THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 9, Issue of February 27, pp. 5315–5322, 1998

Printed in U.S.A.

Potential Role of Protein Kinase B in Insulin-induced Glucose Transport, Glycogen Synthesis, and Protein Synthesis*

(Received for publication, July 24, 1997, and in revised form, October 20, 1997)

Kohjiro Ueki‡§, Ritsuko Yamamoto-Honda‡, Yasushi Kaburagi‡, Toshimasa Yamauchi‡, Kazuyuki Tobe‡, Boudewijn M. Th. Burgering¶, Paul J. Coffer‖, Issei Komuro‡, Yasuo Akanuma§, Yoshio Yazaki‡, and Takashi Kadowaki‡**

From the ‡Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan, §The Institute for Diabetes Care and Research, Asahi Life Foundation, 1-6-6 Marunouchi, Chiyoda-ku, Tokyo 100, Japan, ¶Laboratory for Physiological Chemistry, Utrecht University, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands, and ‖Department of Pulmonary Disease, University Hospital Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands

Various biological responses stimulated by insulin have been thought to be regulated by phosphatidylinositol 3-kinase, including glucose transport, glycogen synthesis, and protein synthesis. However, the molecular link between phosphatidylinositol 3-kinase and these biological responses has been poorly understood. Recently, it has been shown that protein kinase B (PKB/c-Akt/Rac) lies immediately downstream from phosphatidylinositol 3-kinase. Here, we show that expression of a constitutively active form of PKB induced glucose uptake, glycogen synthesis, and protein synthesis in L6 myotubes downstream of phosphatidylinositol 3-kinase and independent of Ras and mitogen-activated protein kinase activation. Introduction of constitutively active PKB induced glucose uptake and protein synthesis but not glycogen synthesis in 3T3L-1 adipocytes, which lack expression of glycogen synthase kinase 3 different from L6 myotubes. Furthermore, we show that deactivation of glycogen synthase kinase 3 and activation of rapamycin-sensitive serine/threonine kinase by PKB in L6 myotubes might be involved in the enhancement of glycogen synthesis and protein synthesis, respectively. These results suggest that PKB acts as a key enzyme linking phosphatidylinositol 3-kinase activation to multiple biological functions of insulin through regulation of downstream kinases in skeletal muscle, a major target tissue of insulin.

Insulin promotes a wide variety of biological responses *in vivo*, including regulation of glucose metabolism and protein synthesis (1, 2). One of the most important metabolic responses by insulin is the stimulation of facilitated glucose transport in muscle and adipose tissues, primarily due to translocation of glucose transporter isoform 4 (GLUT4) from an intracellular pool to the plasma membrane (3, 4). Another major aspect of the glucose metabolism regulated by insulin is the activation of glycogen synthesis through the activation of glycogen synthase (GS)[1] in skeletal muscle (5–8). Insulin also serves as the major regulator of protein synthesis by phosphorylation of eukaryotic initiation factor 4E (eIF-4E)-binding protein 1 (4E-BP1) and its dissociation from eIF-4E, thereby increasing the eIF-4E available for initiation of mRNA translation (9, 10). Many processes of these biological actions in response to insulin have been demonstrated to be regulated by phosphatidylinositol 3-kinase (PI3-K) (11–17). However, the molecular link between PI3-K activation and final biological functions is unknown. Recent reports revealed that protein kinase B (PKB) is regulated by PI3-K through the direct interaction of its pleckstrin homology domain and the phosphorylated products of PI3-K, leading to phosphorylation in its serine/threonine residues and activation by a putative upstream kinase (18–23). Moreover, PKB has been shown to phosphorylate and inhibit glycogen synthase kinase 3 (GSK3) *in vitro* (24), suggesting the possibility that it might be involved in glycogen synthesis regulated by PI3-K. Nevertheless, expression of a constitutively active form of PKB failed to stimulate glycogen synthesis in 3T3L-1 adipocytes, although it enhanced glucose transport activity (25). These findings prompted us to study the roles of PKB in multiple biological responses of insulin. Here, we show that expression of GagPKB (18), a constitutive active form of PKB, promotes glucose transport activity and protein synthesis rate in both 3T3L-1 adipocytes and L6 myotubes, which are demonstrated to be regulated by PI3-K, independent of mitogen-activated protein kinase. We also show that expression of GagPKB promotes glycogen synthesis in L6 myotubes but not in 3T3L-1 adipocytes that lack expression of GSK3. These data suggest that PKB might be a key player in insulin-stimulated multiple metabolic actions in skeletal muscles.

EXPERIMENTAL PROCEDURES

Cell Culture and Adenovirus-mediated Gene Transfer—3T3L-1 cells and L6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) and induced to differentiate into adipocytes and myotubes as described in Refs. 26 and 27, respectively. The differentiated cells were cultured in media containing the adenoviruses for 1 h at 37 °C, and DMEM supplemented with fetal calf serum was added and cultured for 24 h. One day later, when cells were subjected to assays, they were serum-starved for 20 h. The adenoviruses were applied at a concentration of 3×10^8 plaque-forming units/cm^2 dish. Under these conditions, LacZ gene expression was observed in more than 90% of 3T3L-1 cells

* This work was supported by Ministry of Education, Science, Sports, and Culture and Ministry of Health and Welfare of Japan grants (to T. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed: Tel.: +81-3-3815-5411 (ext. 3111); Fax.: +81-3-5689-7209.

[1] The abbreviations used are: GS, glycogen synthase; PI3-K, phosphatidylinositol 3-kinase; PKB, protein kinase B, Rac, related to A and protein kinase C; GSK3, glycogen synthase kinase 3; GLUT4, glucose transporter isoform 4; eIF-4E, eukaryotic initiation factor 4E; 4E-BP1, eIF-4E-binding protein 1; DMEM, Dulbecco's modified Eagle's medium; GagPKB, protein kinase B fused to Gag protein; MAPK, mitogen-activated protein kinase; DNRas, dominant negative Ras; PP1G, regulatory subunit of protein phosphatase 1; PDGF, platelet-derived growth factor; p85, the regulatory 85-kDa subunit of phosphatidylinositol 3-kinase; DG, deoxyglucose; HA, hemagglutinin; KRHB, Krebs-Ringer phosphate-HEPES buffer.

and L6 cells on postinfection from day 1 through day 3 measured by β-galactosidase assay. Expression of GagPKB was confirmed by Western blot analysis and immune complex kinase assay as described below. Expression of dominant negative Ras (DNRas) and that of mitogen-activated protein kinase (MAPK) were confirmed by measurement of MAPK activity in response to insulin as described (28). Insulin-induced MAPK activity was completely inhibited in cells expressing DNRas and increased 5–8-fold in cells expressing MAPK compared with that in cells expressing LacZ.

Recombinant Adenoviruses—cDNA of GagPKB was constructed as described (18). cDNA of DNRas (K-Ras substituted Ser-17 to Asn by polymerase chain reaction method) was kindly provided by Dr. Takai (Osaka University). cDNA of MAPK was constructed as described (28). cDNA of GSK3α (29) was kindly provided by Dr. He (NCI, National Institutes of Health) and fused to hemagglutinin (HA) epitope sequence substituted for its original stop codon (GSK3α-HA). cDNA fragments including the whole coding region of GagPKB, DNRas, MAPK, and GSK3α-HA were introduced to the expression cosmid cassette with blunt end ligation into the *Swa*I site, respectively, as described (30). The recombinant adenoviruses, Adex1CAGagPKB, Adex1CADNRas, Adex1CAMAPK, and Adex1CAGSK3a-HA were constructed by homologous recombination between the expression cosmid cassette and parental virus genome as described (30). The control adenovirus Adex1CALacZ and the cosmid cassette were kindly provided by Dr. Saito (University of Tokyo).

