We identified a potential phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P₃) binding pleckstrin homology domain in the data bases and have cloned and expressed its full coding sequence (LL5β). The pleckstrin homology domain mutants of LL5β that could not bind PtdIns(3,4,5)P₃ were constitutively localized to this vesicle population. Importantly, pleckstrin homology domain mutants of LL5β that could not bind PtdIns(3,4,5)P₃ were constitutively localized to this vesicle population. At increased PtdIns(3,4,5)P₃ levels, it became significantly plasma-membrane localized. The distribution of LL5β in in vitro and in vivo. This interaction could co-localize heterologously expressed γ-filamin with GFP-LL5β in the unidentified vesicles.

Phosphoinositide 3-kinases (PI3Ks)³ 3-phosphorylate phosphoinositides. There are three classes of PI3Ks. The type I enzymes seem to act as PtdIns(4,5)P₂-3-kinases in vivo; they can be activated by a variety of close-to-receptor transduction events and drive accumulation of PtdIns(3,4,5)P₃ in the inner leaflet of the plasma membrane. This PtdIns(3,4,5)P₃ serves as a central recruiting proteins from the cytosol that possess modules, typically PH domains, capable of binding its head group (1).

There are a variety of reagents that can be used to inhibit PI3K activity. Most widely used are wortmannin (2) and LY294002 (3); both of which potently inhibit nearly all classes of PI3K and hence cannot generally be used to implicate a particular PI3K in a process. More specific are receptor tyrosine kinase Tyr → Phe mutants. A number of receptor tyrosine kinases (relevant here, the PDGF β-receptor) are capable of binding type IA PI3Ks at specific tyrosine residues that become phosphorylated following ligand binding (4). Mutation of these tyrosines to phenylalanine blocks type IA PI3K binding and activation but does not affect association of other effectors (1, 5). Stable, clonal cell lines have been created overexpressing wild-type PDGF-β receptors or (Y740F/Y751F) PDGF-β receptors (6) that have allowed the impact of selectivity blocking type IA PI3K activation to be assessed in vivo (7).

There is now a substantial family of PtdIns(3,4,5)P₃-binding proteins that have been shown to translocate to the plasma membrane in response to receptor stimulation of type I PI3K activity, including PKB (8, 9), DAPP-1 (10–13), PDK-1 (14), ARNO (15), ARAP-3 (16), and GRP1 (17). All of the above PI3K effectors bind 3-phosphorylated lipids via a PH domain. PH domains are protein modules of ~100 amino acids that bind a variety of ligands ranging from inositol phosphates and phosphoinositides to possibly G-protein βγ-subunits (18, 19). Those PH domains that bind phosphoinositides specifically form a subset that can be recognized via a consensus sequence of basic residues implicated in binding. Initially this concept was based solely on a limited number of sequence alignments, however, as more phosphoinositide binding PH domains were characterized the early consensus has been evolved and further validated by work that has described the structure of a number of PH domains, some with phosphoinositide-based ligands (20). Many different types of proteins seem to use PH domains as phosphoinositide binding modules including enzymes (e.g. PKB, BTK, Vav, and PDK-1) and adaptor proteins (e.g. DAPP-1).

It is generally thought that phosphoinositide-dependent shifts in signaling proteins from predominantly cytosolic to membrane distributions are, in some way, activating. In the case of PKB this seems to result from co-localization with its

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* This work was supported in part by the Biotechnology and Biological Sciences Research Council SAIN initiative. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** Supported by a Biotechnology and Biological Sciences Research Council studentship.

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The abbreviations used are: P3K, phosphoinositide 3-kinase; PH, pleckstrin homology; GFP, green fluorescent protein; PAE, porcine aortic endothelial; PDGF, platelet-derived growth factor; TRITC, tetramethylrhodamine isothiocyanate; HI-FBS, heat-inactivated fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; PBS, phosphate-buffered saline; RTIC, rhodamine isothiocyanate; GST, glutathione S-transferase; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P₃, phosphatidylinositol 3,4,5-trisphosphate.
upstream regulator PDK-1 combined with increased availability of the site PDK phosphorylates (threonine 308 in PKBα) as a result of PtdIns(3,4,5)P3 binding (21). For DAPP-1, which has been claimed to bind PLC-γ (11), it is presumably the relocation of the PLC-γ, to the cell surface and the location of its phospholipid substrate that could be relevant.

