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Expression Cloning of Protein Targets for 3-Phosphorylated Phosphoinositides*

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The phosphatidylinositol 3-kinase (PI 3′-K) family of lipid kinases play a critical role in cell proliferation, survival, vesicle trafficking, motility, cytoskeletal rearrangements, and oncogenesis. To identify downstream effectors of PI 3′-K, we developed a novel screen to isolate proteins that bind to the major products of PI 3′-K: phosphatidylinositol-3,4-bisphosphate (PtdIns-3,4-P_2) and PtdIns-3,4,5-trisphosphate (PtdIns-3,4,5-P_3). This screen uses synthetic biotinylated analogs of these lipids in conjunction with libraries of radiolabeled proteins that are produced by coupled *in vitro* transcription/translation reactions. The feasibility of the screen was initially demonstrated using avidin-coated beads prebound to biotinylated PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 to specifically isolate the pleckstrin homology domain of the serine/threonine kinase Akt. We then demonstrated the utility of this technique in isolating novel 3′-phosphorylated phosphatidylinositol (3′-PPI)-binding proteins through the preliminary screening of *in vitro* transcribed/translated cDNAs from a small pool expression library derived from mouse spleen. Three proteins were isolated that bound specifically to 3′PPIs. Two of these proteins have been previously characterized as PIP3BP/p42^{IP4} and the PtdIns-3,4,5-P_3-dependent serine/threonine kinase phosphoinositide-dependent kinase 1. The third protein is a novel protein that contains only a Src homology 2 domain and a pleckstrin homology domain; this protein has a higher specificity for both PtdIns-3,4,5-P_3 and PtdIns-3,4-P_2 than for PtdIns-4,5-bisphosphate. Transcripts of this novel gene are present in every tissue analyzed but are most prominently expressed in spleen. We have renamed this new protein PHISH for 3′-phosphoinositide-interacting Src homology-containing protein. This report demonstrates the utility of this technique for isolating and characterizing 3′-PPI-binding proteins and has broad applicability for the isolation of binding domains for other lipid products.

It is now well recognized that dynamic changes in the phosphorylation state of intracellular phosphatidylinositol (PtdIns)[1] play critical roles in mediating many cellular events. Phosphatidylinositol 3′-kinases (PI 3′-Ks) are a subfamily of PtdIns kinases that phosphorylate the 3′-OH (D3) position of PtdIns to create four different PtdIns derivatives: PtdIns-3-P, PtdIns-3,4-P_2, PtdIns-3,5-P_2, and PtdIns-3,4,5-P_3 (reviewed in Refs. 1–3). Nine different isoforms of PI 3′-K have been identified in mammalian cells and they have been grouped into three classes by Domin and Waterfield (4) based on the specific form of PI that is used as a substrate.

Singly phosphorylated PtdIns-3-P is constitutively expressed in cells and is involved in a variety of events associated with membrane protein trafficking (5). Although all classes of PI 3′-Ks can phosphorylate PtdIns to generate this lipid, the majority of PtdIns-3-P is probably produced by Class III PI 3′-Ks, which is specific for PtdIns (1). PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 are generated following stimulation by a wide variety of extracellular stimuli through many diverse classes of receptors. Class I PI 3′-Ks phosphorylate PtdIns-4,5-P_2 to generate PtdIns-3,4,5-P_3, which can be dephosphorylated to PtdIns-3,4-P_2 by the 5′ lipid phosphatase SHIP (6). Alternate pathways to PtdIns-3,4-P_2 have also been described involving phosphorylation of the 4-position of PtdIns-3-P by Class II PI 3′-K or an unidentified PtdIns-3-P 4-kinase, but the extent to which these enzymes contribute to PI-3,4-P_2 synthesis in not clear (7–9).

Class I PI 3′-Ks play critical roles in many essential cellular processes. Perhaps most importantly, these kinases regulate cell survival. Inhibition of class I PI 3′-Ks leads to an induction of programmed cell death or apoptosis, and constitutive unregulated activation of these enzymes or downstream targets of PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 can rescue cells from cell death induced by serum deprivation, loss of matrix attachment, Myc expression, and other apoptotic stimuli (10–14). These kinases also control the activation of many intracellular signaling pathways that regulate cell proliferation, including extracellular regulated kinase/mitogen-activated protein kinase, protein translation factors (*e.g.* eIF-4E), and cyclins/cyclin-dependent kinases (15–19). Also, membrane trafficking events regulated by 3′-PPIs control receptor internalization (20). In addition, PI 3′-Ks are necessary for glucose transporter recruitment to the plasma membrane and regulation of glycogen synthase kinase 3 and phosphofructokinase, indicating that 3′-PPIs are critically involved in insulin-mediated events associated with glucose metabolism (2, 21–23). Integrin affinity modulation is also blocked by

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[1] The abbreviations used are: PtdIns, phosphatidylinositol; ARF, ADP ribosylation factor; EST, expressed sequence tag; MBP, maltose-binding protein; PH, pleckstrin homology; MBP-PH, MBP-Akt PH domain; 3′-PPI, 3′-phosphorylated phosphatidylinositol; PDK1, phosphoinositide-dependent kinase 1; PHISH, 3′-phosphoinositide interacting SH2-containing protein; PI 3′-K, phosphatidylinositol 3′-kinase; PtdIns-3-P, PtdIns-3-monophosphate; PtdIns-3,4-P_2, PtdIns-3,4-bisphosphate; PtdIns-4,5-P_2, PtdIns-4,5-bisphosphate; PtdIns-3,4,5-P_3, PtdIns-3,4,5-trisphosphate; SH2, Src homology 2.

