PIKfyve Lipid Kinase Is a Protein Kinase: Downregulation of 5′-Phosphoinositide Product Formation by Autophosphorylation†

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ABSTRACT: A subset of phosphoinositide 3-kinase family members are dual specificity enzymes; their protein kinase activity is thought to bring about an additional level to their intracellular regulation. Here we have examined whether the 5′-phosphoinositide kinase PIKfyve, reported previously to catalyze the formation of PtdIns 5-P and PtdIns 3,5-P_2 in vitro [Sbrissa et al. (1999) *J. Biol. Chem. 274*, 21589−21597], displays dual specificity. We now report that PIKfyve possesses an intrinsic protein kinase activity inseparable from its lipid kinase activity and, besides itself, can phosphorylate exogenous proteins in a substrate-specific manner. Both the autophosphorylation and transphosphorylation were demonstrated with PIKfyve immunopurified or affinity-purified from heterologously transfected COS cells, infected Sf9 cells, or native 3T3-L1 adipocytes. Conversely, no protein kinase activity was associated with immunopurified lipid kinase dead point (K1831E) or truncated (Δ1812−2052) PIKfyve mutants. PIKfyve autophosphorylation or transphosphorylation engaged Ser but not Thr or Tyr residues. PIKfyve autophosphorylation was largely abrogated upon pretreatment with PIKfyve lipid substrates or phosphatases. The impact of autophosphorylation on the PIKfyve lipid kinase activity was further examined with purified PIKfyve preparations. A decrease of 70% in the lipid product formation was associated with PIKfyve autophosphorylation, which was reversed upon treatment with phosphatases. In the cellular context, PIKfyve, or a fraction of it, was found in a phosphorylated form. Collectively, these results indicate that PIKfyve is a dual specificity kinase, which can generate and relay protein phosphorylation signals to regulate the formation of its lipid products, and possibly other events, in the context of living cells.

Accumulated evidence over the past several years has revealed that proteins, which display homology to the catalytic domain of phosphoinositide kinases (PIKs)[1] can act as lipid kinases, protein kinases, or both (for recent reviews, see refs *1*−*5*). Studies with purified mammalian enzymes led to the categorization of the phosphoinositide kinases into three general families on the basis of their specificity for a particular position: PI 3-Ks, PI 4-Ks, and PI 5-Ks (*3*−*5*). PI 3-kinases, which catalyze the phosphorylation at position D-3 of the inositol ring in PtdIns, PtdIns 4-P, or PtdIns 4,5-P_2, are further subdivided into three classes, I_A and I_B, II, and III, on the basis of their structure, in vitro substrate specificity, and mode of activation. PI 4-Ks are also represented by several types. They all display narrower substrate specificity strictly directed toward position D-4 of PtdIns, but not of PI (i.e., the phosphorylated PtdIns derivatives), and are now called PtdIns 4-Ks (*3*). In addition, position 4 of already phosphorylated PtdIns, but not of pure PtdIns, can be attacked by the enzymatic activity of another subclass of PI kinases, known as type II PIPKs or PIP 4-Ks. Finally, position 5 is targeted by two subclasses of enzymes: PI 5-Ks (*3*−*5*) (or type I PIPKs; *4*) and PIKfyve (*6*). While the latter two subclasses share substantial homology in their catalytic region, they display different substrate specificity. Thus, PI 5-Ks show preferences for PtdIns already phosphorylated at D-4 (*7*, *8*), while PIKfyve prefers PtdIns and 3′-phosphorylated PtdIns (*6*).

A region homologous to the catalytic domain of the p110 catalytic subunit of class I_A PI 3-kinases has been identified in a large group of mammalian proteins, such as ATM (ataxia telangiectesia mutated), ATR (ataxia telangiectesia and Rad3 related), mTOR/FRAP (mammalian target of rapamycin), and DNA-PK (DNA-dependent protein kinase) (*1*, *2*). However, they do not belong to the family of phosphoinositide kinases because none of them exhibit an intrinsic lipid kinase activity. Instead, they possess an intrinsic protein kinase activity directed toward the catalytic subunits themselves or toward other endogenous proteins and are referred to as PI 3-kinase-related protein kinases (*9*). These protein kinases have been reported to be important in mitogenic and growth factor responses (FRAP) or cell cycle (DNA-PK, ATM, and ATR) (*2*, *9*).

A third category of proteins with PIK homology was found to possess dual specificity, with an intrinsic protein kinase activity that is inseparable from their lipid kinase activity (*1*). To date, a protein kinase activity of only PI 3-K family

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[1] Abbreviations: DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FBS, fetal bovine serum; GST, glutathione *S*-transferase; PI, phosphoinositide; PIK, phosphoinositide kinase; PtdIns, phosphatidylinositol; P_2, bisphosphate; P_3, trisphosphate; K, kinase.

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members is documented, that of class I PI 3-Ks being the best characterized. Studies in vitro demonstrate that it is directed toward p85α, p85*β*, p110α, or p110*δ* subunits of the complex p85/p110 (or class I_A PI 3-Ks), and in all instances the phosphorylation results in downregulation of the lipid kinase activity of p110α or p110*δ* (*10*−*12*). Experimental evidence is recently provided to suggest that, at least in the case of p110*δ*, similar regulation may exist in intact cells (*12*). IRS-1 is proposed as a candidate physiological protein substrate of class I_A PI 3-Ks (*13*−*15*), but it is still uncertain whether its phosphorylation reflects the activity of PI 3-Ks themselves. The mammalian p110*γ* subunit of class I_B PI 3-Ks, as well as the yeast vps34p (class III PI 3-K), is also found to autophosphorylate, but this seems to not affect their lipid kinase activities (*16*, *17*). Whether other PI kinases with specificity for positions D-4 and D-5, i.e., PI 4-Ks or PI 5-Ks, display a dual enzymatic activity toward lipids and proteins is presently unknown.

The 5′-phosphoinositide kinase PIKfyve, recently cloned from a mouse adipocyte cDNA library, shares extensive homology with the yeast gene product Fab1p, thought to be important in yeast membrane trafficking (*18*−*21*). PIKfyve is a large protein of 2052 amino acids, which displays an intrinsic, wortmannin-resistant (ID_{50}, 600 nM) lipid kinase activity to generate PtdIns 5-P and PtdIns 3,5-P_2 in vitro (*6*, *18*). Its role in signaling/execution of membrane trafficking events in mammalian cells is suggested by the results of complementation experiments in *fab1*-deficient yeast mutants, demonstrating PIKfyve's ability to suppress the vacuolar defect and restore the PtdIns 3,5-P_2 pool to a certain extent (*22*). We demonstrate here that PIKfyve exhibits an intrinsic protein kinase activity and, besides itself, phosphorylates exogenous substrates in vitro. PIKfyve protein kinase shares many common features with PIKfyve lipid kinase, including requirement of identical key amino acids in the catalytic mechanism, resistance to wortmannin, and preferences for Mn^{2+} vs Mg^{2+}. In the context of intact 3T3-L1 adipocytes PIKfyve, or a subfraction of it, is found to be phosphorylated, and as with the in vitro autophosphorylated PIKfyve, a phosphoserine is predominantly detected. We further show that autophosphorylated PIKfyve downregulates its activity as a lipid kinase in vitro. The possible role of this phenomenon for cellular regulation is discussed.

