Differential Effects of Constitutively Active Phosphatidylinositol 3-Kinase on Glucose Transport, Glycogen Synthase Activity, and DNA Synthesis in 3T3-L1 Adipocytes

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Phosphatidylinositol 3-kinase (PI3K) activation is necessary for many insulin-induced metabolic and mitogenic responses. However, it is unclear whether PI3K activation is sufficient for any of these effects. To address this question we increased PI3K activity in differentiated 3T3-L1 adipocytes by adenovirus-mediated expression of both the inter-SH2 region of the regulatory p85 subunit of PI3K (iSH2) and the catalytic p110α subunit (p110). Coexpression resulted in PI3K activity that exceeded insulin-stimulated activity by two- to fivefold in cytosol, total membranes, and the low density microsome (LDM) fraction, the site of greatest insulin stimulation. While insulin increased glucose transport 15-fold, coexpression of iSH2-p110 increased transport 5.2 ± 0.7-fold with a parallel increase in GLUT4 translocation to the plasma membrane. Constitutive activation of PI3K had no effect on maximally insulin-stimulated glucose transport. Neither basal nor insulin-stimulated activity of glycogen synthase or mitogen-activated protein kinase was altered by iSH2-p110 coexpression. DNA synthesis was increased twofold by insulin in control 3T3-L1 adipocytes transduced with β-galactosidase-encoding recombinant adenovirus, while iSH2-p110 coexpression increased DNA synthesis fivefold. These data indicate that (i) increased PI3K activity is sufficient to activate some but not all metabolic responses to insulin, (ii) activation of PI3K to levels exceeding the effect of insulin in adipocyte LDM results in only a partial stimulation of glucose transport, and (iii) increased PI3K activity in the absence of growth factor or oncprotein stimulation is a potent stimulus of DNA synthesis.

Insulin is pivotal in the regulation of glucose homeostasis. One of the primary actions of insulin is the stimulation of glucose transport into insulin-sensitive tissues by eliciting translocation of the major insulin-responsive glucose transporter, GLUT4, from an intracellular pool to the plasma membrane. Resistance to this stimulatory effect of insulin is a major pathologic feature of both type I and type II diabetes (5, 43). In most insulin-resistant states the expression of GLUT4 in skeletal muscle, the major site of insulin-stimulated glucose disposal, is not reduced (16), suggesting a defect in the signaling cascade or the cellular elements linking the insulin receptor to GLUT4 translocation, fusion with the plasma membrane, and/or activation (15). Although the number of molecules known to be involved in insulin signaling is increasing rapidly (2), the exact signaling pathways responsible for glucose transporter translocation remain to be defined. Ultimately these pathways could be attractive targets for novel therapeutic strategies to reduce or prevent insulin resistance.

Insulin stimulation of phosphatidylinositol 3-kinase (PI3K) was first observed by Ruderman et al. (31). Subsequently, several studies using either the PI3K inhibitors wortmannin and LY 294002 (3, 27) or dominant negative approaches (22) demonstrated that stimulation of PI3K activity is necessary for insulin-stimulated glucose transport. However, recent studies aiming to determine whether PI3K activity is sufficient to stimulate glucose transport have yielded contradictory results. For example, growth factors and cytokines such as platelet-derived growth factor (PDGF) and interleukin 4 stimulate PI3K in 3T3-L1 adipocytes or L6 myoblasts but have minimal or no effect on glucose transport (14, 40). Stimulation of PI3K activity with thiophosphorytrosine peptides results in a small (1.5-fold) increase in basal glucose transport and does not affect maximally insulin-stimulated glucose transport in 3T3-L1 adipocytes (12). In contrast, overexpression of the bovine catalytic p110α subunit (p110) of PI3K in these cells elevates both basal and insulin-stimulated glucose transport dramatically (18). The differences in these results might be explained by differences in the exact subcellular localization of PI3K activity generated by the different techniques utilized. Evidence to support this hypothesis comes from studies showing that insulin, unlike other growth factors, stimulates PI3K activity not only in the plasma membrane (PM) fraction but also in the low density microsomal (LDM) compartment (19, 26, 42) and possibly even in the GLUT4-containing subfraction of the LDM of adipocytes (11). This appears to be unique and might be a key to the specificity of the effect of insulin on glucose transport.

While the majority of previous studies focused on the relationship between PI3K activity and glucose transport, PI3K activity has been demonstrated to be necessary for other metabolic as well as mitogenic responses to insulin. Some studies have shown that inhibition of PI3K activity blocks the effects of insulin on activation of pp70 S6 kinase and DNA synthesis (3), glycogen synthase activity and glycogen synthesis (33), and mitogen-activated protein (MAP) kinase activity (39), although other studies challenge some of these findings (3, 10). Little is known as to whether increased PI3K activity is sufficient for stimulation of these actions of insulin (40). Recent studies have demonstrated that coexpression of the inter-SH2 region of the p85 regulatory subunit of PI3K (iSH2) with the catalytic p110α subunit, either expressed as separate molecules or as a fusion protein, leads to high specific activity of PI3K.
that is sufficient to stimulate signalling paths in COS-7 cells independent of growth factor stimulation (15, 21). The expression of activated PI3K molecules therefore is a powerful tool to evaluate whether PI3K activation is sufficient to stimulate specific cellular responses.