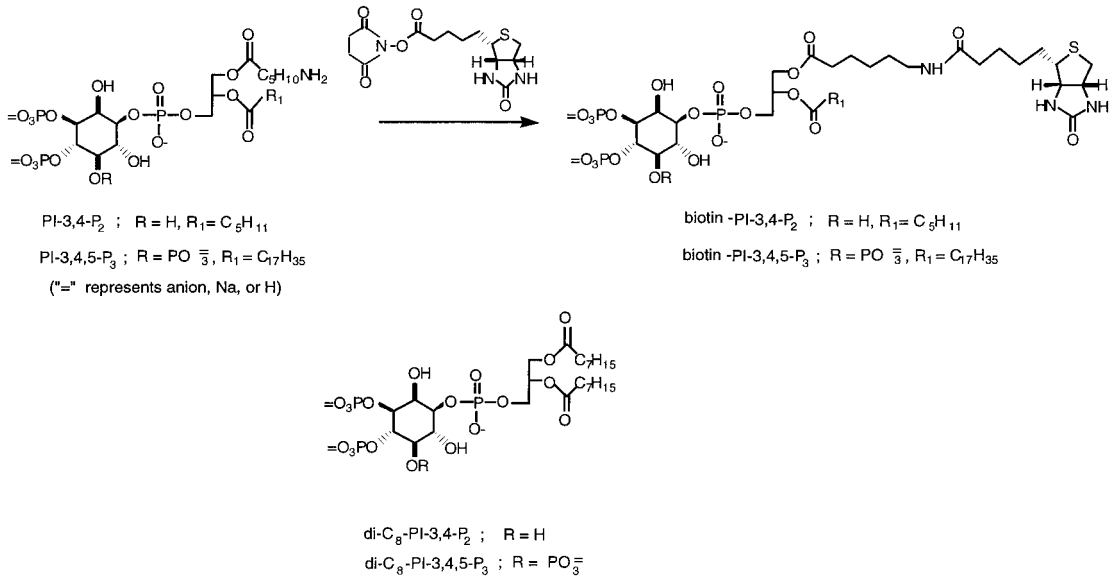

FIG. 1. **Synthesis of biotinylated PIP$_n$ probes and structure of dioctanoyl derivatives.**

pharmacological inhibitors of PI 3′-K, implicating these kinases in critical events associated with leukocyte trafficking and inflammatory responses (24–26). PI 3′-K also plays an important role in regulating cell movement and cytoskeletal rearrangements. For example, 3′-PPIs are necessary for controlling receptor-induced changes in actin assembly, the formation of lamellipodial protrusions, and cell migration through the small GTP-binding protein Rac (27–31).

Because of the central importance of PI 3′-Ks in controlling cell proliferation, survival, and motility, it is likely that class I PI 3′-Ks and 3′-PPI-binding proteins play an important role in the pathogenesis of cancer. Overexpression of PI 3′-K in chicken cells is sufficient to induce cellular transformation both *in vitro* and *in vivo* (32). PI 3′-K has also been implicated in the induction of chronic myelogenous leukemia and acute lymphocytic leukemia by the BCR-ABL oncogene (33, 34). As might be expected from its importance in cellular motility, PI 3′-K has been shown to play a role in tumor invasion and metastasis in several model systems (31, 35–37). Recently, a role for PI 3′-K in human carcinogenesis was demonstrated by the evidence that the tumor suppressor PTEN is a lipid phosphatase that is specific for 3′-phosphate of the inositol head group of and that elimination of the lipid phosphatase activity correlates with the oncogenic potential of PTEN mutants found in human cancers (38–40).

There are several identified protein motifs that bind to 3′-PPIs: PH domains, FYVE domains, SH2 domains, and C2 domains. The primary function of these domains, each of which is approximately 90–120 amino acids in size, is believed to be localization of the protein to high local concentrations of 3′-PPIs found near active signaling complexes at the cell membrane. However, there is evidence indicating that these domains can regulate protein function as well.

The most diverse and best-characterized 3′-PPI binding domains are PH domains, which comprise a large family of binding modules that are known to bind proteins, as well as a wide range of lipids. A subset of PH domains binds with a high affinity to PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 (41, 42). These PH domains are critical for the function of several signaling proteins, including the serine/threonine kinases Akt and phosphoinositidedependent kinase 1 (PDK1), the tyrosine kinase Brutons tyrosine kinase, and the ARF-guanine nucleotide exchange factor Grp1.

FYVE domains are recently characterized domains that contain a zinc finger, associate exclusively with PtdIns-3-P, and are important for vesicle sorting (2). SH2 domains bind primarily to phosphorylated tyrosines; however, the SH2 domains of phospholipase Cγ, the Src tyrosine kinase, and the p85 subunit of PI 3′-K can also bind to PtdIns-3,4,5-P_3 with micromolar affinity (43). C2 domains bind to PtdIns-4,5-P_2, PtdIns-3,4-P_2, and PtdIns-3,4,5-P_3, and the specificity of lipid binding depends upon the local concentration of calcium. C2 domains are found in protein kinase Cs, phospholipase A, and vesicle sorting proteins, such as synaptotagmin (44–46).

Given the diversity of cellular responses dependent on the products of PI 3′-K, it is probable that many as yet unidentified proteins bind to and are regulated by PtdIns-3,4,5-P_3 and PtdIns-3,4-P_2. Identification of these proteins and the elucidation of their role in cellular signaling will be critical to our understanding of these cellular functions as well as of diseases such as cancer. In order to isolate novel 3′-PPI-binding proteins, we developed a screen using *in vitro* coupled transcription/translation technology (47, 48) in conjunction with the use of synthetic biotinylated PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 ligands. We show in this report that this screen can isolate 3′-PPI-binding proteins with high specificity and selectivity from among a vast excess and diversity of other nonspecific proteins. We report an initial demonstration of the effectiveness of the system using the PH domain of the serine/threonine kinase Akt, a known 3′-PPI binder, as a model. Second, we demonstrate the utility of this technique in isolating other 3′-PPI-binding proteins by screening a small pool expression library derived from mouse spleen mRNA. Three 3′-PPI-binding proteins were identified in this initial screen. Two of these proteins have been previously characterized, PtdIns(3,4,5)P_3-binding protein/p42^{IP4} and PDK1, thereby establishing positive controls that the system operates as predicted. Importantly, the third protein is a novel protein of unknown function that contains both a PH domain and an SH2 domain.

