The Interaction of Akt with APPL1 Is Required for Insulin-stimulated Glut4 Translocation*

Received for publication, May 21, 2007, and in revised form, August 16, 2007 Published, JBC Papers in Press, September 11, 2007, DOI 10.1074/jbc.M704150200

Tsugumichi Saito§, Christine C. Jones‡, Shaohui Huang§, Michael P. Czech§ and Paul F. Pilch††

From the ‡Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118 and the §Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01507

APPL1 (adapter protein containing PH domain, PTB domain, and leucine zipper motif 1) is an Akt/protein kinase B-binding protein involved in signal transduction and membrane trafficking pathways for various receptors, including receptor tyrosine kinases. Here, we establish a role for APPL1 in insulin signaling in which we demonstrate its interaction with Akt2 by co-immunoprecipitation and pulldown assays. In primary rat adipocytes and skeletal muscle, APPL1 and Akt2 formed a complex that was dissociated upon insulin stimulation in both tissues. To investigate possible APPL1 function in adipocytes, we analyzed Akt phosphorylation, 2-deoxyglucose uptake, and Glut4 translocation by immunofluorescence following APPL1 knockdown by small interfering and short hairpin RNAs. We show that APPL1 knockdown suppressed Akt phosphorylation, glucose uptake, and Glut4 translocation. We also tested the effect in 3T3-L1 adipocytes of expressing full-length APPL1 or an N- or a C-terminal APPL1 construct. Interestingly, expression of full-length APPL1 and its N terminus suppressed insulin-stimulated 2-deoxyglucose uptake and Glut4 translocation to roughly the same extent (40–60%). We confirmed by cellular fractionation that Glut4 translocation was substantially blocked in 3T3-L1 adipocytes transfected with full-length APPL1. By cellular fractionation, APPL1 was localized mainly in the cytosol, and it showed a small degree of re-localization to the light microsomes and nucleus in response to insulin. By immunofluorescence, we also show that APPL1 partially co-localized with Glut4. These data suggest that APPL1 plays an important role in insulin-stimulated Glut4 translocation in muscle and adipose tissues and that its N-terminal portion may be critical for APPL1 function.

The insulin signaling pathway that leads to the metabolically critical activation of glucose transport in fat and muscle requires the activation of the serine/threonine kinase Akt/protein kinase B (1–3), Akt2/protein kinase B being the Akt isoform responsible for this action of insulin (4–7). Insulin-activated glucose transport results from a vesicular transport process by which the glucose transporter isoform Glut4 moves from an intracellular membrane compartment to the cell surface, where it can clear glucose. An implication from the above data is that the actions of Akt2 are likely to converge on one or more substrates that function at some step in insulin-regulated Glut4 trafficking. So far, AS160 (Akt substrate of 160 kDa) is the only experimentally documented substrate known to play a role in this regard (8). AS160 has the property of a GTPase-activating protein for the Rab family of small GTPases that are known to play an important role in vesicular traffic (9). The GTPase-activating protein activity of AS160 is inhibited by Akt-mediated phosphorylation at several serine residues, and mutation of these sites, followed by introduction of this mutated construct into adipocytes, has a dominant-negative affect and inhibits Glut4 translocation (8).

However, the trafficking of Glut4 is highly complex (recently reviewed in Refs. 10–12), and it remains unclear as to exactly what additional biochemical events are affected by Akt and AS160. Akt kinase activity is dependent on its phosphorylation at Thr308 by PDK1 (phosphoinositide-dependent kinase 1) (13, 14) and at Ser473 by mTOR (mammalian target of rapamycin) (15, 16). PDK1 is activated by binding via its pleckstrin homology (PH) domain to the phosphatidylinositol 1,4,5-trisphosphate generated from phosphatidylinositol 3-kinase-3 kinase that has been activated by binding to tyrosine-phosphorylated IRS protein(s), the direct substrates of the insulin receptor (13, 17–19). The activation of PDK1 is thought to be predominantly or exclusively at the cell surface (17, 20). Akt2 has been documented to act at the plasma membrane in a vesicle fusion step as measured in a cell free assay (21), and this step does not appear to require AS160. Moreover, Akt2 is widely distributed in the cytosol and various membrane fractions of adipocytes, including Glut4-rich vesicles as noted above, where its activity is enhanced by insulin (4, 22). Thus, it is likely that the subcellular localization of Akt2 plays an important role in determining its substrates and its possible multiple modes of action in Glut4 trafficking.