3T3-L1 adipocytes are a useful cell model to study insulin action, but gene transfer into these cells by conventional methods is inefficient. To achieve sufficient levels of expression, either stable transfections or retroviral gene transfer is used in fibroblasts before adipocyte differentiation, but both procedures can alter adipocyte differentiation and growth factor responsiveness, either by the selection process itself or by chronic expression of the transfected gene. We (8) and others (18) have recently demonstrated that recombinant adenosvirus-mediated gene delivery allows rapid and highly efficient gene transfer in terminally differentiated 3T3-L1 adipocytes. Thus, to further clarify the role of PI3K in insulin signalling, we used adenoviral gene delivery to acutely coexpress p110 and iSH2 in 3T3-L1 adipocytes. We achieved levels of PI3K activity that exceed the insulin-stimulated levels of PI3K activity in the cytosol, total membranes and the LDM compartment. This resulted in increased glucone transport, GLUT4 translocation to the plasma membrane, and stimulation of DNA synthesis but did not affect glycosyn thase activity or MAP kinase activation. Thus, activation of PI3K even when achieved in the LDM compartment can mimic some but not all of the metabolic and mito genic effects of insulin.

MATERIALS AND METHODS

Cell culture and transfections. 3T3-L1 cells (ATCC, Rockville, Md.) were grown in Dulbecco’s modified Eagle medium (DMEM) with 10% calf serum, 50 U of penicillin per ml, and 50 μg of streptomycin per ml (all from Life Technologies, Gaithersburg, Md.) per ml at 37°C, 5% CO2. Two days after confluence, differentiation was induced with 0.5 mM 3-isobutyl-1-methyl-xanthine, 0.25 mM dexamethasone (both from Sigma, St. Louis, Mo.), and 1 μg of insulin (porcine, crystalline, gift of R. Chance; Eli Lilly, Indianapolis, Ind.) per ml for 3 days. During and following differentiation DMEM was supplemented with 10% fetal calf serum (Atlanta Biologicals, Norcross, Ga.), and insulin was at 1 μg/ml. Cells were used for experiments at 10 to 20 days after induction of differentiation (8 to 11 days for thymidine incorporation, see below) and only if more than 90% of the cells showed fat droplets. Yields of both protein and DNA (measured as previously described [23]) varied less than 10% for wells of cells used in any one experiment.

293 cells and COS-7 cells were grown in DMEM with 10% fetal calf serum, 50 U of penicillin per ml, and 50 μg of streptomycin per ml (all from Life Technologies, Gaithersburg, Md.) per ml at 37°C, 5% CO2. Two days after confluence, cells were transfected at 90% confluence in serum-free medium using 2.5 μg of DNA and 12.5 μg of Lipofectamine reagent (Life Technologies) per 35-mm plate. Cells were used for experiments after 20 to 25 days of transfection (8 to 11 days for thymidine incorporation, see below) and only if more than 90% of the cells showed fat droplets. Yields of both protein and DNA (measured as previously described [23]) varied less than 10% for wells of cells used in any one experiment.

Plasmids. The plasmid CMV-iSH2 containing the myc tag sequence fused to the coding-terminal sequence of the iSH2 region (amino acids 428 to 678) of the human p53β subunit of PI3K (29) was provided by T. F. Franke, National Cancer Institute, Frederick, Md. The coding region was removed from this plasmid as a SalI-BamHI fragment, blunt ended, and cloned into the plasmid pBluescriptII (provided by H. Bujard, ZMBH, Heidelberg, Germany) using Not linker. A NcoI-HindIII fragment from the resulting plasmid pBluescriptII was cloned into plasmid pAC-CMV-pLpA (1) (provided by C. Newgard, University of Texas Southwestern Medical Center, Dallas, Tex.) resulting in the plasmid pAC-CMVP-pLpA-iSH2.

The plasmid pCMV-110 (29) encoding a constitutively active PI3K was provided by A. Klippel and L. T. Williams, University of California, San Francisco, Calif. (13). The myc-tagged mouse p110 subunit of PI3K was amplified out of this plasmid by PCR (Plnu DNA polymerase [Stratagene], 20 cycles) by using the primers 5′-TGG TGA CCA CCA TGC CTC CAC GAC CAT CTT CCG G 3′ and 5′-ACT CTA GAT CAG TTC AGG TCC TCC TCG G 3′, with the former introducing a Kozak sequence and a KpnI site and the latter introducing an XbaI site. This fragment was cloned into pAC-CMVP-pLpA resulting in the plasmid pAC-CMVP-pLpA-p110.

Generation of recombinant adenovirus and transfection of 3T3-L1 adipocytes. Recombinant adenovirus was generated as previously described (1). Briefly, the plasmids pCMV-pLpA-iSH2 and pAC-CMVP-pLpA-p110 were each cotransfected with the plasmid pCMV-p110 into 293 cells as described above. Cell lysis indicating a recombination event occurred 1 to 2 weeks following cotransfection. Several clones of recombinant virus were checked for the successful integration of the iSH2 or p110 coding regions by Western blotting with lysate of transduced 293 cells. One clone for each of the constructs was amplified further in 293 cell cultures. Purification by cesium chloride centrifugation resulted in high titer stocks of recombinant virus, typically 1 × 109 to 2 × 109 plaque-forming units (PFU) per ml as determined by limiting dilution. The recombinant adenovirus encoding β-galactosidase was obtained by C. Newgard and amplified as described above.

