Phosphoinositide Binding by the Pleckstrin Homology Domains of Ipl and Tih1*

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The Ipl protein consists of a single pleckstrin homology (PH) domain with short N- and C-terminal extensions. This protein is highly conserved among vertebrates, and it acts to limit placental growth in mice. However, its biochemical function is unknown. The closest parologue of Ipl is Tih1, another small PH domain protein. By sequence comparisons, Ipl and Tih1 define an outlying branch of the PH domain superfamily. Here we describe phosphatidylinositol phosphate (PIP) binding by these proteins. Ipl and Tih1 bind to immobilized PIPs with moderate affinity, but this binding is weaker and more promiscuous than that of prototypical PH domains from the general receptor for phosphoinositides (GRP1), phospholipase C δ1, and dual adaptor for phosphoinositides and phosphotyrosine 1. In COS7 cells exposed to epidermal growth factor, green fluorescent protein (GFP)-Ipl and GFP-Tih1 accumulate at membrane ruffles without clearing from the cytoplasm, whereas control GFP-GRP1 translocates rapidly to the plasma membrane and clears from the cytoplasm. Ras*-Ipl and Ras*-Tih1 fusion proteins both rescue cdc25ts Saccharomyces cerevisiae, but Ras*-Ipl rescues more efficiently in the presence of phosphatidylinositol 3-kinase (PI3K), whereas PI3K-independent rescue is more efficient with Ras*-Tih1. Site-directed mutagenesis defines amino acids in the β1-loop1-β2 regions of Ipl and Tih1 as essential for growth rescue in this assay. Thus, Ipl and Tih1 are bona fide PH domain proteins, with broad specificity and moderate affinity for PIPs.

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† The abbreviations used are: PH, pleckstrin homology; PHD, PH domain; BTK, Bruton’s tyrosine kinase; CH1, chains; DAPP1, dual C-terminal extensions. It shares this structure with its closest relative, Tih1, an even smaller protein, which is broadly expressed and not regulated by imprinting (2).

The PH domain is a ~100-amino acid module defined by sequence homology, which has a conserved tertiary structure of a β-sandwich of 3 + 4 stranded β sheets with variable intervening loops, followed by a single amphipathic α helix (3). Most PH domain-containing proteins can bind to certain phosphatidylinositol phosphate lipids (PIP’s), but their binding affinities for the specific products of phosphoinositide metabolism vary greatly (4). For example, the PH domains from phospholipase C δ1 (PLCδ1) bind strongly to PtdIns(4,5)P₂, but not to the downstream metabolites of this compound, PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃, which are produced by the action of phosphatidylinositol 3-kinase (PI3K). Other PH domains, typified by that of the general receptor for phosphoinositides (GRP1), show the opposite pattern of binding affinity, with high affinity for PtdIns(3,4,5)P₃ and only low affinity for PtdIns(4,5)P₂. Based on in vitro and in vivo assays, Kavran et al. (5) classified PH domains into two groups: a small class of PH domains with high affinity and specificity for particular PIPs and a much larger class showing low affinity promiscuous binding to various PIPs. More recently, Maffucci and Falasca (6) have proposed a further division of PH domains into three groups. In this scheme, Group 1 proteins are those with high affinities for specific PIPs; Group 2 proteins have lower but still measurable affinity for phosphoinositides, and show less ability to discriminate among the various PIPs; and Group 3 proteins have minimal affinity for phosphoinositides, with no detectable specificity.

Consistent with their varying interactions with phosphoinositides, and with their occurrence alongside a variety of other modules, PH domains have diverse functions. Among these are...
roles in signal transduction downstream of membrane receptors, control of membrane vesicle trafficking in the cell, targeting of phospholipid-modifying enzymes to cellular membranes, and control of cytoskeleton-membrane interactions (3, 7). These functions are unified by the ability of PH domains to associate with the plasma membrane and/or intracellular membranes through their high or low affinity binding to phosphoinositides. Certain PH domains can also mediate protein-protein interactions, for example that which occurs between the beta-adrenergic receptor protein kinase, 

\[ \text{PKA} \to \text{beta-AR} \]

and the \( \gamma \) subunit of G-proteins (8). But the protein-binding site is distinct from the phosphoinositide binding region, and a PH domain-phosphoinositide interaction may be simultaneously required for physiological function (9).

