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Identification and analysis of PH domain-containing targets of phosphatidylinositol 3-kinase using a novel *in vivo* assay in yeast

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Phosphatidylinositol 3-kinase (PI3K) mediates a variety of cellular responses by generating PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. These 3-phosphoinositides then function directly as second messengers to activate downstream signaling molecules by binding pleckstrin homology (PH) domains in these signaling molecules. We have established a novel assay in the yeast *Saccharomyces cerevisiae* to identify proteins that bind PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 *in vivo* which we have called TOPIS (Targets of PI3K Identification System). The assay uses a plasma membrane-targeted Ras to complement a temperature-sensitive *CDC25* Ras exchange factor in yeast. Coexpression of PI3K and a fusion protein of activated Ras joined to a PH domain known to bind PtdIns(3,4)P_2 (AKT) or PtdIns(3,4,5)P_3 (BTK) rescues yeast growth at the non-permissive temperature of 37°C. Using this assay, we have identified several amino acids in the β1–β2 region of PH domains that are critical for high affinity binding to PtdIns(3,4)P_2 and/or PtdIns(3,4,5)P_3, and we have proposed a structural model for how these PH domains might bind PI3K products with high affinity. From these data, we derived a consensus sequence which predicts high-affinity binding to PtdIns(3,4)P_2 and/or PtdIns(3,4,5)P_3, and we have identified several new PH domain-containing proteins that bind PI3K products, including Gab1, Dos, myosinX, and Sbf1. Use of this assay to screen for novel cDNAs which rescue yeast at the non-permissive temperature should provide a powerful approach for uncovering additional targets of PI3K.

Keywords: CDC25/phosphatidylinositol 3-kinase/phosphoinositide/pleckstrin homology domain/signal transduction

Introduction

A variety of extracellular stimuli and oncogenic viruses mediate many of their cellular effects via activation of a family of lipid kinases known as phosphatidylinositol 3-kinases (PI3K). PI3Ks function to generate intracellular second messengers by phosphorylating the D-3 position of the inositol ring of phosphoinositides (Toker and Cantley, 1997; Vanhaesebroeck *et al*., 1997; Downward, 1998). The cellular responses regulated by PI3K are diverse and include cell growth, inhibition of apoptosis, actin cytoskeletal reorganization, exocytosis and secretion, chemotaxis, and insulin-stimulated translocation of the GLUT4 glucose transporter (Carpenter and Cantley, 1996; Toker and Cantley, 1997; Downward, 1998). However, although PI3K has been known to mediate many of these responses for some time, only recently have some of the downstream targets of PI3K been identified. A major breakthrough which helped identify PI3K targets was the discovery that 3-phosphorylated lipid products generated by PI3K function as second messengers by directly binding Src homology 2 (SH2) and pleckstrin homology (PH) domains in downstream targets (Rameh *et al*., 1995; Lemmon *et al*., 1996; Franke *et al*., 1997b; Frech *et al*., 1997).

PI3Ks can now be divided into three broad classes based on structural characteristics, mode of regulation and substrate preference (Vanhaesebroeck *et al*., 1997). The first class of PI3K molecules was identified based on their activation by both receptor signaling systems and a number of oncogenic viruses, and they can now be divided into two subclasses (A and B) based on the adaptor molecules with which they associate. Class 1A PI3Ks, including p110α, β and δ, were the first to be cloned and are composed of a tightly associated heterodimer of 85 and 110 kDa proteins (Escobedo *et al*., 1991; Otsu *et al*., 1991; Hiles *et al*., 1992; Hu *et al*., 1993). The 85 kDa subunit contains two SH2 domains and functions as a regulatory subunit, coupling the 110 kDa catalytic subunit to phosphotyrosine, containing proteins including tyrosine phosphorylated receptors or intermediate proteins, such as the insulin receptor substrates (IRS) and Gab1 (Carpenter and Cantley, 1996; Vanhaesebroeck *et al*., 1997). In contrast, class 1B PI3Ks, including p110γ, do not heterodimerize with p85 but rather with a recently cloned 101 kDa adaptor molecule that mediates the regulation of p110γ by βγ subunits of heterotrimeric G proteins (Stoyanov *et al*., 1995; Stephens *et al*., 1997). All class 1 PI3Ks exhibit a broad substrate specificity and are able to phosphorylate phosphatidylinositol (PtdIns), PtdIns(4)P and PtdIns(4,5)P_2 *in vitro* (Carpenter and Cantley, 1996; Vanhaesebroeck *et al*., 1997). *In vivo*, however, the likely preferred substrate is PtdIns(4,5)P_2 (Carpenter and Cantley, 1996; Franke *et al*., 1997b; Vanhaesebroeck *et al*., 1997). Class 2 PI3Ks, including the C2-domain-containing PI3K (Cpk), are able to utilize PtdIns and PtdIns(4)P as a substrate but not PtdIns(4,5)P_2. Class 3 PI3Ks are characterized by the yeast VPS34 protein and its mammalian homolog, and utilize only PtdIns as a substrate,

and thus can generate only PtdIns(3)P (for review see Vanhaesebroeck *et al*., 1997).

Several studies have now established that PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 are the second messengers which activate molecules downstream of class 1 PI3K via binding to PH domains of the downstream target molecules. For several of these targets there is now good evidence that direct binding of PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 to PH domains contained within these proteins is critical to their regulation (Klippel *et al*., 1993; Burgering and Coffer, 1995; Franke *et al*., 1995, 1997b; Frech *et al*., 1997; Lemmon *et al*., 1997). PH domains are conserved modules of ~100 amino acids which were first identified as regions that share homology with an internal repeat of pleckstrin, the major substrate of protein kinase C in platelets (Mayer *et al*., 1993; Musacchio *et al*., 1993; Gibson *et al*., 1994; Harlan *et al*., 1994; Lemmon *et al*., 1996). PH domains have now been identified in >100 different proteins, many of which are involved in regulating intracellular signaling pathways or the cytoskeleton (Musacchio *et al*., 1993; Gibson *et al*., 1994; Lemmon *et al*., 1996; Shaw, 1996). A general consensus has emerged that PH domains function to mediate intermolecular interactions and have evolved primarily to regulate protein–lipid interactions, although in some instances PH domains may also mediate protein–protein interactions (Pitcher *et al*., 1995; Lemmon *et al*., 1996; Shaw, 1996). In addition to binding PI3K products, PH domains have also been shown to bind PtdIns(4,5)P_2, although, with the exception of PLCδ, the physiological significance of these interactions are not yet known (Ferguson *et al*., 1995; Lemmon *et al*., 1995, 1997; Chen *et al*., 1997).

While PH domains share only limited amino acid sequence identity and have different ligand binding specificities, their core three-dimensional (3D) structures are remarkably similar (Lemmon *et al*., 1996). PH domains share a common architecture consisting of seven β strands, which form two antiparallel β sheets, with a C-terminal α helix. While the core structure of different PH domains is similar, the loop regions between the β strands are of variable length and recent evidence has indicated that the loops are important in mediating the interaction of several PH domains with Ins(1,4,5)P_3. While the physiological ligand for most PH domains and their role in regulating intracellular signaling molecules are not known, several important examples have been studied. PLCδ is a good example of a case in which binding of a PH domain to PtdIns(4,5)P_2 plays an important role in regulating function, presumably by targeting PLCδ to the plasma membrane where it can interact with its substrate, PtdIns(4,5)P_2 (Lemmon *et al*., 1997). AKT [also known as protein kinase B (PKB)] and BTK are examples of two important PH domain-containing proteins that are regulated by binding PI3K products (Franke *et al*., 1997b; Frech *et al*., 1997; Klippel *et al*., 1997). AKT is a serine/threonine kinase which is activated by PI3K and has recently been shown to mediate the anti-apoptotic response by PI3K (Dudek *et al*., 1997; Franke *et al*., 1997a). BTK is a protein tyrosine kinase which is mutated in X-linked agammaglobulinemia (XLA) (Mattsson *et al*., 1996; Vihinen *et al*., 1996). Mutations in BTK result in a lack of circulating B cells and decreased levels of circulating immunoglobulins due to arrest of B cell development at the pre-B cell stage. Many of the mutations in BTK that lead to XLA are point mutations in the PH domain that impair binding to PtdIns(3,4,5)P_3 (Fukuda *et al*., 1996; Vihinen *et al*., 1996; Hyvonen and Saraste, 1997; Kojima *et al*., 1997). Thus, BTK provides a clear example of failure of a PH domain to bind PtdIns(3,4,5)P_3 leading to disease.

