Phosphoinositide-dependent Kinase-2 Is a Distinct Protein Kinase Enriched in a Novel Cytoskeletal Fraction Associated with Adipocyte Plasma Membranes*

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By recombining subcellular components of 3T3-L1 adipocytes in a test tube, early insulin signaling events dependent on phosphatidylinositol 3-kinase (PI 3-kinase) were successfully reconstituted, up to and including the phosphorylation of glycogen synthase kinase-3 by the serine/threonine kinase, Akt (Murata, H., Hresko, R.C., and Mueckler, M. (2003) J. Biol. Chem. 278, 21607–21614). Utilizing the advantages provided by a cell-free methodology, we characterized phosphoinositide-dependent kinase 2 (PDK2), the putative kinase responsible for phosphorylating Akt on Ser-473. Immunodepleting cytosolic PDK1 from an in vitro reaction containing plasma membrane and cytosol markedly inhibited insulin-stimulated phosphorylation of Akt at the PDK1 site (Thr-308) but had no effect on phosphorylation at the PDK2 site (Ser-473). In contrast, PDK2 activity was found to be highly enriched in a novel cytoskeletal subcellular fraction associated with plasma membranes. Akt isoforms 1–3 and a kinase-dead Akt1 (K179A) mutant were phosphorylated in a phosphatidylinositol 3,4,5-trisphosphate (PIP3)-dependent manner at Ser-473 in an in vitro reaction containing this novel adipocyte subcellular fraction. Our data indicate that this PDK2 activity is the result of a kinase distinct from PDK1 and is not due to autophosphorylation or transphosphorylation of Akt.

Insulin initiates a complex array of intracellular signaling events (1). Upon insulin binding, the receptor becomes autophosphorylated on several critical intracellular tyrosine residues, thereby activating an intrinsic tyrosine kinase that can then phosphorylate cellular substrates. The major proteins that become tyrosine-phosphorylated by the activated insulin receptor include the insulin receptor substrate proteins (2), Shc (3), and Gab1 (4). These proteins do not have enzymatic activity. Rather, they serve as adaptor proteins capable of recruiting numerous downstream effectors that contain Src homology 2 domains (1).

The pathway centered on phosphatidylinositol (PI)3-kinase has emerged as the critical mediator of many of the metabolic responses to insulin, including the stimulation of glucose transport (for review, see Ref. 5). The PI 3-kinase mainly relevant to insulin signaling is a heterodimer consisting of a regulatory subunit (p85) and a catalytic subunit (p110). The regulatory subunit contains two Src homology 2 domains that can interact with tyrosine-phosphorylated adaptor proteins, most notably insulin receptor substrate-1 and -2. The major in vivo substrate for PI 3-kinase is phosphatidylinositol 4,5-bisphosphate, found in the cytoplasmic leaflet of the plasma membrane lipid bilayer. Insulin stimulation, thus, leads to the acute increase in the intracellular level of phosphatidylinositol 3,4,5-trisphosphate (PIP3).

The activity of the serine/threonine kinase Akt (also known as protein kinase B) is markedly stimulated in a PI 3-kinase-dependent manner (6). This phenomenon predominantly relies on the phosphorylation of Akt on two of its amino acid residues, 1) threonine 308 in the activation loop of the kinase catalytic domain and 2) serine 473 in the hydrophobic carboxy-terminal domain (7). The phosphorylation of both of these regulatory sites is inhibitable in vivo by the PI 3-kinase inhibitor wortmannin (8). The protein kinase responsible for phosphorylating Akt on Thr-308 is the recently identified phosphoinositide-dependent kinase 1 (PDK1) (9–11). Despite intense investigative efforts, the kinase responsible for phosphorylating Akt on Ser-473, tentatively termed phosphoinositide-dependent kinase 2 (PDK2), has resisted definitive purification (7, 12, 13). At least three models for Ser-473 phosphorylation have been proposed. Alessi and co-workers (14) demonstrate that PDK1 could be converted in vitro through interaction with a hydrophobic peptide (called PDK1-interacting peptide) into a form capable of phosphorylating Akt on both Thr-308 and Ser-473 (14). Whether this unprecedented mode of regulation occurs in vivo remains unclear. Toker and Newton provide data supporting an Akt autophosphorylation mechanism involving the Ser-473 site (15), similar to that of certain conventional protein kinase C isoforms (16). They suggest that Akt might be partially activated by phosphorylation of Thr-308 due to upstream PDK1, thereby allowing Akt to act upon itself by transferring a phosphate group onto Ser-473 (15). Finally, it is possible for PDK2 to be a distinct kinase yet to be characterized. In cells lacking PDK1, growth factor-stimulated phosphorylation of Akt on Thr-308 did not occur, but phosphorylation of Ser-473 still remained intact, suggesting the existence of a PDK2 kinase distinct from PDK1 (17).