EXPERIMENTAL PROCEDURES

Cloning and Expression of Akt PH Domain—The PH domain of Akt was cloned as a fusion protein to maltose-binding protein (MBP) in the expression vector pMAL2B (New England Biolabs). The N-terminal 130 amino acids of murine Akt1 in the vector pJ3Ω (a gift of P. Tsichlis, Fox-Chase Cancer Center) was amplified by polymerase chain reaction using *Pfu* polymerase with the coding strand primer 5′-cgatcgggatccatggaacag-3′ (upstream of the Myc tag in pJ3Ω and containing a *Bam*HI site) and the noncoding strand primer 5′-ccctgaattctcactgggtga-3′ (from Akt amino acid 130 and encoding an *Eco*RI site). Both maltose-binding protein alone and the MBP-Akt PH domain fusion construct were

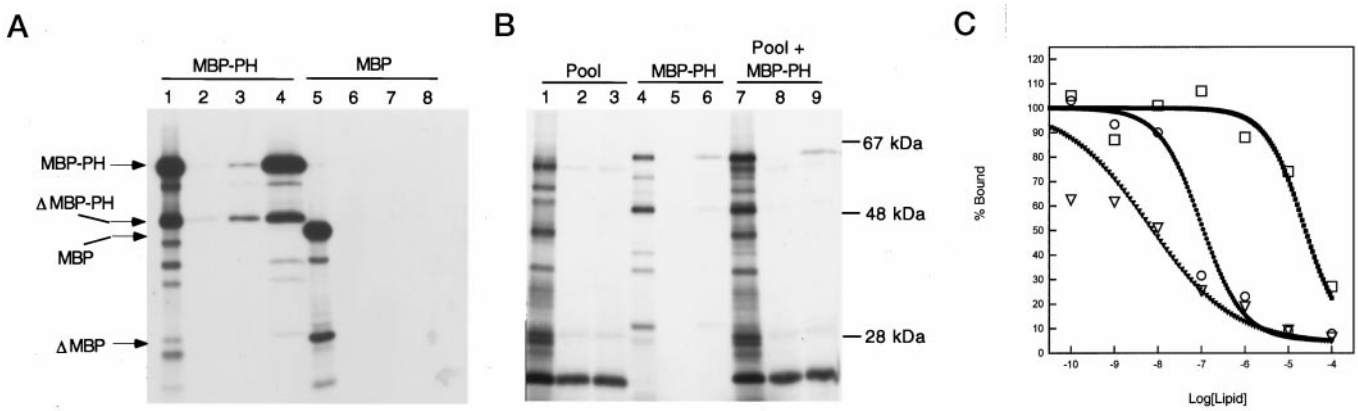

FIG. 2. **The PH domain of Akt binds specifically to avidin beads prebound with biotinylated 3′-PPIs.** *A,* ^{35}S-labeled MBP or MBP-PH (*lanes 1* and *5*) were incubated with avidin beads alone (*lanes 2* and *6*), avidin beads prebound with PtdIns-3,4-P_2-biotin (*lanes 3* and *7*), or avidin beads prebound with PtdIns-3,4,5-P_3-biotin (*lanes 4* and *8*). Proteins were labeled with [^{35}S]methionine by *in vitro* transcription/translation of 0.5 μg of the respective genes in pCS2(+), and the binding reactions were done as described under "Experimental Procedures." Truncated species of the MBP and MBP-PH (Δ*MBP* and Δ*MBP-PH*) result from initiation of translation at start sites after the initial AUG codon. *B,* avidin beads prebound with PtdIns-3,4-P_2-biotin can specifically isolate MBP-PH from a pool of other proteins. 10 ng of MBP-PH DNA and/or 1 μg of DNA from a random pool from the cDNA library was transcribed/translated in the presence of [^{35}S]methionine, and binding reactions were performed as described under "Experimental Procedures." Shown are total labeled proteins (*lanes 1, 4,* and *7*), labeled proteins bound to avidin beads (*lanes 2, 5,* and *8*), and labeled proteins bound to avidin beads prebound with PtdIns-3,4-P_2-biotin (*lanes 3, 6,* and *9*). *C,* PtdIns-3,4,5-P_3 and PtdIns-3,4-P_2 preferentially displace MBP-PH from PtdIns-3,4,5-P_3-biotin. ^{35}S-Labeled MBP-PH was bound to avidin beads coated with PtdIns-3,4,5-P_3-biotin in the presence of the indicated concentrations of PtdIns-4,5-P_2 (*squares*), PtdIns-3,4-P_2 (*circles*), or PtdIns-3,4,5-P_3 (*triangles*) and processed as described under "Experimental Procedures." *Data points* represent the mean of two independent experiments.

subcloned into the *in vitro* translation vector pCS2(+) under the control of the SP6 promoter. The proteins were expressed by transcribing/translating 0.01–1 μg of DNA of MBP or MBP-Akt PH with the Promega TnT coupled reticulocyte lysate system using SP6 RNA polymerase and [^{35}S]methionine (*in vivo* labeling grade, Amersham Pharmacia Biotech) according to the manufacturer's protocol.

Synthesis of Biotinylated and Nonbiotinylated Phosphatidylinositols—All chemically synthesized probes were prepared using a methyl D-glucopyranoside as the chiral starting material for the inositol head group; different phosphate substitution patterns were elaborated using the Ferrier rearrangement of suitably protected glucose-derived precursors (49). Di-C_8 PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 were prepared by modifications (50, 51) of the synthesis of the dipalmitoyl analogs (52, 53). Biotinylated phosphoinositides were prepared from the *sn*-2-aminohexanoyl derivatives of PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 by condensation of the active ester of biotin with the water-soluble PIP_n analog in the presence of a mild base (Fig. 1). The biotinylated probes, bPtdIns-3,4-P_2 and bPtdIns-3,4,5-P_3, were purified by ion exchange chromatography and employed as aqueous solutions for attachment to streptavidin-coated surfaces

Binding and Isolation of Radiolabeled in Vitro Translated Proteins with Biotinylated Phosphoinositides—Avidin beads (Ultralink-immobilized Neutravidin™, Pierce) were washed twice with 5 volumes of wash/binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40 (nonylphenylpolyethylene glycol), 5 mM dithiothreitol). The beads were then reconstituted in a 500 μl of wash/binding buffer, and the biotinylated phosphoinositide was added. Generally, 0.1 μl of 100 μM biotinylated lipid was bound per 1 μl of packed avidin beads. The biotinylated lipid was incubated with the beads for 1–2 h at 4 °C with gentle agitation and then washed twice with a 10× bead volume of wash/binding buffer to remove excess ligand. Control beads without biotinylated lipid were prepared in an identical manner but without the addition of the lipid. 5 μl of the ^{35}S-labeling reaction containing the *in vitro* transcribed/translated protein was then added to the tubes, and the protein was allowed to bind for 2 h at 4 °C with gentle agitation. The tubes were then centrifuged briefly, the beads were washed two times with 0.5 ml of wash/binding buffer, and the bound proteins were eluted by boiling the beads in 20 μl of Laemmli sample buffer containing 5% 2-mercaptoethanol. The proteins were then separated on a 13.75% SDS-polyacrylamide gel electrophoresis gel. Following electrophoresis the gel was soaked in EnHance (NEN Life Science Products) for 1 h and then in a 7.5% w/v solution of PEG-3350 for 1 h. The gel was then dried and subjected to autoradiography.