Antibodies—Rabbit polyclonal anti-PKB antibodies (αPKB-CT) were generated against a peptide corresponding to the sequence of 465–480 human PKB (Upstate Biotechnology Inc.). Rabbit polyclonal anti-p70 S6 kinase antibodies (C-18) were generated against a peptide corresponding to the sequence of 485–502 of rat p70 S6 kinase (Santa Cruz Biotechnology). Anti-PP1G antibodies were generated against a peptide corresponding to the sequence surrounding site 1 of rabbit skeletal muscle PP1G (SPQPSRRGSESSEE) as described (31). Rabbit polyclonal anti-GSK3 antibodies (αGSK3) were generated against a peptide corresponding to the sequence of 462–475 of rat GSK3α, as described (32). GSK3β monoclonal antibodies were generated by immunizing against the N-terminal region (1–160) of rat GSK3β (Transduction Laboratory). Monoclonal anti-HA antibodies (12CA5) were generated against a peptide (YPYDVPDYA) corresponding to the sequence of influenza hemagglutinin (Boehringer Mannheim). Rabbit polyclonal anti-4E-BP1 antibodies were generated against a glutathione *S*-transferase 4E-BP1 fusion protein (33). Immunoprecipitations were performed as described below. Immunoblots were developed using the chemiluminescence Western blotting kit (Boehringer Mannheim) and subjected to autoradiography.

In Vitro Kinase Assays—Cells were starved for 20 h, treated without or with insulin, and lysed with the lysis buffer containing 20 mM Tris-HCl, pH 7.5, 25 mM β-glycerophosphate, 100 mM NaCl, 1 mM sodium orthovanadate, 2 mM EGTA, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were subjected to immunoprecipitation with αPKB-CT, C-18, or αGSK3 followed by PKB kinase assay as described (Cross *et al*. (24, 42)), S6 kinase assay as described (14), or GSK3 kinase assay. Briefly, in PKB and S6 kinase assays, the immunoprecipitates with αPKB-CT or C-18 were washed and resuspended in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiotreitol to which 50 μM ATP, 3 μCi of [γ^{32}P]ATP, and 1 μg of cross-tide in PKB assay or 1 μg of S6 peptide (32-mer peptide from the C-terminal sequence of ribosomal S6 protein; Life Technologies Inc.) in S6 kinase assay had been added. After 20 min at 30 °C, the reaction was stopped, and the aliquots were spotted on squares of P-81 paper, washed, and counted by Cherenkov. GSK3 kinase activity was determined by the method as described (34) with modification. The immunoprecipitates with αGSK3 were resuspended in 25 mM β-glycerophosphate, 40 mM HEPES, pH 7.2, 10 mM $MgCl_2$, and 2 mM protein kinase inhibitor adding 50 μM ATP, 5 μCi of [γ^{32}P]ATP, and 1 μg of phosphoglycogen synthase peptide (Upstate Biotechnology Inc.). After 20 min at 30 °C, the reaction was stopped, and the aliquots were spotted on squares of P-81 paper, washed, and counted by Cherenkov.

2-Deoxyglucose (2-DG) Uptake Assays—2-Deoxyglucose (2-DG) uptake assays were performed as described (26, 27) with modification. Cells were grown in 12-well plates and infected with adenoviruses as described above. Before initiating glucose uptake assays, cells were washed three times with phosphate-buffered saline and incubated in 1 ml of serum-free DMEM for 3 h at 37 °C. Next, cells were washed once with Krebs-Ringer phosphate-HEPES buffer (KRHB) containing 130 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.3 mM $MgSO_4$, 10 mM Na_2HPO_4, and 25 mM HEPES, pH 7.4, and incubated in 1 ml of KRHB containing 0.1% bovine serum albumin without or with 100 nM insulin for 20 min at 37 °C. Glucose uptake was initiated by the addition of 2-deoxy-D-[2,6-^{3}H]glucose to a final concentration of 0.5 μCi for 5 min at 37 °C and terminated by two washes with ice-cold KRHB. Cells were solubilized with 0.4 ml of 0.1% SDS and counted by scintillation counter. Nonspecific glucose uptake was measured in the presence of 20 μM cytochalasin B and 200 μM phloretin and was subtracted from total uptake in each assay to obtain specific uptake.

Glycogen Synthase Assays—Glycogen synthase activity was measured as described previously (14) with slight modifications. Cells were infected with adenoviruses as described above and incubated in serum-free DMEM for 20 h. Then they were washed twice and incubated with KRHB without or with 100 nM insulin for 20 min. Cells were lysed with lysis buffer containing 25 mM Tris-HCl, pH 7.0, 30% glycerol, 10 mM EDTA, 100 mM KF, 1 mM phenylmethylsulfonyl fluoride. The lysates were centrifuged, and 30 μl of the supernatant was added to 60 μl of the assay mixture containing 50 mM Tris-HCl, pH 7.4, 25 mM NaF, 20 mM EDTA, 1 mg/ml glycogen, and 0.1 μCi of UDP-[^{14}C]glucose and with 0.25 or 10 mM glucose 6-phosphate. After incubation at 30 °C for 30 min, aliquots were spotted on 3MM paper (Whatman), washed four times with ice-cold 70% ethanol, and counted radioactivity by scintillation counter.

Assays of Protein Synthesis Rate—The protein synthesis rate was determined by measuring the incorporation of [^{3}H]tyrosine into protein as described (35, 36). Briefly, cells were grown in 6-well dishes and infected with adenoviruses as described above. After starvation in serum-free DMEM for 20 h, cells were incubated in 1 ml of serum-free F-12 (Hams') medium without or with 100 nM insulin for 1 h. Then, the medium was replaced with medium containing the same additions plus L-[2,3,5,6-^{3}H]tyrosine (5 μCi/ml). After 1 h, cells were rinsed twice with ice-cold phosphate-buffered saline and extracted with 1 ml of buffer containing 10 mM Tris-HCl, pH 7.5, 250 mM KCl, 10 mM $MgCl_2$, 6 mM 2-mercaptothanol, 1 mM phenylmethylsulfonyl fluoride before centrifugation. For measurement of radioactivity in total soluble protein, aliquots of the supernatants (30 μg of protein) were spotted on 3MM paper strips, and the strips were kept in boiling 10% trichloroacetic acid for 5 min, washed, and the radioactivity was counted.