Regulation of AKT and BTK via binding of their PH domains to PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 also provide good examples for how PI3K regulates downstream targets. Binding of the PH domains of AKT and BTK to PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 functions to relocalize these proteins from the cytosol to the plasma membrane (Downward, 1998). In the case of AKT, targeting to the plasma membrane allows AKT to be phosphorylated and activated by the protein kinase 3-phosphoinositide-dependent protein kinase (PDK)1, whereas targeting of BTK to the plasma membrane facilitates the phosphorylation and activation of BTK by Src family tyrosine kinases (Rawlings *et al*., 1993; Alessi *et al*., 1997; Downward, 1998; Scharenberg *et al*., 1998; Stephens *et al*., 1998). Binding of the AKT PH domain to PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 contributes to AKT activation by at least two additional mechanisms. First, the binding of AKT's PH domain to PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 is required for AKT to be efficiently phosphorylated and activated by PDK1, perhaps by relieving an inhibitory effect of the AKT PH domain on phosphorylation (Alessi *et al*., 1997; Stephens *et al*., 1998). Secondly, the binding of AKT's PH domain to PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 has been shown to directly activate AKT's kinase activity independently of its effect on phosphorylation (Franke *et al*., 1997b; Frech *et al*., 1997). Other PI3K targets, including the ADP-ribosylation factor (ARF) exchange factors Grp1 and ARNO, and the serine threonine kinase PDK1, are likely to be regulated by similar mechanisms, suggesting that this model will apply to a wide variety of physiologically important PI3K targets (Alessi *et al*., 1997; Klarlund *et al*., 1997, 1998; Stephens *et al*., 1998; Venkateswarlu *et al*., 1998).

The diversity of cellular responses mediated by PI3K suggests that numerous additional downstream targets of PI3K exist. In addition, the detailed structural basis that confers high-affinity binding of PI3K products versus PtdIns(4,5)P_2 or Ins(1,4,5)P_3 to different PH domains remains to be defined. In order to understand the molecular mechanisms whereby PI3K mediates its pleiotropic effects, it is critical to develop new methods to identify proteins which are directly regulated by PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. We have established a novel assay in the yeast *Saccharomyces cerevisiae* which has proved to be very reliable for identifying proteins that bind PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 with high affinity. Using this assay, we have been able to identify several amino acids located in the first and second β strands and the β1–β2 loop that are critical for PH domains to bind PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 with high affinity. We have derived a consensus sequence from this data which predicts high-affinity binding of a subset of PH domains to PI3K products and have proposed a model for how these PH domains bind PI3K products with high affinity. The use of this conserved motif in sequence searches of various databases has enabled us to identify several new

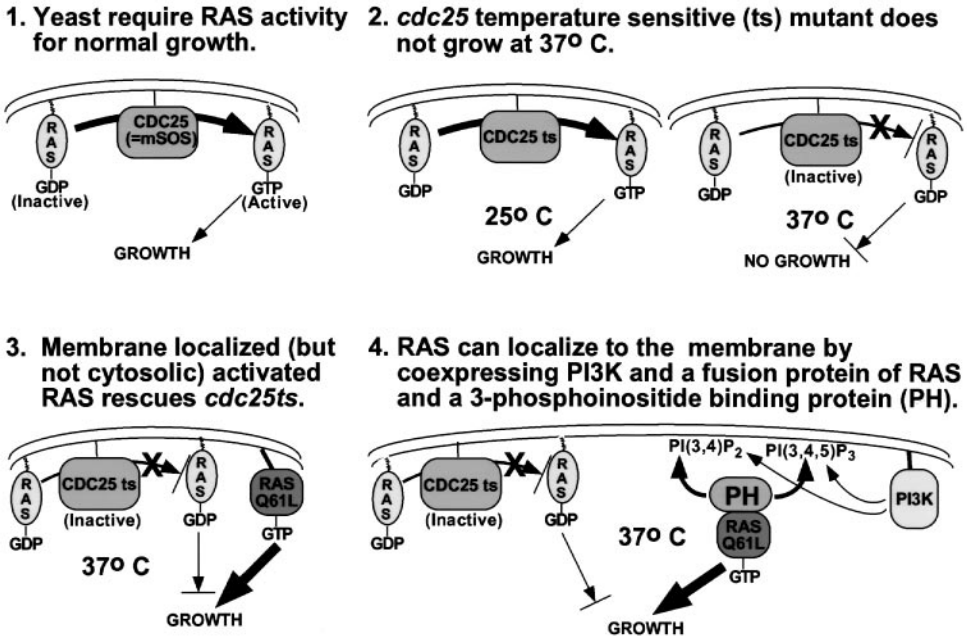


Fig. 1. Schematic diagram of the Target of PI3K Identification System (TOPIS) in yeast to identify 3-phosphoinositide-binding proteins.

PH domains that bind PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 with high affinity and are candidate downstream targets of PI3K.

Results

Identification of PtdIns(3,4)P_2- and PtdIns(3,4,5)P_3-binding proteins in vivo using a novel assay in the yeast S.cerevisiae

PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 function directly as second messengers to activate downstream signaling molecules by binding PH domains in these signaling molecules (Toker and Cantley, 1997; Downward, 1998). However, strategies to identify 3-phosphoinositide-binding proteins have met with only limited success because of the difficulty in studying protein–lipid interactions. We have established a novel assay in the yeast *S.cerevisiae* to identify proteins that bind PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 *in vivo* (Figure 1). The assay we have established takes advantage of a yeast strain which is temperature sensitive for the yeast Ras exchange factor CDC25 (Aronheim *et al*., 1997). At the permissive temperature of 25°C, the CDC25 temperature-sensitive mutant, cdc25ts, is functional and the yeast grow well, but at 37°C, cdc25ts is non-functional and the yeast fail to grow. Expression of membrane-targeted forms of the mammalian Ras exchange factor Son of Sevenless (Sos) or activated Ras complements the cdc25ts allele and restores the viability of these yeast at 37°C (Aronheim *et al*., 1994; A.Aronheim, in preparation). Membrane targeting of these proteins is critical for rescue, and we reasoned that activated Ras or mammalian Sos could be targeted to the yeast plasma membrane in a PI3K-dependent manner by coexpressing an activated form of the p110β catalytic subunit of PI3K together with a fusion protein of activated Ras with a protein which binds PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. A similar system has been used previously to detect protein–protein interactions (Aronheim, 1997; Aronheim *et al*., 1997; A.Aronheim, in preparation). While *S.cerevisiae* contain PtdIns(3)P, they do not have PtdIns(3,4)P_2 nor PtdIns(3,4,5)P_3 (De Camilli *et al*., 1996; Vanhaesebroeck *et al*., 1997). Previous studies have shown that ectopic expression of mammalian p110 in yeast leads to increased levels of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (Kodaki *et al*., 1994a,b).

To test the feasibility of using this assay to identify 3-phosphoinositide binding proteins, we determined whether coexpressing activated p110 with a fusion protein of activated Ras with the PH domains of either AKT or BTK rescued the growth of yeast at 37°C. The PH domain of AKT has previously been shown to bind both PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 with high affinity and the PH domain of BTK has been shown to bind PtdIns(3,4,5)P_3 with high affinity (Fukuda *et al*., 1996; James *et al*., 1996; Salim *et al*., 1996; Franke *et al*., 1997b; Frech *et al*., 1997; Klippel *et al*., 1997; Stephens *et al*., 1998). PI3K can be constitutively activated by adding a farnesylation sequence to localize p110 to the plasma membrane (Logan *et al*., 1997). We predicted that in the presence of activated p110, binding of the PH domain of AKT or BTK to PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 would bring activated Ras to the plasma membrane and that this would allow cdc25ts yeast to grow at 37°C (Figure 1). We found that coexpression of farnesylated p110 and an activated Ras-fusion protein with the AKT or BTK PH domain could complement the cdc25ts mutation, enabling the yeast to grow at the non-permissive temperature (Figure 2A). The ability of these yeast to grow is dependent on PI3K activity because coexpression of a kinase-inactive farnesylated p110 with the same Ras-fusion proteins did not rescue growth at the non-permissive temperature (Figure 2A). In addition, mutation of the conserved arginine in the PH domain of AKT at position 25 to cysteine [AKT(R25C)], which has previously been shown to abolish binding of the AKT PH

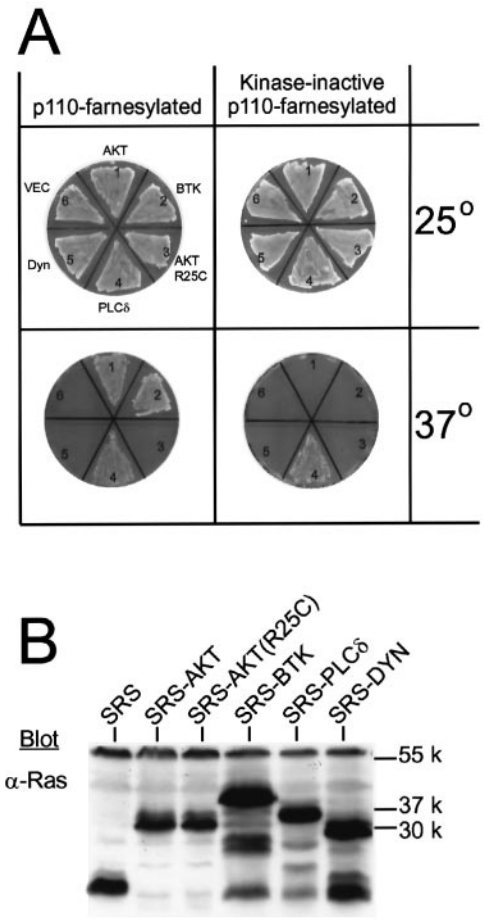


Fig. 2. Complementation of *cdc25ts* yeast at the non-permissive temperature by Ras-fusion proteins containing the PH domains of AKT, BTK and PLCδ. (**A**) *cdc25ts S.cerevisiae* were transformed with the plasmid 3S0B-SRS in which Ras was fused to one of several PH domains as indicated, together with either PYES2-p110-farnesylated or PYES2-kinase-inactive-p110-farnesylated. Transformants were plated on selective media lacking uracil and leucine. Transformation efficiency was assessed by selecting for growth at 25°C for 4 days (upper two plates). Rescue of growth at the non-permissive temperature was assessed by incubating transformants at 25°C for 48 h, and then switching the yeast to 37°C for an additional 48 h (lower two plates). VEC, control vector. (**B**) Western blot of yeast lysates. Yeast were grown to log phase and lysed as described in the Materials and methods. Fifty micrograms of yeast lysates were then separated by SDS–PAGE (12.5%), transferred to nitrocellulose and immunoblotted with anti-Ras antibodies.

domain to PtdIns(3,4)P_2 (Franke *et al*., 1997b), blocked rescue at the non-permissive temperature.