Interactions of PH domains with phosphoinositides have been examined in vitro and in vivo by several complementary approaches. These include direct binding of GST-PH domain fusion proteins to phosphoinositides in lipid vesicles or immobilized on nitrocellulose membranes (5, 10). Biotinylation on nitrocellulose membranes (5, 10) is required for physiological function (9).

Domain-phosphoinositide interaction may be simultaneously required for the protein superfamily, but that they are comparable with other PH domains. The data indicate that Ipl and Tih1, we have used each of these approaches to compare selectivity for the known phosphoinositide lipids. The data indicate that Ipl and Tih1 define an outlying phylogenetic branch of the PH domain superfamily, but that they are bona fide PHP binders, which fit best into the group with moderate affinity and poor selectivity for the known phosphoinositides.

**EXPERIMENTAL PROCEDURES**

**Materials**—PIP strips and PIP arrays were purchased from Echelon Research Laboratories (Salt Lake City, UT); nitrocellulose membranes were from Osmonics Inc. (Westborough, MA); enhanced chemiluminescence reagents, anti-mouse- or anti-rabbit-linked to fluorescein isothiocyanate (Sigma) or TRITC (Southern Biotech Association, Inc.), anti-Ras (sc-29, Santa Cruz Biotechnology Inc.), anti-FLAG M2 (Sigma) and anti-EGFP-C1 (N-terminal GFP, Clontech, Palo Alto, CA), anti-FLAG-tagged constructs. The primer sequences for making the GFP- or EGFP constructs was done using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primer sequences for making the GFP- or EGFP constructs was done using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

**Expression of GST-PH Domains in E. coli**—The pGEX-2TK constructs encoding the PH domains of Ipl, Tih1, GRP1, PLC-1, or DAP1, or the GST vector alone, were transformed into BL21 E. coli (Invitrogen), and a 1-liter culture was grown at 30 °C, with aeration of 0.5. The cells were harvested by centrifugation for 30 min at 4 °C, and washed once with PBS at pH 7.4. The cells were resuspended in 25 ml of ice-cold Buffer A: 10 mM Tris-HCl, pH 7.4, 1 mM NaCl, 1% Triton X-100, 1 mM DTT, 0.5 mM PMFS, and Complete protease inhibitor mixture (one tablet per 25 ml; Roche Diagnostics), lysed by one round of freeze-thawing, and the lysates were sonicated on ice at power 3 (Sonic Dismembrator P500, Fisher Scientific, Pittsburgh, PA) supplemented with 50 μg/ml RNase and 10 μg/ml DNase I. The lysates were centrifuged at 20,000 g for 30 min at 4 °C, and the supernatant was then filtered through a 0.45-μm filter and incubated for 40 min on a rotating platform with 200 μl of glutathione-Sepharose pre-equilibrated in Buffer A. The suspension was centrifuged for 1 min at 3,000 × g, and the beads were washed three times with 15 ml of Buffer A containing 0.5 mM NaCl, 20% glycerol and then a further six times with 15 ml of Buffer B: 20 mM Tris-HCl, pH 7.5, 20% glycerol, 100 mM KCl, 0.1% Triton X-100, 1 mM DTT, 0.5 mM PMFS, and protease inhibitor mixture. The protein was eluted from the resin at 4 °C by incubation with 0.5 ml of Buffer B containing 20 μl glutathione, one, and any contaminating beads were removed by filtration through
a 0.44-μm filter. The eluate was divided into aliquots and stored at −80 °C.