RESULTS AND DISCUSSION

Expression of GagPKB in 3T3L-1 Adipocytes and L6 Myotubes—We investigated insulin-sensitive cell lines, 3T3L-1 adipocytes and L6 myotubes expressing the constitutively active form of PKB (GagPKB) using adenovirus vector (30). Western blot analysis revealed that the amount of GagPKB expressed was comparable to that of endogenous PKB (Fig. 1*A*). PKB activity, assessed as the ability to phosphorylate Crosstide, a peptide corresponding to the sequence surrounding the phosphorylation site Ser-21 in GSK3α (24), was 2–3-fold higher at the basal state in cells expressing GagPKB than in control cells expressing LacZ. In fact, basal PKB activity in cells expressing GagPKB was already comparable to that detected in control cells after insulin stimulation (Fig. 1*B*). Increase in PKB activity was maintained significantly higher after insulin stimulation in cells expressing GagPKB as compared with control cells. Concomitantly, p70 S6 kinase activity in cells expressing GagPKB was increased in both basal and insulin-stimulated states in these cell lines (Fig. 1*C*). In cells expressing LacZ, pretreatment with wortmannin, which completely inhibited PI3-K, also completely inhibited the insulin-induced activation of endogenous PKB (Fig. 1*B*). In cells expressing GagPKB, however, PKB activity was still about 2-fold higher than that in cells expressing LacZ at the basal state without wortmannin, whereas an increase in the kinase activity by insulin stimulation was not noted (Fig. 1*B*). Although not statistically significant, it was noted that wortmannin treatment reduced PKB activity in both cells expressing LacZ and GagPKB in 3T3L-1 adipocytes and L6 myotubes, suggesting the involvement of wortmannin-sensitive pathway in the PKB activation even in the basal condition. Neither expression of DNRas or MAPK nor treatment with PD98059 (MAP kinase kinase inhibitor) affected PKB or p70 S6 kinase activity in either of the cell lines (data not shown), although Ras has been reported to be involved in activation of PKB in some cell lines (19).

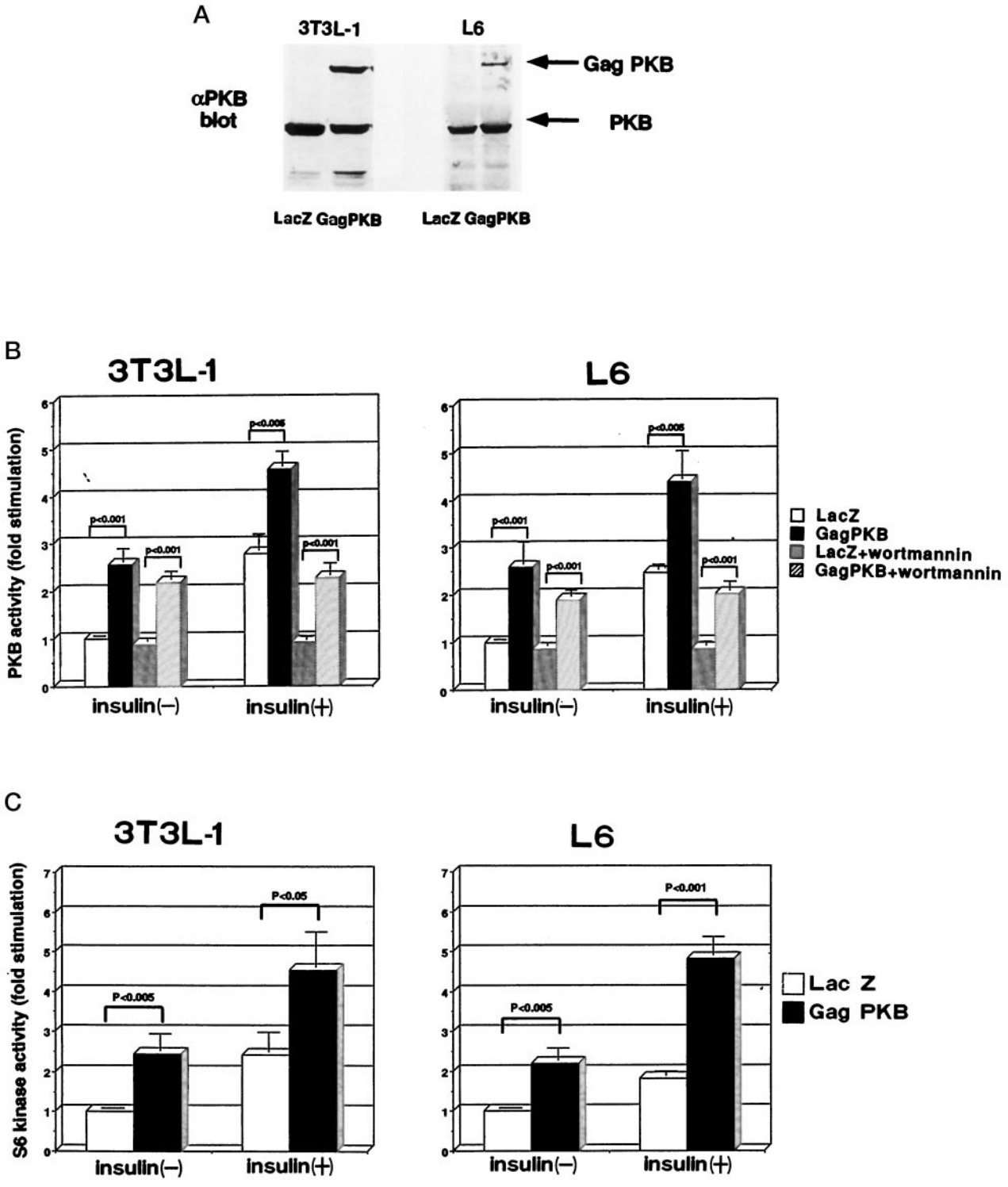

FIG. 1. **Expression of GagPKB by adenovirus-mediated gene transfer increases PKB and S6 kinase activity in 3T3L-1 adipocytes and L6 myotubes.** *A*, expression levels of endogenous PKB and GagPKB in cells expressing the indicated constructs. Cells were cultured and infected with adenoviruses as described under "Experimental Procedures," then lysed and subjected to SDS-polyacrylamide gel electrophoresis (10% gel) followed by Western blotting with the antibodies against the C-terminal region of PKB (αPKB-CT). *B*, *in vitro* kinase assays of PKB immunoprecipitated from serum-starved 3T3L-1 cells or L6 cells expressing the indicated constructs. Serum-starved cells were pretreated without or with wortmannin (30 nM) for 30 min before insulin stimulation. After cells were treated without or with 100 nM insulin for 5 min, they were subjected to immunoprecipitation with αPKB-CT followed by immune complex kinase assay. *C*, *in vivo* activation of p70 S6 kinase by expression of GagPKB in 3T3L-1 cells or L6 cells. Serum-starved cells were treated without or with 100 nM insulin for 30 min and subjected to immunoprecipitation with the antibodies against p70 S6 kinase (C-18) followed by immune complex kinase assay toward S6 peptide (KEAKEKRQEQIAKRRRLSSLRASTSKSGGSQK). The results in *B* and *C* are expressed as the ratio to the value of untreated cells expressing LacZ. Each *bar* represents the mean ± S.E. of more than three independent experiments.

