Localization and Insulin-regulated Relocation of Phosphoinositide 5-Kinase PIKfyve in 3T3-L1 Adipocytes*

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The mammalian phosphoinositide kinase PIKfyve catalyzes the synthesis of phosphatidylinositol 5-P and phosphatidylinositol 3,5-P2, thought essential in cellular functions, including membrane trafficking. To discern the intracellular loci of PIKfyve products' formation, we have examined the localization of PIKfyve protein versus enzymatic activity and a possible acutely regulated redistribution in 3T3-L1 adipocytes. Subcellular fractions of resting cells that were positive for immunoreactive PIKfyve, such as cytosol (~76%), internal structures (low density microsomal fraction (LDM), composed of recycling endosomes, GLUT4 storage compartment, Golgi, and cytoskeletal elements) (~20%), and plasma membrane (~4%), expressed enzymatically active PIKfyve. While the presence of a FYVE finger in PIKfyve predicts early endosome targeting, density gradient sedimentation, immunoadsorption, and fluorescence microscopy analyses segregated the LDM-associated PIKfyve from the membranes of the recycling endosomes and GLUT4. PIKfyve fluorescence staining largely coincided with trans-Golgi network/multivesicular body markers, indicating PIKfyve’s role in the late endocytic/biosynthetic pathways. A subfraction of particulate PIKfyve resisted nonionic detergent treatment, implying association with cytoskeletal structures, previously found positive for key members of the insulin signaling cascade. Upon acute stimulation of 3T3-L1 adipocytes with insulin or pervanadate, a portion of the cytosolic PIKfyve was recruited onto LDM, which was coupled with a commensurate increase of PIKfyve lipid kinase activity and an electrophoretic mobility shift. We suggest the recruited PIKfyve specifies the site and timing of phosphoinositide signals that are relevant to the acute insulin action.

The key function of phosphorylated derivatives of phosphatidylinositol (PtdIns), called collectively PIs, in eukaryotic cell regulation has been recognized for more than 2 decades with the discovery that PtdIns 4,5-P2 is converted by phospholipase C to two second messengers, inositol trisphosphate and diacylglycerol. It has become increasingly clear, however, that PIs have a signaling role in their own right, i.e. they do not require hydrolysis to set in motion cellular processes. In most cases, PIs were found to serve as site-specific signals on membranes that recruit/activate effector protein complexes at the interface with the cytosol. Phosphoinositide signals are used in this way by eukaryotic cells to modulate a large number of responses, such as membrane ruffling, secretion, vesicular trafficking, insulin-regulated membrane translocation of the fat/muscle-specific GLUT4 glucose transporter, cell adhesion, chemotaxis, DNA synthesis, and cell cycle (1–4).

The intracellular PIs identified to date stem from the same precursor, PtdIns, and differ by the degree and position of phosphorylation of PtdIns’ head group. Out of the five candidate phosphorylation positions, only the hydroxyls at positions D-3, D-4, and D-5 are found phosphorylated intracellularly, separately or in all possible combinations, resulting in PtdIns 3-P, PtdIns 4-P, PtdIns 5-P, PtdIns 3,4-P2, PtdIns 3,5-P2, PtdIns 4,5-P2, PtdIns 3,4,5-P3, and PtdIns 3,4,5,6-P4 (2). Among the PI species, PtdIns 3-P and PtdIns 3,5-P2 have attracted increasing attention. They have been identified only recently mainly because PtdIns 5-P and PtdIns 3,5-P2 are poorly separated from PtdIns 4-P and PtdIns 3,4-P2, respectively, under classical HPLC analysis (5–7). An enzyme activity, thought responsible for the main pathway of their biosynthesis in mammalian cells, called PIKfyve (phosphoinositide kinase for five position containing a FYVE finger), has been recently identified by molecular cloning (8). PIKfyve is a large protein of 2052 amino acids, which displays an intrinsic, wortmannin-resistant (ID50, 600 nM) lipid kinase activity to generate PtdIns 5-P and PtdIns 3,5-P2 in vitro (8, 9). While PIKfyve intracellular distribution remains to be determined, of interest is that PIKfyve contains two evolutionarily conserved, plausible membrane-targeting, domains: a FYVE finger, found in other mammalian proteins as a major determinant for localization to early endosomes (10–12), and a DEP domain (named after Dishevelled, Egl-10, and pleckstrin; Ref. 13), reported sufficient for targeting to specific membrane compartments (14, 15). In Saccharomyces cerevisiae, only PtdIns 3,5-P2 and not PtdIns 5-P is documented (16). The enzyme responsible for its biosynthesis is the 2278-amino acid protein Fab1p (16, 17) that, with the exception of the DEP region, shares similar domain architecture and is related in sequence to PIKfyve (8). Fab1p is found associated with fractions enriched for Golgi/endoosome, lysosomal, or cytosolic marker proteins (16). Yeast strains defective in Fab1p, concomitantly with a depletion of the PtdIns 3,5-P2 pool, display severe defects in...
cell growth, expanded vacuoles, which fail to acidify, and defects in MVB formation. Thus, through PtdIns 3,5-P2 production, Fab1p is thought to regulate several steps in yeast membrane trafficking such as internal vesicle formation or cargo selection within the MVB and the recycling/tunover of membranes from the vacular surface to earlier compartments (18). That PIKfyve may play a role in membrane trafficking events in mammals is suggested by the results of complementation experiments in fab1-deficient yeast mutants, demonstrating that PIKfyve exhibits the ability to suppress the vacuolar defect and restore the basal PtdIns 3,5-P2 pool (19). Clearly, these studies underscore the importance of PIKfyve in cellular regulation and suggest that local generation of PtdIns 3,5-P2 and probably PtdIns 5-P may have an important role in signaling and/or execution of membrane trafficking in mammalian cells. Because PtdIns 5-P has never been detected in yeast, it is conceivable this PI derivative, produced presumably by PIKfyve enzymatic activity, has evolved over time to serve specific mammalian cell functions.

