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Live cell imaging with protein domains capable of recognizing phosphatidylinositol 4,5-bisphosphate; a comparative study

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Abstract

Background: Phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] is a critically important regulatory phospholipid found in the plasma membrane of all eukaryotic cells. In addition to being a precursor of important second messengers, PtdIns(4,5)P₂ also regulates ion channels and transporters and serves the endocytic machinery by recruiting clathrin adaptor proteins. Visualization of the localization and dynamic changes in PtdIns(4,5)P₂ levels in living cells is critical to understanding the biology of PtdIns(4,5)P₂. This has been mostly achieved with the use of the pleckstrin homology (PH) domain of PLCδ1 fused to GFP. Here we report on a comparative analysis of several recently-described yeast PH domains as well as the mammalian Tubby domain to evaluate their usefulness as PtdIns(4,5)P₂ imaging tools.

Results: All of the yeast PH domains that have been previously shown to bind PtdIns(4,5)P₂ showed plasma membrane localization but only a subset responded to manipulations of plasma membrane PtdIns(4,5)P₂. None of these domains showed any advantage over the PLCδ1PH-GFP reporter and were compromised either in their expression levels, nuclear localization or by causing peculiar membrane structures. In contrast, the Tubby domain showed high membrane localization consistent with PtdIns(4,5)P₂ binding and displayed no affinity for the soluble headgroup, Ins(1,4,5)P₃. Detailed comparison of the Tubby and PLCδ1PH domains showed that the Tubby domain has a higher affinity for membrane PtdIns(4,5)P₂ and therefore displays a lower sensitivity to report on changes of this lipid during phospholipase C activation.

Conclusion: These results showed that both the PLCδ1PH-GFP and the GFP-Tubby domain are useful reporters of PtdIns(4,5)P₂ changes in the plasma membrane, with distinct advantages and disadvantages. While the PLCδ1PH-GFP is a more sensitive reporter, its Ins(1,4,5)P₃ binding may compromise its accuracy to measure PtdIns(4,5)P₂ changes. The Tubby domain is more accurate to report on PtdIns(4,5)P₂ but its higher affinity and lower sensitivity may limit its utility when phospholipase C activation is only moderate. These studies also demonstrated that similar changes in PtdIns(4,5)P₂ levels in the plasma membrane can differentially regulate multiple effectors if they display different affinities to PtdIns(4,5)P₂.

Background

Phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5) P_2] is the major polyphosphoinositide species found in the plasma membrane (PM) of all eukaryotic cells. This regulatory lipid has several functions in the PM: first, it was identified as the primary substrate of receptor-mediated phospholipase C (PLC) activation, to yield the second messengers, inositol-1,4,5-trisphosphate (Ins P_3) and diacylglycerol [1]. PtdIns(4,5) P_2 is also important for endocytosis of PM proteins through its binding to several clathrin adaptors [2]. Moreover, PtdIns(4,5) P_2 is required for the proper functioning of many ion channels and transporters [3,4] and also contributes to the regulation of actin polymerization [5] and attachment of the PM to the actin cytoskeleton [6]. Although the majority of PtdIns(4,5) P_2 is found in the PM, functional data suggest that the lipid may also regulate signaling complexes in other membranes and even within the nucleus [7]. The pivotal importance and pleiotropic functions of PtdIns(4,5) P_2 have demanded that its distribution and dynamics be followed with subcellular resolution preferentially in living cells. This was finally achieved with the introduction of the PLC δ_1 -PH-domain GFP chimera as a molecular probe to detect PtdIns(4,5) P_2 in eukaryotic cells [8,9].

The PLC δ_1 PH-GFP reporter has since been widely used successfully to monitor PtdIns(4,5) P_2 dynamics under a variety of cellular settings [10]. This reporter has not shown significant amounts of PtdIns(4,5) P_2 in intracellular membranes other than endocytic vesicles in live cells [11], although it detected some of the lipid in internal membranes in an EM application [12]. This could reflect low abundance of PtdIns(4,5) P_2 in internal membranes or a requirement for other components present only in the PM for the PtdIns(4,5) P_2 -dependent membrane recruitment of the PLC δ_1 PH-GFP probe. Moreover, because of its high-affinity binding to Ins P_3 , the interpretation of the data obtained by the use of the PLC δ_1 PH domain has become highly debated [10]. Several studies have shown that Ins P_3 can displace the PLC δ_1 PH-GFP reporter from the membrane without an apparent change in the level of PtdIns(4,5) P_2 [13,14]. Although, Ins P_3 is mostly formed from PtdIns(4,5) P_2 , if the affinity of the probe is significantly higher for the soluble Ins P_3 than for the membrane-bound PtdIns(4,5) P_2 , the translocation of the probe from the membrane to the cytosol will be disproportionately higher than the actual lipid decrease in the membrane [13].

Research in the last 10 years has clearly demonstrated that phosphoinositides may not be the sole determinants of the cellular distribution of phosphoinositide-binding protein-modules [15]. This raises the possibility that functionally distinct inositide pools are differentially reported

on by different protein modules even if they recognize the same phosphoinositide species. Because of these new developments and the limitations of the PLC δ_1 PH-GFP, there is a need to evaluate other potential PtdIns(4,5) P_2 binding PH domains as reporters of the lipid in a true cellular setting. Several protein modules have been shown to recognize PtdIns(4,5) P_2 based on *in vitro* binding assays. Moreover, a detailed analysis of the PH domains identified in *Saccharomyces cerevisiae* also revealed that many PH domains showing relatively limited phosphoinositide binding specificities could detect specific lipid pools probably because of their interaction with other components of signaling domains where these lipids are formed. Therefore, even some of the domains that lack *in vitro* lipid binding specificity might be useful in reporting on some specific signaling inositol lipid pools within the cells. However, to decide whether a reporter is indeed a good sensor of PtdIns(4,5) P_2 one has to investigate the properties of these domains in live cells with controlled manipulation of PtdIns(4,5) P_2 .

In the present study we evaluated several yeast PH-domains characterized in [16] as well as the Tubby domain of the mammalian Tubby protein [17] for their ability to follow PtdIns(4,5) P_2 changes in mammalian cells. Our analysis shows that many but not all of the examined yeast PH domains can follow PtdIns(4,5) P_2 changes, but all have limitations and none is remotely better than the PLC δ_1 PH domain. A more detailed comparison with the Tubby domain shows that while the latter is a good PtdIns(4,5) P_2 reporter, its high affinity to the lipid and slow dissociation can also pose problems leading to underestimation of the PtdIns(4,5) P_2 changes. The similarities yet profound differences in the behavior of these isolated PtdIns(4,5) P_2 domains is a perfect example of how PtdIns(4,5) P_2 can interact and regulate multiple effector proteins simultaneously and yet differentially.

Results

Localization responses of lipid binding domains during manipulation of PM PtdIns(4,5) P_2

Several yeast PH domains showed PM localization in spite of varying *in vitro* inositide binding specificity as described in [16]. A selected panel of these PH domains was tested for their abilities to respond to changes in PM PtdIns(4,5) P_2 levels. In addition, the Tubby domain of the Tubby protein [18], which has been described as a specific PtdIns(4,5) P_2 reporter [19], was analyzed in more detail in comparison to the widely used PLC δ_1 PH-GFP probe. The purpose of these studies was to evaluate the features of these protein domains in a cell line in which the phosphoinositide changes have been well characterized in the same laboratory. Two detailed analysis with similar goals have been recently published, one using the full-length

Tubby protein [20] and the other characterizing a mutant form of the isolated Tubby domain [21].

Table 1. lists the PH domains tested in this study. The probes were expressed in HEK293-AT1 cells, a cell line stably expressing the rat AT_{1a} angiotensin receptor, and in which stimulation with 100 nM AngII causes a robust PLC activation with complete translocation of the PLCδ1-PH-GFP and a ~80% decrease of the ³²P- or [³H]-inositol-labeled PtdIns(4,5)P₂ pools within 30 second stimulation [22]. The recently described rapamycin-inducible 5-phosphatase recruitment system was used to eliminate PtdIns(4,5)P₂ without PLC activation [23]. As shown in Table 1, all of these PH domains showed PM localization in agreement with previous reports [16]. Importantly, none of the domains showed any intracellular localization (apart from nuclear enrichment, see below). Several of the domains lacked a response to PtdIns(4,5)P₂ decrease either evoked by AngII stimulation or by 5-ptase recruitment. These included the PH domains of Cla4, Skm1, and Slm2 (see additional file 1). The other domains, namely: Num1-PH, Slm1-PH and Opy1-PH, displayed a transient, and incomplete translocation from the membrane to the cytosol upon AngII stimulation and except for Slm1p, these domains completely lost their membrane localization in response to the 5-ptase recruitment to the PM (Fig. 1). In case of Slm1-PH the translocation to the cytosol after phosphatase recruitment was partial indicating that the probe still binds to PtdIns4P generated by the 5-ptase. Indeed, the addition of 10 μM wortmannin completely eliminated the remaining localization of the Slm1-PH domain (not shown).

There were several other issues that made some of these domains less than optimal. The Num1-PH domain -

which has the highest specificity of PtdIns(4,5)P₂ binding *in vitro* [16], - showed very poor expression when fused to the C-terminus of GFP. When the PH domain was placed in front of the GFP, its expression has significantly improved, but many cells expressing the domain showed peculiar structures with intense fluorescence. These appeared as vesicles that had just bud off but are still attached to the outer surface of the PM (see additional file 1). The Opy1 PH domain, on the other hand, showed very high affinity to the nucleus and nucleolus. This was partially overcome by placing a nuclear export signal in front of the GFP fusion protein (see additional file 1). These results collectively indicated that the lipid binding characteristics of the yeast PH domains are not identical and in some cases the membrane localization is likely influenced by factors other than phosphoinositides. Having tested the yeast PH domains, we decided to subject the mammalian Tubby domain for a more detailed comparison with the widely used PLCδ1PH-GFP.

Comparative analysis of the Tubby-domain and the PLCδ1-PH domain

1. The Tubby domain binds to the PM with higher affinity

When expressed in HEK293-AT1 cells, a significantly higher fraction of PLCδ1-PH was found cytosolic compared to the Tubby-domain. Fig 2A shows representative images of HEK-293-AT1 cells expressing the two reporters either individually or together, tagged with fluorescent proteins of different colors (Tubby-domain-GFP and PLCδ1-PH-mRFP). To quantify these differences, fluorescent intensity values were recorded along line-intensity histograms taken over the cells (Fig. 2B) and the PM vs. cytoplasmic intensity ratios were calculated. This ratio was substantially higher (6.9 ± 1.23) for the Tubby-domain than for PLCδ1PH-GFP (2.1 ± 0.33 , means \pm S.E.M, n =

Table 1: Localization responses of selected yeast PH domains and the human Tubby domain expressed in HEK293-AT1 or COS-7 cells.