Activation of PKB Mimics Insulin-induced Glucose Transport Activity Regulated by PI3-K both in 3T3L-1 Adipocytes and L6 Myotubes—Expression of GagPKB induced 2-DG uptake in the absence of insulin in 3T3L-1 adipocytes to almost the same levels as detected after insulin treatment of cells expressing LacZ (Fig. 2*A*). This is consistent with the recent results using

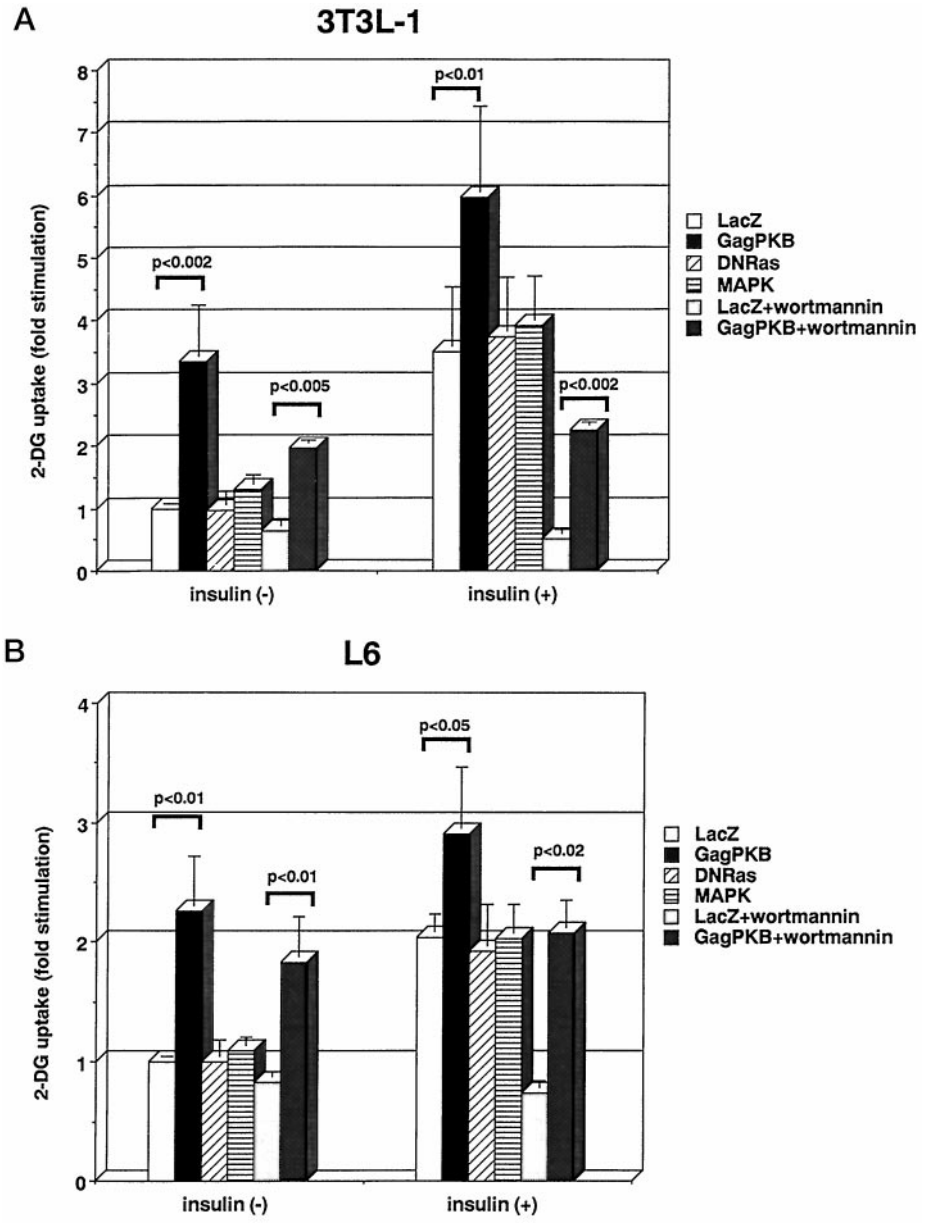

FIG. 2. **Expression of GagPKB enhances glucose transport activity in 3T3L-1 adipocytes and L6 myotubes.** *A*, 2-DG uptake activity in 3T3L-1 adipocytes expressing the indicated constructs. Serum-starved cells were cultured in glucose-free KRHB and pretreated without or with wortmannin (30 nM) for 30 min before insulin stimulation. After cells were treated without or with 100 nM insulin for 10 min, the assays were performed in 3T3L-1 adipocytes as described under "Experimental Procedures." The 2-DG uptake in untreated 3T3L-1 cells expressing LacZ was 0.13 ± 0.02 nmol/min/5 × 10^5 cells. *B*, 2-DG uptake activity in L6 myotubes expressing the indicated constructs. Cells were treated as described above, and the assays were performed as described under "Experimental Procedures." The 2-DG uptake in untreated L6 cells expressing LacZ was 0.07 ± 0.01 nmol/min/5 × 10^5 cells. The results in *A* and *B* are expressed as the ratio to the value of untreated cells expressing LacZ. Each *bar* represents the mean ± S.E. of more than three independent experiments.

another type of constitutively active PKB in 3T3L-1 adipocytes (25) and the findings that expression of GagPKB promotes GLUT4 appearance at the cell surface in isolated adipocytes (37). In L6 myotubes, expression of GagPKB also induced fundamentally similar effects (Fig. 2*B*). Pretreatment with wortmannin, which completely inhibited both PI3-K and insulin-sensitive endogenous PKB (Fig. 1*B*), also completely inhibited 2-DG uptake stimulated by insulin in 3T3L-1 adipocytes and L6 myotubes expressing LacZ (Fig. 2, *A* and *B*). In cells expressing GagPKB treated with wortmannin, basal 2-DG uptake was still higher than that in 3T3L-1 adipocytes and L6 myotubes expressing LacZ, although insulin stimulation was not noted (Fig. 2, *A* and *B*). This was consistent with the results that GagPKB was constitutively active even after wortmannin treatment to the same extent as PKB activity after insulin stimulation in cells expressing LacZ (Fig. 1*B*). In addition, expression of neither DNRas nor MAPK affected 2-DG uptake (Fig. 2, *A* and *B*), indicating that the Ras/MAPK pathway is neither sufficient nor required for glucose uptake by insulin in these cell lines. These findings suggest that PKB activated by insulin via PI3-K might stimulate glucose transport activity independent of Ras/MAPK activation in skeletal muscle and adipose tissue, the major tissues for glucose disposal by insulin.