Similarly, AS160 can be shown to associate with Glut4-containing vesicles (23–25) and might be presumed to function at this cellular locus. Immunohistochemical analysis of AS160 indicated that it has a broad intracellular distribution that only partially overlaps with Glut4 and that it is absent from the plasma membrane (23). Unlike Glut4, it has a widespread tissue distribution (26). Moreover, short hairpin RNA (shRNA)-mediated AS160 knockdown leads to just a partial redistribution of

2 The abbreviations used are: PH, pleckstrin homology; shRNA, short hairpin RNA; TIRF, total internal reflection fluorescence; PTB, phosphotyrosine-binding; siRNA, small interfering RNA; EGF, enhanced green fluorescent protein; mRFP1, monomeric red fluorescent protein 1; ERK1/2, extracellular signal-regulated kinase 1/2.
Glut4 to the cell surface (23, 27). Although this supports the notion that basal AS160 GTPase-activating protein activity prevents Glut4 translocation in part, it suggests there are AS160-independent components of this process. Recent evidence from several laboratories using total internal reflection fluorescence (TIRF) microscopy has provided evidence for several possible loci of insulin action, including the association of Glut4 vesicles with cytoskeletal elements and with vesicle-tethering elements, the fusion of vesicles with the plasma membrane, and Glut4 endocytosis (28–31). Taken together along with biochemical evidence (reviewed in Ref. 12), these data suggest multiple possible modes of action for Akt in Glut4 trafficking, consequently multiple Akt substrates.

In this regard, APPL1 (adapter protein containing PH domain, PTB domain, and Juxte zipper motif J) was first discovered as an Akt-binding protein in yeast two-hybrid assays using Akt2 as bait (32). Subsequently, APPL1 has been implicated as playing a role in the insulin-sensitizing actions of adiponectin in muscle (33). As the definition of APPL1 indicates, it has multiple domains that imply multiple possible cellular functions and loci of action, and these include a BAR (Bin/amphiphysin/Rvs) domain with a possible role in mediating membrane curvature (34–36), the binding of Rab5 (33, 37), and the aforementioned Akt-binding domain as well as PH and PTB sequences. A very recent study has implicated APPL1 in the formation of both G-protein-coupled and tyrosine kinase signaling complexes (38). Accordingly, we sought to determine the possible role of APPL1 in insulin-dependent Glut4 trafficking in adipocytes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dexamethasone, 3-isobutylmethylxanthine, insulin, sodium fluoride, sodium orthovanadate, and fetal bovine serum (Australian origin) were purchased from Sigma. Aprotinin, leupeptin, and pepstatin were obtained from American Bioanalytical (Natick, MA). Calf serum was purchased from Invitrogen, and Dulbecco’s modified Eagle’s medium was from Mediotech (Herndon, VA). The expression vector pcDNA3.1/His was purchased from Invitrogen. Anti-Glut4 antibody used for immunofluorescence and protein G Plus-agarose were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and antibody 1F8 (39) was used for Western blotting. Anti-APPL1 antibody was from Abcam (Cambridge, MA). Anti-phospho-Akt (Ser473) antibody was from Cell Signaling Technology, Inc. (Danvers, MA). Anti-Akt1 and anti-Akt2 antibodies were kind gifts from Dr. Morris J. Birnbaum (University of Pennsylvania).

**Cell Culture**—3T3-L1 preadipocytes were maintained in Dulbecco’s modified Eagle’s medium containing 4.5 g/liter glucose and 1-glutamine supplemented with 10% calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Two days after confluence, cells were induced to differentiate by changing the medium to Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 0.5 mM 3-isobutylmethylxanthine, 1 μM dexamethasone, and 1.7 μM insulin. After 4 days, the induction medium was removed, and cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and insulin.