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§ The abbreviations used are: PI, phosphatidylinositol; PIP3, PI 3,4,5-trisphosphate; CYT, cytosol; Ext, extract; HDM, high density microsomes; HiP, high speed pellet; HiS, high speed supernatant; ILK, integrin-linked kinase; LDM, low density microsomes; LoS, low speed supernatant; PDK, phosphoinositide-dependent kinase; PM, plasma membrane; PM(SW), salt-washed PM; GST, glutathione S-transferase.

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**In Vitro Reconstitution of Akt Phosphorylation**

It is self-evident that these competing models of Akt regulation have mutually exclusive features. However, a clear consensus concerning the nature of PDK2 has not been reached. We chose to address this issue using a novel approach. Using the subcellular components of 3T3-L1 adipocytes, we have recently developed an in vitro assay reconstituting key aspects of PI 3-kinase-dependent insulin signaling (45). We provide evidence obtained through this assay that PDK2 appears to be a cytoskeleton-associated kinase distinct from PDK1.

**EXPERIMENTAL PROCEDURES**

**Cell Culture of 3T3-L1 Adipocytes—**3T3-L1 preadipocytes obtained from the American Type Culture Collection were grown to confluency and 48 h later subjected to differentiation as described previously (18). 3T3-L1 adipocytes were used 10–14 days after initiating differentiation.

**Isolation of Subcellular Components—**Mature 3T3-L1 adipocytes grown on 10-cm dishes were serum-starved overnight. The cells were then rapidly washed three times with ice-cold serum-free Dulbecco's modified Eagle's medium and maintained further for 15 min at 4°C in serum-free Dulbecco's modified Eagle's medium in the absence or presence of 1 μM insulin. Cells were then washed three times with ice-cold phosphate-buffered saline, scraped in 2 ml of ice-cold HES buffer (50 mM HEPES, pH 7.4, 255 mM sucrose, and 1 μM EDTA/dish containing protease inhibitors (0.082 trypsin inhibitory units/ml aprotinin, Sigma), 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml benzamidine, 5 μg/ml trypsin inhibitor, 1 μg/ml chymostatin, 1 μg/ml pepstatin A, and 0.5 mM phenylmethylsulfonyl fluoride) and then homogenized at 4°C by passing the cells 10 times through a Yamato SC homogenizer at a speed of 1200 rpm. The plasma membrane (PM) fraction was obtained by differential centrifugation and sucrose cushion flotation as described previously (19) and designated as either PM (−ins) or PM (+ins) according to whether the starting cell source was exposed to insulin. The low density microsomes (LDM) fraction was obtained from basal cells as described previously (19). PM and LDM, subsequent to their isolation, were resuspended in IC buffer (20 mM HEPES, pH 7.4, 140 mM potassium glutamate, 5 mM NaCl, 1 mM EDTA, and protease inhibitors). A highly concentrated cytosol (CYT) fraction was prepared by washing the 3T3-L1 adipocytes three times with ice-cold IC buffer and then removing the buffer as much as possible by aspiration followed by cell scraping and homogenizing with a ball-bearing homogenizer. The supernatant was recovered after an ultracentrifuge spin for 1 h at 200,000 × g. For the preparation of PM salt-extracted proteins, plasma membranes pelleted after sucrose cushion flotation were resuspended in 200 μl of IC buffer supplemented with NaCl to a final concentration of 1 M. Samples were incubated on ice for 30 min and then subjected to centrifugation in a TLA-100.3 fixed angle rotor at 37,000 × g for 20 min. The pelleted fraction from this spin (PM/SW) was resuspended in IC buffer. The 1 M NaCl was removed from the supernatant (Ext-LoS) using a 1-ml Sephadex G-25 spin column that was pre-equilibrated with 2 ml of IC buffer. Equilibrated columns were centrifuged for 1 min at 300 × g before applying the sample to the top of the resin. Centrifugation of the sample through the spin column for 1 min at 300 × g removed the 1 M NaCl. For certain experiments, Ext-LoS was further centrifuged in a TLA-100.3 fixed angle rotor at 200,000 × g for 1 h to produce a supernatant (Ext-HiS) and a pellet (Ext-HiP). Ext-HiP was resuspended in IC buffer, and Ext-HiS was desalted with a 1-ml Sephadex G-25 spin column.