PH domain	Subcellular Localization	Response to AngII stimulation	Response to PtdIns(4,5)P ₂ elimination by 5-ptase	Presumptive inositide dependence of PM localization
PLCδ1-PH	PM	transient translocation	complete translocation	PtdIns(4,5)P ₂
Cla4p-PH	PM	no change	no change	inositide independent
Num1p-PH	PM	transient translocation	complete translocation	PtdIns(4,5)P ₂
Skm1p-PH	PM	no change	no change	inositide independent
Opy1p-PH	PM	transient translocation	complete translocation	PtdIns(4,5)P ₂
Yil105c/ Slm1p-PH	PM	transient translocation	partial translocation	PtdIns(4,5)P ₂ PtdIns4P
Ynl047/ Slm2p-PH	PM	no change	no change	inositide independent
Tubby domain	PM	transient translocation	complete translocation	PtdIns(4,5)P ₂

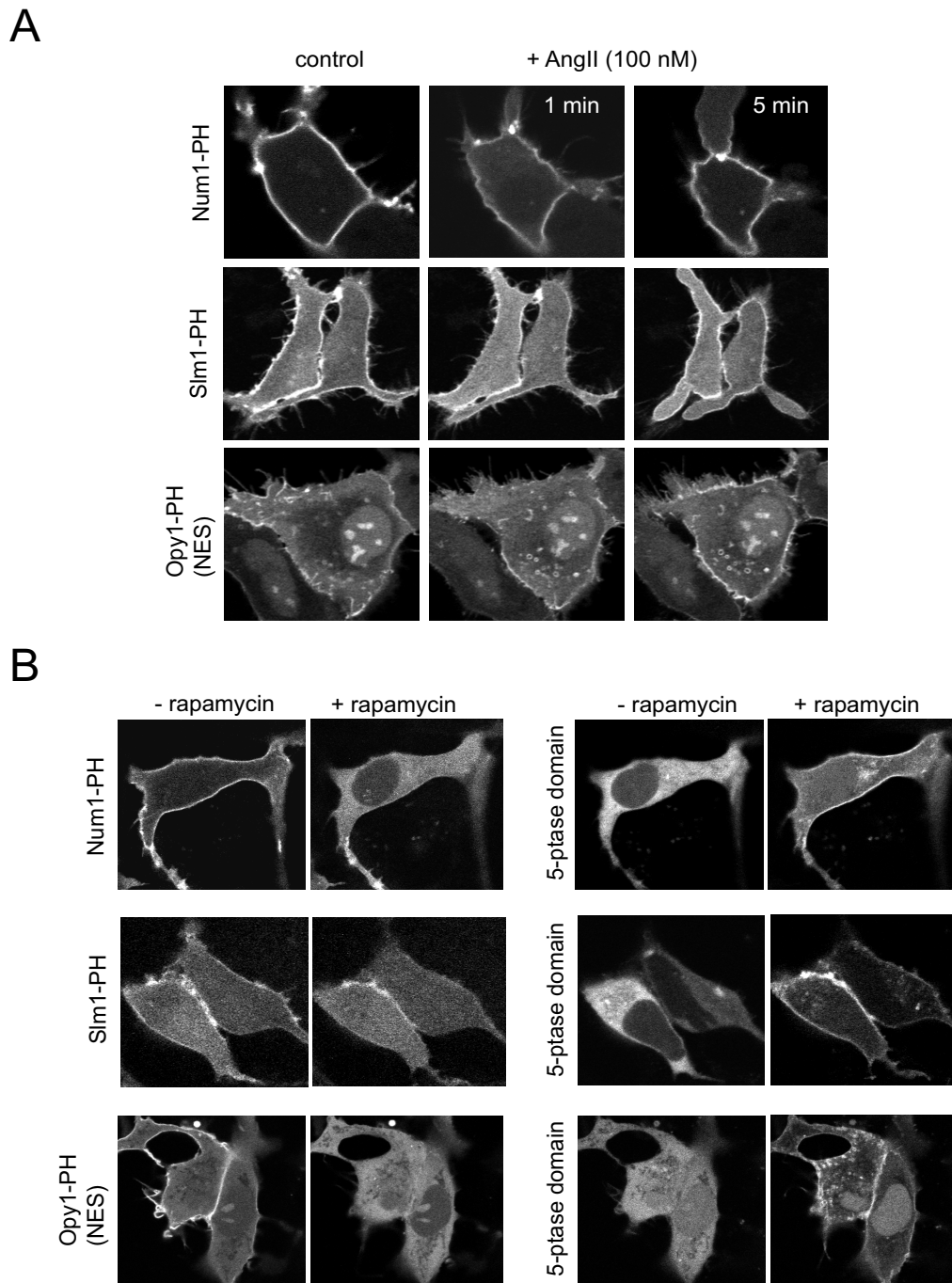


Figure 1
Translocation responses of selected PH domains after PLC activation or phosphoinositide 5-phosphatase action. The GFP-tagged versions of the indicated PH domains were expressed either in HEK293-AT1 cells for AngII (100 nM) stimulation (panel A) or in COS-7 cells for 5-phosphatase action (panel B). AngII stimulation causes rapid PLC activation with a substantial decrease in PtdIns(4,5)P₂ levels (~80% [22]) but these selected probes showed only a small and transient translocation response from the membrane to the cytosol. For 5-phosphatase recruitment, COS-7 cells were transfected with a PM-targeted FRB-CFP and a mRFP-FKBP-12-5-phosphatase domain along with the GFP-tagged PH domain. Rapamycin (100 nM) addition rapidly recruits the 5-phosphatase to the PM (B panel, right) and eliminates PtdIns(4,5)P₂ [23]. Some of the PH domains completely lose localization (Num1 or Opy1) while others (such as Slm1) shows only a partial translocation. See Table I for a complete list of the responses found with the other PH domains.

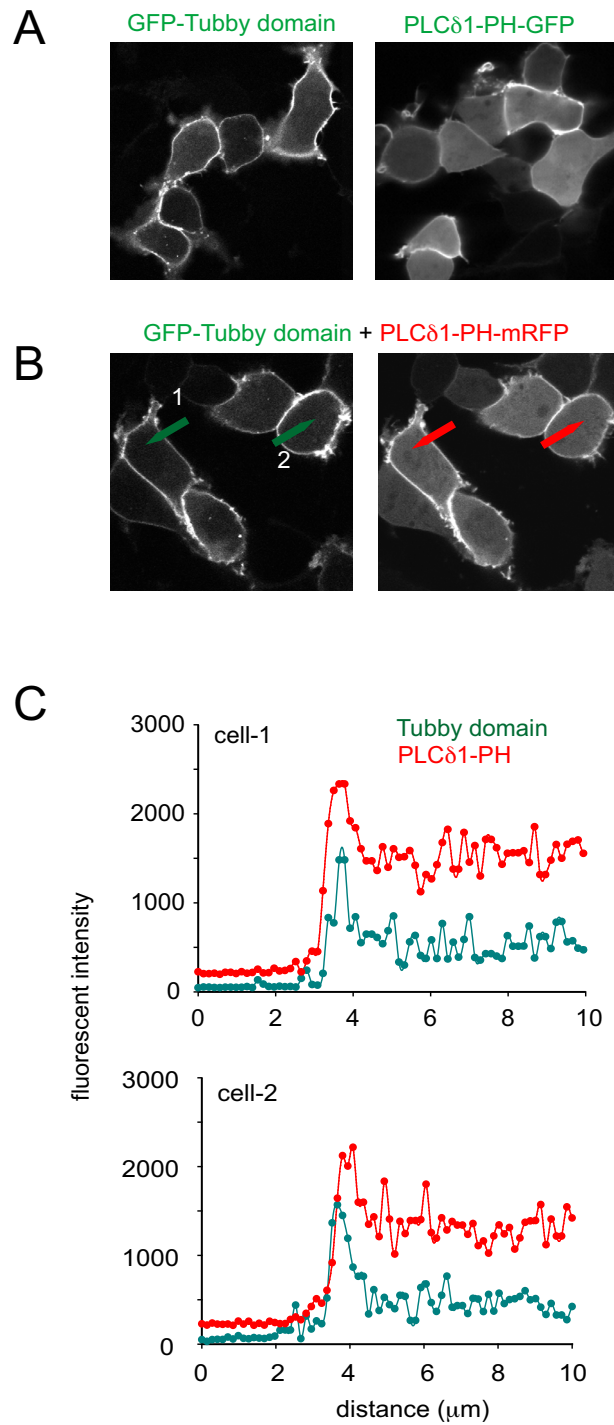


Figure 2
Cellular distribution of the Tubby-domain and PLCδ1PH domain expressed in HEK293-ATI cells. The GFP-Tubby-domain and PLCδ1PH-GFP were expressed either separately (panel A) or were co-expressed (here the PLCδ1PH was tagged with mRFP, panel B) in HEK293-ATI cells. Confocal images show a stronger localization of the Tubby domain to the PM. The PM vs. cytoplasmic fluorescent ratios were calculated from line intensity histograms taken across representative sections of the cells (shown by arrows in the Figures on Panel B). Representative intensity histograms are shown in Panel C from cells 1 and 2 as shown in Panel B. The background fluorescence was subtracted from both the average PM and cytoplasmic fluorescence values when calculating the F_{PM}/F_{cyto} ratios. These values are found in the text.

25). This observation already indicated that the two probes have different apparent affinities to the PM, presumably to PtdIns(4,5)P₂. Based on these results, one would expect to see a competition between the two domains for the plasma membrane PtdIns(4,5)P₂ pools. However, no competition was observed between the two probes. This was consistent with our finding that the cytosol to PM ratio of either probe did not show significant differences depending on the expression level (data not shown). These results suggested that cells can dynamically up-regulate their PtdIns(4,5)P₂ pools when such reporters are expressed and keep a fraction of the lipids sequestered. This could explain the lack of saturation of membrane binding of the domains.