**Plasmid Vectors, shRNA, and Small Interfering RNA (siRNA)**—A human APPL1 construct was made by reverse transcription-PCR from human embryonic kidney cells. Full-length APPL1 and N-terminal (amino acids 1–428) and C-terminal (amino acids 443–709) APPL1 constructs were inserted into the mammalian expression vector pcDNA3.1/His. For shRNA, the designed sense oligonucleotides contained a 4-nucleotide overhang to create a BgiII restriction site (GATC) plus three Cs, followed by a 21-nucleotide sense siRNA sequence (5’-gatc-cccgaggattatgatgacacaagt-3’), a 9-nucleotide loop (TTCAA-GAGA), a 21-nucleotide reverse complementary antisense siRNA sequence (5’-cattctggtgaatcataaatcc), and a polymerase III terminator (TTTTTA). The complementary antisense oligonucleotides contained a 4-nucleotide overhang at the 5’ terminus to create a HindIII restriction site (AGCT), but no 4-nucleotide BgiII restriction site (GATC) at the 3’ terminus. These oligonucleotides were annealed and ligated into pSUPER vector (OligoEngine, Seattle, WA) via BgiII and HindIII sites. The scrambled shRNA sequence (gcgcgcctttgtaggattcg) described previously (40) was used as a control. All plasmid vectors were purified using CsCl gradients. For siRNA, the following cDNA target sequences were used: scrambled siRNA, 5’-caggctcgctttggctggactgg-3’; PTEN siRNA, 5’-gatagagctgctgatataaa-3’; and APPL1 siRNA, 5’-ccacattgactgcaaat-3’.

**Subcellular Fractionation of Rat Adipocytes and 3T3-L1 Adipocytes**—Fractionation was performed as described previously (41, 42). Briefly, epidymidal adipocytes of male Sprague-Dawley rats were removed and digested with collagenase (Roche Applied Science) in Krebs-Ringer phosphate buffer (12.5 mM HEPES, 120 mM NaCl, 6 mM KCl, 1.2 mM MgSO4, 1 mM CaCl2, 0.6 mM Na2HPO4, 0.4 mM NaH2PO4, 2.5 mM glucose, and 2% bovine serum albumin (pH 7.4)) for 45 min at 37 °C. After collagenase treatment, isolated adipocytes were exposed or not to 50 nM insulin for 15 min. Membrane trafficking was stopped with 2 mM KCN, and cells were transferred to HES buffer (5 mM HEPES (pH 7.4), 0.5 mM EDTA, and 250 mM sucrose) and homogenized with a Teflon/glass tissue grinder. For 3T3-L1 adipocytes, the cells were exposed or not to 100 nM insulin for 15 min, collected in HES buffer, and homogenized with a Teflon/glass tissue grinder. Subcellular fractions (plasma membranes and heavy and light microsomes) were obtained by differential centrifugation and resuspended in HES buffer. All buffers used in this work contained a mixture of protease inhibitors consisting of 1 μM aprotinin, 10 μM leupeptin, 1 μM pepstatin, and 5 μM benzamidine (Sigma) and phosphatase inhibitor mixtures 1 and 2 (Sigma).

**Immunoprecipitation**—Protein amounts in various fractions from rat muscle and primary and cultured adipocytes were determined by the BCA method. Cellular protein (100 μg) was mixed with 1 μg of anti-APPL1 antibody and incubated overnight at 4 °C. Then, 10 μl of protein G Plus-agarose was added to these samples and incubated for another 1 h at 4 °C. After the incubation, samples were washed three times with wash buffer (25 mM HEPES, 5 mM EDTA, 1% Triton X-100, 50 mM NaF, 150 mM NaCl, 10 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin, and 1 μM aprotinin A (pH 7.2)). The washed samples were resuspended in SDS sample buffer (125 mM Tris-HCl (pH 6.8), 20% (v/v) glycerol, 4% (w/v) SDS, 100 mM dithi-
Akt2 Interacts with APPL1

FIGURE 1. APPL1/Akt2 interaction is regulated by insulin. A, cytosol from rat extensor digitorum longus from unstimulated (basal (B)) or insulin-stimulated (Ins) animals was immunoprecipitated (IP) with anti-APPL1 antibody as described under “Experimental Procedures.” After SDS-PAGE, samples were immunoblotted (IB) with the indicated antibodies. B, the cytosolic fraction from rat adipocytes stimulated or not with 100 nm insulin was immunoprecipitated with anti-APPL1 antibody. After SDS-PAGE, samples were immunoblotted (IB) with the indicated antibodies. C, the band densities of the immunoblots of Akt2 such as those in A and B were evaluated using NIH Image software. Data are expressed as the means ± S.E. (n = 3 independent experiments). Open bars, basal; closed bars, insulin. D, the cytosolic fraction from rat adipocytes stimulated or not with 100 nm insulin was precipitated with glutathione S-transferase (GST) or glutathione S-transferase-APPL1 fusion protein. For linearity of signal, half of the supernatant and all of the pulldown were subjected to electrophoresis, and calculations were adjusted accordingly. After SDS-PAGE, samples were blotted with anti-Akt2 antibody. Data are expressed as the means ± S.E. (n = 7; unbound value set to 1.0).