The PH domain of PLCδ has been shown to bind PtdIns(4,5)P_2 with high affinity (Lemmon *et al*., 1995). In contrast to PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3, PtdIns(4,5)P_2 is constitutively present in the plasma membrane of yeast and mammalian cells (Carpenter and Cantley, 1990; Stephens *et al*., 1993; Kodaki *et al*., 1994a). Therefore, as an additional control to characterize the assay, we tested whether a fusion protein of activated Ras with the PH domain PLCδ rescued the cdc25ts yeast at the non-permissive temperature in a PI3K-independent manner. A fusion protein consisting of activated Ras and the PH domain of PLCδ rescued the growth of yeast at the non-permissive temperature when coexpressed with either a kinase-active or kinase-defective p110 (Figure 2A), indicating that PLCδ targets Ras to the plasma membrane in the absence of PI3K products. The ability of the PH domains of AKT, BTK and PLCδ to rescue growth at 37°C was specific; transformation of activated Ras alone or of activated Ras fused to a variety of other PH domains did not restore growth of yeast at the non-permissive temperature (Figures 2A, and 5A and B). In addition, we did not detect differences in expression levels of Ras-fusion proteins that would account for the ability to rescue (Figures 2B and 5C). These findings demonstrate that we have developed a simple, easily scorable assay in yeast to identify proteins that bind 3-phosphoinositide products of PI3K. The finding that the PH domain of PLCδ rescues growth of yeast in the absence of PI3K activity suggests that this assay may also identify PH domains that bind PtdIns(4,5)P_2 with high affinity *in vivo*.

A consensus protein sequence identifies a subset of PH domains that bind PI3K products with high affinity

While there is evidence that PH domains mediate protein–protein interaction under some circumstances (Pitcher *et al*., 1995; Yang and Desiderio, 1997), the majority of evidence indicates that PH domains function primarily to regulate interaction with lipid products (Harlan *et al*., 1994; Lemmon *et al*., 1997). Having developed a reliable assay to identify PH domains which bind PI3K products with high affinity, we set out to identify a consensus sequence motif in PH domains that conferred binding to PI3K products. Our hope was that identification of such a consensus sequence would allow us to identify additional PH domains that bind PI3K products with high affinity and thereby identify new candidate downstream targets for PI3K signaling.

To arrive at a consensus protein sequence that conferred binding to PI3K products, we initially compared an alignment of PH domains known to bind PI3K products with high affinity with an alignment of several PH domains that do not (Figure 3). The PH domains included in our initial alignment which had been shown to bind PI3K products included AKT, BTK, ITK, centaurin α and general receptor for phosphoinositides (Grp)1 (Hammonds-Odie *et al*., 1996; James *et al*., 1996; Salim *et al*., 1996; August *et al*., 1997; Klarlund *et al*., 1997; Klippel *et al*., 1997). These PH domains were aligned against the PH domains of PLCδ, dynamin, spectrin and N-pleckstrin. To simplify the alignment, we focused on the first and second β sheets and the loop region between them for two reasons. First, previous experiments had shown that this region is important in mediating binding of the PH domain of PLCδ to Ins(1,4,5)P_3 (Ferguson *et al*., 1995). Secondly, the structure of the PH domain of BTK indicated that its Ins(1,3,4,5)P_4 binding site is similar to the Ins(1,4,5)P_3 binding site on PLCδ (Ferguson *et al*., 1995; Hyvonen and Saraste, 1997). In addition, most of the functional mutations identified in the PH domains of BTK and AKT that impaired binding to PI3K products are localized to this region (Salim *et al*., 1996; Vihinen *et al*., 1996; Hyvonen and Saraste, 1997).

Following alignment of these PH domains it became apparent that several amino acid residues were absolutely conserved in those PH domains which have been shown

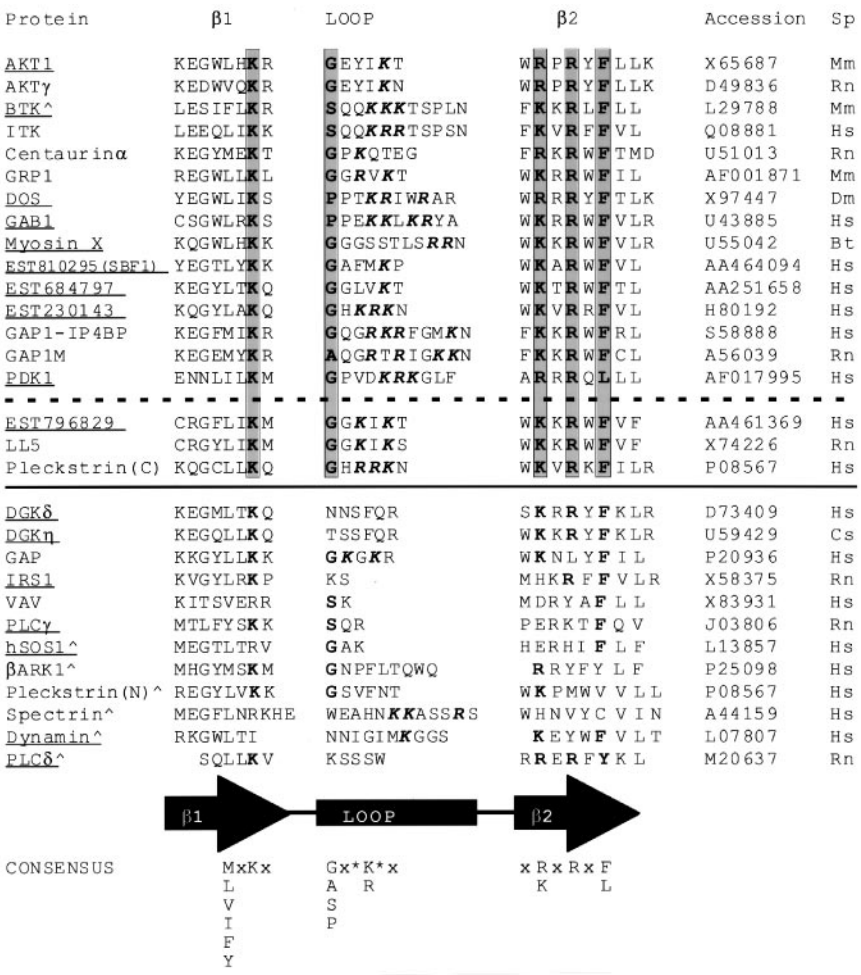


Fig. 3. Alignment of β1 strand, β1–β2 loop and β2 strand of PH domains identifies residues conserved in PH domains that bind PI3K products with high affinity. Alignment of protein sequences was performed as described in Materials and methods. PH domains which have been shown by us and others to bind PI3K products with high affinity are shown above the dashed line. The PH domains between the solid and dashed lines were also identified in searches using the consensus sequence shown at the bottom. We predict that these PH domains will also bind PI3K products with high affinity, but this has not yet been tested or was inconclusive. The PH domains below the solid line are not thought to bind PI3K products with high affinity, based on published results as well as results presented in this manuscript. PH domains that have known structures are indicated by (^). The DDBJ/EMBL/GenBank accession No. and species (Sp) of each sequence is shown on the right. PH domains which are underlined were analyzed in this study. Conserved residues in PH domains that bind to PI3K products with high affinity are shown in bold and shaded. The charged K or R in the loop is in bold italics. AKTγ is included because a mouse cDNA corresponding to the rat AKTγ was identified in initial attempts to screen cDNA libraries to find new PI3K targets. A consensus sequence derived from the conserved residues is shown on the bottom. x, any single residue; *, variable number of residues.

to bind PI3K products, and only partially conserved, if at all, in PH domains that do not bind PI3K products (Figure 3). First, all PH domains that bind PI3K products have a β1–β2 loop of at least 6 amino acids in length and contain at least one positively charged lysine or arginine residue in the β1–β2 loop lying between, but not including, the second and last positions in the loop. Secondly, in the first position of the β1–β2 loop, PH domains that bind PI3K products have an amino acid with a small side-chain, either a glycine, alanine, serine or proline residue (GASP). Thirdly, the β2 strand contains two conserved positively charged amino acids: an arginine or lysine at the +2 position and an arginine at the +4 position from the beginning of this strand. The arginine at the +4 position has previously been shown to be essential for the PH domains of both AKT and BTK to bind PI3K products (Salim *et al*., 1996; Franke *et al*., 1997b). In the crystal structure of Ins(1,4,5)P_3 bound to PLCδ, the arginine at the +4 position makes two hydrogen bonds to the 5-phosphate of Ins(1,4,5)P_3 (Ferguson *et al*., 1995). The importance of the basic residue at the +2 position was suggested in the crystal structure of the BTK PH domain (Hyvonen and Saraste, 1997). From this structure, a lysine at the +2 position (K26 in BTK) is predicted to contribute to the Ins(1,3,4,5)P_4 binding site by contributing to the electrostatic environment near the 1-phosphate. Fourthly, the second-to-last residue in the β1 strand is a highly conserved lysine, which hydrogen bonds to the 4- and 5-phosphates of Ins(1,4,5)P_3 in the PLCδ-PH/Ins(1,4,5)P_3 structure. The highly conserved hydrophobic residue –2 from this lysine in the β1 strand and the phenylalanine in the +6 position of the β2 strand are not predicted to contribute to ligand binding, but rather are likely to be important structural determinants for PH domain folding. Their strong conservation aided in choosing the best possible alignment of the various PH domains.