Competition Binding of Isolated Proteins for Nonbiotinylated Lipids over Biotinylated Lipids—Competition binding experiments were performed exactly as described above for standard binding of radiolabeled proteins to biotinylated lipids except that avidin beads, which had been prebound with the biotinylated diC_7 PtdIns-3,4,5-P_3 or PtdIns-3,4-P_2, were incubated with the *in vitro* translated proteins in the presence of 1 pM to 100 μM of nonbiotinylated lipids (diC_8 forms of PtdIns-3,4,5-P_3, PtdIns-3,4-P_2, and PtdIns-4,5-P_2 (Echelon Research Laboratories, Salt Lake City, UT)). Binding was quantitated by measuring the intensities of bands from the autoradiogram or by scintillation counting of the eluted proteins in Laemmli sample buffer using Opti-fluor (Packard Instrument Co.). Binding curves were modeled by the equation, % Bound = $(IC_{50}/(IC_{50} + C)) \times 100$, where % bound represents the quantity of protein bound to the biotinylated lipid coated beads in the presence of a concentration (*C*) of competing lipid. IC_{50} is the molar excess of competitor C required to reduce the percentage bound to 50% of its maximal value in the absence of competitor.

In Vitro Translation Expression Cloning—A murine spleen cDNA library containing 2×10^5 independent clones was divided into 1700 individual small pools (54). The cDNAs in this library had been cloned into the *in vitro* transcription vector pCS2(+) under control of the SP6 promoter. Individual cDNAs from positive pools were isolated from the other cDNAs of the pool using a 96-well format as described previously (47). The individual cDNAs were sequenced (Harvard Biopolymer Facility) and compared with known sequences by searching the NCBI nr data base using the BLAST algorithm.

Northern Blotting—Northern blotting was performed as described in Ref. 71. A ^{32}P-labeled double stranded DNA probe was made from a polymerase chain reaction fragment from the respective gene using random primed oligonucleotide synthesis (Prime-a-Gene, Promega). Murine tissue RNA was a gift of Dr. W. Swat (Harvard Medical School).

RESULTS AND DISCUSSION

Biotinylated Forms of PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 Specifically Bind to the in Vitro Translated PH Domain of Akt and Can Be Used for Affinity Isolation—In order to test whether cloning of phosphoinositide-binding proteins through coupled *in vitro* transcription/translation expression was feasible, we examined whether biotinylated forms of 3′-PPIs could be used to affinity isolate a known 3′-PPI binding domain that had been produced by a coupled *in vitro* transcription/translation system. We first prebound avidin-coated beads with diC_7-analogs of PtdIns-3,4,5-P_3 and PtdIns-3,4-P_2 that had been biotinylated on the ω-end of the *sn*-1-aminohexanoyl derivatives (see under "Experimental Procedures" and Fig. 1). The beads were then incubated with [^{35}S]methionine-labeled proteins generated by *in vitro* transcription/translation of cDNAs encoding either MBP or MBP fused to the PH domain of Akt. Both genes were

