Binding of multiple ligands to pleckstrin homology domain regulates membrane translocation and enzyme activity of β-adrenergic receptor kinase

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Abstract Pleckstrin homology (PH) domains are discrete structural modules present in numerous proteins involved in signal transduction processes. In the case of the β-adrenergic receptor kinase (βARK), PH domain-mediated binding of two ligands, the βγ subunits of heterotrimeric G proteins (Gβγ) and phosphatidylinositol 4,5-bisphosphate (PIP2), has been shown to be required for the kinase function. In this study, the ability of Gβγ and PIP2 to affect membrane localization of βARK is used to define the ligand binding characteristics of the βARK PH domain. The binding of these ligands to the PH domain of the intact kinase is shown to be cooperative, Gβγ increasing the affinity of the PH domain for PIP2. Notably, although PIP2-dependent membrane association of βARK is observed at high concentrations of this lipid, in the absence of Gβγ, no receptor phosphorylation is observed. Peptides derived from the receptor intracellular loop inhibit the receptor phosphorylation without affecting the membrane translocation of the kinase complex, suggesting that βARK activity does not necessarily correlate with the amount of βARK associated with the membrane. These results point to a distinct role for each PH domain ligand in βARK-mediated receptor phosphorylation. Strikingly, the ligand binding characteristics of the isolated βARK PH domain fused to glutathione *S*-transferase are very different from those of the PH domain of the intact kinase. Thus, in contrast to the native protein, the isolated PH domain binds Gβγ and PIP2 independently and with no apparent cooperativity. That protein environment plays an important role in determining the ligand binding characteristics of a particular PH domain highlights the potential risks of inferring mechanisms from studies of isolated PH domains.

Key words: Pleckstrin homology domain; β-Adrenergic receptor kinase; Heterotrimeric G protein βγ subunit; Phosphatidylinositol 4,5-bisphosphate; Lipid vesicle; Phosphorylation

1. Introduction

Pleckstrin homology (PH) domains are approximately 100–120 amino acid regions of sequence homology found in numerous proteins involved in various signaling pathways [1–7]. Several potential ligands for PH domains have been identified including the βγ subunits of heterotrimeric G proteins (Gβγ) [8–13] or Gβ-like WD40 motif-containing proteins [14,15], phosphatidylinositol 4,5-bisphosphate (PIP2) [16–18], inositol 1,4,5-trisphosphate (IP3) [19–21], and protein kinase C (PKC) [22,23]. Although the physiological relevance of the binding of these ligands remains to be elucidated, the PH domain has been proposed to play a role in membrane recruitment of PH domain-containing proteins, thus targeting them to appropriate cellular compartments or enabling them to interact with other components of the signal transduction pathway.

The ligand binding characteristics of one PH domain, that of the β-adrenergic receptor kinase (βARK), have recently been defined using purified proteins in a reconstituted system. The βARK, a member of the G protein-coupled receptor kinase (GRK) family, phosphorylates and thus desensitizes agonist-occupied G protein-coupled receptors [24–26]. The carboxyl-terminal PH domain of the βARK has been proposed to play an essential role in mediating this process, since in vitro, the simultaneous presence of two PH domain-binding ligands (Gβγ and PIP2) is required for the membrane association of this kinase and β_2-adrenergic receptor (βAR) or muscarinic acetylcholine receptor phosphorylation [27–29].

Three-dimensional structural analyses of several PH domains (i.e. pleckstrin [30], spectrin [31], dynamin [32–34], and phospholipase Cδ [35]) reveal a common structure, a β-barrel of seven antiparallel β-sheets and a carboxyl-terminal amphiphilic α-helix. Interestingly, the phospholipid has been demonstrated to bind a cleft of the β-barrel at the amino-terminus of this domain [16,18] while studies with mutant βARK constructs suggest a Gβγ binding site encompassing the carboxyl-terminal α-helix [8,9,36]. Thus the coordinated binding of two PH domain ligands, one at the carboxyl- and one at the amino-terminus of the βARK PH domain, seems to be essential for kinase localization and function. Whether membrane localization mediated by the coordinated binding of multiple ligands is a feature common to all PH domains remains to be further investigated.