Filter Overlay Assays—To assess the PIP-binding properties of each PH domain, a protein–lipid overlay assay was performed using the GST fusion proteins and PIP strips or PIP arrays. The membranes were blocked in 3% (w/v) fatty acid-free BSA in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% (v/v) Tween 20) for 1 h and then incubated overnight at 4 °C with gentle stirring in the same solution containing either 20 or 200 ng/ml of the indicated GST fusion protein. The membranes were washed six times over 30 min in TBST and then incubated for 1 h with a 1:1000 dilution of anti-GST monoclonal antibody. The membranes were washed as before, then incubated for 1 h with 1.5000 dilution of anti-mouse-herosarder phospholipid conjugate. The membranes were washed six times over 30 min in TBST and then incubated with the indicated GST fusion protein. The membranes were incubated for 40 min in CST at 25 °C then and then heat-treated at 120 °C for 30 min with intermitting mixing. Cells were resuspended in 200 μl of water and plated on glucose minimal medium lacking uracil and leucine (0.12 yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 1% succinic acid, 0.6% NaOH, 2% glucose, amino acid mix lacking leucine and uracil, and 2% agar). To assay for rescue of the cdc25ts allele, transformants were split into two aliquots and plated equably onto two selective plates. Plates were incubated for 48 h at 25 °C, and then one plate was transferred to 37 °C while the other was left at 25 °C. Growth of the transformants was assessed after an additional 48 h for a side-by-side comparison of multiple constructs, the yeast were released in sectors. From the plates at 25 °C, a single colony was grown for 4–5 h in yeast culture medium and stained with DAPI to visualize the membrane ruffles, the cells were counterstained with Alexa Fluor™ 568 phalloidin (Molecular Probes, Eugene, OR), which labels F-actin. In other experiments, the cells were replated on SuperFrost™ Plus microscope slides (Fisher Scientific) at 24 h post-transfection and allowed to grow for another 24 h in Complete medium before immunofluorescence staining.

Isolation of Protein from Yeast and Immunoblotting of Yeast Extracts—Yeast co-transformed with Ras*-PH domain fusion proteins and either active or inactive farnesylated PYES2-p110 were grown to log phase (20 h at 25 °C), lysed with prewarmed (70 °C) cracking buffer (8 n urea, 5% SDS, 40 mM Tris-HCl, pH 6.8, 0.1 mM EDTA, 1% β-mercaptoethanol, 0.4 mg/ml bromphenol blue) by vortexing with acid-washed glass beads (425–600 μm). Proteins were separated by SDS-PAGE and immunoblotted with appropriate antibodies at 1 μg/ml concentration. The blots were then incubated with secondary anti-mouse-herosarder phospholipid antibodies, and Ras*-PHD expression was detected by ECL.

Phylogenetic Analysis of PH Domain Sequences—Taking the PH domain alignment (GenBank™ accession number PF00169) from the PFAM 7.3 data base as guidance, about 600 mammalian PH domain sequences were extracted from the EMBL data bank. Data base entries were manually screened for quality of annotation, and only 245 of them were used for the subsequent analysis. These sequences, along with the human and mouse Ipl and Tih1 PH domain sequences, were aligned using the ClustalW program (28). Phylogenetic analysis of alignment was performed by neighbor-joining method using the MEGA software (29). The bootstrap values were calculated as the percentage of 1000 replicates resulting in a tree identical to the initial one. The bootstrap method was used to estimate the confidence levels of inferred relationships (32). The resulting tree was further processed for easy visualization by MEGA.

**Results**

Evolutionary Conservation of the Ipl and Tih1 PH Domain Sequences—The domain structure of Ipl and Tih1 is shown in Fig. 1A. Both proteins consist of a single PH domain, which is flanked by short N- and C-terminal extensions that lack recognizable functional motifs. A scan of the protein data base (available at smart.EMBL-Heidelberg.de) revealed only one other full-length PH domain protein (out of more than 400 mammalian PH domain sequences) that was similar in size to Ipl and Tih1. This was PHR1, a membrane-associated protein expressed in retinal photoreceptor cells, which does not show comparable functional motifs. A scan of the protein data base (available at smart.EMBL-Heidelberg.de) revealed only one other full-length PH domain protein (out of more than 400 mammalian PH domain sequences) that was similar in size to Ipl and Tih1. This was PHR1, a membrane-associated protein expressed in retinal photoreceptor cells, which does not show comparable functional motifs.
PH domains are highly diverse in sequence. However, there are several conserved amino acids that can act as landmarks to allow the recognition, alignment, and comparison of diverse PH domains. Phylogenetic analysis revealed that Ipl and Tih1 define an outlying branch of the PH domain superfamily (Fig. 1B). Bootstrap values, which show the significance of the corresponding branching point, were high within the groups of similar genes (i.e. Ipl and Tih1) but much lower for the branching points defining the relationships between these groups. This uncertainty in branching order of higher groups suggests early domain duplication and divergence events leading to the current PH domain superfamily gene content. Consistent with this conclusion, the Ipl protein is strikingly conserved across vertebrates, including fish, frog, chicken, mouse, and human (Fig. 1C), with highest conservation within the PH domain and lower conservation in the short N- and C-terminal flanking sequences. Thus, the Ipl gene arose early in vertebrate evolution, and its sequence, particularly within the PH domain, became fixed prior to divergence of the amphibians.