2-Deoxyglucose Uptake—Transfected 3T3-L1 adipocytes were serum-starved for 2 h and incubated in Krebs-Ringer HEPES buffer (121 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO₄, 0.33 mM CaCl₂, and 12 mM HEPES (pH 7.4)) for an additional 2 h at 37 °C. Then, cells were either treated or not with 100 nm insulin for 15 min at 37 °C.

Electroporation—This protocol was performed as described previously (44), and the CsCl plasmid purification routinely results in ≥70% of the cells being transfected (data not shown). Briefly, differentially 3T3-L1 adipocytes were electroporated with 250 µg of plasmid under low voltage conditions (0.16 kV and 950 microfarads) using Gene Pulser II. The cells were split onto collagen-coated dishes. For overexpression experiments, the cells were used after 24–48 h of recovery time. For shRNA and siRNA transfection, the cells were used after 72 h of recovery time. Following transfection, the cells were serum-starved for 4–6 h and stimulated with or without 100 nm insulin for 15 min at 37 °C.

Electroporation was performed as described previously (44), and the CsCl plasmid purification routinely results in ≥70% of the cells being transfected (data not shown). Briefly, differentially 3T3-L1 adipocytes were electroporated with 250 µg of plasmid under low voltage conditions (0.16 kV and 950 microfarads) using Gene Pulser II. The cells were split onto collagen-coated dishes. For overexpression experiments, the cells were used after 24–48 h of recovery time. For shRNA and siRNA transfection, the cells were used after 72 h of recovery time. Following transfection, the cells were serum-starved for 4–6 h and stimulated with or without 100 nm insulin for 15 min at 37 °C.

Electroporation was performed as described previously (44), and the CsCl plasmid purification routinely results in ≥70% of the cells being transfected (data not shown). Briefly, differentially 3T3-L1 adipocytes were electroporated with 250 µg of plasmid under low voltage conditions (0.16 kV and 950 microfarads) using Gene Pulser II. The cells were split onto collagen-coated dishes. For overexpression experiments, the cells were used after 24–48 h of recovery time. For shRNA and siRNA transfection, the cells were used after 72 h of recovery time. Following transfection, the cells were serum-starved for 4–6 h and stimulated with or without 100 nm insulin for 15 min at 37 °C.

Electroporation was performed as described previously (44), and the CsCl plasmid purification routinely results in ≥70% of the cells being transfected (data not shown). Briefly, differentially 3T3-L1 adipocytes were electroporated with 250 µg of plasmid under low voltage conditions (0.16 kV and 950 microfarads) using Gene Pulser II. The cells were split onto collagen-coated dishes. For overexpression experiments, the cells were used after 24–48 h of recovery time. For shRNA and siRNA transfection, the cells were used after 72 h of recovery time. Following transfection, the cells were serum-starved for 4–6 h and stimulated with or without 100 nm insulin for 15 min at 37 °C.

Electroporation was performed as described previously (44), and the CsCl plasmid purification routinely results in ≥70% of the cells being transfected (data not shown). Briefly, differentially 3T3-L1 adipocytes were electroporated with 250 µg of plasmid under low voltage conditions (0.16 kV and 950 microfarads) using Gene Pulser II. The cells were split onto collagen-coated dishes. For overexpression experiments, the cells were used after 24–48 h of recovery time. For shRNA and siRNA transfection, the cells were used after 72 h of recovery time. Following transfection, the cells were serum-starved for 4–6 h and stimulated with or without 100 nm insulin for 15 min at 37 °C.
ters, respectively. Because of chromatic aberration, the red image was focused ~0.4 μm below the green image, which was corrected by a piezoelectric driver (E-662 LVPZT amplifier, Physik Instrumente GmbH & Co., Waldbronn, Germany) coupled to the objective. Thus, for two-color TIRF experiments, one image was taken with a 80-ms exposure, followed by a 200-ms interval during which the laser light and its matching emission filter were selected, and the corresponding focal plane was adjusted piezoelectrically before another 80-ms exposure for the second image. The TIRF microscope is equipped with a Nikon ×60 1.45-numerical aperture objective, and the whole microscope setup is enclosed in a heated chamber with the interior temperature maintained at 35 °C. Resulting TIRF images have 100-nm pixel size. Image analysis was carried out using home-developed tools (available at invitro.umassmed.edu).