Through the application of PD9 inhibitors, some of which have been described above, it has become clear that type I PI3K signaling regulates a huge variety of cellular responses. One of the most widely important of these is cell survival (22). In essence PI3Ks and PKB are thought to supply a signal from some receptors that block cells from undergoing apoptosis (23). These signals operate constitutively in the presence of relatively low levels of survival factors but their inactivation upon factor withdrawal leads rapidly to apoptosis (1).

Filamins are actin-binding proteins that act to stabilize large three-dimensional actin networks, through their ability to dimerize (24, 25). Mammals can make α-, β-, and γ-filamins that possess different tissue distributions. The filamins also seem to bind to a variety of membrane-associated structural or signaling proteins, typically via a region of the molecule that does not interfere with the actin-binding or dimerization domains and these include, cytoplasmic tails of integrins and receptors. Hence filamins can be seen as structural proteins contributing directly to the mechanical properties of the cytoskeleton but also as points at which a variety of cell-surface signals can converge on the actin cytoskeleton.

In this article we describe the identification, cloning, and expression of a PH domain-containing protein that binds PtdIns(3,4,5)P3 and behaves as a PtdIns(3,4,5)P3-effector but also associates with γ-filamin and undergoes a novel redistribution in response to reductions in PtdIns(3,4,5)P3 levels.

**EXPERIMENTAL PROCEDURES**

**Cloning of Human LL5β and Relevant Constructs**—The cDNA encoding the full-length open reading frame of LL5β was obtained via the I.M.A.G.E. clones 531882 (accession numbers, aa116053), 208876 (accession numbers, 165150), which were all obtained from the I.M.A.G.E Consortium (UK HGMP Resource Centre, Hinxton, United Kingdom). The full-length open reading frame of LL5β (3762 bp) was ligated in-frame with an amino-terminal Myc or Glu-Glu tag into pCMV3, generated by a PCR-based mutagenic strategy and ligated in-frame -tubulin (Sigma), anti-vinculin (Sigma), or anti-PMP70 antibody (Sigma), as indicated for 1 h at room temperature. Coverslips were washed three times for 5 min in PBS + 0.5% BSA and then incubated with the appropriate secondary fluorescent isocyanate/RITC-conjugated antibody (1 h at room temperature). Coverslips were then washed four times in PBS + 0.5% BSA (5 min). Cells were fixed in ice-cold 100% methanol for 5 min and rinsed in D.I. Water. For cells transfected with GFP constructs, coverslips were then rinsed in D.I. Water and mounted on slides using Aqua Polymount (Polysciences Inc.). A series of dyes for detection of mitochondria (mitotracker red, 100 nM, 15 min; Molecular Probes), lysosomes (lysotracker red, 50 nM, 30 min; Molecular Probes), and the transferrin receptor conjugated to Texas Red dye (marker for endosomes, 3 min for early endosomes and 10 min for late endosomes) were added to live cells expressing GFP constructs prior to fixation. For all other immunofluorescence studies, cells were fixed and permeabilized in PBS, 0.1% Triton X-100 for 10 min and treated three times in PBS + 1% BSA (w/v) for 30 min at room temperature before being incubated with anti-Myc monoclonal antibody anti-clathrin polyclonal antibody (Santa Cruz Biotechnology), anti-EIA1 monoclonal antibody (Transduction Laboratories), anti-caveolin-1 antibody (Santa Cruz Biotechnology), TRITC-phalloidin (Sigma), anti-α-tubulin (Sigma), anti-vinculin (Sigma), or anti-PMP70 antibody (Sigma), as indicated for 1 h at room temperature. Coverslips were washed three times for 5 min in PBS + 0.5% BSA and then incubated with the appropriate secondary fluorescent isocyanate/RITC-conjugated antibody (1 h at room temperature). Coverslips were then washed four times in PBS + 0.5% BSA (5 min). Cells were fixed in ice-cold 100% methanol for 5 min and rinsed in D.I. Water before being mounted onto slides allowed to dry, and imaged under a Zeiss Axiosfluorescence microscope. Images were captured using a SPOT digital camera (Diagnostic Instruments).