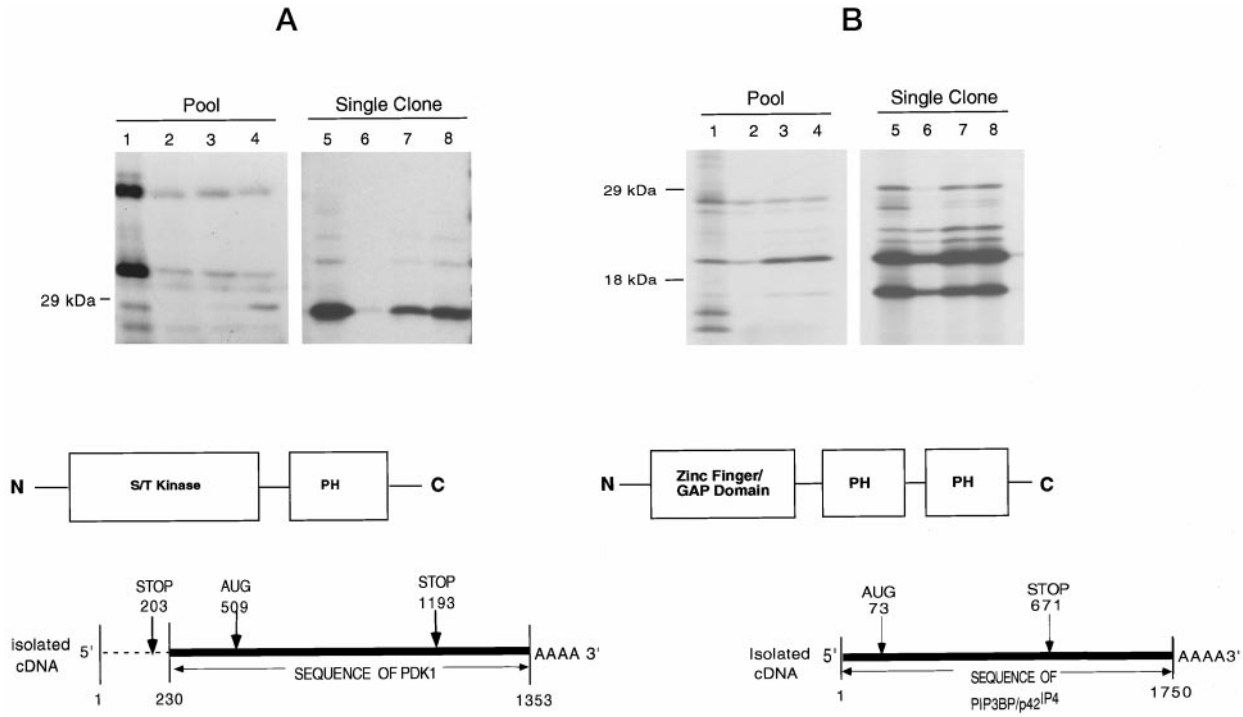

FIG. 3. **Isolation of murine isoforms of PDK1 and PIP3BP/p42^{IP4} from expression library via avidin beads prebound with biotinylated 3′-PPIs.** *A,* PDK1. *Top panel,* specific binding of PDK1 in total pool and as single clone. Shown are total ^{35}S-labeled proteins (*lanes 1* and *5*), labeled proteins bound to avidin beads (*lanes 2* and *6*), labeled proteins bound to avidin beads prebound with PtdIns-3,4-P_2-biotin (*lanes 3* and *7*), and labeled proteins bound to avidin beads prebound with PtdIns-3,4,5-P_3-biotin (*lanes 4* and *8*). *Bottom panel,* protein domain structure of PDK1 and corresponding cDNA fragment isolated from the expression library. Nucleotide positions of putative stop and start codons (AUG) are indicated. *B,* PIP3BP/p42^{IP4}. *Top panel,* specific binding of PIP3BP/p42^{IP4} in total pool and as single clone. Shown are total ^{35}S-labeled proteins (*lanes 1* and *5*), labeled proteins bound to avidin beads (*lanes 2* and *6*), labeled proteins bound to avidin beads prebound with PtdIns-3,4-P_2-biotin (*lanes 3* and *7*), and labeled proteins bound to avidin beads prebound with PtdIns-3,4,5-P_3-biotin (*lanes 4* and *8*). *Bottom panel,* protein domain structure of PIP3BP/p42^{IP4} and corresponding cDNA fragment isolated from the expression library. Nucleotide positions of putative stop and start codons (AUG) are indicated.

cloned into the *in vitro* transcription vector pCS2(+) under the control of the SP6 promoter. The PH domain of Akt is known to bind 3′-PPIs with high affinity and served as a positive control to determine optimal binding conditions and specificity (55–58). As shown in Fig. 2*A,* the fusion protein of MBP-Akt PH bound to avidin beads that were prebound with the biotinylated forms of either PtdIns-3,4,5-P_3 or PtdIns-3,4-P_2. The MBP-PH fusion protein failed to bind to the unmodified avidin beads, and MBP failed to bind to either the avidin beads alone or to the beads prebound with the biotinylated phosphoinositides. These data demonstrate that the biotinylated 3′-PPIs can effectively and specifically isolate an *in vitro* translated form of a phosphoinositide-binding domain.

The goal of this study is to isolate novel 3′-PPI-binding proteins from among pools of *in vitro* transcribed/translated proteins from a small pool expression library. In order to specifically isolate 3′-PPI-binding proteins from among other proteins expressed in the library, it is necessary that the system used for screening have a high specificity for 3′-PPI-binding proteins and a low background affinity for nonspecific binding proteins. The system must also be sufficiently sensitive to detect small amounts of a 3′-PPI-binding protein in a large background of nonspecific binding proteins. To determine whether our system conformed to these criteria, we investigated whether biotinylated PtdIns-3,4,5-P_3 and PtdIns-3,4-P_2 could specifically isolate the MBP-Akt PH domain fusion protein after having been diluted into a pool from the *in vitro* translation library.

There are approximately 100 independent clones in each pool of cDNA from our pCS2 mouse spleen cDNA library, and the total DNA concentration was approximately 1 μg/μl for each pool. Therefore, 10 ng of the plasmid encoding MBP-Akt PH fusion protein was mixed with 1 μg of DNA of a random pool from the library in order to approximate the amount of 3′-PPI-binding protein that would be found in a random pool under the conditions of our screen. As is shown in Fig. 2*B,* biotinylated PtdIns-3,4-P_2-beads captured the MBP-Akt PH fusion protein from among the other proteins in the pool. However, some proteins in the library, *e.g.* the 25-kDa protein, bound to the avidin beads in both the presence and absence of ligand, most likely through a nonspecific hydrophobic interaction. These results provided the proof of concept that this approach is feasible for isolation of 3′-PPI binding domains.

Finally we examined whether our binding conditions allowed the PH domain of Akt to distinguish between phosphoinositides phosphorylated at the 3′ position from phosphoinositides lacking a phosphate at the 3′ position. We performed competition binding experiments using unbiotinylated forms of PtdIns-3,4,5-P_3, PtdIns-3,4-P_2, and PtdIns-4,5-P_2 to compete for biotinylated PtdIns-3,4,5-P_3 binding to the MBP-Akt PH fusion protein (Fig. 2*C*). PtdIns-3, 4,5-P_3 and PtdIns-3,4-P_2 displaced the biotinylated PtdIns-3,4,5-P_3 at concentrations of 1000–10,000-fold lower than PtdIns-4,5-P_2, showing that the specificity of the Akt PH domain for the 3′-phosphate is preserved under our conditions. The data also shows that PtdIns-3,4,5-P_3 binds more tightly to the Akt PH domain than does PtdIns-3,4-P_2, and this is consistent with two published studies on the affinity of the PH domain of Akt for phosphorylated phosphoinositides, which rank the order of affinities as PtdIns-3,4,5-P_3 > PtdIns-3,4-P_2 ≫ PtdIns-4,5-P_2 (55, 56). This result demonstrates that our system preserves the specificity of protein binding to different species of phosphorylated phosphoinositides.

Isolation of the Murine Isoforms of PDK1and PIP3BP/p42^{IP4} by Small Pool Expression Cloning—After an initial screening of 500 pools of the library using the biotinylated phosphoinositides, we isolated three 3′-PPI-binding proteins. We observed a protein of approximately 25 kDa in a single pool that appeared to bind exclusively to biotinylated PtdIns-3,4,5-P_3 (Fig. 3*A, top panel, left*). When the cDNA for the protein was isolated from the other cDNAs in the pool, the expressed purified protein was also found to bind to PtdIns-3,4-P_2 but apparently with a lower affinity than for PtdIns-3,4,5-P_3 (Fig. 3*A, top panel, right*). Sequencing of the cloned cDNA revealed that it was identical to the C-terminal 319 amino acids of murine PDK1 (59). PDK1 is a ubiquitously expressed 559-amino acid (65 kDa) protein that contains an N-terminal serine/threonine kinase domain and a C-terminal PH domain. In agreement with our results, the PH domain of PDK1 has recently been found to bind with a 4-fold higher affinity to PtdIns-3,4,5-P_3 than to PtdIns-3,4-P_2 but with much lower affinity to PtdIns-4,5-P_2 (60). The fragment of PDK1 that we isolated contains the entire C-terminal PH domain and approximately half of the serine/threonine kinase domain (Fig. 3*A, bottom panel*). However, the first 230 nucleotides of the isolated mRNA are not the sequence of PDK1, and the first in-frame AUG codon appears at the end of the kinase domain such that only the entire PH domain of PDK1 is translated. The initial 230 noncoding nucleotides could either be due to an alternative splicing of PDK1 or be an artifact from the construction of the library.