**In Vitro Assay—**Reactions were prepared in a manner described previously (45). Samples were prepared on ice by mixing in various combinations of LDM (−2.5 mM MgCl2 final concentration), CYT (~3 mg/ml final concentration), and PM (~0.5 mg/ml final concentration). Reactions were initiated with the addition of an ATP-regenerating system (final reaction concentrations, 1 mM ATP, 8 mM creatine phosphate, 30 units/ml creatine phosphokinase, and 5 mM MgCl2) and then incubated at 37°C for up to 15 min. The reactions were quenched by the addition of an equal volume of buffer A (50 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM sodium vanadate, 100 mM NaF, and 10 mM sodium pyrophosphate) containing 2% SDS and 1 mM EDTA. Certain in vitro reactions as indicated included 10 μM PIP3 (Calbiochem) in a sonication mixture of 100 μM phosphatidylincholine (Avanti Polar Lipids) and 100 μM phosphatidylserine (Avanti Polar Lipids). All phosphorylated PIP3-containing lipid vesicles were obtained from Calbiochem (18). Certain in vitro reactions were performed in the presence of liver PIP obtained from Avanti Polar Lipids. For the preparation of PDK1-immunodepleted CYT, pre-cleared CYT was incubated for 1.5 h at 4°C with protein G-agarose (Upstate Biotechnology) bound with anti-PDK1 polyclonal sheep IgG (Upstate Biotechnology catalog 006–637; 5 μg of IgG/mg of CYT). For experiments addressing the salt requirement for Akt phosphorylation, the salt content of the IC buffer used in the in vitro reactions (normally 140 mM potassium glutamate and 5 mM NaCl) was altered as indicated.

**Immunoblot Analysis and Immunoprecipitation—**Protein samples from the in vitro assay were subjected to SDS-PAGE and transferred to nitrocellulose. Phospho-specific antibodies recognizing the phosphorylated forms of Akt were obtained from New England Biolabs. Antibodies directed against integrin-linked kinase (ILK), paxillin, and integrin β1 receptor were purchased from Pharmingen. PDK1 antibody used for immunoblot analysis (catalog #06-906) as well as antibodies specific for vinculin, insulin receptor, and cavelin were purchased from Upstate Biotechnology. Actin antibody was obtained from Chemicon. The Arp2 antibody was a kind gift from Dr. John Cooper (Washington University School of Medicine).

Expression of Glutathione S-Transferase (GST)-Akt1 and GST-Akt1(K179A) in 293 Cells—pEG2G-Akt1 and pEG2G-Akt1(K179A) DNA constructs that have been used to overexpress GST-Akt fusion proteins in human embryonic kidney 293 cells were obtained from Dr. Dario Alessi (University of Dundee) (9). 10 μg of pEGFP-C1 (Clontech) were co-transfected into a 6-cm diameter dish of 293 cells using the MBS Mammalian Transfection Kit (Stratagene). Pooled transfectants were selected by growth in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 30 μg/ml G418. GST-Akt1 and GST-Akt1(K179A) fusion proteins were purified from the transfected 293 cells as previously described (9).

**RESULTS AND DISCUSSION**

**Examination of Akt Phosphorylation Using an in Vitro Assay—**Early insulin-stimulated events downstream of PI 3-kinase, including Akt activation, occur with reasonable efficiency in our novel cell-free system (45). This assay involves reconstituting in various combinations three subcellular fractions of 3T3-L1 adipocytes prepared by differential centrifugation; that is, the plasma membrane (PM)−ins or PM (+ins), depending on whether the intact cell source had been exposed to insulin at 4°C before fractionation, LDM, and CYT. After mixing of the components, the in vitro reaction is initiated by the addition of an exogenous ATP source and then incubated at 37°C for up to 15 min. By using this methodology, insulin-stimulated Akt phosphorylation on Thr-308 and Ser-473 is reconstituted in a manner dependent on PI 3-kinase and ATP.

We utilized this system to investigate the molecular regulation of Akt, taking advantage of experimental manipulations made possible by unhindered access to all reaction components. To clarify the role of PDK1 in the phosphorylation of Akt on Ser-473, we performed our in vitro reaction using a CYT fraction from which PDK1 had been immunodepleted. Among the reaction components used, PDK1 was found predominantly in the CYT fraction (Fig. 1A), consistent with localization observed by others (7, 20). The faint band with a slightly retarded mobility observed in the PM fraction might be either a cross-reacting protein or a post-translationally modified form of PDK1. The LDM was essentially devoid of PDK1. Immunodepletion of CYT with an anti-PDK1 antibody successfully removed PDK1 (CYT-PDK1). In contrast, mock immunodepletion of CYT with an irrelevant antibody had no effect on PDK1 content (CYT-CON).

In an in vitro reaction combining immunodepleted CYT with PM, the lack of PDK1 resulted in greatly diminished insulin-stimulated phosphorylation of Akt on Thr-308. However, insulin-stimulated Ser-473 phosphorylation occurred normally (Fig. 1B, left panels). There is some evidence to suggest that the phosphorylation of Akt in vivo takes place after its recruitment to cellular membranes (7). To address the possibility that PDK2 might be membrane-associated, a reaction was performed by combining CYT with PM that had been salt-washed with 1 M NaCl (PM/SW). Salt washing of PM abrogated insulin-stimulated Ser-473 phosphorylation. However, insulin-stimulated Thr-308 still occurred (Fig. 1B, middle panels). The inclusion of LDM to the reaction rescued insulin-stimulated phosphorylation of Akt on Thr-308. However, insulin-stimulated Ser-473 phosphorylation occurred normally (Fig. 1B, right panels). These results are consistent with the idea that insulin-stimulated Akt phosphorylation is dependent on both PDK-dependent and PDK-independent pathways.
Ser-473 phosphorylation from being inhibited by salt extraction of PM (Fig. 1B, right panels).

