,5)P_2	0.04 ± 0.01	3000 ± 2000	ND[b]	ND[b]	–
E345K	4.1 ± 0.1	PM(+)PI(3,4,5)P_3	7 ± 2	1.7 ± 0.1	20 ± 1	0.042 ± 0.007	2.1 ± 0.3
		PM(+)PI(4,5)P_2	0.026 ± 0.005	400 ± 50	53 ± 1	23 ± 1	430 ± 20
E345K/I307A	8 ± 2	PM(+)PI(3,4,5)P_3	14 ± 4	1.7 ± 0.7	35 ± 1	0.16 ± 0.01	4.6 ± 0.1
		PM(+)PI(4,5)P_2	0.035 ± 0.005	800 ± 100	32 ± 1	18 ± 1	550 ± 20
PLCδ1	4 ± 1	PM(+)PI(3,4,5)P_3	ND[b]	>10^4[c]	ND[b]	ND[b]	–
		PM(+)PI(4,5)P_2	0.07 ± 0.01	150 ± 20	180 ± 10	15 ± 1	81 ± 5

[a]The PIP lipid level is 2 mol % (Table 1). [b]Not determined because of insufficient affinity. [c]Lower limit determined as previously described.[2]

domain,[2] side chains with different charges at the putative GRP1 sentry position alter the PIP lipid affinity in the simple fashion expected for direct electrostatic interaction with a negatively charged headgroup. Relative to the neutral sentry glutamine, the negatively charged glutamate lowers affinity for the anionic PIP lipid, while the positively charged lysine increases affinity for the PIP lipid.

To more rigorously test the predictions and generality of the sentry glutamate hypothesis, the remainder of this study focuses on the native E345(WT) and E345K GRP1 PH domains. These two domains exhibit opposite extremes of PIP lipid affinity; moreover, E345K is analogous to the well-characterized, disease-linked AKT1 E17K mutation. The sentry glutamate hypothesis proposes the native E345 residue plays a key role in maintaining specific PI(3,4,5)P_3 targeting in cells by reducing its affinity for constitutive plasma membrane PI(4,5)P_2.[2,31] The hypothesis therefore predicts that the E345K mutation, by significantly increasing the affinity for PI(4,5)P_2, will allow the mutant protein to bind constitutively to plasma membranes in resting cells. Further experiments were conducted to quantitate the PI(3,4,5)P_3 and PI(4,5)P_2 affinities of E345(WT) and E345K GRP1 PH domains in vitro and to compare their plasma membrane targeting in cells. As these experiments proceeded, it became clear that Arf6 binding contributed significantly to GRP1 PH domain targeting in cells as previously reported;[24,25] thus, the E345K/I307A mutant was constructed to clarify the role of Arf6 interactions in the E345K background. Finally, the PI(4,5)P_2-specific PLCδ1 PH domain was included as a control displaying a target lipid preference opposite of that of the PI(3,4,5)P_3-specific E345(WT) GRP1 PH domain.[7,30,32,33] Figure 1 shows the locations in the GRP1 PH domain structure of the E345 and I307 positions targeted for mutation, and of the bound PI(3,4,5)P_3 headgroup (inositol-1,3,4,5-P_4 or IP_4).

The E345K Mutation Enhances GRP1 PH Domain Affinity for PI(4,5)P_2 and PI(3,4,5)P_3, with Important Implications for Target Lipid Specificity. A competitive displacement assay previously developed in our laboratory[13] was used to quantify the affinities of PH domains for PI(3,4,5)P_3 and PI(4,5)P_2 in sonicated unilamellar membrane vesicles. The competitive displacement assay allows determination of equilibrium dissociation constants for membrane-bound target lipids in the range of 0.1−10000 nM, well-suited for measuring the nanomolar affinity of the native GRP1 PH domain for target lipid PI(3,4,5)P_3 in bilayers composed of seven lipids mimicking the lipid composition of the plasma membrane inner leaflet (Table 1).[2] The first step of the assay measured the dissociation constant $K_D(IP_6)$ for the PH domain binding to the competitive inhibitor inositol hexakisphosphate (IP_6) in solution by titration of inhibitor into a sample of free PH domain while the increase in intrinsic tryptophan fluorescence triggered by inhibitor binding was monitored (eq 1). The second step measured the apparent inhibition constant $K_I(IP_6)$ for competitive displacement of the PH domain from the membrane-associated PIP lipid by titration of the inhibitor into a suspension of PH domains bound to vesicles while the decrease in protein-to-membrane FRET was monitored (eq 2). Finally, the standard equation for competitive inhibition (eq 3) was used to calculate the dissociation constant $K_D(PIP_n)$ for the PH domain binding to the membrane-associated target lipid.

Figure 2 summarizes the equilibrium titrations for (i) the native, wild-type GRP1 PH domain (WT), (ii) the single-mutant PH domain (E345K), and (iii) the double-mutant PH domain (E345K/I307A). Shown for each mutant are both the titration quantifying competitive inhibitor IP_6 binding to the free PH domain (Figure 2A−C) and the titration quantifying competitive displacement of the PH domain from membrane-associated PI(3,4,5)P_3 or PI(4,5)P_2 (Figure 2D−F) by IP_6. Table 2 summarizes the resulting equilibrium constants $K_D(IP_6)$, $K_I(IP_6)$, and $K_D(PIP_n)$ for the three GRP1 PH domains, as well as for the PLCδ1 PH domain (titrations not shown).