EXPERIMENTAL PROCEDURES

Materials. PtdIns and phosphatidic acid were from Avanti Polar Lipids Inc.; phosphatidylcholine was from Sigma; diC_{16} PtdIns 3-P as an ammonium salt was from Echelon; [^{32}P]-orthophosphate (8800 Ci/mmol), [γ-^{32}P]ATP (6000 Ci/mmol), and the chemiluminescence detection kit were from DuPont, NEN; horseradish peroxidase-bound anti-rabbit IgG was from Boehringer. Rabbit polyclonal anti-PIKfyve antiserum (R7069) was directed against a recombinant GST fusion protein comprising the N-terminus of PIKfyve (amino acids 1−100). Its ability to immunoprecipitate endogenous PIKfyve was characterized elsewhere (*6*). Rabbit polyclonal anti-HA antiserum was a kind gift from Mike Czech and Joanne Buxton. Viral stock of GST fusion of p110 subunit of PI 3-kinase was a kind gift from Mike Czech and Joe Virbasius (*23*).

Constructs. For expression in Sf9 cells, PIKfyve was cloned into the baculovirus expression vector AcGHLT-A (Pharmingen) in two steps. First, the NH_2-terminal region of the full-length $PIKfyve_S$ cDNA in pBluescript II SK(+) (*18*) was released with *Not*I/*Kpn*I (2.9 kbp fragment) and ligated into the *Not*I/*Kpn*I digest of AcGHLT-A in frame with GST (N-$PIKfyve_S$-AcGHLT-A). Second, the C-terminus of PIKfyve, released by *Kpn*I from pBluescript II SK(+) $PIKfyve_S$ cDNA was ligated into the *Kpn*I digest of N-$PIKfyve_S$-AcGHLT-A.

The generation of $PIKfyve_S$ tagged at the NH_2 terminus with a nine amino acid epitope, YRYDVPDYA, derived from influenza virus hemagglutinin (HA) in the pCMV5 vector has been described elsewhere (*18*). Details for the construction of the pCMV5-HA-tagged version of the deletion mutant PIKfyveΔ1812−2052 are provided in ref *18*. The kinase dead point mutant of PIKfyve was generated by substituting the K^{1831} for E. For this purpose, the *Hin*dIII−*Pst*I fragment of clone K12 (*18*) that encompasses nucleotides 5216−6462 of the PIKfyve sequence (the numbers correspond to GenBank accession number AF102777) was amplified as two PCR fragments, which were subsequently linked in the engineered *Xho*I restriction site (CTCGAG that replaced the original sequence CTGAAG and introduced a single substitution of K to E). The first fragment (416 bp) was amplified with a primer pair consisting of an external sense primer (5′-GCCGAAGCTTCAGGAATGTTG-3′) and an internal antisense primer (5′-GCCGCTCGAGAATGAATCTATC-3′). The second fragment (830 bp) was amplified with an internal sense primer (5′-GCCGCTCGAGCAAATGCCTC-3′) and an external antisense primer (5′-GCCGCTGCAGTCATGGACAGTC-3′). The PCR products were digested with *Hin*dIII−*Xho*I or *Pst*I−*Xho*I, respectively, and then ligated into the *Hin*dIII−*Pst*I cloning sites of pBluescript II SK(+) to confirm the sequence and the desired mutation by sequencing and restriction analysis with *Xho*I. The *Hin*dIII−*Pst*I fragment with the confirmed mutation together with the *Nco*I−*Hin*dIII fragment of PIKfyve−pBluescript II SK(+) (nucleotides 3833−5216) was inserted into the *Nco*I−*Pst*I digest of the pGEM-T vector (Promega). The *Nco*I−*Sal*I fragment of the latter construct together with the *Kpn*I−*Nco*I fragment of PIKfyve−pBluescript II SK(+) (nucleotides 2929−3833) was ligated into the *Kpn*I−*Sal*I digest of pBluescript II SK(+). Finally, the *Kpn*I−*Sal*I fragment of the latter vector was ligated with the N-terminal portion of PIKfyve by inserting the fragment into the *Kpn*I−*Sal*I digest of $PIKfyve_S$−pBluescript II SK(+). HA-tagged $PIKfyve_S^{K1831E}$ was then generated in pCMV5 by a triple ligation using the *Eco*RI and *Sal*I cloning sites of the vector, the double-stranded oligonucleotide encoding for the HA epitope (tailed with *Eco*RI and *Xba*I restriction sites at the 5′ and 3′ ends, respectively), and the *Xba*I−*Sal*I digest of the $PIKfyve_S^{K1831E}$−pBluescript II SK(+) cDNA. The $PIKfyve_S$Δ177−198 mutant was generated by *Pst*I digestion of the N-terminal fragment of PIKfyve−pBluescript II SK(+) (*6*, *18*). The 63 bp fragment encompassing nucleotides 673−736 was eliminated, and the 537 bp fragment was ligated in pBluescript II SK(+). The new truncated N-terminus of PIKfyveΔ177−198 was ligated with the C-terminal part of PIKfyve (*18*) in pBluescript II SK(+) using the unique *Kpn*I restriction site at nucleotide 2929. HA-tagged PIKfyveΔ177−198 was generated in pCMV5 as described above. The expected organization of the mutants was confirmed by restriction endonuclease mapping.

Cell Cultures. Conditions for differentiation of 3T3-L1 mouse fibroblasts into insulin-sensitive adipocytes were as previously described (*24*). Cells were used between 7 and 14 days after the onset of the differentiation program. COS-7 cells were seeded to indicated density in DMEM[1] containing 10% FBS, 50 units/mL penicillin, and 50 μg/mL streptomycin sulfate. Sf9 insect cells were maintained in complete Grace's medium supplemented with 10% FBS and 100 μg/mL gentamicin.

Cell Transfection and Infection. COS-7 cells were seeded at 750 000 cells per 100 mm plate. Transfection with pCMV5-HA-PIKfyve$_S$, point and deletion mutants, or the empty vector was performed by the calcium phosphate precipitation method as we described previously (*24*). Fifty to fifty-five hours posttransfection, the cell lysates were collected in RIPA buffer [50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 1× protease inhibitor cocktail (1 mM phenylmethanesulfonyl fluoride, 5 μg/mL leupeptin, 5 μg/mL aprotinin, 1 μg/mL pepstatin, and 1 mM benzamidine)] and used for immunoprecipitation with anti-HA polyclonal antibodies.

PIKfyve$_S$-AcGHLT-A plasmid was cotransfected with a BaculoGold linearized viral DNA into Sf9 cells following the manufacturer's protocols (Pharmingen). GST−PIKfyve and GST−p110α (*23*) were purified from Sf9 cells at 4−5 days following infection. Cells were collected by centrifugation and lysed in RIPA buffer, supplemented with 1× protease inhibitor cocktail. The lysates were clarified by centrifugation (16000*g*, 15 min, 4 °C) and incubated for 1 h at 4 °C with GSH−agarose beads, and after subsequent washings, the GSH−affinity precipitates were used in lipid or protein kinase assays as described below.

[^{32}P]Orthophosphate Metabolic Labeling of 3T3-L1 Adipocytes. Differentiated 3T3-L1 adipocytes (100 mm dishes) were labeled with [^{32}P]orthophosphate as we previously described (*25*). Briefly, the cells were washed in phosphate-free DMEM and labeled for 12 h in phosphate-free DMEM, containing 0.5% BSA and 2 mM sodium pyruvate. Lysates were collected with RIPA buffer supplemented with 1× protease inhibitor cocktail and phosphatase inhibitors [25 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 50 mM NaF, 2 mM $NaVO_3$, 1 μM pervanadate (*26*), and 50 nM okadaic acid], clarified by centrifugation, and subjected to immunoprecipitation with preimmune or anti-PIKfyve sera as described below.