Previous studies examining the role of the βARK PH domain have focused predominantly on the functional consequences of Gβγ and PIP2 binding [28,29]. Directly examined herein are the roles played by these ligands in mediating membrane association of the βARK, using either the intact kinase or alternatively a βARK PH domain-containing fusion protein. The aim of this study is to address the following specific questions. (i) Can the synergistic enhancement of receptor phosphorylation by PIP2 and Gβγ indeed be accounted for by the cooperative binding of PIP2 and Gβγ to the PH domain and subsequent membrane translocation? (ii) If so, does the isolated PH domain reflect the function of the PH domain

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Abbreviations: PH, pleckstrin homology; Gβγ, βγ subunits of heterotrimeric G proteins; βAR, β_2-adrenergic receptor; GRK, G protein-coupled receptor kinase; βARK, β-adrenergic receptor kinase; PC, phosphatidylcholine; PIP2, phosphatidylinositol 4,5-bisphosphate; PBS, phosphate buffered saline; GST, glutathione *S*-transferase; B1CT, βARK1 carboxyl terminus (residues 467–689 of the βARK)

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in the intact βARK? (iii) Is the PH domain-mediated membrane localization of βARK sufficient to account for the enhanced phosphorylation of the receptor? The results highlight the importance of assessing PH domain function in the context of an intact protein and suggest that Gβγ and PIP2 play coordinated but distinct roles in βARK membrane translocation and βARK-mediated receptor phosphorylation.

2. Materials and methods

2.1. Materials

Bovine βARK was purified from recombinant baculovirus-infected Sf9 cells and Gβγ subunits were purified from bovine brain as previously described [37,38]. Glutathione *S*-transferase (GST) fusion proteins derived from the βARK1 carboxyl-terminus (GST-B1CT) was expressed in and purified from *Escherichia coli* strain NM522 [8,9]. GST fusion protein derived from the third loop of human β_2-adrenergic receptor (βAR-3l) (residues 219–276) was similarly prepared. Synthetic peptides were designed from the third loop of βAR: peptide P1 (residues 219–247 of the βAR) and peptide P2 (residues 248–278 of the βAR). Purified lipids and partially dephosphorylated casein were obtained from Sigma.

2.2. Purification and reconstitution of the βAR

The human βAR was expressed, purified, and reconstituted into vesicles of defined lipid composition as described previously [28,39]. Briefly, baculovirus-infected Sf9 cells were lysed, a membrane fraction prepared and βAR solubilized with 0.25% w/v *n*-dodecyl β-D-maltoside. The βAR was subsequently purified by affinity chromatography on an alprenolol-Sepharose column [39]. Purified receptor was reconstituted into vesicles of defined lipid composition and receptor concentration determined as described in [28,39].

2.3. βARK-mediated βAR phosphorylation

The βAR (3.0 pmol) reconstituted in PC vesicles containing defined concentrations of PIP2 (described in the text and figure legends) was incubated with βARK (6.0 pmol) in either the presence or absence of Gβγ (6.0 pmol). Reactions were performed as described in Pitcher et al. [28], with the exception that the final reaction volume was 30 μl. The final lipid concentration was 1.7 mg/ml in all assays. Thus, the amount of PIP2 in vesicles composed of 95% PC, 5% PIP2, for example, is 2.5 μg. All assays contained 50 μM (−)-isoproterenol. Reactions were incubated at 30°C and stopped after 15 min by addition of an equal volume of SDS sample loading buffer [28]. Reactions were subsequently subjected to electrophoresis on 10% SDS-polyacrylamide gels. Dried gels were quantified by Phosphorimager analysis and exposed to film.

2.4. Lipid vesicle binding assay

The ability of βARK or the GST-B1CT to bind to PC vesicles containing various concentrations of PIP2 was examined using the centrifugation assay described in [28,36]. Briefly, lipid vesicles of defined composition with or without βAR (3 pmol in 30 μl scale) were incubated with purified βARK (6.0 pmol) or the GST-B1CT fusion protein (6.0 pmol) in either the presence or absence of Gβγ (6.0–12.0 pmol) for 10 min at room temperature and an additional 5 min on ice. Following centrifugation, 100 000 rpm (TL-100 rotor) for 15 min at 4°C, the distribution of the βARK or the Gβγ between the supernatant and pellet fractions was determined by Western blot analysis (ECL, Amersham Corp.) using anti-βARKct antibodies [28] or antibodies to the β subunit of Gβγ (DuPont NEN) at a dilution of 1:1000. Blots were subsequently quantified by densitometry.