A common feature among the cellular events regulated by PIs is that they are spatially and temporally restricted. Therefore, the key to our understanding of the molecular mechanism underlying the PI-regulated signals rests upon knowledge of the precise site and timing of the PI generation. Thus far, direct measurement of PI levels in situ meets several restrictions mainly related to the fact that PIs are in low abundance with a rapid turnover and therefore cannot be readily approached by conventional techniques (20). An alternative approach involves following the enzyme activity's intracellular location with the premise that the latter will largely dictate where localized synthesis of the phosphoinositide products can occur. In this study, we undertook biochemical and morphological approaches to characterize the intracellular localization of PIKfyve protein and to relate it to PIKfyve enzymatic activity. We demonstrate here that subcellular fractions of resting 3T3-L1 adipocytes that were found positive for immunoreactive PIKfyve expressed an enzymatically active protein. PIKfyve largely colocalized with markers for Golgi-to-late-endosome traffic but was segregated away from recycling endosomal pathway markers. A subpopulation of the PIKfyve particulate pool was found to be detergent-resistant and cofractionated with structures, possibly cytoskeletal, recently reported positive for key elements of the insulin-signaling circuit relevant to GLUT4 exocytosis in 3T3-L1 adipocytes. We further demonstrate that acute insulin stimulation in this cell type induces spatially localized recruitment of PIKfyve to inner membranes, thus implying PIKfyve lipid products as regulated site-specific signals relevant to acute insulin action.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures, Antibodies, and Fusion Proteins—**Differentiation of 3T3-L1 mouse fibroblasts into insulin-sensitive adipocytes on plates or glass coverslips was described previously (21). Cells were used between 7 and 14 days after the onset of the differentiation program. COS-7 cells were maintained in DMEM, containing 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin sul fate. Rabbit polyclonal anti-PIKfyve antiserum (R7069; East Acres) was directed against a recombinant GST fusion protein comprising the N terminus of PIKfyve (amino acids 1–100). Polyclonal or monoclonal antibodies against GLUT4 (R1288 and 1F8) and IRAP were gifts of Drs. M. Czech, P. Pilch and K. Kandror. Monoclonal anti-TIR (HTR868.4), anti-β2-COP (M3A5), and rabbit polyclonal anti-human CLMP (CLMP) antibodies were gifts of Drs. S. White, I. Trowbridge, T. Kreis, and B. Hoflack, respectively. Anti-PI 3-kinase p85 and anti-phosphotyrosine (4G10) antibodies were provided by Upstate Biotechnology, Inc. (Lake Placid, NY); anti-γ2-FGFR antibody was from Clontech; and human insulin was from Lilly. The EGFP-HA-PIKfyve construct was generated by subcloning the XbaI/SalI digest of pBluescript II SK(+)→PIKfyve cDNA (8) together with the double-stranded oligonucleotide encoding for HA epitope (designed with EcoRI/XbaI restriction sites) into the EcoRI/SalI digest of pEGFP C2 (Clontech).

**Cell Culture, Antibodies, and Fusion Proteins—**3T3-L1 adipocytes (100-mm dish) were serum-deprived for 14 h in DMEM supplemented with 0.5% bovine serum albumin. Cells were stimulated with or without 100 nM insulin or pervanadate (100 μM) for 2 and 30 min, respectively, at 37 °C, washed twice with PBS and once with HES buffer (20 μM Hepes-HCl, pH 7.5, 1 mM EDTA, 255 mM sucrose, containing 1× protease inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 mM benzamidine) and 1× phosphatase inhibitor mixture (25 mM β-glycerophosphate, 10 μg/ml sodium orthovanadate, 5 mM sodium fluoride, 2 μM Na3VO4, and 2 μM NaN3) at 25 °C, and then scraped at 4 °C in HES buffer, supplemented with the inhibitor mixtures. Cells were homogenized by passing the cell suspension six times through a 22-gauge needle at 4 °C. Subcellular fractionation was performed following previous protocols (21) using an SS-34 rotor (Sorvall Instrument Division) in the first spin and Beckman TLA 100.3 rotor (Beckman Instruments Inc.) in the next spins to obtain HDM, LDM, PM, MN, and cytosolic fractions. Pellets were resuspended in HES buffer containing the above inhibitors to a protein concentration of ~2 mg/ml. Aliquots of the fractions were analyzed by immunoblotting or, following solubilization in 1% Nonidet, 0.5% sodium deoxycholate, 150 mM NaCl, were subjected to lipid kinase assay (see below). LDM digestion with alkaline phosphatase (Sigma) or α-phosphatase (Calbiochem) was performed for 40 min at 25 °C. Evaluation of Triton X-100-soluble versus insoluble pools of PIKfyve was performed in 3T3-L1 adipocytes as described previously (22) except for CSK buffer (100 mM NaCl, 300 mM sucrose, 10 mM Pipes, pH 6.8, 3 mM MgCl2, 1 mM EDTA, and 0.5% Triton X-100; Ref. 23) in place of PBS-Triton.

**Immunoadsorption of GLUT4 Vesicles—**Immunopurification of GLUT4-containing vesicles was achieved on anti-GLUT4 IgG as described previously (21). Briefly, LDM fractions derived from insulin-treated or untreated 3T3-L1 adipocytes were resuspended in PBS, supplemented with 1× protease and phosphatase inhibitor mixtures to a final concentration of 1 mg/ml, and then immunoadsorbed (18 h at 4 °C) on anti-GLUT4 IgG (R1288 antisera, purified on protein A-Sepharose 4 Fast Flow; Amersham Pharmacia Biotech) or control rabbit IgG (25 μg each). Protein A-Sepharose CL-4B (Sigma) was added for the final 2 h of the incubation. Following washings, the pellets along with aliquots of the supernatants were subjected either to SDS-PAGE and immunoblotting or to lipid kinase assay, as described below. The potency of anti-GLUT4 to quantitatively immunoadsorb GLUT4 vesicles was analyzed in a parallel experiment, in which the samples were mixed in Laemmli buffer (no boiling) for 30 min at 25 °C and processed further for immunoblotting. Anti-GLUT4 antibodies eliminated ~90% of the GLUT4-vesicle pool in LDM.

**Equilibrium Centrifugation in Self-formed Iodixanol Gradient—**LDM fractions prepared from 3T3-L1 adipocytes and resuspended in HES buffer supplemented with 1× protease and phosphatase inhibitor mixtures were mixed with iodixanol (OptiPrep; Sigma) in a polycrylamide Quick-Seal centrifuge tube (13 × 51 mm; Beckman) to either 14 or 30% iodixanol (on an 18 g/ml sucrose gradient of 196 or 128 samples, respectively), resulting in isosmotic solutions. A self-generating gradient was formed by centrifuging to equilibrium at 4 °C in a VTi 65.2 rotor (Beckman L8-M-55 centrifuge) for 4 h at 53,000 rpm as described (24). Fractions of ~0.35 ml were collected from the bottom of the tube and were analyzed for refractive index (Bausch and Lomb refractometer), protein concentration, and presence of PIKfyve or other proteins by immunoblotting.