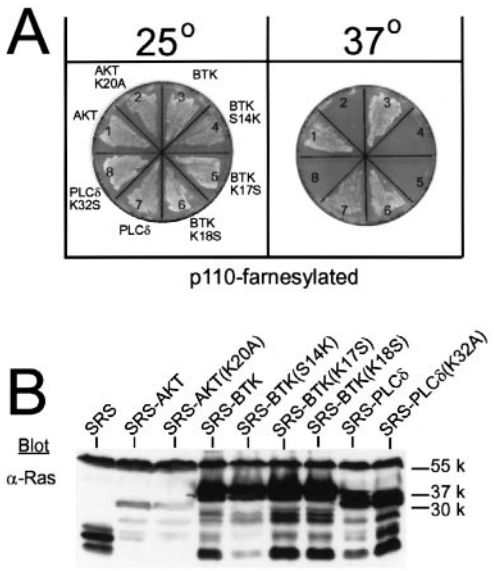


Fig. 4. Mutational analysis of PH domains. Based on the findings in Figure 3, several residues in the PH domains of AKT and BTK which are conserved in PH domains that bind PI3K products with high affinity were mutated. (**A**) The ability of these mutant PH domains to rescue yeast was assessed as described in Figure 2. (**B**) Western blot of yeast lysates as described in Figure 2.

Residues conserved in the β1 and β2 strand and β1–β2 loop are important for binding PI3K products

To confirm experimentally that a positively charged residue in the β1–β2 loop is important for binding of PI3K products by PH domains, we mutated the single lysine in the β1–β2 loop of AKT to alanine (K20A) and each of the first two lysines in the β1–β2 loop of BTK to serine (K17S, K18S), and then tested the ability of these mutants to rescue yeast at the non-permissive temperature (Figure 4). We found that the AKT(K20A) mutation and the BTK(K17S) mutation both completely blocked the PI3K-dependent rescue of yeast at the non-permissive temperature by these PH domains (Figure 4A). In addition, although a mutant BTK(K18S) PH domain rescued yeast at the non-permissive temperature, this rescue was markedly impaired when compared to wild-type BTK; while yeast eventually grew at the non-permissive temperature following transformation with BTK(K18S), the time to onset of visible colonies was delayed by 1–2 days. We do not believe that the lack of growth was due to a deleterious effect of the mutations on proper folding, since the changes are in the unordered loop and should not affect overall structure. Furthermore, protein expression of all mutants in yeast were similar to the wild-type Ras-fusions (Figure 4B). In the crystal structure of BTK, the third lysine in the β1–β2 loop, K19, is ordered and the side-chain extends away from the predicted binding pocket. This residue was not tested. Together, these findings demonstrate that a positive charge in the β1–β2 loop is critical for the PH domains of both AKT and BTK to bind PI3K products.

We next tested whether a small amino acid at the start of the β1–β2 loop was required for PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 binding. The PH domain of PLCδ contains a lysine at this position (K32), and this lysine forms a hydrogen bond with the 4-phosphate of Ins(1,4,5)P_3. The structure of PLCδ bound to Ins(1,4,5)P_3 suggests that the aliphatic portion of the K32 side-chain would clash with a phosphate added to the 3-position of Ins(1,4,5)P_3, providing an explanation for the specificity of PLCδ-PH for Ins(1,4,5)P_3 over Ins(1,3,4,5)P_4 (see Figure 7). To test whether a small side-chain at this position is required for binding PI3K products, we mutated the serine at position 14 in the BTK PH domain to lysine [BTK(S14K)]. In agreement with the hypothesis that a large amino acid at this position interferes with binding PtdIns(3,4,5)P_3, BTK(S14K) did not rescue yeast (Figure 4A). In contrast, the analogous lysine at position 32 in PLCδ is required for binding of the PLCδ PH domain to PtdIns(4,5)P_2; mutation of lysine at position 32 to serine in PLCδ [PLCδ(K32S)] abolished the PI3K-independent rescue of yeast at the non-permissive temperature (Figure 4A). These findings argue that a small residue at the start of β1–β2 loop is required for binding PI3K products. The PH domain of PLCδ requires a lysine at this position to bind PtdIns(4,5)P_2, presumably because lysine 32 forms a hydrogen bond to the 4-phosphate of Ins(1,4,5)P_3 (Ferguson *et al*., 1995). However, hydrogen bonding of the side-chain at position 14 of BTK with the 4-phosphate does not appear to be required for the PH domain of BTK to bind PtdIns(3,4,5)P_3. It is conceivable that hydrogen bonding of the positively charged residue in the β1–β2 loop in BTK to the 3-phosphate in PtdIns(3,4,5)P_3 compensates for the lack of interaction between the S14 in BTK and the 4-phosphate of the inositol ring of PtdIns(3,4,5)P_3 (see Figure 7). Additionally, there may be other determinants in the PH domain of 3-phosphoinositide-binders that compensate for the likely loss of 4-phosphate interaction.

Identification of new PH domains that bind PI3K products using the consensus sequence identified above

The above findings indicate that we have identified a consensus sequence which predicts high-affinity binding of a subset of PH domains to PI3K products. We decided to screen known PH domains for this motif to determine whether additional PH domains that bind PI3K products could be identified. First, we generated a PH domain database by extracting all of the PH domains that we could identify in the DDBJ/EMBL/GenBank protein database as described in Materials and methods. The database was designed to be searchable with specific patterns. Searching this database with the consensus sequence identified in Figure 3 revealed that the PH domains of Gab1, Dos and myosinX all contained this conserved motif (Herbst *et al*., 1996; Holgado-Madruga *et al*., 1996; Raabe *et al*., 1996; Weidner *et al*., 1996; Mermall *et al*., 1998). In agreement with our hypothesis that the amino acid sequence motif which we identified is a good predictor of PH domains which bind PI3K products with high affinity, we found that fusion proteins consisting of activated Ras with the PH domains of Gab1, Dos or myosinX all rescued yeast at the non-permissive temperature in a PI3K-dependent manner (Figure 5A). In addition, the specificity of the interaction was confirmed by making a point mutation in the Dos PH domain, R27C, corresponding to the R25C mutant in the AKT PH domain, and demonstrating that

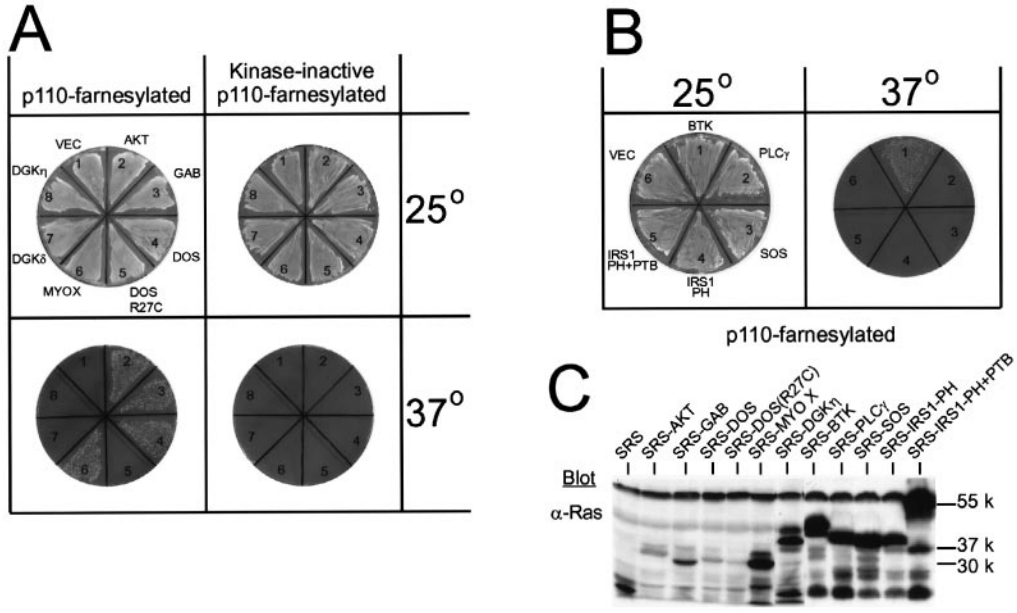


Fig. 5. Several PH domains containing the motif identified in Figure 3 rescue growth of yeast at the non-permissive temperature in a PI3K-dependent manner. Yeast transformation and rescue at the non-permissive temperature were performed as described in Figure 2. The PH domains of Gab1, Dos, and myosinX rescue growth of yeast at non-permissive temperature in a PI3K-dependent manner, whereas a number of other PH domains lacking the motif identified in Figure 3 fail to rescue (**A** and **B**). (**C**) Western blot of yeast lysates as described in Figure 2.