Transduction of differentiated 3T3-L1 adipocytes was performed overnight with constant agitation on a rocking platform in DMEM with 10% fetal calf serum. Recombinant adenoviruses encoding iSH2 and p110 were each used at a concentration of 1 × 109 PFU/ml, resulting in a total concentration of 2 × 109 PFU/ml in cotransduction experiments. β-Galactosidase-encoding recombinant adenovirus was used at a concentration of 2 × 109 PFU/ml. Experiments were performed following an additional 24-h incubation in fresh medium.

Cell fractionation. To generate a total membrane fraction and a cytosolic fraction, COS-7 or 3T3-L1 cells were transfected in DMEM with 0.1% calf serum for 18 h, stimulated overnight with 10 nM insulin for the indicated time, and homogenized by 20 strokes in a Potter homogenizer in PI3K lysis buffer (see below), and centrifuged at 180,000 × g for 75 min. Subcellular fractionation was performed by differential centrifugation as previously described (24). Following separation of cytosolic and high-density membrane fractions by centrifugation, the density of the middle band in sucrose gradients was found to be 2.1 g/ml. After fractionation, isolates were resuspended in equal volumes of PI3K lysis buffer. A total of 40,000 cells was used for each experiment. Fractions were assayed for two enzymes that were either present at high levels in the fraction or were enriched above controls. The enzymes chosen were cytochrome c reductase, which is a marker for the PM, and the ras GTPase activating protein (rGAP) that is present in the cytosol and cytoskeleton. Each fraction was assayed for cytochrome c reductase activity and ras immunoreactivity. The fractionation procedure cytochrome c reductase activity and ras immunoreactivity were determined in aliquots of the fractions obtained from four different cell lines (Table 1). Cytochrome c reductase activity, measured as previously described (4), was highest in the PM fraction, followed by the PM and HDM fractions, respectively, and the homogenate. This indicated a sevenfold enrichment of this marker in the PM fraction compared with homogenate and virtually no enrichment in the HDM fraction. Ras immunoreactivity was determined by Western blotting with a monoclonal pan-ras antibody which detects H-, K-, and N-ras (Santa Cruz Biotechnology, Santa Cruz, Calif.). Denstometry units per microgram of membrane protein showed the highest values for the PM fraction, followed by the HDM fraction, homogenate, and the LDM fraction. Taken together, these data indicate some cross contamination of the PM and HDM fractions but little contamination of the LDM fraction with the PM or HDM markers. GLUT4 immunoreactivity quantitated in fractions from cells in the basal state and expressed as densitometry units per microgram of protein was 5.4 ± 0.3-fold higher (mean ± standard error of the mean [SEM]) in the LDM compared with the PM fraction (see Fig. 4, lanes 1 and 5) and 17-fold enriched in the LDM fraction compared with the homogenate. Since membranes from different experiments were run on different gels, the exact densitometry units cannot be used to express mean values for GLUT4, but the fold increase for each experiment can be computed. The decrease in GLUT4 in the LDM fraction

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Immune-reactivity</th>
<th>Cytochrome c reductase (μmol/min/mg)</th>
<th>Protein yield (μg/10-cm-diameter dish)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM</td>
<td>35.6 ± 3.8</td>
<td>1.27 ± 0.11</td>
<td>103 ± 3</td>
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<tr>
<td>LDM</td>
<td>2.7 ± 0.2</td>
<td>0.92 ± 0.10</td>
<td>197 ± 13</td>
</tr>
<tr>
<td>HDM</td>
<td>14.2 ± 1.8</td>
<td>5.41 ± 0.59</td>
<td>72 ± 6</td>
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<tr>
<td>Homogenate</td>
<td>5.3 ± 0.5</td>
<td>0.76 ± 0.05</td>
<td>4,410 ± 236</td>
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* Four sets of differentiated 3T3-L1 adipocytes in the basal state were subject to subcellular fractionation by differential centrifugation as described in Materials and Methods. To characterize the fractions, ras and GLUT4 immunoractivity quantitated in fractions from cells in the basal state and expressed as densitometry units per microgram of protein load. All values are expressed as means ± standard error of the mean.
and its increase in the PM fraction in response to insulin (see Fig. 4) is consistent with no major cross contamination of the LDM and PM fractions. Both protein recoveries (micrograms per 100-mm-diameter well) for each fraction and distribution of the markers were not altered by transduction with recombinant adenovirus.

Glucose transport. Glucose transport activity was determined as previously described (7). Following incubation in serum-free DMEM for 3 h, 3T3-L1 adipocytes were washed twice with PBS and incubated for 30 min with or without 100 nM insulin in glucose-free MEM. 2(3H)Glucose, a product of glucose-6-phosphate dehydrogenase (NEIN, Boston, Mass.), 0.33 μCi per 35-mm-diameter well, was added to a final concentration of 100 μM for an additional 10 min. Transport was stopped by putting the cells on ice and adding 1:1 (vol/vol) ice-cold phenol solution (82 mg/liter in PBS). Cells were washed three times with cold PBS, dried, and lysed in 1 N NaOH. Aliquots of this lysate were used for liquid scintillation counting and determination of DNA content as previously described (23). Wortmannin (stock, 10 mM in dimethyl sulfoxide, Sigma) was used at a final concentration of 1 μM for 30 min and was added after cells were stimulated for 15 min with insulin.