**Transient Transfection and Fluorescence Microscopy—**COS-7 cells seeded on 100-mm dishes (for biochemistry) or 22 × 22 mm coverslips (for microscopy) were transfected with CDNA constructs indicated in the figure legends by LipofectAMINE (Life Technologies, Inc.) or the calcium precipitation method as described previously (22) and then processed for immunoprecipitation (see below) or fluorescence microscopy. Twenty h post-transfection with pEGFP-HA-PIKfyve, 5 μg each. Protein A-Sepharose CL-4B (Sigma) was added for the final 2 h of the incubation. Following washings, the pellets along with aliquots of the supernatants were subjected either to SDS-PAGE and immunoblotting or to lipid kinase assay, as described below. The potency of anti-GLUT4 to quantitatively immunoadsorb GLUT4 vesicles was analyzed in a parallel experiment, in which the samples were mixed in Laemmli buffer (no boiling) for 30 min at 25 °C and processed further for immunoblotting. Anti-GLUT4 antibodies eliminated ~90% of the GLUT4-vesicle pool in LDM.

**Photobleaching and Fluorescence Recovery Microscopy—**COS-7 cells seeded on 100-mm dishes (for biochemistry) or 22 × 22 mm coverslips (for microscopy) were transfected with CDNA constructs indicated in the figure legends by LipofectAMINE (Life Technologies, Inc.) or the calcium precipitation method as described previously (22) and then processed for immunoprecipitation (see below) or fluorescence microscopy. Twenty h post-transfection with pEGFP-HA-PIKfyve, 5 μg each. Protein A-Sepharose CL-4B (Sigma) was added for the final 2 h of the incubation. Following washings, the pellets along with aliquots of the supernatants were subjected either to SDS-PAGE and immunoblotting or to lipid kinase assay, as described below. The potency of anti-GLUT4 to quantitatively immunoadsorb GLUT4 vesicles was analyzed in a parallel experiment, in which the samples were mixed in Laemmli buffer (no boiling) for 30 min at 25 °C and processed further for immunoblotting. Anti-GLUT4 antibodies eliminated ~90% of the GLUT4-vesicle pool in LDM.
β-COP antibodies and detection by Cy3-coupled goat anti-rabbit IgG (Kirkegaard & Perry Laboratories) or Texas red-coupled goat anti-mouse IgG (Molecular Probes), respectively, was performed in formaldehyde-fixed transacted COS-7 cells (20 h post-transfection with LipofectAMINE). Double staining with affinity-purified anti–GLUT4 monoclonal antibody (1F8) and anti-PIKfyve polyclonal antibodies in 3T3-L1 adipocytes was as previously described (8). Coverslips were mounted on slides using the Slow Fade Antifade Kit (Molecular Probes). Fluorescence analyses were performed with a confocal microscope (Zeiss LSM 310) using a 63X/1.4 immersion lens.

**Immunoblotting and Immunoprecipitation**—For immunoblotting, protein samples were solubilized by SDS-PAGE and then transferred onto nitrocellulose membranes as previously described (8). The blots were saturated with blocking buffer and probed (16 h at 4 °C) with the antibodies indicated in the figure legends. After washes, bound antibodies were detected with horseradish peroxidase-bound anti-rabbit IgG or anti-mouse IgG (Roche Molecular Biochemicals) and a chemiluminescence kit (PerkinElmer Life Sciences). In some experiments, cytosol, LDM, or PM fractions were subjected to lipid kinase assay in 25 mM HEPES buffer, pH 7.4, containing 100 mM lipid substrate (synthetic diC 16 PtdIns 3-P as an internal standard, detailed elsewhere). Its identity and purity was confirmed by HPLC analysis performed with an HPLC equipped with a SP-Hypersil C18 reverse-phase column (250 × 4.6 mm, 5-μm particle size). For immunoprecipitation, the RIPA (50 mM Tris/HCl, pH 8.0, containing 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 1 mM EDTA) or 3T3-L1 adipocytes lysis buffer was precleared by centrifugation at 4 °C, and the supernatants were then incubated (16 h at 4 °C) with the antibodies indicated in the figure legends or with preimmune serum. Protein A-Sepharose beads were washed as described elsewhere (9) and subjected to lipid kinase assay in 25 mM HEPES buffer, pH 7.4, containing 100 mM lipid substrate (synthetic dic16, PtdIns 3-P as an ammonium salt; a gift from Echelon), 2.5 mM MnCl2, 2.5 mM MgCl2, 50 μM ATP, and γ32P]ATP (12.5 μCi). Following incubation for 15 min at 30 °C, the lipids were extracted and analyzed by TLC under previously described conditions (9). Generated radioactive PtdIns 3,5-P2 product was detected by autoradiography and quantified by radioactive counting. Its identity and purity was confirmed by HPLC analysis performed in the presence of appropriate internal standards, detailed elsewhere (9). In some experiments, cytosol, LDM, or PM fractions were subjected directly to the lipid kinase assay without immunoprecipitation. In this case, following 3T3-L1 adipocyte fractionation, described under “Cell Treatment and Subcellular Fractionation,” aliquots of the fractions (20 μl) were mixed with 30 μl of the lipid kinase reaction mixture and processed as described above.

**Other Methods**—Pervanadate was generated from vanadate and H2O2, mixed prior to experiments in a molar ratio of 1:2 as described (25). Fraction density was calculated from the refractive index (26). Protein concentration in all samples was determined by a bicinchoninic acid protein assay kit (Pierce). Protein levels on the immunoblots were quantified on a laser densitometer (Molecular Dynamics, Inc., Sunnyvale, CA) by area integration scanning. Several exposures of each blot were quantified to ensure the linearity of the chemiluminescence signal on the film.

**RESULTS**

**Biochemical Fractionations Indicate Cytosolic, Cytoskeleton-associated, and Inner Membrane-bound Components of PIKfyve**—Subcellular distribution was first analyzed by immunoblotting utilizing different cell fractionation techniques in 3T3-L1 adipocytes and using anti-PIKfyve antibodies directed against a glutathione S-transferase fusion of the PIKfyve N-terminal 100 amino acids. The ability of anti-PIKfyve antibodies to specifically recognize endogenous PIKfyve in 3T3-L1 adipocytes and other cells by Western blotting or immunoprecipitation was characterized previously (8, 9). To determine the soluble versus particulate pools of PIKfyve, 3T3-L1 adipocytes, rat1 fibroblasts, or COS-7 cells were fractionated into cytosol, total membranes, and nuclear pellets. A significant fraction of the immunoreactive PIKfyve was found to reside in the cytosol under resting conditions (70–80%). The remaining PIKfyve was found associated with the total membrane fractions; no detectable levels were documented in the nuclear pellets of either cell type (not shown). To gain insight into the nature of the membranes/structures with which PIKfyve is associated, we took advantage of previously well defined protocols for differential centrifugation of 3T3-L1 adipocytes. This fractionation enables isolation of five fractions enriched in marker proteins for PM-enriched fraction; M/N; endosomes, Golgi elements, and endoplasmic reticulum (HDM); recycling endosomes, Golgi and cytoskeletal elements, and intracellular GLUT4 storage compartment (LDM); and soluble fraction (cytosol) (21, 27). The immunoreactive PIKfyve was detected predominantly in cytosol and LDM and at low levels in the PM-enriched fraction (Fig. 1), with an average relative distribution of 77, 20, and 3%, respectively, as expressed per equal number of cells. HDM and M/N fractions showed no detectable PIKfyve levels (Fig. 1). Lack of significant contamination of the PM-enriched fraction with intracellular membranes was evidenced by the absence of a signal for the endosomal marker protein Rab4 upon immunoblotting (not shown).