this Dos mutant did not rescue (Figure 5A). We were also able to identify additional novel PH domain-containing proteins that bound PI3K products by searching the Expressed Sequence Tag (EST) and DDBJ/EMBL/GenBank databases. Using this approach, four additional PH domains which contained the consensus PI3K binding motif were identified (Figure 3). Three of the four, EST810295, EST230143 and EST684797, rescued in a PI3K-dependent manner suggesting that they also bind PI3K products with high affinity (Figure 3 and data not shown). While this paper was in review, the full-length clone of EST810295 was identified as a myotubularin family member called SET domain Binding Factor 1 (Sbf1) (Cui *et al*., 1998). The fourth PH domain, EST796829, rescued yeast in a PI3K-independent manner and we cannot conclude whether it indeed binds 3-phosphoinositides. Thus, using our consensus, we have identified six additional PH domains which bind PI3K products with high affinity, and we predict that an additional three (LL5, c-Pleckstrin and EST796829) will also bind. The ability of these PH domains to bind PI3K products has not been previously appreciated.

From these results, we would also predict that several other PH domains bind PI3K products with high affinity. These PH domains include PDK1, GAP1M and GAP1-IP4BP (see Figure 3). Interestingly, PDK1, GAP1M and GAP1-IP4BP have all now been shown to bind PI3K products or Ins(1,3,4,5)P_4 with high affinity, providing further validation that our motif identifies PH domains which bind PI3K products (Fukuda and Mikoshiba, 1997; Lockyer *et al*., 1997; Stephens *et al*., 1998).

As a further means to validate the sequence motif described above, we also tested several additional PH domains that contained some, but not all, of the conserved residues in our consensus (Figure 5A and B). When tested for 3-phosphoinositide binding using our system, none of these PH domains were found to rescue yeast at the non-permissive temperature either in the presence or absence of p110 (Figure 5A and B). The failure of these Ras-fusion proteins to rescue yeast at the non-permissive temperature was not due to different levels of protein expression in yeast (Figure 5C). While these findings do not rule out the possibility that PH domains such as Sos and PLCγ are able to bind and be regulated by PtdIns(4,5)P_2 or PtdIns(3,4,5)P_3 *in vivo* (see Discussion), they suggest that they bind these lipid products with a significantly lower affinity than, and in a manner that may be distinct from, PLCδ or BTK.

PH domains that rescue yeast are also localized to the plasma membrane in mammalian cells by an activated PI3K

To verify that our findings in yeast can be extrapolated to mammalian cells, we determined whether several of the new PH domains which rescued cdc25ts yeast in a PI3K-dependent manner also bound PI3K products in mammalian cells. Cos cells were transfected with either a myc epitope-tagged EST684797 or a Green Fluorescent Protein (GFP)–Gab1 PH domain fusion together with p110-farnesylated or a kinase-inactive p110-farnesylated. Membrane localization was determined using confocal microscopy. The expression of farnesylated p110 has previously been shown to increase PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 levels (Logan *et al*., 1997). In agreement with studies in yeast, both PH domains targeted to the plasma membrane as well as to intracellular membranes when co-transfected with p110-farnesylated but not kinase-inactive p110-farnesylated (Figure 6). Translocation of both PH domains to the plasma membrane was observed in >80% of the transfected cell population when these PH domains were transfected with p110-farnesylated (data not shown). The specificity of membrane targeting in response to PI3K was further demonstrated by showing that a point mutation in EST684797, corresponding to

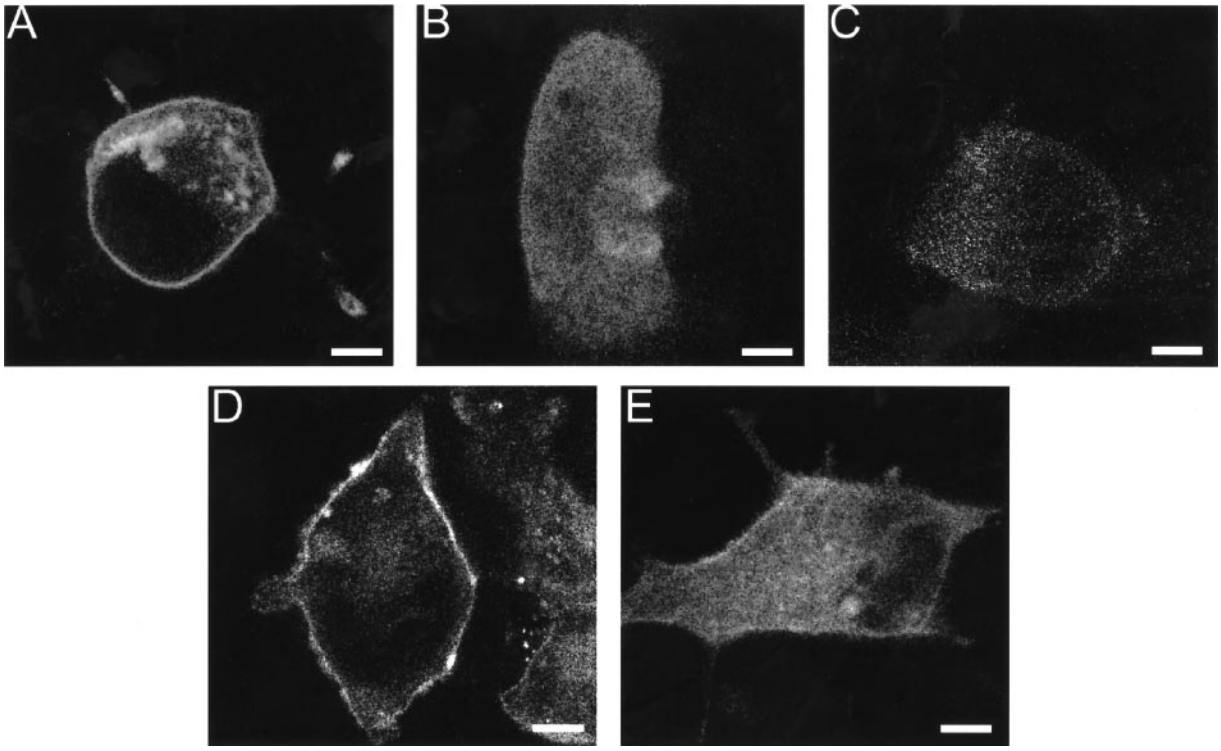


Fig. 6. Several PH domains that rescue yeast are also targeted to the plasma membrane by activated PI3K in mammalian cells. (**A**–**C**) Cos cells were transfected with myc-epitope tagged EST684797 together with p110-farnesylated (A) or a kinase-inactive p110-farnesylated (B). (C) EST684797 containing a point mutation corresponding to the R25C mutation in AKT was transfected with p110-farnesylated. Cells were serum starved for 24 h, fixed, and EST684797 was detected using the anti-myc antibody 9E10 and a Texas Red conjugated secondary antibody. (**D** and **E**) Cos cells were transfected with GFP–Gab1PH together with p110-farnesylated (D) or a kinase-inactive p110-farnesylated (E). Cells were serum starved and fixed as in (A). All slides were then analyzed by confocal microscopy using a Molecular Dynamics laser scanning system. Scale bars, 5 microns. Western blot analysis of transfected cell lysates demonstrated that p110-farnesylated and kinase-inactive p110-farnesylated were expressed at equal levels (data not shown).

the R25C mutation in AKT, blocked plasma membrane targeting by PI3K (Figure 6C). These findings confirm that studies in yeast identify PH domains that also bind PI3K products with high affinity in mammalian cells and indicate that these PH domains are sufficient, by themselves, to target proteins to membranes in mammalian cells by binding PI3K products.

As an additional means to confirm that the PH domain of EST684797 specifically bound PI3K products with high affinity, we measured binding affinities of the PH domain of EST684797 to inositol phosphates using isothermal titration calorimetry (Lemmon *et al*., 1995). Using this assay, we found that the PH domain of EST684797 bound the inositol headgroups of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 with a K_d of 43 and 49 nM, respectively. The K_d of the PH domain of EST684797 for other lipid headgroups including PtdIns(4,5)P_2 and PtdIns(3)P was at least 40-fold higher (J.Kavran and M.Lemmon, in preparation). The binding affinity of the PH domain of EST684797 for Ins(1,3,4,5)P_4 was similar to the binding affinity of the BTK and Grp1 PH domains to Ins(1,3,4,5)P_4. Together, these findings confirm that our sequence motif and yeast TOPIS assay can be used successfully to identify new PH domains that specifically bind PI3K products with high affinity in mammalian cells.