To further elucidate the difference in the membrane binding properties, namely, the association/dissociation rates and mobility of the two probes, we performed fluorescence recovery after photobleaching (FRAP) analysis in HEK293-AT₁ cells expressing the GFP-tagged versions of the respective reporters. Selected areas of the PM were bleached and the recovery of fluorescence was recorded (see additional file 3 and 4). The mobile fraction- and T_{1/2} values were calculated as described in the Materials and Methods. There was a small but statistically significant difference between the mobile fraction values of Tubby-GFP (73.8 ± 2.33, S.E.M., n = 90) and PLCδ₁PH-GFP (79.8 ± 2.67, S.E.M., n = 90, expressed as % of prebleach values). (p < 0.05). In contrast, the T_{1/2} values showed more than a two fold difference between the two reporters [3.1 ± 0.33 sec (S.E.M., n = 90) and 1.2 ± 0.09 sec (S.E.M., n = 90) for the Tubby-GFP and PLCδ₁PH-GFP, respectively]. This difference was highly significant (p < 0.0001). In fact, the T_{1/2} value of the Tubby domain was close to that measured previously for the membrane anchored GFP-CAAX domain [24] (the T_{1/2} value for PLCδ₁PH-GFP in that study was found identical to our current measurement). These results were consistent with a higher affinity of the Tubby-domain-GFP probe to its PM binding partner, most likely reflecting its slower dissociation rate. It is important to note that in a FRAP analysis the recovery of the fluorescence in the bleached area can occur as a result of both lateral diffusion (starting from the borders of the bleached area) and dissociation from the membrane to the cytosol and rapid re-association (which should not differ regardless of the site within the bleached area). This feature was elegantly utilized to determine the diffusion properties of several GFP-fused PH domains in a recent study [25]. In the present analysis we selected the central area within a larger bleached region (additional file 2) to minimize the effects of lateral diffusion. Yet, the slower recovery of the Tubby domain was consistent with a rate similar to that of simple lateral diffusion.

2. Response to agonist-induced PLC activation

Initial experiments showed that while PLCδ₁PH-GFP showed a full translocation in > 90% of cells from the membrane to the cytosol after 100 nM AngII stimulation, the Tubby domain-GFP construct had much more variable responses ranging from no detectable change to a full translocation. For a better comparison of the two probes during PLC activation, the GFP-tagged Tubby-domain and the mRFP-tagged PLCδ₁PH domain were co-expressed in HEK293-AT₁ cells. The dynamics of the redistribution of the two probes during this process was then followed after stimulation with different concentrations of AngII. At high concentrations of AngII (0.1-1 μM) both reporters translocated rapidly from the membrane to the cytosol. However, while the translocation of PLCδ₁PH-GFP was almost always complete, that of the Tubby domain was often partial. When the AngII concentration was lowered to 30 nM, there was a significantly longer delay in the translocation of the Tubby-domain compared to that of the PLCδ₁PH domain (Fig. 3, Panel A). Moreover, while a complete translocation of PLCδ₁PH-GFP always occurred at this concentration of AngII stimulation, the Tubby-domain either failed to translocate or did it only partially or with a significant time delay. As shown in Fig. 3B, the average time required for half maximal translocation of PLCδ₁PH-mRFP was 3.8 ± 0.3 sec, while that of the Tubby-domain was 6.7 ± 0.3 sec (Means ± S.E.M, n = 54) (p < 0.001).

To determine the extent of maximum translocation of the reporters, high concentration of ionomycin (10 μM) was added after AngII stimulation. The high Ca²⁺ concentration attained with this amount of ionomycin activates endogenous PLC enzymes even without G-protein activation, completely eliminating both PtdIns(4,5)P₂ and PtdIns4P from the cells [9,26]. This ensures displacement of all expressed reporters from the membrane allowing us to determine the fraction of the probe that showed translocation during agonist stimulation. As with the agonist, a significant delay in the translocation of the Tubby-domain (T_{1/2} = 27.2 ± 2.62 sec, n = 54) compared to that of the PLCδ₁PH-GFP (half max 14.0 ± 1.54 sec, n = 54) (p < 0.001) was observed upon ionomycin addition (Fig. 3B). Moreover, in the case of the PLCδ₁PH-GFP the cytoplasmic fluorescent intensity increases after AngII and ionomycin were comparable (92% vs 100%), whereas in the case of the Tubby-domain-GFP, the intensity increase after AngII was only 30% of that observed after ionomycin. This confirmed the observation that 30 nM AngII did not displace the Tubby-domain completely from the PM. It is worth noting that lower concentration of ionomycin (1 μM) that releases Ca²⁺ and induces capacitative Ca²⁺ entry evoked only a small or no response in cases of either reporter in naïve cells in agreement with other reports [21].

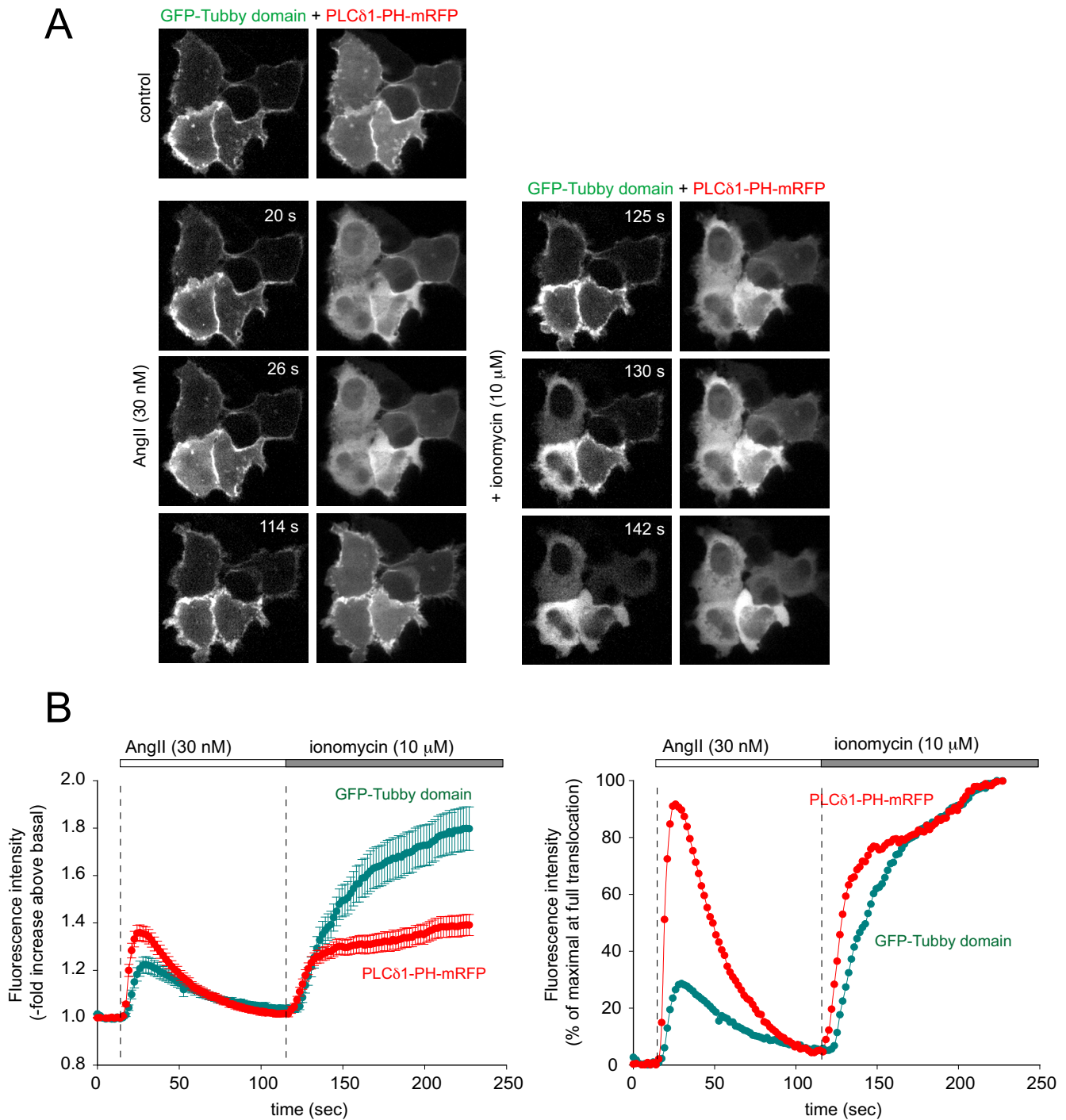


Figure 3
Translocation responses of Tubby-domain and PLC δ 1PH domain in AngII and ionomycin stimulated HEK293-ATI cells. HEK293-ATI cells were co-transfected with the GFP-Tubby-domain and the PLC δ 1PH-mRFP constructs. After 24 h, cells were analyzed by confocal microscopy. Cells were stimulated with 30 nM AngII (at 16 s) followed by 10 μ M ionomycin (at 117 s) as indicated. Panel A shows a representative image series recorded at the indicated time points. Panel B shows the average responses (cytoplasmic fluorescence increase) of 50-54 cells (mean \pm S.E.M) (left) and the average curve normalized to the maximal cytoplasmic fluorescence attained after ionomycin treatment (full translocation) (Panel B, right). Note the significantly slower and only partial response of the Tubby domain after AngII stimulation.

In a subsequent set of experiments, we examined the response of the reporters to overexpression of a constitutively active Gq (Gq*). Gq mediated PLC activation has been the proposed mechanism by which the transcription factor Tubby is released from the PM allowing its diffusion into the nucleus [18]. As in the previous experiments, the two reporters tagged with different color fluorescent proteins were co-expressed with the constitutively active α subunit of Gq*. In a large number of cells, the decrease of PtdIns(4,5)P₂ due to PLC β activation was apparent, shown by a full or partial translocation of the PLC δ 1PH-GFP. The PM vs. cytoplasm ratio of the probe was 1.0 ± 0.10 in a selected panel of cells, which is essentially a full translocation. However, in the very same cells, the Tubby-domain was only partially translocated to the cytosol. The PM vs cytoplasm ratio was reduced to 2.3 ± 0.21 from its control levels (6.9 ± 1.23). Fig. 4, Panel A shows representative images: one, where the Tubby-domain localization is clearly visible, while the PLC δ 1PH domain is completely cytosolic (Panel A, upper images), and another, where both reporters are cytosolic (Panel A, lower images). Importantly, even a low concentration of AngII (10 nM) rapidly displaced the remaining Tubby-domain from the PM indicating that Gq* has sensitized the system for a Gq-coupled receptor agonist (Fig. 4, Panel B).

3. Response to PtdIns(4,5)P₂ dephosphorylation

Next, we studied the translocation of the probes when PtdIns(4,5)P₂ was eliminated with the help of a polyphosphoinositide 5-phosphatase (5-ptase). For this, we used the rapamycin-induced recruitment of the type IV 5-ptase to the PM that can acutely eliminate PtdIns(4,5)P₂ [23] in COS-7 cells. We chose COS-7 cells because expression of the four constructs required for these studies did not yield high enough levels in HEK293 cells to see a robust 5-phosphatase response. Cells were transfected with the PM targeted FRB-CFP, the 5-ptase-domain fused to FKBP12-cerulean, Tubby-domain-GFP and PLC δ 1PH-mRFP. Addition of 100 nM rapamycin causes rapid heterodimerization of the membrane-anchored FRB with the FKBP12 protein fused to the cytosolic 5-ptase domain causing rapid translocation of the 5-ptase domain to the PM. (In this transfection regime the translocation of the enzyme cannot be followed as both proteins are tagged with a version of the CFP to keep the other colors for the Tubby domain and PLC δ 1PH domain). Rapamycin led to the prompt and complete loss of both the Tubby-domain and PLC δ 1PH domain PM localization (Fig. 5A). Unlike with agonist or ionomycin stimulation, there was no significant difference between the speed and extent of translocation of the two reporters (Fig. 5B). The half maximal translocation of Tubby-domain occurred at 10.9 ± 2.8 sec and for the PLC δ 1PH domain this was 10.4 ± 3.1 , respectively (n = 10) (N.S. difference).