The subcellular fractionation in 3T3-L1 adipocytes presented above implies that particulate PIKfyve mainly populates the intracellular GLUT4 storage compartment, Golgi/endosomes, and/or the cytoskeleton. To distinguish among these possibilities and to determine the relationship of the PIKfyve compartment to the other compartments, we performed a series of LDM fractionations using equilibrium sedimentation in self-formed iodixanol gradients, shown previously to effectively separate iodixanol gradients, shown previously to effectively separate GLUT4 vesicles, endosomal membranes, the bulk of other membranes, and cytoskeletal elements (24). Upon sedimentation in 14% iodixanol, the GLUT4 storage compartment is reportedly resolved into two distinct pools, containing different levels of the TR recycling endosomal marker, TGN markers, or core proteins of the transport machinery (24). In agreement with these studies, we have obtained two peaks of GLUT4 vesicles, evidenced by the Western blotting of the fractions collected from LDM equilibrium sedimentation at a starting concentration of 14% iodixanol (Fig. 2A). Under these conditions, PIKfyve was found to slightly overlap with the fractions containing the immunoreactive GLUT4 (Fig. 2A) or IRAP, which reside exclusively on GLUT4 vesicles, and TR (see below). Intriguingly, PIKfyve almost fully cofractionated with the p85 subunit of class IA PI 3-kinase detected in the denser bottom part of the 14% iodixanol gradient (Fig. 2A).

Higher starting concentrations of iodixanol generate shallower gradients at a lower refractive index, resulting in a
greater separation of the denser particles with small differences in density (26). Under a better resolution of the GLUT4/endosomal compartments versus the denser structures, we sought to examine first whether the observed slight overlap between the first GLUT4 peak and PIKfyve would persist and, second, the extent of overlap between p85 PI 3-kinase and PIKfyve. It is worth emphasizing that the combined phosphorylation of D-3 and D-5 in PtdIns is a result of a joint action of two lipid kinases; hence, a colocalization of PI 3-kinase and PIKfyve may facilitate the fidelity for the product’s synthesis. Equilibrium sedimentation at 30% iodixanol distributed the LDM into three main protein peaks (Fig. 2C). Immunoblotting of the collected fractions indicated that TIR, IRAP, and GLUT4 cofractionated with the third protein peak at the top of the gradient at a density of 1.13–1.08 g/ml (Fig. 2B; fractions 14–16), in agreement with the reported density of GLUT4 vesicles (28). The immunoreactive PIKfyve peak appeared clearly segregated from the above fractions, spanning fractions 5–12 (density 1.21–1.16 g/ml), comprising parts of the first and the second protein peaks (Fig. 2B). The immunoreactive p85 cosedimented entirely with the first protein peak (Fig. 2B; fractions 2–8; density 1.30–1.19 g/ml), indicating that while the fractions enriched in PIKfyve and PI 3-kinase largely overlap, they do not colocalize identically. Clearly, the results of the equilibrium sedimentation in density gradients demonstrate that PIKfyve is segregated from the membranes of the recycling endosomal system and GLUT4 storage compartment, sedimenting with denser structures enriched in class IA PI 3-kinases.

Electron microscopy studies in 3T3-L1 adipocytes document that a subfraction of LDM structures that sediments at the bottom of density gradients is composed of cytoskeleton and macromolecular protein complexes and, to a much lesser extent, of membranes (27). Signaling intermediates, including those related to the insulin signaling cascade, such as class IA PI 3-kinase and IRSs (29, 30), are enriched in this subfraction. Because they appear to be Triton X-100-resistant, it has been suggested that these proteins associate with cytoskeleton rather than with membranes (27, 31). The sedimentation of PIKfyve within the denser structures and its large cofractionation with p85, documented above, suggests that a pool of the particulate PIKfyve can also be attached to the cytoskeletal elements. We tested this hypothesis by two independent biochemical approaches. In the first, we examined the resistance of LDM-associated PIKfyve to Triton X-100 solubilization. About 60% of the LDM-associated PIKfyve was recovered in the Triton-insoluble fraction (Fig. 3A). In the second approach, we first liberated the soluble and membrane-associated proteins from intact cells with Triton X-100-containing cytoskeletal buffer and then collected the cytoskeletal network retained on the dish. When 3T3-L1 adipocytes were treated in this manner, a significant pool of PIKfyve equal to 10% of the total immunoreactive PIKfyve was detected in the Triton-insoluble pellet.
(Fig. 3B). Collectively, these data indicate that a subfraction of particulate PIKfyve resists Triton solubilization, suggesting a potential PIKfyve attachment to cytoskeletal elements.