Collectively, the data strongly suggest that PDK2 might be a kinase distinguishable from PDK1. This was supported by the fact that independent manipulations (i.e. immunodepletion of PDK1 and salt extraction of PM) segregated these two kinase activities in complementary fashion (i.e. inhibiting PDK1 while leaving PDK2 intact and vice versa). In contrast to the predominantly cytosolic localization of PDK1, PDK2 appeared to be associated with PM and LDM and was largely absent from the cytosol.

ILK has been recently identified as a candidate for PDK2. The activity of ILK is apparently increased by insulin stimulation in a PI 3-kinase-dependent manner (21). Using transfected cells, S. Dedhar and co-workers provide evidence suggesting that ILK can phosphorylate Akt on Ser-473 (22). However, the data regarding ILK are not conclusive. There is even uncertainty that ILK is a functional kinase, as several critical residues normally found in the catalytic domain of protein kinases are not conserved in ILK (23). ILK may, thus, regulate the phosphorylation of Akt on Ser-473 through an indirect mechanism (23).

To address the role of ILK in our system, immunoblot analysis using an ILK antibody was performed on 50 μg of each of the subcellular fractions (Fig. 1C). ILK was found enriched in the PM fraction relative to the CYT and LDM. However, the amount of ILK in the cytosolic fraction was significant considering the fact that our in vitro reactions typically contained five times the amount of cytosolic proteins relative to PM proteins. This observation is inconsistent with our expected profile for PDK2, which should be absent from the cytosol. In addition, although the amount of ILK in the PM was reduced with salt washing, there was still a significant amount remaining. This behavior did not correlate with the PDK2 activity that was observed. These data indicated that the presence of ILK might not be sufficient for Ser-473 phosphorylation, but they did not rule out the possibility that ILK might be a necessary cofactor.

The localization of PDK2 was also independently confirmed by another approach. We investigated whether the addition of exogenous PIP3 to our in vitro reaction could bypass the requirement for PI 3-kinase altogether, thus allowing the phosphorylation of Akt to occur in a non-insulin-dependent manner (Fig. 2). The addition of PIP3 to CYT alone resulted in the efficient phosphorylation of Thr-308, consistent with the localization of PDK1 predominantly in the cytosol. However, the phosphorylation of Ser-473 occurred only marginally, consistent with PDK2 being largely absent from the cytosol. When PIP3 was added to a reaction containing CYT and LDM, the Thr-308 signal was not further enhanced relative to that produced by a reaction containing CYT alone. In contrast, PIP3 addition to CYT and LDM produced a robust Ser-473 signal, confirming the presence of PDK2 activity associated with the LDM. Heating the LDM at 65 °C for 10 min completely inhibited the PIP3-stimulated Ser-473 activity, suggesting that a catalytic protein was responsible for the PDK2 activity in the LDM rather than an ancillary thermostable cofactor (data not shown). Similar effects were observed when PIP3 was added to a reaction containing CYT and PM. The presence of PM significantly enhanced the phosphorylation of Ser-473 but not Thr-308. Salt extraction of the LDM did not affect Thr-308 phosphorylation and only marginally reduced Ser-473 phosphorylation. Approximately 50% of the total protein in the LDM could be extracted with 1 μM NaCl. In contrast, salt extraction of the PM almost completely suppressed PIP3-induced Ser-473 phosphorylation but had no effect on Thr-308 phosphorylation. In this case, ~30% of the PM proteins were removed with the salt wash. The data again support the idea that PDK2, in contrast to PDK1, was mainly associated with the membrane fractions and absent in soluble form.

In addition to PIP3, we tested other phosphatidylinositol lipids for their ability to stimulate Akt phosphorylation. Only PIP3 and PI 3,4-bisphosphate, but not PI 4,5-bisphosphate, PI 3-phosphate, or PI, could stimulate Thr-308 and Ser-473 phosphorylation (Fig. 3), confirming the class Ia PI 3-kinase-dependent nature of both PDK1 and PDK2 (5). Notably, the PDK2 activity associated with the PM did not significantly differ in lipid specificity compared with the PDK2 activity associated with the LDM.