PI3K assay. Total PI3K activity in membrane and cytosolic fractions was assayed as previously described for cytosol (25) by the borate thin-layer chromatography method described by Walsh et al. (37), which allows separation of phosphorylatedinositol 3-phosphate and phosphorylatedinositol 4-phosphate. Following overnight incubation in DMEM with 0.1% calf serum, cells were or were not stimulated with 100 nM insulin and homogenized directly in PI3K lysis buffer (20 mM Tris-Cl, 140 mM NaCl, and 10% gycerase [pH 7.4], containing 1 mM sodium orthovanadate, 0.5 mM EDTA, 2% each of apotinin and leupeptin per ml, and 0.1 mM dithiothreitol). Cytosol and total membranes were prepared as described in “Cell fractionation” above. For determination of PI3K activity in PM and LDM fractions, cells were homogenized and fractionated in HES buffer as described above, and fractions were resuspended in PI3K lysis buffer.

Aliquots of fractions in a total volume of 25 μl of PI3K lysis buffer were allowed to warm to room temperature for 5 min and were then mixed with 25 μl of a lipid-ATP mix containing 500 μg of phosphatydilinositol per ml, 80 μM ATP, 0.8 μM of [γ-32P]ATP (3000 Ci/mmol, NEN) per μl, 20 μM HEPES (pH 7.5), 50 mM NaCl, 12.5 mM MgCl2, and 0.015% Nonidet P-40. To inhibit some of the PI4K activity in membrane fractions, adenosine was added to a final concentration of 200 μM (20). The reaction was stopped after 5 min by addition of 80 μl of 1 N HCl, and the phosphoh lipids were extracted with 160 μl of chloroform-methanol (1:1, vol/vol). Phosphatidylinositol-monophosphate in 40 μl of organic phase was separated by borate thin-layer chromatography on chloroform-methanol (1:1, vol/vol). Phosphatidylinositol-monophosphate and was added after cellswere stimulated for 15 min with insulin.

DNA synthesis. DNA synthesis was determined as previously described (38). Differentiated 3T3-L1 adipocytes were incubated in serum-free DMEM with 1% bovine serum albumin (BSA) with or without 100 nM insulin for 15 h. After this time, the medium was replaced by DMEM containing 2% BSA. After 30 min, 25 μl of [3H]thymidine was added (0.25 μCi per 16-mm-diameter well). After an additional 1-h incubation cells were washed three times with cold PBS and lysed in sodium dodecyl sulfate (SDS) (1 mg/ml). Lysates were precipitated with trichloroacetic acid, filtered through 0.25 μm nitrocellulose filters, and was added after cells were stimulated for 15 min with insulin.

Glycogen synthase activity. Glycogen synthase activity was determined as previously described (35). Briefly, following incubation in serum- and glucose-free DMEM for 3 h, cells were or were not stimulated with 100 nM insulin for 30 min and homogenized in 100 mM NaF–10 mM EDTA (pH 7.0) with a Potter homogenizer. After the homogenates were centrifuged at 7,000 × g for 15 min, the ability of the supernatant to stimulate incorporation of [3H]UDP-glucose into glycogen was determined in the presence and absence of glucose-6-phosphate, and the activity ratio (activity without glucose-6-phosphate/activity with glucose-6-phosphate) were calculated. Wortmannin was used as described in “Glycogen transport” above.

Western blotting. Polysacrylamide gel electrophoresis (PAGE) and Western blotting were performed as previously described (24, 36) by using 1.5-mm thick minigels (Novex, San Diego, Calif.), nitrocellulose membranes (pore size, 0.45 μm; Schleicher & Schuell, Keene, N.H.), and the Mini Trans-blot Transfer cell (Bio-Rad). Proteins were separated by SDS-PAGE and blotted onto nitrocellulose (0.2 μM) membranes. Proteins were visualized using enhanced chemiluminescence (Amersham). Blots and autoradiograms were scanned by using an Agfa PhotoLook software to adjust brightness and contrast, and a Lintronix 2000 printer.

RESULTS

Expression of iSH2, p110, and p110* in COS-7 cells. The plasmids pACCMV.pLPa-iSH2, pACCMV.pLPa-p110, and pCCG-110* were transiently transfected into COS-7 cells, and expression was examined by Western blotting and PI3K assay in a cytosolic fraction and a membrane fraction prepared 2 days after transfection. Figure 1A shows the results of an anti-myc Western blot. Specific bands appeared at 35 kDa for the myc-tagged iSH2 region and at 115 kDa for the myc-tagged p110. Bands at 40 kDa and 85 kDa are nonspecific. Expression levels of p110 increased in cytosol when this protein was coexpressed with the iSH2 region (Fig. 1A, compare lanes 3c and 4c), a finding that was also observed in 3T3-L1 adipocytes (data not shown). This might be explained by increased stability of the iSH2-p110 heterodimer compared with p110 alone.

PI3K assays performed on these fractions showed that iSH2 (Fig. 1B, lanes 2) or p110 (lanes 4) expressed alone did not increase (iSH2) or increased minimally (p110 in the membrane fraction) total PI3K activity. In contrast, coexpression of both proteins (lanes 3) increased activity in both the cytosolic and membrane fractions to an extent comparable to the activity associated with a recently described (13) constitutively active PI3K (Fig. 1B, lanes 5). However, following immunoprecipitation with an anti-myc antibody, some PI3K activity was associated with the iSH2 region and at 115 kDa for the myc-tagged p110. Bands at 40 kDa and 85 kDa are nonspecific. Expression levels of p110 increased in cytosol when this protein was coexpressed with the iSH2 region (Fig. 1A, compare lanes 3c and 4c), a finding that was also observed in 3T3-L1 adipocytes (data not shown). This might be explained by increased stability of the iSH2-p110 heterodimer compared with p110 alone.