Distribution of PIKfyve Lipid Kinase Activity among Adipocyte Subcellular Fractions—Often the presence of an enzyme in a particular compartment does not necessarily imply a site of catalytic action. Therefore, we examined in parallel the PIKfyve lipid kinase activity in the subcellular fractions. 3T3-L1 adipocytes were homogenized in HES buffer and fractionated to obtain M/N, LDM, PM, and cytosol or treated with Triton X-100-containing cytoskeletal buffer to collect the soluble proteins and the Triton-insoluble cytoskeletal elements. Anti-PIKfyve immunoprecipitates of the solubilized fractions were analyzed for lipid kinase activity in the presence of PtdIns 3-P and \([\gamma^{32}P]ATP\) as substrates. Generated PtdIns 3,5-P\(_2\) was separated by TLC and confirmed by HPLC (Fig. 4). Control experiments revealed a linear relationship between the protein amounts (25 \(\mu\)g to 2 \(\mu\)g) subjected to immunoprecipitation with anti-PIKfyve and the PtdIns 3,5-P\(_2\) production for each one of the fractions (not shown). Except for the PM-enriched fraction, the basal PIKfyve activity measured in the individual fractions principally paralleled the immunoreactive PIKfyve amounts. However, while the distribution ratio of the immunoreactive PIKfyve among cytosol, LDM, and PM was 4:1:0.18 as normalized per cell number (Fig. 1), the distribution ratio of the PIKfyve lipid kinase activity among those fractions was 4:1:0.8 under these conditions (Fig. 4A). For yet-to-be identified reasons, the PM-enriched fraction showed significantly higher PIKfyve specific activity (~5-fold). The relative distribution of PIKfyve lipid kinase activity between the Triton-soluble and Triton-insoluble pools of 3T3-L1 adipocytes principally mirrored the PIKfyve relative amounts (Fig. 4B). Thus, the majority of the activity (87%) was recovered in the Triton-soluble pool. The Triton-insoluble cytoskeletal network showed ~13% of the enzymatic activity (Fig. 4B), which was equivalent to the estimated PIKfyve protein levels (10%; see above). These results demonstrate that all fractions found positive for immunoreactive PIKfyve express enzymatically active protein and indicate that PIKfyve protein presence in a particular fraction most probably reflects a site of its action.

Characterization of PIKfyve Protein Localization by Confocal Microscopy—The biochemical studies presented above document that particulate PIKfyve is composed of two pools: Triton-soluble, membrane-associated; and Triton-insoluble, attached, presumably, to cytoskeletal elements. To further discern and define the PIKfyve-containing elements/membranes, fluorescence studies were performed using confocal microscopy. We took advantage of a heterologous cell system transiently expressing PIKfyve full-length protein tagged on its N terminus with GFP. We first confirmed the expression of the predicted protein by immunodetecting a 230-kDa band corresponding to EGFP-PIKfyve only in the lysates derived from the transfected cells (Fig. 5A). Expressed HA-tagged PIKfyve served as a size marker control (Fig. 5A). We next verified that the localization of EGFP-PIKfyve reflected that of the endogenous protein by several ways. First, we compared the subcellular distribution of the endogenous PIKfyve with that of ectopically expressed PIKfyve\(_{WT}\) in COS-7 cells by immunoblotting. Endogenous PIKfyve, EGFP-PIKfyve\(_{WT}\), or HA-PIKfyve\(_{WT}\) partitioned among cytosol, intracellular membrane, and plasma membrane fractions at identical ratios of 70:28:2. Second, because the low endogenous levels of PIKfyve in COS cells (32) prevent a convincing detection by immunofluorescence microscopy, we compared the immunofluorescence signals associated with the authentic PIKfyve in adipocytes with that of expressed EGFP-PIKfyve in transfected COS cells. In both cell types, PIKfyve displayed a characteristic discrete punctate pattern in the cell periphery (Fig. 5B, b, e, h, k, n, and p). Diffuse staining, indicative of soluble PIKfyve populations, was also observed, consistent with the biochemical detection of PIKfyve cytosolic populations. Finally, loss of the peripheral puncta and appearance of an exclusive diffuse staining was associated with the expression of a EGFP-PIKfyve mutant with a deleted FYVE finger, determined as an intracellular localization signal in other studies.\(^2\) Together, these results imply that the localization of het-

\(^2\) A. Shisheva, D. Sbrissa, and O. C. Ikonomov, unpublished data.
erologously expressed EGFP-PIKfyve in COS cells reflects that of the endogenous protein.

We first examined the localization of EGFP-PIKfyve in the context of the recycling endosomal pathway. To label the membranes of the endosomal recycling system, transfected COS cells were allowed to internalize Texas red-Tf for 15 min at 37 °C. The cells were then chilled and processed for fluorescence microscopy. Irrespective of the similar vesicular-like pattern of appearance of the fluorescent signals associated with EGFP-PIKfyve and Texas red-Tf, yellow color was practically undetectable upon overlay of the two images (Fig. 5B). Accordingly, and in agreement with our previous observations (8), the punctate staining of the native PIKfyve documented in 3T3-L1 adipocytes was largely negative for GLUT4 (Fig. 5B). Thus, consistent with the biochemical studies presented above, PIKfyve is segregated from the recycling endosomal pathway, defined by TIR and GLUT4.

We next determined whether PIKfyve localizes to compartments along the endocytic pathway but distal to the early endosomal recycling compartment. PIKfyve distribution to lysosomes was monitored by dextran fluorescent conjugates, which reach the lysosomes by pinocytosis and largely accumulate there, resisting enzymatic cleavage. COS cells expressing EGFP-PIKfyve were allowed to take up Texas red-dextran and then chased for 2 h and processed for fluorescence microscopy. Upon merging of the EGFP-PIKfyve and Texas red-dextran images, it was apparent that the two punctate patterns of distribution were largely different (Fig. 5B). PIKfyve-positive/dextran negative structures were principally observed, and vice versa.

In both mammalian and yeast cells, the endocytic pathway merges with the biosynthetic pathway at MVBs (used here as a synonym of prelysosomal or late endosomal compartments) and, subsequently, their distinct cargo transit together to the lysosome (33). Possible localization of PIKfyve to MVBs was examined using CI-MPR, which sorts and delivers newly synthesized lysosomal enzymes from TGN to late endosomes. Electron microscopy studies indicate that, at least in kidney cells (as are the COS cells), the late endosomes are the predominant CI-MPR-positive compartment, while the lysosomes themselves are CI-MPR-negative (34). Intriguingly, on the merged image a substantial population of vesicles positive for both CI-MPR and EGFP-PIKfyve, as judged by the appearance of yellow, were visible (Fig. 5B). Distinct populations of single-positive green- and red-staining vesicles were also observed (Fig. 5B), indicating that while the two proteins largely overlap, a unique localization pattern is associated with each one of them. Documented overlap between PIKfyve and CI-MPR marker is consistent with the notion that a subfraction of membrane-bound PIKfyve localizes to TGN/MVB. In line with this notion was the detection of few vesicles positive for both PIKfyve and the Golgi marker β-COP (Fig. 5B). However, the inability of brefeldin A, a drug that causes a rapid loss of Golgi as a distinct organelle (22), to change substantially the EGFP-PIKfyve punctate pattern (not shown) places the majority of the particulate PIKfyve at MVBs.

The biochemical characterization of a PIKfyve pool resistant to Triton extraction (Fig. 3) was confirmed by fluorescence microscopy. We observed a largely preserved punctate pattern of the fluorescence associated with EGFP-PIKfyve following brief Triton X-100 extraction prior to cell fixation (Fig. 5B). Collectively, the results of these approaches support the conclusion that a subpopulation of an active PIKfyve enzyme associates with detergent-resistant cytoskeletal structures through a binding mechanism, which is also Triton-resistant.