**Predicted PIP-binding Sequences in Ipl and Tih1**—Because the phosphoinositide binding affinity and specificity of several well-studied PH domains depends on the amino acid identities in the β1-loop1-β2 region, we next focused on sequences in this region. As shown in Fig. 1D, the presence of sequence landmarks allows an unambiguous alignment of Ipl and Tih1 with other PH domains that have known PIP-binding properties. Two features are noteworthy: First, based on x-ray crystallography and site-directed mutagenesis, a series of amino acids at the beginning of the β2 sheet define the ability of PH domains to bind with high affinity to PtdIns(3,4,5)P3 and PtdIns(3,4)P2. In Fig. 1D, **yellow** represents interaction with the 1-phosphate; **red**, with the 3-phosphate; **green**, with the 4-phosphate; and **blue**, with the 5-phosphate (34). Several of these critical residues are present at the corresponding positions in Ipl and Tih1 (boldface in Fig. 1D), suggesting that these two proteins will bind to PIPs. A hint of binding preference can also be gleaned from the alignments: notably, the bulky tryptophan residue at position 24 in Tih1 and position 35 in Ipl aligns with the tryptophan at position 36 in the PtdIns(4,5)P2-binder PLCβ.

The presence of this bulky moiety, which creates steric hindrance in PLCβ, excluding PtdIns(3,4,5)P3 binding, contrasts with the uniform presence of a smaller residue (Thr or Asn) at this position in the PH domains that bind selectively to PtdIns(3,4,5)P3. Finally, DYN1 and PLS-N are examples of PH domains. Phylogenetic analysis of PH domains. The original tree was assembled using MEGA software for 245 sequences of PH domains; only 38 of them are shown here. Numbers at the internal nodes represent bootstrap branching quality estimations for the corresponding nodes. C, sequence alignment of vertebrate IPL proteins. Human (h), mouse (m), chicken, frog (*Xenopus laevis*), medakafish, and zebrafish sequences are shown. Identities are in white lettering on a black background, and similarities are in gray. The GenBank™ accession numbers are: human (AF001294) and mouse (AF002708). The complete frog (*X. laevis*) IPL sequence was corrected by sequencing reverse transcription-PCR products from Xenopus stage 20 RNA (GenBank™ accession number: BK792027). The sequence alignment was performed using PIMA1.4 (40) and was adjusted manually. The PH domain is underlined. D, alignment of the Ipl and Tih1 β1 strand, variable loop 1 (VL1), and β2 strands with those of other PH domains that bind PI3K products with high, moderate, or low affinity. The amino acid making contacts with phosphate groups of PIPs are color-coded: **yellow** represents interaction with the 1-phosphate; **red**, with the 3-phosphate; **green**, with the 4-phosphate; and **blue**, with the 5-phosphate. Residues making double contacts are shaded in the corresponding two colors. Site-directed mutagenesis of Arg-38 (pink) also resulted in reduced Ins(1,4,5)P3 binding.

**Fig. 1.** Sequence analysis of the Ipl and Tih1 PH domains. A, domain structure of Ipl and Tih1. The PH domain was defined by SMART version 3.4 (available at smart.embl-heidelberg.de/smart). B, sequence alignment of vertebrate IPL proteins. Human (h), mouse (m), chicken, frog (*Xenopus laevis*), medakafish, and zebrafish sequences are shown. Identities are in white lettering on a black background, and similarities are in gray. The GenBank™ accession numbers are: human (AF001294) and mouse (AF002708). The complete frog (*X. laevis*) IPL sequence was corrected by sequencing reverse transcription-PCR products from Xenopus stage 20 RNA (GenBank™ accession number: BK792027). The sequence alignment was performed using PIMA1.4 (40) and was adjusted manually. The PH domain is underlined. D, alignment of the Ipl and Tih1 β1 strand, variable loop 1 (VL1), and β2 strands with those of other PH domains that bind PI3K products with high, moderate, or low affinity. The amino acid making contacts with phosphate groups of PIPs are color-coded: **yellow** represents interaction with the 1-phosphate; **red**, with the 3-phosphate; **green**, with the 4-phosphate; and **blue**, with the 5-phosphate. Residues making double contacts are shaded in the corresponding two colors. Site-directed mutagenesis of Arg-38 (pink) also resulted in reduced Ins(1,4,5)P3 binding.
domains that bind to known phosphoinositides with very low affinity. Based on the sequence alignments (Fig. 1D), Ipl and Tih1 do not resemble these proteins.