**Confocal Image Analysis of Live Cells**—Cells were transfected, cultured on sterile glass coverslips, and treated as described above. For imaging, coverslips were mounted on the stage of an Olympus 1× 7 microscope interfaced with an UltraView confocal system. The cells were imaged at 37 °C using a thermostatically controlled cell chamber and incubated in PAE salt solution (25 mM Hepes, pH 7.4, 1.8 mM CaCl2, 5.37 mM KCl, 0.81 mM MgSO4, 112.5 mM NaCl, 25 mM N-glucose, 1 mM NaHCO3, and 0.1% (w/v) fatty acid-free BSA). Freeze-fracture images of GFP filamin-containing Point mutants were obtained using an UltraView confocal microscope (PerkinElmer Life Sciences). GFP fluorescence was excited at 488 nm and the emission was collected at wavelengths >505 nm using a long pass filter. Typically, 12 bit ~600 × 400 pixel images were captured every 2–3 s.

**Northern Blot Analysis**—A 745-bp fragment encoding the unique NH2 terminus of LL5β was used as a probe for Northern blot analysis. The probe was labeled with [32P]dCTP (Amersham Biosciences) and the Prime-a-Gene labeling system (Promega). The radioactive probe was applied to multiple tissue Northern blots containing RNA from various human tissues obtained from Clontech and carried out according to the recommended protocol.

**Phosphoinositide Binding Specificities of LL5β**—10× 100% COS-7 cells were transfected by electroporation with 20 μg of the DNA construct encoding Myc-tagged LL5β. Cells were allowed to recover in DMEM containing 10% HI-FBS in two 15-cm diameter dishes for 48 h and were washed and lysed with 5 ml/dish of lysis buffer (1% Nonidet P-40, 20 mM Hepes (pH 7.5), 0.12 mM NaCl, 5% EDTA, 5 mM EGTA, 5 mM β-glycerophosphate, 1 mM orthovanadate, 10 mM NaF). Lysates were centrifuged at 100,000× g for 1 h (2°C). Supernatants were then mixed with 50 μl free dipalmitoyl phosphatidylcholine vesicles containing phosphoinositides (16) on ice for 20 min. The probe was labeled with [32P]dCTP (Amerham Biosciences) and the Prime-a-Gene labeling system (Promega). The radioactive probe was applied to multiple tissue Northern blots containing RNA from various human tissues obtained from Clontech and carried out according to the recommended protocol.

**Purification of LL5β Interacting Partners**—Recombinant proteins, GST-LL5β and GST-LL5βPH, were expressed in bacteria and purified
on GS-Sepharose beads. The glutathione-Sepharose beads bound to GST LL5β and GST-LL5βPH were used to “pull down” interacting proteins from COS-7 cell lysates. Previously seeded COS-7 cells were washed twice in PBS and left in Met- and Cys-free DMEM for 35 min. Then, 0.2 nCi of [35S]methionine and [35S]cysteine (Amersham Pharmacia Biotech) was added 16 h prior to lysis. Lysates were centrifuged for 10 min at 4°C at 13,000 x g, and supernatants were transferred to 2 µg of GST LL5β and GST-LL5βPH purified on 30 µl of glutathione-Sepharose beads, and allowed to mix for 2 h at 4°C. Four washes were then carried out in lysis buffer (30 mM Hepes (pH 7.4), 10 mM NaF, 5 mM β-glycerophosphate, 1 mM MgCl2, 1 mM EGTA, 1% Nonidet P-40, 110 mM NaCl, 5 mM NH4 bicarbonate (pH 8), then soaked in 100% acetonitrile for 5 min, and dried in a 5200 centrifugal concentrator for 20 min. 10 µg of excision grade trypsin (Sigma) in 25 mM NH4 bicarbonate (pH 8) was added to the dried gel slices and digested at 37°C for 16 h. Extraction of peptides was carried out by soaking the gel slices in 50% acetonitrile, 5% trifluoroacetic acid for 30 min with gentle agitation. A second extraction was carried out as above and the combined extracts were dried as before for 1 h. The dried sample was then reconstituted by adding 4 µl of 50% acetonitrile, 0.1% trifluoroacetic acid. The generated peptides were then analyzed by Applied Biosystems by a mass spectrometer (Qstar Pulsar).