Immunoprecipitation and Immunoblotting. PIKfyve proteins (native, wild type, or mutants) were immunoprecipitated from 3T3-L1 adipocytes or from COS-7 cells transiently expressing the HA-epitope-tagged PIKfyve$_S^{WT}$, PIKfyve$_S^{K1831E}$, or PIKfyve$_S$ deletion mutants, using polyclonal anti-PIKfyve N-terminal specific or anti-HA antibodies. Control immunoprecipitates on preimmune serum or anti-HA immunoprecipitates from control transfections were run in parallel. Prior to the immunoprecipitations, the cells were lysed in RIPA buffer supplemented with 1× protease inhibitor cocktail. The lysates were precleared by centrifugation for 15 min in a microfuge at 4 °C and incubated with immune or preimmune sera for 16 h at 4 °C. Protein A−Sepharose CL-4B was added in the final 1.5 h of incubation at 4 °C. Immunoprecipitates or GSH−affinity precipitates were washed with RIPA buffer and then solubilized in Laemmli sample buffer and analyzed by SDS−PAGE (6% acrylamide) or subjected to additional washes prior to their assay for lipid or protein kinase activities. For Western blot analysis, after SDS−PAGE, the proteins were transferred onto nitrocellulose membranes. The blots were saturated with blocking buffer under previously specified conditions (*6*, *18*) and blotted (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 and a chemiluminescence kit.

Autophosphorylation and Transphosphorylation Assays. The protein kinase assay was carried out with the native or recombinant PIKfyve immunopurified from 3T3-L1 adipocytes and transfected COS-7 cells or affinity-purified from infected Sf9 cells on GSH−agarose beads as described above. After adsorption, the protein A−Sepharose or GSH−agarose beads were washed three times with RIPA buffer supplemented with 1× protease inhibitors and then three times with 50 mM HEPES, pH 7.4. The beads were then preincubated for 10 min at 25 °C with several agents indicated in the figure legends in 50 mM HEPES, pH 7.4. In some experiments protein kinase activity of native or recombinant PIKfyve was measured in the presence of exogenous substrates. In this case, the washed beads were preincubated with histone or β-casein (Sigma) (each at 800 μg/mL) in 50 mM HEPES, pH 7.4, for 10 min at 25 °C. The standard phosphorylation reactions in the presence or absence of exogenous substrates were according to our previously published conditions for insulin receptor kinase (*27*). Briefly, the reaction was initiated by addition of the ATP/ion mix, composed (unless otherwise stated) of [γ-^{32}P]-ATP (3−6 μCi), 25 μM ATP, 2.5 mM $MnCl_2$, and 12 mM $MgCl_2$ in 50 mM HEPES, pH 7.4, continued for 15 min at 25 °C (unless otherwise stated), and stopped on ice. The supernatants were removed and solubilized in Laemmli sample buffer for analysis of the exogenous substrate phosphorylation by SDS−PAGE (15% acrylamide) and autoradipography (Kodax X-OmatAR). The beads were washed three times with ice-cold PBS containing 50 mM NaF and 10 mM $Na_4P_2O_7$, boiled in Laemmli sample buffer, and analyzed by SDS−PAGE (6% acrylamide). Resolved proteins, transferred onto Immobilon P (Millipore), were analyzed by autoradiography and immunoblotting. The radioactive bands were cut and subjected to phosphoamino acid analysis, described below. To analyze the capacity of alkaline (Sigma) or λ phosphatases (Calbiochem) to dephosphorylate the ^{32}P-labeled PIKfyve, following autophosphorylation in the presence of [γ-^{32}P]ATP described above, the immunoaffinity or affinity PIKfyve complexes were washed with "alkaline phosphatase buffer" (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, and 10 mM $MgCl_2$) or "λ phosphatase buffer" (50 mM Tris-HCl, pH 7.8, 5 mM DTT, 2 mM $MnCl_2$, and 100 μg/mL BSA) and then incubated in the presence or absence of the enzymes (50 units each) in the corresponding buffer for 30 min at 30 °C. The samples were then washed as described above and analyzed by SDS−PAGE and, after electrotransfer to membranes, by autoradiography and immunoblotting.

Lipid Kinase Assay and TLC Resolution of Lipid Products. After immunoprecipitation the beads were washed once with RIPA buffer, twice with 50 mM HEPES, pH 7.4, 1 mM EDTA, and 150 mM NaCl, three times with 100 mM Tris-

HCl, pH 7.5, and 500 mM LiCl, twice with 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 1 mM EDTA, and twice with "assay buffer" (25 mM HEPES, pH 7.4, 120 mM NaCl, 5 mM β-glycerophosphate, and 1 mM DTT containing typically 2.5 mM $MgCl_2$ and 2.5 mM $MnCl_2$) (*6*). The kinase reaction (50 μL final volume) was carried out for 15 min at 37 °C in the assay buffer supplemented with 50 μM ATP, [γ-^{32}P]ATP (12.5 μCi), and 100 μM PtdIns (sonicated prior to use in 20 mM HEPES, pH 7.5, 1 mM EDTA). The lipids were extracted, applied on TLC plates (Whatman, PE SIL G, 250 μm), and separated by chromatographic solvent systems as we described previously (*6*). Generated radioactive products were detected by autoradiography and quantified by laser densitometry (Molecular Dynamics) and radioactive counting (*6*).

In some experiments PIKfyve lipid kinase activity was measured following PIKfyve prephosphorylation with cold ATP and dephosphorylation with alkaline or λ phosphatases. For this purpose, following washes described above, the immunoaffinity or affinity beads were incubated with or without 200 μM cold ATP in the assay buffer for 1 h at 25 °C. The beads were then washed twice with λ phosphatase buffer or alkaline phosphatase buffer and incubated at 30 °C for 30 min in 250 μL of the corresponding buffers in the presence or absence of λ or alkaline phosphatases (50 units each). The beads were then washed once with the corresponding buffer for each phosphatase and then washed three times in lipid kinase assay buffer prior to their analyses for lipid kinase activity described above.

Phosphoamino Acid Analysis. The samples for phosphoamino acid analysis (in vitro autophosphorylation or substrate phosphorylation reactions and immunoprecipitated PIKfyve from ^{32}P-labeled 3T3-L1 adipocytes) were resolved by SDS−PAGE, electrotransferred, and analyzed by autoradiography. The strips, corresponding to the radioactive bands of PIKfyve or histone, were cut from the Immobilon P membrane and hydrolyzed at 110 °C for 1 h in 6 N HCl. Upon drying (SpeedVac, Savant), the hydrolyzed material was resuspended in 6 μL of a standard phosphoamino acid mixture (*28*) and spotted onto a cellulose thin-layer plate (Merck) along with 2 μg of each individual phosphoamino acid standard and 6 μL of the standard phosphoamino acid mixture. The plate was run on a high-voltage thin-layer electrophoresis system at 20 mA and 1.5 kV for 1 h in one dimension under previously described conditions (*28*). The plate was then dried, sprayed with 0.25% (w/v) ninhydrin, and analyzed by autoradiography.