Activation of PKB Mimics Insulin-induced Glycogen Synthesis Regulated by PI3-K in L6 Myotubes but Not in 3T3L-1 Adipocytes—Next, we investigated GS activity regulating glycogen synthesis, a major glucose utilization pathway by insulin. In 3T3L-1 adipocytes, expression of GagPKB did not affect either basal or insulin-stimulated GS activity (Fig. 3*A*), whereas inhibition of PI3-K by wortmannin resulted in complete inhibition of insulin-dependent GS activity in cells expressing either LacZ or GagPKB (Fig. 3*A*). Moreover, expression of neither DNRas nor MAPK affected GS activity (Fig. 3*A*). These results suggest that a PI3-K-dependent but PKB-independent pathway plays a key role in activation of GS activity in 3T3L-1 adipocytes. In contrast, expression of GagPKB in L6 myotubes enhanced basal GS activity to essentially the same levels as the insulin-stimulated activity in cells expressing LacZ. Increase in insulin-stimulated activity in cells expressing GagPKB compared with cells expressing LacZ did not reach statistical significance (Fig. 3*B*). After treatment with wortmannin in L6 myotubes, GS activity in cells expressing GagPKB still remained significantly higher than that in cells expressing LacZ (Fig. 3*B*), suggesting that wortmannin inhibited

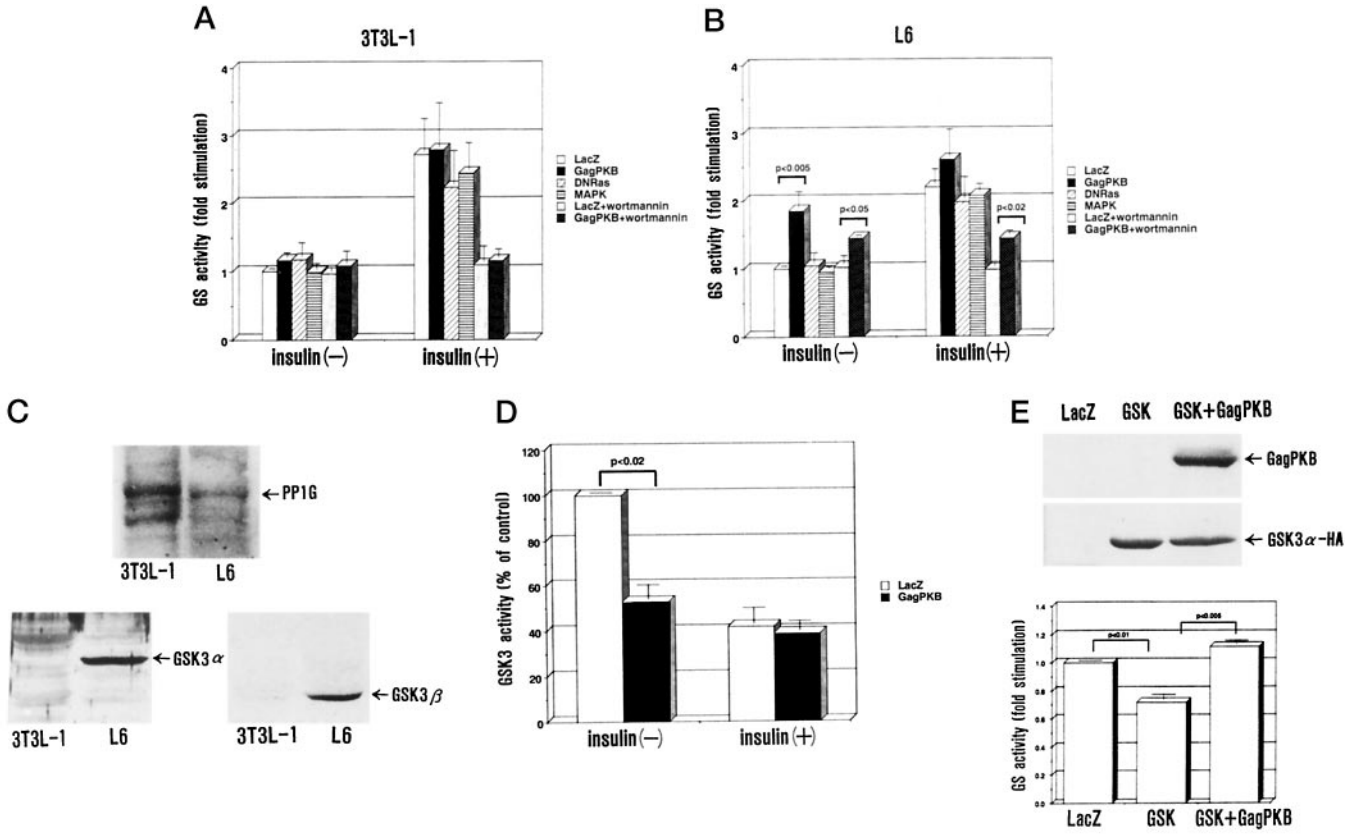

FIG. 3. **Expression of GagPKB increases GS activity in L6 myotubes via inhibition of GSK3 activity, but does not affect GS activity in 3T3L-1 adipocytes.** *A*, GS activity in 3T3L-1 adipocytes expressing the indicated constructs. Serum-starved cells were washed with KRHB and pretreated without or with wortmannin for 30 min before being treated without or with 100 nM insulin for 20 min. Cells were then lysed and subjected to assay of GS activity as described under "Experimental Procedures." Each result was converted to the activity ratio determined by dividing the activity measured with 0.25 mM glucose 6-phosphate (ligand-dependent activity) by the activity measured with 10 mM glucose 6-phosphate (total activity). The activity ratio in untreated 3T3L-1 cells expressing LacZ was 0.23 ± 0.04. *B*, GS activity in L6 myotubes expressing the indicated constructs. Cells were treated and subjected to the assays as described above. The activity ratio in untreated L6 cells expressing LacZ was 0.06 ± 0.01. The results in *A* and *B* are expressed as the ratio to the value of untreated cells expressing LacZ. Each *bar* represents the mean ± S.E. of more than three independent experiments. *C*, detection of PP1G and GSK3 in 3T3L-1 adipocytes and L6 myotubes. Cell lysates containing 30 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis followed by Western blot analysis with anti-PP1G antibodies (7% gel), anti-GSK3α antibodies, or anti-GSK3β antibodies (10% gel). *D*, inhibition of GSK3 kinase activity by insulin or expression of GagPKB. Serum-starved cells were treated without or with 100 nM insulin for 10 min. Cells were immunoprecipitated with anti-GSK3α antibody, and the immunoprecipitates were subjected to determination of kinase activity, as described under "Experimental Procedures." The results are expressed as per cent of maximum to the value of untreated cells expressing LacZ. Each *bar* represents the mean ± S.E. of more than three independent experiments. *E*, GS activity in 3T3L-1 adipocytes affected by expression of GSK3 or both GSK3 and GagPKB. Cells were cultured and infected with adenoviruses as described under "Experimental Procedures" then lysed and subjected to SDS-polyacrylamide gel electrophoresis (10% gel) followed by Western blotting with αPKB-CT or 12CA5 (*upper panels*). GS activity in untreated cells expressing the indicated constructs was measured (*lower panel*). The activity ratio in cells expressing LacZ was 0.21 ± 0.03. The results are expressed as the ratio to the value of cells expressing LacZ. Each *bar* represents the mean ± S.E. of more than three independent experiments.

both PI3-K and the insulin-induced activation of endogenous PKB but that constitutively active GagPKB could mimic PI3-K-dependent GS activation in L6 myotubes. In L6 myotubes as well as 3T3L-1 adipocytes, expression of neither DNRas nor MAPK affected GS activity (Fig. 3*B*). These findings indicate that constitutively active PKB is sufficient to stimulate GS activity in L6 myotubes and that the Ras/MAPK pathway is not involved in GS activation.