RESULTS

**APPL1/Akt2 Interaction Is Regulated by Insulin**—APPL1 is expressed to significant levels in skeletal muscle and other tissues, including heart, testis, and pancreas, and in hepatocytes (32), but its expression has not heretofore been demonstrated in adipocytes. Thus, we sought to determine whether this adapter protein is present in adipocytes and if it interacts with Akt2 basally and under conditions that lead to Glut4 translocation, viz. insulin exposure in fat and muscle. By co-immunoprecipitation, there was a robust interaction of APPL1 with Akt2 under basal conditions in muscle (Fig. 1A) and fat (Fig. 1B), and remarkably, insulin disrupted this interaction almost completely (Fig. 1, A–C). We next determined the extent of APPL1/Akt interaction by a pulldown assays and determined that ~20% of Akt2 bound to APPL1 under basal conditions (Fig. 1D), which is significant in light of the small amount of Akt2 that is required for maximal glucose transport activation (see “Discussion”) (47). We then sought to determine whether this interaction might have functional consequences for signaling and Glut4 trafficking by reducing the interaction using APPL1-directed siRNA and shRNA.

**APPL1 Knockdown Inhibits Akt Phosphorylation, Insulin-stimulated Glucose Transport, and Glut4 Translocation**—We employed both siRNA and shRNA strategies to knock down APPL1 in 3T3-L1 adipocytes, and as shown in Fig. 2 (A and B), both strategies led to a ~50% decrease in APPL1 expression compared with the scrambled RNA controls (upper panels), with no effect being observed upon adiponectin or ERK1/2 expression. This knockdown resulted in a 50% decrease in insulin-independent Akt phosphorylation and had no effect on overall Akt2 expression (middle and lower panels). There was no effect on insulin-stimulated ERK1/2 phosphorylation under these conditions. Moreover, insulin-dependent glucose transport was decreased significantly (50–70%) by both experimental strategies (Fig. 3, A and B). As an additional assay, the insulin-dependent translocation of Myc-tagged Glut4 was measured.
and found to be decreased to approximately the same extent as glucose transport by APPL1-directed siRNA (Fig. 3C).

**Overexpression of APPL1 or Its N-terminal Domain Inhibits Insulin-stimulated Glucose Transport and Glut4 Translocation**

To further investigate the function of APPL1, full-length APPL1, an N-terminal APPL1 construct containing the BAR and PH domains, and a C-terminal APPL1 construct containing the PTB domain were overexpressed in 3T3-L1 adipocytes by electroporation. The transfection efficiency was ~80% (44), and the amount of exogenous APPL1 construct expressed increased total APPL1 by ~2-fold (Fig. 4A, inset). Glucose uptake was then monitored before and after insulin stimulation, and as shown in Fig. 4A, adipocytes transfected with full-length (wild-type) His-APPL1 (inset) showed a 60% decrease in 2-deoxyglucose uptake upon insulin stimulation compared with cells expressing vector only. A similar effect of the N-terminal construct was also observed, whereas the C-terminal construct was without statistically significant effect on glucose uptake. To confirm that full-length APPL1 and its N terminus were affecting glucose transport by Glut4, transporter translocation to the cell surface was assessed by the number of cells that showed rim fluorescence after insulin treatment (Fig. 4B). Similar to the results for glucose uptake, wild-type and N-terminal APPL1 significantly inhibited Glut4 translocation, whereas the C-terminal construct was without effect (Fig. 4C).

Finally, we measured Glut4 translocation by cellular fractionation of 3T3-L1 cells overexpressing full-length APPL1, and we observed a diminished Glut4 translocation (Fig. 4D) to essentially the same extent observed in the other assays (Fig. 4A and C). These data further demonstrate that APPL1 plays a significant role in Glut4 translocation in 3T3-L1 adipocytes.

We also monitored the effects of transfecting wild-type APPL1 and the N- and C-terminal constructs on insulin-dependent Akt phosphorylation. However, the former two inhibited Akt phosphorylation by only 10–15%, which seems insufficient to account for the more extensive inhibition of glucose transport and Glut4 translocation (Fig. 4, C and D). Thus, it seems likely that the localization of APPL1-Akt2 complexes is the most important aspect of its potential regulatory function.