RESULTS

PIKfyve Autophosphorylates in Vitro. At present, only members of the PtdIns 3-K family are found to possess dual specificity toward lipid and protein substrates. To determine whether PIKfyve, which displays specificity for the D-5 position of PtdIns and PtdIns 3-P, exhibits a protein kinase activity toward itself or other proteins, PIKfyve immunoprecipitates derived from 3T3-L1 adipocytes were first subjected to an autophosphorylation assay in the presence of [γ-^{32}P]ATP, Mg^{2+}, and Mn^{2+} to test for autokinase activity. The autoradiographic detection of the SDS−PAGE-resolved autophosphorylation reaction revealed two principal phosphoprotein products in the PIKfyve immunoprecipitates,

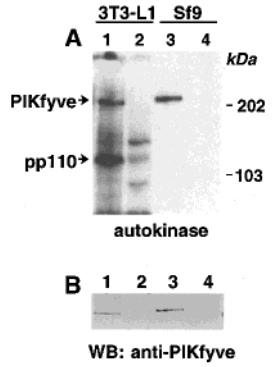

FIGURE 1: PIKfyve autophosphorylates in vitro. Native PIKfyve, immunoprecipitated and immobilized on protein A−Sepharose (lane 1), or recombinant GST−PIKfyve on GSH−agarose beads (lane 3) were prepared from 3T3-L1 adipocyte or infected Sf9 cells, respectively, as described under Experimental Procedures. Precipitates were subjected to an in vitro autophosphorylation reaction in 50 mM HEPES, pH 7.4, with [γ-^{32}P]ATP (3 μCi), 25 μM ATP, 2.5 mM $MnCl_2$, and 12 mM $MgCl_2$ for 15 min at 25 °C. The reactions were stopped and washed with ice-cold PBS containing 50 mM NaF and 10 mM $Na_4P_2O_7$. SDS−PAGE-resolved proteins, transferred onto Immobilon P membrane, were analyzed by autoradiography (A) and immunoblotting with 1:5000 anti-N-terminal PIKfyve antibodies (B). Preimmune immunoprecipitates from 3T3-L1 adipocyte lysate (lane 2) and GSH affinity-purified beads from Sf9 cell lysate infected with a control vector (lane 4) were analyzed similarly. Shown are an autoradiogram (A) and a chemiluminescence detection (B) of a representative blot out of four to ten experiments with similar results.

not seen in the immunoprecipitates with the corresponding preimmune serum (Figure 1A, lanes 1 vs 2). The first one migrated at ~110 kDa, referred to herein as pp110, and is discussed below. The second band displayed the electrophoretic mobility of PIKfyve, just below the 202 kDa protein marker. It aligned identically with the PIKfyve protein band, detected by immunoblotting of the same membrane with anti-PIKfyve antibodies (Figure 1B). This result indicates that PIKfyve is largely phosphorylated under the conditions of the assay.

To delineate between PIKfyve autophosphorylation and phosphorylation by a PIKfyve-associated protein kinase that may be present in PIKfyve immune complexes of 3T3-L1 adipocytes, we examined the ability of recombinant PIKfyve proteins, expressed in infected insect or transfected mammalian cells, to autophosphorylate. Sf9 cells were infected with baculoviruses, mediated the expression of either PIKfyve or a control protein in the same vector, and the proteins were subsequently affinity-purified on GSH−agarose beads. Bound proteins were then used in a autokinase assay under standard conditions. A phosphorylated band with the electrophoretic mobility of GST−PIKfyve was documented only in PIKfyve-infected Sf9 cells (Figure 1A, lanes 3 vs 4). Its identity as the PIKfyve protein was confirmed by Coomassie staining (not shown) and immunoblotting of the same membrane with anti-PIKfyve antibodies (Figure 1B). Accordingly, only PIKfyve immunoprecipitates derived from COS-7 cells transfected with PIKfyve^{WT} but not with PIKfyve lipid kinase dead mutants displayed the ability to self-phosphorylate (Figure 2, see below). Collectively, these results demonstrate that PIKfyve lipid kinase displays an intrinsic protein kinase activity directed toward itself.

The stoichiometry of PIKfyve autophosphorylation was measured in the presence of excess of ATP (50 μM, specific

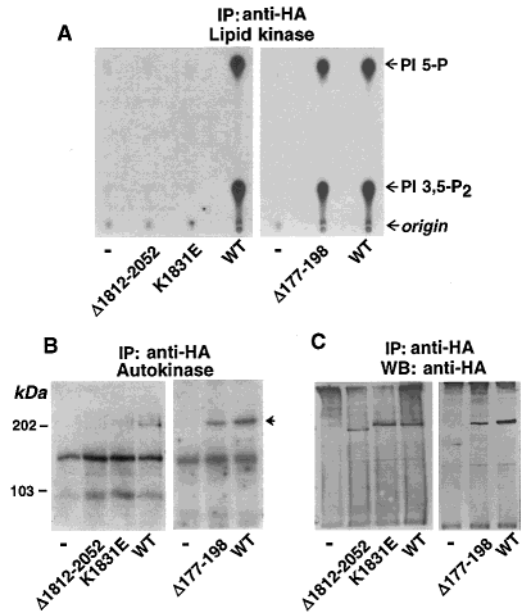

FIGURE 2: PIKfyve autophosphorylation activity is inseparable from its lipid kinase activity. COS-7 cells were transfected with the indicated pCMV5−HA−PIKfyve constructs or the empty vector. Parallel anti-HA immunoprecipitates (IP) derived from cell lysates were analyzed in lipid kinase assay as described in Experimental Procedures (A), in autokinase assay using the conditions described in the legend to Figure 1 (B), or for protein expression (C). Shown are autoradiograms following TLC separation of radiolabeled PtdIns 5-P and PtdIns 3,5-P_2 products from cells transfected with the empty vector (−), the C-terminal kinase domain deletion mutant (Δ1812−2052), the FYVE finger deletion mutant (Δ177−198), the point mutant (K1831E), or wild-type (WT) PIKfyve (A), autoradiograms of the autokinase reaction resolved by SDS−PAGE and transferred onto membranes (B), and a chemiluminescence detection of the same blots presented in panel B, probed with anti-HA antibodies at 1:1000 dilution (C). Shown are representative experiments out of three with similar results. The arrows in panel A depict PtdIns 5-P (PI 5-P) and PtdIns 3,5-P_2 products (PI 3,5-P_2) confirmed by HPLC analysis (*6*). The arrowhead in panel B shows PIKfyve autophosphorylated bands.

activity 0.0015 μCi/pmol) with recombinant PIKfyve, affinity-purified from infected Sf9 cells. Approximately 2.2 pmol of phosphate was incorporated into 1 pmol of PIKfyve protein.

PIKfyve Protein Kinase Is Inseparable from Its Lipid Kinase Activity. To reveal whether the same amino acids mediate both the lipid phosphorylation and the autophosphorylation, we analyzed the effects of PIKfyve point and truncated mutants on the PIKfyve lipid and protein kinase activity. Alignment of several protein and lipid kinases pinpointed Lys^{1831} of PIKfyve as a candidate ATP-binding residue (*18*, *29*, *30*). It corresponds to K^{72} of protein kinase A and is conserved in many mammalian and yeast PI kinases, including PI 5-Ks, PIP 4-Ks, PtdIns 4-Ks, and PI 3-Ks in mammals or Vps34p, Mss4p, and the PIKfyve homologue Fab1p in yeast (*30*). Mutation of K^{72} in protein kinase A or the counterparts in PI 5-Ks or PIP 4-Ks was found to result in catalytically inactive forms (*29*−*31*). The effect of K^{1831} to E substitution in PIKfyve was first examined on the ability of PIKfyve to catalyze the formation of PtdIns 5-P and PtdIns 3,5-P_2 in vitro. COS-7 cells transiently transfected with the HA-tagged version of $PIKfyve^{WT}$ or $PIKfyve^{K1831E}$ were lysed, and anti-HA-immunoprecipitated PIKfyve proteins were analyzed for lipid kinase activity. As expected, $PIKfyve^{WT}$ displayed a catalytic activity to generate PtdIns 5-P and PtdIns 3,5-P_2 from native PtdIns substrate preparations, as we previously reported (Figure 2A and ref *6*). Most importantly, this enzymatic activity was completely abolished in the HA immunoprecipitates derived from HA-$PIKfyve^{K1831E}$ expressing cells (Figure 2A). Similarly, no lipid kinase activity was documented in the HA immunoprecipitates of COS cell lysates expressing a truncated version of the PIKfyve catalytic domain ($PIKfyve^{\Delta1812-2052}$), in agreement with our previous studies (Figure 2A and ref *6*). The autokinase activity of these PIKfyve proteins paralleled that of the lipid kinase. PIKfyve autophosphorylation was documented only with the HA immunoprecipitates of $PIKfyve^{WT}$ but not with those of $PIKfyve^{K1831E}$ or $PIKfyve^{\Delta1812-2052}$ mutants (Figure 2B), under almost identical expression levels in COS cells (Figure 2C). Concordantly, a PIKfyve mutant with a truncation (Δ177−198) that does not affect its lipid kinase activity (Figure 2A) displayed the $PIKfyve^{WT}$ capacity to autophosphorylate (Figure 2B) proportionally to its expression level (Figure 2C). These results demonstrate that the two catalytic mechanisms use the same C-terminal phosphoinositide kinase homology domain and utilize K1831 as a key amino acid, possibly in the ATP binding.