**Effect of Chloride on PDK1 and PDK2 Activities**—The definitive identification of PDK2 has remained elusive despite in-
tensive efforts by many investigators over the past several years (7, 12, 13). The enrichment of PDK2 in membrane fractions may contribute to technical difficulties experienced in attempts at purifying this activity. Our initial efforts at reconstituting PDK2 activity from the salt extract of membranes were unsuccessful until we discovered that high concentrations of chloride (>100 mM) could completely inhibit PDK2 activity. This observation is illustrated in Fig. 4. In vitro reactions containing PM and CYT were carried out in the presence of 140 mM potassium glutamate, 5 mM NaCl (KGlu), 140 mM potassium acetate, 5 mM NaCl (KaAc), 140 mM KCl, 5 mM NaCl (KCl), or 145 mM NaCl. Insulin-stimulated phosphorylation of both Thr-308 and Ser-473 were almost completely suppressed in the presence of 145 mM chloride (Fig. 4, top panels). To address whether PDK1 and PDK2 activities were directly inhibited by chloride as opposed to an indirect effect involving an earlier signaling step, in vitro reactions were performed using exogenous PIP3 (Fig. 4, lower panels). The robust PIP3-induced Ser-473 phosphorylation observed in the presence of potassium glutamate or potassium acetate was completely inhibited at high chloride concentrations. The PIP3-stimulated Thr-308 phosphorylation was also severely inhibited at high chloride concentrations but was readily apparent with longer film exposures. The effect of chloride is probably not observed in vivo because the intracellular concentration of this ion is low (<5 mM). Nevertheless, buffers containing chloride in excess of 100 mM are routinely used in kinase assays and protein purification, which may in part explain some of the past difficulty in identifying PDK2.

Characterization of Salt-extracted PDK2 Activity from the PM—To distinguish the possibility of PDK2 activity being irreversibly inhibited by 1 M NaCl as opposed to simply being extracted and removed from the PM, it was necessary to rescue the lost PDK2 activity by adding back the extracted proteins to the salt-washed PM. After the PM was salt-washed for 30 min in 1 M NaCl, the extracted proteins were recovered in the supernatant after centrifugation at 37,000 × g for 20 min and desalted using a Sephadex G-25 spin column as described under “Experimental Procedures.” As shown in Fig. 5A, salt washing of the PM almost completely inhibited insulin-stimulated phosphorylation of Ser-473 but had no effect on insulin-stimulated Thr-308 phosphorylation. Adding the desalted protein extract (Ext-LoS) back to the reaction containing the salt-washed PM and the cytosol recovered the PDK2 activity but had no effect on the PDK1 activity. The rescue result was confirmed using PIP3 to stimulate Akt phosphorylation (Fig. 5B). Again, we found that washing the PM with high salt almost completely suppressed PIP3-induced Ser-473 phosphorylation but exerted little effect on Thr-308 phosphorylation.

Adding the extracted proteins back to the salt-washed PM and CYT recovered the stimulated PDK2 activity but had minimal effect on Thr-308 phosphorylation. Thus, the data suggest that PDK2 could be dissociated from the plasma membrane fraction by salt extraction in functional form.

As shown in Fig. 1, PDK1 is mainly localized in CYT after our fractionation protocol. Its indisputably soluble nature is supported by the fact that it resists pelleting by centrifugation at 200,000 × g during the preparation of CYT. The PDK2 activity, localized to the PM fraction, can be separated from the bulk of the PM with 1 M NaCl followed by low speed centrifugation. Peripheral membrane proteins, once separated from membranes with 1 M NaCl, generally behave as soluble proteins and remain in the supernatant after centrifugation at 200,000 × g. To address whether PDK2 that resides in the PM fraction is a peripheral membrane protein, the 1 M NaCl PM
Fig. 6. PDK2 activity extracted from PM with high salt can be pelleted at high speed. In vitro reactions containing different subcellular components were incubated for 15 min at 37 °C. PM(SW) are plasma membranes that were salt-washed in 1 M NaCl for 30 min and then recovered as a pellet by centrifugation at 37,000 × g for 20 min. Ext-LoS refers to proteins found in the supernatant after the 37,000 × g centrifugation step. Centrifugation of Ext-LoS at 200,000 × g for 1 h resulted in a pellet (Ext-HiP) and a supernatant (Ext-LoS). PIP3 + or − denotes whether 10 μM PIP3 was added or not to the reaction. The reaction samples were subjected to immunoblot analysis using phospho-Akt antibodies. A, various extracts (Ext-LoS, Ext-HiP, and Ext-HiS) were tested for PDK2 activity in a PIP3-stimulated reaction containing PM(SW) and Cyt. B, various extracts (Ext-LoS, Ext-HiP, and Ext-HiS) were tested for PDK2 activity in a PIP3-stimulated reaction containing Cyt alone.