Coexpression of iSH2 and p110 increases PI3K activity above insulin-stimulated levels in 3T3-L1 adipocytes. Based on the results obtained in COS-7 cells, recombiant adenoviruses encoding iSH2 and p110 were prepared. Figure 2A shows the effect of iSH2 and p110 expression in differentiated 3T3-L1 adipocytes. The cells were transduced overnight with 109 PFU of recombinant adenovirus per ml encoding either iSH2 or p110, a combination of both viruses (total concentration, 2 × 109 PFU/ml), or 2 × 109 PFU of recombinant virus per ml encoding β-galactosidase as a control. Exposure to up to 2 × 109 PFU of recombinant virus per ml overnight did not change the morphological appearance of the cells. With 109 PFU of recombinant virus per ml, transduction efficiency was >90% as assessed by β-galactosidase staining. Cotransduction with the two viruses encoding β-galactosidase and iSH2 (each at 109 PFU/ ml) simultaneously did not reduce either the percentage of cells transduced as assessed by staining for galactosidase or the level of expression as assessed by Western blotting for iSH2 compared with cells transduced with one of the viruses alone.
rate experiments, with PI3K activity expressed as a fold of basal activity in the cytosolic fraction.

To further elucidate the exact intracellular localization of the membrane-associated activity, PI3K was measured in subcellular fractions of differentiated 3T3-L1 adipocytes. Figure 2B and C show that in control cells transduced with β-galactosidase-encoding adenovirus, insulin stimulated PI3K activity in the LDM fraction by 3.7 ± 0.4-fold (mean ± SEM, n = 3) with a much smaller effect in the PM fraction, which is consistent with other recent reports showing that the majority of insulin-stimulated PI3K activity is in the LDM compartment (19, 26). Similarly in cells coexpressing iSH2 and p110, stimulation of PI3K activity was primarily in the LDM fraction. The level of PI3K activity generated in the LDM fraction by this coexpression was ~60% higher than that resulting from insulin stimulation in the β-galactosidase-expressing control cells, and it tended to increase further with insulin treatment. Quantitation of three separate experiments is shown in the right panel of Fig. 2C. Results are expressed as the fold stimulation above basal PI3K activity in the PM fraction.

Figure 2D shows Western blots of aliquots of the identical fractions used for PI3K activity determination (Fig. 2A and B), probed for p110 with a monoclonal anti-myc antibody (top) or for iSH2 and p85 with a polyclonal p85 antiserum (bottom). The lanes are loaded with protein amounts that correspond to equal numbers of cells to parallel Fig. 2A and B. As the immunizing peptide used to generate the p85 antiserum overlaps partially with the inter-SH2 region of the p85 regulatory subunit of PI3K, both full-length endogenous p85 and the iSH2 region were detected by this antiserum. Immunoreactivity for endogenous p85 was high in cytosol and the LDM fraction and lower in the PM fraction. Expression of the iSH2 construct was highest in cytosol, lower in the LDM fraction, and lowest in the PM fraction. The increase of p85 immunoreactivity in cytosol from cells coexpressing iSH2 and p110 was also observed in cells overexpressing p110 alone but not in cells expressing only iSH2 (data not shown). p110 was most abundant in cytosol, and surprisingly, p110 was nearly absent from the PM fraction, despite the presence of both endogenous p85 and transduced iSH2.

iSH2 expression and iSH2-p110 coexpression increase glucose uptake. The effect of coexpression of iSH2 and p110 on 2-deoxyglucose uptake in 3T3-L1 adipocytes is shown in Fig. 3. Figure 3A shows that β-galactosidase-encoding adenovirus did not affect basal or insulin-stimulated glucose uptake. Coexpression of iSH2 and p110 resulted in PI3K activity that was about twofold higher than insulin-stimulated activity in cytosol and three- to fourfold higher than that in total membranes, p110 expressed alone elevated total PI3K activity about twofold in the membrane fraction but not in cytosol, while the iSH2 region expressed alone had no effect. The left panel of Fig. 2C shows quantitation from three separate transfections.

As shown in Fig. 2A the β-galactosidase-encoding virus did not alter basal or insulin-stimulated (15 min, 100 nM) PI3K activity in cytosol or membranes, and it was therefore used as a control in all subsequent experiments. Insulin treatment resulted in a two- to threefold stimulation of total PI3K activity in both cytosol and total membranes from untransduced cells and β-galactosidase-expressing cells (Fig. 2A and C). Figure 2C shows mean values plus SEM (n = 3) for cells transduced with β-galactosidase-encoding or iSH2-p110-encoding adenoviruses. Coexpression of iSH2 and p110 resulted in PI3K activity that was about twofold higher than insulin-stimulated activity in cytosol and three- to fourfold higher than that in total membranes, p110 expressed alone elevated total PI3K activity about twofold in the membrane fraction but not in cytosol, while the iSH2 region expressed alone had no effect. The left panel of Fig. 2C shows quantitation from three separate transfections.
important to note that the standard concentration of iSH2- and p110-encoding adenovirus used in our experiments (10⁹ PFU/ml each) results in stimulation of PI3K activity in the LDM fraction to a level ~60% greater than that elicited by insulin (Fig. 2C). Nevertheless, this does not result in maximal stimulation of glucose transport. The probability that iSH2-p110 coexpression is activating the same pathways as insulin is supported by the fact that maximally insulin-stimulated glucose uptake was not altered by iSH2-p110 coexpression. Thus, the effects of iSH2-p110 coexpression and insulin stimulation were not additive.