RESULTS

Cloning, Tissue Distribution, and Lipid Binding Properties of LL5β—Using the consensus Lys-Xaa-Gly/Ser-Xaa(6–11)-Arg/Lys-Xaa-Arg-Phe/Leu in 1996 (26) we identified a partial human protein sequence in the NCB1/EMBL protein expressed sequence tag data base encoding the COOH-terminal domain of a protein, previously called LL5 (rat) (27), that we predicted would bind PtdIns(3,4,5)P3. Sequencing the relevant Image clone (820502) revealed upstream overlaps with further expressed sequence tags and iteration identified a potential up-stream start codon with an in-frame stop immediately upstream. Although we began searching for the human orthologue of rat LL5, we ended up with the open reading frame of a paralogue of the human orthologue of rat LL5. The predicted open reading frame was for a 160-kDa protein (see Fig. 1A) and a full-length clone (accession number AJ496194) was created from Image clones 531883, 208876, and 82052. The predicted sequence tag data base encoding the COOH-terminal domain of a protein, previously called LL5 (rat) (27), that we predicted would bind PtdIns(3,4,5)P3. Sequencing the relevant Image clone (820502) revealed upstream overlaps with further expressed sequence tags and iteration identified a potential up-stream start codon with an in-frame stop immediately upstream. Although we began searching for the human orthologue of rat LL5, we ended up with the open reading frame of a paralogue of the human orthologue of rat LL5. The predicted open reading frame was for a 160-kDa protein (see Fig. 1A) and a full-length clone (accession number AJ496194) was created from Image clones 531883, 208876, and 82052. The protein contains a single spectrin repeat and a COOH-terminal PH domain. The same PH domain was also identified as a potential PtdIns(3,4,5)P3-binding protein in a screen by Isakoff et al. (26) (who called the host protein LL5β) and a closely related molecule LL5γ, which is the human orthologue of the rat LL5. We will retain the nomenclature Dowler et al. (28) applied to the PH domain of this protein and hence will term it LL5β. LL5α and LL5β are different proteins that occur at different locations and have less than 70% identity at the protein level. A 745-bp probe from the NH2-terminal region of LL5β, which would not recognize LL5α, was used to analyze a Northern blot prepared from human tissues. A 6-kb band was detected in a number of tissues with the highest levels found in heart, kidney, and placenta (see Fig. 1B).

Expression plasmids encoding NH2-terminal Myc- and GFP-tagged LL5β (and various constructs, see below) were prepared and transiently transfected into COS-7 and PAE cells. Anti-
Myc-immunoblots of appropriately transfected PAE or COS-7 cells revealed a 160-kDa protein (see Fig. 2A).

We analyzed the lipid binding specificity of LL5β in vitro. Cytosolic fractions were prepared from COS-7 cells transfected with Myc-LL5β and mixed with Affi-Gel beads covalently attached to PtdIns(3,4,5)P3 (16). The beads were washed, and bound proteins were eluted and immunoblotted with anti-Myc antibody. This revealed that -1-2% of input LL5β was recovered on the beads. Various competing, free phosphoinositides were added to the binding reactions and this revealed that PtdIns(3,4,5)P3 most effectively displaced LL5β from the beads (see Fig 2B); indicating that LL5β can bind PtdIns(3,4,5)P3 selectively under these assay conditions. Mutations in the PH domain predicted to disrupt lipid binding (LL5βΔPH and LL5β-K1162A/R1163A) abolished LL5β binding to the PtdIns(3,4,5)P3 beads (Fig. 2C).

Distribution of LL5β in Cells—We transiently expressed Myc- or GFP-LL5β in PAE cells stably overexpressing the PDGFβ receptor. In the presence of serum or after only short periods of serum starvation (up to 6 h), the LL5β constructs appeared predominantly cytosolic in fixed cells. After prolonged serum starvation (8 h or more) a significant proportion of Myc- or GFP-LL5β become particulate apparently at the expense of the cytosolic pool in both living or fixed cells. After approximately 6 h of serum starvation, when the protein was predominantly cytosolic, stimulation with PDGF resulted in a partial translocation of both Myc- and GFP-LL5β to the edge of the cell (Fig. 3). This event was observed in both living and fixed cells using confocal and standard epifluorescence microscopes. The translocation was apparently more prolonged than that displayed by proteins such as DAPP-1 or PKB studied under similar conditions. Furthermore, it appeared that the peripheral accumulation of LL5β constructs correlated with reductions in both its cytosolic and particulate pools (also see below). PAE cells were similarly transfected with a GFP-tagged construct containing a 160-kDa protein (see Fig. 2A).