extract (Ext-LoS) was subjected to centrifugation at 200,000 × g for 1 h. The resulting pellet (Ext-HiP) and supernatant (Ext-HiS) were then tested for PDK2 activity in a PIP3-stimulated reaction containing PM(SW) and Cyt, and extract (Fig. 6A). The PDK2 activity removed from the PM with 1 M NaCl was completely recovered by adding back the high speed pellet (Ext-HiP) but not the high speed supernatant (Ext-HiS) to the reaction containing PM(SW) and Cyt. In a reaction containing Cyt alone supplemented with Ext-HiP (Fig. 6B), PIP3-stimulated PDK2 site phosphorylation of Akt occurred efficiently, suggesting that Ext-HiP was sufficient to support PDK2 activity in our assay and did not require other factors associated with the PM. Thus, 1 M NaCl appeared to alter the sedimentation property of PDK2, allowing its segregation from the bulk of the membrane fraction using low speed centrifugation. However, salt-extracted PDK2 pelleted at 200,000 × g and, therefore, did not behave as a typical soluble protein. PDK2 in the PM may be associated with a subpopulation of low density membrane vesicles that resists pelleting at low speed in 1 M NaCl. The PDK2 activity associated with the PM may in fact be identical to that associated with the LDM. It is entirely possible that homogenization of the cell may cause PDK2 to segregate into two subcellular fractions. The mechanism by which PDK2 associates with these membrane fractions is unclear. PDK2 may be part of a higher order macromolecular structure distinct from membranes, such as cytoskeletal elements, which would be expected to pellet following high speed ultracentrifugation.

We decided to test whether Ext-HiP was abundant in proteins associated with the cytoskeleton. A comparison of 50 μg of PM, PM(SW), and Ext-HiP by immunoblot analysis (Fig. 7) revealed that Ext-HiP was greatly enriched in paxillin, vinculin, actin, and the actin-associated protein Arp3 but was not enriched in the integrin-β1 receptor. Paxillin, vinculin, and the integrin β1 receptor are three proteins localized to focal adhesions (24, 25), attachment points connecting the cell cytoskeleton to the extracellular matrix. The physical link between the integrin receptor, a PM integral membrane protein, and the actin cytoskeleton is provided by a number of cytoskeleton-interacting proteins including vinculin, talin, paxillin, α-actinin, and filamin (25). Paxillin migrates as a diffuse band on SDS-PAGE with a molecular mass of 65–70 kDa due to tyrosine and serine/threonine phosphorylation (26). There is evidence to suggest that serine phosphorylation within the LIM domain 3 of paxillin might regulate its focal adhesion localization (27). In our system paxillin appeared as two discrete bands on SDS-PAGE (Fig. 7). The slower migrating band, which was highly enriched in Ext-HiP, may represent a highly serine-phosphorylated form of paxillin that localizes to focal adhesions. The faster migrating band, which was de-enriched in Ext-HiP, may represent a non-phosphorylated form not present in focal adhesions. ILK, which has also been shown to be present at focal adhesions (28), was not enriched in Ext-HiP. A comparison between PM and PM(SW) indicated that 1 M NaCl removed some ILK from the membrane, but the ILK extracted with high salt behaved like a typical peripheral membrane protein and predominantly localized to Ext-HiS and not to Ext-HiP (data not shown). Consistent with our earlier observations, the distribution of ILK did not correlate with the observed PDK2 activity. The insulin receptor remained associated with the PM(SW) and was largely absent from Ext-HiP. Caveolae, cholesterol-rich invaginations abundant in the plasma membranes of 3T3-L1 adipocytes, are thought to play an important role in the insulin-stimulated CAP (Cbl-associating protein) pathway (29). Caveolin, a major protein in caveolae (30), was not enriched in Ext-HiP. We concluded from these results that PIP3 remained the majority of the plasma membrane consisting of the phospholipid bilayer, integral membrane proteins such as the insulin and the integrin β1 receptors, and caveolae, whereas Ext-HiP, the fraction that contained the bulk of the PDK2 activity in the PM, was enriched in cytoskeletal elements, some of which are found in focal adhesions. Incubation in 1 M NaCl disrupted the association between the cytoskeleton and the plasma membrane, thereby allowing them to be segregated by centrifugation. The actin cytoskeleton and focal adhesions are known to be important in PI 3-kinase-dependent signaling. Adhesion to the extracellular matrix causes a rapid elevation of PI 3-kinase lipid products, activation of Akt (31, 32), and rearrangement of the cytoskeletal architecture (31). PTEN, a 3′ lipid phosphatase, interacts with focal adhesion kinase and can dephosphorylate both PIP3 and focal adhesion kinase and thereby negatively regulate the extracellular matrix-dependent PI 3-kinase/Akt survival pathway (33). Depolymerization of actin filaments by cytochalasin D inhibits insulin-stimulated phosphorylation of Akt in 3T3-L1 adipocytes by...
Fig. 8. PDK2 activity migrates from the PM to the LDM subcellular fraction upon disruption of the actin cytoskeleton. 3T3-L1 adipocytes were treated or not with 2 μM cytochalasin D for 2.5 h at 37 °C. CYT, PM, Ext-HiP, and LDM were prepared from control and treated-cells. A, in vitro reactions containing different subcellular components were incubated for 15 min at 37 °C in the presence or absence of 10 μM PIP3. An asterisk (*) signifies which component in a reaction was derived from cytochalasin D-treated cells. Reactions were subjected to immunoblot analysis using phospho-Akt antibodies. B, quantification of three independent experiments. Phosphorylations were normalized to (+PIP3) controls. Statistical analyses were done using Student’s t test (two sample assuming equal variance). # denotes statistical significance (p < 0.05).