Ins(1,4,5)P₃ and PtdIns(4,5)P₂ binding properties of the Tubby domain

Previous reports have suggested that the Tubby domain does not bind InsP₃ although no direct binding data have been presented [18]. To compare the inositol phosphate and inositol lipid binding characteristics of these reporters, we created the Tubby-domain-GFP fusion protein for bacterial expression in a similar manner as described for the PLC δ 1PH-GFP [27]. First, the binding of [³H]-Ins(1,4,5)P₃ to the two recombinant proteins was examined under identical conditions. As shown in Fig. 6, Panel A, PLC δ 1PH-GFP showed significant InsP₃ binding that was displaced by increasing amounts of the unlabeled ligand (as described in [27]). In contrast, very little (if any) [³H]-Ins(1,4,5)P₃ binding was found to the Tubby-domain-GFP protein. However, binding of Tubby-domain-GFP and PLC δ 1PH-GFP to lipid vesicles containing PtdIns(4,5)P₂ was very similar suggesting that the lack of InsP₃ binding was not due to misfolding of the Tubby-domain. However, while the PLC δ 1PH domain could be displaced from the PtdIns(4,5)P₂ containing vesicles by increasing concentrations of InsP₃, the Tubby-domain showed no such displacement with InsP₃ (Panel B). These experiments showed that Tubby-domain indeed shows no significant InsP₃ binding.

Inhibitory effects of the PLC δ 1PH-GFP and Tubby-domain-GFP on Ca²⁺ signaling

Expression of protein domains that bind to PtdIns(4,5)P₂ and/or InsP₃ can exert an inhibitory effect on Ca²⁺ signaling. Binding to PtdIns(4,5)P₂ can sequester a fraction of the lipid in the membrane and also hamper its availability to PLC enzymes. Binding and buffering of InsP₃, on the other hand, will delay the onset of the Ca²⁺ release and also inhibit coupling between the individual Ca²⁺ release events causing a slower and smaller cytoplasmic Ca²⁺ increase [28,29]. We, therefore, compared the effects of overexpression of the Tubby-domain-mRFP (a pure PtdIns(4,5)P₂ binder), PLC δ 1PH-mRFP (a mixed PtdIns(4,5)P₂ and InsP₃ binder) and the p130PH-mRFP (a clear InsP₃ binder with similar affinity to that of the PLC δ 1PH domain [28]) on the agonist-induced Ca²⁺ signals. Red versions of these constructs were used not to interfere with the Ca²⁺ measurements with Fura-2. At the end of the experiments high concentration of ionomycin (10 μ M) was used to release the constructs to the cytosol so that their red fluorescence intensities within the individual cells could be comparably determined as a quantitative measure of their expression level. COS-7 cells stimulated via their endogenous P_{2Y} receptor were used in these studies because ATP causes modest PLC activation and InsP₃ increases, where interference from the expressed constructs could be better assessed than in the HEK293-AT₁ cells, where the robust PLC activation can override the inhibitory effects of these molecules.

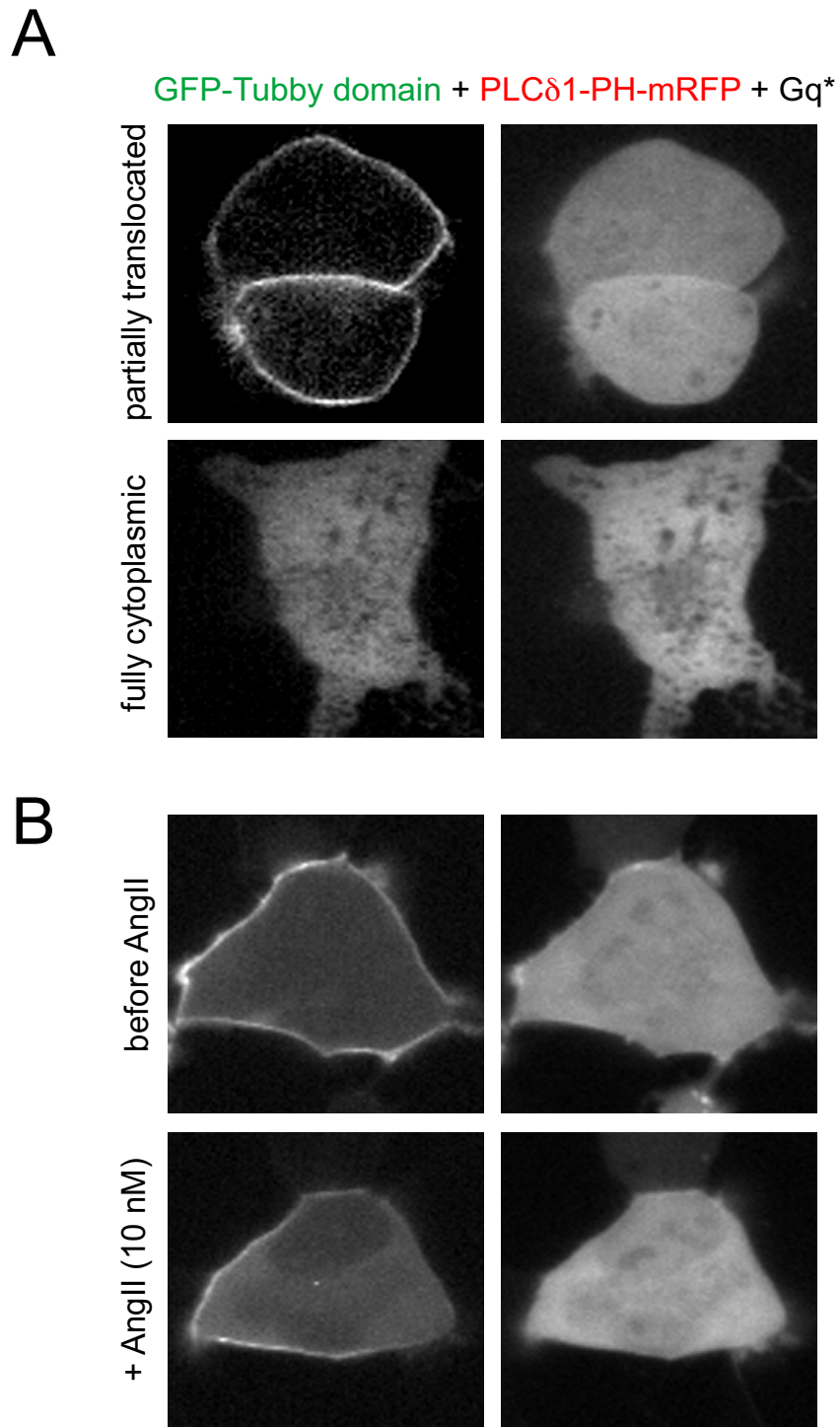


Figure 4
Distribution of the Tubby-domain and PLC δ 1PH domain in cells expressing a constitutively active Gq protein.
 HEK293-AT1 cells were co-transfected with the GFP-Tubby-domain, PLC δ 1-PH-GFP plasmids together with a constitutively active Gq α -subunit (Q209L). Panel A shows cells in which the PLC δ 1-PH localization is completely lost while that of the Tubby domain is still quite substantial (upper) while in some cells both construct completely lose membrane localization (lower). Panel B shows an example where a cell that still shows substantial localization of the Tubby domain loses its PM localization after stimulation with even a low concentration (10 nM) of AngII.

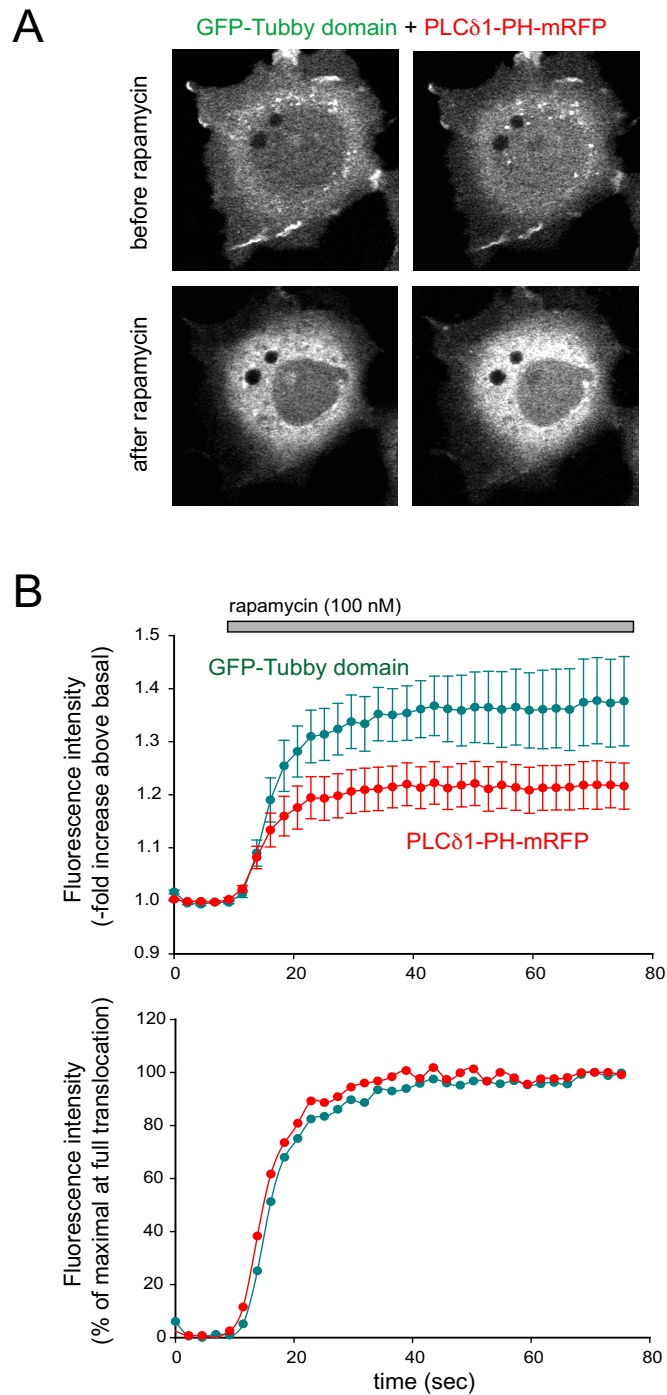


Figure 5
Translocation responses of the Tubby domain and PLC δ 1PH domain after rapid dephosphorylation of PtdIns(4,5)P $_2$ at the PM. COS-7 cells were co-transfected with the PM-targeted FRB tagged with CFP (not shown), the type-IV 5-ptase domain tagged with cerulean (not shown), the GFP-Tubby-domain and PLC δ 1PH-mRFP. After 24 h, live cells were analyzed by confocal microscopy. Addition of 100 nM rapamycin induces translocation of the 5-ptase to the PM, causing a complete loss of both PtdIns(4,5)P $_2$ reporter localization. Panel A shows representative images before and after rapamycin addition. Panel B shows a full time course of cytoplasmic fluorescence intensity changes based on the average of 10 cells (Mean \pm S.E.M.) expressed as a -fold increase over initial values (upper) or as a percent of the maximal response (lower panel, means are shown only).