Coexpression of iSH2 and p110 induces GLUT4 translocation to the plasma membrane. Figure 4 shows a representative Western blot of GLUT4 in subcellular fractions of control cells expressing β-galactosidase and cells coexpressing iSH2-p110. Quantitation of Western blots from three different fractionations showed a 4.2 ± 0.1-fold increase of GLUT4 immunoreactivity in the PM fraction with insulin stimulation and a corresponding decrease of GLUT4 in the LDM fraction. iSH2 and p110 coexpression resulted in a 3.0 ± 0.2-fold increase in GLUT4 in the PM fraction, which was also accompanied by a decrease of GLUT4 in the LDM fraction. With insulin treatment of cells overexpressing iSH2-p110 there was a 4.0 ± 0.4-fold increase in GLUT4 in the PM fraction. Both insulin stimulation and iSH2-p110 coexpression also caused translocation of GLUT1 to the PM fraction (data not shown). The overall expression of either GLUT4 or GLUT1 was not altered in cells expressing β-galactosidase or iSH2-p110 compared
with nontransduced control cells as assessed by Western blotting of total membrane fractions (data not shown). Thus, the changes in glucose uptake described above can be explained by glucose transporter translocation.

**Glycogen synthase activity is not altered by iSH2-p110 coexpression.** Figure 5 shows glycogen synthase activity in differentiated 3T3-L1 adipocytes. Glucose 6-phosphate independent activity is expressed as a percentage of total glycogen synthase activity in both nontransduced (not shown) and β-galactosidase-transduced cells, insulin-stimulated glycogen synthase activity ~threefold. Transduction with β-galactosidase-encoding adenovirus had no effect on basal or insulin-stimulated glycogen synthase activity. Wortmannin abolished this insulin effect completely, but coexpression of iSH2 and p110 did not increase basal activity. The apparent small decrease in insulin-stimulated activity in cells coexpressing iSH2 and p110 compared with β-galactosidase-expressing cells did not reach statistical significance. These data suggest that activation of PI3K is necessary but not sufficient for activation of glycogen synthase.

iSH2 expression and iSH2-p110 coexpression stimulate DNA synthesis. DNA synthesis was measured as [3H]thymidine incorporation in differentiated 3T3-L1 adipocytes. As

**FIG. 3.** 2-Deoxyglucose uptake in differentiated 3T3-L1 adipocytes expressing β-galactosidase (β-gal), iSH2, and/or p110. (A) Cells were or were not transduced overnight with recombinant adenovirus encoding the indicated proteins. After an additional 24 h, which included 3 h of serum-free incubation, cells were or were not stimulated with 100 nM insulin for 30 min. 2-Deoxyglucose uptake was determined over a 10-min period as described in Materials and Methods. iSH2-p110 coexpression increased basal glucose transport significantly (P < 0.01) compared with nontransduced or β-galactosidase-expressing cells. (B) Following transduction and serum-free incubation as described above, cells were incubated in the absence (first six bars) or presence (last two bars) of 100 nM insulin for 15 min. Either 1 μM wortmannin (Wortm.) (black bars) or vehicle dimethyl sulfoxide (grey bars) was added for an additional 30 min before 2-deoxyglucose uptake was determined. The results shown are representative of 5 to 7 (panel A) or 2 to 3 (panel B) independent experiments, each performed in triplicate. Data are presented as means ± SEM; P values indicate the level of statistical significance.

**FIG. 4.** Translocation of GLUT4 to the plasma membrane induced by insulin or iSH2-p110 coexpression. Differentiated 3T3-L1 adipocytes were transduced overnight with recombinant adenoviruses encoding either β-galactosidase (β-gal) or iSH2 and p110 (Transd., transduction). Following an additional 24-h incubation including an overnight serum-starvation, cells were or were not stimulated with 100 nM insulin and subcellular fractions were prepared as described in Materials and Methods. PM (5 μg) and LDM (4 μg) fractions were resolved by SDS-PAGE, electrophoretically transferred to nitrocellulose membranes, and probed with a polyclonal GLUT4-antis serum. Molecular mass markers in kilodaltons are indicated on the left. This Western blot represents results from three independent experiments.