PDGF stimulation commenced and viewed under a confocal microscope.

Fig. 2. Immunoblot of Myc-tagged full-length and in vitro lipid binding specificity of LL5β. A, immunoblot of Myc-tagged full-length LL5β. Myc-LL5β was transiently transfected into PAE cells. Cell lysates were immunoblotted with anti-Myc antibodies. B, in vitro lipid binding specificity of LL5β. Recombinant, Myc-tagged LL5β prepared in COS-7 cells were mixed with 50 μl dipalmitoyl forms of free, competing phosphoinositides, PtdIns(3,4,5)P3, PtdIns(3,4)P2, PtdIns(3,5)P2, PtdIns(3)P, PtdIns(4,5)P2, PtdIns(3,5)P2, PtdIns(3,4)P2, and PtdIns(5)P for 10 min and then transferred to PtdIns(3,4,5)P3 beads, allowed to mix for 1 h, washed, and proteins were eluted with SDS sample buffer. The first lane was loaded with a sample of Myc-LL5β equivalent to 1% of that included in the binding assays. Myc-tagged LL5β was detected by immunoblotting with anti-Myc antibodies. C, binding of Myc-LL5βΔPH and Myc-LL5β-K1162A/R1163A expressed in COS-7 cell lysates to PtdIns(3,4,5)P3 beads. Proteins were detected by immunoblotting with anti-Myc antibodies.

Fig. 3. The effects of PDGF stimulation on the subcellular localization of GFP-LL5β in PAE cells. PAE cells were transiently transfected with GFP-LL5β. After recovery and serum starvation, the cells were stimulated with PDGF (10 ng/ml). A, live cells were viewed with a confocal microscope. Images were captured at the indicated times after PDGF stimulation commenced. B, cells were fixed at the indicated times after PDGF stimulation commenced and viewed under a confocal microscope.

Authors of this work provided the following information: 2 P. J. Cullen, personal communication.
lar vesicular compartment, apparently identical to that seen in cells after prolonged serum starvation, became decorated (Fig. 4A, see also Supplementary Material for a video showing the effects of wortmannin on the distribution of GFP-LL5β in living PAE cells). We have not seen this phenomenon before in the context of similar experiments with PAE cells studying proteins such as PDK-1, P RB, ARAP-3, PReX-1, and DAPP-1 (10, 14, 16, 29).

Interestingly, with PAE cells transiently expressing GFP-LL5β in the presence of 30 μM LY294002 (where the inhibition of PI3Ks was substantial but not complete), PDGF stimulation caused a significant redistribution of GFP-LL5β from the vesicular pool into the cytoplasmic fraction without a clearly detectable accumulation near the plasma membrane. Transient expression of GFP-LL5β in a PAE cell line expressing (Y740F/Y751F)-PDGF-β-receptors (unable to bind and activate type I PI3Ks) revealed that GFP-LL5β was constitutively associated with intracellular vesicles in the absence of wortmannin or LY294002. In an attempt to assess whether other procedures, potentially capable of reducing cellular PtdIns(3,4,5)P₃ levels, could cause this shift of LL5β constructs into a particulate compartment, we serum-starved and/or detached PAE cells transiently expressing GFP-LL5β and held them in suspension. Both treatments significantly increased the proportion of GFP-LL5β in the vesicular compartment, this was seen most clearly in cells that were detached and in the absence of serum (Fig. 4, C and D).

We examined the distribution of GFP-LL5β in transiently transfected COS-7 cells to check whether this phenomenon is cell-type specific. We found that the construct was very largely cytosolic in both living and fixed cells and treatment with PI3K inhibitors wortmannin or LY294002 lead to a reduction in cytosolic staining and the decoration of an intracellular vesicular compartment (data not shown).