3. Results and discussion

The simultaneous binding of Gβγ and PIP2 to the PH domain of the βARK has been shown to enhance βARK-mediated βAR phosphorylation by facilitating the membrane localization of this kinase [28]. This Gβγ- and PIP2-dependent association of the βARK with lipid vesicles has been utilized in this study to examine the ligand binding characteristics of

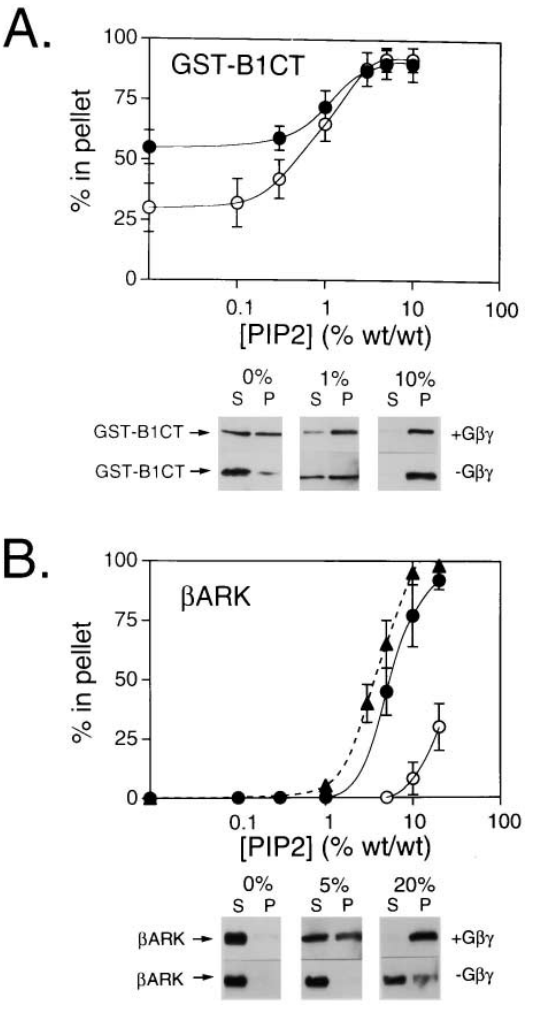

Fig. 1. PIP2- and Gβγ-dependent membrane association of GST-B1CT (A) and βARK (B). Large unilamellar vesicles composed of PC containing the indicated concentrations of PIP2 were prepared as described in Section 2. Following incubation with either the intact βARK or the GST-B1CT vesicles (± Gβγ) were recovered by centrifugation and the distribution of the βARK proteins between the supernatant and pellet fractions determined by Western blot analysis (see Section 2). The results shown are expressed as the percent βARK or GST-B1CT associated with the pellet and represent the means ± S.E.M. of three separate determinations. Incubations were performed in the absence of Gβγ (○), in the presence of an equimolar ratio of Gβγ:βARK protein (200 nM Gβγ) (●), or in the presence of 2-fold molar excess of Gβγ:βARK protein (400 nM Gβγ) (△). A representative Western blot showing the distribution of the βARK or the GST-B1CT between the supernatant (s) and pellet (p) fractions at various PIP2 concentrations is also shown. The association of Gβγ with vesicles regardless of lipid composition was assessed by Western blot analysis using the anti-Gβ antibodies (data not shown).

the βARK PH domain. Essentially all Gβγ were found to associate with vesicles of any lipid compositions utilized in this study according to the Western blot analysis. As shown in Fig. 1B, the simultaneous presence of both PH domain ligands is required for effective membrane localization of the βARK. Addition of Gβγ alone or low concentrations of PIP2 (5% or less) fail to promote membrane association of the kinase. Some PIP2-mediated membrane association of the βARK is observed at high concentrations of this lipid (10% or higher) in the absence of Gβγ, however, the coordinated