**FIG. 5.** Glycogen synthase activity is not altered by iSH2-p110 coexpression. Differentiated 3T3-L1 adipocytes were transduced overnight with recombinant adenovirus encoding either β-galactosidase (β-gal) or iSH2 and p110. After an additional 24-h incubation including a 3-h incubation in serum- and glucose-free DMEM, cells were or were not stimulated with 100 nM insulin for 30 min and lysed. Following centrifugation at 7000 × g for 15 min, the ability of the supernatant to stimulate incorporation of UDP-glucose into glycogen was determined in the presence and absence of glucose 6-phosphate as described in Materials and Methods. For the last two bars, 1 μM wortmannin was added for 30 min starting 15 min after initiation of insulin stimulation. Results are expressed as means plus SEM of activity ratios for three independent experiments, each performed in duplicate. The percent activity was determined as activity without glucose 6-phosphate/activity with glucose 6-phosphate × 100.
shown in Fig. 6, insulin stimulated DNA synthesis by ~0.3 pmol/h per 16-mm-diameter well in both nontransduced cells and cells expressing β-galactosidase. However, β-galactosidase transduction increased basal DNA synthesis compared with that of nontransduced cells. Transduction with p110-encoding adenovirus did not further increase basal DNA synthesis, while expression of iSH2 led to a 2.5-fold increase, and coexpression of iSH2 and p110 resulted in a sixfold increase of DNA synthesis compared with that in β-galactosidase-transduced cells. Insulin had no further stimulatory effect in iSH2-p110-coexpressing cells. These results indicate that elevated PI3K activity is a more potent stimulus of DNA synthesis than insulin stimulation in 3T3-L1 adipocytes.

Increased PI3K activity is not sufficient to stimulate MAP kinase. Activation of MAP kinase was assessed by Western blotting by using an antibody against a dually phosphorylated peptide that corresponds to the active form of MAP kinase. As can be seen in Fig. 7 (left panel), insulin increased MAP kinase activation in cytosol from both β-galactosidase-expressing control adipocytes and cells coexpressing iSH2 and p110, while activation of MAP kinase in cytosol from nonstimulated cells was comparably low. Expression of MAP kinase as assessed by Western blotting with a polyclonal MAP kinase antiserum that recognizes both nonphosphorylated and phosphorylated forms was not altered by transduction with β-galactosidase or iSH2-p110 (Fig. 7, right panel). These data suggest that the increase in PI3K activity caused by coexpression of iSH2 and p110 is not sufficient to activate MAP kinase.

DISCUSSION

This study demonstrates that constitutive activation of PI3K is sufficient to stimulate some but not all of the effects of insulin that have been shown to be dependent on PI3K activation. In particular, we show that elevation of PI3K activity above insulin-stimulated values in cytosol, membranes, and the LDM compartment by adenovirus-mediated expression of iSH2 and p110 results in stimulation of DNA synthesis exceeding insulin-stimulated values but elicits only one-third of the effect of insulin on glucose transport and no effect on the activation of glycogen synthase or MAP kinase.

Previous studies which aimed to elucidate whether PI3K activation is sufficient to stimulate glucose transport used two different approaches: (i) PI3K was activated by PDGF or interleukin-4 in insulin-sensitive cells (14, 34) or (ii) PI3K was activated independent of growth factor or cytokine stimulation either by thio-phosphotyrosine peptides or by adenovirus-mediated gene transfer of the p110 subunit (12, 18). Investigations using the first approach came to the conclusion that PI3K activity is not sufficient to stimulate glucose transport substantially, but more recent findings indicate that the subcellular localization of growth factor (e.g., PDGF)-activated PI3K activity does not match the localization of insulin-stimulated activity (26). In addition, as pointed out by Herbst et al. (12), certain growth factors or cytokines might also activate signaling pathways that inhibit stimulation of glucose transport.

Using thio-phosphotyrosine peptides to stimulate PI3K activity, Herbst et al. concluded that increased PI3K activity can stimulate glucose transport to up to 20% of maximally insulin-stimulated levels with no additive effect in the presence of maximally stimulating insulin concentrations (12). These findings differ only quantitatively and not qualitatively from our results. The discrepancy between the magnitudes of the effect of PI3K activation on glucose transport might well be explained by the level of PI3K activity achieved or by the subcellular localization of the activity. While we achieved PI3K activity above insulin-stimulated levels in cytosol, membranes, and specifically in the LDM fraction, Herbst et al. induced cytosolic levels that match insulin-stimulated activity and provided no data on membrane-associated activity. Using a lower concentration of adenoviruses encoding iSH2 and p110, we too found smaller increases in basal glucose transport (data not shown), but this finding is difficult to interpret as the percentage of cells transduced decreased below 90%.

A study published during preparation of the manuscript (18) shows results that are more difficult to explain. Overexpressing only the bovine catalytic p110α subunit of PI3K, fused to a 14-amino-acid tag derived from the C terminus of GLUT2, by
using adenovirus-mediated gene transfer in 3T3-L1 adipocytes. Katagiri et al. (18) showed activation of glucose transport that exceeded insulin-stimulated transport in control cells. Furthermore, insulin-stimulated glucose transport increased twofold in p110-transduced cells compared with control cells. Unfortunately it is not demonstrated whether these effects are reversible with wortmannin treatment. As we show in COS-7 cells and 3T3-L1 adipocytes in this study, and as other groups have demonstrated in COS-7 cells (20, 21), p110 exhibits relatively low specific activity. We do not see any effect of mouse p110α overexpression on glucose transport or on DNA synthesis, which appears to be more sensitive to PI3K activation (Fig. 6). Another recent study reports no substantial effects of high level overexpression of p110 on pp70 S6 kinase, Akt/Rac, or Jun N-terminal kinase activation, while a constitutively active PI3K with high specific activity activates all of these signalling molecules (21). In the same study, Klipple et al. demonstrate that membrane localization of a PI3K with low specific activity is sufficient to stimulate downstream signalling. It might therefore be speculated that the bovine p110α used by Katagiri et al. (18), either by itself or due to the attached GLUT2 tag, localizes in the membrane of the mouse cell line 3T3-L1, thus increasing its efficiency to stimulate downstream signaling.