**FIG. 5.** Confocal microscopy documents PIKfyve as peripheral puncta, excluded from recycling endosomes and lysosomes, partially overlapping with MPR marker and largely Triton-resistant. A, COS-7 cells were transfected with pEGFP-HA-PIKfyve, pCMV5-HA-PIKfyve, or the empty vectors, lysed and immunoprecipitated as indicated. SDS-PAGE and immunoblotting reveals expression of anti-GFP-reacting protein with the expected size (230 kDa) as related to HA-PIKfyve (200 kDa) (arrows). B, details of cell processing for confocal microscopy are given under “Experimental Procedures.” a–c, double immunofluorescence staining for GLUT4 (a), PIKfyve (b), and composite (c) in 3T3-L1 adipocytes reveals practically no overlap. d–f, COS-7 cells transfected with EGFP-PIKfyve (e; shown are two cells expressing PIKfyve at different levels) were allowed to take up Texas red-Tf (15 min; 37 °C) (d). No colocalization is documented upon overlap of both images (j; g–i), COS-7 cells transfected with EGFP-PIKfyve (k) were allowed to take up Texas red-dextran (g). Only a single yellow particle could be observed on the composite image (i, j–l, double staining of EGFP-PIKfyve-transfected COS cells (k) with anti-MPR antibodies (j) reveals a substantial overlap, indicated in l. m–o, double staining of EGFP-PIKfyve-transfected COS cells (w; shown are three cells expressing PIKfyve) with anti-β-COP antibody (m) reveals a limited overlap, indicated in o. p and q, COS-7 cells transfected with EGFP-PIKfyve were extracted for 60 sec with Triton X-100 (0.5%) prior to fixation (p). A phase-contrast image illustrates the peripheral distribution of the Triton-resistant EGFP-PIKfyve puncta relative to the whole cell (q). Bar, 10 μm.

PIKfyve LDM Recruitment in Response to 3T3-L1 Adipocyte Stimulation—Relocation upon cellular stimulation appears to be a common theme in cellular regulation. Class I PI 3-kinase(s) for example is recruited to a variety of intracellular locations in response to cell stimulation with growth factors, including insulin, where it increases the local concentration of key lipid products (20, 30). In the case of PIKfyve, regulated specific intracellular targeting may selectively facilitate its interactions with PtdIns 3-P substrates, given the fact that its other favorite substrate, PtdIns, is about 400 times more abundant (32). This consideration, together with the apparent existence of cytosolic and particulate PIKfyve pools, suggests the possibility that PIKfyve may relocate upon cellular stimulation. To test this hypothesis, we examined the levels of PIKfyve in the subcellular fractions (PM, HDL, LDM, MN, and cytosol) derived from serum-deprived 3T3-L1 adipocytes acutely stimulated with insulin. At the basal state, the immunoreactive PIKfyve was distributed mainly between LDM and cytosol as nonphosphorylated or poorly phosphorylated forms (Fig. 6, and see below). Importantly, insulin action in 3T3-L1 adipocytes resulted in a 2-fold increase (2.15 ± 0.29; mean ± S.E., n = 7) of the immunoreactive PIKfyve in LDM with a commensurate decrease in the cytosol (Fig. 6A). Concordantly, similar recruitment (2.5-fold) was triggered upon cell stimulation with pervanadate (Fig. 6B), a well known insulin-mimetic agent shown to increase IR phosphotyrosine content (and that of other proteins) (25). No insulin- or pervanadate-dependent changes in the PIKfyve levels were documented in the remaining fractions (not shown). Intriguingly, the insulin- or pervanadate-dependent recruitment of PIKfyve onto LDM was accompanied by a decrease in the PIKfyve electrophoretic mobility, causing the immunoreactive PIKfyve to appear on the gels as a clear cut doublet (Fig. 6, A and B). It is noteworthy that this bandshift was observed only in LDM; no significant changes in the electrophoretic mobility were detected in the immunoreactive PIK-
FIG. 5—continued

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B

Endosomal Marker

GLUT4

Transferrin

Dextran

M6PR

β-COP

PIKfyve/GFP-PIKfyve

Composite

+ Triton X-100
fyve from cytosol (Fig. 6, A and B) or PM fraction (not shown) upon 3T3-L1 adipocyte stimulation.

Since this mobility shift is suggestive of an IR-induced covalent modification such as phosphorylation, we next examined the phosphorylation status of PIKfyve in the fractions. The profile and subcellular distribution of tyrosine-phosphorylated proteins observed in response to insulin versus control was largely in agreement with previous observations in this cell type (27, 35). We readily detected the 170-kDa phosphotyrosine band (IRSs) in LDM and cytosol and 95-kDa phosphotyrosine band (IRβ-subunit) in PM and, to a lesser extent, in LDM by probing with two anti-phosphotyrosine antibodies, 4G10 and 4G10.

FIG. 6. Insulin and pervanadate induce a recruitment of the cytosolic PIKfyve to LDM, coupled with PIKfyve hyperphosphorylation and proportional changes in PIKfyve lipid kinase activity. A and C, serum-deprived 3T3-L1 adipocytes treated or not with insulin (100 nM; 7 min) were fractionated to obtain LDM and cytosol. Proteins (55 μg of LDM and 200 μg of cytosol) were resolved by SDS-PAGE and immunoblotted. B, 3T3-L1 adipocytes treated or not with pervanadate (PV; 100 μM; 20 min) were fractionated to obtain LDM and cytosol. Proteins (200 μg) were resolved by SDS-PAGE and immunoblotted. Both treatments render PIKfyve doublet form (indicated). D, equal amounts of LDM fraction derived from insulin-treated adipocytes were subjected or not to dephosphorylation for 40 min at 25 °C with alkaline phosphatase (AP; 50 units) or λ-phosphatase (λP; 50 units) in 10 mM Tris-HCl buffer, pH 7.4, containing 50 mM NaCl, 10 mM MgCl2 and 1 mM dithiothreitol (for AP) or 50 mM Tris-HCl, containing 2 mM MnCl2 and 5 mM dithiothreitol (for λP). Proteins were analyzed by SDS-PAGE and immunoblotting. A–D, representative immunoblots of 3–7 independent experiments for each panel. E, serum-deprived 3T3-L1 adipocytes treated or not with insulin (100 nM; 7 min) were fractionated to obtain LDM and cytosol. Detergents were added to each fraction as described under "Experimental Procedures," and after centrifugation, aliquots of the clear lysates (cytosol (400 μg) and LDM (50 μg), equivalent to one-fourth of a 100-mm dish), were immunoprecipitated with anti-PIKfyve. The immunoprecipitates were subjected to lipid kinase assay with PtdIns 3-P substrate as described in the legend to Fig. 4. Shown are an autoradiogram of a TLC plate of a representative experiment (upper panel) and quantitation of the PIKfyve activity in four independent cellular fractionations (lower panel). PIKfyve activity in the indicated fractions is expressed as fold alteration of the basal activity in cytosol. For each experiment, the activity was expressed per equal number of cells.
crease in the LDM fraction upon insulin is visible in the supernatants of adsorption experiment out of two with similar results. PIKfyve indicated. Shown are chemiluminescence detections from a single immunoblotting with antibodies as indicated. Shown are chemiluminescence detections from a single immunoadsorption experiment out of two with similar results. PIKfyve increase in the LDM fraction upon insulin is visible in the supernatants (lane 2 versus lane 1).