Comparison of the equilibrium dissociation constants for competitive inhibitor IP_6 reveals that the E345K mutation significantly increases the affinity of the free GRP1 PH domain for IP_6. Thus, the free E345K domain exhibits a nearly 5-fold higher IP_6 affinity than the WT domain [$K_D(IP_6)$ values of 4.1 ± 0.1 and 20 ± 3 μM, respectively]. By contrast, the I307A mutation at the Arf6 interaction site has little or no effect on inhibitor binding, such that the IP_6 affinity of the E345K/I307A mutant [$K_D(IP_6)$ = 8 ± 2 μM] is within 2-fold of that of E345K.

Comparison of the equilibrium dissociation constants for binding of the PH domain to PI(3,4,5)P_3 or PI(4,5)P_2 embedded in the plasma membrane-like bilayer reveals that the E345K mutation significantly increases the affinity of the GRP1 PH domain for both of these PIP lipids (Table 2). The native WT domain binds its membrane-associated target PI(3,4,5)P_3 with high affinity (K_D = 14 ± 8 nM) and binds membrane-bound PI(4,5)P_2 more than 200-fold more weakly (K_D = 3000 ± 2000 nM). At the opposite extreme, the PLCδ1 PH domain binds its target PI(4,5)P_2 with a relatively high affinity (K_D = 150 ± 20 nM) while exhibiting a much weaker affinity for PI(3,4,5)P_3 (K_D > 10^4 nM). In the GRP1 PH domain, the E345K charge reversal mutation yields an 8-fold

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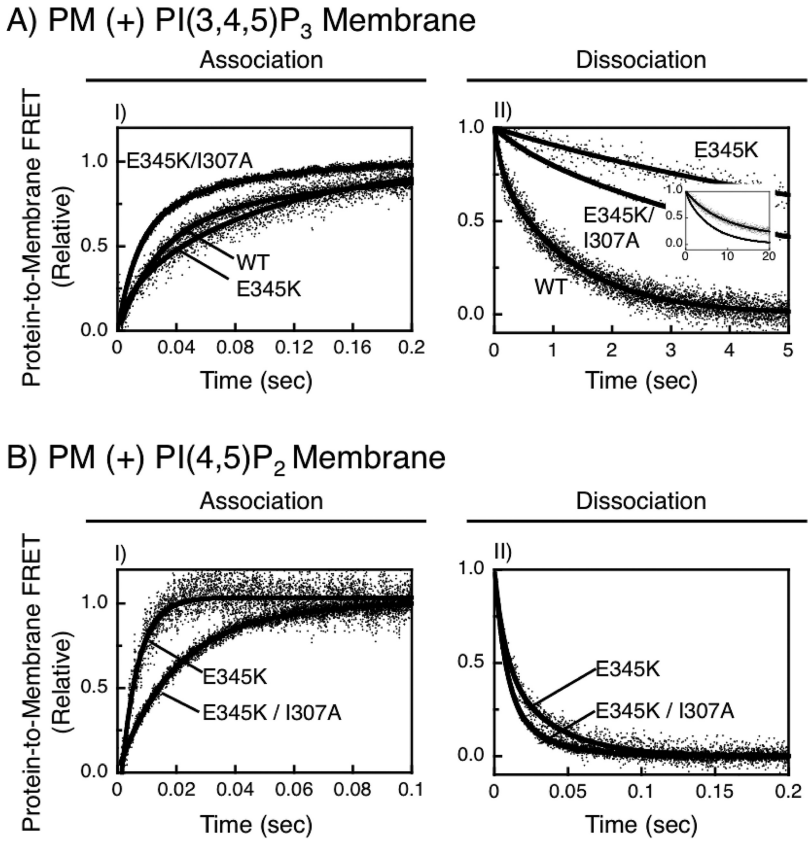

Figure 3. Association and dissociation rate measurements. Shown are association and dissociation reactions of the indicated PH domains binding to $PI(3,4,5)P_3$ (A) or $PI(4,5,)P_2$ (B) embedded in plasma membrane-like bilayers. In all cases, the final protein and lipid concentrations were identical to those in the equilibrium competitive displacement assays (Figure 2). Reactions were initiated by rapid mixing of either the free PH domain with membranes (association) or the prebound PH domain−membrane complex with IP_6 (dissociation) as described in Materials and Methods. Note that the time axis is optimized for each panel. For each reaction, at least five shots were conducted at 25 °C in a physiological buffer and then averaged to give the representative trace. Bold curves indicate best fit double-exponential functions, which yielded dominant fast (association) and slow (dissociation) components used to determine on and off rate constants as previously described.[13] For each rate constant, at least three independent measurements were averaged to produce the parameters summarized in Table 2. The inset shows the full time courses of the two mutant dissociation reactions.