To further compare the characteristics of PIKfyve lipid and protein kinase activities, we examined the in vitro sensitivity of PIKfyve autophosphorylation to agents such as wortmannin, Triton X-100, and adenosine, shown previously to affect PIKfyve lipid kinase activity to certain extents (*6*). PIKfyve immunoprecipitates derived from 3T3-L1 adipocytes were incubated with the indicated agents prior to their analyses by autokinase assay, SDS−PAGE, and autoradiography. Similarly to PIKfyve lipid kinase activity (*6*), the PIKfyve autophosphorylation was largely wortmannin-resistant. Thus, concentrations of 5−100 nM wortmannin that completely inhibit the protein kinase activity of class I PI 3-Ks (*10*−*12*, *16*) did not alter PIKfyve autophosphorylation (Table 1 and see Figure 6C). At 1 μM wortmannin, PIKfyve phosphorylation was inhibited by 20% (Figure 3A and Table 1). However, as opposed to PIKfyve lipid kinase, PIKfyve autophosphorylation was insensitive to low concentrations of Triton X-100 (0.1%) while more affected than the lipid kinase by adenosine (300 μM) (Figure 3A and Table 1). These results imply that differences depending upon the nature of the substrate may give some variations in PIKfyve lipid vs protein kinase activity.

We next determined the requirement of PIKfyve autophosphorylation for divalent cations. An important property of the PIKfyve lipid kinase activity in vitro is the requirement for Mn^{2+}, which supports PIKfyve lipid kinase activity much better than Mg^{2+} over a wide concentration range (*6*, *32*). Similarly, the in vitro autophosphorylation of PIKfyve immunopurified from 3T3-L1 adipocytes was largely Mn^{2+}-dependent. The level of phosphorylation in the presence of 5 mM Mn^{2+} alone exceeded ∼6-fold the levels in the presence of equimolar Mg^{2+} alone (Figure 3B and Table 1). Together, the data presented in Figures 1−3 and Table 1 indicate that both activities by PIKfyve are based on a similar catalytic mechanism.

In all experiments utilizing 3T3-L1 adipocytes, a radiolabeled phosphoprotein band with a mobility of ∼110 kDa, pp110, was found to co-immunoprecipitate with PIKfyve.

Table 1: Effect of Different Agents on PIKfyve's Autokinase and Lipid Kinase Activities[a]

	autokinase activity (%)	lipid kinase activity[b] (%)
no addition	100	100
inhibitors		
wortmannin, 20 nM	100 ± 5	100 ± 5
wortmannin, 400 nM	90 ± 6	70 ± 5
wortmannin, 1000 nM	80 ± 6	20 ± 3
adenosine, 300 μM	59 ± 5	80 ± 6
Triton X-100, 0.1%	125 ± 15	5
cations		
Mg^{2+}, 0.5 mM	100	70 ± 5
Mg^{2+}, 5 mM	220 ± 20	100 ± 5
Mn^{2+}, 0.05 mM	300 ± 35	100 ± 8
Mn^{2+}, 0. 5 mM	670 ± 62	600 ± 70
Mn^{2+}, 5 mM	1250 ± 100	700 ± 80
Mg^{2+} + Mn^{2+}, 12 mM + 2.5 mM	670 ± 68	ND

[a] PIKfyve immune complexes from 3T3-L1 adipocytes were preincubated in the absence or presence of the indicated inhibitors and subjected to autokinase assay in the presence of 12 mM Mg^{2+} and 2.5 mM Mn^{2+} as described in Experimental Procedures. Alternatively, PIKfyve immunoprecipitates were analyzed in a autokinase assay in the presence of the indicated ions. The data are expressed as a percentage of the PIKfyve autophosphorylation in the absence of inhibitors or as a percentage of the PIKfyve autophosphorylation in the presence of 0.5 mM Mg^{2+} as indicated and are presented as the mean ± SE (n = 3). [b] Data are from refs *6* and *32*, where the phosphorylation of the D-5 position in PtdIns 5-P and PtdIns 3,5-P_2 was quantified.

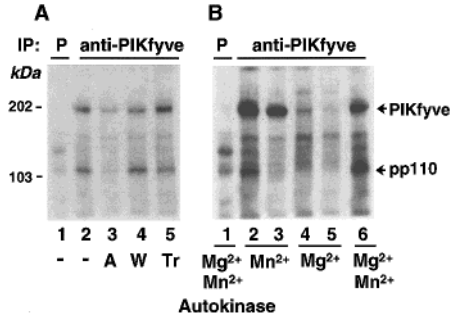

FIGURE 3: PIKfyve autophosphorylates in a wortmannin-resistant, Mn^{2+}-dependent manner. Immunoprecipitates (IP) with anti-PIKfyve N-terminal immune (anti-PIKfyve) or preimmune (P) sera were derived from 3T3-L1 adipocyte lysates and subjected to autophosphorylation assay under the conditions given in the legend to Figure 1, except for the presence of final concentrations of 300 μM adenosine (A), 1 μM wortmannin (W), or 0.1% (w/v) Triton X-100 (Tr) (A), or under similar conditions (lanes 1 and 6), except the final concentrations of the indicated ions were 0.5 mM (lanes 3 and 5) and 5 mM (lanes 2 and 4) (B). SDS−PAGE-resolved labeled proteins were transferred onto Immobilon P membrane and subjected to autoradiography as described in Figure 1. Shown are representative autoradiogams out of three to four experiments for each condition with similar results.

While the identity of this phosphoprotein and the potential role of PIKfyve protein kinase in its phosphorylation are not clear at present, it is worth emphasizing that, while PIKfyve autophosphorylation required only Mn^{2+}, pp110 phosphorylation was best supported in the presence of both Mn^{2+} and Mg^{2+} (Figure 3B). It is also interesting that pp110 phosphorylation largely paralleled PIKfyve autophosphorylation with respect to sensitivity to adenosine or resistance to wortmannin and Triton X-100 (Figure 3A).

PIKfyve Autophosphorylation Downregulates PIKfyve Lipid Product Formation. Having established PIKfyve autophosphorylation in vitro, we next addressed the question of its impact on the lipid kinase activity. Parallel PIKfyve immunoprecipitates of 3T3-L1 adipocytes were phosphorylated in the presence of [γ-^{32}P]ATP or cold ATP for increasing periods of time up to 60 min. At the indicated time interval the reactions were stopped with EDTA. The "hot" samples were then analyzed by SDS−PAGE to determine the level of ^{32}P label incorporated into PIKfyve, while the "cold" samples were subjected to lipid kinase assay. Results illustrated in Figure 4A,B clearly demonstrate that the time-dependent increase of PIKfyve autophosphorylation is accompanied by a corresponding decrease in PIKfyve lipid kinase activity. Next, we made use of the fact that the in vitro autophosphorylated PIKfyve is highly sensitive to treatment with either alkaline or λ phosphatases. As demonstrated in Figure 4C−E, the ^{32}P radioactivity incorporated upon autophosphorylation of either affinity-purified recombinant PIKfyve or native PIKfyve immunoprecipitated from 3T3-L1 adipocytes was almost completely abolished upon treatments with alkaline or λ phosphatases. Therefore, PIKfyve purified from either insect cells (Figure 4F) or 3T3-L1 adipocytes (Figure 4G) was first prephosphorylated in the presence of cold ATP, Mn^{2+}, and Mg^{2+}. The samples were then incubated in the absence or presence of λ phosphatase, which, as discussed above (Figure 4C,D), decreases PIKfyve autophosphorylation to almost undetectable levels. After washing, the lipid kinase activity was measured in the presence of PtdIns as a substrate. The phosphorylated PIKfyve exhibited 3−4-fold less activity to catalyze both PtdIns 5-P and PtdIns 3,5-P_2 formation vs the nonphosphorylated form (Figure 4F,G). More importantly, the inhibitory effect was reversed upon treatment of the prephosphorylated PIKfyve with the phosphatases (Figure 4F,G). Together, the results are consistent with the conclusion that PIKfyve autophosphorylation downregulates its enzymatic activity as a lipid kinase, which may be an important regulatory mechanism in the context of living cells.