We also tested whether Myc-LL5β-K1162A/R1163A (double point mutation in the PH domain predicted to abolish PtdIns(3,4,5)P₃ binding) in PAE (Fig. 4B) and COS-7 cells. Both constructs adopted a constitutive vesicular distribution in both PAE and COS-7 cells. These distributions in PAE cells were unaffected by PDGF (not shown).

Together these results suggest that overexpressed LL5β can translocate to the plasma membrane in response to PI3K activation but that under conditions of low PtdIns(3,4,5)P₃, LL5β becomes associated with a vesicular compartment. This does not appear to be an artifact of inhibition of PI3K activity as a number of inhibitory strategies are effective nor is the vesicular compartment made up of aggregated protein because LL5β enters the compartment rapidly (15 min), can apparently move back into the cytosolic compartment under certain conditions, and the decorated vesicles move actively around the cell in a manner akin to vesicles like endosomes (see Supplementary Materials). Furthermore, our results with the ΔPH and LL5β-K1162A/R1163A constructs suggest that it is likely that this process represents the association of LL5β with a pre-existing organelle (unless they are formed specifically in the presence of these constructs) and that the key event is that LL5β “perceives” that cellular PtdIns(3,4,5)P₃ is low, rather than the levels of PtdIns(3,4,5)P₃ are actually low.

We consider the best working explanation for these data is that even at relatively low cellular levels of PtdIns(3,4,5)P₃ LL5β can cycle on and off the plasma membrane from the cytosol in a PH domain-dependent manner (without substantial accumulation at the plasma membrane) and that this proc-
ess leads to modification of LL5β (e.g. phosphorylation or association of a protein) that prevents it becoming localized into the vesicular compartment and has a lifetime of roughly 15 min. The cytosolic pool of LL5β can undergo a net translocation to the plasma membrane in response to receptor activation of type 1 P3Ks.

**The Nature of the Vesicle Compartment That Can Be Decorated by LL5β**—The work we described above suggests that the “vesicular” LL5β is unlikely to be a aggregated/denatured protein. This view is also supported by the dynamic, jittering movements of GFP-LL5β-associated structures in the presence of wortmannin or of GFP-LL5β-K1162A/R1162A decorated structures. We attempted to co-localize GFP-LL5β with a variety of markers in wortmannin-treated PAE cells. We used Texas Red-conjugated transferrin to label early (3 min incubation with cells) and late (10 min incubation with cells) endosomes, an antibody against early endosomal autoantigen 1 as an alternative marker for early endosomes, lysotracker as a marker for lysosomes, anti-caveolin antibodies to identify caveolae, anti-clathrin antibodies to decorate clathrin-coated pits, anti-PMP70 to label peroxysomes, mitotracker to label mitochondria, anti-vinculin antibodies to identify focal adhesions, and phalloidin to identify filamentous actin fibers. The GFP-LL5β did not co-localize with any of these markers (see Supplementary Material).

We used latrunculin B to disassemble actin fibers in PAE cells. It had no effect on the distribution of GFP-LL5β-decorated vesicles (see Supplementary Material). We co-transfected cells with Arf-6 and GFP-LL5β and localized the Arf-6 with anti-Arf-6 antibodies and RITC secondary antibodies; the constructs did not co-localize (see Supplementary Material). We have previously observed that DAPP-1 becomes localized to an endosomal compartment in the presence of PDGF. We co-transfected PAE cells with GFP-DAPP-1 and Myc-LL5β/K1162A/R1163A, stimulated with PDGF and detected the LL5β construct via an anti-Myc antibody and a RITC secondary antibody. The internalized GFP-DAPP-1 did not co-localize with the vesicular LL5β construct (data not shown). Finally we used a dominant-negative dynamin construct that we have previously used to establish that DAPP-1 is localized to an dynamin-sensitive fashion and is an effective inhibitor of dynamin-mediated membrane internalization (dynamin and dynamin mutant was a kind gift of H. McMahon). In an experiment where the dynamin point mutant blocked internalization of co-transfected GFP-DAPP-1 in response to PDGF it had no effect on the formation or distribution of GFP-LL5β-decorated structures in the presence of wortmannin (data not shown). We have not yet positively identified the vesicle compartment that is labeled by LL5β constructs although the number markers we have failed to co-localize suggest that it is a tightly defined subpopulation.