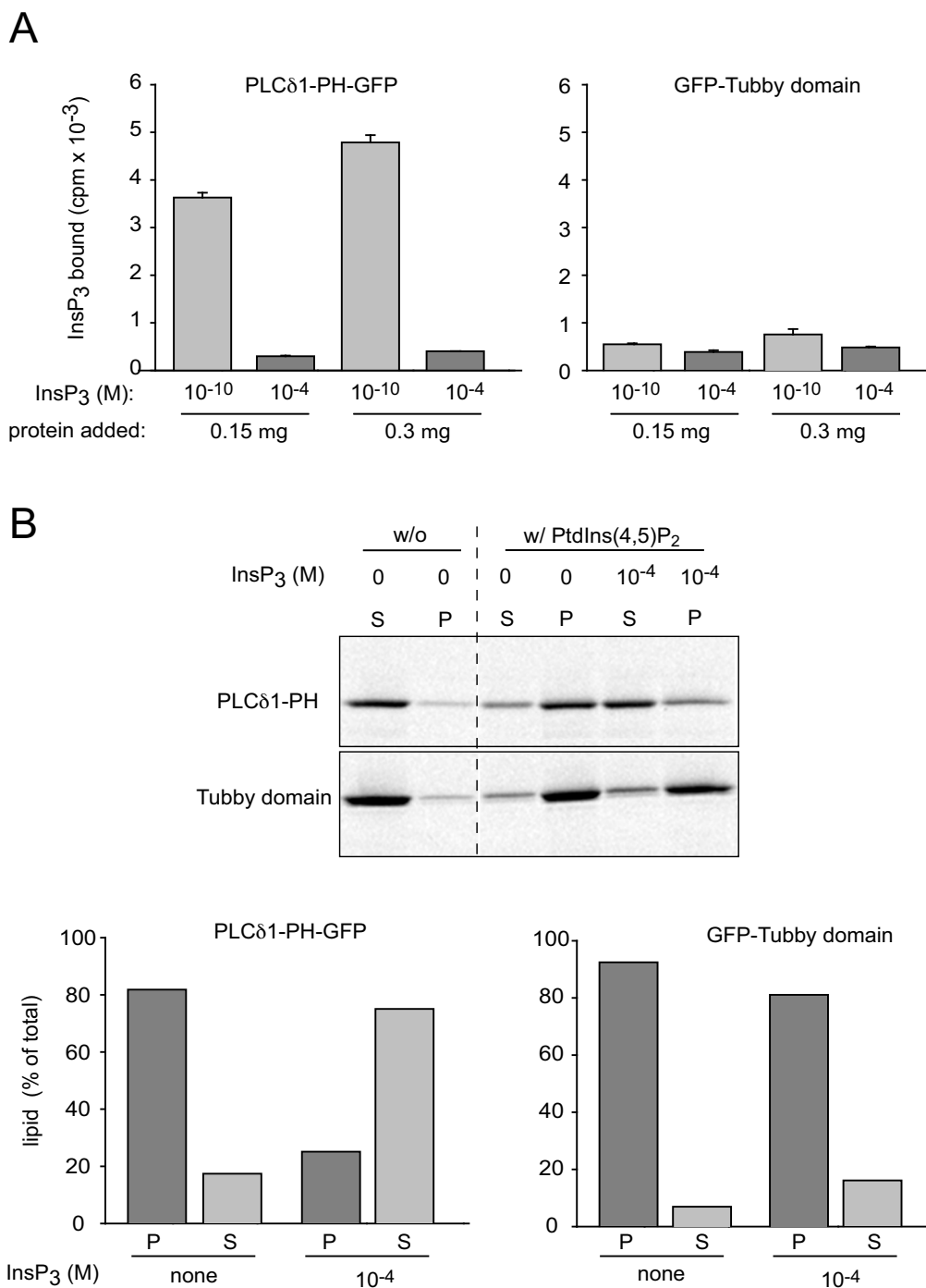


Figure 6
InsP₃ and PtdIns(4,5)P₂ binding of recombinant PLCδ1PH-GFP and GFP-Tubby-domains. Panel A: InsP₃ binding assays were performed at room temperature using [³H]-Ins(1,4,5)P₃ in the presence or absence of the indicated concentrations of unlabeled InsP₃ as detailed under "Experimental Procedures". Means ± range of 2 experiments performed in duplicate are shown. Panel B shows the binding of recombinant proteins to lipid vesicles containing phosphatidyletanolamine in the absence or presence of PtdIns(4,5)P₂ and InsP₃. Vesicles were pelleted with ultracentrifugation and "S" and "P" represent the soluble (unbound) and pellet-associated (bound) GFP fusion protein, respectively, analyzed by a PhosphorImager after SDS-PAGE. The result of one of two experiments with identical results is shown. Note the negligible InsP₃ yet very good PtdIns(4,5)P₂ binding of the Tubby domain. The latter shows only a minor sensitivity to the presence of InsP₃ in contrast to PLCδ1PH-GFP.

Recorded cells were grouped into four categories based on their expression level of the respective constructs (mRFP) and their averaged Ca^{2+} responses were calculated. As shown in Fig. 7, the largest effects on delaying the Ca^{2+} signal and desynchronizing the response was observed with the p130PH-mRFP construct. It is clear (as observed and reported in [29]) that this construct delays and inhibits the Ca^{2+} signal very potently. There was very little (if any) difference between the PLC δ 1PH and Tubby domains in their effects on Ca^{2+} signaling; both domains showed inhibition, but only at higher expression levels. The delay in the Ca^{2+} rise was slightly bigger in the case of the PLC δ 1PH-mRFP construct (Fig. 7; Table 2 shows the numerical values of the Ca^{2+} signaling parameters.).

Effects of $\text{Ins}(1,4,5)\text{P}_3$ buffering on the translocation responses of the two domains

Since the PLC δ 1PH domain has significant InsP_3 affinity, large InsP_3 increases can promote the translocation of the PLC δ 1PH-GFP fusion protein from the membrane to the cytosol [13]. Based on our *in vitro* InsP_3 binding experiments, the Tubby domain does not have such an InsP_3 binding property. As shown above and in [27,29], overexpression of the p130 PH domain binds InsP_3 and buffers its agonist-induced increases. To determine whether such InsP_3 buffering has an impact on the translocation of the two studied $\text{PtdIns}(4,5)\text{P}_2$ reporters, the GFP-tagged versions of either the Tubby-domain or the PLC δ 1PH domain were co-expressed with p130-PH-mRFP in HEK293-AT1 cells and their translocation responses were followed upon the addition of 100 nM AngII. Again, to determine the extent of maximum translocation of the reporters, high concentration of ionomycin (10 μM) was added after AngII stimulation. To decrease the speed and the amplitude of InsP_3 changes, these experiments were performed at room temperature as opposed to 35 °C. This condition also ensured that the PLC δ 1PH domain response is not examined at saturation.

As shown in Fig. 8, the translocation responses of the PLC δ 1PH domain and Tubby domain at room temperature showed differences similar to those observed with the 30 nM AngII at 35 °C. The amplitude of the Tubby domain translocation was only partial, while that of PLC δ 1PH was complete relative to the maximal response evoked by high concentration of ionomycin. Comparison of the responses of the respective constructs in the presence or absence of the p130PH-mRFP showed that InsP_3 buffering in these cells had only small (if any) effect on the translocation responses. In both cases the amplitude of the AngII response was slightly smaller, slower and more prolonged in the p130PH-mRFP expressing cells, which was consistent with a buffered InsP_3 (and resultant cytoplasmic Ca^{2+} kinetics). The fact that both constructs showed the same alteration of the translocation responses

suggests that it is the slight distortion of the Ca^{2+} signal rather than the buffered InsP_3 change per se that was responsible for these changes. It is important to note that in contrast to the ATP-stimulated COS-7 cells in which PLC activation is modest (and cannot even be detected by PH-domain translocation), the InsP_3 changes and the translocation responses are a lot more robust in the AngII-stimulated HEK293-AT1 cells and, in the latter, p130PH-expression has only a modest effect on the AngII induced Ca^{2+} signal (Varnai and Balla unpublished observations). However, while the COS-7 cells are ideally suited to determine the effects of InsP_3 binding or impaired substrate access of PLC when the cells express the $\text{PtdIns}(4,5)\text{P}_2$ reporters, the HEK293-AT1 cells are more useful to determine the effects of InsP_3 buffering on reporter translocation because their supramaximal InsP_3 responses can be significantly blunted before affecting their cytoplasmic Ca^{2+} signals. These results together suggested to us that at least in our cellular model, the InsP_3 changes primarily affect the translocation responses of either domain via their effects on cytoplasmic Ca^{2+} -PLC rather than via direct displacement.

Discussion

The present study was designed to widen the repertoire of phosphoinositide binding modules capable of providing information on $\text{PtdIns}(4,5)\text{P}_2$ changes in mammalian cells. So far most such studies have utilized the PLC δ 1PH-GFP construct, which does not detect $\text{PtdIns}(4,5)\text{P}_2$ in cellular location other than the PM and also could suffer from overestimating $\text{PtdIns}(4,5)\text{P}_2$ decreases, due to a displacing effect of InsP_3 . The extent of this distortion may vary from cell-type to cell-type and also depends on expression levels [30] and, therefore, has been a matter of dispute [10,31].