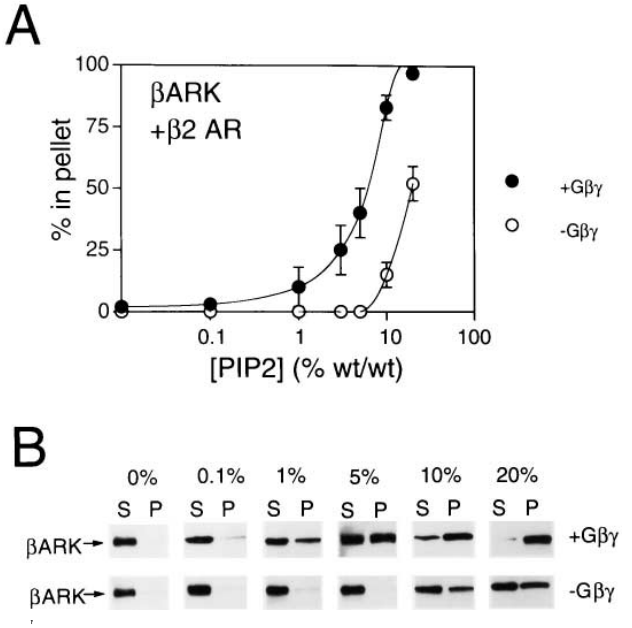

Fig. 2. PIP2- and Gβγ-dependent membrane association of βARK in the presence of βAR. A: Membrane association of the βARK with βAR-containing vesicles. Purified βAR reconstituted in vesicles containing PC and PIP2 at the concentrations indicated were incubated with purified βARK, the vesicles were recovered by centrifugation, and the distribution of the kinase determined as previously described (see Section 2). The results shown are expressed as the percentage of the βARK associated with the vesicle (pellet) fraction ± S.E.M. of three separate determinations. Incubations were performed in the absence of Gβγ (○), or the presence of an equimolar ratio of Gβγ:βARK (200 nM Gβγ) (●). B: A representative Western blot showing the distribution of the βARK (±Gβγ) between the supernatant (s) and pellet (p) fractions at various PIP2 concentrations.

presence of both Gβγ and PIP2 dramatically increases the amount of membrane-associated βARK and the apparent affinity of βARK for PIP2. The apparent EC_{50} for PIP2 decreases from, respectively, >20% to approximately 5% PIP2 in the absence and presence of 200 nM Gβγ (Fig. 1B). Binding of Gβγ appears to increase the affinity of the PH domain of the βARK for PIP2. From these experiments, it cannot be clearly distinguished from the possibility that PIP2 functions to increase the affinity of βARK for Gβγ. Nonetheless, direct assessment of the Gβγ- and PIP2-mediated membrane association of the βARK provides a clear demonstration of the coordinated binding of these two ligands to the βARK PH domain. Previous studies using wild type or mutated PH domain fusion proteins demonstrated that the PH domain of the βARK is the sole region of the enzyme that interacts with both Gβγ and PIP2 [28,36], excluding the possibility that the cooperativity is due to binding of the ligands to regions other than the PH domain.

Interestingly, comparison of the ligand binding properties of the βARK PH domain in the intact enzyme (Fig. 1B) with the ligand binding properties of the βARK PH domain in a GST fusion protein (Fig. 1A) reveals striking differences. The PH domain of the βARK when expressed as a GST fusion protein (GST-B1CT) binds both Gβγ and PIP2 independently and without apparent cooperativity (Fig. 1A). Thus in the absence of Gβγ, 3% PIP2 is sufficient to promote complete membrane association of the GST-B1CT (Fig. 1A). Similarly, membrane association of the GST-B1CT was observed in the absence of PIP2 upon addition of Gβγ (Fig. 1A). The amount of membrane associated fusion protein increases from 30 ± 10% to 55 ± 7% on addition of 200 nM Gβγ (Fig. 1A). These results are consistent with previous studies demonstrating either PIP2 [16,36] or Gβγ [8,36] binding to PH domain-containing fusion proteins. The binding of GST-B1CT to the vesicles in the absence of PIP2 and Gβγ (30 ± 10%) turned out to be a non-specific binding that could be removed by gel filtration column [40]. The ability of the GST-B1CT to bind either Gβγ or PIP2 clearly distinguishes this protein from the intact βARK. The markedly different binding characteristics of the βARK PH domain in either the intact enzyme or the GST fusion protein highlight the difficulties of inferring PH domain function in the absence of the appropriate protein context, although the possibility that it is due to the difference in the way of preparation (i.e. Sf9 cells or *E. coli* cells) cannot be excluded. These results reveal an important, and potentially general, caveat: that the behavior of an isolated PH domain does not necessarily reflect the function of that same PH domain in the context of an intact protein. The dose-dependent membrane association of βARK by PIP2 and Gβγ, measured here using direct binding studies, suggests that the synergistic enhancement of receptor phosphorylation reported by Pitcher et al. [28] and DebBurman et al. [29] can indeed be accounted for by the Gβγ/PIP2-mediated membrane association of the βARK.