PY20 (Fig. 6C, and data not shown). However, neither one of the antibodies documented convincingly an insulin-dependent tyrosine phosphorylation of PIKfyve in several experiments, in which the insulin-regulated increase in phosphotyrosine content of IRSs and IR was dramatic (Fig. 6C). These results indicate that PIKfyve LDM recruitment/bandshift triggered by the tyrosine-phosphorylated IRS most probably involves phosphorylation of PIKfyve on Ser/Thr. To test this hypothesis, LDMs derived from insulin-stimulated 3T3-L1 adipocytes were treated with alkaline phosphatase or protease and phosphatase inhibitors, immunoadsorbed on anti-GLUT4 IgG (lanes 3 and 6) or rabbit IgG (lanes 1 and 2) and immobilized on protein A-Sepharose CL-4B beads. Washed immune complexes, together with aliquots of the supernatants from the nonimmune IgG (lanes 1 and 2) were subjected to SDS-PAGE and consecutive immunoblotting with antibodies as indicated. Shown are chemiluminescence detections from a single immunoadsorption experiment out of two with similar results. PIKfyve increase in the LDM fraction upon insulin is visible in the supernatants (lane 2 versus lane 1).

The observed insulin-dependent relocation/mobility shift of a PIKfyve is undetectable in immunopurified GLUT4 vesicles. Analysis of the fractions from the iodixanol gradients of LDM, however, did not detect significant differences in PIKfyve distribution from basal versus insulin-treated 3T3-L1 adipocytes (Fig. 2, and data not shown). Considering the fact that PIKfyve is of relatively low abundance (silver stain experiments, not shown) it is possible that its absence in the GLUT4-positive gradient fractions is due to detection limitations. Therefore, we addressed the possibility of PIKfyve recruitment onto GLUT4 vesicles by an alternative approach exploring GLUT4 vesicle immunoadsorption on anti-GLUT4 antibodies. PIKfyve presence in the immunopurified GLUT4 vesicles was then examined by both immunoblotting and enzymatic activity. Irrespective of insulin treatment, the immunoadsorbed GLUT4 vesicles neither contained detectable levels of immunoreactive PIKfyve (Fig. 7) nor generated detectable amounts of PIKfyve products, PtdIns 5-P or PtdIns 3,5-P2 (not shown). Both the immunoreactive PIKfyve (Fig. 7) and the products of its enzymatic activity were recovered only in the GLUT4 vesicle supernatant. By contrast, known residents of GLUT4 vesicles, such as IRAP and TIR, were readily detected, as was their insulin-dependent decrease due to GLUT4 vesicle’s departure to PM (Fig. 7), in agreement with previous studies (reviewed in Ref. 3). Together, the data indicate that while PIKfyve function may be essential for insulin action on GLUT4 translocation, this is not achieved by PIKfyve’s direct presence in the GLUT4 compartment.

**DISCUSSION**

In this study, we examined the intracellular protein localization and the enzymatic activity distribution of the recently cloned phosphoinositide 5-kinase PIKfyve (8) as a first step toward assessment of the plausible intracellular sites of generation and action of its lipid products, PtdIns 5-P and PtdIns 3,5-P2. To this end, we have applied biochemical fractionation and immunofluorescence microscopy and utilized specific anti-PIKfyve antibodies to detect the protein and measure its lipid kinase activity in basal or stimulated cells expressing native or recombinant PIKfyve. Our studies unequivocally demonstrate presence of an enzymatically active PIKfyve in resting cells, distributed in at least two pools: cytosolic and associated with particulate structures/membranes. Because PIKfyve does not contain a transmembrane domain (nor is there any evidence for
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post-translational modification that would enable it to associate with membranes), PIKfyve retention on the particulate fractions should result from interactions with membrane proteins, lipids, or cytoskeletal elements. Both authentic and heterologously expressed PIKfyve displayed typical peripheral punctate staining reminiscent of endosomes but were found largely excluded from the early endosomal and recycling pathway. Rather, PIKfyve colocalized with a Golgi-to-late-endosome marker, MPR, suggesting a role in the later steps of the endocytic and/or biosynthetic pathway. The punctate pattern of PIKfyve fluorescence appearance was remarkably stable to agents known to disrupt cellular membranes, implying PIKfyve attachment, at least in part, to the Triton-insoluble cytoskeletal network. We show further that in fractionated resting 3T3-L1 adipocytes PIKfyve protein and activity were present mostly in the cytosol and to a lesser extend in LDM but upon insulin stimulation underwent an acutely directed re-recruitment to LDM linked with hyperphosphorylation that increased its levels and lipid kinase activity more than 2-fold. These results provide support for the hypothesis that the regulated local production of PtdIns 3,5-P2 and/or PtdIns 5-P at a particular intracellular site is important for acute insulin action.

Particulate PIKfyve Is Segregated from the Early Endosomal/Recycling Compartment—Association of PIKfyve with the early endosome recycling compartment was expected because most of the mammalian FYVE finger-containing proteins, including EEA1, Hrs, and Ankhzn, are localized to early endosomes (10–12). Moreover, the morphological appearance of the native PIKfyve in adipocytes or heterologously expressed PIKfyve in COS cells as discrete peripheral puncta was highly reminiscent of endosomes (Ref. 8 and Fig. 5). Several lines of experimental evidence presented in this study clearly exclude the recycling endosomes as a principal site of PIKfyve residency. Thus, upon LDM fractionation by equilibrium sedimentation in density gradients, the immunoreactive PIKfyve was recovered in a distinct population of dense structures, segregated from the recycling endosome membrane markers TIR, IRAP, or GLUT4. Next, PIKfyve protein and activity were undetectable in the immunopurified intracellular GLUT4 storage compartment, which contains recycling endosomal markers to some extent. Finally, membranes of the recycling endosomal pathway, defined by fluorescently labeled Tf or anti-GLUT4, were largely negative for the punctate pattern associated with EGFP-PIKfyve or immunoreactive adipocytic PIKfyve. The possibility that PIKfyve may label a functionally distinct compartment within the earlier stages of TfR recycling was ruled out in an experiment in which Texas red-Tf was allowed to internalize for only 3 min in EGFP-PIKfyve-transfected COS cells (not shown). Together, these data are consistent with the notion that, while PIKfyve and its lipid products may be operational in the endocytic pathway, PIKfyve is not associated with the membranes of the early endosomal/recycling pathway.