**LL5β-binding Proteins**—In the context of our hypothesis that the P13K-dependent redistribution of LL5β to a vesicular compartment might be dependent/blockd by an LL5β-associated protein we attempted to isolate potential binding partners. We prepared GST-LL5β and GST-LL5βΔPH in bacteria and derived glutathione-Sepharose beads loaded with them, GST alone, or GST-SHIP-1 as a control. These protein-loaded beads were mixed with aliquots of lysates made from [35S]methionine-labeled COS-7 cells, washed, the bound protein was resolved by SDS-PAGE, and the gel was dried and autoradiographed. A 260-kDa protein was recovered specifically with GST-LL5β and GST-ΔPH-LL5β (Fig. 5A). Two-dimensional isoelectric focusing and SDS-PAGE could not further resolve the 260-kDa protein band (not shown). The preparation was scaled up without [35S]methionine and the final one-dimensional SDS-PAGE gel was stained with Coomassie Brilliant Blue (Fig. 5B). The 260-kDa protein band was excised, in-gel digested with trypsin, and the resulting peptides were ultimately analyzed by electrospray mass spectrometry (Q star Pulsar i). Four peptides were selected for fragmentation and the patterns of the m/z ratios were used to reconstruct their sequences. Those sequences were used to interrogate the NRDB (nonredundant data base maintained by the European Bioinformatics Institute) and they identified γ-filamin. This assignment was confirmed by a γ-filamin-specific monoclonal antibody and a pan-filamin antibody preparation in immunoblots of the 260-kDa protein eluted from the GST-LL5β affinity supports (Fig. 5C).

We tested whether γ-filamin could interact with LL5β in vivo. (Glu-Glu)-tagged LL5β and γ-filamin were transfected individually and together into COS-7 cells (note, transfection with γ-filamin did not substantially increase the amount of total γ-filamin in the cells). Lysates were prepared and immuno-precipitated with anti-(Glu-Glu) monoclonal antibody covalently attached to protein G-Sepharose. γ-Filamin was only immunoprecipitated in the presence of (Glu-Glu)-LL5β (Fig.
LL5β, a PIP₃ and γ-Filamin-binding Protein

**Fig. 6. Interaction of γ-filamin and LL5β in vivo assessed by immunoprecipitation and immunocytochemistry.** A, interaction of γ-filamin with (Glu-Glu)-tagged LL5β in *vivo*. (Glu-Glu)-LL5β and γ-filamin were transfected individually and together into COS-7 cells. The cell lysates were then immunoprecipitated with anti-(Glu-Glu) beads. The samples of the supernatant and pellet resulting from the immunoprecipitation and 1% of the cell lysate included in each assay were immunoblotted. The upper panel shows detection of (Glu-Glu)-LL5β with an anti-(Glu-Glu) antibody and the lower panel shows detection of γ-filamin with a γ-filamin-specific antibody. B, colocalization of GFP-LL5β and γ-filamin in wortmannin-treated COS-7 cells. GFP-LL5β and γ-filamin were transiently transfected into COS-7 cells and plated onto coverslips. 12 h later, cells were treated with wortmannin (100 nM, 15 min). The panel in green shows vesicular structures decorated by GFP-LL5β following wortmannin treatment. The panel in red shows γ-filamin distribution in the same cells using a γ-filamin-specific antibody. The panel on the right is a merged image and shows an enlarged area with colocalization of GFP-LL5β and γ-filamin in the vesicular structures. C, localization of GFP-LL5β and γ-filamin in COS-7 cells. GFP-LL5β and γ-filamin were transiently transfected into COS-7 cells and plated onto coverslips, left in 10% serum, and fixed 12 h later. The panel in green shows distribution of GFP-LL5β. The panel in red shows distribution of γ-filamin (in absence of wortmannin) and the merged image on the right shows the distinct localizations of the two proteins.