To investigate a potential role for the receptor substrate in modulating βARK distribution, binding assays were performed using lipid vesicles containing purified βAR and various concentrations of PIP2 (Fig. 2). Under these conditions, the Gβγ- and PIP2-dependent membrane association of the βARK is somewhat enhanced but not significantly as compared with that obtained in the absence of receptor (compare Fig. 1BFig. 2A). Thus the presence of the receptor substrate in either the agonist-occupied (data not shown) or -unoccupied (Fig. 2) conformations has itself no significant effect on βARK distribution except at 1% PIP2. These results contradict previous studies in which heat inactivation of receptor impaired translocation of the βARK/Gβγ complex, suggesting that the presence of receptor enhances βARK translocation in the presence of Gβγ [27]. The reconstituted vesicles in this study, however, consist of more defined lipid composition, not like the crude phospholipids or rod outer segment utilized before. Therefore, it may not be surprising to see less effect of receptor on βARK translocation under these conditions.

Interestingly, although significant membrane association of the βARK is observed at high PIP2 concentrations in the absence of Gβγ (Fig. 1BFig. 2), no agonist-dependent receptor

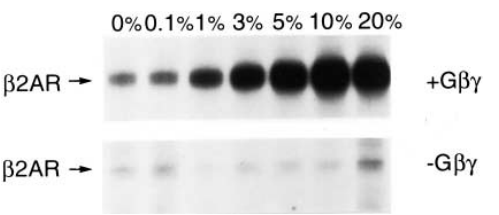

Fig. 3. PIP2-dependent βAR phosphorylation by βARK in the presence or absence of Gβγ. Autoradiograph showing Gβγ-dependent βARK-mediated βAR phosphorylation at various concentrations of PIP2. Phosphorylations were performed as described in Section 2. A representative autoradiogram is shown. Similar results were obtained in four separate experiments.

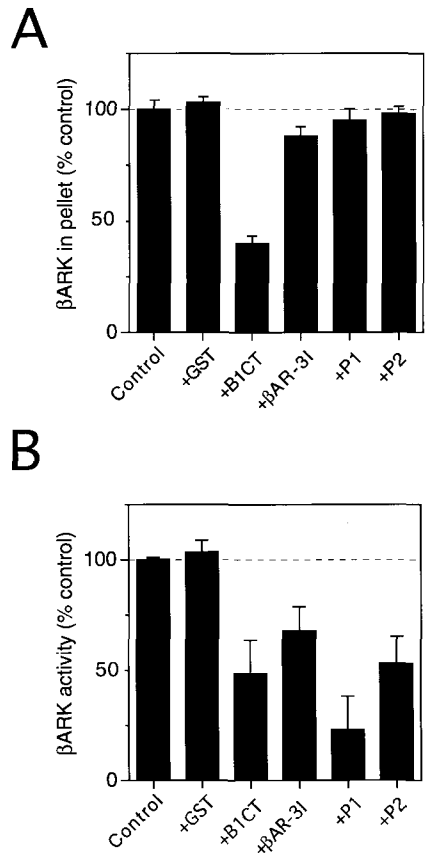

Fig. 4. Effect of a βAR third loop fusion protein and peptides on Gβγ/PIP2-mediated βARK membrane association and receptor phosphorylation. A: Inhibition of Gβγ/PIP2-mediated βARK translocation to vesicles composed of 95% PC and 5% PIP2 by fusion proteins (5 μM) and synthetic peptides (1 mM). Experiments were performed as described in Section 2 and results are expressed as percent βARK associated with the vesicle pellets under the conditions indicated. The amount of βARK present in the pellet fraction in the presence of 5% PIP2 and 200 nM Gβγ and in the absence of added fusion protein or peptide is taken as 100%. The results represent the means ± S.E.M. of three separate determinations. B: Inhibition of Gβγ/PIP2-dependent βARK-mediated βAR phosphorylation by fusion proteins (5 μM) and synthetic peptides (1 mM). The results are expressed as percent βARK activity where the incorporation of ^{32}P into the βAR in the absence of peptide additions is taken as 100%. The percent βARK activity is shown as means ± S.E.M. of three separate determinations. GST, glutathione *S*-transferase; βAR-3I, βAR fusion protein encoding residues 219–276 of the βAR (the third intracellular loop); P1, peptide encoding residues 219–247 of the βAR; P2, peptide encoding residues 248–273 of the βAR.