PIKfyve in MVB Function—Genetic studies in yeast predict that Fab1p function, while unessential for the anterograde protein traffic to the vacuole (lysosome) along all known vacuolar transport pathways, plays a certain role in MVB protein sorting (16, 18). Because a correct MVB function is also dependent upon class E VPS gene products, it is proposed that localized production of the Fab1p product PtdIns 3,5-P2 recruits/activates class E Vps proteins from the cytoplasm to drive different stages in the MVB sorting pathway (18). A possible role for PIKfyve and, therefore, the localized production of PtdIns 3,5-P2 and/or PtdIns 5-P in the MVB function in mammalian cells is predicted in the present study by the demonstration that a large proportion of PIKfyve populates MVBs, evidenced by several criteria. Thus, similarly to MVBs (36), PIKfyve-containing structures are labeled poorly for recycling receptors such as TIR. Next, MVBs are negative for lysosomal markers (33) as are the PIKfyve-containing structures (Fig. 5B, g–i). Finally, as with MVBs (33), PIKfyve containing structures are positive for the TGN-to-late endosome marker CI-MPR. These data indicate that a substantial population of PIKfyve is localized to MVB and are consistent with the notion of PIKfyve’s role in the late compartments of the endocytic and/or biosynthetic pathway in mammalian cells, a hypothesis that should be rigorously tested in future studies.

PIKfyve and Cytoskeleton in Insulin Action on GLUT4 Translocation—In insulin-sensitive muscle and fat, GLUT4 is responsible for transporting the vast majority of glucose into the cell (3, 37). While it is now clear that insulin evokes GLUT4 translocation from the intracellular storage compartment to the fat/muscle cell surface, the exact molecular mechanism is still elusive. Resting upon solid experimental support from numerous studies, it is now well accepted that insulin-regulated GLUT4 exocytosis is directly dependent on the enzymatic activity of class I phosphatidylinositol 3-kinase(s) and the production of PtdIns 3,4,5-P3 (30, 37). A first clue that PIKfyve function may also be important in GLUT4 membrane dynamics came from experiments demonstrating enriched expression of both PIKfyve mRNA and protein in primary fat cells and adipocytes in culture (8, 32). In fact, PIKfyve was originally identified through a search of fat/muscle molecular elements, which, similarly to the fat/muscle-specific GLUT4, are expressed in a tissue-specific manner (38). Furthermore, PIKfyve displays an intrinsic activity to generate PtdIns 5-P and PtdIns 3,5-P2, and both PtdIns P3,5-P2 are now identified in resting 3T3-L1 adipocytes (9, 39). Intriguingly, it has recently become apparent that insulin-induced GLUT4 translocation is extremely sensitive to dephosphorylation of position D-5 in PtdIns 3,4,5-P3 (40). Because the intracellular PtdIns 3,4,5-P3 production is apparently a result of joint actions of class I phosphatidylinositol 3-kinase(s) and other lipid kinases with a specificity for positions D-4 and D-5, a role for PIKfyve function in insulin action on GLUT4 could be expected. Intriguingly, our recent studies detected complexes of PIKfyve with the class I phosphatidylinositol 3-kinase(s) in lysates of 3T3-L1 adipocytes (41), suggesting plausible collaboration of these two kinases in the generation of the insulin-relevant lipid messengers. PIKfyve’s role in acute insulin actions is further corroborated by the present data, demonstrating an acute insulin-regulated recruitment/bandshift of the soluble PIKfyve onto the LDM pool to increase the local synthesis of PtdIns 3,5-P2 (Fig. 6). This phenomenon is observed not only with insulin but also upon 3T3-L1 adipocyte stimulation with the insulinomimetic agent pervanadate, which increases the IR-phosphotyrosine content (25), implying that a signal issued by tyrosine-phosphorylated IR is relayed to Ser/Thr-hypophosphorylated and recruit PIKfyve. It is tempting to speculate that hyperphosphorylated PIKfyve tethers to key LDM structures specifying the site and timing of high local PI production. Together, our findings are consistent with a model whereby a fraction of the soluble PIKfyve becomes phosphorylated in an insulin-dependent manner. Phosphorylated PIKfyve molecules are then recruited to insulin-sensitive intracellular sites, which harbor and/or receive other insulin-stimulated signaling molecules, i.e. PI 3-Ks, possibly jointly delivered with PIKfyve. Locally generated lipids then serve as signaling mediators of insulin end point responses. Our recent observation that increased levels of PIKfyve by adenovirus-mediated gene delivery in insulin-sensitive 3T3-L1 adipocytes mimic typical insulin-regulated physiologi-
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... is consistent with this model. The challenge remains to identify the PIKfyve Ser/Thr kinase, which is predicted in this study, as well as plausible partners of PIKfyve recruitment to LDM.

While a correlation of the insulin-dependent recruitment/mobility shift of PIKfyve protein and activity on LDM within the time course of insulin-regulated GLUT4 exocytosis could be documented, the causal relationship is somewhat hampered by the fact that PIKfyve is largely excluded from the intracellular GLUT4 storage compartment. This notion is supported here by a variety of approaches, discussed above. Moreover, a potential PIKfyve recruitment into the GLUT4 compartment upon acute cell stimulation with insulin was also below the detection limit of different approaches. However, recent independent experimental evidence lends support to the notion that key elements of the insulin signaling cascade relevant to GLUT4 exocytosis, such as IRSs and PI 3-kinase activity, are in fact outside the GLUT4 compartment. Thus, in 3T3-L1 adipocytes, both PI 3-kinase and IRS proteins cofractionate with the cytoskeletal-like insoluble proteinaceous particles of LDM but not with GLUT4 (27, 31), similarly to PIKfyve. Next, microinjected probes that specifically interact with the intracellular PI 3-kinase such as IRSs and PI 3-kinase activity, are in fact outside the GLUT4 compartment. Thus, in 3T3-L1 adipocytes, both PI

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