**PIP Binding by the Ipl and Tih1 PH Domains in Overlay Assays**—To directly assess the PIP binding properties of Ipl and Tih1, we carried out protein-lipid overlays, using GST fusion proteins containing the PH domains of Ipl and Tih1 to probe nitrocellulose membranes containing bound PIPs. GST alone and GST fusions of the PH domains of GRP1, PLC/H9254, and DAPP1 were used as controls. The controls gave results consistent with their known binding specificities: GST alone did not bind; GST-GRP1 bound primarily to PtdIns(3,4,5)P3, GST-PLC81 bound primarily to PtdIns(4,5)P2, and GST-DAPP1 bound to both PtdIns(3,4)P2 and PtdIns(3,4,5)P5 (Fig. 2A). At moderately high concentrations (200 ng/ml), both GST-Ipl and GST-Tih1 were capable of binding to most PIPs, including PtdIns(4,5)P2 and PtdIns(3,4,5)P5, as well as several of the PtdIns monophosphates (Fig. 2A), but, in contrast to the control GRP1 and PLC8 constructs, neither GST-Ipl nor GST-Tih1 bound specifically to any single PIP (Fig. 2A). The higher affinity of the GRP1 and PLC8 constructs for their cognate PIPs was emphasized by repeating the binding assay using PIP arrays, which contained serial dilutions of the PIPs (Fig. 2B). Notably, although GST-Ipl bound well to PtdIns(3,4,5)P5 and to PtdIns monophosphates when it was overlaid at a concentration of 200 ng/ml, it bound poorly at 20 ng/ml, a protein concentration that was sufficient for strong and specific binding to PtdIns(3,4,5)P5. Tih1 did not show a preference for binding PtdIns(3,4,5)P5 at either protein concentration and, instead, showed promiscuous PIP binding at both concentrations (Fig. 2B).

**Fig. 2.** Protein-lipid overlay assays. A, analysis using PIP strips. Membranes containing the indicated phospholipids (100 pmol per spot) were incubated with the purified GST fusion proteins at 200 ng/ml. The membranes were washed, and GST fusion proteins were detected using anti-GST antibody. The duplicated PIPs (PtdIns(4)P and PtdIns(4,5)P2) are from an animal source (lower spot) and synthetic (upper spot). B, analysis using PIP arrays. The indicated phospholipids were spotted onto a nitrocellulose membrane in concentrations from 100 to 1.6 pmol per spot in a serial 2-fold dilution. Membranes were incubated with the purified GST fusion proteins at the indicated concentrations. FL, full-length protein; PH, PH domain only.
Ipl and Tih1 PH Domains Accumulate at Membrane Ruffles of COS7 Cells in Response to EGF—Because the lipid binding assay showed that both Ipl and Tih1 have a detectable affinity for PIPs, we next asked whether the Ipl and Tih1 PH domains associated with cell membranes in vivo. First, the subcellular localization of GFP-, Myc-His-, and FLAG-tagged Ipl and Tih1 proteins was surveyed in transfected COS7 cells, growing in the continuous presence of serum. All of the Tih1 constructs were observed concentrated at membrane ruffles, in a pattern identical to that produced by GFP-PLCγ1 and unlike that produced by the low affinity PIP binder, GFP-DYN1 (data not shown). The tagged Ipl constructs also highlighted membrane ruffles but were more prominent in the cytoplasm. This is consistent with the predominantly cytoplasmic localization of endogenous Ipl protein, which we previously documented by immunohistochemistry in placental trophoblast and yolk sac epithelial cells (1, 2).