phosphorylation is observed under these conditions (Fig. 3). Thus, conditions which result in the membrane association of equivalent amounts of the βARK (i.e. 5% PIP2/200 nM Gβγ or ~15% PIP2 alone) lead to distinct functional consequences, receptor phosphorylation being observed only in the presence of Gβγ. Regardless of the presence of Gβγ and PIP2, the phosphorylation is always agonist-dependent. These results suggest that PH domain-mediated localization of the βARK to the membrane is insufficient to account for the dramatic enhancement of βARK-mediated βAR phosphorylation observed in the presence of Gβγ and PIP2. βARK-catalyzed phosphorylation of a soluble substrate (casein) was unaffected when assayed under these conditions in either the presence or absence of Gβγ (data not shown) [28]. Thus, no inhibition was observed at the concentrations of PIP2 used in this study, demonstrating that these effects on receptor phosphorylation are not the results of an inhibitory effect of PIP2 on βARK activity. The binding of Gβγ to the βARK appears to play an important role in specifically targeting the enzyme complex to its receptor substrate. Requirement of Gβγ for receptor phosphorylation is consistent with the paper by Pitcher et al. [28]. The apparent difference from previous studies that observed phosphorylation in the absence of Gβγ has been shown to be due to the crude phospholipid vesicles utilized in those studies [28].

In an attempt to distinguish PH domain-mediated membrane localization of the βARK from PH domain-mediated receptor targeting, the effects of various fusion proteins or peptides on βARK membrane association (Fig. 4A) or βARK-mediated βAR phosphorylation (Fig. 4B) were investigated. Peptides derived from various intracellular loops of the βAR have previously been demonstrated to inhibit βARK-mediated βAR phosphorylation in vitro [41]. These peptides, which are not βARK substrates, block formation of the βARK/receptor substrate complex. A GST fusion protein encompassing the third loop of the βAR (βAR-3I), together with two synthetic peptides derived from the βAR third loop (P1 and P2), were tested for their ability to block Gβγ- and PIP2-mediated membrane association. The βAR-3I fusion protein (5 μM), peptide P1 (1 mM) and peptide P2 (1 mM) inhibited βARK-mediated βAR phosphorylation as previously reported [28,41] (Fig. 4B) while having no significant effect on the Gβγ- and PIP2-dependent membrane association of this kinase (Fig. 4A). Neither the βAR-3I, P1 or P2 inhibited βARK-mediated phosphorylation of casein (data not shown), indicating that these peptides specifically inhibit interaction of the βARK with its receptor substrate. The ability to selectively inhibit receptor targeting of the βARK without affecting the membrane localization of this kinase demonstrates the separate contributions of these two processes to the PH domain-mediated enhancement of βARK activity. Also demonstrated is that βARK activity does not necessarily correlate with the amount of βARK associated with the membrane. In contrast to the βAR peptides, the GST-B1CT (PH domain fusion protein) inhibits both membrane association of the βARK and βARK-mediated receptor phosphorylation, demonstrating that PH domain is crucial for both membrane association and receptor phosphorylation (Fig. 4). That this inhibition was dependent on the presence of the βARK PH domain, and thus reflects the ability of this protein to competitively inhibit the binding of Gβγ and PIP2 to the βARK, is demonstrated by the inability of GST alone to inhibit either of these processes. Therefore, although PIP2 binding to the PH domain is sufficient for βARK to be translocated to the membrane, Gβγ/PIP2/βARK complex formation is necessary to recognize the receptor substrate for phosphorylation.