Another 3′-PPI-binding protein identified in our screen was a protein of approximately 25 kDa that bound tightly to both biotinylated PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 but also had some residual binding to the avidin beads (Fig. 3*B, top panel*). Upon isolation and sequencing of the cDNA from the pool, the gene was found to encode the C-terminal 210 amino acids of a protein that has >95% amino acid homology to 42-kDa inositol-1,3,4,5-tetrakisphosphate-binding proteins isolated from both porcine brain, called p42^{IP4} (61), and bovine brain, called PIP3BP (62). PIP3BP/p42^{IP4} is a protein of 374 amino acids that contains an N-terminal zinc-finger domain that has homology to GTPase activating proteins for the ARF family of small G-proteins and two tandem PH domains at the C-terminal end of the protein (Fig. 3*B, bottom panel*). The fragment of the gene that we isolated contains the entire C-terminal PH domain and approximately half of the N-terminal PH domain but contains none of the putative ARF-GTPase-activating protein domain. The C-terminal PH domain contains a consensus sequence for high affinity binding of 3′-PPIs (41, 42). The N-terminal PH domain does not contain this sequence, but two studies have shown that it displays some specificity for 3′-PPIs (62, 63).

The function of PIP3BP/p42^{IP4} is currently unknown, although it is highly expressed in the brain and is thought to play a role in vesicle and membrane transport because of the putative ARF-GTPase-activating protein domain. Interestingly, a closely related yeast protein called Gcs1 has been shown to be an ARF-GTPase-activating protein and is important in proper cytoskeletal organization and actin polymerization in yeast (64). A recent study has also shown that PIP3BP is localized to the nucleus but translocates to the plasma membrane upon activation of PI 3′-K; however, the functional significance of both the nuclear localization and the PI 3′-K-dependent translocation is unknown (65).

Isolation of 3′-Phosphoinositide-interacting SH2-containing Protein (PHISH), a Novel 3′-PPI-binding Protein Containing Both PH and SH2 Domains—We isolated a novel 3′-PPI-binding protein from the library that migrated at approximately 30 kDa and, similar to PIP3BP/p42^{IP4}, bound tightly to both biotinylated PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 but not to the avidin beads alone (Fig. 4*A*). In competition binding studies, the *in vitro* transcribed/translated protein was found to bind with equal affinity to both PtdIns-3,4,5-P_3 and PtdIns-3,4-P_2 but with significantly lower affinity to PtdIns-4,5-P_2, thus demonstrating the specificity of the protein for 3′-PPIs (Fig. 4*B*).

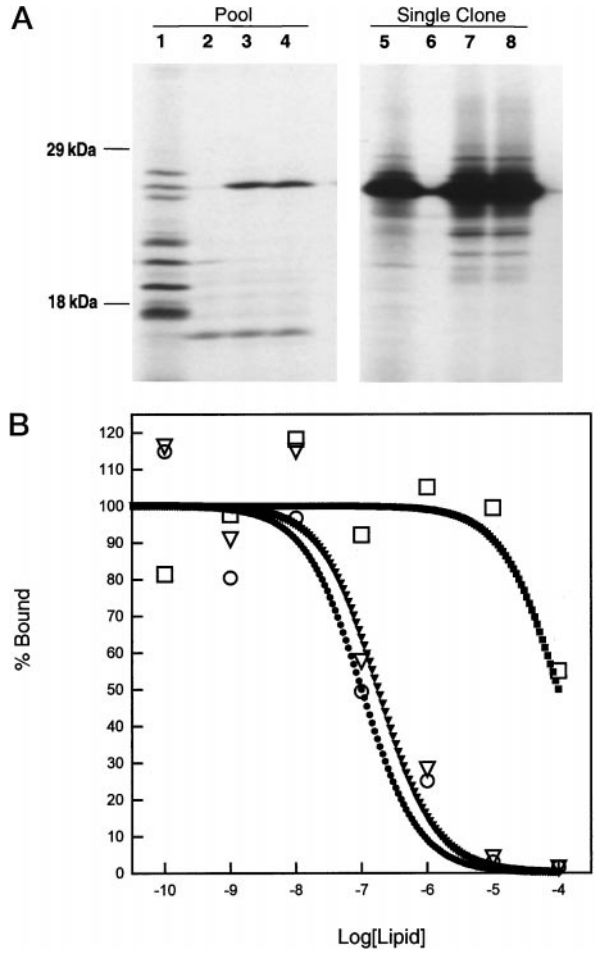

FIG. 4. **Isolation of PHISH from expression library via avidin beads prebound with biotinylated 3′-PPIs.** *A,* specific binding of PHISH in total pool and as single clone. Shown are total ^{35}S-labeled proteins (*lanes 1* and *5*), labeled proteins bound to avidin beads (*lanes 2* and *6*), labeled proteins bound to avidin beads prebound with PtdIns-3,4-P_2-biotin (*lanes 3* and *7*), and labeled proteins bound to avidin beads prebound with PtdIns-3,4,5-P_3-biotin (*lanes 4* and *8*). *B,* PtdIns-3,4,5-P_3 and PtdIns-3,4-P_2 preferentially displace PHISH from PtdIns-3,4,5-P_3-biotin bound to avidin beads. ^{35}S-labeled PHISH was bound to avidin beads coated with PtdIns-3,4,5-P_3-biotin in the presence of the indicated concentrations of PtdIns-4,5-P_2 (*squares*), PtdIns-3,4-P_2 (*circles*), or PtdIns-3,4,5-P_3 (*triangles*) and processed as described under "Experimental Procedures." *Data points* represent the mean of two independent experiments.