Discussion

Although a number of previous studies have demonstrated interactions between phosphoinositides and several different PH domains, the physiological relevance of many of these interactions is unknown. Moreover, inconsistencies exist in the reported literature regarding phospholipid binding to specific PH domains, with some studies demonstrating interaction of a specific PH domain with a particular phosphoinositide while other studies fail to find the same interaction (Lemmon *et al*., 1996). It has been previously noted that *in vitro* binding assays of proteins in aqueous solutions to lipid suspensions may markedly overestimate the true affinity of a particular lipid for a protein (Irvine and Cullen, 1996). This overestimation may arise due to the phospholipid headgroups preferring to interact with hydrophobic regions in the protein rather than dissociating into a hydrophilic solution. Thus, many interactions that may be observed *in vitro* may never occur *in vivo*. Moreover, it is becoming increasingly evident that several factors, including buffer compositions used in the binding assays, lipid composition of phospholipid vesicles, the particular binding assay used, and the protein folding and expression of specific PH domains, may all affect *in vitro* phosphoinositide-binding results (Salim *et al*., 1996; Zheng *et al*., 1996; Kubiseski *et al*., 1997; Rameh *et al*., 1997). Therefore, the establishment of an easy and reliable assay that identifies binding of PH domains to phosphoinositides *in vivo*, such as the assay described here, is clearly needed.

The target of the PI3K identification system (TOPIS) that we have established in yeast is simple to perform and to standardize between samples, since it involves transforming a yeast strain which is temperature sensitive

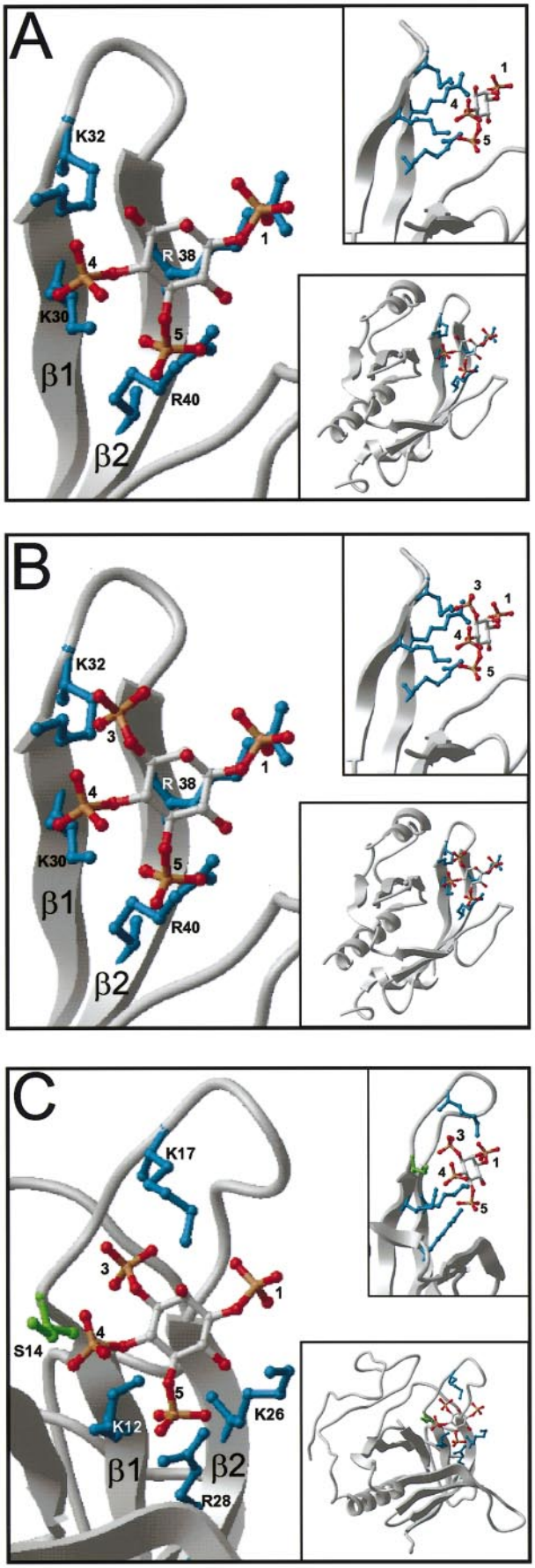
for *CDC25* with a construct that expresses activated Ras fused with a PH domain of interest. This assay is not subject to many of the limitations encountered in the *in vitro* phosphoinositide binding assays and it avoids the need to purify PH domains and to prepare phospholipid vesicles containing various amounts of the phosphoinositide of interest. The reliability of our assay in detecting interactions of PH domains with the PI3K products PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, as well as with PtdIns(4,5)P_2, which is constitutively present in the plasma membrane, has been established using several criteria. First, the PH domains of AKT and BTK, which have previously been shown to bind PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 with high affinity (Fukuda and Mikoshiba, 1996, 1997; Salim *et al*., 1996; Franke *et al*., 1997b; Frech *et al*., 1997; Rameh *et al*., 1997), both rescued growth of yeast at the non-permissive temperature in a PI3K-dependent manner. On the other hand, the PH domain of PLCδ, which has previously been shown to bind PtdIns(4,5)P_2 with high affinity (Lemmon *et al*., 1995), rescued yeast in a PI3K-independent manner. Secondly, point mutations that have been shown to impair binding of these PH domains to phosphoinositides *in vitro* abrogated rescue of yeast by these PH domains. Thirdly, we have used our assay to identify several known and unknown PH domains that bind PI3K products. Using *in vitro* binding assays, we have confirmed that several of these PH domains specifically bind PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 with high affinity. Moreover, we have tested two of these new PH domains for binding PI3K products in mammalian cells and have found that they are sufficient, by themselves, to target proteins to membranes in mammalian cells by binding PI3K products.

One example that highlights some of the potential problems encountered when assessing interactions between PH domains and phosphoinositides using *in vitro* binding assays is demonstrated by studies on the PH domain of Sos. The PH domain of Sos has been reported to bind PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 with a relatively high affinity in *in vitro* binding studies. However, while three studies demonstrated high-affinity interactions between the Sos PH domain and PtdIns(4,5)P_2, only one study found an interaction with PtdIns(3,4,5)P_3 (Klarlund *et al*., 1997; Koshiba *et al*., 1997; Kubiseski *et al*., 1997; Rameh *et al*., 1997). The failure of the Sos PH domain to rescue yeast in our system suggests that Sos does not bind either phospholipid *in vivo* with an affinity comparable to that of either PLCδ or BTK, despite these other studies showing similar *in vitro* binding affinities. We believe that our failure to identify a high-affinity interaction in yeast between the Sos PH domain and either PtdIns(4,5)P_2 or PtdIns(3,4,5)P_3 is because the results obtained in yeast may more accurately reflect *in vivo* interactions between the Sos PH domain and phosphoinositides in mammalian cells. A recent study by Chen *et al*. (1997) demonstrated that while the Sos PH domain may target Sos to the plasma membrane, this function is independent of binding either PtdIns(4,5)P_2 or PtdIns(3,4,5)P_3 *in vivo*. It is possible that in the case of Sos, *in vitro* binding artifacts may have markedly overestimated the affinity of the Sos PH domain for PtdIns(4,5)P_2 or PtdIns(3,4,5)P_3. The solution structure of the Sos PH domain also supports the idea that the PH domain of Sos binds ligands other than PtdIns(4,5)P_2 (Koshiba *et al*., 1997). This structure revealed that while the Sos PH domain contains a characteristic PH fold, it also contains a unique N-terminal α helix and a long unstructured β3–β4 loop which are absent from other PH domains but which are conserved among all Sos proteins. Thus, these findings suggest that unique regions in the Sos PH domain may mediate interactions with novel targets.

One potential limitation of our assay is its inability to detect low-affinity interactions between PH domains and phosphoinositides. The finding that the PH domains which rescued in our assay have all been shown to bind specific phosphoinositides with high affinity, while several PH domains that have been shown to bind PtdIns(4,5)P_2 or PtdIns(3,4,5)P_3 with only lower affinities failed to rescue, suggests that our assay may be best at detecting only high-affinity interactions of a specific phosphoinositide with a PH domain (K_d of <1 μM). However, while the physiological importance of high affinity interactions between PH domains and phosphoinositides has become apparent over the past several years, the physiological relevance of many low-affinity interactions is unknown.

Some low-affinity interactions between PH domains and phosphoinositides may have important biological functions, and these interactions may not be detected using our assay. For example, a PH domain that binds a phosphoinositide with low affinity could play an important role in facilitating membrane targeting by cooperating with other membrane targeting domains (Lemmon *et al*., 1997). This may be the case for the PH domain of PLCγ, which does not rescue yeast in our assay. A recent study has demonstrated that interaction of the PH domain of PLCγ with PtdIns(3,4,5)P_3 is necessary for the platelet-derived growth factor (PDGF) receptor to fully activate PLCγ (Falasca *et al*., 1998). However, the SH2 domains of PLCγ probably contribute to plasma membrane targeting both by binding the tyrosine phosphorylated receptor and by directly binding PtdIns(3,4,5)P_3 (Bae *et al*., 1998; Falasca *et al*., 1998).