Because the interpretation and comparison of the immunofluorescence patterns in fixed cells at a single time point is subjective, we wished to follow the movement of the tagged Ipl and Tih1 PH domains in response to growth factor stimulation in living cells. For this purpose, we imaged GFP-tagged constructs in transfected COS7 cells before and after exposure to epidermal growth factor (EGF). Control constructs were GFP-GRP1, which, due to its selective binding to PtdIns(3,4,5)P3, rapidly translocates from the cytoplasm to the plasma membrane in response to EGF (24), and GFP-DYN1, which has a low affinity for PIPs and should not alter its distribution in response to growth factor signaling. As illustrated in Fig. 3A and expressed quantitatively in Fig. 3B, GFP-GRP1 showed the expected rapid translocation in response to EGF, with cytoplasmic clearing and accumulation of signal along the entire plasma membrane. GFP-DYN1, the negative control construct, showed the expected lack of movement after EGF addition (Fig. 3, A and B). The findings with GFP-Ipl and GFP-Tih1 did not match either of these patterns. Instead, both of these
proteins accumulated at the membrane, but only in structures that obviously corresponded to ruffles (Fig. 3, A and B). The GFP-Ipl and GFP-Tih1 proteins showed little evidence for rapid clearance from the cytoplasm (Fig. 3, A and B). In some cells, GFP-Tih1, and to a lesser extent in GFP-Ipl, also showed local concentrations at the membrane even in serum-starved quiescent cells at the zero time point (see legend of Fig. 3B).

We also attempted to assess the effects of mutations at critical amino acid residues on the movement of GFP-Ipl in this assay. Quantitative differences were observed, which were not as amenable to scoring as the qualitative differences seen between GFP-GRP1 and GFP-Ipl. Like wild-type Ipl, a GFP-Ipl-K38A mutant remained predominantly in the cytoplasm upon EGF activation. Although some accumulation at ruffles was observed after EGF exposure, the signal was less intense as compared with that seen with the wild-type Ipl construct (data not shown). For all of the constructs, movement of the tagged proteins was blocked by PI3K inhibitor wortmannin, which, as expected, also blocked membrane ruffling (Fig. 3B, legend).

Overall, these results are consistent with the information from the in vitro binding assays. For rapid cytoplasm to plasma membrane translocation to occur, the PH domain must have a

Fig. 4. Placental tissue fractionation. Western blotting for Ipl, phospho-Akt, and β1-integrin in fractions obtained from mouse placentas. A parallel Coomassie Blue-stained gel shows equivalent protein loading in each lane.

Fig. 5. Growth-rescue of cdc25ts yeast by Ras*-PH domain fusion proteins. A, cdc25ts yeast transformed with DYN (1, 2), BTK (3, 4), PLC51 (5, 6), Tih1 (7, 8), or Ipl (9, 10). Robust PI3K-independent rescue is seen with Tih1. In contrast, Ipl produces a partial rescue, which is slightly stronger in the presence of active PI3K. This partial PI3K dependence was reproducibly observed in five independent transformants. B, mutational analysis of the Ipl-PH domain: Ipl-wt (9, 10); Ipl-K38A (11, 12); Ipl-R39A (13, 14); Ipl-K638R93A (15, 16); and Ipl-R27L (17, 18). All mutants have a reduced ability to rescue yeast growth, and the Ipl-R39A mutant accentuates the partial PI3K dependence of the rescue. C, mutational analysis of the Tih1-PH domain: PLC51 (5, 6); Tih1-wt (7, 8); Tih1-K27A (19, 20); Tih1-R28A (21, 22); and Tih1-K27A/R28A (23, 24). All mutants have a reduced ability to rescue yeast growth. All constructs were tested in the presence of either active (1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, and 23) or inactive (2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24) PI3K.
much greater affinity for PIP3 than for PIP2. Clearly, neither Ipl nor Tih1 meet this requirement. However, the fact that GFP-Ipl and GFP-Tih1 accumulate at membrane ruffles under conditions that do not promote membrane recruitment of GFP-DYN1 suggests that the Ipl and Tih1 PH domains have a higher affinity for PIP3 than does the PH domain from DYN1. 