The gene fragment containing the sequence for the protein was approximately 1.3 kilobases in length and encoded an open reading frame from the beginning of the fragment to a stop codon at nucleotide position 927 (Fig. 5). A putative Kozak initiator (ATG) codon for methionine is present at position 87, and initiation of translation from this codon is consistent with the observed the size of the translated protein (280 amino acids and 30 kDa). However, because the sequence upstream of this ATG did not contain any in-frame stop codons, it was difficult to determine whether the isolated gene fragment encoded the entire coding sequence of the gene or whether more coding sequences existed upstream.

The amino acid sequence contains coding regions for both an SH2 domain and a C-terminal PH domain. The SH2 domain, which encompasses nucleotides 200–470, is most similar to the

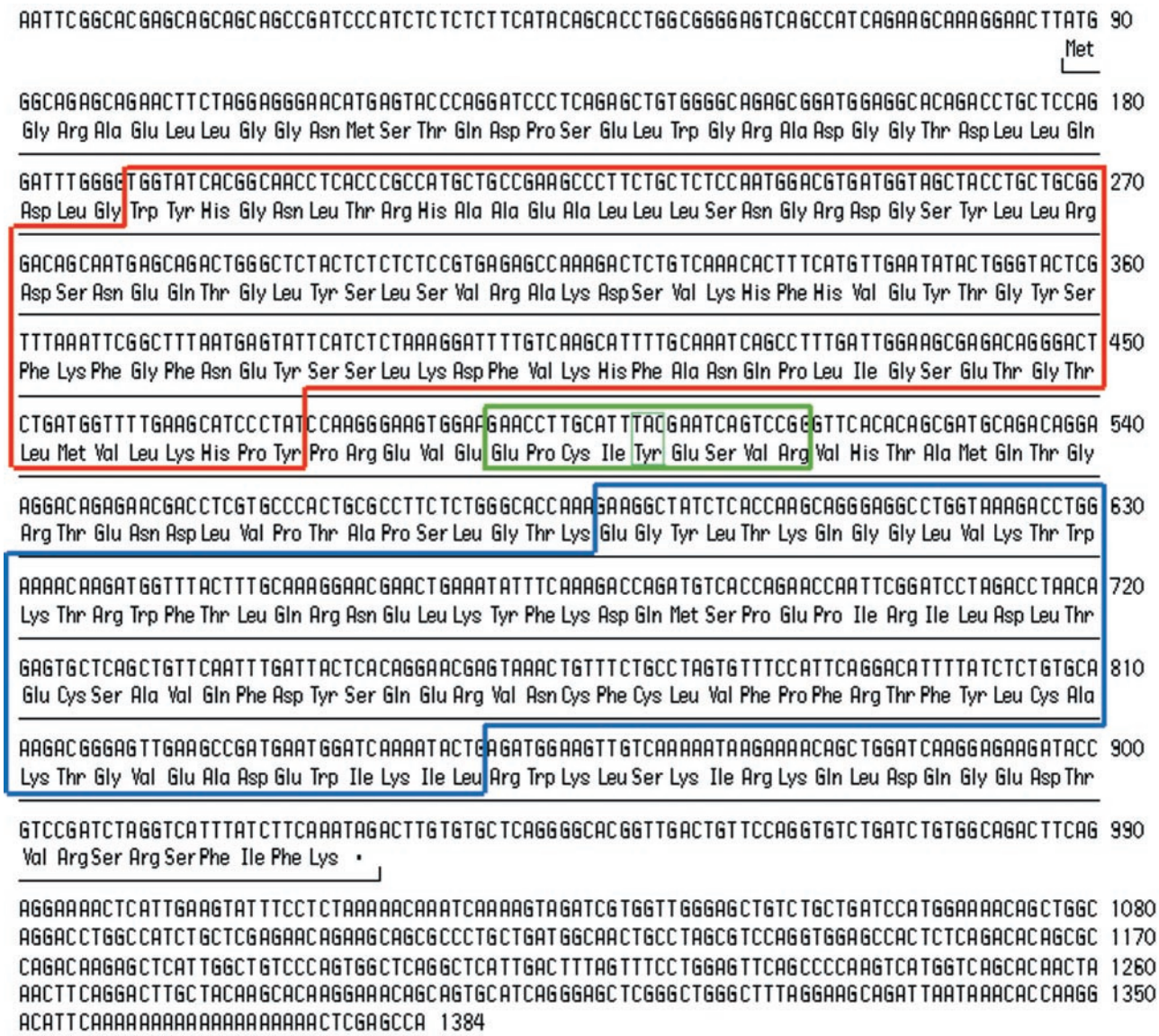

FIG. 5. **Nucleotide and amino acid sequence of murine PHISH.** The sequence of PHISH was isolated via expression cloning. The putative SH2 domain is outlined in *red,* the predicted tyrosine phosphorylation site is outlined in *green,* and the putative PH domain is outlined in *blue*.

SH2 domain of the neural adaptor protein Nck (35% identical, 59% homologous at the amino acid level). The PH domain, which encompasses nucleotides 590–840, is most similar to the PH domain of Akt (39% identical, 63% homologous at the amino acid level) and contains the consensus sequence for high affinity binding of 3′-PPIs (42). There is also a tyrosine (Tyr-139) located between the SH2 and PH domains that could be phosphorylated in stimulated cells because the sequence surrounding this tyrosine is a putative consensus motif for phosphorylation by tyrosine kinases (66). The existence of a putative phosphotyrosine-binding SH2 domain, a 3′-PPI-binding PH domain, and a sequence for phosphorylation of tyrosine strongly suggests that this protein plays a role in cellular signaling. We have named this protein PHISH for 3′-phosphoinositide-interacting SH2-containing protein.

In order to determine the tissue distribution of PHISH, we performed Northern blots on total RNA from several murine tissues, spleen, brain, heart, lung, thymus, and lymph using a probe derived from nucleotides 87–927, the putative coding region of the protein. Two RNA species that are approximately 1.2–1.4 kilobases were detected. These could represent products of alternative splicing of the gene. The larger of the two transcripts was detected in all tissues, and the smaller of the two transcripts was highly expressed in spleen and at lower levels in both heart and lung tissue (Fig. 6). *In vitro* transcription of the cDNA for PHISH isolated from the expression library also produced two transcripts that were approximately 1.2–1.4 kilobases in size. The major transcript is very close in size to the larger of the two transcripts present in all murine tissues tested, whereas the minor product is close in size to the smaller transcript present in heart, lung, and spleen. This data suggests that the gene fragment isolated from the expression cloning library encodes the complete sequence of the gene.