Predicted physiological relevance of the PIKfyve autophosphorylation for the PIKfyve lipid kinase activity was further elaborated by examining the effect of PIKfyve lipid substrates PtdIns and PtdIns 3-P on the enzyme autophosphorylation status. Preincubation of immunopurified adipocytic PIKfyve with PtdIns (Figure 5A,B) or PtdIns 3-P (not shown) rendered an enzyme with a dramatically decreased autokinase activity. The rate of the autokinase reaction was diminished ~3-fold under these conditions (Figure 5B). Other phospholipids tested such as phosphatidic acid and phosphatidylcholine were with no significant effect (Figure 5C), indicating a specificity of the PIKfyve autophosphorylation inhibition induced by the PIKfyve lipid substrates. While the structural mechanism of this effect is unclear, these studies imply plausible lipid substrate-dependent upregulation of the PIKfyve lipid kinase activity by hampering the enzyme autophosphorylation.

PIKfyve Not Only Autophosphorylates but Also Transphosphorylates Protein Substrates. While PIKfyve function as a protein kinase toward itself was well documented in Figures 1−5, it remained to be identified whether PIKfyve can phosphorylate exogenous protein substrates. Two such substrates, widely used in the analysis of Ser/Thr protein kinases, were examined here: casein and histone. PIKfyve,

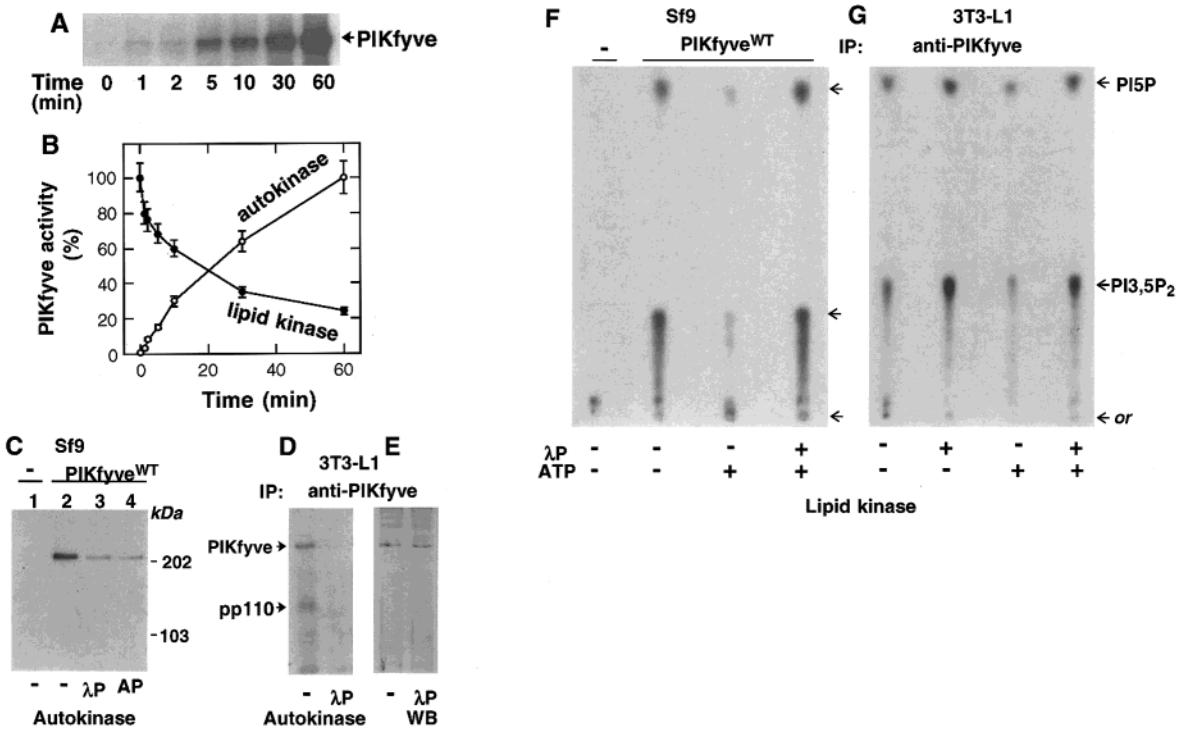

FIGURE 4: PIKfyve autophosphorylation downregulates PIKfyve lipid kinase activity. (A and B) Parallel anti-PIKfyve immunoprecipitates of 3T3-L1 adipocytes were subjected to autokinase assay for different time intervals in a reaction mixture consisting of 50 mM HEPES, pH 7.4, 5 mM $MnCl_2$, and 50 μM ATP supplemented or not with [γ-^{32}P]ATP (6 μCi) as described in Experimental Procedures. The reactions were stopped and washed with ice-cold PBS supplemented with 50 mM NaF, 10 mM EDTA, and 10 mM $Na_4P_2O_7$. The set of samples containing [γ-^{32}P]ATP was subjected to SDS−PAGE, electrotransfer, and autoradiography (A). At the indicated incubation intervals the samples with cold ATP were subjected to lipid kinase assay in the presence of PtdIns as a substrate as described in Experimental Procedures (B). The autokinase activity is expressed as a percentage of the 60 min time point; the lipid kinase activity, as a percentage of the 0 min time point. (C) Recombinant PIKfyve isolated from Sf9 cells and in vitro autophosphorylated on GSH−agarose beads with [γ-^{32}P]ATP (10 μCi) as described in Figure 1 was subsequently treated with (lanes 3 and 4) or without (lane 2) 50 units of the indicated phosphatase (AP, alkaline phosphatase; λP, λ phosphatase) in the appropriate phosphatase buffer, washed, and subsequently processed by SDS−PAGE, electrotransfer, and autoradiography as described in Experimental Procedures. Sf9 cells infected with a control vector were autophosphorylated in parallel (lane 1). (D) Native PIKfyve from 3T3-L1 adipocyte immunoprecipitates (IP), subjected to in vitro autophosphorylation under conditions described in the legend to Figure 1, was processed for autoradiography similarly to the samples in (C). (E) Immunoblot of the membrane presented in (D) with anti-N-terminal PIKfyve antiserum at 1:5000 and chemiluminescence detection. (F and G) PIKfyve lipid kinase activity was measured following prephosphorylation of affinity-purified recombinant PIKfyve (F) or native immunoaffinity-purified 3T3-L1 adipocyte PIKfyve (G) with or without cold ATP (200 μM) for 1 h at 25 °C in lipid assay buffer and subsequent treatment with or without λ phosphatase as detailed under Experimental Procedures. Panels show representative experiments out of two to six for each one of the conditions. λP-dephosphorylated PIKfyve exhibits a (3.2 ± 0.3)-fold (mean ± SE) higher enzymatic rate for D-5 phosphorylation vs autophosphorylated PIKfyve. Abbreviations: PI5P, PtdIns 5-P; $PI3,5P_2$, PtdIns 3,5-P_2.

immunopurified from 3T3-L1 adipocytes, was subjected to a protein kinase assay in the presence of either substrate. The reactions were then pelleted, and both the supernatants and the pellets were analyzed by autoradiography and immunoblotting after SDS−PAGE and electrotransfer onto membranes. While casein was ineffective as a substrate for PIKfyve protein kinase activity, histone was found to be readily phosphorylated (Figure 6A). Thus, the level of radioactivity incorporated into histone was ~6-fold higher with the PIKfyve immunoprecipitates vs the immunoprecipitates with a preimmune serum, used as a negative control (Figure 6A,B). Although the efficiency of this transphosphorylation was low, collectively these data indicate that PIKfyve displays the capacity to phosphorylate not only itself but also other protein substrates.