Low-affinity interactions between PH domains and phosphoinositides may also play important roles in activation which are independent of membrane targeting. For example, the interaction of the PH domain of dynamin with PtdIns(4,5)P_2 or PtdIns(3,4,5)P_3 has been shown to activate the intrinsic GTPase of dynamin (Salim *et al*., 1996; Barylko *et al*., 1998) and the interaction of the PH domain of Vav with PtdIns(3,4,5)P_3 has been shown to increase Rac guanine nucleotide exchange activity of Vav as well as to facilitate Vav tyrosine phosphorylation by Lck (Han *et al*., 1998). In addition, the interaction of the PH domain of Sos with PtdIns(3,4,5)P_3 may activate Rac guanine nucleotide exchange activity of the Sos Dbl homology domain (Nimnual *et al*., 1998). We currently favor the hypothesis that high-affinity interactions between a PH domain and a phosphoinositide predominantly function to target a protein to a membrane compartment where it can then function and/or become activated (Downward, 1998). These interactions can be detected using our assay in yeast and this should allow for identification of PH domains which are, by themselves, sufficient for targeting a protein to the membrane. On the other hand, low-affinity interactions of some PH domains with phosphoinositides may function predominantly to induce a conformational change, independent of membrane targeting, to facilitate



activation of an associated enzyme activity. These low-affinity modulatory interactions are unlikely to be detected using our assay. A second limitation of our assay is the inability to distinguish between binding to PtdIns(3,4)P_2 versus PtdIns(3,4,5)P_3.

By aligning the first and second β strands and the β1–β2 loop of several PH domains that bind PI3K products with high affinity, we identified several amino acid residues that are conserved among these PH domains. We found that PH domains which bound PI3K products with high affinity all contained a positively charged residue in the β1–β2 loop, and the β1–β2 loops in these PH domains were at least six amino acids in length. In addition, all PH domains contained a small amino acid at the start of the β1–β2 loop, two positively charged amino acids at conserved positions in the second β strand, and a positive charge in the second to last position of the first β strand. Using several criteria we have been able to establish the importance of these residues in binding PI3K products. First, site-directed mutagenesis of several of these residues abrogated rescue of yeast, indicating that binding to PI3K products was substantially inhibited by these mutations. Secondly, through computer database searches we have identified several known and several new PH domains that contain this motif. Nearly all these PH domains were subsequently found to rescue yeast at the non-permissive temperature in a PI3K-dependent manner, suggesting that they are binding PI3K products with high affinity. Thirdly, a number of PH domains lacking this motif did not rescue yeast.

Based on the published data on the structure of the PH domain of PLCδ complexed with Ins(1,4,5)P_3, the structure of the BTK PH domain, and the results presented here, we have constructed a theoretical model for how PH domains bind PI3K products with high affinity (Figure 7) (Ferguson *et al*., 1995; Hyvonen and Saraste, 1997). We propose that high-affinity binding of PH domains to PI3K products *in vivo* requires a set of specific interactions between negatively charged phosphates in the polar head groups of PtdIns(3,4,5)P_3 with several basic residues in

Fig. 7. Theoretical model for interaction between BTK and Ins(1,3,4,5)P_4. Ribbon diagrams are shown with certain key residues highlighted as ball and stick models as indicated. Lysine and arginine residues are in blue, and serine is green. (**A**) The solved crystal structure of the PH domain of PLCδ complexed with Ins(1,4,5)P_3. (**B**) Theoretical model for interaction between PLCδ complexed with Ins(1,3,4,5)P_4. Note that the lysine (K32) in the first position of the β1–β2 loop is in position to block access of the 3-phosphate in Ins(1,3,4,5)P_4 to the binding site. (**C**) Diagram depicting a theoretical model for the interaction between the PH domain of BTK and Ins(1,3,4,5)P_4. In contrast to K32 of PLCδ, the serine (S14) in the first position of the β1–β2 loop allows access of the 3-phosphate in Ins(1,3,4,5)P_4 to this binding site. In addition, lysine at position 17 in the β1–β2 loop is in position to contact the 3-phosphate of Ins(1,3,4,5)P_4 to stabilize the interaction. The model is based on the BTK PH domain crystal structure coordinates but has been modified by replacing C28 with R and energy minimizing the β1–β2 loop in the presence of Ins(1,3,4,5)P_4 ligand as described in Materials and methods. The structure of the BTK PH domain has been energy minimized in the presence of ligand between residues 13–25. Three views for each structure are shown: the larger structure is face on; the upper inset is the same structure rotated counterclockwise on the *y*-axis 55°; the lower inset is the face on view of the complete PH domain to provide orientation. Ligand atoms are colored as follows: carbon, white; oxygen, red; phosphorous, orange.

conserved positions in the β1–β2 strand and loop region. In addition, high-affinity binding of PH domains to PI3K products requires that a PH domain contain a small amino acid at the start of β1–β2 loop to accommodate the 3-phosphate in a binding pocket. Placing Ins(1,3,4,5)P_4 into the Ins(1,4,5)P_3 binding site in the PLCδ PH domain resulted in a very energetically unfavorable interaction because the presence of a bulky lysine residue (K32) at the start of the β1–β2 loop sterically hinders access of the 3-phosphate in Ins(1,3,4,5)P_4 to this binding site (Figure 7B). On the other hand, the presence of a serine residue which lacks a bulky side-chain at this position in BTK allows the 3-phosphate in Ins(1,3,4,5)P_4 to gain access to this binding site when modeled in using the same procedure (Figure 7C). One contact which is critical for stabilizing the interaction is likely to be between a positively charged residue in the β1–β2 loop and the 3-phosphate (Figure 7C). The requirement for a large β1–β2 loop of at least six amino acids, which we identified in our consensus motif, would then be necessary to allow sufficient flexibility for the loop to first bend around the 3-phosphate and then for the positive charge in this loop to contact the 3-phosphate (Figure 7C). Based on the findings presented here, we would predict that low-affinity interactions between PH domains and PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 will require a different set of interactions. The validity of this model awaits structural data on different PH domains complexed with Ins(1,3,4,5)P_4.

It is probable that residues in other parts of the PH domain, and not contained in our motif, are also important for PH domains to bind PI3K products with high affinity. For example, additional interactions between the β3–β4 loop and Ins(1,3,4,5)P_4 also contribute to high-affinity binding of PH domains to PI3K products; K53 in BTK is critical for the BTK-PH domain to bind PtdIns(3,4,5)P_3 (Hyvonen *et al*., 1995). However, the finding that similar residues in the first and second β strands and β1–β2 loop are conserved among a subset of PH domains that bind PI3K products with high affinity suggests that all these PH domains bind PI3K products in a similar manner. This finding is in contrast to results that have been obtained for binding of PtdIns(4,5)P_2 with several PH domains. For example, while the PH domains of Sos, PLCδ and N-Pleckstrin all utilize the β1–β2 and β3–β4 loops to bind Ins(1,4,5)P_3, different basic residues in each of these PH domains are involved in Ins(1,4,5)P_3 binding (Harlan *et al*., 1994, 1995; Ferguson *et al*., 1995; Koshiba *et al*., 1997).

At present, we do not know the biological roles of the new PH domains which bind PI3K products with high affinity that we have identified using our assay. It is intriguing to speculate that PH domains present in these molecules may target these molecules to a membrane compartment where they can then function by a PI3K-dependent mechanism. For example, the family of unconventional myosins, of which myosinX is the only member with a PH domain, is thought to play important roles in organelle trafficking and movement (Mermall *et al*., 1998). Binding of the myosinX PH domain to PI3K products may function to localize myosinX to specific organelles where it may then regulate subcellular trafficking of that organelle. Both Dos and Gab1 belong to an expanding class of molecules which are tyrosine phosphorylated by receptor tyrosine kinases and function as adaptors to activate signaling pathways by recruiting signaling molecules containing SH2 domains (Herbst *et al*., 1996; Holgado-Madruga *et al*., 1996; Raabe *et al*., 1996; Weidner *et al*., 1996). Stabilization and/or recruitment of Gab1 and Dos to the plasma membrane via an interaction of their PH domains with PI3K products could facilitate signaling by enhancing their phosphorylation by RTKs. Alternatively, sustained recruitment of these molecules to membranes may facilitate signaling by the SH2 domain-containing proteins that bind Dos and Gab1 in stimulated cells. In preliminary experiments, however, treatment of cells with the PI3K inhibitor wortmannin did not significantly decrease Gab1 tyrosine phosphorylation by either the PDGF or insulin receptors (data not shown). Nevertheless, the finding that only a small number of PH domains rescued in our system, coupled with the finding that many other PH domains which specifically bind PI3K products with high affinity are important targets for regulation by PI3K, suggests that these new proteins are also targets for regulation by PI3K.

The TOPIS assay should also prove useful for screening new proteins which bind PI3K products with high affinity. We have constructed Ras and Sos fusions with various cDNA libraries and using this assay have screened for cDNAs that rescue yeast at the non-permissive temperature in a PI3K-dependent manner. Preliminary results have indicated that this approach can be used successfully to screen for new cDNAs that bind PI3K products. Identification of downstream targets of PI3K is essential for understanding how PI3K mediates its numerous effects in cells. The use of the TOPIS assay described in this report to screen for new cDNAs encoding proteins which bind PI3K products, together with database searches with the motif we identified, should provide valuable tools for uncovering important downstream targets of PI3K.