In a thorough recent study, several PH domains of *S.cerevisiae* have been described as capable of phosphoinositide binding, although only one, the Num1p PH domain, showed decent $\text{PtdIns}(4,5)\text{P}_2$ binding specificity based on several *in vitro* binding assays [16]. However, *in vivo* studies in yeast mutants that allowed specific manipulations of phosphoinositides concluded that PM targeting of several of these PH domains (Num1p, Cla4p, Skm1p, Slm1p, and Slm2p) showed $\text{PtdIns}(4,5)\text{P}_2$ dependence, while the membrane recruitment of the Opy1p-PH domain was found to be independent of phosphoinositides. We extended these studies to mammalian cells and characterized the $\text{PtdIns}(4,5)\text{P}_2$ dependence of the membrane association of these PH domains using controlled manipulations of $\text{PtdIns}(4,5)\text{P}_2$. As described before, all of these PH domains showed PM localization and none was recruited to any intracellular membranes, although some (such as the Opy1p PH domain) also showed nuclear or nucleolar localization. The expression of some

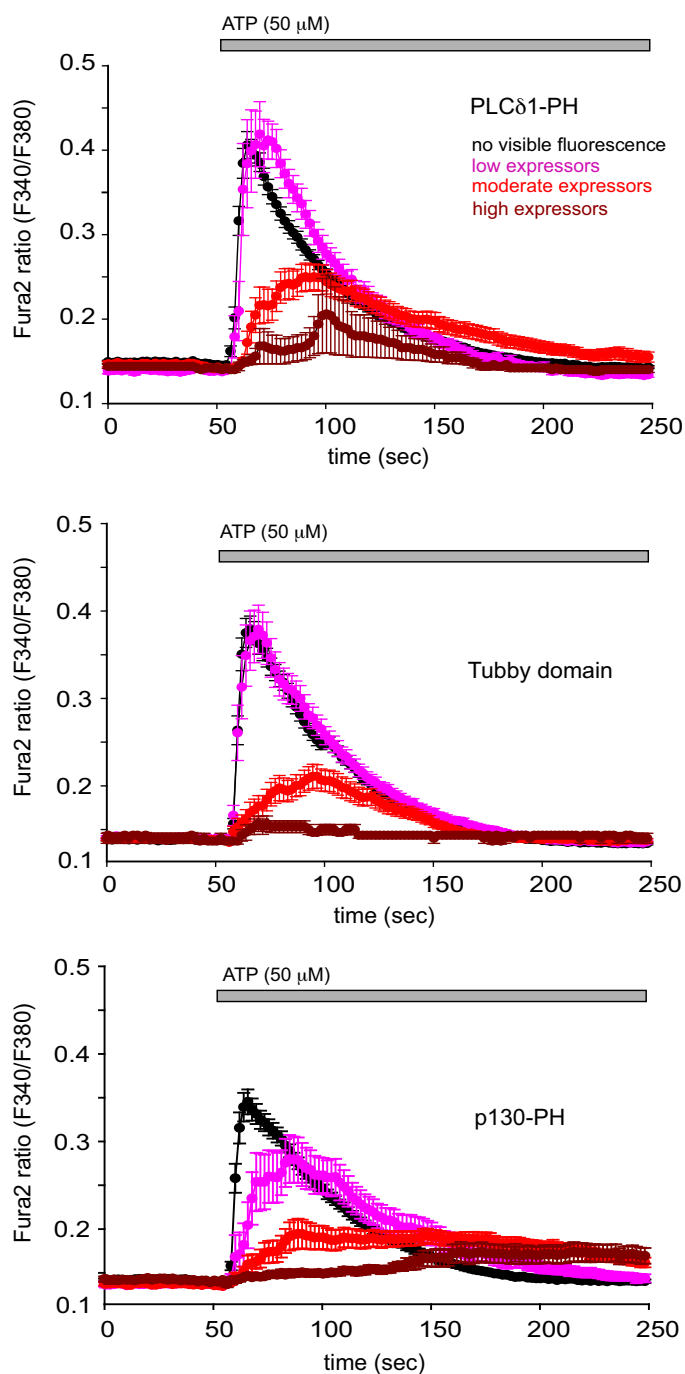
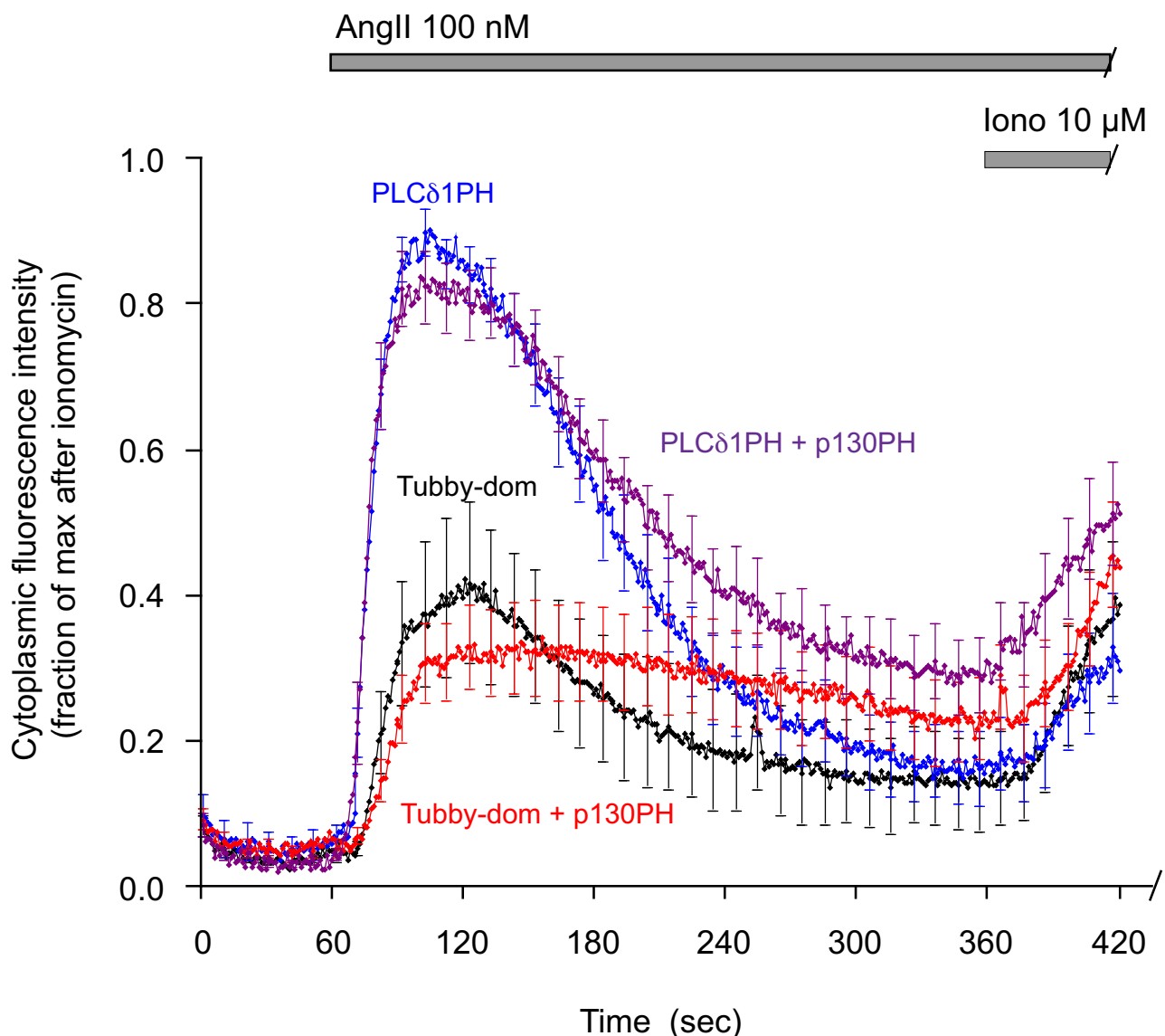


Figure 7
Inhibition of agonist-induced Ca²⁺ signaling by expressed mRFP-Tubby-domain, PLCδ1PH-mRFP and p130-PH-mRFP. COS-7 cells were transfected with the indicated mRFP fusion constructs, and their cytosolic Ca²⁺ responses were analyzed by ratiometric Ca²⁺ imaging using Fura2. The amount of fluorescent protein was determined after the release of all membrane bound constructs by the addition of high concentration of ionomycin at the end of each experiment. ATP-induced Ca²⁺ responses were grouped into four categories according to expression levels of the red fluorescence and the responses were averaged (see Table 2 for these values in each group in arbitrary units). Note that each construct interferes with the Ca²⁺ signal in these COS-7 cells where the ATP-induced PLC activation and InsP₃ increase is relatively modest. Such inhibitory effects are substantially smaller in HEK293-AT1 cells that show robust PLC activation (see Fig. 8). Table 2 shows the numerical values of the Ca²⁺ signaling parameters.

**Figure 8**

Effects of InsP_3 buffering on AngII-induced Tubby-domain and PLC δ 1PH translocation responses. HEK293-AT1 cells were transfected with the indicated GFP reporter constructs alone or together with p130PH-mRFP. After 24 hours, cells were examined in a Zeiss Live 5 DuoScan confocal microscope at room temperature. Cells were stimulated with 100 nM AngII at 60 second followed by 10 μM ionomycin at 360 sec. Data were collected at 1 fps rate and the cytoplasmic fluorescence intensity was analyzed in selected regions of interest outside the nucleus. Values were normalized for minimum and maximum fluorescence values for each run and these values were averaged. Means \pm S.E.M. are shown obtained from: $n = 10$ (Tubby domain alone), $n = 12$ (PLC δ 1PH alone), $n = 21$ (Tubby domain + p130PH-mRFP) and $n = 20$ (PLC δ 1PH + p130PH-mRFP) cells. The traces were truncated at 420 sec to better illustrate the AngII-induced changes.

of these PH domains (namely the Num1p) was very low and required switching the GFP around to improve expression. Adding a nuclear export signal to the GFP-Opy1p-PH construct allowed a better assessment of its membrane binding properties. Remarkably, the behavior of these constructs did not completely match with that described in yeast cells. For example, the PH domains of

Cla4p, Skm1p, and Slm2p showed no detectable decrease during PLC activation or after elimination of PtdIns(4,5) P_2 via a 5-phosphatase. In contrast, the Opy1p PH domain followed the changes in PtdIns(4,5) P_2 in spite of its apparent failure to do so in yeast. Unfortunately, the Num1p PH domain that binds PtdIns(4,5) P_2 with the highest specificity in yeast cells did not show any features

Table 2: Ca²⁺ signaling parameters in ATP-stimulated COS-7 cells expressing either one of the mRFP-Tubby-domain, PLC δ 1PH-mRFP and p130-PH-mRFP proteins

Construct	mRFP fluorescence (AU)	T _{1/2} activation* (sec)	N =
Tubby-domain	< 100	6.0 ± 0.3**	109
	101-1000	6.0 ± 1.8	31
	1001-5000	13.5 ± 3.5	40
	5001-10000	12.0 ± 1.5	4
PLC δ 1PH	< 100	3.5 ± 0.4	99
	101-1000	6.5 ± 1.1	20
	1001-5000	12.5 ± 3.9	34
	5001-10000	18.0 ± 6.1	5
p130-PH	< 100	3.5 ± 0.4	91
	101-1000	12.0 ± 4.9	16
	1001-5000	19.5 ± 5.3	39
	5001-10000	> 100	17

* The lag time of the Ca²⁺ response was defined as the time it takes for a cell to reach half-maximum of its Ca²⁺ peak after addition of 50 μ M ATP.