**Cellular Localization of APPL1**—Previous cellular localization studies of wild-type APPL1 and N- and C-terminal constructs in HeLa cells indicated their distribution to be widespread with appreciable amounts in cytosolic, internal membrane, and nuclear fractions (37). Western blotting following 3T3-L1 cellular fractionation showed APPL1 in nuclear, cytosolic, and internal membrane fractions, with very little at the plasma membrane (Fig. 5A). This gel was loaded by equal protein amounts in each fraction, and the cytosol had a much higher protein content and therefore contained the great majority of APPL1 protein. We verified this with proportional protein loading (see Fig. 6) (data not shown). Thus, there was translocation of APPL1 to the light microsomal fraction upon insulin treatment, presumably from the cytosolic pool, consistent with a possible role for APPL1 in Glut4 vesicles. In confirmation of this possibility, TIRF microscopy of EGFP-tagged APPL1 revealed a mainly cytosolic and internal membrane localization with some overlap with Glut4-containing vesicles (Fig. 5B). These likely represent endocytic structures, as they were co-stained with Rab5 (37) (data not shown).

We confirmed that the localization of APPL1 is regulated by insulin in primary adipocytes from rats. Adipocytes were isolated and treated or not with insulin, and the localization of
APPL1 was detected by Western blotting following fractionation. As shown in Fig. 6, APPL1 localization was primarily cytosolic, with appreciable amounts in the nuclear/mitochondrial fraction, and no detectable signal was observed in the plasma membrane and heavy microsomal fractions. Moreover, APPL1 showed a relatively weak signal in the light microsomal fraction, which substantially increased with insulin. Taken together, our data support the notion that the localization of APPL1 complexes may be an important factor in APPL1 regulatory function for Glut4 trafficking.

**DISCUSSION**

We have shown for the first time that the interaction of Akt2 with APPL1 is regulated by insulin in a negative fashion, where ligand causes the nearly complete dissociation of the complex (Fig. 1). The interaction of APPL1 and Akt has functional consequences for Glut4 translocation, as APPL1 knockdown inhibits glucose transport and Glut4 translocation (Fig. 3). Similar to what was seen for APPL1 distribution in epidermal growth factor-treated HeLa cells (37), insulin causes a redistribution of APPL1 in primary and cultured adipocytes (Figs. 5A and 6). In addition and also as is the case in HeLa cells, chronic insulin treatment of 3T3-L1 cells (6–24 h) causes a redistribution of APPL1 to the nucleus (data not shown), indicating a possible chronic mode of APPL1 action.

To determine what part or domain of APPL1 might mediate these effects, we expressed wild-type APPL1 and N- and C-terminal APPL1 constructs and determined that the former two inhibit glucose uptake and Glut4 translocation, whereas the latter is without affect (Fig. 4). The latter sequence contains the PTB domain, which therefore appears unnecessary for the effects of APPL1 on Glut4 translocation. On the other hand, the N terminus contains the BAR and PH domains, and the latter domain is necessary for Akt2 and PDK1 activation as well as for Glut4 translocation. The interaction of PH domains in APPL1, Akt2, and PDK1 with
Akt2 Interacts with APPL1

It is also possible that APPL1 knockdown disrupts insulin receptor trafficking that may be required for insulin signaling to Akt2. APPL1 interacts with Rab5 (37), and Rab5 knockdown inhibits the ability of insulin to activate Akt, albeit in insulin receptor-transfected NIH3T3 cells (48). Nevertheless, Rab5 is a marker of early endosomes (49), and newly internalized, endosomal insulin receptor has enhanced kinase activity (50), needed to activate Akt.

There is relatively limited literature regarding APPL1 and Akt, but in the context of yeast two-hybrid assays (32) and when overexpressed in human embryonic kidney 293 cells (51), it is the C terminus that seems to mediate the interaction of these two proteins. Others have found more complex interactions of APPL1 with various receptors and receptor complexes (33, 38, 52), and thus, the nature and composition of APPL1-Akt complexes may depend on the cellular context. Moreover, the levels of expression may also influence the subsequent cellular response. In any case, it seems reasonable to hypothesize that by binding to proximal PH domains, these two proteins could directly interact, as must PDK1 and Akt for the former to phosphorylate the latter. Subsequent to its phosphorylation, Akt undergoes a conformational change, dissociate from the complex, and interact with downstream substrates. Further work will be necessary to reveal these details, but regardless, some clear conclusions remain. Akt2 robustly interacts with APPL1 in primary adipocytes and skeletal muscle, and insulin treatment disrupts this interaction. Knockdown of APPL1 inhibi-
its glucose uptake and Glut4 translocation, implicating an important regulatory role for this interaction.