Upon searching the human EST data base with the nucleotide sequence of PHISH, we found that PHISH had an 87% nucleotide sequence identity with that of a human EST derived from stem cells (locus *AF150266*). This EST encodes a cDNA that is 1.4-kilobases long and contains entire coding region of PHISH. The sequences of PHISH and the human EST differ significantly outside of the protein coding sequence. Moreover, three codons upstream from the putative start codon in the human EST are in frame stop codons. The high degree of sequence homology between PHISH and the human EST implies that they encode species-specific homologues of the same

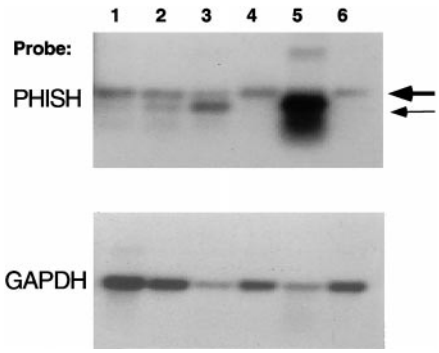

FIG. 6. **Northern blot of mRNA from various murine tissues hybridized to probes derived from PHISH and glyceraldehyde-3-phosphate dehydrogenase.** Murine tissue RNA (10 μg per lane) was subjected to agarose gel electrophoresis, transferred to nylon membrane, and hybridized to ^{32}P-labeled probes derived from the coding regions of PHISH or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as described under "Experimental Procedures." *Lane 1,* brain; *lane 2,* heart; *lane 3,* lung; *lane 4,* lymph node; *lane 5,* spleen; *lane 6,* thymus. *Arrows* represent the mobility of the major (*large arrow*) and minor (*small arrow*) products of an *in vitro* transcription reaction of the PHISH gene isolated from the expression library.

protein and that the gene for PHISH isolated from the expression screen encodes the entire protein.

Because PHISH contains only a PH domain and an SH2 domain, it is possible that PHISH functions as an adaptor protein. The PH domain could dock PHISH to 3′-PPI generated at membrane receptor complexes, and the SH2 domain could recruit phosphotyrosine containing protein(s) to such complexes. It is interesting to note that Skolnik and co-workers (42, 67) have isolated the PH domain of EST684797 from a homology search of human ESTs for PH domains that would be predicted to bind tightly to 3′-PPIs. They have also shown that the PH domain of EST684797, which is the human homolog of PHISH, binds tightly and selectively to PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 but not to PtdIns-4,5-P_2.

These studies demonstrate that coupled *in vitro* transcription/translation libraries can be used in conjunction with affinity isolation technology using synthetic phosphoinositides to isolate 3′-PPI binding domains. Recently, several other methods have been described to isolate and clone 3′-PPI-binding proteins. One example is the use of resins coupled to phosphorylated inositol phosphates, such as inositol 1,3,4,5-tetrakisphosphate, or inositol phosphates linked to a glycerol moiety, to extract proteins from tissue extracts (61, 62, 68). Using these techniques, PIP3BP and p42^{IP4} were extracted from total brain extracts. However, proteins expressed in low abundance are difficult to isolate by this procedure, and furthermore, cDNA cloning requires multiple steps following affinity isolation.

Expression cloning of genes from cDNA libraries can circumvent some of the limitations of protein affinity isolation techniques. Two expression cloning techniques have been developed recently to identify 3′-PPI-binding proteins. The first involved the screening of a λgt11 library with a crude mixture of brain phospholipids that had been phosphorylated *in vitro* by PI 3′-K (69). The screening of two murine-derived cDNA libraries (from brain and adipocytes) yielded only one protein that bound tightly to PtdIns-3,4,5-P_3. This protein, called Grp1 (*g*eneral *r*eceptor for phosphoinositides), contained a PH domain and an ARF-guanine nucleotide exchange factor domain that catalyzes 3′-PPI-dependent nucleotide exchange on mammalian ARFs (70). However, no other 3′-PPI-dependent binding protein was isolated in the screen, possibly due to improper folding of the proteins.

Another recent expression cloning strategy for isolating 3′-PPI-binding proteins is the use of a modified yeast two-hybrid system (42). In this system, genes from a mammalian cDNA library were fused to a constitutively active Ras. The fusion proteins were then tested for their ability to rescue a temperature-sensitive phenotype, due to a defect upstream of Ras, when coexpressed with constitutively active PI 3′-K. This system worked well in experiments in which constitutively active Ras was fused to PH domains already known to bind to 3′-PPIs. However, when tested with an expression library in yeast, only Aktγ was isolated.

The screen described here is the first to isolate multiple 3′-PPI binding targets from an expression library, including a novel protein. The basis for the better efficiency of this screen may stem from the fact that *in vitro* translated proteins are more likely to be properly folded. In addition, many internal initiation sites for translation allow for the independent and unoccluded expression of PH and other 3′-PPI binding domains. We also used analogs of PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 that are synthetically pure and, as a result of modification in a distal region of the acyl chain, closely resemble the phosphorylated phosphatidylinositols that occur naturally. It has been shown that although the main binding of the protein is to the inositol head group, the contribution of the glycerol chain and the fatty acid side chains of the phosphatidylinositol are essential for specificity and tight binding to the protein (41). Our screening technique is not without drawbacks. For example, there can be nonspecific binding of proteins to the avidin-coated beads, and this nonspecific binding may obscure the binding of other 3′-PPI-binding proteins with the same electrophoretic mobility. In addition, we isolated only C-terminal PH domains, possibly because our library was made using oligo(dT) to prime cDNA synthesis from the 3′ poly(A) tail of mRNAs. In addition, our screen thus far has isolated only strong binders of 3′-PPIs, indicating that our binding conditions may be too stringent to accommodate weaker binding proteins.

In summary, we have described a novel and effective way of isolating and cloning 3′-PPI-binding proteins from expression libraries. The technique described here has broad applications for the isolation of binding partners for other phosphoinositide polyphosphates or other lipid products.

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Note Added in Proof—While this manuscript was in review, Alessi and co-workers (72) identified the cDNA for the mouse isoform of DAPP1, which is identical to the cDNA of PHISH described in our report. The National Center for Biotechnology Information accession number for mouse DAPP1 gene is *AF163255*.

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Expression Cloning of Protein Targets for 3-Phosphorylated Phosphoinositides

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