Activation of PKB Inhibits GSK3 Activity in L6 Myotubes—What is it that causes the difference between 3T3 L-1 adipocytes and L6 myotubes? GS activation by insulin is thought to be mediated by promotion of dephosphorylation and activation of GS due to either activation of protein phosphatase 1 by phosphorylation of its G subunit (PP1G) (6, 38, 39) or inactivation of GSK3 by phosphorylation (8, 24, 40) or both. We addressed the possibility that the difference in the relative abundance of PP1G and GSK3 between 3T3L-1 adipocytes and L6 myotubes might lead to alterations of the predominant signaling pathways regulating GS activity by insulin. Thus, we investigated the expression levels of PP1G and GSK3 by Western blot and compared the two cell lines. Fig. 3*C* shows that PP1G was expressed in both 3T3L-1 adipocytes and L6 myotubes and seemed to be more abundant in the former. In contrast, expression of GSK3 (both α and β) was observed only in L6 myotubes but not in differentiated 3T3L-1 adipocytes as described previously (41). These findings raise the possibility that PKB might stimulate GS activity by inactivation of GSK3 in L6 myotubes, and this mechanism might not work in 3T3L-1 adipocytes. In fact, expression of GagPKB significantly inhibited GSK3 activity at the basal state to essentially the same levels as insulin-stimulated GSK3 activity in cells expressing LacZ in L6 myotubes (Fig. 3*D*), whereas the activity could not be detected in 3T3L-1 adipocytes, even at the basal state (data not shown). Moreover, expression of GSK3α resulted in a decrease in basal GS activity (about 30%) in 3T3L-1 adipocytes compared with cells expressing LacZ, consistent with the results in 293 cells (40), whereas such suppression of GS activity was not observed in cells coexpressing GSK3 and GagPKB (Fig. 3*E*). From these data, we conclude that PKB stimulates GS activity by regulating GSK3 activity in L6 myotubes. Our data

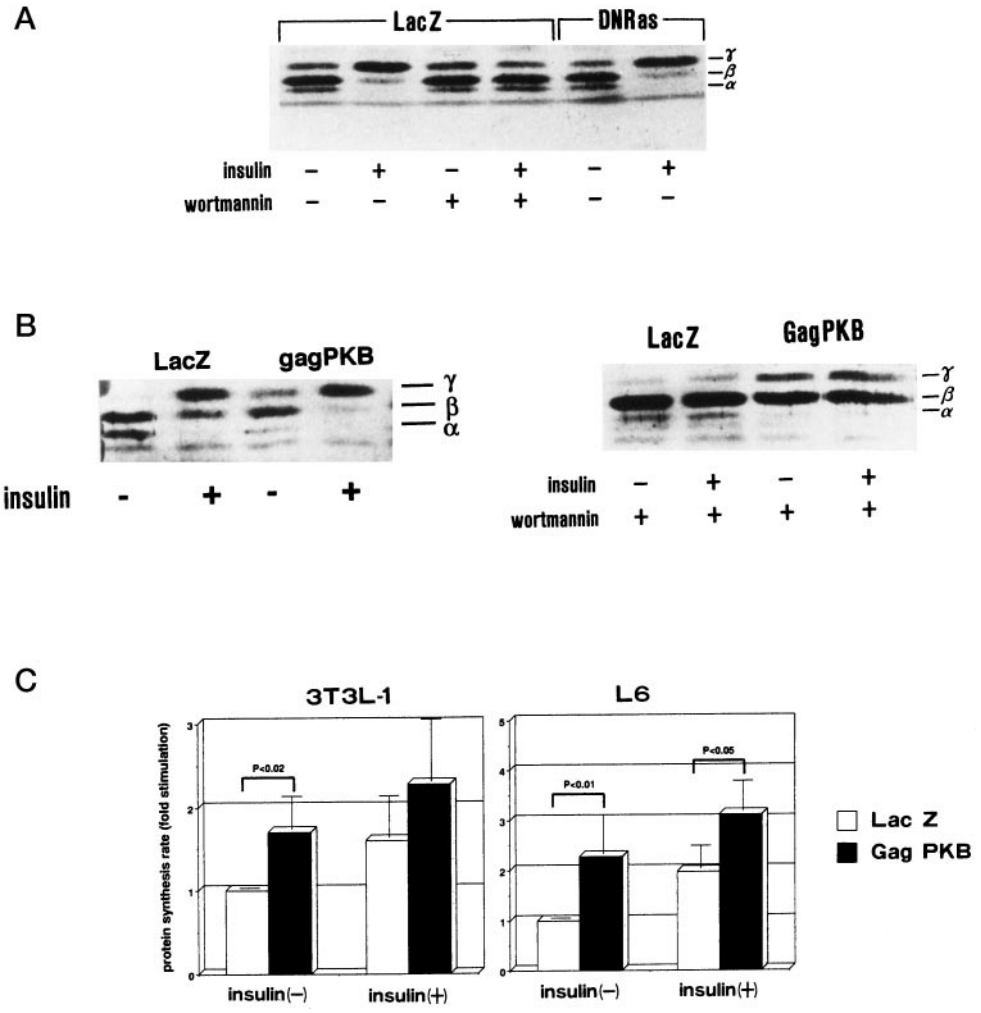

FIG. 4. **The effect of expression of GagPKB on phosphorylation of 4E-BP1 and protein synthesis.** *A*, inhibition of phosphorylation of 4E-BP1 by wortmannin but not by DNRas in 3T3L-1 adipocytes. *B*, the effect of expression of GagPKB on phosphorylation of 4E-BP1 in 3T3L-1 adipocytes. Serum-starved cells were pretreated without or with wortmannin (30 nM) then treated without or with 100 nM insulin for 15 min, lysed, and subjected to Western blot analysis by anti-4E-BP1 antibodies (13% gel). *C*, the effect of expression of GagPKB on the incorporation of [^{3}H]tyrosine into protein. Serum-starved cells were subjected to determination of protein synthesis rate as described under "Experimental Procedures." The results are expressed as the ratio to the value of untreated cells expressing LacZ. Each *bar* represents the mean ± S.E. of more than three independent experiments.

also suggested that regulation of GS activity by PP1G via PI3-K, independent of PKB, might be dominant in 3T3L-1 adipocytes. This may explain the lack of stimulation of glycogen synthesis by a constitutively active PKB in 3T3L-1 adipocytes (this study and Ref. 25). Taken together, these findings suggested the possibility that PKB might act as a key regulator in glycogen synthesis in skeletal muscle. Recent studies have demonstrated that GSK3 is detected, and the kinase activity is negatively regulated by insulin in isolated fat cells (42, 43), different from the results with 3T3L-1 adipocytes shown in this study. It suggests the possibility that PKB/GSK3 signaling pathway in adipose tissue might be also involved in the regulation of glycogen synthesis *in vivo*.