**Values are calculated from cells expressing the mRFP-tagged proteins at the indicated intensities. Note the largest effects of the p130-PH-mRFP protein that does not bind to the membranes but binds InsP₃ with comparable affinity to the PLC δ 1PH-mRFP [27].

that would make it a great substitute for PLC δ 1PH-GFP. First, its expression was modest and in cells showing higher expression, it created bright vesicular structures budding off the plasma membrane. Moreover, it showed only a modest translocation from the membrane to the cytosol after PLC activation. One of the probes, the Slm1p-PH appeared to be a mixed reporter of PtdIns(4,5)P₂ and PtdIns4P in the membrane. This domain was only partially displaced from the membrane after 5-phosphatase recruitment, suggesting that it may still be binding to the PtdIns4P that is generated by the 5-phosphatase.

The discrepancy between the probes behavior in the yeast and mammalian cells is not unprecedented. For example, we did not observe Golgi localization of the PtdIns4P reporter OSH2-PH in mammalian cells [22] while it clearly showed the Golgi pool in yeast [16,32]. This is suggestive of a more complex mechanism of membrane recruitment of the PH domains, involving not only phosphoinositides but probably other proteins (or anionic lipids) as interacting partners. Whether a mammalian protein can substitute for the yeast protein in the protein-protein interaction with a yeast PH domain probably varies from one domain to another, making the outcome difficult to predict. Nonetheless, these studies have concluded that none of the predicted PtdIns(4,5)P₂ recognizing probes from the yeast PH domain collection have detected other pools of this lipid in intracellular compart-

ments and none has shown any obvious advantage over the mammalian ones to be used in imaging studies. This is in contrast to PtdIns4P recognizing PH domains of the yeast that have been successfully used in mammalian cells [22,32,33].

Another PtdIns(4,5)P₂ reporter characterized in this study was the Tubby domain of the mammalian transcription factor Tubby protein. The Tubby domain was described as a high affinity PtdIns(4,5)P₂ binding module found at the C-terminus of the Tubby protein and being responsible for its lipid binding and membrane localization [18]. It was also claimed as one that does not bind InsP₃, although direct experimental evidence for this has not been available in published literature. The Tubby domain has already been used as a PtdIns(4,5)P₂ reporter [19] and two recent studies have examined the usefulness of the full-length Tubby protein [20] or a mutant form of the Tubby domain [21] as a PtdIns(4,5)P₂ probe in comparative studies similar to ours. Our results with the wild-type Tubby domain had several similarities, but also notable differences to the results described in those studies.

Firstly, both studies found that the Tubby protein as well as the Tubby domain has higher affinity to the PM than PLC δ 1PH-GFP. In fact, the wild-type Tubby domain was found to have high enough affinity that it did not show agonist-induced responses in many cells prompting the authors to create a mutant (R332H) with a reduced affinity that was found useful in their studies [21]. Interestingly, in the cells used in our studies the same mutation completely eliminated the membrane localization of the Tubby domain (Szentpetery and Balla unpublished observation) making it unsuitable for further studies. We did not have an explanation for this discrepancy other than the different fluorescent proteins used in the two studies and that the placing of the fluorescent protein relative to the Tubby domain was different. Quinn et al. used eYFP, while we used eGFP fusion constructs and we used GFP in front of the Tubby domain whereas the Quinn study found the mutant Tubby construct having YFP at its C-terminus a more suitable one. Since the dimerization tendency of YFP is larger than that of GFP [34] it is possible that the higher apparent affinity of the constructs in the Quinn study reflects a dimerization of the fusion proteins, which could explain these differences. Indeed, when we generated the same Tubby domain constructs fused to YFP (still the fluorescent protein in front) we found that the R332H mutant did show some membrane localization, especially in COS-7 cells but not as much as shown in the Quinn study (Szentpetery and Balla, unpublished observation). Although this slight localization does not match those described by Quinn et al., the higher dimerization tendency of YFP still may play into the apparent affinity of the mutant Tubby construct.

Nevertheless, a higher affinity of the full-length Tubby protein (fused to eGFP) to the membrane was also shown in the Nelson study and manifested as a rightward shift (compared to the PLC δ 1PH-GFP response) in the dose-response curve with muscarinic agonist measuring the translocation of the Tubby protein from the membrane to the cytosol. Our results are in good agreement with these studies as we also found a significantly higher fraction of the Tubby domain at the PM than with PLC δ 1PH, and documented a substantial difference between the agonist-sensitivities of the Tubby domain and PLC δ 1PH-GFP translocation responses. Moreover, our FRAP analysis showed that the dissociation of the Tubby domain from the membrane is significantly slower than that of the PLC δ 1PH-GFP.

All three studies showed that Tubby domain (or the full-length Tubby protein) displayed no sensitivity to InsP $_3$ in agreement with the original claims [18]. The present study also showed it with direct binding assays using recombinant Tubby domains. Quinn et al. showed that diffusion of InsP $_3$ into the patch pipette caused no translocation of the Tubby domain mutant, while making the PLC δ 1PH-GFP fully translocate to the cytosol [21]. The Nelson study, on the other hand, used overexpression of an InsP $_3$ 3-kinase to limit InsP $_3$ increases as described in their earlier studies [35,36]. They found that in contrast to PLC δ 1PH-GFP, the Tubby domain translocation after agonist stimulation was insensitive to InsP $_3$ 3-kinase overexpression [20]. However, any reduction in InsP $_3$ increase (whether converted to InsP $_4$, or InsP $_2$ or being buffered by an InsP $_3$ binding domain) also reduces the Ca $^{2+}$ signal and as a corollary will limit PLC activation. This was most likely the case in the Quinn study where overexpression of the InsP $_3$ 5-phosphatase eliminated the translocation responses of both PtdIns(4,5)P $_2$ probes [21]. This study also found that the Tubby domain translocation was slightly more sensitive to inhibition by Ca $^{2+}$ depletion than that of the PLC δ 1PH construct. This finding agreed with our observation that the Tubby domain had a slower response to the cytoplasmic Ca $^{2+}$ increase than the PLC δ 1PH-GFP, consistent with an increased "resistance" of the Tubby-domain-covered PtdIns(4,5)P $_2$ to PLC-mediated hydrolysis. It is worth noting that both the Quinn study and ours used HEK293 cells.

In contrast to these findings, a striking reduction was observed in neuroblastoma cells in the translocation responses of the PLC δ 1PH domain, but not those of the Tubby protein, when InsP $_3$ 3-kinase was expressed, with a strong reduction in the cytoplasmic Ca $^{2+}$ response [20]. This seemingly contradicted the higher Ca $^{2+}$ requirement of the Tubby domain translocation also observed in that same study. One possible explanation for this apparent contradiction is, if the magnitudes of InsP $_3$ increases are

much larger in the neuronal cells used in the Nelson study than those observed in HEK293 cells. This is not an unreasonable assumption, since N1E-115 neuroblastoma cells require much higher InsP $_3$ increases to induce Ca $^{2+}$ signaling than other cell types [37]. In search of an explanation for this unique feature, Watras et al. has identified a protein in neurons that binds the InsP $_3$ receptor to significantly decrease its InsP $_3$ sensitivity [37]. This could make neuroblastoma cells (and perhaps other neuronal cell lines) less sensitive to InsP $_3$ increases requiring them to generate a lot more InsP $_3$ in response to agonists that could significantly displace the PLC δ 1PH-GFP from the PM. Additionally, any change in the relative proportion of PtdIns(4,5)P $_2$ vs. InsP $_3$ can contribute to a larger InsP $_3$ sensitivity of the PLC δ 1PH translocation as demonstrated in mathematical modeling studies [30].

The question remains whether the tighter binding of the Tubby domain to the membrane is related to its lower affinity to InsP $_3$. A significant amount of free InsP $_3$ present in the cytosol could indeed partially displace the PLC δ 1PH-GFP from the membrane while not affecting the binding of the Tubby domain. However, overexpression of either the InsP $_3$ kinase [20], the InsP $_3$ 5-phosphatase [21] or an InsP $_3$ binding domains had little if any impact on the basal localization of the PLC δ 1PH-GFP. Moreover, the slightest increases in free InsP $_3$ are immediately detected by the InsP $_3$ -R in the form of Ca $^{2+}$ release. Therefore, we do not favor an explanation that assumes significant free InsP $_3$ levels in the cytoplasm of quiescent cells. We would rather assume that the tighter binding of the Tubby domain to the membrane reflects a genuinely higher affinity either to PtdIns(4,5)P $_2$ itself, or to the lipid together with some additional membrane component(s). This is consistent with the slower dissociation rate as well as the stronger resistance of the PtdIns(4,5)P $_2$ pool covered with the Tubby domain to respond to PLC activation. The fact that the 32 P-phosphate or 3 H-*myo*-inositol labeled HEK293-AT1 cells (the same cells used in this study) lose about 80-90% of their labeled PtdIns(4,5)P $_2$ within 30 sec of stimulation with 100 nM AngII [22], yet the Tubby domain responds only in a fraction of cells stimulated with the same dose of agonist, suggest that the Tubby domain probably underestimates the changes in PtdIns(4,5)P $_2$ because of its interference with PLC activation (PLC breaks down a larger fraction of PtdIns(4,5)P $_2$ that is not bound to the Tubby domain). Therefore, while the PLC δ 1PH domain may overestimate PLC activation because of its InsP $_3$ sensitivity, the Tubby domain may underestimate it because of its higher apparent PtdIns(4,5)P $_2$ affinity. In fact, in the cells used in these studies (HEK293-AT1 and COS-7), the inhibitory effects of the two domains on Ca $^{2+}$ signaling were very comparable: in one case due to the combined effects on InsP $_3$ quenching and PtdIns(4,5)P $_2$ binding (PLC δ 1PH-GFP),

while in the other case because of a tighter PtdIns(4,5)P₂ binding (Tubby domain). The slight delay in the onset of Ca²⁺ rise caused by the expression of the PLCδ1PH-mRFP but not the mRFP-Tubby domain is certainly another indication that the PLCδ1PH domain binds InsP₃. Importantly, we did not see a significant difference between the two domains in their sensitivity to expression of InsP₃ buffering constructs, essentially both being affected probably due to interference with the Ca²⁺ signal.