**Ipl Is Found in the Cytoplasmic Fraction of Mouse Placenta Lysates**—Consistent with the results using tagged constructs, immunolocalization of native Ipl protein in mouse placenta and yolk sac shows predominantly cytoplasmic staining, with rare nuclear staining (1, 2). To check for Ipl bound to cellular membranes, we prepared nuclear, cytoplasmic, and membrane fractions by differential centrifugation of mouse placental lysates (obtained at 12.5 days postcoitum). The integral membrane protein β1-integrin was used as a control marker for the membrane fractions. β1-Integrin is also present in rough endoplasmic reticulum, which is contiguous with the outer nuclear membrane, and therefore the nuclear fraction was also positive for this marker (Fig. 4). In contrast, Ipl was only detected in the cytoplasmic fraction (Fig. 4). The PH domain protein Akt, which transiently translocates to the membrane when it is phosphorylated by growth factor-activated PI3K, was also detectable (as phospho-Akt) only in the cytoplasmic fraction under these fractionation conditions (Fig. 4). Equivalent loading of different fractions was confirmed by Coomassie Blue staining. This experiment excludes stable interactions of Ipl with cellular membranes but, given the co-fractionation of Ipl with phospho-Akt, it does not exclude transient interactions of the Ipl PH domain with membrane lipids.

**The Ipl and Tih1 PH Domains Rescue cdc25ts Yeast When Present as Ras* Fusion Proteins**—The filter binding and *in vivo* translocation assays agreed in that both suggested a moderate but measurable affinity of the Ipl PH domain for PIP3, with a poor ability to discriminate among the different phosphorylated forms. To investigate this further, we used Ras*-PH domain fusion constructs to rescue the growth of the *cdc25ts* mutant *S. cerevisiae* (26). This assay tests the ability of the PH domain of interest to bring activated Ras to the yeast plasma membrane, either constitutively or in response to D3-phosphoinositides produced by PI3K. We fused the PH domains

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**Table I**

| Assay                  | Ipl                                      | Tih1                                      |
|------------------------|------------------------------------------|-------------------------------------------|
| Protein-lipid overlay  | Moderate affinity, low specificity PIP binding. | Moderate affinity, low specificity PIP binding. |
|                        | Preferential binding to PtdIns(3,4,5)P₃ at high protein concentration. |                                           |
| Agonist-dependent membrane translocation | Delayed, agonist stimulated (wortmannin-sensitive) accumulation at membrane ruffles. | Bulk of the signal remains cytoplasmic. |
|                       |                                          |                                           |
| *cdc25ts* yeast rescue | Partial rescue; PI3K-independent, but stronger than the PH domain. | Strong PI3K-independent rescue. |

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**Fig. 6. Amounts of Ras*-PH domain fusion proteins produced in yeast.** *A*, expression of Ras*-PH domain proteins in the presence or absence of active PI3K detected by anti-Ras antibody. *B*, comparison of the expression of Ras*-PH domain proteins between native Tih1 and the Tih1 mutants. This blot contains lysates from yeast with active PI3K. *C*, comparison of expression of Ras*-PH domain proteins between native Ipl and the Ipl mutants. This blot contains lysates from yeast with active PI3K.
of Ipl and Tih1 to activated (Q61L) Ras* and matched these constructs to control proteins containing the PH domains of BTK, which binds PtdIns(3,4,5)P3 with high affinity and specificity, PLCγ1, which binds PtdIns(4,5)P2 with high affinity and specificity, and DYNI, which binds phosphoinositides only weakly. Both Ras*-Ipl and Ras*-Tih1 rescued cdc25ts S. cerevisiae in the presence and absence of active PI3K (Fig. 5A). This PI3K-independent rescue was executed more efficiently by the Tih1 fusion protein, compared with Ras*-Ipl, as indicated by more rapid growth of the yeast. Furthermore, rescue by Ras*-Ipl was slightly stronger when this construct was co-expressed with active PI3K, as compared with inactive PI3K. The control constructs gave the predicted results: PI3K-dependent rescue by Ras*-BTK; PI3K-independent rescue by Ras*-PLCγ1, and, significantly, no rescue by the negative control, Ras*-DYNI (Fig. 5A). These results provide additional in vivo support for placing Tih1 and Ipl in the category of PH domain proteins that can bind PIPs with moderate affinity.