greater affinity for the native target $PI(3,4,5)P_3$ (K_D = 1.7 ± 0.1 nM) relative to that of WT (Table 2). Strikingly, the E345K mutation also generates a nearly 8-fold higher affinity for $PI(4,5)P_2$ (K_D = 400 ± 50 nM) compared to that of WT (Table 2), yielding a $PI(4,5)P_2$ affinity within 2.6-fold of that of the PLCδ1 PH domain (Table 2). Because the latter domain is widely used as a $PI(4,5)P_2$ sensor and binds to constitutive plasma membrane $PI(4,5)P_2$ in cells, the E345K mutant is expected to target constitutively to the plasma membrane. Finally, the Arf6 interaction mutant E345K/I307A exhibits affinities for $PI(3,4,5)P_3$ and $PI(4,5)P_2$ (K_D values of 1.7 ± 0.7 and 800 ± 100 nM, respectively) that are indistinguishable, within a factor of 2, from the corresponding affinities for $PI(3,4,5)P_3$ and $PI(4,5)P_2$ observed for the E345K mutant (Table 2). It follows that, in the absence of Arf6, the I307A mutation has little effect on binding of the E345K mutant to target lipids.

Kinetic Analysis Reveals That E345K Triggers PIP Lipid Affinity Increases Primarily by Slowing Off Rates. In principle, the E345K mutation could increase the equilibrium affinities of the GRP1 PH domain for $PI(3,4,5)P_3$ and $PI(4,5)P_2$ by speeding association kinetics or slowing dissociation kinetics. To resolve these possibilities, where feasible the on and off rates were measured for the wild-type and E345K mutant PH domains binding to membrane-embedded $PI(3,4,5)P_3$ and $PI(4,5)P_2$ by stopped-flow fluorescence, using protein-to-membrane FRET to monitor membrane association or dissociation. Such kinetic measurements were straightforward for the interaction of wild-type and E345K domains with $PI(3,4,5)P_3$, because both proteins bind this lipid tightly [kinetic parameters could not be determined for the wild-type protein binding to $PI(4,5)P_2$ because of the low affinity].

Association reactions were conducted by rapidly mixing the free PH domain with target membranes and then monitoring the approach to equilibrium. Under the saturating conditions employed, the off rate is negligible and the approach to equilibrium yields the true on rate. Figure 3A and Table 2 show that all three of the GRP1 PH domains, regardless of whether they lack or possess the E345K mutation or the I307A Arf6 interaction mutation, exhibit similar on rates for $PI(3,4,5)P_3$ within a narrow 2.5-fold range [k_{on} values from $(14 \pm 1) \times 10^6$ to $(35 \pm 1) \times 10^6$ M^{-1} s^{-1}]. Moreover, the on rates of the E345K mutant and the E345K/I307A double mutant for both $PI(3,4,5)P_3$ and $PI(4,5)P_2$ fall within a similar 3-fold range [k_{on} values from $(20 \pm 1) \times 10^6$ to $(53 \pm 1) \times 10^6$ M^{-1} s^{-1} (Table 2)]. The observed similarities in the measurable on rates of GRP1 PH domains for $PI(3,4,5)P_3$ and $PI(4,5)P_2$ likely arise