Fig. 9. Akt1–3 can be phosphorylated on Ser-473 by ExtHiP in a PIP3-dependent manner. Ext-HiP was prepared from PM as described under “Experimental Procedures.” In vitro reactions containing various combinations of Ext-HiP, 10 μM Akt1, -2, or -3 (Upstate Biotechnology), and 10 μM PIP3 were incubated for 15 min at 37 °C. The reaction samples were analyzed by immunoblot analysis using phospho-Akt antibodies.

blocking the synthesis of 3-phosphoinositides (34). Based on these data, it is not surprising that PDK2 may colocalize with proteins associated with the cytoskeleton.

To strengthen the connection between PDK2 activity and the actin cytoskeleton, we performed in vitro reactions using subcellular fractions isolated from both control and cytochalasin D-treated 3T3-L1 adipocytes. It has been reported that disrupting the actin cytoskeleton with 2 μM cytochalasin D for 2.5 h at 37 °C will inhibit both insulin-stimulated Akt phosphorylation (34) and glucose transport by ~50% in 3T3-L1 adipocytes (34, 35). In vitro reactions containing different combinations of CYT, PM, Ext-HiP, and LDM, prepared from control and treated cells, were incubated for 15 min at 37 °C in the presence or absence of 10 μM PIP3. Samples were analyzed for Akt phosphorylation by immunoblot analysis using phospho-Akt antibodies (Fig. 8). We found that Thr-308 phosphorylation was independent of the source (control or cytochalasin D-treated cells) of the individual components. This result was consistent with PDK1 having a cytosolic localization uninfluenced by actin. Ser-473 phosphorylation, however, was dependent on

whether the components originated from either control or cytochalasin D-treated cells. Reactions containing CYT prepared from control cells and either PM or Ext-HiP (Ext) derived from cytochalasin D-treated cells (denoted by an asterisk) had a statistically significant decrease in PIP3-dependent Ser-473 phosphorylation (~25%) than those reactions consisting of all control components. Conversely, reactions containing control CYT and LDM from cytochalasin D-treated cells had an ~25% higher PIP3-dependent Ser-473 phosphorylation than the corresponding control reaction. We conclude from this study that disruption of the actin cytoskeleton in 3T3-L1 adipocytes causes a redistribution of PDK2 from the PM to the LDM upon fractionation. This result provides a reasonable explanation as to why PDK2 activity was originally found in both the PM and LDM (Fig. 2). PDK2 fractionates with the PM due to its asso-
cation with the actin cytoskeleton. The population of PDK2 found in the LDM presumably is not associated with cytoskeletal elements but still may be part of a large macromolecular complex since it can be pelleted with a high centrifugal force.

Next we tested whether Ext-HiP could directly phosphorylate exogenous Akt1–3 at the PDK2 site in a PIP3-dependent manner in the absence of CTY (Fig. 9). No detectable Ser-473 (or equivalent residue for Akt2 and Akt3) phosphorylation was present in a reaction containing Ext-HiP alone, indicating that this fraction did not contain Akt. Akt1, Akt2, and Akt3 added to reactions as purified recombinant proteins did not exhibit PIP3-dependent PDK2 site phosphorylation in the absence of Ext-HiP. Each of the isoforms, however, was phosphorylated at the PDK2 site in a PIP3-dependent manner in the presence of Ext-HiP. Ext-HiP contained no detectable PIP3-stimulated PDK1 activity, which supports our earlier results demonstrating that PDK1 is mainly cytosolic. It should be noted that the Thr-308 phospho-Akt antibody used in this study was unable to detect phosphorylation of Akt3.

Although the above experiments suggest that the membrane-associated PDK2 activity in our system is a novel kinase, they do not rule out the possibility that the observed activity is not the result of a novel kinase per se but, rather, to a cofactor required for the autophosphorylation of Ser-473. To address this possibility, Akt1 and a kinase-dead Akt1(K179A) mutant were expressed as GST fusion proteins in human embryonic kidney 293 cells. Phosphorylation of the two fusion proteins were studied both in vitro with intact 293 cells and in vitro using the purified fusion proteins, Ext-HiP, and PIP3 (Fig. 10). Treatment of transfected 293 cells with insulin resulted in the phosphorylation of both GST-Akt1 (wild type (WT)) and the GST-Akt1(K179A) kinase-dead mutant (K179A) on Ser-473 and Thr-308 (Fig. 10A). These results are consistent with a previous study that showed that a hemagglutinin-tagged kinase-dead mutant of Akt1 (HA-Akt1(K179A)) is phosphorylated on Ser-473 and Thr-308 in response to insulin in transfected 293 cells (8). GST fusion proteins were then purified, and the phosphorylation reactions were carried out in an in vitro reaction in the presence of Ext-HiP. Ext-HiP was able to phosphorylate both wild type GST-Akt1 and kinase-dead GST-Akt1(K179A) on Ser-473 in response to PIP3 (Fig. 10B). No detectable Ser-473 phosphorylation (Ser-P) was observed in reactions containing Ext-HiP, GST-WT, or GST-K179 alone. The phosphorylation of kinase-dead GST-Akt1(K179A) on Ser-473 by Ext-HiP demonstrates conclusively that Ext-HiP contains a PDK2 “kinase” and not a cofactor necessary for the autophosphorylation of Ser-473. PDK1, through its interaction with PDK1-interacting fragment, can be converted in vitro into a form capable of phosphorylating Akt on both Thr-308 and Ser-473 (14). Interestingly, it has been reported as an unpublished observation that PDK1 complexed with PDK1-interacting fragment is only capable of phosphorylating catalytically inactive mutants of Akt in vitro on Thr-308, but not on Ser-473 (36). This observation coupled with the absence of PIP3-dependent Thr-308 phosphorylation in reactions containing Ext-HiP and purified Akt1–3 (Fig. 9) strongly suggests that PDK2, found in Ext-HiP, is not a modified form of PDK1.