Materials and methods

Yeast methods

The yeast strain referred to in the text as *cdc25ts* was *S.cerevisiae* strain 352-15A2 (*MATa, ade5, cdc25-2, his7, met10, trp1, ura3-52*) which contains a temperature sensitive allele of *CDC25* (Aronheim *et al*., 1997). Yeast were cotransformed with 3 μg of each plasmid or 10 μl of miniprep DNA by a modified lithium acetate (LiAc) protocol (Gietz and Schiestl, 1995). Briefly, an overnight culture of *cdc25ts* yeast, grown at 25°C, was diluted in 50 ml YPD medium to a density of 5×10^6 cells/ml. Yeast were grown to a density of 2×10^7 cells/ml at 25°C. Cells were washed once with 25 ml water, then once with 1 ml of 100 mM LiAc. Cells were resuspended in a total volume of 500 μl of 100 mM LiAc and split into 50 μl aliquots. Cells were pelleted and the supernatant was removed. To each tube was added, in order, 240 μl 50% PEG3350, 36 μl 1M LiAc, 25 μl salmon sperm DNA (2 mg/ml) and 50 μl of plasmid DNA in water. After vigorous mixing, yeast were incubated for 30 min at 25°C and then heat shocked at 42°C for 20 min. Cells were resuspended in 200 μl water and plated on glucose minimal medium lacking uracil and leucine. To assay for rescue of the *cdc25ts* allele, transformants were split in half and plated equally on two selective plates. Plates were incubated for 48 h at 25°C, and then one plate was transferred to 37°C and the other left at 25°C. After an additional 48 h, growth of most transformants was easily assayed. For several (EST810295), incubation for a further 48 h at 37°C was required to see growth.

Plasmid constructs

To generate the PYES2-p110-farnesylated plasmid, a *Sma*I–*Kpn*I fragment of the 5′ end of HA-tagged p110β (Hu and Schlessinger, 1994) was subcloned into a *Hin*dIII(blunted)–*Kpn*I site in PYES2 (Invitrogen), which contains the *GAL1* promoter and *URA3* gene. A *Kpn*I fragment

from HA-p110β, containing the remaining coding region of p110β, was then ligated in. Finally, a *Bgl*II fragment containing the Ras farnesylation sequence was ligated into the *Bgl*II site at the 3′ end of the p110β coding sequence. To generate the kinase-inactive p110β vector, the *Kpn*I fragment from the kinase-inactive HA-p110β was used in the above subcloning (Logan *et al*., 1997)

The 3S0B-SRS (for 3 stops, zero *Bam*HIs) vector is a derivative of pADNS, a multicopy yeast expression vector containing the *ADH1* promoter and the *LEU2* gene (Colicelli *et al*., 1989). To generate 3S0B, the two *Bam*HI sites in pADNS were destroyed by digestion with *Bam*HI, blunting with Klenow and religation. The vector was digested with *Hin*dIII–*Not*I and a double-stranded oligonucleotide linker was ligated in to destroy the original *Not*I site and generate three stop codons in all three frames following the *Not*I site in the linker. The 2 oligonucleotides were 5′(AGCTTATCGCGGCCGCTAGATAGATAG) and 5′(GCGGCCCTATCTATCTAGCGGCCGCGATA). Next, the *Hin*dIII–*Not*I region from the yeast two-hybrid vector VP16 was ligated in (Vojtek *et al*., 1993), providing a unique *Bam*HI site between the *Hin*dIII–*Not*I. Finally, a *Hin*dIII–*Bam*HI fragment of activated Q61L c-Ha-Ras (A.Aronheim, in preparation) was ligated into the *Hin*dIII–*Bam*HI site of 3S0B to generate 3S0B-SRS.

To generate 3S0B-SRS–PH domain fusions, the PH domains of the proteins indicated were amplified by overlapping PCR to add a Myc-epitope tag and restriction sites to each PH domain. The 5′ oligonucleotides contained a *Bam*HI or *Not*I site in frame with Ras. The PCR products were subcloned into the 3S0B-SRS vector with either *Bam*HI–*Not*I or *Not*I alone. Site-directed mutagenesis was performed by overlapping PCR.

Except where noted, the cDNA used to PCR the various PH domains correspond to the sequences shown in Figure 3. The region used for each PH domain are as follows: AKT1(1–106), BTK(1–174), Dos(1–111), Gab1(1–116), DGKδ(1–164), DGKη (1–150), IRS1-PH(1–113), IRS1(PH+PTB) (1–309), PLCγ(15–150), hSOS1(423–554), Dynamin(511–629) and PLCδ(1–140). AKT AH domain(1–147) was also used with results similar to AKT(1–106). For ESTs which have an incomplete PH domain sequence reported, additional sequence was obtained by searching the EST database for overlapping fragments. EST clones were obtained from Genome Systems. For myosinX, human EST649664 (DDBJ/EMBL/GenBank accession No. aa216552) which was >95% identical at the amino acid level to the bovine myosinX PH domain, was amplified over the region corresponding to amino acids 1203–1312 in the bovine sequence.

To express EST684797 in mammalian cells, myc-epitope tagged EST684797 was cloned into the vector pRK5 (Isakoff *et al*., 1996). GFP–Gab1PH was expressed using the vector pEGFP–C2 (Clontech).

Isolation of protein from yeast and immunoblotting of yeast extracts

Yeast transformed with 3S0B-SRS encoding fusion proteins of Ras with various PH domains and PYES2-p110-farnesylated were grown to log phase and lysed as described (Superti-Furga *et al*., 1996). Proteins were separated by SDS–PAGE and after transfer to nitrocellulose were immunoblotted with anti-Ras antibodies (Skolnik *et al*., 1993).

Mammalian cell culture, transfections and immunofluorescence microsocopy

Cos cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Cos cells were transfected with 1 μg of EST684797-myc or GFP–Gab1PH together with 1 μg of p110-farnesylated or kinase-inactive p110-farnesylated using DEAE dextran. One day after transfection, cells were split onto coverslips and grown for 24 h in DMEM containing 10% FBS followed by 24 h in 0.5% FBS. Transfected cells were then fixed in 4% formaldehyde in PBS and then permeablilzed using 0.2% Triton X-100/100 mM glycine/1 mM $CaCl_2$ in PBS. GFP–Gab1PH was directly visualized by confocal microscopy. EST684797 was detected by confocal microscopy using the anti-myc monoclonal antibody 9E10 and a secondary anti-rabbit IgG antibody coupled to Texas Red. Cells were visualized using an upright SARASTRO 2000TM CLSM (Molecular Dynamics).

Pattern searches of databases

For generation of PH domain database, protein domains likely to have the PH β-barrel fold were identified by starting with the largest possible seed group of sequences likely to have the PH β-barrel fold and generating a quantitative score for a whole-domain comparison between a candidate sequence or sequence fragment and sequences in the seed group of PH β-barrel fold sequences. For the seed group, we used the group of protein sequences classified as PH domains in the Prosite Database (Bairoch *et al*., 1997). A limited number of sequences strongly suspected in recent literature to be PH domains were manually added to the seed group. The most commonly used sensitive score of similarity between sequences is the BLAST Probability based on Karlin and Altschul (1990) statistics. However, this score is a local alignment score and cannot determine the protein domain boundaries when a high score is encountered. Recently, structural significance statistics were derived for global (whole sequence) alignments by exhaustive cross-comparison of all the protein domains in the database of experimentally solved 3D structures (Abagyan and Batalov, 1997) These statistics provide an appropriate quantitative probability score for extracting all the sequence fragments which are likely to be PH domains from the non-redundant database of all deposited protein sequences (NCBI). Sequence fragments with a probability score of less than 0.0001 were thus collected and added to the seed group to construct the pattern-searchable database.

Structure-based multiple sequence alignment of PH domains

The available solved PH domain structures for Pleckstrin (PDB code 1pls), PLC-δ (1mai), BTK(1btk), Spectrin(1mph), Dynamin(1dyn) and βARK1 (1BAK) were manually aligned according to their 3D structures. For each sequence without a solved 3D structure, the highest-probability global pairwise alignment (Abagyan and Batalov, 1997) to one of the five structures was determined. This alignment was then projected onto the multiple structural alignment through this closest sequence, and the final alignment was manually adjusted. This procedure is in contrast to traditional multiple alignment algorithms in which the noise of weak alignments is averaged into the residue positional information. Since several of the PH domains in the structural alignment are very weakly related by sequence, the error introduced by averaging in the sequence signal from the whole group would be significant and detrimental.

Molecular modeling of BTK in complex with Ins(1,3,4,5)P_4

Using internal coordinate representations of molecular structures with ideal covalent geometry (Abagyan *et al*., 1993), the Ins(1,4,5)P_3 ligand from PLCδ was placed into the BTK structure based on the structural superposition of the two 3D structures. The BTK structure was first modified by replacing cysteine 28 in the BTK structure with arginine as is found in the wild-type sequence. The ligand was then modified from Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 by addition of a phosphate group at the 3′ position in a conformation symmetrical to the phosphate at the 5′ position. The backbone loop of residues 13–25 in BTK and all the side-chains within 4 Å of the loop were then energy minimized by a previously described loop modeling procedure (Cardozo *et al*., 1995). Representation of the molecules studied in this work and graphical output was performed by the ICM program (Abagyan *et al*., 1993).

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