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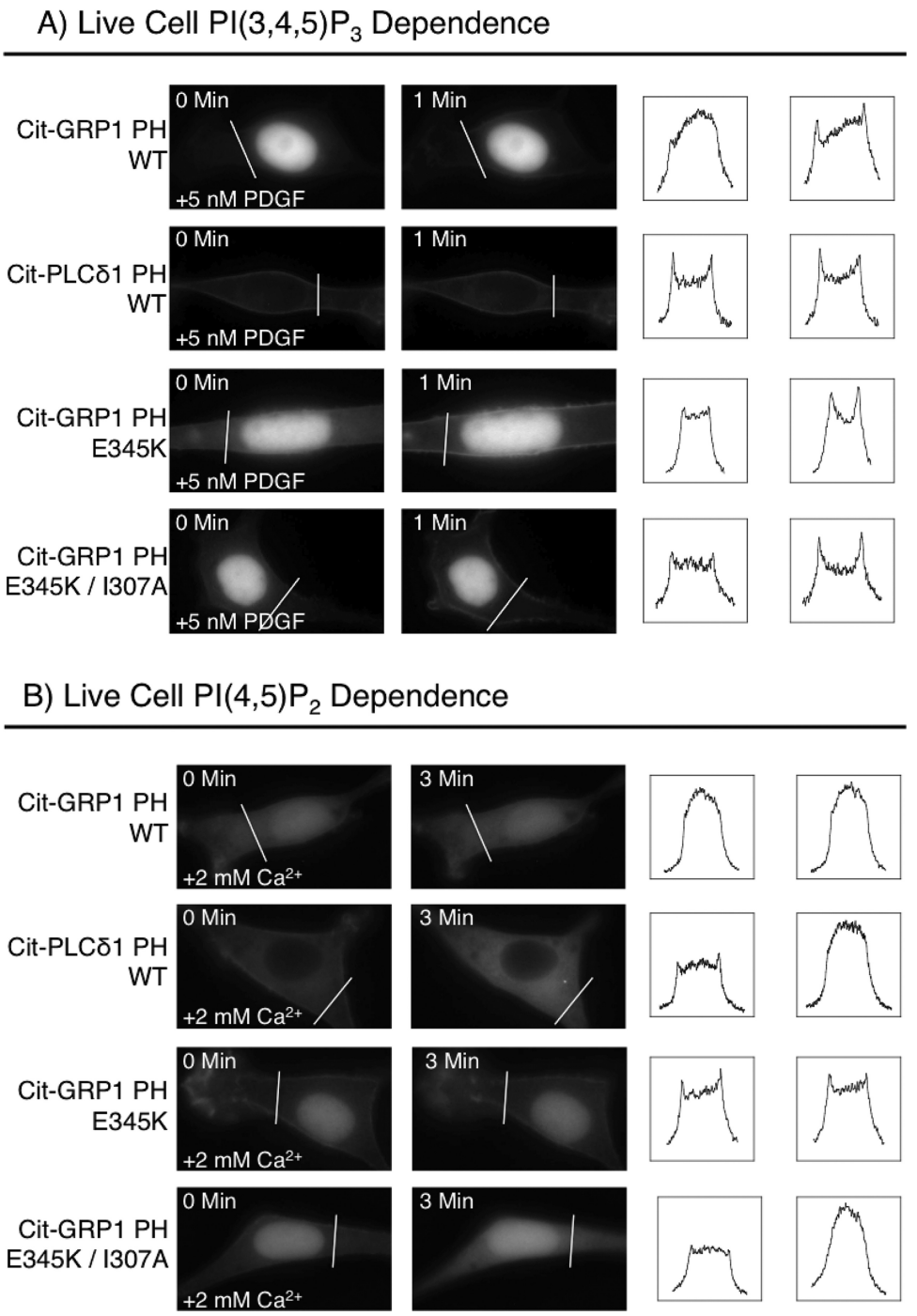

Figure 4. PIP lipid dependence of plasma membrane targeting in NIH 3T3 cells. (A) Dependence of membrane targeting on $PI(3,4,5)P_3$. Shown are the time zero and 1 min images for the indicated citrine-coupled PH domains. Serum-starved NIH 3T3 cells were stimulated with 5 nM PDGF at time zero and imaged at multiple time points (see Materials and Methods). (B) Dependence of membrane targeting on $PI(4,5)P_2$ for the indicated citrine-coupled PH domains. Shown are the time zero and 3 min images for the indicated citrine-coupled PH domains. Serum-starved NIH 3T3 cells were incubated in 20 μM ionomycin in Ca^{2+}-free buffer and then stimulated at time zero with 2 mM extracellular Ca^{2+} to trigger cytoplasmic Ca^{2+} influx and Ca^{2+}-activated $PI(4,5)P_2$ hydrolysis[45] with imaging at multiple time points (see Materials and Methods). Each set of images is a representative example of responses observed for 12 or more cells. To assist visualization of the protein movements between the plasma membrane and cytoplasm, we plotted fluorescence intensities both before (left box) and after (right box) treatment for pixels defined by the indicated line across each cell.

from the rate-determining electrostatic search mechanism that the PH domain is known to employ to find its rare target lipid, because this search mechanism relies on interactions between the protein and background PS lipids.[13,34] Such PS-based searching is expected to yield similar on rates for different PIP target lipids as long as they are present at similar mole percents, as in this study where $PI(3,4,5)P_3$ and $PI(4,5)P_2$ were always at 2 mol % and PS was always at 21 mol %.

Dissociation reactions were conducted by stopped-flow mixing preformed PH domain−membrane complexes with

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excess competitive inhibitor, IP_6, thereby ensuring that membrane dissociation is essentially irreversible. As shown in Figure 3A (II), the E345K mutation slows the dissociation of the GRP1 PH domain from $PI(3,4,5)P_3$-containing membranes by a factor of 18-fold (k_{off} = 0.042 ± 0.007 s^{-1}) relative to that of the wild type (k_{off} = 0.79 ± 0.01 s^{-1}) (Table 2). The rate of dissociation of the E345K mutant from $PI(4,5)P_2$ is well within the measurable range but is 540-fold faster (k_{off} = 23 ± 1 s^{-1}) than its rate of dissociation from $PI(3,4,5)P_3$ (k_{off} = 0.042 ± 0.007 s^{-1}) (Table 2). Interestingly, the E345K mutant exhibits a $PI(4,5)P_2$ off rate nearly the same (k_{off} = 23 ± 1 s^{-1}), within 1.5-fold, as that of the PLCδ1 PH domain (k_{off} = 15 ± 1 s^{-1}), helping to explain the similar $PI(4,5)P_2$ affinities of the two PH domains (Table 2). The effect of I307A on the dissociation kinetics of the E345K mutant is minimal, such that the E345K/I307A off rates for $PI(3,4,5)P_3$ and $PI(4,5)P_2$ are within 3.8-fold of the corresponding E345K off rates (Table 2).