Using an entirely different approach, Hill et al. (37) have recently characterized a protein associated with plasma membrane rafts of HEK 293 cells that has Akt-Ser-473 kinase activity. This enzyme can constitutively phosphorylate Akt1–3 as well as the kinase-inactive K179A mutant and the T308A Akt1 mutant. Similar to our work, this protein is distinct from PDK1 and ILK and can be extracted from the bulk PM with high salt. Once released from the membrane with high salt, gel filtration analysis revealed that the PDK2 activity eluted as an ~550-kDa protein complex. It was not reported, however, whether this large protein complex was soluble at 200,000 \times g. To make a direct comparison, we tested whether our PDK2 activity was soluble at centrifugation conditions employed by Hill et al. (37), i.e. 100,000 \times g in 1 \text{m} \text{NaCl}. PM were washed in 1 \text{m} \text{NaCl} for 30 min and then centrifuged at 37,000 \times g (37P in Fig. 11). The resulting supernatant was successively recenterfuged at 100,000 \times g (100P) for 30 min and then at 200,000 \times g (200P) for 1 h. All pellets were tested for PIP3-dependent Akt-Ser-473 activity in an in vitro reaction containing CTY (Fig. 11). Compared with the control reaction of PM and CTY, the majority of the PIP3-dependent PDK2 activity was soluble at 100,000 \times g but could be pelleted at 200,000 \times g.

Based on the similarities in the properties of PDK2 activity observed in our system and those recently reported (37), it is quite possible that the two proteins are identical. We have shown that our PDK2 activity was PI 3-kinase-dependent, whereas Hill et al. (37) report their activity as being constitutively active. We have deliberately pursued a PDK2 activity sensitive to insulin and PIP3 to eliminate kinase activities that might be irrelevant to insulin action in vitro. Hill et al. (37) also provide evidence that PDK2 is associated with lipid raft domains in HEK293 cells. The amount of experimental data in recent years documenting the relationship between lipid rafts and insulin signaling is considerable (for review, see Ref. 38). We have shown that PDK2 activity in our system, once released from the PM with high salt, co-sediments with proteins associated with the actin cytoskeleton. As discussed above, there is good evidence linking the actin cytoskeleton and PI 3-kinase-dependent signaling events. The two observations are not mutually exclusive. Previous studies show that the actin cytoskeleton can regulate or stabilize interactions of specific proteins with cholesterol-rich microdomains (39–41). It has also been shown that integrins, upon activation, will mobilize to lipid rafts, suggesting that rafts may positively regulate integrin function (42). Interestingly, PI 3-kinase, lipid rafts, and the actin cytoskeleton have all been shown to be important in insulin-stimulated Glut4 translocation. In addition to the PI 3-kinase-dependent insulin pathway, a PI 3-kinase-independent pathway has recently been described that involves the insulin-stimulated tyrosine phosphorylation of cbl, its recruitment to lipid rafts, and the activation of the small GTPase, TC10 (29). The actin-regulatory neural Wiskott-Aldrich syndrome protein (N-WASP) lies downstream of TC10 to mobilize cortical F-actin, an event necessary for the Glut4 translocation process (43, 44). Based on all of the above observations, it seems plausible that PDK2 may be found associated with the actin cytoskeleton that underlies lipid rafts.

In summary, we have exploited the unique advantages offered by a cell-free methodology to make several novel observations about the regulation of Akt. Our results strongly suggest that PDK1 and PDK2 are distinct kinases. In addition, PDK1 is predominantly localized to the cytosol, whereas PDK2 is associated with both the PM and LDM. Further characterization of the PM fraction using high salt extraction and differential centrifugation revealed that PDK2 activity co-sediments with a subpopulation of the PM fraction that is enriched in proteins associated with the actin cytoskeleton, including proteins present in focal adhesions but that this fraction is devoid of PDK1 activity. We are hopeful that future analysis of Ext-HiP may eventually lead to the unambiguous identification of PDK2.

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