Activation of PKB Mimics Insulin-induced Protein Synthesis both in 3T3L-1 Adipocytes and L6 Myotubes—The stimulation of protein synthesis is an important and early response by insulin and is observed in a wide variety of cell types (44). The initiation phase of translation is rate-limiting, and the regulation by insulin is exerted at this step. The most important event is phosphorylation of 4E-BP1 (PHAS-I) in response to insulin and its dissociation from eIF-4E/4E-BP1 complex, leading to mRNA translation. MAPK was reported to phosphorylate 4E-BP1 *in vitro* and to abolish binding of 4E-BP1 to eIF-4E (10). However, recent studies revealed that insulin promotes the phosphorylation of multiple sites in 4E-BP1, one of which is likely to involve PI3-K-p70 S6 kinase, since it occurs in a rapamycin-sensitive and MAPK-independent manner (33, 45–48). 4E-BP1 detected by Western blot appears as three bands, designated α, β, and γ, representing different extents of phosphorylation in 3T3L-1 adipocytes (Fig. 4*A*), as described (36). Indeed, wortmannin inhibited the increase in the amount of γ band in response to insulin, but expression of DNRas did not alter phosphorylation levels of 4E-BP1 (Fig. 4*A*), suggesting that such phosphorylation by insulin is PI3-K-dependent and MAPK-independent. We next investigated the effect of GagPKB, a putative regulatory molecule of downstream effectors of PI3-K such as p70 S6 kinase, on phosphorylation of 4E-BP1 and protein synthesis in response to insulin in both cell lines. Expression of GagPKB resulted in much higher phosphorylation of 4E-BP1 in basal and insulin-stimulated states. In cells expressing GagPKB, the basal levels of phosphorylation of 4E-BP1 were much higher than in cells expressing LacZ, even after treatment with wortmannin (Fig. 4*B*), suggesting that GagPKB can stimulate 4E-BP1 phosphorylation downstream of PI3-K. In L6 myotubes, essentially the same results were observed (data not shown). These data suggested that activa-

tion of PKB might promote protein synthesis by phosphorylating 4E-BP1. Expression of GagPKB indeed increased the protein synthesis rate in both cell lines (Fig. 4*C*). Recently, a hypothetical model for the pathways promoting phosphorylation of 4E-BP1 in response to insulin has been proposed (46, 48, 49). According to this model, insulin stimulates phosphorylation on multiple sites of 4E-BP1 that might be mediated by the putative proline-directed serine/threonine kinase. Rapamycin potently inhibits this serine/threonine kinase, thereby inhibiting phosphorylation of both 4E-BP1 and p70 S6 kinase. (Very recently, Brunn *et al*. (50)) has demonstrated that TOR (target of rapamycin) may, in fact, directly phosphorylate PHAS-I.) Since expression of GagPKB stimulated 4E-BP1 phosphorylation and p70 S6 kinase activity, which were both inhibited by rapamycin in cells expressing LacZ (data not shown), PKB may activate the common kinase downstream of PI3-K, phosphorylating both 4E-BP1 and p70 S6 kinase in a TOR-sensitive fashion; phosphorylation of 4E-BP1 and phosphorylation and activation of p70 S6 kinase could cause a rise in cap-dependent mRNA translation and phosphorylation of ribosomal S6 protein, respectively, leading to increased protein synthesis.

Skeletal muscle is the primary site for insulin-stimulated glucose uptake, and a large part of the glucose incorporated into muscle cells is deposited as glycogen in response to insulin (51, 52). Thus, glucose uptake and glycogen synthesis in skeletal muscle play a pivotal role in blood glucose homeostasis in human. Protein synthesis is also promoted by insulin, predominantly in skeletal muscle (36, 53). Numerous reports have suggested the physiological role of PI3-K in these three most important biological effects of insulin (11–17, 33, 46–48). However, the molecular mechanisms downstream of PI3-K remain unclear. Our report provides the first evidence that PKB activity is sufficient to activate glucose uptake, glycogen synthesis, and protein synthesis *in vivo* in L6 myotubes, suggesting the possibility that PKB might regulate these three major biological responses to insulin in skeletal muscle. Our data also revealed that PKB might be the molecular link between PI3-K and these biological effects by insulin. However, they do not rule out either the possible role of other PKB-independent pathways or the importance of cooperation with other molecules such as PI3-K. Indeed, platelet-derived growth factor (PDGF) as well as insulin does activate PKB (18, 19). However, PDGF almost never affects glucose metabolism and is generally believed not to mediate significant stimulation of GLUT4 translocation, although overexpression of PDGF receptor was reported to promote GLUT4 translocation by PDGF in Chinese hamster ovary cells (54). With respect to glucose transport, translocation of PI3-K to the target such as GLUT4 vesicle in response to insulin has been reported to occur but not to PDGF (55, 56). Thus, in the case of PKB, targeting to the same compartment as PI3-K might be necessary for these biological effects by insulin. In fact, GagPKB is mainly localized in the plasma membrane, low density microsomal membrane, and high density microsomal membrane. This subcellular distribution is similar to that of p85 subunit of PI3-K (38) and might make it possible for GagPKB to mimic the biological actions of insulin. However, our data do not imply that all the downstream actions of PI3-K are mediated by PKB. For instance, expression of GagPKB did not affect thymidine incorporation into DNA in 3T3L-1 adipocytes,[2] whereas expression of constitutively active PI3-K promoted that in those cells (17), suggesting that signals downstream of PI3-K diverge into at least two pathways; PKB-dependent and -independent.

[2] K. Ueki, R. Yamamoto-Honda, Y. Kaburagi, T. Yamauchi, K. Tobe, B. M. Th. Burgering, P. J. Coffer, I. Komuro, Y. Akanuma, Y. Yazaki, and T. Kadowaki, unpublished data.

Acknowledgments—We thank P. Cohen for anti-PP1G antibody, N. Sonenberg for anti-4E-BP1 antibody, Y. Takai for the construct of DNRas, X. He for the construct of GSK3α, I. Saito for adenovirus vector, and S. Kakinuma for technical assistance.