Wherever possible, the measured on and off rate constants were used to calculate a kinetic dissociation constant for each protein−target lipid combination [$K_D(PIP_n) = k_{off}/k_{on}$]. As shown in Table 2, the K_D values calculated from kinetic constants are within 4-fold of the K_D values measured in equilibrium studies. Such good agreement validates both the equilibrium and kinetic measurements.

Examination of the on and off rate constants of the E345K mutant reveals that its higher affinity for $PI(3,4,5)P_3$ over $PI(4,5)P_2$ arises primarily from a 540-fold slower $PI(3,4,5)P_3$ off rate. Notably, however, the major functional difference between the wild-type GRP1 PH domain and the E345K mutant (see cell studies below) arises from the increased affinity of the mutant for $PI(4,5)P_2$. While it is not possible to directly determine the kinetic mechanism of this 8-fold affinity increase because of our inability to measure the WT $PI(4,5)P_2$ on rate, the $PI(4,5)P_2$ on rates of the wild-type and E345K PH domains are likely to be similar. Recall that the $PI(3,4,5)P_3$ on rates of the wild type and E345K, as well as the $PI(3,4,5)P_3$ and $PI(4,5)P_2$ on rates of the E345K and E345K/I307A mutants, are all similar because of the rate-limiting electrostatic search mechanism (see above). Assuming the WT $PI(4,5)P_2$ on rate falls in the same range because of its standard rate-limiting search process, it would follow that the 8-fold higher affinity of the E345K mutant for $PI(4,5)P_2$ arises from an approximately 8-fold slower dissociation from $PI(4,5)P_2$ relative to that of WT.

Together, the in vitro equilibrium and kinetic studies indicate the E345K putative sentry glutamate mutation in the GRP1 PH domain behaves like the oncogenic, E17K sentry glutamate mutation in the AKT1 PH domain that we characterized previously.[2] In both the E345K GRP1 and E17K AKT1 PH domain mutants, the glutamate to lysine charge reversal mutation significantly increases the affinities for both $PI(3,4,5)P_3$ and $PI(4,5)P_2$. Notably, the resulting affinity of the E345K GRP1 PH domain for $PI(4,5)P_2$, like that of the E17K AKT1 PH domain, is remarkably similar to the affinity for $PI(4,5)P_2$ of the PLCδ1 PH domain, the standard $PI(4,5)P_2$ sensor employed in many in vitro and live cell studies.[2,30] It was previously observed that the E17K AKT1 PH domain is bound to the plasma membrane in the absence of a $PI(3,4,5)P_3$ signal (1) because of its interaction with $PI(4,5)P_2$ (2). This in vitro evidence led us to predict that the E345K GRP1 PH domain would display a similar constitutive targeting to plasma membrane $PI(4,5)P_2$ in resting cells.

Live Cell Studies Confirm the GRP1 E345K PH Domain Is Targeted to the Plasma Membrane by $PI(4,5)P_2$. Live cell imaging was used to visualize the intracellular targeting patterns of the PH domains studied in vitro while the plasma membrane levels of $PI(3,4,5)P_3$ and $PI(4,5)P_2$ were varied. Each PH domain was fused at its N-terminus to citrine fluorescent protein (Cit), expressed in serum-starved NIH 3T3 fibroblasts, and imaged in live cells by wide field fluorescence microscopy. Resting cells were serum-starved to minimize stimulation by attractants and growth factors in the media, ensuring low basal plasma membrane levels of the signaling lipid $PI(3,4,5)P_3$.

Figure 4A summarizes the cellular distributions of PH domains in resting cells and in activated cells with high levels of plasma membrane $PI(3,4,5)P_3$. In resting cells, the wild-type GRP1 PH domain Cit−WT fusion is cytoplasmic with no detectable plasma membrane binding (Figure 4A,B). In contrast, the Cit−E345K mutant fusion protein is significantly targeted to the plasma membrane, consistent with its increased in vitro affinity for constitutive plasma membrane $PI(4,5)P_2$ (Figure 4A,B and Table 2). As expected, the PLCδ1 PH domain is also targeted to the plasma membrane in resting cells because of its high affinity for constitutive $PI(4,5)P_2$ (Figure 4A,B and Table 2). Global extracellular addition of PDGF activates phosphatidylinositol 3-kinase (PI3K) and generates plasma membrane $PI(3,4,5)P_3$.[35] The resulting $PI(3,4,5)P_3$ signal recruits the wild-type PH domain to the plasma membrane and drives additional plasma membrane recruitment of the E345K mutant (Figure 4A). The observed $PI(3,4,5)P_3$-stimulated recruitment of the wild-type and E345K PH domains to the plasma membrane is consistent with the high $PI(3,4,5)P_3$ affinities observed for both domains in vitro (Table 2). The E345K/I307A mutant behaves like the E345K mutant, exhibiting both detectable plasma membrane binding in resting cells (Figure 4A,B) and enhanced membrane targeting during a $PI(3,4,5)P_3$ signal (Figure 4A).