6A). About 5% of the total γ-filamin was recovered in the washed (Glu-Glu)-LL5β immunoprecipitates. This indicates that γ-filamin and LL5β can interact in *vivo*. We examined the distribution of γ-filamin and its relationship to LL5β in COS-7 (Fig. 6, B and C) and PAE cells (data not shown). We transiently transfected cells with GFP-LL5β and/or γ-filamin (detected by γ-filamin-specific antibody and a RITC-labeled secondary antibody). In cells co-transfected with GFP-LL5β and γ-filamin in the presence of wortmannin, it was clear that 30–40% of the γ-filamin-positive structures were also positive for the GFP-LL5β vesicular compartment (Fig. 6B). In cells transfected with γ-filamin alone, or co-transfected with GFP-LL5β and γ-filamin but not treated with wortmannin, the γ-filamin adopted a punctate distribution that was insensitive to wortmannin and were clearly smaller than those that contained both GFP-LL5β and γ-filamin (Fig. 6C). In cells co-transfected with γ-filamin and GFP-LL5β and treated with wortmannin, the structures that were only positive for γ-filamin were the same size as the γ-filamin-positive structures in cells transfected with γ-filamin alone. These results suggest that LL5β and γ-filamin can co-localize in both COS-7 and PAE cells in the presence of wortmannin and that γ-filamin localization is dictated by LL5β and wortmannin. This indicates that the interaction between LL5β and γ-filamin (in the presence of wortmannin) leads to the targeting of γ-filamin into the vesicular compartment by LL5β and that γ-filamin is not responsible for directing or blocking the movement of LL5β into a vesicular compartment.

**DISCUSSION**

These results imply that LL5β has the potential to act as a PH domain-containing PI3K effector that can translocate to the plasma membrane in response to receptor activation of type I PI3Ks. However, at low levels of PtdIns(3,4,5)P₃ or when the PH domain of LL5β is unable to bind PtdIns(3,4,5)P₃, LL5β is directed to a vesicular compartment. We consider the simplest explanation for these events, bearing in mind that unstimulated cells contain low levels of PtdIns(3,4,5)P₃ and that PH domain/PtdIns(3,4,5)P₃-mediated membrane recruitment is probably a dynamic process with turnover times of the order of a maximum of 1–105, that PH domain/PtdIns(3,4,5)P₃-mediated signaling through LL5β blocks targeting of LL5β to a vesicular compartment. This signal could be a modification to LL5β (e.g. phosphorylation, dephosphorylation, or association of a protein) that is reversed in the absence of reinforcing signals in a time scale of 10–30 min. The outcome is that LL5β shows a dramatic change in distribution as the cellular levels of PtdIns(3,4,5)P₃ alter in the low basal range.

Much literature has shown how many cells require PI3K signals to survive in several different contexts. Notable among these are serum or growth factor starvation and detachment from a substrate. We have noted that LL5β redistributes relatively (cf. events like apoptosis) rapidly under these conditions; however, it is completely unclear whether the changes in LL5β distribution have a cause/effect relationship with these survival pathways.

LL5β can bind γ-filamin. Our results indicate that this is not
a PI3K-regulated interaction and that it appears to serve to redistribute γ-filamin, although we have not yet established whether LL5β can recruit γ-filamin to the plasma membrane in a PI3K-dependent manner. This contrasts with a number of examples of proteins that bind filamins and as a result are targeted to the actin-containing cytoskeleton e.g. SHIP-1 (30).

In the light of the fact that γ-filamin serves as a stabilizer and organizer of the actin cytoskeleton, this interaction may be important for the role of γ-filamin. This view is strengthened by the result of a recent study that has suggested that filamin A is effectively down-regulated and as a result cell migration is reduced by interaction with L-FILIP (31). Interestingly, L-FILIP targets filamin A into an undefined punctate intracellular organelle, where the filamin A is degraded. It will be important to investigate the effects of LL5β on γ-filamin degradation and whether FILIP family proteins target filamins into a related intracellular compartment.

Acknowledgments—We thank Philip Jackson of Applied Biosystems for identification of the interacting protein peptides by electrospray-sequencing technology, Peter Lipp for use of confocal microscopes, and Nick Ktistakis for discussions and useful reagents. We also thank Dominic Chung (University of Washington) for the gift of γ-filamin cDNA, Louis M. Kunkel (Howard Hughes Medical Institute) for the γ-filamin-specific antibody, Dieter O. Furst (University of Potsdam) for filamin antibody, and Harvey McMahon (LMB, Cambridge) for the Dynamin constructs.

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J. Biol. Chem. 2003, 278:1328-1335.
doi: 10.1074/jbc.M208352200 originally published online October 9, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M208352200

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