An important additional question is whether pp110 found coprecipitated with PIKfyve from 3T3-L1 adipocyte lysates (Figures 1A and 3) is a substrate of PIKfyve protein kinase. Several experimental and theoretical considerations point to the PIKfyve partnership with class I_A PI 3-kinases (*6*, *32*, *33*), suggesting a plausible identity of pp110 with the p110 subunit of PI 3-Ks. Therefore, we examined whether the p110α subunit of class I_A PI 3-kinases is a substrate for PIKfyve protein kinase activity. We utilized p110α and PIKfyve recombinant proteins, both affinity purified from infected Sf9 cells in an in vitro protein kinase assay and used wortmannin as a selective inhibitor of p110α protein kinase (*10*−*12*). The result demonstrated no connection between those two lipid kinases in their protein kinase mode of action. The radioactive levels of both enzymes were unaltered in the presence of the other protein kinase, nor was p110 phosphorylation reinstated upon a selective p110 protein kinase inhibition by wortmannin (Figure 6C). These results indicate that pp110, coprecipitated with and phosphorylated in the PIKfyve immunoprecipitates, displays properties distinct from the p110α subunit of PI 3-kinases, thus making pp110 identification an important objective.

PIKfyve Autophosphorylates and Transphosphorylates on a Serine Residue(s). The nature of PIKfyve autokinase activity was further investigated. Phosphoamino acid analysis of PIKfyve, affinity purified from Sf9 cells or immunopurified from 3T3-L1 adipocytes and then phosphorylated in

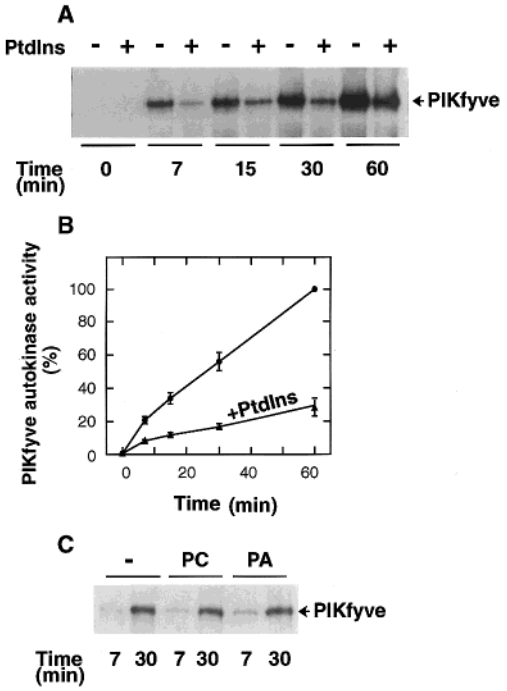

FIGURE 5: PtdIns attenuates PIKfyve autophosphorylation. PIKfyve immunoprecipitates prepared from cleared 3T3-L1 adipocyte lysates were assayed for autokinase activity as described in Experimental Procedures in 50 mM HEPES, pH 7.4, containing 5 mM $MnCl_2$ and 50 μM [γ-^{32}P]ATP (6 μCi). Samples were preincubated with or without 100 μM PtdIns, phosphatidic acid (PA), or phosphatidylcholine (PC) as indicated for 10 min at room temperature prior to addition of radiolabel. At the indicated time intervals the reactions were stopped with ice-cold PBS/50 mM NaF/10 mM $NaPP_i$/10 mM EDTA, washed once with the same, boiled in sample buffer, and resolved by SDS−PAGE. Following electrotransfer onto a nitrocellulose membrane, radiolabeled PIKfyve bands were detected by autoradiography (A and C). (B) Quantitation of the PIKfyve autokinase activity in the absence or presence of PtdIns, expressed as a percentage of the 60 min time point value in the absence of PtdIns (mean ± SE; $n = 3$).

vitro, revealed PIKfyve autophosphorylation exclusively on Ser (Figure 7). In dephosphorylated preparations phospho-Ser was hardly detectable. Similarly, phosphoamino acid analysis of the in vitro phosphorylated histone by the PIKfyve immunoprecipitates (see Figure 6A) identified only Ser as the phosphorylated amino acid (not shown). Together, these results allow the classification of PIKfyve as a phosphoinositide kinase with an intrinsic protein−serine kinase activity.

PIKfyve Phosphorylation Status in Vivo. Having established a PIKfyve autophosphorylation on Ser in vitro, we next addressed the question of PIKfyve phosphorylation status in vivo. Lysates of 3T3-L1 adipocytes metabolically labeled with [^{32}P]orthophosphate were immunoprecipitated with preimmune serum or anti-PIKfyve antiserum and analyzed by SDS−PAGE and autoradiography. Results illustrated in Figure 8A clearly demonstrate that PIKfyve, or a subfraction of it, is a phosphoprotein in vivo. Phosphoamino acid analysis of the in vivo phosphorylated PIKfyve documents principally phosphoserine and no detectable signals at the position of P-Thr or P-Tyr (Figure 8B). Collectively, these results identify PIKfyve as a serine-phosphorylated protein in the cellular context and indicate possible contribution of the PIKfyve autophosphorylation capacity, established in vitro, in its phosphorylation in vivo.

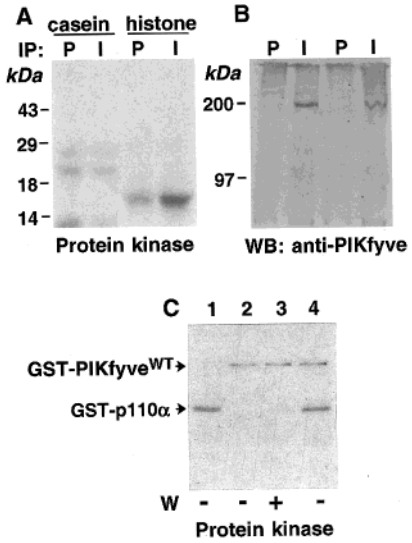

FIGURE 6: PIKfyve lipid kinase phosphorylates not only itself but also exogenous substrates. (A and B) Anti-PIKfyve N-terminal immune (I) or preimmune (P) immunoprecipitates (IP) derived from 3T3-L1 adipocytes were subjected to in vitro protein kinase assay in the presence of either histone or β-casein as indicated in Experimental Procedures. The supernatants (A) and the protein A−Sepharose beads (B) were resolved by SDS−PAGE and after electrotransfer were analyzed by autoradiography (A) or probed with anti-N-terminal PIKfyve antiserum at 1:5000 (B). Shown are an autoradiogram (A) and a chemiluminescence detection (B) of a representative experiment out of three with identical results. (C) Recombinant GST−PIKfyve on GSH−agarose beads (lanes 2−4) was subjected to in vitro protein kinase assay in the presence (lanes 3 and 4) or absence (lane 2) of affinity-purified GST−p110α fusion protein on GSH−agarose beads provided as exogenous substrate (lane 1). Where indicated, wortmannin (W) was added at 50 nM. Shown is an autoradiogram of a representative experiment out of two with identical results.