To directly test the hypothesis that the E345K mutant targets to plasma membrane $PI(4,5)P_2$ in resting cells, Ca^{2+} and ionomycin were added globally to increase the cytoplasmic Ca^{2+} concentration and activate PLCδ1 hydrolysis, thereby destroying the plasma membrane $PI(4,5)P_2$.[29,30] Figure 4B shows the effect of the resulting loss of $PI(4,5)P_2$ on the cellular distribution of each PH domain. The Ca^{2+}−ionomycin treatment fully relocates the PLCδ1 PH domain from the plasma membrane to the cytoplasm, confirming that the treatment destroys the $PI(4,5)P_2$ lipid this domain targets (Figure 4B). By contrast, the treatment only partially relocates the E345K domain to the cytoplasm (Figure 4B) unless the I307A mutation known to weaken the PH domain−Arf6 interaction[24,25] is present. Thus, the E345K/I307A double mutant is fully relocated to the cytoplasm by the Ca^{2+}−ionomycin treatment (Figure 4B). These observations demonstrate that $PI(4,5)P_2$ binding and Arf6 interactions both make important contributions to the plasma membrane affinity and bound state lifetime of the E345K mutant, consistent with the previous conclusion that the native GRP1 PH domain is a dual $PI(3,4,5)P_3$ and Arf6 sensor.[24,25]

Overall, the live cell findings strongly support the conclusion that the E345K sentry glutamate mutation in the GRP1 PH domain, like the corresponding E17K mutation in the AKT1 PH domain, drives constitutive plasma membrane targeting via its enhanced affinity for $PI(4,5)P_2$. As a result, the E345K GRP1 PH domain, like the E17K AKT1 PH domain, localizes

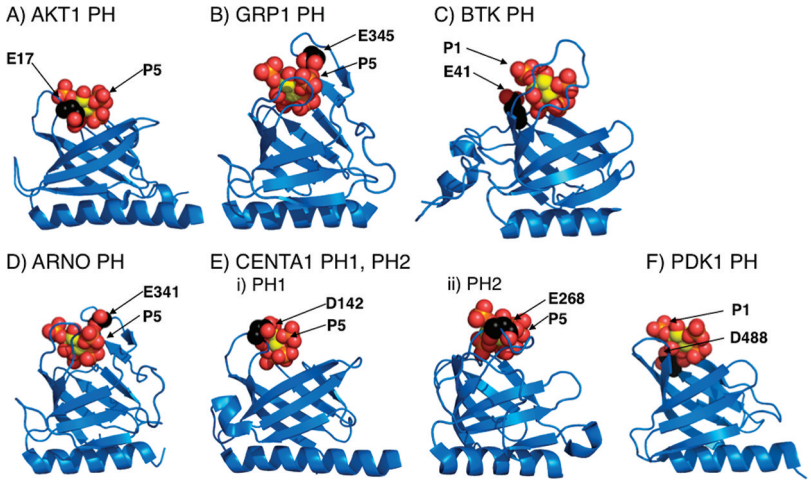

Figure 5. Comparison of all six $PI(3,4,5)P_3$-specific PH domains for which cocrystal structures with the bound $PI(3,4,5)P_3$ headgroup are currently available.[17,25,26,35−38] Highlighted are the putative sentry residue and the headgroup phosphate predicted to have the strongest electrostatic interactions with the sentry side chain carboxylate (see Discussion).

to the plasma membrane even in resting cells lacking a $PI(3,4,5)P_3$ signal.

DISCUSSION

This study extends our previous analysis of the molecular mechanism of the E17K mutation in the AKT1 PH domain to the analogous E345K mutation in the GRP1 PH domain.[2] Together, these two studies strongly support the conclusion that a sentry glutamate adjacent to the $PI(3,4,5)P_3$ binding pocket plays a crucial role in reducing the affinities of both PH domains for constitutive plasma membrane $PI(4,5)P_2$, thereby ensuring that recruitment of the PH domain to the plasma membrane occurs only during a $PI(3,4,5)P_3$ signal. Like the AKT1 E17K mutation, the GRP1 E345K mutation at the sentry glutamate position (i) increases the affinity for soluble IP_6 and for membrane-bound $PI(3,4,5)P_3$ and $PI(4,5)P_2$ in vitro, (ii) yields a $PI(4,5)P_2$ affinity in vitro similar to that of the PLCδ1 PH domain, the prototypical $PI(4,5)P_2$ sensor, and (iii) yields binding to constitutive plasma membrane $PI(4,5)P_2$ in resting cells, as observed for the control PLCδ1 PH domain but not for the WT GRP1 PH domain.