Acknowledgments—We thank Dr. Steve Gygi (Harvard Medical School) and Mark Jedrychowski (Boston University School of Medicine) for mass spectrometry analysis of potential Akt substrates done early in this study.

REFERENCES

1. Koh, A. D., Summers, S. A., Birnbaum, M. J., and Roth, R. A. (1996) J. Biol. Chem. 271, 31372–31378

2. Tanti, J. F., Grilli, S., Gremeaux, T., Coffer, P. J., Van Obberghen, E., and Le Marchand-Brustel, Y. (1997) Endocrinology 138, 2005–2010

3. Krook, A., Kawano, Y., Song, X. M., Endic, S., Roth, R. A., Wallberg-Henriksson, H., and Zierath, J. R. (1997) Diabetes 46, 2110–2114

4. Calera, M. R., Martinez, C., Liu, H., Jack, A. K., Birnbaum, M. J., and Pilch, P. F. (1998) J. Biol. Chem. 273, 7201–7204

5. Hill, M. M., Clark, S. F., Tucker, D. F., Birnbaum, M. J., James, D. E., and Macaulay, S. L. (1999) Mol. Cell. Biol. 19, 7771–7781

6. Cho, H., Mu, J., Kim, J. K., Thorvaldsen, J. L., Chu, Q., Crenshaw, E. B., III, Kaestner, K. H., Bartolomei, M. S., Shulman, G. I., and Birnbaum, M. J. (2001) Science 292, 1728–1731

7. Jiang, Z. Y., Zhou, Q. L., Coleman, K. A., Chouinard, M., Boese, Q., and Czech, M. P. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 7569–7574

8. Sano, H., Kane, S., Sano, E., Miinea, C. P., Asara, J. M., Lane, W. S., Garner, C. W., and Liendhard, G. E. (2003) J. Biol. Chem. 278, 14699–14602

9. Behnia, R., and Munro, S. (2005) Nature 438, 597–604

10. Ishiki, M., and Klip, A. (2005) Endocrinology 146, 5071–5078

11. Watson, R. T., and Pessin, J. E. (2006) Trends Biochem. Sci. 31, 215–222

12. Huang, S., Lifshitz, L. M., Jones, C., Bellve, K. D., Standley, C., Fonseca, S., Corvera, S., Fogarty, K. E., and Czech, M. P. (2007) Mol. Cell. Biol. 27, 3456–3469

13. Mitsuuchi, Y., Johnson, S. W., Sonoda, G., Tanno, S., Golenis, E. A., and Testa, J. R. (1999) Oncogene 18, 4891–4898

14. Gallop, J. L., Jao, C. C., Vallis, Y., Butler, P. J., Evans, P. R., and McMahon, H. T. (2006) Science 303, 495–499

15. Gallop, J. L., Jao, C. C., Kent, H. M., Butler, P. J., Evans, P. R., Langen, R., and McMahon, H. T. (2006) EMBO J. 25, 2898–2910

16. Coffer, P. J., Jin, J., and Woodgett, J. R. (2002) Biochem. Soc. Symp. 69, 93–99

17. Frolov, V. A., Matsumoto, H., Zimmerberg, J., Cushman, S. W., and Frolov, V. A. (2005) J. Biol. Chem. 280, 415–422

18. Kublaoui, B., Lee, J., and Pilch, P. F. (1995) J. Biol. Chem. 270, 710–714

19. Chawla, A., Corvera, S., Fogarty, K. E., and Czech, M. P. (2002) J. Biol. Chem. 277, 710–714

20. Schmoranzer, J., Goulian, M., Axelrod, D., and Simon, S. M. (2000) J. Cell Biol. 151, 1053–1062

21. Nechamen, C. A., Thomas, R. M., Cohen, B. D., Acevedo, G., Poulikakos, P. I., Testa, J. R., and Dias, J. A. (2004) Biochim. Biophys. Acta 1694, 22115–22118

22. Jiang, Z. Y., Chawla, A., Bose, A., Way, M., and Czech, M. P. (2002) J. Biol. Chem. 277, 509–615