Despite these differences, a common finding in all of these studies is critically important: regardless of the manner in which PI3K is activated or the level to which glucose transport is increased by PI3K activation, insulin further stimulates glucose transport. It is possible that other signalling pathways need to be stimulated in parallel in order to achieve maximal stimulation of glucose transport. However, it is also possible that PI3K activity is sufficient to stimulate maximal glucose transport only when localized in sufficient amounts to a specific subcellular compartment. Insulin, but not other growth factors, stimulates PI3K activity in the LDM compartment (26). However, we demonstrate that even when coexpression of iSH2 and p110 generates PI3K activity in the LDM compartment that is higher than that induced by insulin, the effect on glucose transport is smaller than that of insulin. A possible explanation is that in a specific subfraction of the LDM compartment, insulin induces PI3K activity to a greater extent than iSH2-p110 coexpression. Future studies therefore will need to define the localization of insulin-stimulated PI3K activity more precisely, and initial steps in this direction have been carried out (11). It is of interest that with iSH2-p110 coexpression the increase of GLUT4 translocation is proportionally greater than the increase in glucose transport. One possible explanation is that not all the GLUT4 which is detected by immunoblotting in the PM fraction from cells coexpressing iSH2-p110 is fully fused in a functionally active state (15). Possibly, insulin stimulation results in activation of additional signals which facilitate GLUT4 vesicle fusion and/or GLUT4 activation. However, it is also possible that this quantitative difference is due to differences in the sensitivities of the assays used.

Stimulation of DNA synthesis and glycogen synthase activity by insulin depend on PI3K, as demonstrated in experiments with the inhibitors wortmannin and LY 294002 (3, 33). One study which attempts to elucidate whether PI3K is sufficient to stimulate glycogen synthesis demonstrates that PDGF-induced activation of PI3K leads to only a minor increase in glycogen synthesis (40). However, this does not take into account the fact that PDGF and insulin stimulate PI3K in different compartments. We demonstrate that stimulation of glycogen synthase activity by insulin is not only blocked by preincubation with wortmannin, as described previously (33), but also is completely reversed with a 30-min wortmannin incubation following preincubation with insulin. Despite this dependency on PI3K activity, glycogen synthase activity is not stimulated by elevated PI3K activity. It might be argued that increased PI3K activity could initially lead to activation of glycogen synthase but that the continuous presence of activated PI3K leads to a desensitization. We find this unlikely, since insulin stimulation of glycogen synthase in cells coexpressing iSH2 and p110 is not impaired (Fig. 5).

In contrast to glycogen synthase activity, DNA synthesis is increased by coexpression of iSH2 and p110 to levels above those induced by insulin. Thus, increased PI3K activity, in the absence of growth factor or oncogene stimulation, is a potent stimulus of DNA synthesis. Several growth factors and oncogene products are known to regulate polyphosphoinositide metabolism (28), and the potential impact of PI3K activity on cell transformation (17, 32) is underscored by the fact that PI3K inhibitors are being tested as antiproliferative agents (28). Since other growth factors stimulate PI3K and 10% calf serum stimulates four- to fivefold more DNA synthesis in 3T3-L1 fibroblasts than 1 μM insulin (3), the high level of DNA synthesis in iSH2-p110-expressing cells compared with insulin stimulation might not be surprising. It is, however, surprising that expression of iSH2 alone, in addition to its small effect on glucose transport, increases DNA synthesis substantially while not elevating total PI3K activity. As some PI3K activity is associated with immunoprecipitated iSH2, we speculate that the effects of iSH2 are caused by a redistribution of PI3K activity rather than by an increase in activity.

The hierarchy in the interaction between the signalling cascade involving PI3K and the p21ras-Raf-MAP kinase cascade has attracted intense debate. While the physical interaction of the effector domain of p21ras with p110 has been demonstrated (30, 34), data suggest PI3K may be either upstream or downstream of p21ras (9, 13, 30, 41). This controversial finding might be explained by tissue-specific differences in interactions of signalling molecules (6). In 3T3-L1 adipocytes, the PI3K inhibitor LY294002 blocks activation of p21ras and decreases MAP kinase activation by insulin (6). However, we do not see MAP kinase activation by increased PI3K activity alone, suggesting that other signalling events need to be stimulated in parallel. Similarly, activated PI3K was found not to affect MAP kinase activation in COS-7 cells (21). Indeed, the activation of PI3K might have a more permissive role for p21ras activation by functional interaction with the GTPase-activating protein in 3T3-L1 adipocytes (6).

Several new questions arise from our findings. The exact intracellular localization of PI3K activity necessary for stimulating specific metabolic responses and DNA synthesis is still unclear, and it will be especially exciting to determine whether targeting of PI3K activity to specific compartments can selectively elicit DNA synthesis or metabolic effects. Also undetermined are which of the phospholipid products of PI3K are necessary and which downstream signals require activation to elicit specific responses. For insulin actions such as stimulation of glycogen synthase for which activation of PI3K activity is not sufficient, the additional molecules which need to be coactivated remain to be determined. Adenovirus-mediated gene transduction is a powerful tool to address these questions in fully differentiated insulin target cells. The current demonstration of differential effects of constitutively active PI3K on DNA synthesis and specific metabolic pathways will lead to deeper insights into the specificity of growth factor signalling.

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