The constitutive plasma membrane targeting observed for the sentry glutamate mutants AKT1 E17K and GRP1 E345K arises from their increased $PI(4,5)P_2$ affinities, and not from their increased $PI(3,4,5)P_3$ affinities. Thus, in resting cells, starvation to minimize growth factors in the media, thereby suppressing plasma membrane $PI(3,4,5)P_3$, does not block the constitutive plasma membrane targeting of either PH domain mutant (ref 2 and Figure 4). Instead, in vitro binding measurements reveal that the affinities of AKT1 E17K and GRP1 E345K PH domains for $PI(4,5)P_2$ are increased to a level sufficient to bind the constitutive plasma membrane $PI(4,5)P_2$ in resting cells, like the PLCδ1 PH domain (ref 2 and Table 2). The AKT1 E17K mutation increases the affinity of the PH domain for $PI(4,5)P_2$ at least 80-fold, from a native K_D of $>10^4$ nM to a K_D of 130 ± 30 nM for the mutant, indistinguishable from the affinity of the PLCδ1 PH domain for $PI(4,5)P_2$.[2] The GRP1 E345K mutation increases $PI(4,5)P_2$ affinity approximately 8-fold, yielding a K_D of 400 ± 50 nM in vitro (Table 2), again similar to the affinity of the PLCδ1 PH domain for $PI(4,5)P_2$. Moreover, in cells, the affinity of the GRP E345K PH domain for $PI(4,5)P_2$ is expected to be further enhanced by interactions with plasma membrane-associated Arf6 (refs 24 and 25 and below). Consistent with this picture, the constitutively plasma-membrane targeted AKT1 E17K, GRP1 E345K, and PLCδ1 PH domains are displaced from the plasma membrane upon introduction of high cytoplasmic Ca^{2+} concentrations to activate phospholipase C activity and thereby hydrolyze the $PI(4,5)P_2$ pool (ref 2 and Figure 4B), although the GRP1 E345K PH domain is fully displaced by $PI(4,5)P_2$ hydrolysis only when its interaction with Arf6 is weakened by incorporation of the I307A mutation (Figure 4B). In short, the evidence indicates that the primary physiological role of the native sentry glutamate of AKT1 and GRP1 is to minimize binding to constitutive plasma membrane $PI(4,5)P_2$ in resting cells. Secondarily, the sentry glutamate also reduces the effective $PI(3,4,5)P_3$ affinity, and this affinity tuning could further optimize cellular responses to $PI(3,4,5)P_3$ signals.

More broadly, these findings have widespread implications for $PI(3,4,5)P_3$-specific PH domains. Currently, there are seven $PI(3,4,5)P_3$-specific PH domains bound to the $PI(3,4,5)P_3$ headgroup for which structures are available. Figure 5 illustrates the confirmed sentry glutamates of AKT1 [E17, Protein Data Bank (PDB) entry 1H10[27]] and GRP1 (E345, PDB entries 1FGY and 1FHX[18,26]) PH domains and shows that the other five known structures also possess a putative sentry glutamate (or, in two cases, a putative sentry aspartate): (i) Bruton's tyrosine kinase (BTK, E41, PDB entry 1B55[36]), (ii) Arf nucleotide-binding-site opener (ARNO, E341, PDB entry 1U27[37]), (iii and iv) centaurin α1 PH1 and PH2, respectively (CENTA1, D142 and E268, respectively, PDB entry 3LJU[38]), and (v) 3-phosphanositide-dependent kinase 1 (PDK1, D488, PDB entry 1W1D[39]). It follows that an acidic sentry side chain is a common, perhaps ubiquitous, feature of $PI(3,4,5)P_3$-specific PH domains. However, these acidic sentry residues may have arisen from convergent evolution, because their locations in the PH domain architecture are not conserved. Examination of Figure 5 reveals that the proposed sentry residues are distributed among different loops and are disposed differently relative to the $PI(3,4,5)P_3$ headgroup. In five cases (AKT1, GRP1, ARNO, and CENTA1 PH1 and PH2), the sentry side chain carboxylate is predicted to have the strongest repulsive electrostatic interaction with the 5-phosphate of the $PI(3,4,5)P_3$ headgroup because of proximity or, in certain cases, screening

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of other proximal phosphates by intervening basic side chains. In the remaining cases (BTK and PDK1), the side chain carboxylate is predicted to interact most strongly with the 1-phosphate.

The different locations of sentry glutamates are likely to yield subtle mechanistic differences as illustrated by a comparison of AKT1 and GRP1. In both cases, the mobile side chain carboxylate of the sentry glutamate can closely approach the 5-phosphate, but AKT E17 is on the $\beta1-\beta2$ loop[27] and GRP1 E345 is on the $\beta6/\beta7$ loop[18] on opposite sides of the inositol ring. The E17K AKT1 PH domain mutation increases the affinity for $PI(3,4,5)P_3$ by a factor of 8-fold and increases the affinity for $PI(4,5)P_2$ by a much greater factor of 80-fold. By contrast, the E345K GRP1 PH domain mutation increases the affinity for both $PI(3,4,5)P_3$ and $PI(4,5)P_2$ by the same factor, 8-fold. The simplest explanation for the more dramatic effect of the E17K mutation on the affinity for $PI(4,5)P_2$ is that the sentry glutamate of the AKT1 PH domain prevents the binding of the $PI(4,5)P_2$ headgroup in an alternative orientation that would yield significantly tighter binding, while this alternative $PI(4,5)P_2$ orientation is not accessible in the GRP1 PH domain.