Two recent papers studying the effect of PIP2 addition on the βARK concluded that this lipid directly inhibits βARK activity [42,43]. These results apparently contradict those presented in this and a previous paper [28], in which PIP2 is shown to be required for Gβγ-dependent βARK-mediated receptor phosphorylation. This apparent contradiction turned out to arise as a consequence of the differences in PIP2 concentration and of the experimental differences in vesicle preparations [44]. In contrast to the receptor-containing vesicles composed of a mixture of PC and PIP2 [28,44], when PIP2 vesicles were exogenously added into the receptor-reconsti-

tuted vesicles, it appeared to sequester the PH domain from the receptor environment resulting in inhibition of the phosphorylation [42,43]. Most recently, DebBurman et al. [29] demonstrated that at low concentrations PIP2 activated βARK via an interaction with the βARK PH domain, while at high concentrations PIP2 inhibited βARK activity apparently via another mechanism.

The concentrations of PIP2 required for efficient membrane association of the βARK in this in vitro assay is still somewhat higher than those found physiologically (i.e. range between 0.25% and 1.0% of the total plasma membrane [45]), however the simplified lipid environment was required to examine how the two PH domain ligands interact with the βARK PH domain. Interestingly, the results obtained using small unilamellar vesicles, which were prepared by simple sonication, appeared to be quite different from those obtained in this study in which larger unilamellar vesicles were utilized. Thus, the translocation of βARK to the sonicated vesicles was observed with EC_{50} 0.6% ± 0.1 of PIP2 in the absence of Gβγ and 0.07% ± 0.02 of PIP2 in the presence of an equimolar ratio of Gβγ:βARK (data not shown). The binding appears to be cooperative in a fashion similar to the results obtained in Fig. 1B. Previously, the binding of GST-B1CT to PIP2 was observed at 1% PIP2 vesicles [16], consistent with these results. The EC_{50} values for the reconstituted vesicles in this study are higher, but these vesicles reflect a more physiological environment. Nonetheless, in the future, the in vivo significance of PIP2 binding to the βARK PH domain must be examined by determining the in vivo function of modified βARK in which PIP2-binding sites are mutated, since the model membrane utilized here represents an oversimplification of the in vivo situation in that it lacks other lipids such as phosphatidylserine that have previously been shown to enhance βARK activity [29]. Thus it remains to be determined whether a more complex lipid environment influences the affinity of the βARK PH domain for PIP2.

The data presented in this paper serve to elucidate the ligand-binding characteristics of the PH domain of βARK. Binding of Gβγ and PIP2 to the PH domain of this protein occurs cooperatively, with Gβγ binding increasing the affinity for PIP2. Furthermore, the PH domain-mediated binding of Gβγ and PIP2 serves two coordinated but distinct functions: localization of the βARK to the membrane and additionally targeting of this complex to its receptor substrate. PH domain-mediated membrane association alone appears insufficient to account for the dramatically enhanced βARK-mediated βAR phosphorylation observed upon addition of Gβγ and PIP2. It remains to be elucidated whether a general feature of PH domains is the binding of multiple ligands. If this is indeed the case then the βARK PH domain may provide a general paradigm in which one, or multiple, ligands dictate the membrane localization of the PH domain-containing protein while a specific ligand localizes these proteins to their appropriate cellular targets.

Comparison of the ligand-binding characteristics of the PH domain of the βARK in two different protein environments, the intact kinase and a GST fusion protein, reveal important and significant differences. The independent binding of Gβγ and PIP2 to the βARK fusion protein contrasts sharply with the cooperative binding of these PH domain ligands to the intact enzyme. That PH domain ligand-binding characteristics depend on protein context represents an important and potentially general caveat to be considered when investigating the functional characteristics of isolated PH domains.

The following novel conclusions emerge from this study. First, the binding of Gβγ and PIP2 to the PH domain of βARK is cooperative, Gβγ increasing the affinity of the PH domain for PIP2, accounting for the enhanced receptor phosphorylation by these ligands. Second, the markedly different binding characteristics of the PH domain in the intact enzyme or the isolated PH domain highlight the potential difficulties of inferring PH domain function in the absence of the appropriate protein context. Third, the PH domain-mediated membrane localization of the βARK by PIP2 alone is insufficient to account for the enhanced phosphorylation of the receptor substrate. Finally, the PH domain-mediated binding of PIP2 and Gβγ serves two coordinated but distinct functions: localization of the βARK to the membrane and targeting of this complex to its receptor substrate. The in vivo significance of PIP2 or Gβγ binding to the βARK PH domain remains to be elucidated.

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