Mutations of Positively Charged Amino Acids in Loop 1–β2 Sheet Abrogate Yeast Rescue—To obtain additional evidence for binding of Ipl and Tih1 to PIPs via their predicted PH domains, we carried out site-directed mutagenesis, and repeated the yeast growth rescue assays. In these experiments, we altered a binding of Ipl and Tih1 to PIPs via their predicted PH domains, and specificity, PLCγ/H9254, corresponding native PH domain constructs (Fig. 6, A–C). To exclude the trivial possibility that these findings might be based on poor expression of the mutant constructs, we subjected lysates of the yeast transformants to Western blotting with an anti-Ras antibody. This showed that, except for a poorly expressed double-amino acid mutant of Tih1 (K27A/R28A), all of the mutant constructs evaluated in the yeast rescue assay were expressed in amounts comparable to the corresponding native PH domain constructs (Fig. 6, A–C). The data from each of the three assays are summarized in Table I. The findings are quite consistent between these systems and, taken as a whole, suggest that binding of the Ipl and Tih1 PH domains to PIPs is sufficiently strong to be relevant to the biological functions of these proteins.

**DISCUSSION**

The Ipl gene was originally found in a chromosomal walk, which was undertaken to identify and characterize novel imprinted genes in a megabase-scale imprinted domain on human Chr11p15.5 and distal mouse Chr7 (35). Since then, substantial information has accrued concerning the tissue-specific expression and functional imprinting of this gene, and the creation of Ipl-knockout mice has established that at least one of its functions is to regulate the growth of the placenta (1, 2). Biochemical information is needed to explain this genetic finding, and a starting point is provided by the domain structure of the Ipl protein. This structure, which is shared by the closest Ipl homologue, Tih1, is remarkably simple, consisting of a single predicted PH domain with short N- and C-terminal extensions. The results shown here verify that this central region acts as a bona fide PH domain, which is capable of interacting with phosphoinositide lipids, both in direct binding assays and in membrane translocation assays, as well as functionally in a yeast growth rescue assay. Overall, the findings suggest that the Ipl and Tih1 PH domains bind to known PIPs with moderate affinity and low specificity. Whether this promiscuous binding reflects the true physiological targets of proteins in this group is not known, and they may have a high affinity for a yet unidentified lipid, or even protein, ligand. For example, recent work has shown that the tagged PH domain of human oxysterol binding protein accumulates at the yeast Golgi apparatus partly in response to the presence of PtdIns(4)P and partly in response to an unidentified factor that is dependent on the Golgi ATPase ArfIp (36).

As summarized in Table I, our data also show clear differences in the behavior of the Ipl versus Tih1 PH domains. Consistent with these differences, Ipl and Tih1 have different patterns of tissue-specific expression (2), and no detectable growth abnormality was found in Tih1-null concepti (2). In addition, the Ipl protein is highly conserved in vertebrate evolution, presumably reflecting a conserved biological function, but we have not been able to identify Tih1 orthologues in non-mammalian vertebrates.

Further progress in understanding the biochemical functions of Ipl and Tih1 will depend on localizing the native proteins within the cell at high resolution and on identifying any proteins or other lipids with which they interact. For most PH domain-containing proteins, such protein-protein interactions do not occur via the PH domain, but instead involve different modules elsewhere in the protein. For example, Lnk associates with the ABP-280 actin binding protein via an interdomain stretch of 56 amino acids between its PH and SH2 domains (37), several GRB family proteins bind to cytoplasmic domains of growth factor receptors via their SH2 domains (38), and Gab1 binds to PI3K and intracellular domains of some growth factor receptors via phosphorylated tyrosine residues outside of its PH domain (39). Neither Ipl nor Tih1 show clear evidence for protein-protein binding motifs, except possibly for proline-containing sequences near their C termini. Additional work will be required to determine whether their short C- or N-terminal extensions, or their PH domains, are able to mediate protein-protein interactions. An alternative possibility, which is suggested by the lack of additional functional modules in the small Ipl and Tih1 proteins, is that these proteins may act as natural “dominant-negatives” to dampen some biological processes by binding to PIPs and thereby excluding PIP binding “effector” proteins.

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