REFERENCES

1. Lee, J., and Pilch, P. F. (1994) *Am. J. Physiol.* **266,** C319–C334
2. White, M. F., and Kahn, C. R. (1994) *J. Biol. Chem.* **269,** 1–4
3. Cushman, S. W., and Wardzala, L. J. (1980) *J. Biol. Chem.* **255,** 4758–4762
4. Suzuki, K., and Kono, T. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 2542–2545
5. Parker, P. J., Caudwell, F. B., and Cohen, P. (1983) *Eur. J. Biochem.* **130,** 227–234
6. Hubbard, M. J., and Cohen, P. (1989) *Eur. J. Biochem.* **186,** 711–716
7. Shulman, R. G., Bloch, G., and Rothman, D. L. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 8535–8542
8. Lawrence, J. C. J., and Roach, P. J. (1997) *Diabetes* **46,** 541–547
9. Lin, T. A., Kong, X., Haystead, T. A., Pause, A., Belsham, G., Sonenberg, N., and Lawrence, J. C. J. (1994) *Science* **266,** 653–656
10. Pause, A., Belsham, G. J., Gingras, A. C., Donze, O., Lin, T.-A., Lawrence, J. C. J., and Sonenberg, N. (1994) *Nature* **371,** 762–767
11. Hara, K., Yonezawa, K., Sakaue, H., Ando, A., Kotani, K., Kitamura, T., Kitamura, Y., Ueda, H., Stephens, L., Jackson, T. R., Hawkins, P. T., Dahnd, R., Clark, A. E., Holman, G. D., Waterfield, M. D., and Kasuga, M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 7415–7419
12. Okada, T., Kawano, Y., Sakakibara, T., Hazeki, O., and Ui, M. (1994) *J. Biol. Chem.* **269,** 3568–3573
13. Cheatham, B., Vlahos, C. J., Cheatham, L., Wang, L., Blenis, J., and Kahn, C. R. (1994) *Mol. Cell. Biol.* **14,** 4902–4911
14. Yamamoto-Honda, R., Tobe, K., Kaburagi, Y., Ueki, K., Asai, S., Yachi, M., Shirouzu, M., Yodoi, J., Akanuma, Y., Yokoyama, S., Yazaki, Y., and Kadowaki, T. (1995) *J. Biol. Chem.* **270,** 2729–2734
15. Martin, S. S., Haruta, T., Morris, A. J., Klippel, A., Williams, L. T., and Olefsky, J. M. (1996) *J. Biol. Chem.* **271,** 17605–17608
16. Tanti, J. F., Gremeaux, T., Grillo, S., Calleja, V., Klippel, A., Williams, L. T., Van Obberghen, E., and Le Marchand-Brustel, Y. (1996) *J. Biol. Chem.* **271,** 25227–25232
17. Frevert, E. U., and Kahn, B. B. (1997) *Mol. Cell. Biol.* **17,** 190–198
18. Burgering, B. M., and Coffer, P. J. (1995) *Nature* **376,** 599–602
19. Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. (1995) *Cell* **81,** 727–736
20. Kohn, A. D., Kovacina, K. S., and Roth, R. A. (1995) *EMBO J.* **14,** 4288–4295
21. Datta, K., Bellacosa, A., Chan, T. O., and Tsichlis, P. N. (1996) *J. Biol. Chem.* **271,** 30835–30839
22. Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B. A. (1996) *EMBO J.* **15,** 6541–6551
23. Franke, T. F., Kaplan, D. R., Cantley, L. C., and Toker, A. (1997) *Science* **275,** 665–668
24. Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovic, M., and Hemmings, B. A. (1995) *Nature* **378,** 785–789
25. Kohn, A. D., Summers, S. A., Birnbaum, M. J., and Roth, R. A. (1996) *J. Biol. Chem.* **271,** 31372–31378
26. Frost, S. C., and Lane, M. D. (1985) *J. Biol. Chem.* **260,** 2646–2652
27. Klip, A., Li, G., and Logan, W. J. (1984) *Am. J. Physiol.* **247,** E291-E296
28. Ueki, K., Matsuda, S., Tobe, K., Gotoh, Y., Tamemoto, H., Yachi, M., Akanuma, Y., Yazaki, Y., Nishida, E., and Kadowaki, T. (1994) *J. Biol. Chem.* **269,** 15756–15761
29. He, X., Saint-Jeannet, J. P., Woodgett, J. R., Varmus, H. E., and Dawid, I. B. (1995) *Nature* **374,** 617–622
30. Miyake, S., Makimura, M., Kanegae, Y., Harada, S., Sato, Y., Takamori, K., Tokuda, C., and Saito, I. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 1320–1324
31. Cohen, P., Gibson, B. W., and Holmes, C. F. (1991) *Methods Enzymol.* **201,** 153–168
32. Yu, J. S., and Yang, S. D. (1994) *J. Biol. Chem.* **269,** 14341–14344
33. Beretta, L., Gingras, A. C., Svitkin, Y. V., Hall, M. N., and Sonenberg, N. (1996) *EMBO J.* **15,** 658–664
34. Moxham, C. M., Tabrizchi, A., Davis, R. J., and Malbon, C. C. (1996) *J. Biol. Chem.* **271,** 30765–30773
35. Kanamoto, R., Utsunomiya, K., Kameji, T., and Hayashi, S. (1986) *Eur. J. Biochem.* **154,** 539–544
36. Yamauchi, T., Tobe, K., Tamemoto, H., Ueki, K., Kaburagi, Y., Yamamoto Honda, R., Takahashi, Y., Yoshizawa, F., Aizawa, S., Akanuma, Y., Sonenberg, N., Yazaki, Y., and Kadowaki, T. (1996) *Mol. Cell. Biol.* **16,** 3074–3084
37. Tanti, J. F., Grillo, S., Gremeaux, T., Coffer, P. J., Van Obberghen, E., and Le Marchand Brustel, Y. (1997) *Endocrinology* **138,** 2005–2010
38. Ingebritsen, T. S., and Cohen, P. (1983) *Science* **221,** 331–338
39. Dent, P., Lavoinne, A., Nakielny, S., Caudwell, F. B., Watt, P., and Cohen, P. (1990) *Nature* **348,** 302–308
40. Eldar Finkelman, H., Argast, G. M., Foord, O., Fischer, E. H., and Krebs, E. G. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 10228–10233
41. Benjamin, W. B., Pentyala, S. N., Woodgett, J. R., Hod, Y., and Marshak, D. (1994) *Biochem. J.* **300,** 477–482
42. Cross, D. A., Watt, P. W., Shaw, M., van der Kaay, J., Downes, C. P., Holder, J. C., and Cohen, P. (1997) *FEBS Lett.* **406,** 211–215
43. Moule, S. K., Welsh, G. I., Edgell, N. J., Foulstone, E. J., Proud, C. G., and Denton, R. M. (1997) *J. Biol. Chem.* **272,** 7713–7719
44. Kimball, S. R., Vary, T. C., and Jefferson, L. S. (1994) *Annu. Rev. Physiol.* **56,** 321–348
45. Graves, L. M., Bornfeldt, K. E., Argast, G. M., Krebs, E. G., Kong, X., Lin, T. A., and Lawrence, J. C. J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 7222–7226
46. von Manteuffel, S. R., Gingras, A. C., Ming, X. F., Sonenberg, N., and Thomas,

G. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 4076–4080
47. Azpiazu, I., Saltiel, A. R., DePaoli Roach, A. A., and Lawrence, J. C. Jr. (1996) *J. Biol. Chem.* **271,** 5033–5039
48. Fadden, P., Haystead, T. A. J., and Lawrence, J. C. Jr. (1997) *J. Biol. Chem.* **272,** 10240–10247
49. von Manteuffel, S. R., Dennis, P. B., Pullen, N., Gingras, A. C., Sonenberg, N., and Thomas G. (1997) *Mol. Cell. Biol.* **17,** 5426–5436
50. Brunn, G. J., Hudson, C. C., Sekulic, A., Williams, J. M., Hosoi, H., Houghton, P. J., Lawrence, J. C., and Abraham, R. T. (1997) *Science* **277,** 99–102
51. Baron, A. D., Brechtel, G., Wallace, P., and Edelman, S. V. (1988) *Am. J. Physiol.* **255,** E769–E774
52. Shulman, G. I., Rothman, D. L., Jue, T., Stein, P., DeFronzo, R. A., and Shulman, R. G. (1990) *N. Engl. J. Med.* **322,** 223–228
53. Stirewalt, W. S., Wool, I. G., and Cavicchi, P. (1967) *Proc. Natl. Acad. Sci. U. S. A.* **57,** 1885–1892
54. Quon, M. J., Chen, H., Lin, C. H., Zhou, L., Ing, B. L., Zarnowski, M. J., Klinghoffer, R., Kazlauskas, A., Cushman, S. W., and Taylor, S. I. (1996) *Biochem. Biophys. Res. Commun.* **226,** 587–594
55. Ricort, J. M., Tanti, J. F., Van Obberghen, E., and Le Marchand Brustel, Y. (1996) *Eur. J. Biochem.* **239,** 17–22
56. Heller-Harrison, R. A., Morin, M., Guilherme, A., and Czech, M. P. (1996) *J. Biol. Chem.* **271,** 10200–10204