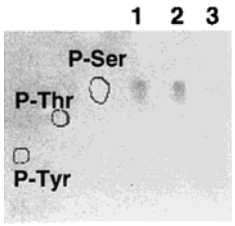

FIGURE 7: PIKfyve autophosphorylates on serine. In vitro autophosphorylated native 3T3-L1 adipocyte or recombinant PIKfyve were resolved by SDS−PAGE, electrotransferred onto Immobilon P, located by autoradiography, cut from the membrane, hydrolyzed for 1 h in 6 N HCl, and processed for phosphoamino acid analysis by high-voltage thin-layer electrophoresis on cellulose TLC plates along with phosphoamino acid standards detailed in Experimental Procedures. Shown is an autoradiogram of a representative experiment out of two with identical results. Lanes: 1, pooled 3T3-L1 PIKfyve bands isolated from lanes 2−4 in Figure 3A; 2, GST−PIKfyve from lane 2 in Figure 4C; 3, pooled GST−PIKFyve phosphatase-treated bands from lanes 3 and 4 in Figure 4C (P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine).

DISCUSSION

Here we report on the dual specificity of the recently identified 5′-phosphoinositide kinase PIKfyve. Besides inositol lipids, PIKfyve phosphorylates in vitro protein substrates including itself. Both PIKfyve autophosphorylation and transphosphorylation occur on a serine residue(s), allowing the classification of PIKfyve's second enzymatic activity as a protein−serine kinase. PIKfyve autophosphorylation downregulates the PIKfyve lipid kinase activity in vitro and can

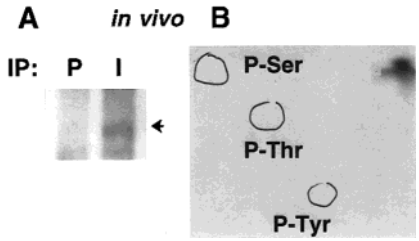

FIGURE 8: In vivo PIKfyve is a phosphoprotein phosphorylated on a serine residue(s). (A and B) Differentiated 3T3-L1 adipocytes were metabolically labeled with [^{32}P]orthophosphate for 12 h at 37 °C. Cell lysates were immunoprecipitated (IP) with preimmune (P) or PIKfyve immune (I) sera and then subjected to SDS−PAGE, electrotransfer onto Immobilon P, and autoradiography. The band corresponding to PIKfyve (indicated by an arrow) was cut and processed for phosphoamino acid analysis as described in the legend to Figure 7. Shown are autoradiograms of the membrane after SDS−PAGE and transfer (A) and of the thin-layer plate after phosphoamino acid analysis (B).

be modulated by the PIKfyve lipid substrates PtdIns and PtdIns 3-P. PIKfyve, or a fraction of it, is found in a phosphorylated form in the cellular context. These results suggest that PIKfyve is subject to regulatory autophosphorylation and that it may influence cellular metabolism by its enzymatic actions as both a lipid kinase and a protein kinase.

Except for wortmannin resistance (*10*−*12*, Figures 3A and 6C, and Table 1), there are many similarities between the enzymatic actions of class I_A PI 3-Ks and PIKfyve as protein kinases. Thus, as with the p110α and p110δ subunits (*10*−*12*), PIKfyve autophosphorylates in vitro (Figures 1−4), and this autophosphorylation engages serine residue(s) (Figure 7). Next, like the autophosphorylation of p110α and p110δ subunits (*10*−*12*), the PIKfyve autophosphorylation is far better supported by Mn^{2+} vs Mg^{2+} (Figure 3B and Table 1), a characteristic more typical for Tyr kinases than for Ser/Thr kinases. Furthermore, as in the in vitro autophosphorylation of either p110α or p110δ subunits (*10*−*12*), the autophosphorylated PIKfyve downregulates its lipid kinase activity (Figure 4). Importantly, in the case of p110δ this phenomenon has been recently demonstrated to occur in vivo (*12*), indicating the physiological relevance of the regulatory autophosphorylation. It is consistent with a mechanism in which the addition of a negatively charged phosphate group(s) hampers the binding of the negatively charged lipid substrates, resulting in attenuation of the PIKfyve lipid kinase activity. Conversely, presence of the PIKfyve lipid substrates hampers the enzyme autophosphorylation (Figure 5), thus enhancing their own phosphorylation. Finally, as reported for the p110 subunits (*10*−*12*), PIKfyve can transphosphorylate exogenous protein substrates (Figure 6A), suggesting that in living cells these enzymes can relay lipid as well as protein phosphorylation signals to differentially regulate cellular events. Support for this hypothesis has been recently provided by studies in which the lipid and protein kinase activity of PI 3-kinase have functionally been separated (*34*). The authors demonstrate that, while a lipid kinase inactive hybrid of PI 3-Kγ with a preserved protein kinase activity contributes to MAP kinase activation, it fails to stimulate protein kinase B, known to be sensitive to PI 3-K lipid products (*34*).

While regulatory Ser autophosphorylation leading to downregulation of PIKfyve lipid kinase activity was demonstrated in vitro (Figures 4 and 5), the impact of this phenomenon in the cell context remains to be identified. The data documented here identifying PIKfyve as a phosphoprotein in the cellular context phosphorylated mainly on a serine residue(s) (Figure 8) are consistent with the notion that the autoregulation observed in a cell-free system is relevant in vivo. The fact that class I_A PI 3-Ks are believed to be regulated by a similar mechanism in living cells (*10*−*12*) is intriguing in light of the expected joint action of PI 3-Ks and PIKfyve in generating the PtdIns 3,5-P_2 lipid product. In fact, recent experimental evidence implicates the members of class I_A PI 3-Ks as cellular partners of PIKfyve (*33*). Possible association of PIKfyve and PI 3-Ks will give rise to PtdIns 3-P, which represents only a small fraction of PtdIns, and will ensure an efficient generation of PtdIns 3,5-P_2 in situ. Conversely, autophosphorylation of PI 3-Ks and PIKfyve, under as yet to be identified mechanisms, will simultaneously shut off both enzymatic activities necessary for the PtdIns 3,5-P_2 formation. Theoretically, autophosphorylated PIKfyve not only may display loss of the PIKfyve lipid kinase activity but can acquire substrate-specific protein kinase activity. The above-mentioned findings with the PI 3-kinase hybrid (*34*) clearly demonstrate that lipid and protein kinase activities could generate distinct forward signals, further supporting the concept that protein kinase activities of the lipid kinases, including PIKfyve, play a physiological role. Whether PIKfyve possesses a protein kinase activity in vivo and, if yes, what is the identity of its cellular protein substrate(s) are questions that should be rigorously tested. It is also possible that autophosphorylated PIKfyve may change its intracellular localization, leading to different protein interactions and, hence, transmitting signals through distinct pathways. Further insight into these issues should be forthcoming as we learn more about the PIKfyve Ser phosphorylation site(s) in vivo and in vitro. Serine is the most abundant amino acid in the deduced amino acid sequence of PIKfyve; 205 serine residues (*18*) making the identification of the exact phosphorylation site(s) a technical challenge. Specific antibodies directed to this phosphorylation site(s) will resolve many questions related to the in vivo function of PIKfyve autophosphorylation.

In conclusion, the results presented here suggest that PIKfyve protein kinase activity may be an integral part of the enzyme physiological function and regulation. The occurrence and relevance of PIKfyve protein kinase activity in the cellular context remain to be elucidated, as is the identification of its downstream protein substrates. Noteworthy, a recent study (*35*), published while this paper was at a final stage of preparation, has demonstrated that type 1 PIKs can also downregulate their lipid kinase activities by autophosphorylation, suggesting a general physiological mechanism by which the lipid kinases are regulated by protein phosphorylation.

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