The strikingly similar, 8-fold affinity increases observed for the interactions of the E17K AKT1 PH domain with $PI(3,4,5)P_3$ and the GRP1 PH domain with both $PI(3,4,5)P_3$ and $PI(4,5)P_2$ may share similar mechanisms. In principle, at least three electrostatic interactions could be involved, but only one is favored by the data. (i) In crystal structures of the apo state, the AKT1 or GRP1 sentry glutamate forms a salt bridge to a nearby basic side chain required for PIP headgroup coordination (E17 to K14 or E345 to R349, respectively). This salt bridge must be broken for the PIP headgroup to bind, yielding an energy barrier for binding that is eliminated by the E17K or E345K mutation. However, the kinetic analysis indicates this barrier is insignificant because the measured target lipid on rates (Table 2) are largely unaffected by the sentry mutations. The findings are consistent with the previously documented rate-determining, electrostatic search process that involves membrane-embedded PS and defines the target lipid association kinetics.[13,34] It follows that an apo state salt bridge does not play a central role in sentry glutamate function. (ii) When bound to its target lipid on the membrane surface, the AKT1 or GRP1 PH domain interacts electrostatically with negatively charged lipids, primarily phosphatidylserine (PS). This interaction would be repulsive for the native sentry glutamate, but the E17K or E345K mutation would convert this repulsive interaction into an attractive interaction, thereby stabilizing the bound state and increasing target lipid affinity. Such a picture is disfavored by the observation that a membrane is not required for the affinity increase, because both mutations trigger affinity increases for soluble IP_6 similar in magnitude to the affinity increases observed for membrane-embedded PIP lipids (ref 2 and Table 2). (iii) Instead, the molecular basis of sentry function appears to be the electrostatic interaction between the sentry side chain carboxylate and the PIP lipid headgroup. The sentry glutamate side chain is presumably dynamic, and the AKT1 and GRP1 cocrystal structures with a $PI(3,4,5)P_3$ headgroup analogue both indicate the side chain carboxylate can approach the inositol 5-phosphate of the target headgroup well within the ~8 Å/D limit for significant electrostatic interactions at physiological ionic strengths. The resulting carboxylate−phosphate charge repulsion would generate an energy barrier for PIP headgroup binding that would be converted to a favorable electrostatic interaction by the E17K or E345K mutation. This picture explains the higher affinities for $PI(3,4,5)P_3$, $PI(4,5)P_2$, and IP_6 observed for the AKT1 and GRP1 sentry lysine mutants, as well as the slower $PI(3,4,5)P_3$ dissociation kinetics observed for these mutants (ref 2 and Table 2).

Thus, the native sentry glutamate is a negative regulator of $PI(4,5)P_2$ binding, and charge reversal to lysine enhances both the $PI(4,5)P_2$ and $PI(3,4,5)P_3$ affinities. These observations may provide a clue about the evolution of $PI(3,4,5)P_3$-specific PH domains. If ancestor cells possessed sufficiently low constitutive levels of $PI(4,5)P_2$ and generated smaller $PI(3,4,5)P_3$ signals, the original PH domains may have required lysine or arginine residues at the position now occupied by the sentry glutamate. Modern $PI(3,4,5)P_3$-specific PH domains that encounter high levels of $PI(4,5)P_2$, however, require the sentry glutamate to ensure that plasma membrane targeting occurs only during a $PI(3,4,5)P_3$ signal. The glutamate also moderates the high affinity for $PI(3,4,5)P_3$ generated by the multiple basic residues that coordinate $PI(3,4,5)P_3$ in the headgroup binding pocket. In the case of the GRP1 PH domain, one of these residues is a His side chain (His355) that may further modulate $PI(3,4,5)P_3$ targeting by introducing a strong pH sensitivity.[40]

The physiological impact of the glutamate to lysine charge reversal mutation at the sentry glutamate position has been previously demonstrated for full-length AKT1 E17K, because this mutant is an oncogenic kinase,[1,41] and recently confirmed.[42] The constitutive plasma membrane targeting of AKT1 E17K leads to hyperactivation of its kinase activity, transformation of tissue culture cells, multiple human cancers, and, in the case of Proteus syndrome, tissue overgrowth.[1,3] Similarly, while the BTK E41 residue has not been directly confirmed as a sentry glutamate because of a lack of quantitative equilibrium and kinetic analysis, the BTK E41K mutation does lead to $PI(4,5)P_2$ binding in vitro as well as constitutive targeting to the plasma membrane and transformation of tissue culture cells.[36,43,44] Thus, the available evidence suggests that BTK E41 is a sentry glutamate and that its primary role is to minimize $PI(4,5)P_2$ binding in resting cells, as demonstrated for AKT1 E17 and GRP1 E345. While to date only the AKT1 E17K mutation has been directly linked to disease, the ability of charge reversal mutations at sentry glutamate positions to trigger constitutive plasma membrane targeting suggests that future studies may well uncover diseases linked to the GRP1 E345K or BTK E41K mutation, or to sentry charge reversals in other PH domains.

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■ ABBREVIATIONS

AKT1, protein kinase B, isoform 1; PDK1, phosphoinositide-dependent protein kinase 1; PLCδ1, phospholipase C δ, isoform 1; PH, pleckstrin homology; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; dPE, dansyl-PE; PI(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; DTT, dithiothreitol; IP_6, inositol hexakisphosphate.

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