An important observation of the present study was the delayed Tubby response relative to that of the PLCδ1PH domain during PLC activation but not when the 5-phosphatase was recruited to the PM. This finding may simply suggest that the InsP₃ increase does indeed contribute to PLCδ1PH translocation. However, it is also possible that the endogenous PLC activation mechanism is more sensitive to masking of PtdIns(4,5)P₂ by the Tubby domain than the catalysis by the recruited 5-phosphatase. The fact that the Tubby domain does not bind the soluble head-group, InsP₃, even though it binds PtdIns(4,5)P₂ with higher affinity, already suggests that the binding force of the Tubby domain must receive significant contributions from interactions with the glycerol backbone. If this were indeed the case, the Tubby domain would obscure the phosphodiester group more and with that could block the access of PLC to this site.

Lastly, these studies have shown a perfect example of how the same lipid, PtdIns(4,5)P₂ can regulate several effectors in the same cell simultaneously and yet differently. Just by having two different apparent affinities of two reporters used in the present study - mimicking two endogenous binding partners - is sufficient to elicit a differential response to the same PLC activation. This was also convincingly demonstrated in the experiments where the Kir6.2 K_{ATP} channel [20] or the KCNQ M-channel [38] (two known PtdIns(4,5)P₂ regulated K⁺ channels) responses were correlated with PtdIns(4,5)P₂ changes assessed by the fluorescent reporters.

There are several other conclusions highlighted by these studies. Firstly, all probes based on binding to PtdIns(4,5)P₂ (or to any other lipid) show some bias depending on its affinity to the lipid. Secondly, the experimental system may also determine the extent to which the probe manifests its limitation. Thirdly, even the fluorescent protein used a fusion partner can modify the utility of a bioprobe. These should be all reminders that we need to be cautious of selecting a probe and consider it as "the" gold standard.

Conclusion

The present study analyzed a set of PtdIns(4,5)P₂ reporters some selected from yeast PH domains and one compris-

ing of the Tubby domain of the transcription factor, Tubby. All of these fluorescent probes confirmed that PtdIns(4,5)P₂ shows highest abundance in the PM and that this lipid is undetectable in intracellular membranes. Most of the yeast-derived PH domains were found less useful, some expressing at low levels or showing weak membrane localization, others causing membrane blebbing. Detailed comparison of the PLCδ1PH-GFP and the Tubby domain showed that both reporters are suitable to follow PtdIns(4,5)P₂ changes in living cells, but the Tubby domain is less sensitive because of its higher affinity to PtdIns(4,5)P₂. PLCδ1PH domain was found to be the most sensitive reporter but because of its binding to InsP₃ some of its translocation is caused by InsP₃ increases that can become a problem especially in certain cell types. The differential responses of the two domains to PtdIns(4,5)P₂ changes helps us understand and envision the principles of inositide regulation of multiple effectors.

Methods

Materials

AngiotensinII (human octapeptide) was from Bachem (Torrance, CA), rapamycin was from Calbiochem (San Diego, CA) or LC Biochemicals (Woburn, MA). All other chemicals were of the highest purity grades.

DNA constructs

The PLCδ1-PH-GFP and its color variants and the p130-PH-mRFP constructs have been described previously [24,27]. The C-terminal (243-505) Tubby domain of the mouse Tubby protein fused at its N-terminus to GFP was a generous gift from Dr. L. Shapiro (Mount Sinai School of Medicine, NY) [18]. The Tubby domain was also subcloned into the mRFP-C1 vector using the Sall-BamHI sites. For bacterial expression of the recombinant proteins, the GFP-Tubby-domain was amplified with the primer pairs, 5'-ATATCATATGCTGAGCAAGGGCGAG-GAGC-3' and 5'-ATATGCGGCCGCCCTCGCAG-GCCAGCTTGCTGTC-3' and the PCR product cloned into the pET-23a bacterial expression vector between the NdeI and NotI sites. The PLCδ₁-PH-GFP fusion protein for bacterial expression has been described previously [27].

Transfection of cells for confocal microscopy and intracellular Ca²⁺ measurements

For single cell analysis HEK293-AT1 (a HEK293 cell line stably expressing the rat AT_{1a} angiotensin receptor) or COS-7 cells were used. Cells were plated onto 25-mm-diameter circular glass cover slips at a density of 3 × 10⁵ cells/dish one day before transfection with plasmid DNAs (0.5-1 μg/dish) using the Lipofectamine2000 reagent (Invitrogen) and OPTI-MEM (Invitrogen). One day after transfection, cells were washed twice with a modified Krebs-Ringer solution, containing 120 mM NaCl, 4.7 mM KCl, 1.2 mM CaCl₂, 0.7 mM MgSO₄, 10 mM glucose, 10

mM Na-Hepes, pH 7.4 and the coverslip was placed into a metal chamber that was mounted on a heated stage with the medium temperature kept at 33°C. Note that some experiments were performed at room temperature. Cells were incubated in 1 ml of the Krebs-Ringer buffer and the stimuli were added in 200 µl warm buffer removed from the cells. Cells were examined in an inverted microscope under a 60× oil-immersion objective (LSM 510-META; Carl Zeiss MicroImaging, Inc.) equipped with an objective heater (Biotech).

FRAP analysis

FRAP experiments were performed in a Zeiss Live5 DuoScan Confocal Microscope system in which continuous fast scanning is possible while photobleaching with another laser. Images were collected at 0.1-0.5 s intervals and selected regions in the PM were photobleached at 100% power of the bleaching laser. After background subtraction, two parameters were calculated from the intensity curves obtained in the center of a larger bleached area: the mobile fraction (MF), which represents the percent recovery after photobleaching compared to the initial fluorescence value before photobleaching; and a $T_{1/2}$ value, which is the time required to reach half maximal recovery.

Cytoplasmic Ca²⁺ studies

For Ca²⁺ measurements, cells were loaded with 3 µM fura-2/AM (45 min, room temperature) in a Hepes-based M-199, containing 200 µM sulfapyrazone and 0.06% pluronic acid. After loading, Ca²⁺ measurements were performed at room temperature in the same modified Krebs-Ringer solution containing no Ca²⁺. An inverted microscope (IX70; Olympus) equipped with a Lambda DG-4 illuminator (Sutter Instruments) and a Micromax 1024 BFT camera (Roper Scientific) and the appropriate filter sets were used for Ca²⁺ analysis. Data acquisition and processing were performed by the MetaFluor software (Molecular Devices).

In vitro Ins(1,4,5)P₃ and PtdIns(4,5)P₂ binding assays

Recombinant proteins were produced in the BL-21-DE3 strain of Escherichia coli (Invitrogen). Bacterial cells were grown to A₆₀₀: 0.6 at 37°C and induced with 300 µM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 18-20°C for 7 h. Bacterial lysates were prepared by sonication in lysis buffer (20 mM NaCl and 20 mM Tris, pH 8.0) followed by centrifugation at 10,000 × g for 30 min at 4°C. The supernatant was incubated with Ni²⁺-NTA-agarose beads (Qiagen) in the presence of 5 mM imidazole for 1 h at 4°C. The beads were washed three times with lysis buffer, and the recombinant proteins were eluted with the same buffer containing 1 M imidazole. The concentration of recombinant proteins was assessed by quantifying the bands of Coomassie Blue-stained SDS gel containing the

recombinant proteins using bovine serum albumin as a standard.

The in vitro InsP₃ binding assay and the InsP₃ displacement from PtdIns(4,5)P₂ binding were performed as described previously [27]. Briefly, the incubation buffer of the in vitro InsP₃ binding assay contained 50 mM Na-Hepes, pH 7.4, 50 mM KCl, 0.5 mM MgCl₂, and 0.01 mM CaCl₂. About 0.2 µg of soluble recombinant proteins was incubated in 50 µl of this buffer with 0.74 kBq (0.5 nM) [³H]Ins(1,4,5)P₃ with increasing concentrations of unlabeled InsP₃ for 10 min on ice. The binding reaction was terminated by adding 5 µl of human γ-globulin (10 mg/ml) and 50 µl polyethylene glycol 6000 (30%). Tubes were left on ice for 5 min and then centrifuged at 10,000 × g for 10 min. The pellets were dissolved in 0.1 ml of 2% SDS, and their radioactivity was counted in a liquid scintillation counter.

Binding to phospholipids was performed with mixed lipid vesicles. 110 µg of PtdIns(4,5)P₂ and 1.4 mg of phosphatidylethanolamine (bovine liver; Avanti) were mixed and dried under a nitrogen stream followed by high vacuum, and the dried mixtures were suspended to a final total lipid concentration of 2 mM (PE) in 20 mM Hepes, pH 7.2, 100 mM NaCl, 2 mM EGTA, 0.1 µg/ml bovine serum albumin by bath sonication. 5 µl of the purified GFP fusion protein (~1 µg) and 5 µl of buffer or Ins(1,4,5)P₃ were added to 90 µl of phospholipid vesicles. The reaction mixture was incubated at 30°C for 10 min followed by ultracentrifugation at 85,000 rpm for 20 min at 4°C. The 100-µl supernatant was mixed with 30 µl of 5× Laemmli buffer, and the pellet was resuspended in 100 µl of incubation buffer followed by the addition of 30 µl of 5× Laemmli buffer. After vortexing, 40 µl of each fraction was loaded onto a 12% Tris glycine gel without boiling and separated by SDS-PAGE at 4°C. After electrophoresis, gels were analyzed in a Storm 860 PhosphorImager (Molecular Dynamics) using blue fluorescence screening for quantitation of the GFP fusion protein band in the gel. Western blot analysis was also performed on parallel samples using the purified polyclonal antibody against GFP (Clontech).

Abbreviations

GFP: Green fluorescent protein; InsP₃: Inositol 1,4,5-trisphosphate; PtdIns: Phosphatidylinositol; PtdIns(4,5)P₂: Phosphatidylinositol 4,5-bisphosphate; PLC: Phospholipase C; PM: Plasma membrane.

Authors' contributions

ZS has created constructs, run most of the experiments, analyzed data, participated in experimental planning, wrote draft of the manuscript, and prepared raw figures. AB created constructs, prepared recombinant proteins and

run the InsP₃ and lipid binding assays. YJK has run some of the experiments and analyzed the corresponding data. ML has provided all yeast constructs, discussed data and their implications, read the manuscript. TB has designed experiments, trained ZS to run microscopy experiments, participated in microscopy sessions, analyzed data, completed the writing of the manuscript and prepared the final figures. All authors have seen and approved the final version of the manuscript.

Additional material

Additional file 1

Figure S1. This is a Figure showing the distribution of some of the GFP-tagged PH domain constructs

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Additional file 2

Figure S2. This is a Figure showing the FRAP analysis performed with the PLCδ1PH and the Tubby domain.

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Additional file 3

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Additional file 4

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